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Impact of amino acid replacements on *in vitro* permeation enhancement and cytotoxicity of the intestinal absorption promoter, melittin

Sam Maher[a], Marc Devocelle[b], Sinéad Ryan[a], Siobhán McClean[c], David J. Brayden[a,*]

[a] *UCD School of Agriculture, Food Science and Veterinary Medicine and UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland*
[b] *Centre for Applied Science for Health, Centre for Synthesis and Chemical Biology, Department of Pharmaceutical & Medicinal Chemistry, Royal College of Surgeons in Ireland, Ireland*
[c] *Centre for Applied Science for Health, Institute of Technology Tallaght, Dublin 24, Ireland*

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ABSTRACT

Melittin is an amphipathic α-helical peptide known to cause the non-cell selective perturbation of cell membranes, especially erythrocytes. The well characterised interaction of the peptide with phospholipid bilayers has led to its use as a model to study lipid-peptide interactions. In recent years, melittin has emerged as a potential intestinal absorption promoter that increases paracellular marker permeability across both *in vitro* and *in situ* intestinal drug delivery models. Like many other promoters, inherent toxicity limits the drug delivery potential of melittin. The purpose of this study was to examine the effect of amino acid modifications of melittin on viability and drug permeation in human intestinal epithelial cell monolayers (Caco-2), where each structural change made to the peptide is known to reduce the cytolytic action of the peptide on cell membranes composed of zwitterionic phospholipids. Each of the 4 peptide analogues (PA) demonstrated reduced cytotoxicity in the methylthiazolyldiphenyl-tetrazolium bromide (MTT) conversion assay and lactate dehydrogenase (LDH) membrane integrity assay, which was correlated with a reduction in amphipathicity and hydrophobicity, as measured by RP-HPLC. The selected amino acid changes however, also attenuated the epithelial permeation enhancement activity of melittin, as measured by transepithelial electrical resistance (TEER) and flux of FITC-dextran-4 kDa across Caco-2 monolayers. This data suggests that the cytolytic action of melittin is responsible in part for permeation enhancement and that these effects are related to transcellular perturbation in addition to effects on tight junctions.

1. Introduction

Oral bioavailability of large hydrophilic molecules is limited by poor absorption across the intestinal epithelium. The majority of these molecules, which include most biotech cargoes, must therefore be delivered by injection at significant inconvenience to patients. For some drugs, patients must endure regular discomfort to prevent serious illness while for other drugs used in non-life threatening indications, the necessity for repeat injections can affect their commercial success, even if they have superior efficacy and safety to an established oral therapy. Continuing growth in the biotech industry has led to an increase in the number of licensed parenterals. One of the major challenges in biopharmaceutical development therefore continues to be the need for effective oral drug delivery systems.

In recent years there has been a renewed interest in use of oral drug delivery platforms that incorporate absorption promoters. The earliest promoters tested in animal models included surfactants, salicylates and chelating agents, which were not realistic candidates to promote drug absorption in man because of their inherent toxicity (reviewed in Maher et al., 2008; Muranishi, 1990, 1987; Swenson and Curatolo, 1992). The latest generation of oral absorption promoters selectively modulate paracellular permeability without overt toxicity and some may be good candidates for future development (reviewed in Gonzalez-Mariscal et al., 2005, 2008; Johnson et al., 2008; Kondoh and Yagi, 2007; Kondoh et al., 2008). Of these, there are Peptide-based analogues modeled on polypeptide toxins, including the claudin modulator, C-terminal of the *Clostridium perfringens* enterotoxin (C-CPE) (Kondoh et al., 2006), and the synthetic peptide fragment AT1002, modeled on the active moiety of *zonula occludens* toxin from *Vibrio cholera* (Motlekar et al., 2005). Elucidation of structure-activity relationships also led to the design and optimization of a tight junction

Abbreviations: HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; HOBt, *N-hydroxybenzotriazole*; DIEA, *N*,*N*′*-diisopropylethylamine*; MBHA, 4-methylbenzhydrylamine; Fmoc, 9-fluorenylmethoxycarbonyl; NMP, *N-methylpyrrolidone*; t-Bu, t-*butyl*; Boc, t-*butoxycarbonyl*; Trt, trityl; TFA, trifluoroacetic acid; O-*t*Bu, *t*-butoxy.

* Corresponding author. Tel.: +353 1 7166013; fax: +353 1 7166219.
E-mail address: david.brayden@ucd.ie (D.J. Brayden).

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(TJ) modulating peptide, analogous to the first loop of occludin, which has strong promoting activity (Tavelin et al., 2003). A major factor that contributed to both the promoting action and initial safety profiles of these new generation peptide-based promoters has been the ability to tailor their structure to improve these features. Modeling this approach, we examined the effect of selected structural modifications to the α-peptide, melittin, the principle cell lytic component in the venom of the arthropod, *Apis mellifera* and a recently established intestinal absorption promoter.

Melittin is a model amphipathic α-peptide, which promotes transmucosal flux of model paracellular markers across both *in vitro* and *in situ* drug delivery models. In Caco-2 monolayers, the peptide caused a rapid and reversible drop in TEER accompanied by flux of a number of paracellular polar sugars (Liu et al., 1999; Maher et al., 2007b; Schasteen et al., 1992). Likewise, in isolated rat and human colonic mucosae, the peptide increased the permeation of [^{14}C]-mannitol and FITC-dextran-4 kDa (FD4) (Maher et al., 2007a, 2009a). In rat intestinal instillations, melittin significantly increased both jejunal and colonic bioavailability (F) of FD4 and it also improved mannitol absorption in rat jejunal single pass perfusions (Maher et al., 2009c). Despite this epithelial permeating action, the potential of this peptide as an enhancer is hampered from a safety perspective. Cytotoxicity of melittin has been reported in a large number of cells including erythrocytes (Tosteson et al., 1985), lymphocytes (Pratt et al., 2005), cervical (Zhu et al., 2007) and intestinal epithelial cells (Maher and McClean, 2006). In most cases this cytotoxicity can be attributed to the non-selective perturbation of mammalian cell membranes. In intestinal epithelial cells melittin caused leakage of lactate dehydrogenase (LDH) and cell death by necrosis at concentrations in the same order as those required to increase permeation (Maher and McClean, 2006, 2008). It seems that there is a close relationship between the cytotoxic potential of melittin and its capacity to increase permeation.

The effect of even minor structural modification of melittin can have a large impact on the cytolytic action of the peptide (reviewed in Dempsey, 1990; Raghuraman and Chattopadhyay, 2007). In general, a reduction in amphipathicity, hydrophobicity and helicity reduces the cell perturbation capacity of melittin. The four peptide analogues (PA) selected for this study were based on previous experiments demonstrating that changes to the physicochemical properties required for cytolysis reduced the hemolytic action of melittin (Asthana et al., 2004; Blondelle and Houghten, 1991; Subbalakshmi et al., 1999). While it was anticipated that selected modification of amino acids of melittin would reduce the cytotoxicity of the peptide in intestinal epithelial cells, the impact on its intestinal permeating activity was unknown. The purpose of this study was therefore to examine the impact of specific chemical modifications of melittin on both cytotoxicity and intestinal permeation in Caco-2 monolayers, in an attempt to widen the margin between enhancement and cytotoxicity.

2. Materials and methods

2.1. Peptide synthesis, purification and characterization

Fmoc-protected amino acids, coupling reagents and the Rink Amide MBHA resin were purchased from Novabiochem (Nottingham, UK). All other reagents and solvents were purchased from Aldrich (Dublin, Ireland) and used without further purification. Peptides were prepared by standard solid phase peptide synthesis (Merrifield, 1986) according to the Fmoc-*t*Bu strategy (Carpino and Han, 1972) with HBTU/HOBt/DIEA coupling chemistry, in NMP solvent. Single coupling cycles (except for L^{13}, double coupling cycle) using a 10-fold excess of Fmoc-amino acid derivatives to resin-bound peptide were used. The side chain protecting groups were *t*Bu for Ser and Thr; Trt for Gln; Boc for Lys and Trp; Pbf for Arg. The syntheses were carried out on a 1.0×10^{-4} mol scale. Assembly of the amino acid sequences, starting from a Rink Amide MBHA resin, were carried out on an automated peptide synthesizer (Applied Biosystems 433A). Peptides were deprotected and cleaved from the synthesis resin using a mixture of 82.5% trifluoroacetic acid, 5% water, 5% triisopropylsilane, 5% thioanisole, 2.5% EDT, at RT for 2.5 h. The peptides were precipitated and washed three times with 10 ml aliquots of diethyl ether. They were then dried, dissolved in distilled water and lyophilized. Chromatographic analysis and purification were performed on a BioCAD SPRINT Perfusion Chromatography Workstation (*PerSeptive Biosystems*) using Gemini columns (Phenomenex, 110 Å, 5 μm, C18, 4.6 mm × 250 mm, 250 mm × 100 mm, for the analytic and semi-preparative columns, respectively). Buffers used were mobile phase A: 0.1% TFA in HPLC grade water; mobile phase B: 0.1% trifluoroacetic acid in acetonitrile with a gradient: 2–60% B in 18 column volumes (analytical) or 5 column volumes (semi-preparative) with a flow rate: 1 ml/min (analysis) or 5 ml/min (semi-preparative) and single wavelength detection at 214 nm. Purified peptides were finally characterised by matrix-assisted laser desorption/ionization Time-of-flight (MALDI-TOF) mass spectroscopy using the α-cyano-4-hydroxy-cinnamic acid matrix.

2.2. HPLC: assessment of peptide hydrophobicity

HPLC retention times from analytical reverse phase chromatography are often used to assess the hydrophobicity and amphipathicity of α-helical peptides (Blondelle and Houghten, 1991, 1992; Kim et al., 2005). RP-HPLC was carried out with a Varian 920 HPLC equipped with a Luna 5 μ C18(2) column 250 mm × 4.6 mm (Phenomenex, UK). Gradient elution at a flow rate of 1 ml/min was performed with mobile phase A, containing 0.1% (v/v) TFA in H_2O and mobile phase B containing 0.1% (v/v) TFA in acetonitrile. The gradient sequence was 20% B from 0 to 5 min, 20–60% B from 5 to 35 min, 60% B from 35 to 40 min and 60–40% B from 40 to 45 min. Samples were detected at a UV absorbance wavelength of 215 nm.

2.3. Cell culture

Caco-2 cells (passage 30-50, ECACC, UK) were cultured in DMEM containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine and 1% (v/v) non-essential amino acids. Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. Viability of the test cells prior to use exceeded 99%, as determined by trypan blue dye exclusion.

2.4. Cytotoxicity assays

The effect of each melittin analogue on cell viability was measured at selected concentrations using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) conversion assay. Caco-2 cells (2×10^4 cells) incubated with test agent for 24 h were incubated with fresh culture media containing MTT (500 μg/ml) for 3 h. Media was then aspirated and replaced with DMSO after which the plates were shaken for 2 min to solubilise the formazan crystals and the optical density (OD) was measured at 550 nm. The effect of each test agent on membrane integrity was measured by the LDH release assay. After incubation of Caco-2 cells (2×10^4 cells) with test agent, the multi-well plate was centrifuged ($2000 \times g$) for 5 min and the enzyme activity of LDH was subsequently measured in supernatants treated with an equal volume of LDH assay substrate solution for 30 min (TOX-7; Sigma, Ireland). The reaction was stopped by the addition of HCl (0.1 M) and the stoichiometric formation of chromogenic tetrazolium dye

Table 1
Structural modifications made to melittin and their effect on hydrophobicity as measured by RP-HPLC.

Peptide sequence	Molecular weight (Da)	Modification (designation)	Relative hydrophobic index
GIGAVLKVLTTGLPALISWIKRKRQQ	2846.48	Synthetic melittin	1
GIGAVLKVLTTGLP-LISWIKRKRQQ	2775.42	(PA-1)[a]	0.941
GIGAVLKVLTTGLPALIS-IKRKRQQ	2660.29	(PA-2)[a]	0.891
GIGAV**A**KVLTTG**A**PALISWIKRKRQQ	2762.34	(PA-3)[b]	0.836
––––––––––GLPALISWIKRKRQQ	1793.19	(PA-4)[a]	0.739

[a] Amino acid deletion.
[b] Amino acid substitution.

was measured spectrophotometrically at 490 nm in a multi-well plate reader. Percent LDH release was measured relative to that induced by Triton X-100 (1%, v/v).

2.5. Hemolysis assay

The hemolytic activity of each test peptide was measured spectrophotometrically using a hemoglobin release assay, as previously described (Shin et al., 2001). Briefly, fresh defibrinated sheep erythrocytes (Cruinn Diagnostics, Ireland) were rinsed three times with normal saline (0.9%, w/v), resuspended at 4% (v/v) in saline and incubated with an equal volume of test agent for 1 h at 37 °C. The cell suspension was then centrifuged at 1000 × *g* for 5 min after which an aliquot of the supernatant was transferred to a new multi-well plate with hemoglobin release measured spectrophotometrically at 414 nm. Percent hemolysis was calculated from the equation; % hemolysis = (OD 414 nm test agent–OD 414 nm saline control)/(OD 414 nm Triton X-100 (0.1%)–OD 414 nm saline control).

2.6. Measurement of TEER and FD4 permeability in Caco-2 monolayers

Caco-2 cells (5×10^5 cells) were seeded on to 12 mm Transwell® polycarbonate inserts (Corning Costar Corp., USA) and grown for 21 days, renewing the culture medium every other day. Transepithelial electrical resistance (TEER) of each monolayer was measured with an EVOM voltohmmeter with a chopstick-type electrodes (World Precision Instruments, UK). The corrected TEER value was calculated by subtracting the resistance of a blank filter from the monolayer, normalized as $\Omega\,cm^2$, and expressed as percent change in TEER measured prior to the addition of test agent.

Permeability of Caco-2 monolayers was measured with FITC-dextran of 4 kDa (FD4) (Sigma, Ireland). Monolayers were equilibrated with pre-warmed Hank's balanced salt solution (HBSS) without phenol red and supplemented with glucose (11 mM) and HEPES (25 mM, pH 7.4), after which FD4 (0.5 mg/ml) was added to the apical chamber. The apical (10 μl) and basolateral (0.75 ml) $t = 0$ samples were withdrawn before the addition of test agent, while every subsequent basolateral sample ($t = 30, 60, 90, 120, 150, 180$) was measured after the addition of test agent. Fluorescence (λ ex/λ em 480/520 nm) was measured in a spectrofluorimeter (MD Spectramax, Gemini). The apparent permeability coefficient (P_{app}) for FD4 was calculated according to the equation; P_{app} (cm/s) = $(dQ/dt)(1/A\,C_0)$, where dQ/dt is the transport rate (mol/s); A is the surface area of the monolayer (cm^2), and C_0 is the initial concentration in the donor compartment (mol/ml) (Hubatsch et al., 2007).

2.7. Data analysis

Unless otherwise stated, all experiments were carried out on three independent occasions and data was expressed as the mean ± standard error of the mean. Statistical significance was measured by two-tailed Student's *t*-tests using GraphPad Prism 5® software and was designated at the level of $P < 0.05$.

3. Results

3.1. Synthesis, purification and characterization of melittin analogues

Each of the synthetic peptides listed in Table 1 demonstrated purity in excess of 95% and their theoretical molecular weight corresponded to the actual value obtained by MALDI-TOF mass spectroscopy. Peptide analogue-1 differed from native melittin by an alanine deletion at position-15, peptide analogue-2 was the deletion analogue of tryptophan at position 19, peptide analogue-3 was a double substitution of heptadic leucine at 6 and 13 with alanine and peptide analogue-4, was a melittin analogue truncated at its 11 N-terminal residues (Table 1). In order to validate the synthesis, purification and characterization of the synthetic peptides, a commercially available melittin was included in the study (Serva, Germany). Both the native and our synthetic melittin had a molecular weight of 2846.5 Da in mass spectroscopy, had purity in excess of 95% by RP-HPLC and behaved identically in cytotoxicity and intestinal permeability assays, thus validating the peptide synthesis.

3.2. Effect of melittin analogues on cytotoxicity of intestinal epithelial cells and erythrocytes

Native melittin caused a concentration dependent decrease in Caco-2 cell viability in MTT assay after 24 h incubation, with an IC_{50} value of 2.5 μM (Fig. 1a). Each of the peptide analogues tested had lower cytotoxicity against Caco-2 cells in the MTT assay than the native peptide (Fig. 1a). Omission of alanine at position 15 (PA-1) or tryptophan at position 19 (PA-2) reduced the cytotoxicity of melittin by 3.5- and 4.5-fold, respectively, while "PA-3 with substitution of leucine at positions 6 and 13 did not lead to appreciable cytotoxicity at the highest concentration tested" (Fig. 1a). Deletion analogue of the hydrophobic N-terminal (1–11) sequence of melittin (yielding PA-4) was not cytotoxic in Caco-2 cells even at 50 μM (data not shown). The reduction in cell viability in MTT assay was correlated with leakage of LDH (Fig. 1b). Similar to the MTT assay, native melittin reduced membrane integrity at a lower concentration than any of the peptide analogues (Fig. 1b). Native melittin and PA-1 were the only peptides that significantly increased LDH release from Caco-2 cells after 24-h incubation (Fig. 1b). PA-2-to-PA-4 did not alter membrane integrity of Caco-2 cells in the LDH release assay (Fig. 1b). The effect of each peptide on hemolysis of defibrinated sheep erythrocytes was also measured after 1 h incubation (Fig. 2). Hemolytic activity of native melittin was significantly greater than each of the peptide analogues, matching the effects seen in Caco-2 cells. Of the analogues, PA-1 was the most hemolytic, inducing ~40% hemolysis at a concentration of 11 μM (Fig. 2), while PA-4 did not cause hemolysis of sheep erythrocytes at concentrations up to 50 μM (data not shown).

3.3. Impact of structural modification of melittin on its intestinal permeating activity

TEER and permeability to FD4 across Caco-2 monolayers was used as a measure of intestinal permeating activity. Melittin (3 μM)

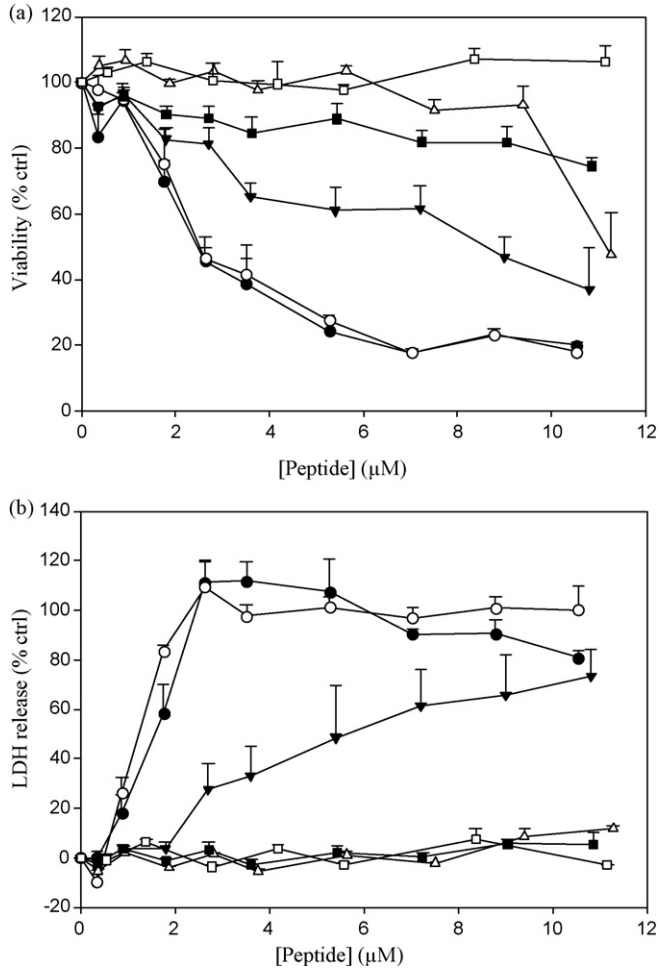

Fig. 1. Effect of structural modification of melittin on cytotoxicity in Caco-2 cells after 24-h incubation. Viability and membrane integrity were measured by (a) MTT assay and (b) LDH assay, respectively. Each value represents the mean ± SEM (triplicate, $n = 3$). Symbols represent: (●) native melittin; (○) synthetic melittin; (▼) PA-1; (△) PA-2; (■) PA-3; (□) PA-4.

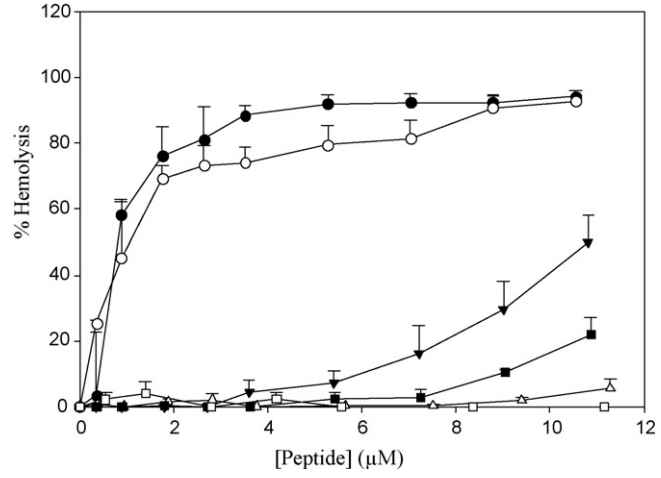

Fig. 2. Hemolytic activity of melittin and four structural analogues in defibrinated sheep erythrocytes after 1 h incubation. Hemolytic positive and negative controls were Triton X-100 (0.1%, v/v) and saline (0.9%). Each value represents the mean ± SEM (triplicate, $n = 3$). Symbols represent: (●) native melittin; (○) synthetic melittin; (▼) PA-1; (△) PA-2; (■) PA-3; (□) PA-4.

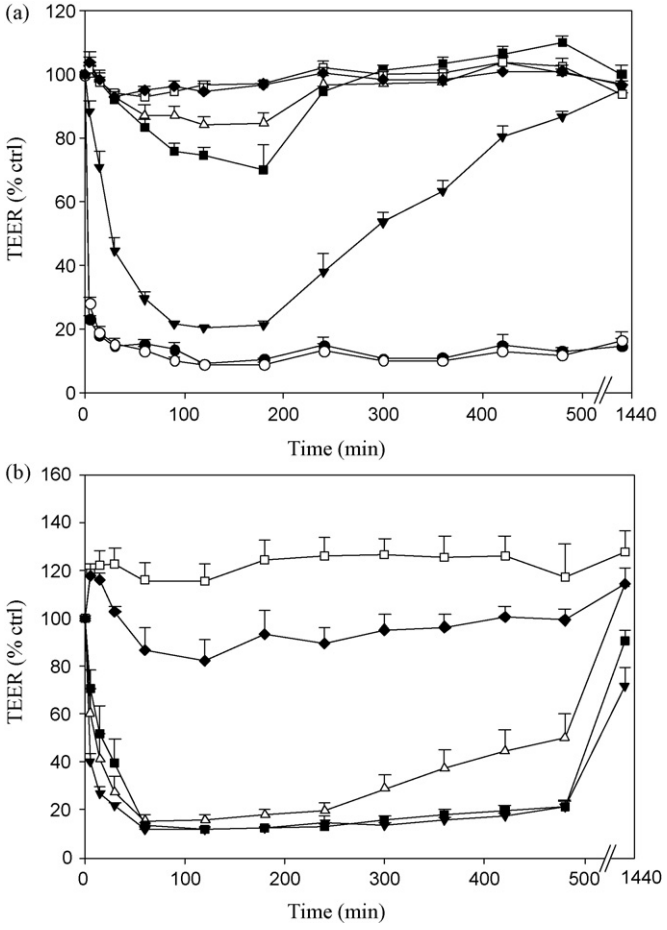

Fig. 3. Effect of structural modification of melittin on TEER in Caco-2 monolayers over 24 h. TEER was periodically measured in Caco-2 monolayers treated with 3 μM (a) or 10 μM (b) of each test peptide and expressed as percent change over time (min). Each value represents the mean ± SEM (triplicate, $n = 3$). Symbols denote: (♦) media control; (●) native melittin; (○) synthetic melittin; (▼) PA-1; (△) PA-2; (■) PA-3; (□) PA-4.

induced a rapid drop in TEER across Caco-2 monolayers which did not recover over 24 h (Fig. 3a). The drop in TEER was accompanied by a 21-fold increase in the apical-to-basolateral flux of FD4 over 3 h ($P < 0.0001$) (Fig. 4). The structural modifications of melittin how-

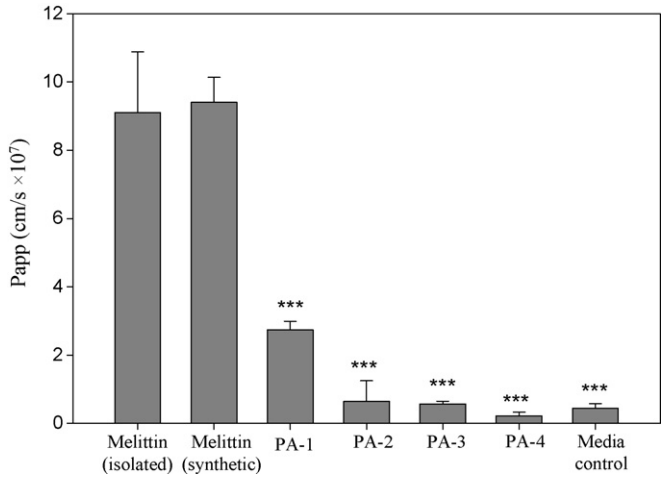

Fig. 4. Impact of structural modification of melittin (3 μM) on P_{app} (cm/s) of FD4 across Caco-2 monolayers over 3 h. Each value represents the mean ± SEM (triplicate, $n = 3$) (***$P < 0.0001$ compared with synthetic and/or native melittin).

ever, reduced the permeation enhancement capacity of the peptide in Caco-2 monolayers (Figs. 3 and 4). PA-1 led to a transient drop in TEER that recovered over 24 h (Fig. 3). Even at the higher concentration of 10 μM, the actions of PA-1 on TEER partially recovered over 24 h (Fig. 3b). Compared with untreated control, PA-1 led to a 6-fold increase in P_{app} of FD4 ($P < 0.05$). More importantly, its effects on the P_{app} were 3.5 times less than native melittin ($P < 0.0001$) (Fig. 4). PA-2, PA3 and PA4 did not decrease TEER across Caco-2 monolayers at 3 μM, a concentration at which the native peptide irreversibly reduced TEER (Fig. 3a). At the higher concentration of 10 μM, PA-2 and PA3 transiently reduced TEER, but PA-4 had no effect (Fig. 3b). Compared with control, PA-2, PA-3 and PA-4 had no effect on the P_{app} of FD4 (Fig. 4). Furthermore, these three modifications had 14-, 16- and 44-fold lower promoting action on FD4 flux than the native peptide, respectively ($P < 0.0001$) (Fig. 4).

RP-HPLC retention times are often used to measure α-helical peptide amphipathicity and hydrophobicity (Blondelle and Houghten, 1991; Kim et al., 2005). In general, the greater percentage of mobile phase that is required to elute a peptide from a hydrophobic stationary phase is indicative of greater hydrophobicity and amphipathicity. With this in mind, we measured the percentage of organic solvent required to elute melittin and the four peptide analogues from a C18 reverse phase column and calculated their observed hydrophobicity as a fraction of the native peptide. The elution order was ranked in order of native melittin > PA-1 > PA-2 > PA-3 > PA-4 (Table 1), indicating the native peptide was the most lipophilic of the group, while PA-4 was the most hydrophilic. This order was therefore consistent with the order that the analogues reduced cell viability (Fig. 1a), membrane integrity (Figs. 1b and 2), and reduced epithelial permeability (Figs. 3 and 4).

4. Discussion

The intestinal absorption promoting action of melittin has been demonstrated in intestinal cell cultures, isolated colonic mucosae and in rat instillations and perfusions (Liu et al., 1999; Maher et al., 2007a,b, 2009a, 2009c; Maher and McClean, 2006; Schasteen et al., 1992). Since inhibition of calmodulin attenuated the effect of melittin on TEER and P_{app} of mannitol in isolated rat colonic mucosae (Maher et al., 2007a), it was thought that the promoter could act, in part, through the paracellular pathway. However, the peptide's widely reported non-cell selective cytolytic actions also suggest it could also act through transcellular perturbation. With this in mind, we made selected modifications to melittin to reduce its capacity to cause transcellular perturbation and monitored the effect of these changes on its epithelial permeation enhancement action. It was hoped that such modification would reduce the cytotoxicity of the peptide while preserving its paracellular permeation-promoting activity.

A number of eloquent studies examining the interaction of melittin with biological membranes have shown that hydrophobicity, amphipathicity, and helicity are important structural elements in the peptide's perturbation of zwitterionic phospholipid bilayers (e.g. Asthana et al., 2004; Blondelle and Houghten, 1991; Oren and Shai, 1997; Zhu et al., 2007). In general, decreasing lipophilicity, amphipathicity as well as disrupting the α-helical structure decreases the cytolytic action of melittin towards these mammalian cell plasma membranes. The PA with deletion of hydrophobic alanine at position 15 (denoted PA-1) had 8-fold lower hemolytic activity than native melittin (Blondelle and Houghten, 1991). In the same study, deletion of tryptophan at position 19 (PA-2) reduced the hemolytic activity of melittin by 124-fold. These modifications altered the hydrophobic and amphipathic character of melittin, which is known to be involved in the cytolytic potential of α-helical peptides. Indeed, the omission of any hydrophobic amino acid in the α-helix alters the orientation of other amino acids in the peptide, which can subsequently disrupt its interaction with other peptide monomers and the plasma membrane of susceptible cells (Blondelle and Houghten, 1991; Zhu et al., 2007). The PA-3 was an analogue with substitution of leucine with alanine at positions 6 and 13 which disrupts the peptides leucine zipper motif (Asthana et al., 2004). The omission of heptadic leucine dramatically alters the cytolytic action of the peptide. These leucine substitutions significantly reduced the hemolytic action of the peptide without any effect on antimicrobial activity (Asthana et al., 2004; Zhu et al., 2007). Mutation of the leucine zipper motif reduced the α-helical content from 75% to 8%, and also disturbed the tetrameric helical assembly, which is critical to the cytolytic action of the peptide on zwitterionic membranes. Mutation of even leucine at position-13 alone led to a 254-fold reduction in hemolytic activity of melittin (Blondelle and Houghten, 1991). Disruption of the leucine zipper motif also reduced melittin cytotoxicity in mouse fibroblasts and human cervical cells (Zhu et al., 2007). The final modification made to melittin (PA-4) was omission of the 11-amino acid hydrophobic N-terminus sequence, yielding the 15-residue hydrophilic C-terminus sequence. PA-4 has previously been shown to have 300-fold less hemolytic activity than the native peptide, and it was the least toxic analogue tested in the current study (Subbalakshmi et al., 1999; Yan et al., 2003). Similar to PA-1-to-PA-3, the reduced capacity of PA-4 to form α-helical and multimeric structures abrogated the cytolytic action of the peptide in zwitterionic plasma membranes.

In light of the previous studies that illustrate the impact of structural modification on the hemolytic activity of melittin, reduced cytotoxicity in intestinal epithelial cells in the current study was anticipated. Each of the peptide analogues had significantly less cytotoxicity than the native peptide. Furthermore, PA-4 which had lowest reported hemolytic activity (Subbalakshmi et al., 1999), also had the lowest cytotoxicity in Caco-2 cells. The reduction in cell viability as measured by MTT assay correlated well with the reduction in membrane integrity in LDH assay in Caco-2. Compared with previous studies, PA-1 had greater hemolytic action than PA-2, while in this study PA-1 also had greater cytotoxicity in Caco-2 cells (Blondelle and Houghten, 1991). The order of hemolytic activity for each peptide analogue was similar to previously published reports and the decrease in hemolytic activity followed a reduction in amphipathicity as measured by RP-HPLC.

Having made the desired structural modifications to melittin that reduced the peptide's cytotoxicity in intestinal epithelial cells, we examined whether the capacity of the peptide to form defects in the plasma membrane were involved in permeation promotion. Unfortunately, all of the selected changes to melittin reduced the epithelial permeation-enhancing action of melittin in Caco-2 monolayers. In fact, the promoting action of the peptide was inversely correlated with reduction in cytotoxicity. Furthermore, reduced cytotoxicity and intestinal permeation by melittin was also correlated with lower retention times in RP-HPLC analysis, a marker of reduced amphipathicity and hydrophobicity (Blondelle and Houghten, 1991; Kim et al., 2005). Of the peptide analogues screened, PA-1 retained a degree of cytotoxicity and a capacity to increase monolayer permeability to FD4, while PA-2-to-PA-4 had considerably less cytotoxicity and no effect on FD4 flux across Caco-2 cells. This study would therefore suggest that the promoting action of melittin is intimately related to its capacity to perforate the plasma membrane. Intestinal absorption promoters can increase permeability across epithelia by either opening TJs at the paracellular pathway or by destabilizing the plasma membrane therefore decreasing resistance to transcellular permeation. Both the toroidal pore and carpet models that describe the interaction of melittin with model membranes indicate the peptide has the capacity to cause transcellular perturbation (Fig. 5) (Oren and Shai,

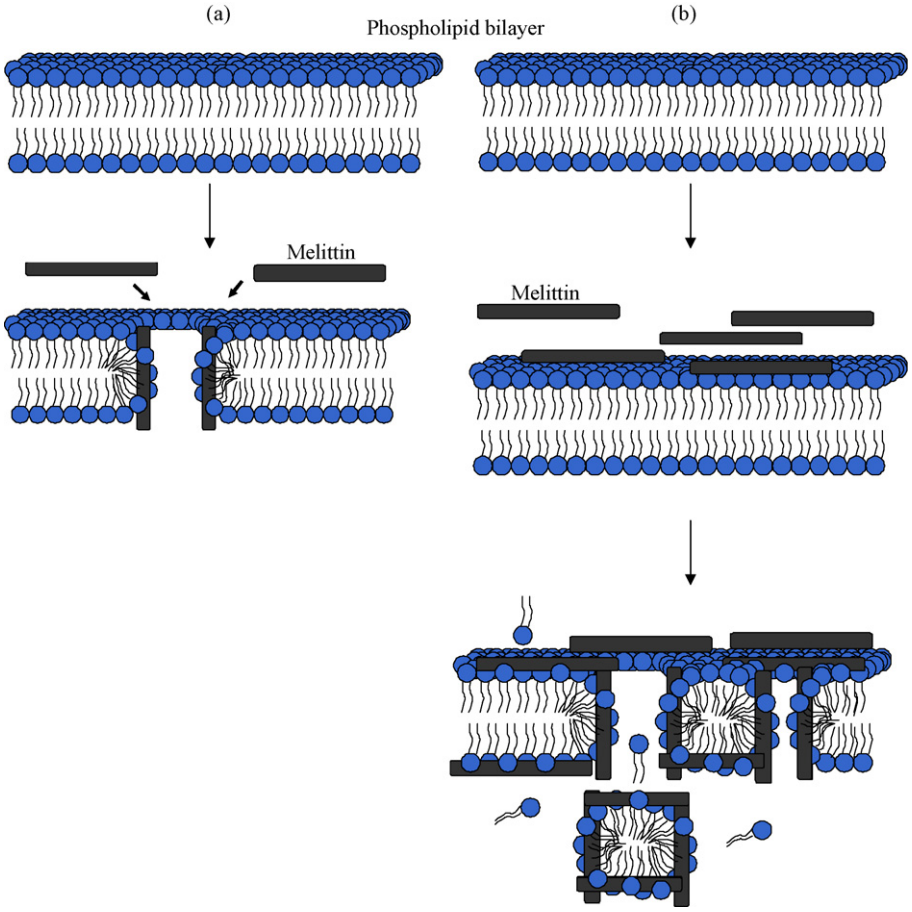

Fig. 5. (a) Toroidal pore and (b) carpet models proposed to describe the perturbation of susceptible plasma membranes by melittin. In the toroidal pore model, the peptide helices align perpendicular to the lipid bilayer and upon insertion the bilayer bends continuously through the pore so that the water core is lined by peptides and the phospholipid head groups (Yang et al., 2001). In the carpet model peptide monomers cover the bilayer and at a threshold concentration disintegrate it like a detergent with the possible formation of micelles (Oren and Shai, 1997). These models provide evidence that melittin can destabilize and solubilise lipid bilayers. These models, along with the data in the current study showing the concurrent reduction in cytolytic activity and permeability enhancing action following chemical modification, suggest melittin could increase epithelial permeability in part through the transcellular pathway.

1997; Yang et al., 2001). Cellular necrosis caused by melittin in gastrointestinal goblet cells provides further evidence that the actions of the peptide could be mediated by transcellular perturbation (Maher and McClean, 2008). To add further weight to the hypothesis that melittin acts primarily by transcellular perturbation we synthesised the all-D isomer to examine whether the promoter has receptor mediated enhancement that is isomer specific. Synthesis of peptides with all D-amino acids can, in some cases, effect their interaction with receptor and/or enzyme activity. In the current study, the all-D isomer behaved in a similar fashion to the native peptide in cytotoxicity assays and permeation assays (data not shown) suggesting that either the transcellular mode of action is responsible for the promoting action of the peptide or that the receptor that it targets is isomer independent. The D-isomer had similar hemolytic action to the native peptide in previous assay which indicates that the all D-melittin actions on cell perturbation are not disrupted (Wade et al., 1990). However, it is also worth noting that all D-melittin binds to calmodulin similarly to the native peptide (Fisher et al., 1994), and since calmodulin inhibition attenuates the promoting action of melittin in isolated rat colonic mucosae (Maher et al., 2007a), the D-isomer could retain paracellular promoting action. The all D-isomer of melittin could be a more viable option for promoting drug absorption in the protease rich small intestine, since D-peptide analogues of melittin are resistance to protease activity (Wade et al., 1990).

In the case of transcellular enhancement by mild surfactants like sodium caprate (C_{10}), a potential drawback may be membrane solubilisation at the high concentrations necessary for oral drug absorption (Maher et al., 2009b), which is also a distinct possibility with melittin since detergent-like actions are described in the carpet model (Fig. 5b). However, despite this transcellular mode of action in vivo, the clinical candidate promoter C_{10} has been licensed for use in a rectal suppository and is in clinical trials to promote the oral absorption of oligonucleotides (Tillman et al., 2008), bisphosphonates (Leonard et al., 2006) and other poorly absorbed Class III drugs (Amory et al., 2009; Leonard et al., 2006). Furthermore, a degree of transcellular promotion may be necessary for effective oral drug delivery since it is possible that TJ openings in leaky intestinal epithelia could be limited in capacity. Nevertheless, the fact that reducing melittin cytotoxicity impinges on its capacity to increase FD4 permeation could hamper the peptide's potential as a drug delivery agent.

In conclusion, the non-cell selective cytolytic action of melittin is a prerequisite for its intestinal permeating action, suggesting the promoter acts predominantly through the transcellular pathway. Structural changes to melittin that reduced the peptide's cytotoxicity in intestinal epithelial cells abolished the peptides capacity to improve flux of paracellular marker solutes. The close relationship between cytotoxicity and permeation enhancement demonstrated here could limit the potential of melittin as a drug delivery agent.

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