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Melittin exhibits necrotic cytotoxicity in gastrointestinal cells which is attenuated by cholesterol

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ARTICLE INFO

Article history:
Received 6 September 2007
Accepted 18 October 2007

Keywords:
Melittin
Necrosis
Paracellular permeability enhancer
Cholesterol
Cytotoxicity
Antimicrobial peptide
Intestinal epithelial cells

ABSTRACT

Melittin, a cationic antimicrobial peptide isolated from the venom of *Apis mellifera*, has shown potential as a permeability enhancer, transiently increasing intestinal permeability and enhancing the absorption of paracellular markers. Although it is cytotoxic to eukaryotic cells, its cytotoxicity is significantly lower in polarised epithelia compared to non-polarised cells. The aim of this study was to explore the mechanism of melittin cytotoxicity in gastrointestinal cells and to determine whether cytotoxicity was mediated by a necrotic or an apoptotic pathway. The role of cholesterol in melittin cytotoxicity was also examined. Using four distinct assays for apoptosis, phosphatidylserine translocation, caspase activation, DNA ladder formation and cell cycle analysis, no evidence of apoptotic pathway for cell death was observed with any of these approaches. It can therefore be concluded that cytotoxicity was likely to be mediated by necrosis in gastrointestinal epithelial cells. However, at low concentrations of melittin (<1 μM), BRDU uptake was enhanced, demonstrating proliferative effects of melittin at sub-lethal concentrations. Furthermore, melittin cytotoxicity was further enhanced by depletion of cholesterol, using methyl-β-cyclodextrin, indicating that cholesterol depleting agents could be contradictory to its potential as an enhancer. Overall, although melittin appears to stimulate necrosis, with careful dosage selection the peptide could be considered for the oral delivery of poorly bioavailable drugs.
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1. Introduction

The barrier function of the GI tract, controlled by tight junctions, limits the absorption of macromolecules and water miscible solutes into the body [1]. The delivery of peptides and proteins to sites along the GI tract without digestion has been successful, however, poor absorption restricts biotech preparations reaching efficacious concentrations. Invasive delivery methods, such as intravenous and subcutaneous, can lead to decreased patient compliance, particularly with chronic therapies [2]. One alternative is the co-administration of a therapeutic preparation with a molecule that enhances oral bioavailability through transient disruption of tight junctions [3].

Melittin is a cationic and amphipathic antimicrobial peptide isolated from the venom of *Apis mellifera* (honey bee) [4]. At sub-toxic concentrations, melittin is a potent gastrointestinal absorption enhancer of class III type molecules *in vitro*. Melittin induced a concentration-dependant decrease of transepithelial electrical resistance (TER) in gastrointestinal models, which was reversible [5–7]. This

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doi:10.1016/j.bcp.2007.10.029

was accompanied by an increase in apical-basolateral flux of a range of fluorescent dextrans, depolymerisation of actin and a decrease in tight junction associated proteins [7]. The absorption enhancing properties of melittin were also shown across sections of rat colonic mucosae, and the goblet cell culture model, HT29-MTX-E12 with no loss of viability [6].

The diverse activities of melittin have recently been comprehensively reviewed [8]. Melittin disrupts the plasma membrane of susceptible bacterial cells through the formation of transmembrane pores, detergent-like damage to the plasma membrane, and/or damage to respiratory metabolism with a breakdown of electrochemical gradients [9,10]. In contrast to other antimicrobial peptides, melittin has demonstrated cytotoxicity in epithelial (intestinal [5]) and haematopoietic cells (thymocytes [11] and erythrocytes [12]) at, or around, published antimicrobial concentrations [5]. In addition, melittin also interacts with a broad range of metabolic functions in mammalian cells *in vitro* [11,13–19]. While the antimicrobial activity of melittin is similar to classic antibiotics, the lack of selective toxicity limits the use of this peptide as an anti-infective. However, the use of melittin as a permeation enhancer has a broader therapeutic index, particularly in polarised monolayers and may be useful in oral formulations [6].

Although melittin cytotoxicity was not apparent in polarised monolayers or in rat colonic mucosae at permeability enhancing concentrations, a loss of viability was observed at high concentrations across rat colonic epithelium [6] and in non-polarised, log growing intestinal epithelial cells [5]. Understanding the mechanism by which melittin leads to cell death in gastrointestinal cells is important in determining its potential toxicity. Apoptosis is a physiological mode of cell death wherein the cell takes part in its controlled death. The process of apoptosis regularly occurs *in vivo* and is part of the regular replenishment of the gastrointestinal epithelium. A drug which results in necrosis, an accidental cell death, can result in cell lysis, and the release of inflammatory mediators. In the GI tract, necrosis can lead to the uncontrolled disruption of intestinal barrier function, enhancing bacterial translocation and sepsis, an undesirable property. There is no one specific approach to differentiate between apoptosis and necrosis, therefore the mechanism of cytotoxicity was assessed by four distinct methods: alterations in phospholipids location, apoptotic caspase activity and traditional DNA ladder formation, cell cycle alterations.

Furthermore, the apparent stimulatory effects of sub-toxic concentrations of melittin were examined in HT29 and Caco-2 cells through DNA synthesis assays. Previously, melittin has been shown to enhance viability of gastrointestinal cells in MTT assays, and thymocytes independently. The effect sub-lethal concentrations of melittin on DNA synthesis was evaluated. Finally, since an increasing number of drugs, and particularly nutraceuticals, have been shown to reduce cholesterol in the body [20,21], the effect of reducing cholesterol on the cytotoxicity of melittin was investigated as a potential contraindication to the use of this peptide as a permeation enhancer.

This study provides information on the interaction of melittin with target cells, which will aid in the design of absorption enhancing analogues with greater stability, and reduced cytotoxicity.

2. Materials and methods

2.1. *Reagents*

Melittin (Serva Inc., Germany) was purchased as a lyophilised powder and exhibited purity in excess of 98% by μRPC and SDS PAGE. The peptide was reconstituted in sterile ultra pure water and frozen in single-use aliquots. The cholesterol absorbing molecule, methyl-β-cyclodextrin (MβD), was used according to reported concentrations [22]. All other reagents were purchased from Sigma-Ireland unless otherwise stated.

2.2. *Tissue culture*

Caco-2 cells (P50-70, ECACC, UK) were grown in Dulbecco's modified eagles medium (DMEM) supplemented with 2 mM L-glutamine, 1% (v/v) non-essential amino acids and 10% (v/v) foetal bovine serum (FBS). HT29 cells (P141-155, ECACC, UK) were grown in McCoy's 5a medium with 2 mM L-glutamine and 10% (v/v) FBS. Both cell lines were grown at 37 °C in a humidified atmosphere with 5% CO_2. Viability of test cells prior to use exceeded 99%, as determined by exclusion of the vital dye, trypan blue.

2.3. *Phosphatidylserine translocation*

During the early stages of apoptosis phosphatidylserine is translocated from the inner leaflet of the plasma membrane to the extracellular surface without altering membrane integrity [23]. Externalisation of phosphatidylserine was detected with annexin-FITC, counterstaining for damage to membrane integrity (necrosis) with propidium iodide (PI). For flow cytometric analysis, HT29 cells seeded at a density of 1×10^6 cells were treated with melittin for a range of time intervals over 24 h. Following incubation, cells were recovered from the substrata by scraping, centrifuged at $200 \times g$ for 5 min, and treated with annexin-FITC and PI, according to the manufacturer's instructions (Annexin-FITC/PI Roche, UK). Cell samples were analysed using a FACScalibur™ flow cytometer (BD, UK), equipped with an argon-ion laser tuned to 488 nm and Cell Quest™ software. Histogram plots of FL1 and FL2 signal area versus cell number, corresponding to FITC and PI fluorescence respectively were saved for statistical analysis. For microscopic analysis, cells were grown on sterile coverslips (1×10^6 cells), treated with test peptide and stained with annexin-FITC/PI (Roche, UK). Coverslips were then mounted on glass slides with 10% (v/v) glycerol and examined by fluorescence microscopy (Nikon Eclipse 80i, Nikon UK).

2.4. *Total caspase activity*

The upregulation of cysteine-dependant aspartate-specific proteases (caspases) was examined with the universal caspase substrate (L-asp)$_2$ rhodamine (D_2R) [24] by flow cytometry. HT29 cells were seeded at a density of 5×10^5 cells, treated with test compound for 24 h, harvested and treated with caspase substrate (caspSCREEN, Alexis, UK). The cell density was adjusted to 1×10^5 cells, permeabilised with 10mM dithioreitol (DTT) followed by the addition of D_2R substrate for 30 min at 37 °C in the dark. Cells were analysed using a

FACScalibur™ flow cytometer, preparing histogram plots and data analysed using Cell Quest™ software.

2.5. DNA fragmentation

Chromosomal DNA from test cells (1×10^6 cells) treated with melittin or vincristine or from untreated cells was isolated using the Apoptotic DNA-Ladder Kit (Roche, UK). Floating cells were pooled with trypsinised adherent cells and collected by centrifugation. Cells were lysed by addition of an equal volume of lysis buffer containing 6 M guanidine–HCl, 10 mM urea, 10 mM Tris–HCl, and 20% (w/v) triton X-100 (pH 4.4). Cell lysates were applied to a glass fiber column with high affinity for DNA in the presence of chaotropic salts [25]. DNA was eluted under centrifugation with prewarmed Tris (10 mM, pH 8.5, 70 °C) and analysed by gel electrophoresis (1% (w/v) agarose), staining with ethidium bromide and visualising nucleic acids with a Transluminator (Pharmacia, UK).

2.6. Cell cycle analysis

HT29 cells (1×10^6 cells) treated with a range of peptide concentrations for 24 h were washed with phosphate buffered saline (PBS), pH 7.4 and harvested by trypsinisation. Cells were recovered by centrifugation at $1500 \times g$ for 10 min. The effect of each peptide on cell cycle population distribution was examined using flow cytometry [26]. Cell suspensions were permeabilised by the vigorous addition of nine volumes of ice cold 70% (v/v) ethanol and stored at −20 °C for a minimum of 24 h. The cells were then rinsed with PBS, to remove residual ethanol and resuspended in 20 μg/ml propidium iodide (PI, Molecular Probes, UK), and 0.2mg/ml RNaseA in PBS and incubated in the dark at ambient temperature for 30 min. Cell cycle distributions were examined on a FACScalibur™ flow cytometer using Cell Quest software™ (Becton Dickinson, UK).

2.7. DNA synthesis studies

The effect of sub-toxic concentrations of melittin on DNA synthesis were examined with a colorimetric 5-bromo-2-deoxyuridine (BRDU) incorporation ELISA [27]. Briefly, HT29 or Caco-2 cells were treated with melittin for 24 h followed by the addition of 10 μM BRDU for 5 h at 37 °C. Following the removal of melittin and BRDU, cells were rinsed, permeabilised and fixed. Incorporated BRDU was detected by ELISA with a peroxidase conjugated anti-BRDU monoclonal antibody with colourimetric (TMB) detection. Results were expressed as BRDU incorporation as percentage control.

2.8. Measurement of Caco-2 cell cholesterol

Cholesterol from Caco-2 or HT29 cells (5×10^5 cells) was isolated as previously described [22]. Cells with and without 2 h pre-treatment with MβD (5 mM) were rinsed with ice cold PBS (pH 7.4) and scraped into lysis buffer (1% (v/v)) Triton X-100, 150 mM NaCl, 2 mM EDTA, 25 mM HEPES (pH 7.4), 2 mM sodium orthovanadate, and 1× Complete® protease inhibitor). After 30 min on ice, samples were sonicated for three bursts (3 s each) at 50% amplitude capacity (Microson® XL2020, USA). Samples were then assayed for cholesterol using the Amplex® red cholesterol detection assay (Invitrogen, UK) using a Perkin-Elmer™ spectrofluorimeter at Ex/Em of 530/585nm ($n = 3$, replicates of 6). Total cellular cholesterol was normalised to total cellular protein, and expressed as percentage of untreated control.

2.9. Cytotoxicity assay

Cytotoxicity of melittin on Caco-2 and HT29 cells with and without MβD pre-treatment was assessed using the MTT (methylthiazolydiphenyl-tetrazolium bromide) conversion assay as previously described [5] ($n = 3$, replicates of 5).

2.10. Statistical analysis

All experiments were carried out on a minimum of three independent occasions unless otherwise stated. All figures are expressed as the mean ± S.E.M. Statistical deductions were

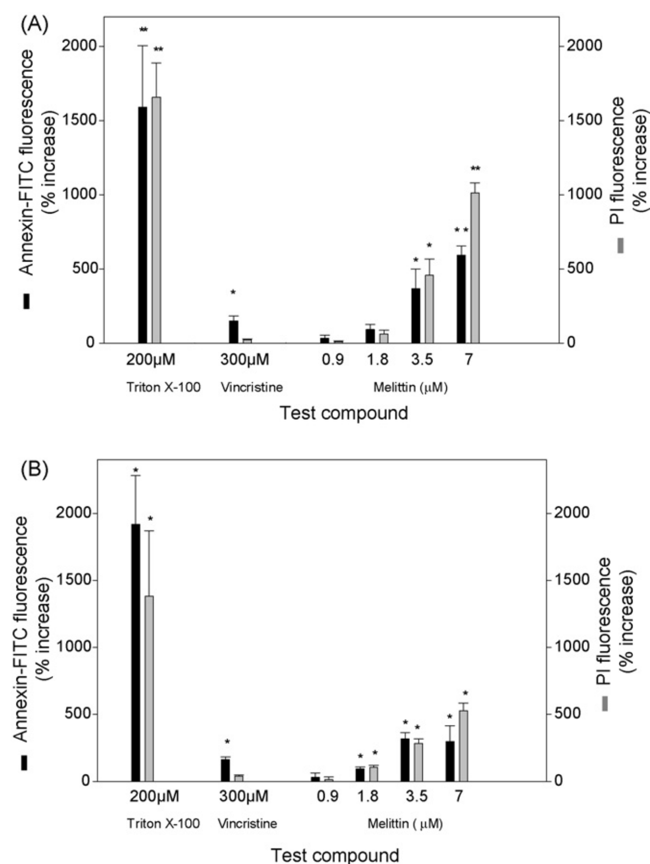


Fig. 1 – Flow cytometry analysis of the mechanism of melittin cytotoxicity through phosphatidylserine translocation, an early apoptotic marker in HT29 cells following 4 h (A) and 7 h (B) treatment. Vincristine (300 μM) and Triton X-100 (200 μM) were used as positive controls for apoptosis and necrosis, respectively. Each experiment was carried out in duplicate, on at least four independent occasions. Bars indicate fluorescence intensity as a percentage of untreated control ± S.E.M. Statistically significant differences relative to controls are denoted with *$P < 0.05$ and **$P < 0.01$.

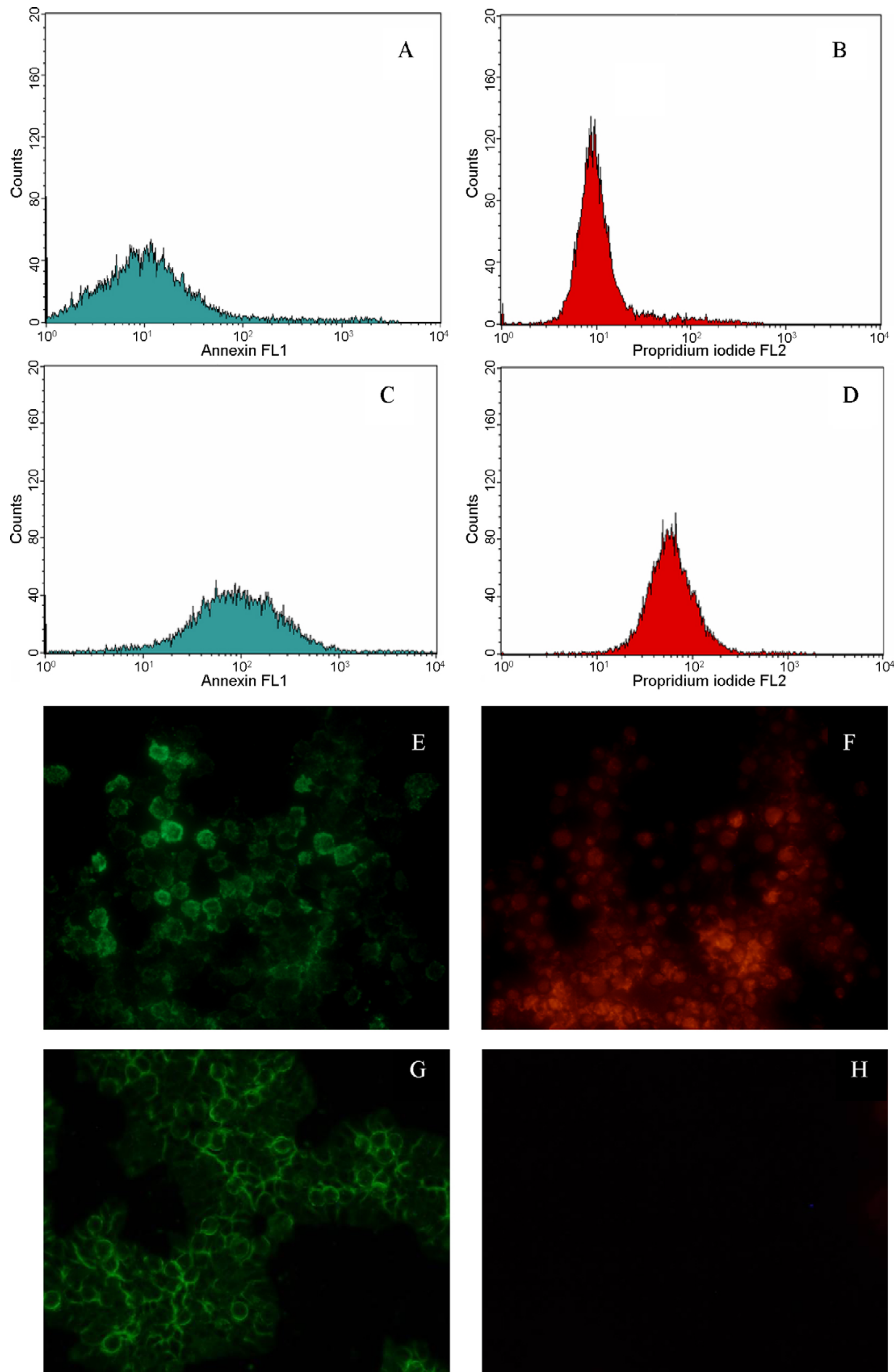


Fig. 2 – Representative data on the effect of melittin on plasma membrane phosphatidylserine localisation. Histogram plots showing media-treated control HT29 cells analysed for annexin-FITC (A) and PI (B) localisation; HT29 cells treated with 3.5 μM melittin analysed for annexin-FITC (C) and PI (D) localisation. Fluorescence micrographs (40×) of HT29 cells treated with melittin and stained with annexin-FITC (E) and PI (F) or treated with 300 μM vincristine and stained with annexin-FITC (G) and PI (H).

carried out by unpaired Student's *t*-test or one-way ANOVA with Minitab® software.

3. Results

3.1. Phosphatidylserine translocation

We have previously shown that the IC_{50} of melittin in Caco-2 cells, as determined by MTT assay, was 1.8 μM [5], therefore the concentrations of melittin chosen in these studies were 0.5, 1.0 and 2.0 times this IC_{50} value.

To assess whether melittin induced apoptotic or necrotic cell death in gastrointestinal cells, the translocation of phosphatidylserine to the extracellular leaflet of the plasma membrane, a marker of early apoptosis, was detected with the phosphatidylserine-binding protein, annexin, conjugated to the fluorophor FITC. Since necrotic cell death leads to a loss of plasma membrane integrity, this can result in an increase in annexin-FITC binding to phosphatidylserine through internalisation. The counter stain, PI, which is only taken up by cells which have lost integrity, was used to detect necrosis. HT29 cells treated with a range of melittin concentrations increased both annexin-FITC and PI population fluorescence in a concentration-dependant fashion on histogram plots, indicative of necrosis (Fig. 1). The increase in annexin FITC and PI in HT29 cells treated with melittin observed at 4 h (Fig. 1A) was unchanged at 7 h (Fig. 1B). In the same experiment, dilute Triton X-100 also increased annexin-FITC and PI, albeit with greater increases in fluorescence than those induced by melittin ($P < 0.01$). The apoptotic positive control, vincristine (300 μM), significantly increased annexin-FITC fluorescence ($P < 0.05$) without altering PI fluorescence at both 4 and 7 h (Fig. 1).

The melittin-induced increase in annexin FITC and PI in the cell population, shown on histogram plots (Fig. 2A–D), were also visualised by fluorescence microscopy (Fig. 2E and F). HT29 cells treated with melittin (3.2 μM) showed an increased intracellular fluorescence of both annexin-FITC and PI with localisation of annexin-FITC near the plasma membrane and PI intracellularly. Pro-apoptotic concentrations of vincristine lead to strong localisation of annexin-FITC at the plasma membrane of HT29 cells (Fig. 2G), while no increase in PI fluorescence was noted for vincristine treated HT29 cells, under identical microscopic conditions (Fig. 2H).

3.2. Total caspase activity

To provide additional evidence of the mode of cytotoxicity of melittin in HT29 cells, the activity of apoptotic caspase enzymes was assessed after 24 h. The result presented in Fig. 3 shows that melittin did not initiate caspase enzymes in HT29 cells at concentrations close to, and over twice, the IC_{50} value. In contrast, HT29 cell populations treated with vincristine (300 μM) for 24 h lead to a significant shift in the rhodamine fluorescence ($P < 0.01$) illustrating that caspase activation in HT29 cells could result in cleavage of D_2R, releasing rhodamine with subsequent fluorescence.

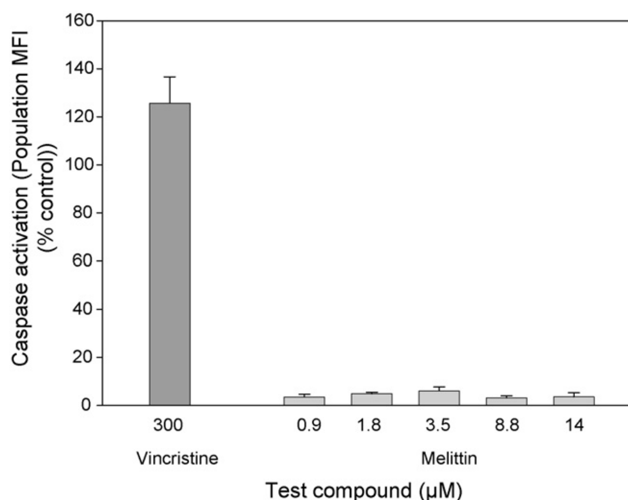


Fig. 3 – Effect of melittin (0.9–14 μM) or vincristine on the induction of pro-apoptotic caspase enzymes in HT29 cells using D_2R with FACS analysis. Results are expressed as mean % untreated control ± S.E.M., from five independent experiments, carried out in duplicate.

3.3. DNA fragmentation

Digestion of internucleosomal DNA by caspase-activated-endonucleases is a late stage event in apoptotic cell death. Isolated DNA from HT29 cells treated with melittin or vincristine was analysed by gel electrophoresis. Melittin

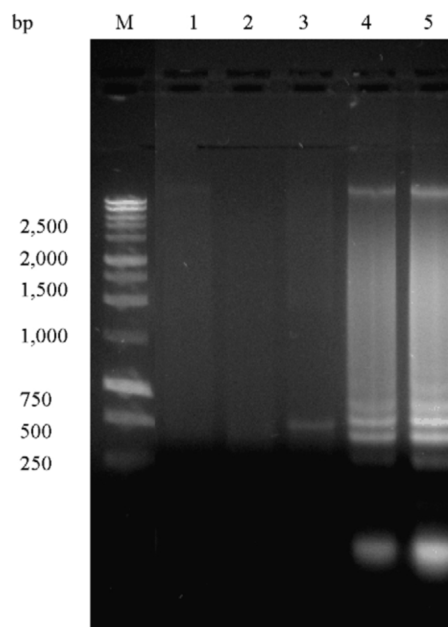


Fig. 4 – Effect of melittin and vincristine on DNA fragmentation in HT29 cells treated for 24 h and analysed on 1.5% agarose gels. Lane M contains 150–2000 bp ladder; lanes 1–5 contain DNA from HT29 cells treated with melittin (lanes 1–3: 1.8 μM, 3.5 μM, 7 μM, respectively; or vincristine (lanes 4 and 5: 150 and 300 μM, respectively). Results are representative of two independent experiments.

treatment (0.87–5.3 μM) did not result in the fragmentation of genomic DNA in HT29 cells after 24 h (Fig. 4) nor was fragmentation observed at earlier times of 4 and 7 h (data not shown). In contrast, the positive control, vincristine, was capable of inducing apoptosis in HT29 cells, forming 180 bp multimeric bands (Fig. 4).

3.4. *Cell cycle analysis*

The effect of melittin on cell cycle events was examined for evidence of apoptotic cell death. HT29 cells were exposed to melittin (1.8–5.3 μM) for 24 h and the percentage of cells entering each phase of the cell cycle was examined on

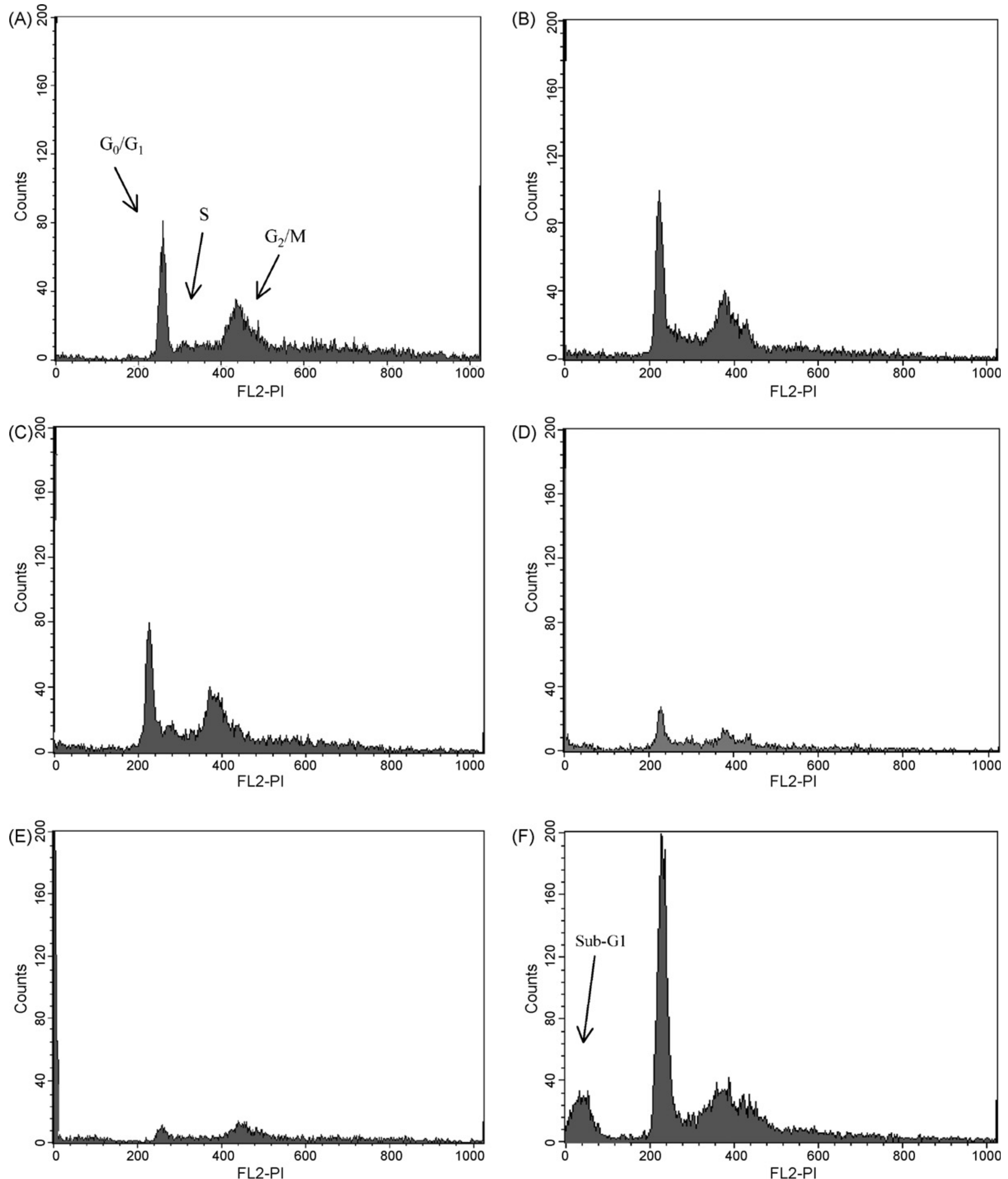


Fig. 5 – Effect of melittin on cell cycle progression in HT29 cells following 24 h treatment. Cell cycle distributions of permeabilised and PI stained cells were determined with flow cytometry analysis. Panels correspond to media control (A), and 1.8 μM (B), 3.5 μM (C), 5.3 μM (D), and 7 μM (E) melittin. The appearance of sub-G_1 phase cells following treatment with the positive apoptotic control gossypol (10 μM) after 24 h is shown in panel (F). Histograms are representative of three independent experiments.

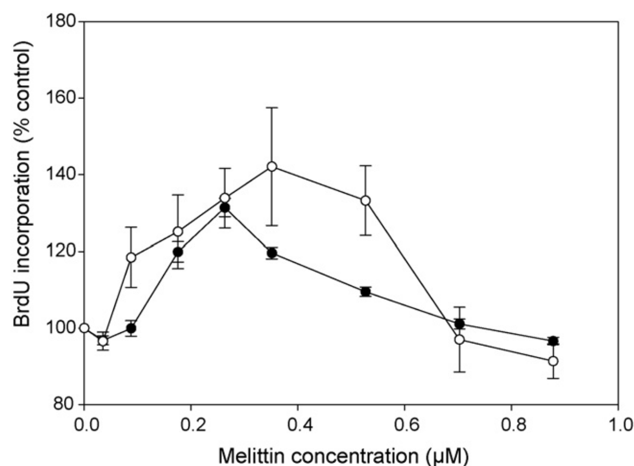
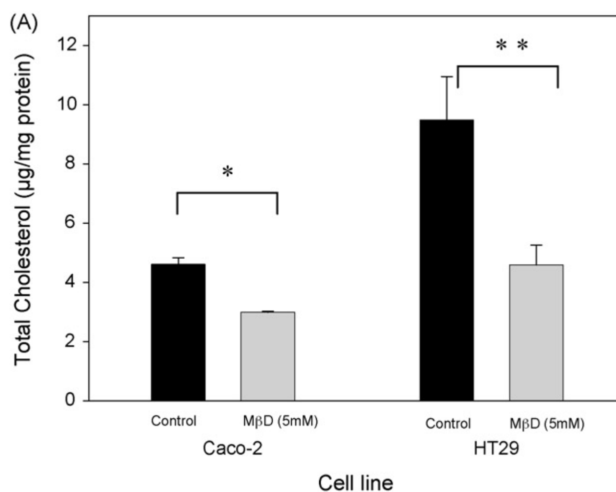


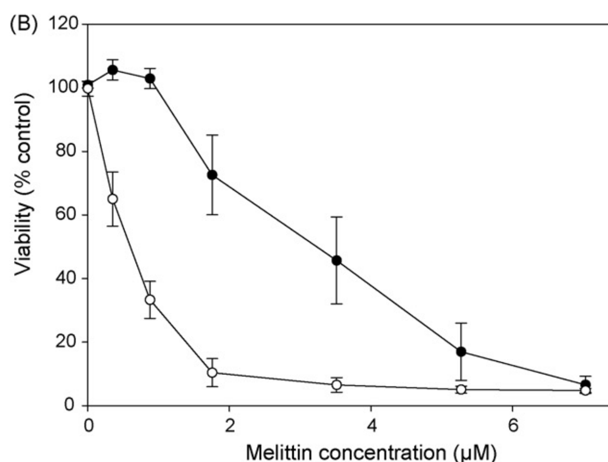
Fig. 6 – Effect of sub-lethal concentrations of melittin on DNA synthesis in HT29 (○) and Caco-2 (●) cells using the BrdU incorporation ELISA. Both HT29 and Caco-2 cells treated with melittin for 24 h resulted in a low-concentration dependant increase in BrdU incorporation while at higher concentrations of melittin (over 0.6 μM) stimulation of DNA synthesis was not seen. Bars indicate ± S.E.M. (*n* = 3, triplicate).

histogram plots (Fig. 5). Necrotic cell death results in the post-lytic fragmentation of nucleic acids into non-specific fragment lengths. Histogram plots showing HT29 cells treated with melittin (0.87–5.3 μM) did not initiate significant phase accumulation or the activation of endonucleases associated with sub-G_1 peak formation after 24 h treatment (Fig. 5). Although a significant concentration dependant decrease in DNA was seen, this was representative of necrotic disruption or secondary necrosis. The histogram profile of melittin was comparable to dilute concentrations of Triton X-100 (data not shown) and different to the apoptotic agent gossypol (Fig. 5F). Consistent with previous reports, pro-apoptotic concentrations of gossypol increase the population of cells resting in G_0/G_1 phase of the cell cycle and the appearance of sub-G_1 phase cells, following 24 h exposure [28,29]. Taken together, these results suggest that treatment of HT29 cells with melittin over 24 h results in necrotic cell death under the experimental conditions.

3.5. *DNA synthesis studies*

Sub-lethal concentrations of melittin have been reported to stimulate growth of gastrointestinal cells in MTT assay [5].The effect of melittin on DNA synthesis following 24 h treatment was assessed using a non-radiolabelled nucleotide incorporation assay in both Caco-2 and HT29 cells. Melittin induced a significant concentration-dependent stimulation of DNA synthesis of the order of 20–40% at low concentrations up to 0.4 μM in HT29 and 0.3 μM in Caco-2 cells. Above these concentrations (>0.6 μM) melittin reduced DNA synthesis consistent with the cytotoxicity data obtained in MTT assay (Fig. 6).





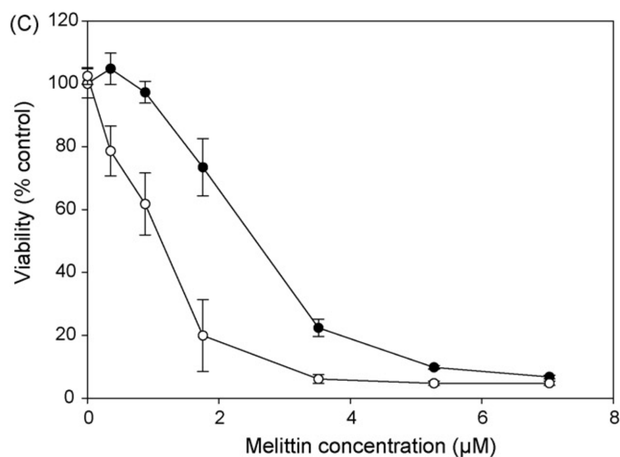


Fig. 7 – Effect of reduced cholesterol on cytotoxicity of melittin in HT29 and Caco-2 cells after 24 h using the MTT conversion assay. (A) Reduction of cellular cholesterol by 5 mM MβD in HT29 and Caco-2 cells, determined by Amplex red cholesterol assay (*n* = 3, replicates of 6); *P < 0.05; **P < 0.01. Cytotoxicity of melittin on HT29 (B) and Caco-2 (C) cells assayed by MTT assay; cells treated with melittin only (dark circles) or cells pre-treated with MβD for 2 h (open circles). Bar indicates ± S.E.M. (*n* = 3, replicates 5).

3.6. Cholesterol reduction assay

Methyl-β-cyclodextrin significantly reduces cholesterol in mammalian plasma membranes [22,30]. Total cell cholesterol was significantly reduced in both Caco-2 ($P < 0.05$) and HT29 ($P < 0.01$) cells when pre-treated with MβD (5 mM) for 2 h. Cholesterol was reduced by 34% in Caco-2 and 48% in HT29 cells within 2 h (Fig. 7A). Treatment with MβD alone did not reduce the viability of either cell line after 2 h, or following subsequent 24 h incubation with media alone, when tested in MTT assay (data not shown). The depletion of cellular cholesterol significantly enhanced the cytotoxicity of melittin in both cell lines (Fig. 7B and C). In HT29 cells depletion of cholesterol resulted in the IC_{50} for melittin being reduced over five-fold from 3.2 μM to 0.6 μM ($P < 0.01$) while in Caco-2 cells, the IC_{50} was reduced two-fold from 2.58 μM to 1.13 μM after 24 h treatment ($P < 0.05$).

4. Discussion

The cytotoxicity of melittin has previously been attributed to both necrotic and apoptotic cell death. The induction of apoptosis by melittin, has recently been reported in hepatic [31], and smooth muscle cells [32]. The venom of *Apis mellifera* that contains ∼50% (dry weight) melittin triggers apoptosis in breast [33], and lung [34] cells as well as synovial fibroblasts [35]. This induction of apoptosis was not directly attributed to melittin due to the extensive number of other molecules found in bee venom. In three independent reports, melittin was found to induce necrosis of rat thymocytes [11,36,37]. Melittin has also induced necrosis in murine skeletal muscle [38]. The necrotic-like lytic effects of melittin have also been shown in erythrocytes [12], lymphocytes [39], and lymphoblastoid cells [17] as well as in rat alveolar primary cultures [40].

There is no one definitive approach to distinguish between apoptosis and necrosis, however, in this study, no evidence of apoptosis was identified in any of the four distinct assays. Firstly, the dual increase in annexin-FITC and PI fluorescence induced by melittin indicated an alteration in the integrity of the plasma membrane which allowed absorption of annexin-FITC which binds to phosphatidylserine on the inner leaflet of the cell and PI intercalation with nucleic acids. In the apoptotic pathway, phosphatidylserine is translocated from the cytosolic to the extracellular membrane surface by a scramblase without any loss in membrane integrity and hence PI can not penetrate the cell [41]. The concentrations of melittin used in these annexin/PI assays ranged from those which disrupt membrane integrity of gastrointestinal cells (>1 μM) and erythrocytes to concentrations that do not alter membrane integrity (<1 μM), and it was demonstrated that sub-lytic concentrations of melittin were also not found to induce apoptosis.

In caspase activity assays, no apparent upregulation of the cysteine proteases was observed at cytotoxic concentrations of melittin. Furthermore, melittin did not lead to digestion of internucleosomal DNA, a caspase activated event, as demonstrated experimentally by a lack of 180–200 bp mono- and oligo-nucleosomal fragments on agarose gels. The activation of caspase-3 inactivates the inhibitor of CAD (caspase activated DNase) which translocates the nucleus resulting in fragmentation [42,43]. In addition, melittin did not statistically alter the progression of HT29 cells through phases of the cell cycle. The lack of sub-G_1 peak 24 h post-drug treatment further indicates melittin does not induce apoptotic cell death [44]. Although a concentration dependant decrease in DNA was seen in HT29 cells at 24 h, this is likely to be due to non-specific digestion of DNA often attributed to necrosis [44–46]. Apoptosis has been observed in the absence of caspase activation or in the absence of DNA ladder formation. In addition, the appearance of a sub-G_1 peak is not exclusively definitive of apoptosis. However, the consistent lack of any of these classic apoptotic markers in the four different assays strongly suggests that cytotoxicity in gastrointestinal cells is not attributable to the machinery involved in cell division and ultimately attributable to apoptotic cell death.

It is clear that membrane active lytic peptides such as melittin [12] and magainin [47,48] can rapidly disrupt susceptible mammalian membranes leading to cell death. Examination of the structural activity relationship between melittin and bacterial or mammalian cell membranes can shed further light on the peptide's mechanisms of cytotoxicity. The disruption of bacterial cells by melittin is thought to occur via one of two alternative models. The torroidal pore model [9] involves an electrostatic interaction between cationic melittin and anionic bacterial phospholipids, which forms a monolayer of phospholipids from the outer to inner leaflet of the membrane resulting in formation of channels/pores. Alternatively, the carpet model has also been used to describe the disruption of membranes [49]. In this model, higher concentrations of peptide form a carpet-like layer at the membrane surface, and at a threshold concentration the peptide inserts into the membrane leading to extensive perturbation with micellisation. Selective toxicity of antimicrobial peptides for bacterial cells over mammalian cells is based on the outer leaflet of mammalian cells having more neutral and positively charged phospholipid composition, opposing the initial electrostatic interaction [50]. In bacterial cells, anionic phospholipids represent the greatest concentration of cellular lipids. In contrast, while the phospholipid composition of mammalian cells varies, they normally have zwitterionic phosphatidylcholine and sphingomyelin on the outer leaflet and anionic phosphatidylserine on the inner leaflet [51,52]. The presence of cholesterol in eukaryotic membranes can also be attributed to the reduced cytotoxicity of AMPs [30,53,54].

Although membrane damage plays a role in cytotoxicity, it is likely that non-membrane targets are also involved in cell death, particularly in light of the interaction of the peptide with many facets of mammalian metabolism. These include the activation of PLA_2 [55], phospholipase D [56], as well as inhibition of PLA_2 [19], inhibition of calmodulin [13,57,58], protein kinase C [15], Na-, K-ATPase, and Ca^{2+}-ATPase transport pumps [14,16] among others. Indeed, the necrotic cytotoxicity induced by melittin was somewhat unexpected as both calmodulin, and ATPase pumps have been implicated in the activation of apoptosis [59,60].

Sub-lethal concentrations of melittin increased incorporation of labelled nucleotides in both HT29 and Caco-2 cells in a concentration dependant fashion. We have shown that

sub-lethal concentrations of melittin promoted cell viability in HT29 and Caco-2 cells [5]. Treatment of Caco-2 monolayers with low concentrations of melittin also increased the expression of the tight junction protein, zonula occluden-1 (ZO-1) [7] and electrogenic chloride secretion on basolateral application to rat colonic mucosae [61]. At low concentrations melittin was also shown to stimulate growth of thymocytes while at higher concentrations it caused necrotic cell death [11]. The study of melittin's proliferative effects is difficult, considering the peptide's antagonistic interaction with many mammalian receptors that are inconsistent with growth stimulation. Overall, however, it is clear from this study that different concentrations of melittin have contrasting effects on the growth of gastrointestinal cells.

The presence of cholesterol in eukaryotic membranes can also contribute to selective toxicity of antimicrobial peptides [30,53,54,62]. Cholesterol is an essential molecule in the structure of mammalian plasma membranes but its role in cardiovascular disease has led to a number of pharmaceutical preparations (e.g. statins) and nutraceuticals (plant sterols) which reduce cholesterol in the body. In the current study, a 32% reduction in Caco-2 cell cholesterol increased cytotoxicity by two-fold, while a 48% reduction in HT29 cholesterol led to a five-fold increase in melittin cytotoxicity. This is similar to the effects seen in human erythrocytes, where a reduction in membrane cholesterol by 55% led to a five-fold increase in hemolytic activity of melittin [30]. Furthermore, addition of cholesterol to PC liposomes reduced the leakage induced with melittin by 20-fold [62]. Reduction of membrane cholesterol has also been shown to enhance cytolytic activity of magainin peptides [53,54]. Considering bacteria do not possess cholesterol, the exact role, if any, of this sterol in selective toxicity remains to be seen. The data presented in this study, together with work on other linear amphipathic antimicrobial peptides, support previous observations that cholesterol protects mammalian cells from the cytotoxic lytic effects of linear amphipathic antimicrobial peptides. Should melittin be used successfully in oral drug delivery, agents that reduce cholesterol in the plasma membrane of human cells may be contraindicatory to the peptide's use.

Melittin stimulates necrosis in HT29cells, a passive process that has been described as a cell's response to gross injury and often induced by an overexposure to cytotoxic agent [45]. This observation implies that with careful dosage the peptide may be considered for further investigations as a potential enhancer of the absorption of drugs with poor oral bioavailability. The use of dietary supplements and drugs which reduce cholesterol in gastrointestinal cells may enhance cytotoxicity of melittin, limiting its use in drug delivery. The stimulation of mammalian cell proliferation and DNA synthesis at low concentrations of melittin warrants further study to evaluate the cell machinery involved.

Acknowledgements

This research was supported by the Programme for Research in Third Level Institutes (PRTLI) as administered by the HEA, Ireland. The authors express their gratitude to James Reilly (ITT Dublin) for assistance with statistical analysis.

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