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Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro

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ABSTRACT

Antimicrobial peptides (AMPs) are a diverse group of proteinaceous compounds ranging in size, complexity and antimicrobial spectrum. The activity of AMPs against gut pathogens warrants the study of the interaction of AMPs with the mammalian gastrointestinal tract. In particular, the investigation of the in vitro cytotoxicity of these peptides is critical before they can be considered in clinical infections. The cytotoxicity of gallidermin, nisin A, natural magainin peptides, and melittin was investigated in two gastrointestinal cell models (HT29 and Caco-2) with the MTT conversion assay, neutral red dye uptake assay and compared with that of vancomycin. The hemolytic activities were also investigated in sheep erythrocytes and the effect of AMPs on paracellular permeability was examined by transepithelial resistance (TEER) and TEM. Gallidermin was the least cytotoxic AMP followed by nisin A, magainin I, magainin II and melittin. Melittin and nisin were the only peptides to result in significant hemolysis. However, while nisin caused hemolysis at concentrations which were 1000-fold higher than those required for antimicrobial activity, melittin was hemolytic at concentrations in the same order of magnitude as its antimicrobial activity. Melittin was the only AMP to affect paracellular permeability. Long term melittin treatment also resulted in loss of microvilli, an increase in cell debris and destruction of intestinal tight junctions and cell–cell adhesion. Gallidermin shows most promise as a therapeutic agent, with relatively low cytotoxicity and potent antimicrobial activities. Melittin, while showing little potential as an antimicrobial agent, may have potential in delivery of poorly bioavailable drugs.

1. Introduction

Antimicrobial peptides (AMPs) represent a novel class of antibiotics with potent antimicrobial activity against a range of infectious bacteria including multi-resistant isolates. Despite impressive antimicrobial activity, the therapeutic potential of these peptides is currently limited to specific infections due to high production costs and their stability in biological fluids [1,2]. The high concentration estimated for systemic infections, although not uncommon in conventional antibiotics, is currently not cost effective with AMPs [3]. Many therapeutic applications to date have focused on infections of epithelial and topical barriers, however more recently pharmaceutical companies are developing peptides of greater potential for systemic infections [4,5].

The development of novel drug delivery systems for release of AMPs to infectious sites within the gastrointestinal tract [6] allows AMPs to be considered for use in gastrointestinal

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infections. The delivery of the lantibiotic nisin A to the site of colonic infections has been achieved and is currently under patent [7]. A cocktail preparation of magainin peptides, nisin A, and gallidermin has the potential for the treatment of a broad range of infectious organisms that are often not self-limiting. These include vancomycin resistant *Enterococci*, *Escherichia coli*, *Clostridium difficile*, *Helicobacter pylori* and *Bacillus* species [8–13]. The ability to deliver these AMPs to sites in the gastrointestinal tract and their potent activities against gut pathogens warrants the study of their interaction with the gastrointestinal tract.

A number of models have been proposed that describe the activity of different peptides. The most notable mechanisms of pore formation include barrel stave [14], torroidal pore [15,16], carpet [17], aggregate channel [18], and wedge models [19], with certain peptides thought to act via two or more models. Despite the specific controversy there is a consensus that AMPs selectively disrupt bacterial plasma membranes resulting in cell death. AMPs can possess additional mechanisms of antimicrobial activity. For example, nisin A from *Lactococcus lactis* and gallidermin produced by *Streptococcus gallinarium* inhibit peptidoglycan synthesis [20], while pleurocidin from *Pleuronectes americanus* has been shown to inhibit nucleic acid and protein synthesis [21]. The physiochemical characteristics, most notably the hydrophobicity [22], of these peptides suggests they may interact with mammalian cell membranes of the GI tract with undesired toxicity. The specific interaction of AMPs with mammalian proteins involved in metabolism [23], as well as translocation and up-regulation of apoptosis through the disruption of the mitochondria has been demonstrated [24].

It is therefore critical to assess the interaction of these peptides with intestinal epithelial cells to evaluate their selective toxicity before they can be considered for delivery to infection sites. This study compares the cytotoxicities of natural magainin peptides, nisin A, gallidermin, and melittin on two robust intestinal epithelial cells. These cells frequently encounter xenobiotics and therefore toxicity demonstrated in them may potentially translate to toxicity in other epithelial cells. The MTT conversion assay and neutral red dye uptake assay was used to evaluate the cytotoxicity of AMPs while the effect of these peptides on the plasma membrane integrity of gastrointestinal cells was monitored with the LDH release assay. The effect of peptides on intestinal epithelial integrity was measured using transepithelial electrical resistance (TEER) and visualised with TEM. Here we also compare the AMPs to the conventional antibiotic, vancomycin, routinely used in GIT infections. It is hoped that this study will provide data for the pre-clinical and clinical dosage of AMPs in gastrointestinal infections

2. Materials and methods

2.1. Peptides

All AMPs used were commercial preparations. Gallidermin (Alexis Corporation, UK), Magainin I and magainin II (Anaspec Inc., USA), Nisin A (Nutrition 21, USA), Melittin (Serva Inc, Germany), Vancomycin (Sigma–Aldrich, Ireland) were purchased as lyophilised powders with purity in excess of 95%. A concentrated stock solution of each test compound was prepared in sterile water and stored at −20 °C until use. Daunorubicin (Sigma–Aldrich, Ireland) an antineoplastic anthracycline was used as a positive control in cytotoxicity assays. Peptide purity was verified by μRPC and SDS PAGE [25]. Molecular weight determination for final verification of peptide preparation was carried out using MALDI-TOF.

2.2. Cell culture

HT29 (American Type Culture Collection, USA) and Caco-2 (European Collection of Animal cell Cultures, UK) cells are both human intestinal epithelial cell lines and were used between passages 30–50. HT29 cells were grown in McCoy's 5a media with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal bovine serum. Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal bovine serum (Sigma–Aldrich, Ireland). Both cell lines were grown at 37 °C in a humidified atmosphere with 5% CO_2. The viability of the test cells prior to cytotoxicity assay exceeded 99%, as determined by exclusion of the vital dye trypan blue.

2.3. Antimicrobial activity

The antimicrobial potency of each peptide/control preparation was determined prior to cytotoxicity assays using a modified National Centre for Clinical Laboratory Standard (NCCLS) protocol for aerobic bacteria [26]. *Micrococcus luteus* (ATCC 9341) was used as a Gram positive indicator organism as suggested for various antimicrobials by the American and European Pharmacopoeias [27]. Briefly, 5×10^4 bacterial cells (*M. luteus* or *E. coli* (ATCC 10536)) were seeded per well of a microtiter plate and treated with varying concentrations of freshly prepared peptide in tryptic soy broth (TSB) for 21 h. Following incubation, the absorbance as a measure of cell growth and viability was measured in a microtiter plate assay at 550 nm. The data was plotted and the IC_{90} was calculated.

2.4. Cytotoxicity assays

The cytotoxic potential of test compounds was determined following incubation of exponentially growing cells using the MTT assay. This method is based on the reduction of the tetrazolium salt, methylthiazolyldiphenyl-tetrazolium bromide (MTT) into a crystalline blue formazan product by the cellular oxidoreductases of viable cells [28]. The resultant formazan crystal formation is proportional to the number of viable cells. Following 24 h incubation, cells were washed to remove test compounds and were then incubated with 200 μl of fresh culture media and 20 μl of MTT (5 mg/ml) in 0.1 M PBS, pH 7.4 at 37 °C in a humid atmosphere with 5% CO_2 for 4 h. Media was then gently aspirated from test cultures and 100 μl of dimethyl sulphoxide (DMSO) was added to all wells. The plates were then shaken for 2 min and the absorbance was read at 550 nm in a microtiter plate reader. The IC_{50} was defined as the concentration of test compound required to reduce the absorbance of the MTT-formazan crystals by 50%, indicating 50% cell deactivation.

The cytotoxicity of test compounds was verified with the neutral red dye uptake assay (Sigma–Aldrich, Tox-4). Neutral red is a chromogenic vital dye which is actively transported into the lysosome of viable cells. The cellular dye uptake is proportional to the number of viable cells. Again, test compound was removed from treated test cultures at 24 h and cells were incubated with 200 μl of fresh media with 10% (w/v) neutral red solution to a final concentration of 0.033% for 3 h (HT29) or 30 min (Caco-2) at 37 °C in a humid atmosphere with 5% CO_2. Media was then carefully removed and cells were washed with Hanks balanced salt solution. Incorporated dye was then solubilised by addition of 100 μl of an aqueous solution containing 50% (v/v) ethanol, 1% (v/v) acetic acid. Plates were shaken and absorbance read at 550 nm in a microtiter plate reader. The IC_{50} was defined as the concentration of test compound required to reduce the absorbance of the Neutral red dye uptake by 50%.

The effect of test compounds on plasma membrane integrity of model cell lines was examined by the LDH release assay (Tox-7, Sigma–Aldrich). Cell supernatants were analysed for the enzymatic activity of the enzyme LDH, which under control conditions is exclusively found intracellularly. The data was plotted as drug concentration versus relative percentage lysis (0.1% (v/v) Triton X100). Briefly, test cultures, grown in culture media without phenol red, were removed from the incubator and centrifuged in a plate spinner at 250 × *g* for 4 min. Aliquots of test culture supernatants were treated with enzymatic assay reagent for 25 min. The reaction was stopped by the addition of 0.1N HCl. Absorbance was subsequently measured in a microtiter plate reader at 490 nm. The IC_{50} was defined as the concentration of test compound that stimulated an absorbance equal to 50% total lysis stimulated by 0.1% (v/v) Triton X100.

2.5. *Haemolysis assays*

Hemolytic potential of test compounds was measured spectrophotometrically using a haemoglobin release assay [29]. Fresh defibrinated sheep erythrocytes (Cruinn Technology, Ireland) were rinsed three times with PBS (35 mM phosphate buffer, 150 mM NaCl, pH7.0), centrifuged for 15 min at 900 × *g* and resuspended 4% (v/v) in PBS. Samples (100 μl) of the suspension were plated in 96-well microtiter plates. Red cells were treated with selected peptide concentrations, incubated for 1 h at 37 °C, and then centrifuged at 1000 × *g* for 5 min. Aliquots of the supernatant were then transferred to a fresh 96-well microtiter plate, where haemoglobin release was monitored spectrophotometrically in a microtiter plate at 414 nm. Percent hemolysis was calculated as follows: % hemolysis = (A_{414} peptide soln − A_{414} PBS)/(A_{414} 0.1% Triton X100 − A_{414} PBS). Where zero and 100% hemolysis were determined in PBS and 0.1% Triton X100, respectively.

2.6. *Epithelial integrity*

The effect of test compounds on intestinal epithelial integrity was assessed using trans-epithelial electrical resistance (TEER). Caco-2 cells (P35-45) were seeded in 0.4 μm, 12 mm Transwell polycarbonate inserts (Corning Costar Corp., USA) at a density of 5 × 10^5 cells/insert. The cells were grown on filters for 21 days with the tissue culture media changed on alternate days. The differentiated monolayers were washed with Hanks balanced salt solution (HBSS) at 37 °C prior to treatment with a range of pre-warmed test compounds delivered to the apical surface of the monolayers. TEER measurements were taken periodically over a 24 h period using an EVOM volt–ohm meter with a chopstick-type electrode (World Precise Instruments, USA). The resistance of the monolayer was obtained by subtracting the intrinsic resistance of the system (filter insert alone) from the total resistance (monolayer plus filter insert). The data was displayed as percentage TEER relative to untreated controls at time zero.

The effect of test compound on ultrastructural morphology and intestinal epithelial integrity was assessed using transmission electron microscopy (TEM) [30]. Polarised Caco-2 cells grown for 21–25 days on Transwell polycarbonate inserts were treated with test compound at twice the IC_{50} for 24 h. The cells were immediately washed three times with HBSS and fixed with 2.5% (v/v) glutaraldehyde. The sections were embedded in epoxy resin, mounted on 300 mesh copper grid, stained with uranyl acetate and lead citrate and examined.

Table 1 – In vitro antibacterial activities of the antimicrobial peptides as determined by a modified NCCLS method

Antimicrobial peptide	IC_{90} (μM)	
	E. coli (ATCC 10536)	*M. luteus* (ATCC 9431)
Nisin A	18.7	0.13
Gallidermin	20.6	0.12
Vancomycin	57.8	0.45
Melittin	5.7	8.80
Magainin I	7.9	76.10
Magainin II	18.7	>80.0

Table 2 – Cytotoxicities of AMPs on intestinal epithelial cells following 24 h incubation as determined by MTT assay and neutral red dye uptake assay

Drug	IC_{50} (μM)			
	MTT conversion		Neutral red dye uptake	
	HT29	Caco-2	HT29	Caco-2
Gallidermin	231.0	210.5	>230.0	>230.0
Daunorubicin	31.5	61.1	86.0	58.1
Vancomycin	>6000.0	>6000.0	4500.0	>6000.0
Magainin I	65.0	66.3	75.5	94.9
Magainin II	81.0	79.9	>100.0	>100.0
Nisin A	89.9	115.0	104.5	132.2
Melittin	1.2	1.8	5.6	4.7

All values represent the mean the IC_{50} value of three independent experiments carried out with five replicates of each. The mean IC_{50} values (the AMP concentration required to reduce cell activity to 50% of untreated controls) were calculated from three independently derived full concentration response curves. Greater than symbol indicates the IC_{50} was not reached up to the concentration shown.

2.7. Statistical analysis

Unless otherwise stated all experiments were carried out in replicates of five on at least three independent occasions and data expressed as mean ± standard deviation. Statistical significance was determined using analysis of variance with Minitab®.

3. Results

3.1. Antimicrobial activities of the AMPs

Gallidermin and nisin A showed the greatest antimicrobial activity in *M. luteus* with activities in the nanomolar range and both lantibiotics showed at least 100-fold less activity against *E. coli* (Table 1). In addition, these two prokaryotic AMPs were more potent than Vancomycin to both *M. luteus* and *E. coli* in NCCLS assay. The eukaryotic peptides melittin, magainins I and II showed considerably lower antimicrobial activity than nisin A and gallidermin. Melittin showed antimicrobial activity in the low micromolar range with both indicator organisms. Magainin I and magainin II showed antimicrobial activity in the low micromolar range against *E. coli* (IC_{90} 7.89 and 18.7 μM, respectively) with substantially lower potency in the Gram positive *M. luteus* (IC_{90} 76.1 and >80 μM).

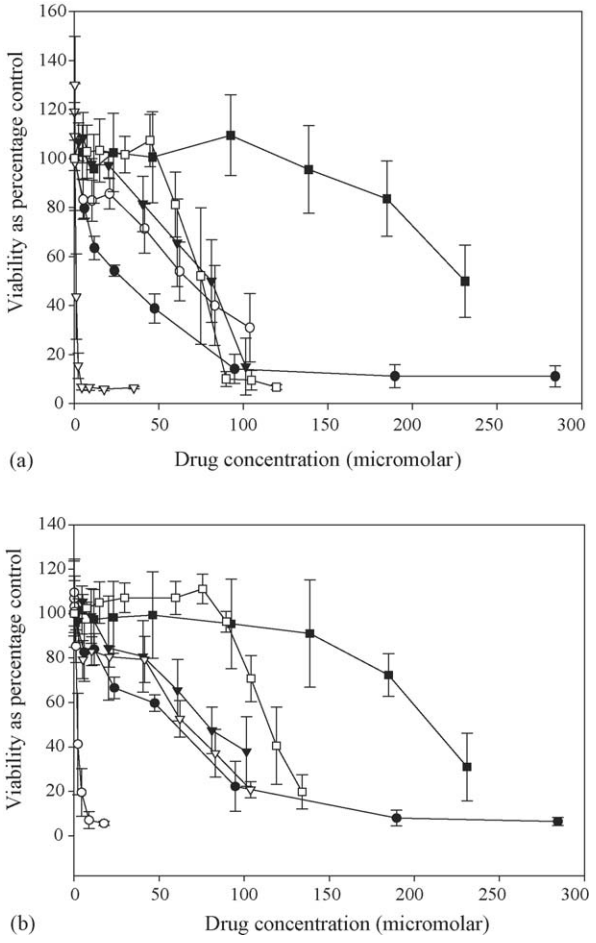

Fig. 1 – Effect of the AMPs on the viability of HT29 (a) and Caco-2 (b) cells following a 24 h incubation, using the MTT assay. The symbols represent responses to the following treatments in HT-29 cells: daunorubicin filled circles, melittin clear triangles, magainin II filled triangles, magainin I open circles, gallidermin filled squares, nisin A open squares and in Caco-2 cells: daunorubicin filled circles, melittin clear circles, magainin II filled triangles, magainin I open triangles, gallidermin filled squares, nisin A open squares. The results represent the mean ± S.E.M. of at least three independent experiments.

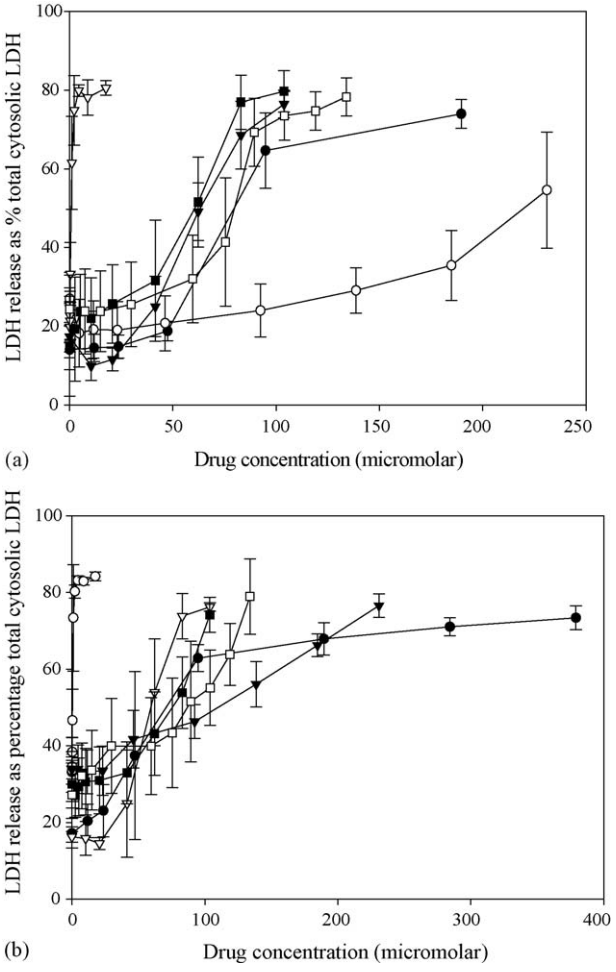

Fig. 2 – Effect of a series of AMPs on the integrity of the plasma membrane of HT29 cells (a) and Caco-2 cells (b) following 24 h incubation using the LDH release assay. The symbols represent responses to the following treatments: In HT29 cells Daunorubicin filled circles, gallidermin open circles, magainin II filled triangles, melittin clear triangles, magainin I filled squares, nisin A clear squares. In Caco-2 cells daunorubicin filled circles, melittin clear circles, gallidermin filled triangles, magainin I clear triangles, magainin II filled squares, nisin A clear squares. The results represent the mean ± S.E.M. of three independent experiments.

3.2. Cytotoxicities of AMPs in intestinal epithelial cells in vitro

Cytotoxicities of each of the five AMPs were comparable in the two distinct epithelial cell lines, HT29 and Caco-2. Gallidermin exhibited the lowest level of cytotoxicity for a cationic AMP under the assay conditions, with two-fold lower toxicity than FDA approved nisin A, and up to three-fold lower than the eukaryotic peptides magainin I, magainin II and positive control (Table 2). This two-fold difference in cytotoxicity of gallidermin over nisin A, magainin I and magainin II was found to be statistically significant in MTT assay ($P < 0.01$). The comparison of the in vitro cytotoxicity between magainin peptides and melittin showed a statistical difference ($P < 0.001$). Nisin A, magainin I and magainin II showed comparable IC_{50} values in both MTT and NR assay in HT29 cells, however a two-fold difference in IC_{50} value was observed for nisin A over magainin I in Caco-2 cells (Table 2). Melittin was the most cytotoxic AMP with 20-fold greater toxicity than any other peptide in the MTT assay. Indeed, Melittin was more toxic than the anticancer drug and known cytotoxic agent, Daunorubicin. The conventional antibiotic, Vancomycin, was found to be non-toxic in MTT conversion assay up to concentrations of 6 mM (Fig. 1).

3.3. Effect of AMPS on lactate dehydrogenase release

A concentration-dependent increase in extracellular LDH was observed with all cationic AMPs, thus indicating each peptide caused a loss of plasma membrane integrity (Fig. 2). In contrast, Vancomycin was not found to compromise the plasma membrane integrity of HT29 or Caco-2 cells up to concentrations of 6 mM. HT29 and Caco-2 cells treated with melittin resulted in a significantly higher LDH release at a lower concentration than those observed with all other AMPs and/or with daunorubicin ($P < 0.01$). HT29 cells treated with gallidermin showed a significantly lower LDH release when compared with all other peptides ($P < 0.05$). The loss of plasma membrane integrity in both HT29 and Caco-2 cells occurred at lower drug concentrations than those observed for both the

Table 3 – Haemolytic potential of AMPs on live sheep erythrocytes following a 1 h exposure to either 0.5×, 1× or 2× the respective IC_{50} concentration obtained in the MTT assay on Caco-2 cells

Drug	Relative % hemolysis		
	$(1/2) \times IC_{50}$[a]	IC_{50}[a]	$2 \times IC_{50}$[a]
Daunorubicin	0	0	0
Melittin	1.5 ± 3.5	4.01 ± 5.71	18.16 ± 16.31
Magainin I	0	2.1 ± 3.1	1.9 ± 3.7
Magainin II	0.75 ± 0.57	1.45 ± 1.10	1.89 ± 1.75
Nisin A	1.36 ± 2.31	2.45 ± 3.41	12.14 ± 10.10
Gallidermin	0.12 ± 0.2	1.36 ± 0.6	4.09 ± 1.2
Vancomycin	0.9 ± 1.3	1.19 ± 3.4	0.56 ± 1.3

Data are expressed as mean percentage hemolysis ± S.E.M. for three separate experiments.

[a] The concentration used relates to the respective IC_{50} values obtained in the MTT assay carried out on Caco-2 cells.

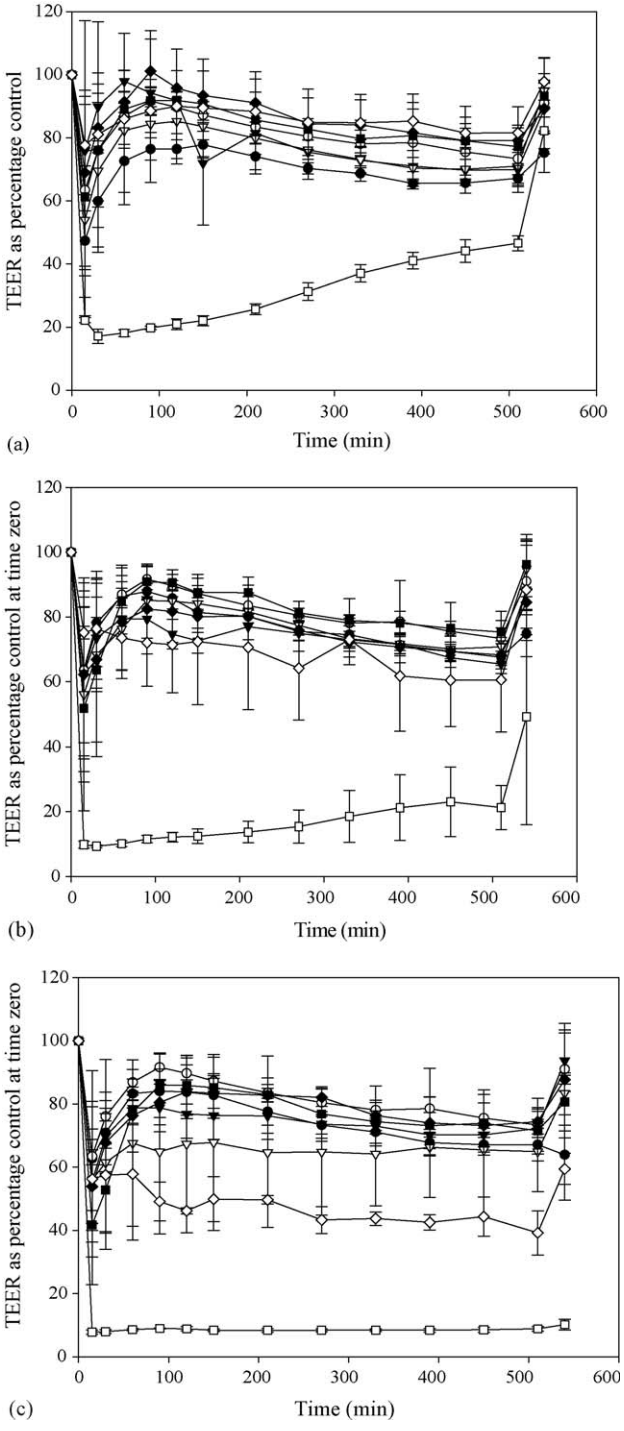

Fig. 3 – Effect of AMPs on intestinal epithelial integrity over a period of 24 h as determined by transepithelial resistance measurements. Polarised monolayers of Caco-2 cells treated with half the IC_{50} (a), the IC_{50} (b), and twice the IC_{50} (c) value, respectively, as obtained from the MTT assay for Caco-2 cells. Daunorubicin filled circles, control clear circles, magainin I filled triangles, magainin II clear triangles, gallidermin filled squares, melittin clear squares, nisin A filled diamonds, vancomycin clear diamonds. The results represent the mean ± S.E.M. of three independent experiments.

MTT and NR assays with the exception of daunorubicin and magainin I. This greater sensitivity to plasma membrane effects suggests that a loss of plasma membrane integrity is the primary mode of toxicity in nisin A, magainin II, melittin and gallidermin.

3.4. *Effect of AMPs on erythrocyte hemolysis*

In order to compare the cytotoxicity obtained in gut epithelial cells with red blood cell lysis, an erythrocyte hemolysis assay was performed at concentrations which directly related to the IC_{50}s obtained in the MTT assay on Caco-2 cells. Daunorubicin was found to be non-hemolytic at each concentration examined (Table 3). All peptides exhibit minimal hemolysis at concentrations tested, with the exception of melittin and nisin A. When erythrocytes were treated at concentrations equivalent to double their respective IC_{50} values, melittin showed $18.16 \pm 16.31\%$ relative hemolysis compared with 0.1% (v/v) Triton X100 while nisin A treatment resulted in $12.14 \pm 10.10\%$ following 1 h incubation.

3.5. *Effect of AMPS on intestinal epithelial integrity as determined by transepithelial resistance measurements*

The in vitro effect of AMPs on intestinal epithelial integrity was determined using TEER measurements on differentiated Caco-2 cells. Intestinal models were treated with a range of concentrations of test compounds relative to the cytotoxicity as determined by MTT assay (see Table 2). The TEER of the untreated monolayers was $\sim 1671 \pm 95\ \Omega\ cm^2$ at 21 days post-plating on filters. Melittin was the only AMP to have a significant effect on TEER (Fig. 3). Within 15 min of addition of

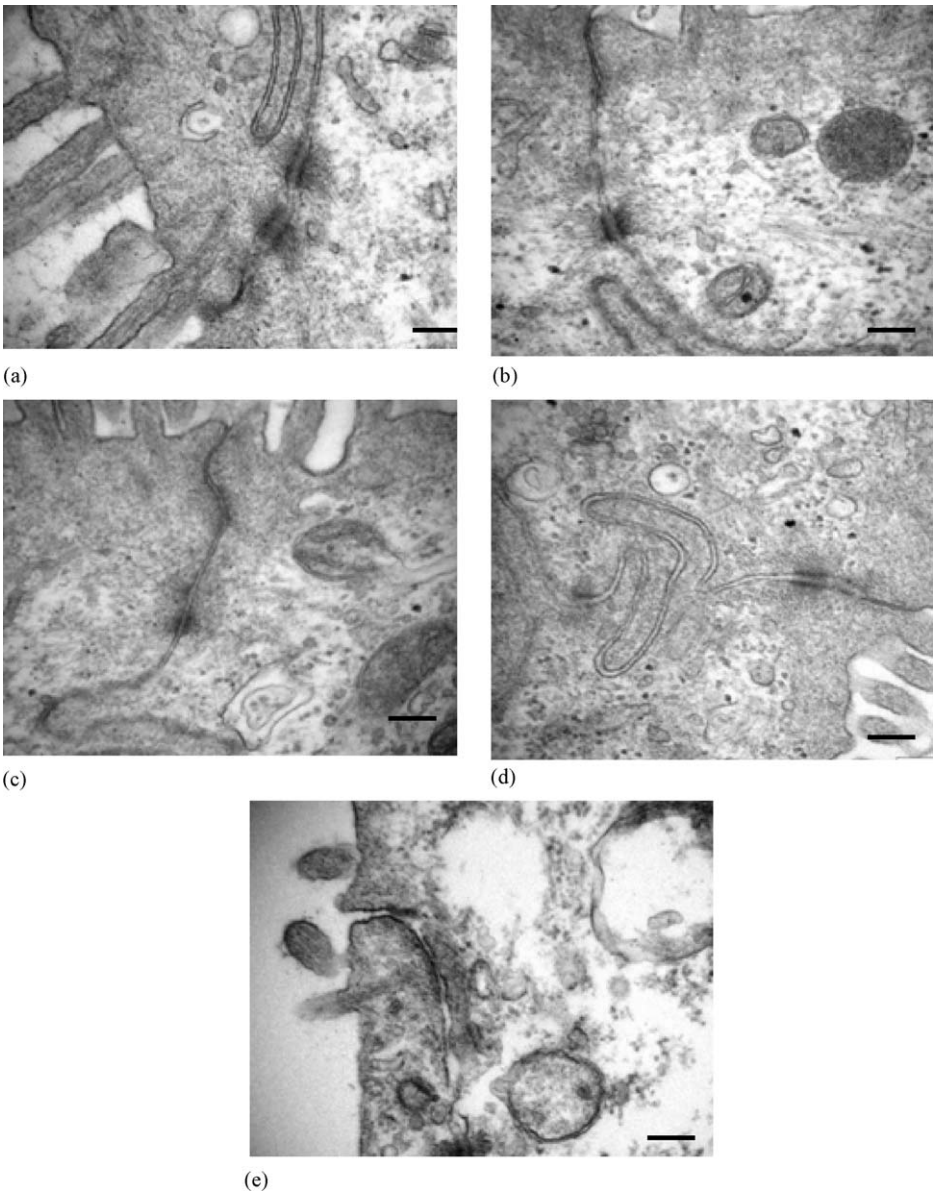

Fig. 4 – (a)–(e) Electron micrographs of polarised Caco-2 cell models after 24 h treatment with selected AMPs at twice the IC_{50} achieved in MTT assay for Caco-2 cells as follows: (a) media treated control, (b) magainin II, (c) nisin A, (d) gallidermin, and (e) melittin, respectively. Bar = 200 nm in all cases.

0.8 μM melittin (half the IC_{50} concentration observed in MTT assay) a 78% reduction in TEER was observed. A complete recovery of epithelial integrity was observed over 24 h. Monolayers treated with 1.8 μM Melittin (IC_{50} value) showed a 89% reduction in TEER with only a intermediary recovery to 46.9% of control over 24 h. Monolayers treated with 3.6 μM of melittin (twice the IC_{50} value) showed a 91% reduction in epithelial integrity which was irreversible over 24 h. Vancomycin resulted in intermediary effects on TEER values in a concentration dependent manner. Nisin, magainin I, gallidermin, magainin II and daunorubicin all elicited negligible effects on differentiated Caco-2 cells, which were not concentration dependent.

Caco-2 cells treated with magainin I, nisin A, and gallidermin for 24 h showed all the features of polarised Caco-2 cells, tight junctions, cell-to-cell adhesion and microvilli at their apical surface and were indistinguishable from untreated control (Fig. 4(a)–(d)). Melittin treatment of Caco-2 cells, however, had a pronounced effect on cellular morphology (Fig. 4e) with loss of microvilli observed after 24 h treatment with 3.6 μM of melittin. Barrier integrity was also affected by melittin (3.6 μM) with opening of tight junctions and dilation of cell–cell junctions. Melittin treatment also resulted in destruction of intracellular organelles and a build up of cellular debris.

4. Discussion

A relatively small amount of cytotoxicity data is available on cationic AMPs and their interaction with human cells. These peptides have potential in the treatment of a vast array of clinical conditions in particular those associated with epithelial barriers. With potent activity towards gastrointestinal pathogens nisin A, magainin peptides and gallidermin represent an option on the treatment of GI tract infections [8–13]. This study is the first report comparing the cytotoxicities of a range of different prokaryotic and eukaryotic AMPs on intestinal epithelial cells. Gallidermin, the lantibiotic produced from *S. gallinarium*, was one of the most potent antimicrobials and the least toxic of all the ribosomally synthesised AMPs tested in the two distinct intestinal epithelial cell lines. In addition, it was more effective than Vancomycin. This was included in the study in order to compare AMPs with an FDA approved antibiotic used in treatment of various infections including gastrointestinal. Although vancomycin is classed as an AMP, it differs from the other peptides within this study due to its monocyclic structure, lower molecular weight, the fact that it is non-ribosomally synthesised and does not induce pore formation. Vancomycin was not toxic to either HT29 or Caco-2 cells at concentrations up to up to 6 mM in MTT assay. Nisin A, an FDA approved food preservative, exhibited significantly greater toxicity than gallidermin in both cells ($P < 0.05$). The increased cytotoxicity of nisin A could be associated with the increased hydrophobicity, however the exact mechanism by which toxicity differs deserves further study. The cytotoxicity of nisin A in vaginal [31], colonic and kidney [32], epithelial cells has been reported. Significant toxicity was reported in Vero cells over 48 h between 0.85 and 3.4 μM [32], whereas significant toxicity was not demonstrated in vaginal epithelial cells up to concentrations of 95 μM [31]. These differences in toxicity compared to the current study may be associated with different cell lines used, impurities in the nisin A preparations used, or a potential loss of activity following purification [33,34]. Partially purified food grade nisin whose contents are described [35], were used in both of the earlier studies. The exact purity of nisin A used in each study was not stated making comparisons difficult. Cytotoxicity studies carried out by Kordel and Sahl, showed 75 mM nisin A induced 90% efflux of intracellular ATP in human lung fibroblasts after 13 min [36] which is substantially higher than the concentration required for 75% efflux of intracellular LDH in intestinal epithelial cells following 24 h incubation. However, this difference may be due to many parameters including eukaryotic cell type, exposure times, assay differences, as well as the medium used to apply the peptides. We have shown that nisin A possessed lower toxicity than magainin I and magainin II in MTT conversion assay with both HT29 and Caco-2 cells. The cytotoxicity of magainin I and magainin II has previously been assessed in a series of cell lines and solid tumors; however as with nisin, direct comparisons are difficult. It is apparent that intestinal epithelial cells have greater sensitivity to magainin I than U937 cells over a shorter incubation period (24 h versus 72 h) [37]. In contrast, intestinal cells appear to possess lower toxicity towards magainin II than U937 cells over a shorter incubation period (24 h versus 72 h). Magainin II demonstrated cytotoxicity in intestinal epithelial cells which was comparable with that of lung epithelial cells ($IC_{50} \sim 100$ μg/ml, 48 h) [38]. Further cytotoxicity studies on magainin II were carried out by Haimovich and Tanaka, where exclusion of propridium iodide formed the basis of the cytotoxicity assay [39]. This study shows toxicity in both normal and transformed cells in excess of 500 μg/ml. Magainin analogues have been investigated in cervical epithelial cells with a view to development of a contraceptive where in particular Magainin A was considered selectively toxic for the specific application [40–43]. The magainin peptides possess greater cytotoxicity and lower antimicrobial potency than the lantibiotics which may be a limiting factor in their application in systemic infection, however they may be used as therapeutics in non-systemic infections such as gastrointestinal infections, vaginal infections, and infections of the skin.

Melittin and magainin are believed to act via the ''torroidal pore'' mechanism of pore formation [15,16,44]. However, their in vitro cytotoxicities are drastically different ($P < 0.001$). Melittin is the principle cytotoxic component in European Honey bee venom (*Apis meliffera*). In addition to potent haemolytic activity melittin is also a calcium dependent antagonist of the multifunctional protein calmodulin and has also has also been demonstrated to inhibit the Na^+ K^+ ATPase pump [45,46]. The more potent cytotoxicity of melittin over other AMPs in the study suggests melittin has multiple mechanisms of toxicity including cytolytic activity. The positive control used for the cellular toxicity assays was daunorubicin, an antineoplastic, anthracycline with potent ability to inhibit topoisomerase II thereby inducing apoptosis. It may cause a necrotic loss of plasma membrane integrity at high concentrations or by secondary necrosis

[47]. Each AMP/control induced cell lysis in intestinal epithelial cells following a 24 h exposure (Fig. 2). This loss of plasma membrane integrity suggests that the peptides caused a fatal necrosis, however the potential for a secondary necrosis following an apoptosis is currently being studied.

Overall, the antimicrobial activity demonstrated by each peptide against indicator bacteria (Table 1) and a range of gastrointestinal pathogens [8–13], is significantly lower than the cytotoxicity in HT29 and Caco-2 cells, particularly in the case of the lantibiotics nisin A and gallidermin. This selective toxicity suggests these peptides have potential in clinical infections.

The hemolytic activity of membrane-active AMPs against erythrocytes is often used as a measure for their cytotoxicity and to estimate their therapeutic index. Comparing the % hemolysis observed at respective IC_{50} value for each AMP demonstrated minimal that all peptides exhibited lower toxicity (less than 2% hemolysis) against sheep erythrocytes than human intestinal epithelial cells. This may be related to different plasma membrane compositions of normal and transformed mammalian cells [24], and metabolic activity, as well as the particular assay used for comparison. Magainin I was considered non-hemolytic up to the highest concentration tested of 130 μM (313 μg/ml), which is 2-fold more than their IC_{50} value in Caco-2 cells. This lower toxicity in two different cell types is probably related to different plasma membrane compositions including differing cholesterol [48] concentrations. A lack of hemolysis by magainin I and magainin II has previously been demonstrated up to 150 μg/ml [49], however, we have shown minimal hemolysis up to 313 and 400 μg/ml of magainins I and II, respectively. Melittin was found to cause $18.16 \pm 16.31\%$ hemolysis of sheep erythrocytes at 2.4 μM. This is considerably lower hemolysis than achieved by Tosteson et al. [50], with human cells but comparable to those observed by Zasloff [49]. This is likely to be as a result of lower levels of phosphate used in the former study [49]. The replacement of cationic sodium with anionic phosphate reduces the maximum extent of lysis without any effect on the rate of hemolysis [49]. The IC_{90} for the antimicrobial activity for melittin towards *E. coli* and to *M. luteus* was comparable to the concentration required to induce hemolysis in eukaryotic cells. That hemolysis was observed at the effective concentration for antimicrobial activity, confirms that melittin does not have potential in infection treatment. In the case of nisin A, the concentration required to induce 12.14% hemolysis was 1000-fold greater than the antimicrobial IC_{90} value. Hence the hemolysis should not cause concern due to the massive selective toxicity associated with the peptide. Kordel and Sahl, showed substantially lower levels of toxicity with nisin A in red blood cell lysis, only 6% following 30 min incubation with 1 mM Nisin [36], however, again these differences may be associated with the different nisin A preparations. In either case the amount of nisin needed to cause hemolysis are considerably higher than the antimicrobial activity of the AMP. Vancomycin was found to be non-hemolytic up to concentrations of 12 mM, as expected because vancomycin is not a pore forming AMP. Gallidermin treatment resulted in only 4% hemolysis at 3850 times its IC_{90} value in NCCLS assay (Table 3), indicating substantial selective toxicity and again confirming its potential as a therapeutic agent.

TEER has been well correlated with the change in paracellular permeability of a cell monolayer [51]. The effect of AMPs on cell–cell adhesion and tight junction integrity is critical if the peptides are to be considered in treatment of bacterial infections of epithelial barriers. This is particularly relevant in the gastrointestinal tract, where damage to either TJ integrity or cell-to-cell adhesion may cause serious irreversible damage. The lack of effect of magainin I, magainin II, gallidermin and nisin A shows that these peptides do not have potential to disrupt intestinal epithelial integrity and suggests that they may be suitable in the treatment of gastrointestinal tract infections. This was unexpected as polycations have previously been shown to open tight junctions [52]. Significantly, the lack of disruption of the epithelial barrier was confirmed visually by TEM. The damage to the epithelial integrity induced by melittin over a range of concentrations confirms the peptide is not suitable for the treatment of either topical or systemic infections. However, it is very interesting that melittin induced a substantially greater reduction in TEER than two previously studied cell penetrating peptides (CPP); transportan, and penetratin in similar experiments [53], and that effect was reversible. Molecules such as these are very useful in the delivery of drugs across various epithelia by acting as paracellular permeability enhancers (PPEs). The current study shows melittin also has the potential to mediate drug uptake as previously demonstrated by Liu et al. [54]. However, further studies are required to determine whether an optimal, non-toxic concentration can be obtained with sufficient opening of tight junctions and timely closure. Overall these data show that melittin, or its analogues, may be useful in the delivery of poorly bioavailable hydrophilic drugs.

Overall, we have shown that gallidermin possesses the greatest level of selective toxicity of the tested AMPs. This suggests potential for gallidermin as a therapeutic antimicrobial agent. Furthermore nisin, and magainin peptides, despite possessing lower selective toxicity than gallidermin, also exhibit low cytotoxicity in gastrointestinal cells and therefore may be useful, alone or in combination, against range of clinical infections of the GI tract. Furthermore the potent cytotoxicity and ability of melittin to modify intestinal epithelial integrity suggests the AMP is not suitable for therapeutic use as an antimicrobial agent, but its ability to act as a paracellular permeability enhancer warrants further studies, where melittin derivatives of reduced toxicity may maintain a permeability enhancing role.

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REFERENCES

[1] Hancock RE, Scott MG. The role of antimicrobial peptides in animal defenses. Proc Natl Acad Sci USA 2000;97: 8856–61.
[2] Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis 2001;1:156–64.
[3] Zasloff MA. The commercial development of the antimicrobial peptide pexiganan. In: Lohner K, editor. Development of novel antimicrobial agents: emerging strategies. Horizon Press; 2001. p. 260–70.
[4] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2002;415:389–95.
[5] Zhang L, Falla TJ. Cationic antimicrobial peptides—an update. Expert Opin Investig Drugs 2004;13:97–106.
[6] Kato Y, Sugiyama Y. Targeted delivery of peptides, proteins, and genes by receptor-mediated endocytosis. Crit Rev Ther Drug Carrier Syst 1997;14:287–331.
[7] Sakr AA, Habib WAA. Oral formulation for treatment of bacteria-induced diseases of the colon. US Patent 5,958,873 (1997).
[8] Kellner R, Jung G, Horner T, Zahner H, Schnell N, Entian KD, et al. Gallidermin: a new lanthionine-containing polypeptide antibiotic. Eur J Biochem 1988;177:53–9.
[9] Brumfitt W, Salton MR, Hamilton-Miller JM. Nisin, alone and combined with peptidoglycan-modulating antibiotics: activity against methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci. J Antimicrob Chemother 2002;50:731–4.
[10] Zasloff M, Martin B, Chen HC. Antimicrobial activity of synthetic magainin peptides and several analogues. Proc Natl Acad Sci USA 1988;85:910–3.
[11] Moore KS, Bevins CL, Brasseur MM, Tomassini N, Turner K, Eck H, et al. Antimicrobial peptides in the stomach of *Xenopus laevis*. J Biol Chem 1991;266:19851–7.
[12] Bartoloni A, Mantella A, Goldstein BP, Dei R, Benedetti M, Sbaragli S, et al. In-vitro activity of nisin against clinical isolates of *Clostridium difficile*. J Chemother 2004;16:119–21.
[13] Mota-Meira M, LaPointe G, Lacroix C, Lavoie MC. MICs of mutacin B-Ny266, nisin A, vancomycin, and oxacillin against bacterial pathogens. Antimicrob Agents Chemother 2000;44:24–9.
[14] Baumann G, Mueller P. A molecular model of membrane excitability. J Supramol Struct 1974;2:538–57.
[15] Matsuzaki K, Murase O, Fujii N, Miyajima K. An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. Biochemistry 1996;35:11361–8.
[16] Ludtke SJ, He K, Heller WT, et al. Membrane pores induced by magainin. Biochemistry 1996;35:13723–8.
[17] Shai Y. Molecular recognition between membrane-spanning polypeptides. Trends Biochem Sci 1995;20:460–4.
[18] Hancock RE, Chapple DS. Peptide antibiotics. Antimicrob Agents Chemother 1999;43:1317–23.
[19] Driessen AJ, van den Hooven HW, Kuiper W, van de Kamp M, Sahl HG, Konings RN, et al. Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. Biochemistry 1995;34:1606–14.
[20] Chatterjee C, Paul M, Xie L, van der Donk WA. Biosynthesis and mode of action of lantibiotics. Chem Rev 2005;105:633–84.
[21] Patrzykat A, Friedrich CL, Zhang L, Mendoza V, Hancock RE. Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. Antimicrob Agents Chemother 2002;46:605–14.
[22] Hwang PM, Vogel HJ. Structure–function relationships of antimicrobial peptides. Biochem Cell Biol 1998;76: 235–46.
[23] Hait WN, Grais L, Benz C, Cadman EC. Inhibition of growth of leukemic cells by inhibitors of calmodulin: phenothiazines and melittin. Cancer Chemother Pharmacol 1985;14:202–5.
[24] Papo N, Shai Y. Host defense peptides as new weapons in cancer treatment. Cell Mol Life Sci 2005;62:784–90.
[25] Swank RT, Munkres KD. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal Biochem 1971;39: 462–77.
[26] National Committee for Clinical Laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically-Second Edition: Approved Standard M7-A2. NCCLS, Villanova, PA, USA; 1990.
[27] Tang JS, Gillevet PM. reclassification of ATCC 9341 from *Micrococcus luteus* to *Kocuria rhizophila*. Int J Syst Evol Microbiol 2003;53(Pt 4):995–7.
[28] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983;65:55–63.
[29] Shin SY, Lee SH, Yang ST, Park EJ, Lee DG, Lee MK, et al. Antibacterial, antitumor and hemolytic activities of alpha-helical antibiotic peptide, P18 and its analogs. J Pept Res 2001;58:504–14.
[30] McClean S, Prosser E, Meehan E, O'Malley D, Clarke N, Ramtoola Z, et al. Binding and uptake of biodegradable poly-DL-lactide micro- and nanoparticles in intestinal epithelia. Eur J Pharm Sci 1998;6:153–63.
[31] Aranha C, Gupta S, Reddy KV. Contraceptive efficacy of antimicrobial peptide nisin: in vitro and in vivo studies. Contraception 2004;69:333–8.
[32] Murinda SE, Rashid KA, Roberts RF. In vitro assessment of the cytotoxicity of nisin, pediocin, and selected colicins on simian virus 40-transfected human colon and Vero monkey kidney cells with trypan blue staining viability assays. J Food Prot 2003;66:847–53.
[33] Liu W, Hansen JN. Some chemical and physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis*. Appl Environ Microbiol 1990;56:2551–8.
[34] Rollema HS, Kuipers OP, Both P, de Vos WM, Siezen RJ. Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering. Appl Environ Microbiol 1995;61:2873–8.
[35] Cleveland J, Chikindas M, Montville TJ. Multimethod assessment of commercial nisin preparations. J Ind Microbiol Biotechnol 2002;29:228–32.
[36] Kordel M, Sahl HG. Susceptibility of bacterial, eukaryotic, and artificial membranes to the disruptive action of the cationic peptides pep5 and nisin. FEMS Microbiology Lett 1986;34:139–44.
[37] Cruciani RA, Barker JL, Zasloff M, Chen HC, Colamonici O. Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. Proc Natl Acad Sci USA 1991;88:3792–6.
[38] Baker MA, Maloy WL, Zasloff M, Jacob LS. Anticancer efficacy of Magainin2 and analogue peptides. Cancer Res 1993;53:3052–7.
[39] Haimovich B, Tanaka JC. Magainin-induced cytotoxicity in eukaryotic cells: kinetics, dose-response and channel characteristics. Biochim Biophys Acta 1995;1240:149–58.
[40] Clara A, Manjramkar DD, Reddy VK. Preclinical evaluation of magainin-A as a contraceptive antimicrobial agent. Fert Steril 2004;81:1357–65.
[41] Reddy KV, Aranha C. Studies on safety aspects of contraceptive Magainin-A in rabbits. Indian J Exp Biol 2000;38:1217–21.

[42] Reddy KV, Shahani SK, Meherji PK. Spermicidal activity of Magainins: in vitro and in vivo studies. Contraception 1996;53:205–10.
[43] Reddy VR, Manjramkar DD. Evaluation of the antifertility effect of magainin-A in rabbits: in vitro and in vivo studies. Fert Steril 2000;73:353–8.
[44] Yang L, Harroun TA, Weiss TM, Ding L, Huang HW. Barrel-stave model or toroidal model? A case study on melittin pores. Biophys J 2001;81:1475–85.
[45] Baker KJ, East JM, Lee AG. Mechanism of inhibition of the Ca(2+)-ATPase by melittin. Biochemistry 1995;34:3596–604.
[46] Cuppoletti J, Malinowska DH. Interaction of polypeptides with the gastric (H+ + K+)ATPase: melittin, synthetic analogs, and a potential intracellular regulatory protein. Mol Cell Biochem 1992;114:57–63.
[47] Masquelier M, Zhou QF, Gruber A, Vitols S. Relationship between daunorubicin concentration and apoptosis induction in leukemic cells. Biochem Pharmacol 2004;67:1047–56.
[48] Raghuraman H, Chattopadhyay A. Cholesterol inhibits the lytic activity of melittin in erythrocytes. Chem Phys Lipids 2005;134(2):183–9.
[49] Zasloff M. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci USA 1987;84:5449–53.
[50] Tosteson MT, Holmes SJ, Razin M, Tosteson DC. Melittin lysis of red cells. J Membr Biol 1985;87:35–44.
[51] Mine Y, Zhang JW. Surfactants enhance the tight-junction permeability of food allergens in human intestinal epithelial Caco-2 cells. Int Arch Allergy Immunol 2003;130:135–42.
[52] Ranaldi G, Marigliano I, Vespignani I, Perozzi G, Sambuy Y. The effect of chitosan and other polycations on tight junction permeability in the human intestinal Caco-2 cell line(1). J Nutr Biochem 2002;13:157–67.
[53] Lindgren ME, Hallbrink MM, Elmquist AM, Langel U. Passage of cell-penetrating peptides across a human epithelial cell layer in vitro. Biochem J 2004;377: 69–76.
[54] Liu P, Davis P, Liu H, Krishnan TR. Evaluation of cytotoxicity and absorption enhancing effects of melittin—a novel absorption enhancer. Eur J Pharm Biopharm 1999;48: 85–7.