High Content Analysis to Determine Cytotoxicity of the

Antimicrobial Peptide, Melittin and Selected Structural Analogues

Edwin G. Walsh[1*], Sam Maher[1*], Marc Devocelle[2], Peter J. O'Brien[3], Alan W. Baird[1], David J. Brayden[1†]

[1]UCD School of Veterinary Medicine and UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland; [2]Centre for Synthesis and Chemical Biology, Department of Pharmaceutical & Medicinal Chemistry, Royal College of Surgeons in Ireland, Ireland [3]Advanced Diagnostics Laboratory, Unit 0.13, Nova UCD, Belfield Innovation Park, University College Dublin, Belfield, Dublin 4, Ireland.

[*] Both authors contributed equally

[†]Author to whom all correspondence should be addressed:
UCD School of Veterinary Medicine
Belfield
Dublin 4
Tel: +353 1 7166013
Fax: +353 1 716 6219
Email: david.brayden@ucd.ie

Abbreviations:　　　　　HBTU: *O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-
hexafluoro-phosphate
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
CN: Cell number
PMP: Plasma membrane permeability
NI: Nuclear intensity
NA: Nuclear area
IC: Intracellular calcium
MMP: Mitochondrial membrane potential
FCCP: carbonyl cyanide p-trifluoromethoxy-phenylhydrazone

ABSTRACT

Antimicrobial peptides (AMPs) are naturally occurring entities with potential as pharmaceutical candidates and/or food additives. They are present in many organisms including bacteria, insects, fish and mammals. While their antimicrobial activity is equipotent with many commercial antibiotics, current limitations are poor pharmacokinetics, stability and potential toxicology issues. Most elicit antimicrobial action via perturbation of bacterial membranes. Consequently, associated cytotoxicity in human cells is reflected by their capacity to lyse erythrocytes. However, more rigorous toxicological assessment of AMPs is required in order to predict potential failure at a later stage of development. We describe a high-content analysis (HCA) screening protocol recently established for determination and prediction of safety in pharmaceutical drug discovery. HCA is a powerful, multi-parameter bioanalytical tool that amalgamates the actions of fluorescence microscopy with automated cell analysis software in order to understand multiple changes in cellular health. We describe the application of HCA in assessing cytotoxicity of the cytolytic α-helical peptide, melittin, and selected structural analogues. The data shows that structural modification of melittin reduces its cytotoxic action and that HCA is suitable for rapidly identifying cytotoxicity.

INTRODUCTION

Antimicrobial peptides (AMPs) are an emerging group of natural host-defence peptides that have clinical potential as novel antibiotics for treatment of systemic, enteric or topical microbial infections [1], and at the same time have use as food additives, mostly as preservatives of canned food (e.g. Nisin; Danisco, UK) [2]. There have been a number of candidate AMPs in clinical development for microbial infections (reviewed in [3]). One such example is Pexiganin® (Magainin Pharmaceuticals, USA), a magainin analogue, although it failed a Phase III trial as a topical agent for treatment of diabetic foot ulcers [4, 5]. Literature describing novel antimicrobial peptides have typically outlined their isolation, synthesis and physicochemical properties, as well as their spectrum of antimicrobial action and with mechanistic study of cell membrane perturbation [6]. Assessment of AMP cytotoxicity potential is usually determined by measuring their capacity to perturb neutral and cationic model membranes or erythrocytes, as well as their role in regulation of host immunity [7]. Although such assays are pertinent because some AMPs are thought to act exclusively through perturbation of phospholipid bilayers [8], subtle cytotoxicity should also be examined in mammalian cells in order to understand how AMPs act on a range of cellular processes in sub-lethal concentrations, and not just by their ability to cause cytolysis. This is important because determination of drug safety is multi-factorial and depends on maintenance of virtually all cellular events [9].

Selecting the right candidate for further pre-clinical and clinical development is often challenging, especially from large synthetic peptide libraries or where rational structural

modification of an AMP can lead to large groups of candidate peptides with potent antimicrobial action [10], all with variable degrees of cytotoxicity to human cells. For example, structure activity studies on the AMP, melittin, have generated large numbers of structural analogues [11]. Melittin is an amphipathic cationic linear 26-mer α-peptide that has cell penetrating activity as well as well-known cytotoxicity in erythrocytes [12] and enterocytes [13] and other mammalian cells [14]. Alongside phospholipase A2, it is a major constituent of the venom of *Apis Mellifera,* and has been found to cause apoptosis and necrosis in different cells [15, 16], as well as agonist and antagonist actions on many cellular proteins including phospholipase A2 and ATPases (reviewed in [14]). Melittin itself cannot be used clinically as an antimicrobial because it is equally damaging to mammalian cells. Single amino acid modifications of melittin significantly reduced hydrophobicity, amphipathicity and helicity, all of which reduced the peptide's hemolytic action, but did not have as significant an effect on its antimicrobial activity [11, 17, 18].

HCA screening to determine drug-induced toxicity is a recently established pre-clinical cell-based technology platform that is used to understand subtle changes in cell health in discovery programmes, with the aim of reducing toxicity-associated attrition in the clinic [19, 20]. Unlike conventional cytotoxicity assays such as the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) conversion assay, which detects overt, gross cytotoxicity, HCA provides pre-lethal toxicity assays that have high sensitivity and specificity [9, 19, 21]. HCA combines flow cytometry with automated fluorescence microscopy and advanced image analysis software, permitting quantitative analysis of multiple cellular events in single cells (Fig. 1). The HCA instrument reads metrics in

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individual cells in multiwell plate formats with sensitive fluorescent dyes, inverted fluorescent microscopy and suitable software to quantify changes in fluorophore staining. Sensitivity of HCA for detection of cytotoxicity was an order of magnitude greater than that of the MTT assay in assessing cytotoxicity of the polymer, poly((2-dimethylamino)ethyl methacrylate) (pDMAEMA) in Caco-2 and U937 cells [22]. For the HCA cytotoxicity protocol for AMPs, we used methodology developed by O'Brien *et al* (2006) [21]. Screening involved detecting changes in cell number (CN), nuclear area (NA), nuclear intensity (NI), mitochondrial membrane potential (MMP), plasma membrane permeability (PMP) and intracellular calcium (IC). These parameters measure selected cellular metabolic events and were optimised for HCA because they are hallmarks of cell health [9, 19].

In order to better understand the structural activity relationships (SARs) involved in the cytotoxic effects of this AMP, and also to illustrate the capacity of HCA as a tool for the rapid delineation of such SARs, we examined the cytotoxicity of melittin and four structural analogues in Caco-2 cells after 24 and 72 hours. The duration of exposure was chosen to encompass acute and chronic exposure because 75% of molecules typically do not express acute cytotoxicity [9]. Human intestinal Caco-2 cells were selected for assessment because of current interest in use of AMPs both to treat enteric infections and as potential food additives.

MATERIALS AND METHODS

Peptide synthesis, purification and characterisation

Coupling reagents, Fmoc-protected amino acids, and Rink Amide MBHA resin were purchased from Novabiochem (Merck, UK). All other reagents and solvents were purchased from Sigma-Aldrich (Ireland). Peptides were prepared by standard solid-phase peptide synthesis [23] according to the Fmoc-*t*-Bu strategy [24] with HBTU/HOBt/DIEA coupling chemistry in NMP solvent. Single coupling cycles (except for L13, 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, was 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 (TFA), 5% water, 5% triisopropylsilane, 5% thioanisole, 2.5% EDT, at room temperature for 2.5 hours. 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, 5µm, C18, 4.6mm×250mm, 250mm×100mm, 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% TFA in acetonitrile with a gradient: 2–60% B in 18 column volumes (analytical) or 5 column volumes (semi-preparative) with a flow rate: 1ml/min (analysis) or 5ml/min (semi-preparative) and single wavelength detection at 214 nm. Following purification by

preparative RP-HPLC, each peptide was dried by lyophilisation, weighed on an analytical balance and reconstituted in sterile ultrapure water for purity analysis by analytical RP-HPLC, in which the purity of each peptide exceeded 95%. Purified peptides were finally characterised by matrix-assisted laser desorption/ionization Time-of-flight (MALDI-TOF) mass spectrometry using the α-cyano-4-hydroxy-cinnamic acid matrix.

Cell culture

Caco-2 cells were purchased from the European Collection of Animal Cell Cultures (UK) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with foetal bovine serum (10% (v/v)), L-glutamine (2mM), penicillin (100U) / streptomycin (100µg/ml) and 1 % (v/v) non-essential amino acid cocktail. Cells were maintained in vented 75 cm^2 flasks in a humidified cell culture incubator with 5% CO_2 at 37°C. Viability of cells at the point of seeding onto plates exceeded 99%, as determined by haemocytometry with trypan blue dye exclusion.

HCA screening assay

Cytotoxicity of melittin and its structural analogues on Caco-2 cells was examined in a HCA protocol that has been validated in the sensitive and selective *in vitro* assessment of hepatotoxicity [21]. Caco-2 cells were seeded in 96-well cell-culture plates at a density of 3×10^3 cells for 24 hours to adhere, and subsequently treated with ten concentrations (0.1-50µM) of melittin or peptide analogues (diluted two-fold), in duplicate for 24 and 72 hours in an incubator (37°C, 5% CO_2/95% O_2). Positive controls including carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP) (a mitochondrial membrane

uncoupler, 100µM), ionomycin (a calcium ionophore, 20µM) and Triton® X-100 (a detergent, 0.05% (v/v)), were included [21]. Prior to data acquisition, each well was incubated with a dye cocktail mixture containing a final concentration of Hoechst (0.8µM) (measure of CN, NI and NA), Fluo-4 AM (1µM) (measure of IC), TOTO®-3 iodide (1µM) (measure of PMP) and tetramethyl rhodamine methyl ester (TMRM; 20nM) (measure of MMP) for 60 min at 37°C and protected from light. Data were captured for each plate at 10x (for cell counts) and 20x (for all other cytotoxic metrics) objective magnification in the selected excitation and emission wavelengths of Hoechst (Ex/Em 360/460nm), Fluo-4 AM (Ex/Em 480/535nm), TMRM (Ex/Em 535/600nm) and TOTO®-3 iodide (Ex/Em 620/700nm). Exposure times were optimized for Caco-2 cells for Hoechst (150 ms), Fluo-4 AM (500 ms), TMRM (600 ms) and TOTO®-3 iodide (200 ms). For each well, six random field-of-view images were acquired to examine each parameter except cell number, where ten fields of view were used. Data for CN, NA, NI, MMP, PMP and IC were acquired on an In Cell® 1000 High Content Analyzer (GE Healthcare, UK). This instrument analyses epifluorescence of individual cell events using an automated micro-plate reader analyzer interfaced with a PC (Dell XW6200 Workstation).

Statistical analysis

All experiments were carried out with technical replicates on three independent occasions. Data exported from the In Cell® 1000 workstation software were illustrated using GraphPad 5.0® Prism software, and expressed as the mean ± SEM. Statistical

analysis was carried out in GraphPad® by analysis of variance (ANOVA) with $P \leq 0.05$ selected as the criterion of statistical significance.

RESULTS

Synthesis, characterisation and purification of melittin analogues

Purity of each synthetic peptide was $\geq 95\%$ and their integrity was confirmed by matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectroscopy (data not shown). Peptide analogue-1 (PA-1) had an alanine deletion at position 15; analogue-2 (PA-2) had a tryptophan deletion at position 19, while analogue-3 (PA-3) was a double substitution of heptadic leucine at positions 6 and 13 with alanine. Analogue-4 (PA-4) was truncated at its 11 N-terminal residue (Table I). Peptide synthesis was validated by inclusion of commercially obtained melittin purified by chromatography (Serva, Germany); both the native and synthetic melittin had identical cytotoxicity in MTT assay (data not shown).

Effect of melittin analogues on viability of Caco-2 cells measured by HCA

Compared to untreated control, FCCP decreased the MMP of Caco-2 cells by 30% following 10 min incubation (P<0.0001) (Fig. 2a, b). Triton X-100 and ionomycin likewise increased PMP and IC by 3- and 5- fold, respectively (P<0.0001) (Figs. 2 c, d and e, f, respectively). These data indicate that Caco-2 cells respond to an expected exogenous challenge by positive control drugs. None of the peptides significantly altered CN after 24 hours incubation (Fig. 3, Table II). The parameters significantly affected by

low concentrations of native melittin were NA and MMP (1.6µM; P<0.05). At the higher concentration of 3.1µM, NI, IC and PMP were significantly increased. Representative images depicting melittin-induced changes to each cell metric are illustrated in Fig. 4a-c. Upon addition of melittin (3.1µM, Fig. 4b), there was a noticeable increase in IC (green) and PMP (magenta) compared with control (Fig. 4a). After further increases in melittin concentration (6.3µM; Fig. 4c), there were even more significant morphological changes to cell shape and NA as well as more obvious increases in PMP (magenta) and IC (green), and decreases in the MMP (red) (Fig 4c). The data for the 24 hour incubation indicate that melittin alters each cell metric in a concentration dependent fashion, except for CN.

The effects of PA-1-to-PA-4 on Caco-2 cell viability are summarised in Table II. The initial metrics to change in cells treated with PA-1 for 24 hours were NA, NI and PMP (6.3µM) (Fig. 3). The effect of PA-1 on IC was only noted at the highest concentration (100µM) (Fig. 3, Table II). In the case of PA-2, MMP of Caco-2 cells was reduced at 3.1µM after 24 hours, and all other parameters were significantly changed at 12.5µM, which was a 4-fold higher concentration than native peptide (Fig. 3, Table II). The actions of PA-3 on parameters measured in Caco-2 cells broadly varied from changes in IC (6.3µM) to changes in MMP (100µM), and its affects on each parameter were greater than native melittin, PA-1 and PA-2 (Fig. 3, Table II). PA-4 did not affect most parameters measured even at the highest concentration (100µM) (Fig. 3, Table II). Fig. 4c illustrates modest effects of PA-4 on cell parameters following 24 hour incubation. These data indicate that PA-1-to-PA-3 cause a concentration-dependent alteration in most cell

parameters at 24 h, and that the trend follows the order of PA-1=PA-2>PA-3>PA-4. In summary, PA-1 induced similar changes in cell parameters to native melittin, whereas the other analogues had reduced cytotoxic actions at lower concentrations.

72 hour incubation of cells with melittin (3.1µM) and PA-1 (3.1µM), PA-2 (12.5µM) and PA-3 (25µM) led to a significant decrease in CN (P< 0.001) (Fig. 4, Table II). PA-4 had no effect on CN up to a concentration of 100µM. The initial cellular metrics that melittin affected at 72 hours were NI and IC (both 1.6µM) followed by CN, NA, PMP (all 3.1µM), and then MMP (6.3µM) (Fig. 4). Fig. 3e-g illustrates the effect of melittin on each parameter after 72 hours. The most noteworthy effects of melittin (3.1-6.3 µM) are decreased CN and MMP, increased IC as well as alteration in cell and nuclear shape. The effect of the peptides on CN decreased in the order melittin = PA-1<PA-2<PA-3<PA-4 (Table II). Such a trend was also observed for all other parameters with the exception of IC, which was affected to a similar extent by each PA (Table II). At 72 hours exposure, PA-4 did not affect CN, PMP, NA, NI or MMP. However, although it altered NI (6.3µM) and IC (0.2µM), the effects were concentration-independent and significant only at two outlying concentrations. Fig. 4h is a representative image showing that PA-4 had no effect on each parameter even at the highest concentration (100µM). In summary, 72 hour treatment with melittin also led to a concentration dependent decrease in each of the cell metrics, including CN. Similar to the shorter incubation time, the concentration required to effect each measured metric increased in the order of melittin>PA-1>PA-2>PA-3> PA-4. The only exception was IC which was affected by each peptide analogue,

suggesting structural changes do not alter the ability of melittin to interfere with calcium homeostasis.

DISCUSSION

The ability of a membrane-active, cationic AMP to lyse erythrocytes is a relevant initial assay that is aimed at understanding their capacity to kill mammalian cells, but falls short of predicting overall cytotoxicity of AMPs. No single parameter can be used to define the cytotoxicity of a drug. The ideal properties of optimally effective assays have been reviewed [19]: the assay must be sensitive enough to detect lethal and sub-lethal cytotoxicity for a wide range of concentrations; it must predict acute and chronic cytotoxicity, and it should measure multiple parameters. The current HCA screen was established and optimised in Pfizer (USA), where subtle hepatotoxicities of 243 drug substances were evaluated and compared to known human toxicity [21]. Comprehensive assessment of cytotoxicity combined with automation and high throughput are suited to drug discovery and permit drug prioritisation and optimisation during drug screening. The number of natural peptides listed in AMP databases is into the thousands, and increases year-on-year [25, 26], and there is potential for even larger numbers of AMPs to arise from synthetic AMPs generated rationally or by serendipity from recombinant micro-organisms (e.g. of lantibiotics [27]) or from synthetic combinatorial libraries [28]. Because efficacy of these AMPs can be rapidly determined in multi-well HTS assays (e.g. National Clinical Laboratory standards), it is paramount that such an equally rapid

cytotoxicity screen can be employed to detect selective toxicity for bacterial over human cells

Cytotoxicity of melittin is well established in a range of mammalian cells, notably erythrocytes [12]. Its toxicity has also been detected in hepatocytes [16], enterocytes [13], intestinal goblet cells [13], and astrocytomas [29]. Incubation of Caco-2 cells for 24 hours with melittin caused a concentration-dependent decrease in PMP and MMP, the former suggesting it could cause cellular necrosis, similar to findings in human intestinal goblet cells (HT29) using annexin-5-FITC/Propidium iodide staining [15]. The only parameter not significantly affected after 24 hours by the AMP was CN; however, this was also the case with the non-ionic surfactant and cell-penetrant, Triton X-100. It is not uncommon for such membrane perturbants to reduce cell viability by first altering plasma membrane permeability [30]. After lysis, the subsequent actions of a surface active agent are solubilisation of the lamellar bilayer structure into mixed micelles and only then is the cell structure disbanded [30, 31]. We previously hypothesized that cationic melittin could more readily dissipate the anionic mitochondrial membrane compared with that of the more neutral cell-plasma membrane leading to induction of apoptosis [15]. This process can be followed by phosphatidylserine (PS) translocation from the inner leaflet of the phospholipid bilayer to the outer leaflet, reducing the charge on the cell membrane, and ear-marking the outer membrane for subsequent lysis by extracellular peptide. Chronology of the data indicated that melittin altered Caco-2 MMP before PMP, and therefore this could be an initial process in cell membrane perturbation.

The effects of melittin on calcium homeostasis are well established. It increased IC in osteoblast-like cells [32], fibroblasts [33], hepatocytes [16, 34] and mast cells [35]. In human osteoblasts, melittin increased IC levels only when calcium was present extracellularly and not from intracellular stores [32]. Melittin also inhibits Ca^{2+}-ATPase in the sarcoplasmic reticulum, suggesting modulation of IC is not just related to cell membrane perturbation [36]. The peptide (1.8-3.5µM) did not alter membrane integrity of HepG2 cells when stained with annexin-FITC/PI dual staining, indicating an apoptotic process rather than necrosis [16]. Further assessment indicated that melittin increased IC followed by activation of a signaling cascade involving CAMKII-TAK1-MKK-JNK/p38. The authors also noted a decrease in MMP, activation of caspase-3, capase-9 and PARP in HepG2 cells, as well as release of cytochrome c from isolated mitochondria, suggesting involvement of the mitochondrial oxidative pathway. The lack of effect on membrane permeability in liver cells contrasted from our data with Caco-2 cells. In another report, melittin did not lead to DNA fragmentation in a range of cell types, even at concentrations where there was a significant loss of plasma membrane integrity and an increase in IC [37]. Apoptosis was not detected in intestinal goblet cells by four methods for detecting programmed cell death [15], but did however detect necrosis by annexin-FITC/PI dual staining. There are multiple studies showing different effects of melittin in different cell types (reviewed in [14, 38]). What is clear is that the actions of micromolar concentrations of melittin are mediated, in part, through increases in IC, decreased MMP and loss of membrane integrity, which may lead to concentration-, time- and cell type-dependent induction of cell death.

Activation of phospholipase A2 was not investigated in this study. However, the cytotoxicity profile of melittin, which was inhibited by gangliosides, was pharmacologically separated from melittin's stimulatory action on phospholipase A2 [35]. Furthermore, the cytotoxicity of melittin appears to be separate from its capacity to activate $G\alpha_i$ and $G\alpha_o$, and appears to be a direct action of melittin since it has been observed in cell free systems [39]. Our results do not preclude contributions to cell damage via other pathways and some of these may act in synergy [40].

Structural modification of melittin attenuated its cytotoxicity in Caco-2 cells after both 24 and 72 hours. The selected modifications did not have the same affect on the antimicrobial action of melittin and therefore such findings encourage the molecular engineering of AMPs to generate non-toxic peptides which retain antibiotic function [41]. PA-1 had a comparable minimum inhibitory concentration (MIC) against *S. aureus* [11]; PA-2 had 4-fold higher MIC against *S. aureus* [11]; PA-3 had the same MIC as the native peptide when tested against *E. coli*, *B. subtilis* and *S. aureus* [18] and PA-4 had 3-7 times lower MIC against *E. coli*, *B. subtilis* and *S. aureus* [17]. For most metrics, there was a reduction in cytotoxicity in the order of melittin>PA-1>PA-2>PA-3>PA-4, correlating with reduction in hydrophobicity index (see Table I and reference [42]) but different to the order that antimicrobial activity changes suggesting different modes of membrane perturbation for bacterial and mammalian cells. Altering hydrophobicity, amphipathicity and/or helicity of melittin reduces the cytolytic action of melittin on mammalian membranes [11, 18, 43, 44]. PA-1 and PA-2 are deletion analogues that had 8-fold and 124-fold less hemolytic activity than native melittin [11], are less hydrophobic and

demonstrate lower cytotoxicity by MTT assay [42]. Similarly, PA-1 was also less sensitive than melittin in three of the six measured parameters in HCA screening of Caco-2 cells after 24 and 72 hours. Reduced cytotoxicity of each PA correlated with reduced hydrophobicity. There were however, signs that these analogues could still have undesired cellular effects because they also increased IC at 72 hours [18]. Disruption of the leucine zipper motif in PA-3 reduced the α-helical content from 75% to 8% which was reflected in the significant attenuation in cytotoxicity in Caco-2 cells. PA-4 had 300-fold reduction in hemolytic activity compared with native melittin [17, 45], and it was the least cytotoxic analogue tested here. Similar to PA-1-to-PA-3, the reduced capacity of PA-4 to form α-helical and multimeric structures abrogated its cytolytic action. The PA-4 truncation also reduced the hydrophobicity of melittin by the greatest percentage as measured by elution from a reverse phase HPLC separation (Table I and [42]). Melittin has also been investigated as a drug delivery agent that can boost permeation of poorly absorbed drugs across the intestinal epithelium [46]. The same structural modifications reduced its capacity to promote drug permeation through transcellular pathways. The current data corroborates these findings, since PMP changes are characteristic of agents that act transcellularly [42].

In a recent HCA toxicity assessment we also evaluated the cytotoxicity of patulin, another toxin that has been found to alter permeability in Caco-2 cells [47]. Patulin is a small polyketide that is structurally distinct from melittin, yet comparison is relevant in the context of antimicrobial action and effects on intestinal permeability [47, 48]. The same concentrations of melittin and patulin affected PMP, IC and NA, but melittin

perturbed MMP at a 2-fold lower concentration. Patulin reduced CN at nearly 4-fold lower concentrations than melittin, indicating that the mycotoxin is more cytotoxic than melittin in Caco-2 cells [47].

Direct comparison of HCA cytotoxicity assessment with MTT in Caco-2 can be made for melittin, PA-1 and PA-2. Their respective IC_{50} values in the MTT assay were 2.5μM, 8.6μM and 11.2μM at 24 hours [42]. This was in contrast to 1.6μM (MMP), 6.3μM (PMP) and 3.1μM (MMP) by HCA screening at 24 hours. The HCA also highlights that while the cytotoxicity of each peptide analogue is reduced, they can still influence IC which suggests they could have undesired effects *in vivo*; this was something that the MTT assay did not flag. Also noteworthy is that HCA was more sensitive than the MTT assay, despite melittin's actions on mitochondria, the target of enzymes for the MTT assay. The current HCA assay has also highlighted effects of melittin and its analogues on cellular parameters at concentrations below those that are hemolytic. Advancement of these peptides to preclinical assessment based purely on haemolysis is therefore not recommended and further study is required to establish their therapeutic potential and safety.

In conclusion, melittin is cytotoxic to Caco-2 cells and uses complex mechanisms involving dissipation of MMP, elevation of IC and increased PMP. Selected structural modifications that attenuated the hemolytic action of melittin also reduced its cytotoxicity in Caco-2 cells. There was evidence from HCA that these analogues do not have as high a degree of separation between their antimicrobial action and toxicity, as previously

demonstrated by haemolysis. The data for each peptide indicates that cytotoxicity primarily involves membrane perturbation. HCA screening is therefore a powerful tool that permits sensitive and specific detection of cytotoxicity in target cells and aids understanding of a wide range of cellular processes that could be affected by AMPs.

ACKNOWLEDGEMENTS

REFERENCES

[1]   Zhang L, Falla TJ. Cationic antimicrobial peptides - an update. Expert Opin Investig Drugs 2004;13:97-106.
[2]   Settanni L, Corsetti A. Application of bacteriocins in vegetable food biopreservation. Int J Food Microbiol 2008;121:123-38.
[3]   Zhang L, Harris S, Falla TJ. Taoiip, in: Gallo, R. (Ed.), Antimicrobial peptides in human health and disease, Horizon Biosciences, UK 2005, chapter 12 pp. 331-360.
[4]   Zasloff M, The commercial development of the antimicrobial peptide pexiganin, in: Lohner K., (Ed), Development of novel antimicrobial agents: emerging strategies, Horizon Press, UK, 2001, Chapter 17 pp. 261-270.
[5]   Islam K, Hawser SP. MSI-78 Magainin Pharmaceuticals. IDrugs 1998;1:605-9.
[6]   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 U S A 1987;84:5449-53.
[7]   Yang D, Oppenheim JJ, Multiple functions of antimicrobial peptides in host immunity. In Hancock RD, D. (eds) in Mammalian Host Defence Peptides. (Cambridge Univ. Press, New York, AMCM Series, 2004), pp39-68.
[8]   Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 2005;3:238-50.
[9]   O'Brien P, Haskins JR, In vitro cytotoxicity assessment, In Taylor DL, Haskins JR, Giuliano KA (Eds.) High content screening (Methods in molecular biology), Humana Press, New Jersey, USA, 2007 Vol. 356 pp 415-425.
[10]   Blondelle S, Synthetic combinatorial libraries: an emerging approach towards identification of novel antibiotics in: Lohner K., (Ed), Development of novel

antimicrobial agents: emerging strategies, Horizon Press, UK, 2001, Chapter 17 pp. 261-270.

[11]   Blondelle SE, Houghten RA. Hemolytic and antimicrobial activities of the twenty-four individual omission analogues of melittin. Biochemistry 1991;30:4671-8.

[12]   Tosteson MT, Holmes SJ, Razin M, Tosteson DC. Melittin lysis of red cells. J Membr Biol 1985;87:35-44.

[13]   Maher S, McClean S. Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro. Biochem Pharmacol 2006;71:1289-98.

[14]   Raghuraman H, Chattopadhyay A. Melittin: a membrane-active peptide with diverse functions. Biosci Rep 2007;27:189-223.

[15]   Maher S, McClean S. Melittin exhibits necrotic cytotoxicity in gastrointestinal cells which is attenuated by cholesterol. Biochem Pharmacol 2008;75:1104-14.

[16]   Wang C, Chen T, Zhang N, Yang M, Li B, Lu X, et al. Melittin, a major component of bee venom, sensitizes human hepatocellular carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by activating CaMKII-TAK1-JNK/p38 and inhibiting IkappaBalpha kinase-NFkappaB. J Biol Chem 2009;284:3804-13.

[17]   Subbalakshmi C, Nagaraj R, Sitaram N. Biological activities of C-terminal 15-residue synthetic fragment of melittin: design of an analog with improved antibacterial activity. FEBS Lett 1999;448:62-6.

[18]   Asthana N, Yadav SP, Ghosh JK. Dissection of antibacterial and toxic activity of melittin: a leucine zipper motif plays a crucial role in determining its hemolytic activity but not antibacterial activity. J Biol Chem 2004;279:55042-50.

[19]   O'Brien P, High content analysis of sublethal cytotoxicity in human HepG2 hepatocytes for assessing potential and mechanism for chemical- and drug-induced human toxicity, In: Haney, SA, (Ed.), High content screening (science, techniques, and applications), Wiley, New Jersey, 2008, pp. 293-315.

[20]   Xu JJ, Diaz D, O'Brien PJ. Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. Chem Biol Interact 2004;150:115-28.

[21]   O'Brien PJ, Irwin W, Diaz D, Howard-Cofield E, Krejsa CM, Slaughter MR, et al. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Arch Toxicol 2006;80:580-604.

[22]   Rawlinson LA, O'Brien PJ, Brayden DJ. High content analysis of cytotoxic effects of pDMAEMA on human intestinal epithelial and monocyte cultures. J Control Release 2010 146:84-92.

[23]   Merrifield B. Solid phase synthesis. Science 1986;232:341-7.

[24]   Carpino LA, Han GY. 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. J Org Chem 1972;37:3404-9.

[25]   Hammami R, Zouhir A, Le Lay C, Ben Hamida J, Fliss I. BACTIBASE second release: a database and tool platform for bacteriocin characterization. BMC Microbiol 2010 10:22.

[26] Wang G, Li X, Wang Z. APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 2009;37:D933-7.

[27] Field D, Quigley L, O'Connor PM, Rea MC, Daly K, Cotter PD, et al. Studies with bioengineered Nisin peptides highlight the broad-spectrum potency of Nisin V. Microb Biotechnol 2010 3:473-86.

[28] Blondelle SE, Houghten RA. Novel antimicrobial compounds identified using synthetic combinatorial library technology. Trends Biotechnol 1996;14:60-5.

[29] Lee GL, Hait WN. Inhibition of growth of C6 astrocytoma cells by inhibitors of calmodulin. Life Sci 1985;36:347-54.

[30] Helenius A, Simons K. Solubilization of membranes by detergents. Biochim Biophys Acta 1975;415:29-79.

[31] Lichtenberg D. Characterization of the solubilization of lipid bilayers by surfactants. Biochim Biophys Acta 1985;821:470-8.

[32] Chu ST, Cheng HH, Huang CJ, Chang HC, Chi CC, Su HH, et al. Phospholipase A2-independent Ca2+ entry and subsequent apoptosis induced by melittin in human MG63 osteosarcoma cells. Life Sci 2007;80:364-9.

[33] Mix LL, Dinerstein RJ, Villereal ML. Mitogens and melittin stimulate an increase in intracellular free calcium concentration in human fibroblasts. Biochem Biophys Res Commun 1984;119:69-75.

[34] Li B, Gu W, Zhang C, Huang XQ, Han KQ, Ling CQ. Growth arrest and apoptosis of the human hepatocellular carcinoma cell line BEL-7402 induced by melittin. Onkologie 2006;29:367-71.

[35] Nishikawa H, Kitani S. Gangliosides inhibit bee venom melittin cytotoxicity but not phospholipase A(2)-induced degranulation in mast cells. Toxicol Appl Pharmacol. 2011 252(3):228-36

[36] Baker KJ, East JM, Lee AG. Mechanism of inhibition of the Ca(2+)-ATPase by melittin. Biochemistry 1995;34:3596-604.

[37] Duke RC, Witter RZ, Nash PB, Young JD, Ojcius DM. Cytolysis mediated by ionophores and pore-forming agents: role of intracellular calcium in apoptosis. Faseb J 1994;8:237-46.

[38] Dempsey CE. The actions of melittin on membranes. Biochim Biophys Acta 1990;1031:143-61.

[39] Higashijima T, Ross EM. Mapping of the mastoparan-binding site on G proteins. Cross-linking of [125I-Tyr3,Cys11]mastoparan to Go. J Biol Chem 1991;266:12655-61.

[40] King TP, Jim SY, Wittkowski KM. Inflammatory role of two venom components of yellow jackets (Vespula vulgaris): a mast cell degranulating peptide mastoparan and phospholipase A1. Int Arch Allergy Immunol 2003;131:25-32.

[41] Pandey BK, Srivastava S, Singh M, Ghosh JK. Inducing toxicity by introducing a leucine zipper like motif in frog antimicrobial peptide, magainin 2. Biochem J. 2011 436(3):609-20

[42] Maher S, Devocelle M, Ryan S, McClean S, Brayden DJ. Impact of amino acid replacements on in vitro permeation enhancement and cytotoxicity of the intestinal absorption promoter, melittin. Int J Pharm 387:154-60.

[43]   Oren Z, Shai Y. Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: structure-function study. Biochemistry 1997;36:1826-35.

[44]   Zhu WL, Song YM, Park Y, Park KH, Yang ST, Kim JI, et al. Substitution of the leucine zipper sequence in melittin with peptoid residues affects self-association, cell selectivity, and mode of action. Biochim Biophys Acta 2007;1768:1506-17.

[45]   Yan H, Li S, Sun X, Mi H, He B. Individual substitution analogs of Mel(12-26), melittin's C-terminal 15-residue peptide: their antimicrobial and hemolytic actions. FEBS Lett 2003;554:100-4.

[46]   Maher S, Wang X, Bzik V, McClean S, Brayden DJ. Evaluation of intestinal absorption and mucosal toxicity using two promoters. II. Rat instillation and perfusion studies. Eur J Pharm Sci 2009;38:301-11.

[47]   Maher S, Collin D, Walsh E, Mohan H, Edvardsson A, Winter D, et al. Effect of the mycotoxin patulin on permeability of isolated rat colonic mucosa and high content analysis in Caco-2 cells. Toxicol lett 2011 (Submitted)

[48]   Maher S, Kennelly R, Bzik VA, Baird AW, Wang X, Winter D, et al. Evaluation of intestinal absorption enhancement and local mucosal toxicity of two promoters. I. Studies in isolated rat and human colonic mucosae. Eur J Pharm Sci 2009;38:291-300.

Fig. 1: Schematic representation of HCA. Instrumentation exploits the combined power of high throughput multi-well automation with epifluorescence microscopy as well as the capability of multi-wavelength fluorescent dye analysis. (a) Cells treated in multiwell plates and stained with fluorescent dye cocktail, (b) plate is docked in the live cell

chamber at 37ºC for kinetic analysis, (c) images are acquired at 10x or 20x magnification, (d-e) images are then subjected to segmentation analysis and large volumes of quantitative data are generated for both individual cells and cell populations; this is used in tandem with the fluorescent micrographs taken during acquisition.

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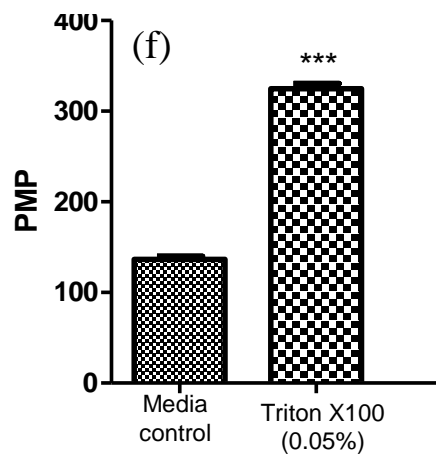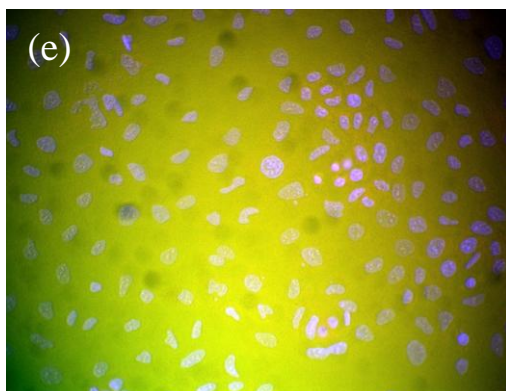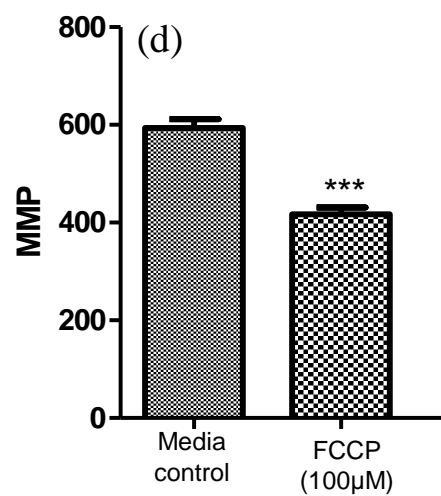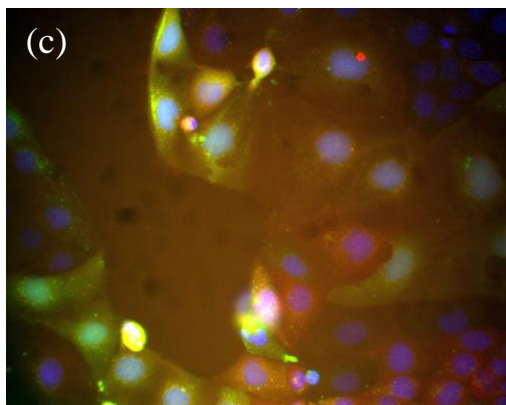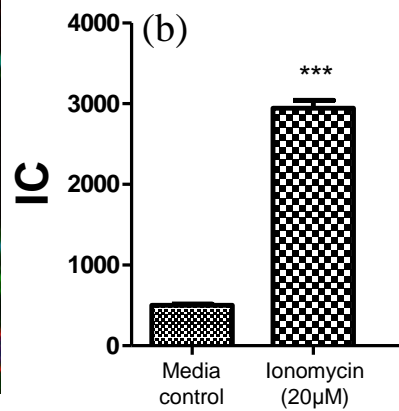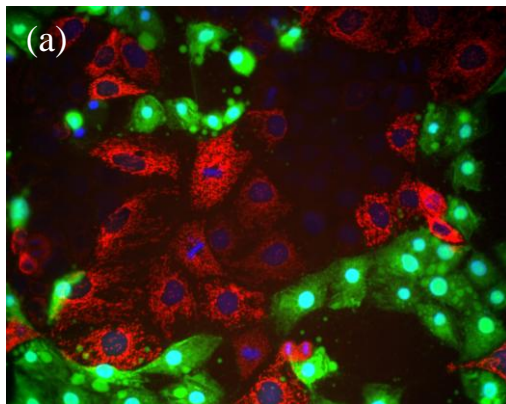
Fig. 2: (a-b) ionomycin (20µM), (c-d) Triton X100 (0.05% (v/v)) and (e-f) FCCP (100µM) for determination of optimal fluorescence exposure times during HCA method validation and within each experiment to verify the cell capacity to respond to each measure metric. Panels (a, c, e) are representative photomicrographs and (b, d, f) are collated, quantitative fluorescence intensity (y-axis) data processed for each field of measurement. Each fused fluorescent micrograph was acquired at 20x objective magnification and pseudo-coloured accordingly; Hoechst® (nuclear staining; blue), Fluo-4 AM (green; measure of IC), TOTO®-3 iodine (magenta; measure of PMP) and TMRM (red; measure of MMP). Each value represents the mean fluorescence intensity ± SEM. (*** P< 0.0001)

Melittin   PA-01   PA-02   PA-03   PA-04

Mean Fluorescence Intensity

NA

NI

MMP

IC

PMP

CN

Concentration [log M]
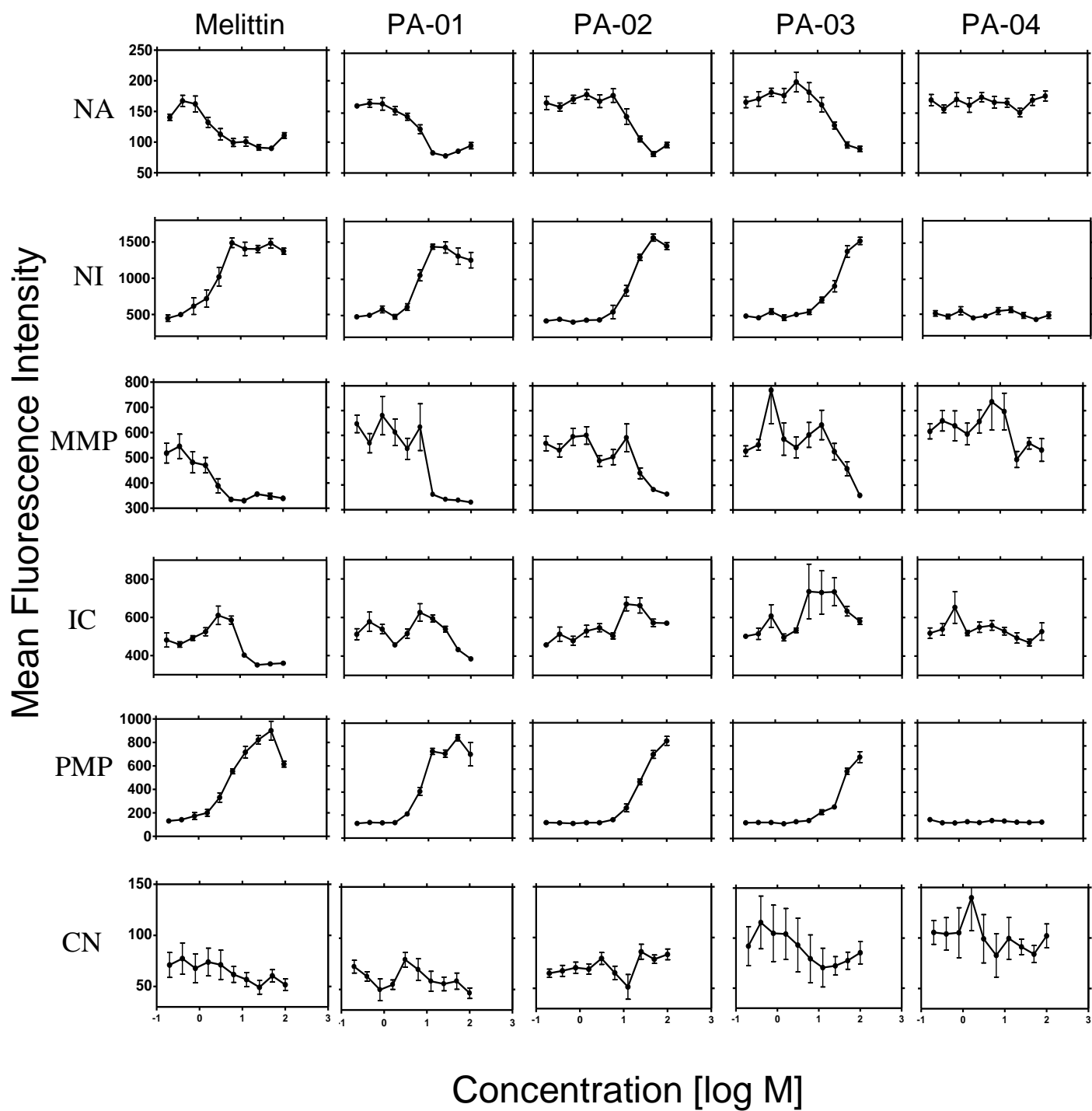
Fig. 3: Cytotoxicity of melittin and analogues on Caco-2 cells after 24 hour incubation. Rows are CN, NA, NI, MMP, IC, and PMP. Columns are (i) melittin, (ii) PA-01, (iii) PA-02, (iv) PA-03 and (v) PA-04. Each value represents the mean fluorescence intensity ± SEM.
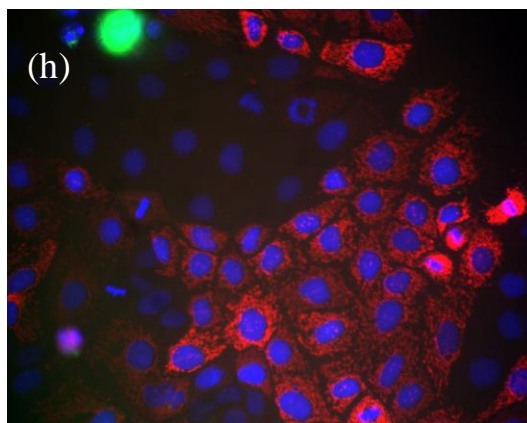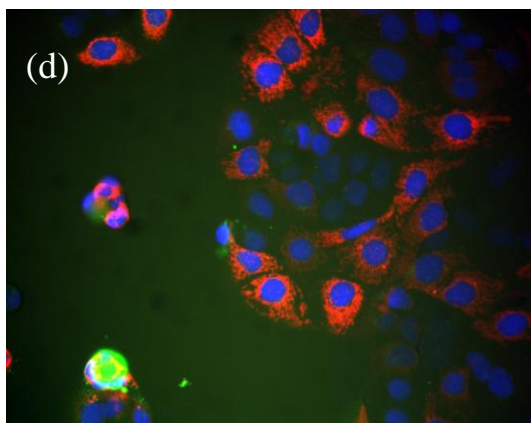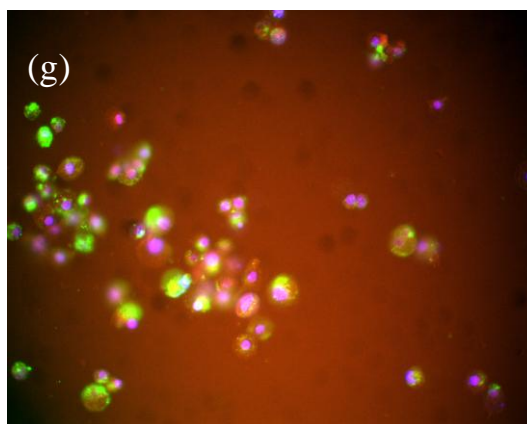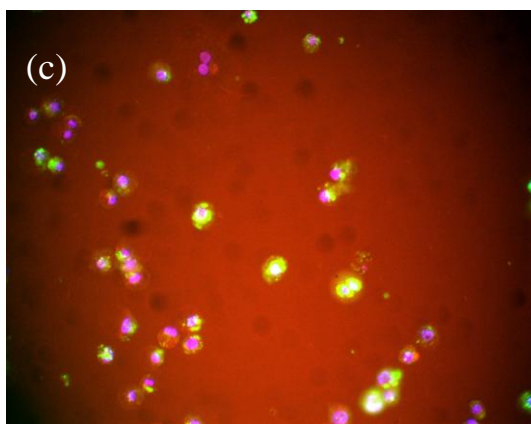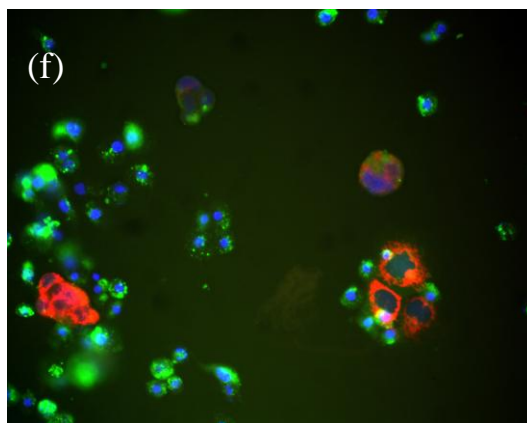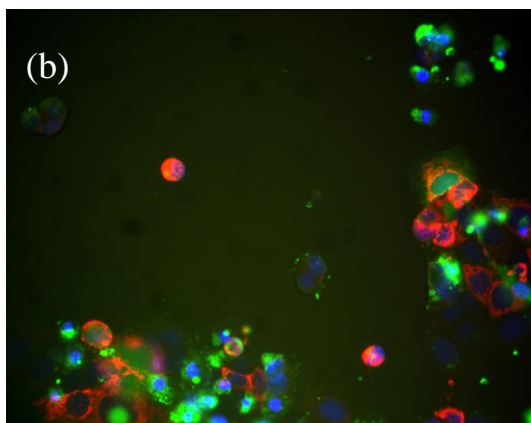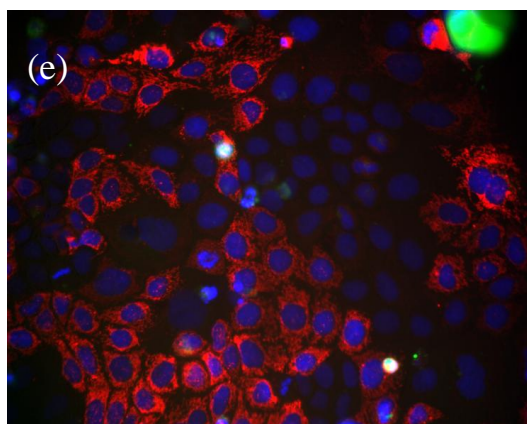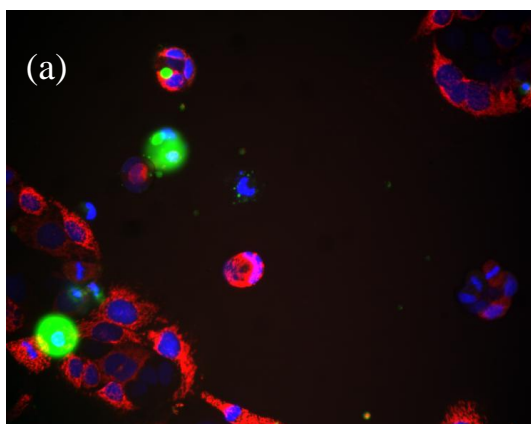
Fig. 4: Selected photomicrographs representing metrics affected by melittin and PA-04 on Caco-2 cells over time. Panels (a-d) show Caco-2 cells treated for 24 hours with (a) media (b) melittin (3.125μM), (c) melittin (6.3μM), (d) PA-04 (100μM). Panels (e-h) show Caco-2 cells treated for 72 hours with (e) media (f) melittin (3.125μM), (g) melittin (6.3μM), (h) PA-04 (100μM). Each fused fluorescent micrograph was acquired at 20x objective magnification and pseudo-coloured accordingly; Hoechst® (nuclear staining; blue), Fluo-4 AM (green; measure of IC), TOTO®-3 iodine (magenta; measure of PMP) and TMRM (red; measure of MMP).
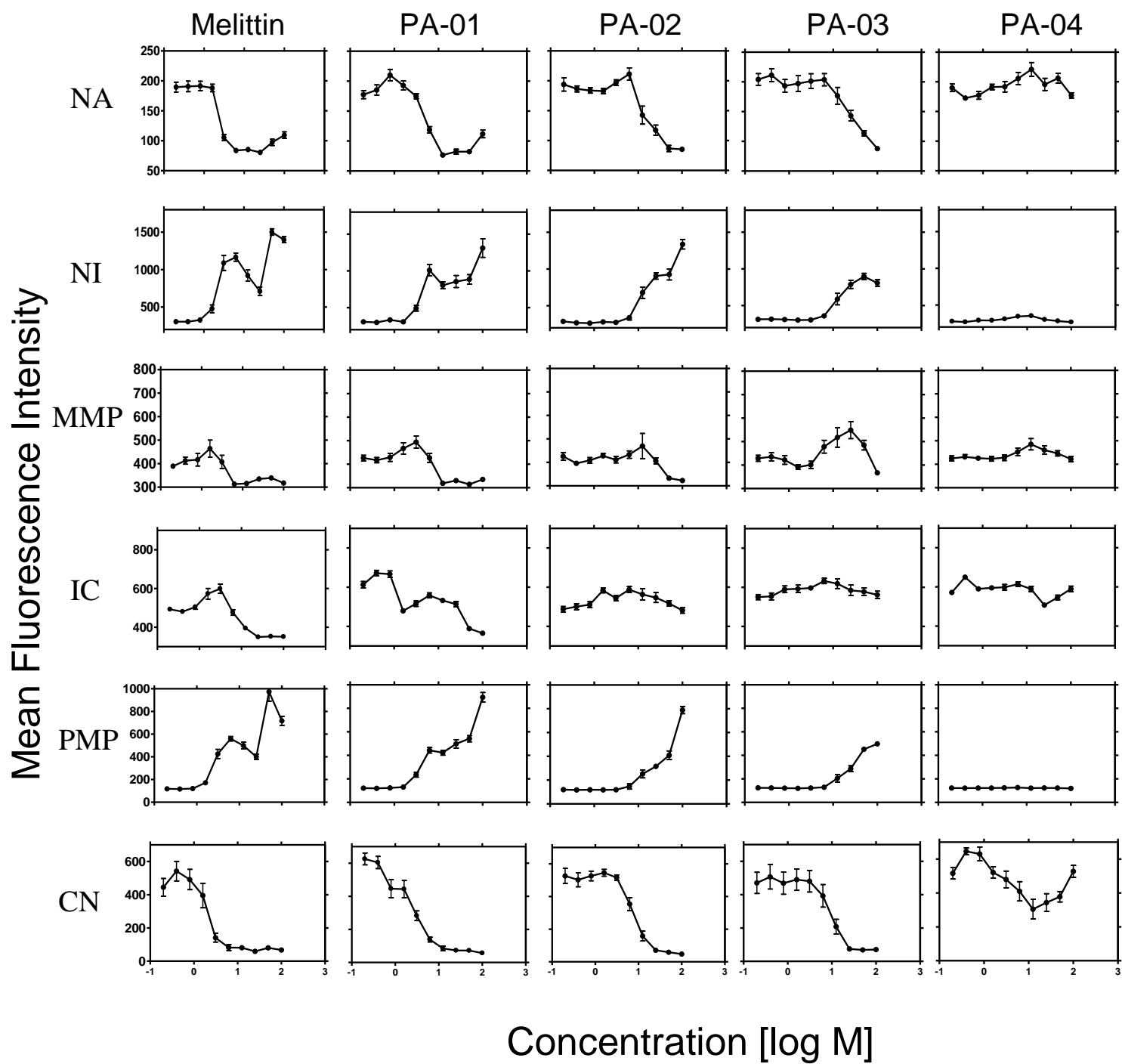
Fig. 5: Cytotoxicity of melittin and analogues on Caco-2 cells after 72 hour incubation. Rows are (a) CN (b) NA, (c) NI, (d) MMP, (e) IC and (f) PMP. Columns are (i) melittin,

(ii) PA-01, (iii) PA-02, (iv) PA-03 and (v) PA-04. Each value represents the mean

fluorescence intensity $\pm$ SEM.

Table I: Properties of melittin and four structural analogues, including; sequence, molecular weight, designation, relative hydrophobic index and cytotoxic inhibitory concentration (IC_{50} (μM)) value as determined by MTT assay in Caco-2 cells following 24 hour incubation (modified from [19]).

Peptide Sequence	Molecular Weight (Da)	Modification (designation)	Relative Hydrophobic Index	IC_{50} from MTT (24 h) (μM)
GIGAVLKVLTTGLPALISWIKRKRQQ	2846	Synthetic melittin	1	2.48
GIGAVLKVLTTGLP─LISWIKRKRQQ	2775	* (PA-1)	0.94	8.6
GIGAVLKVLTTGLPALIS─IKRKRQQ	2660	* (PA-2)	0.89	11.2
GIGAV**A**KVLTTG**A**PALISWIKRKRQQ	2762	** (PA-3)	0.84	>12
──────────GLPALISWIKRKRQQ	1793	* (PA-4)	0.74	>12

*   Amino acid deletion
**   Amino acid substitution

Table II: Comparison of the cytotoxic effect of melittin and four structural analogues on

Caco-2 cells following 24 and 72 hour incubation. Each concentration (μM) represents

the first concentration where there was an effect on the measured metric. Arrows (↑) indicate the effect was an increase in value (* P < 0.01; ** <0.001 and *** P < 0.0001).

Peptide Analogue	NA		NI		MMP		IC		PMP		CN	
	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h
Melittin	1.6 *	3.125 ***	3.125(↑) ***	1.6 (↑) *	1.6 *	6.25 *	3.125(↑) ***	1.6(↑) ***	3.125(↑) **	3.125(↑) ***	-	3.125 ***
PA-1	6.25 **	6.25 ***	6.25(↑) ***	6.25(↑) ***	12.5 *	12.5 ***	100 **	0.4(↑) ***	6.25(↑) ***	3.125(↑) **	-	3.125 **
PA-2	12.5 *	12.5 ***	12.5(↑) ***	12.5(↑) ***	3.125 **	50*	12.5(↑) ***	1.6(↑) ***	12.5(↑) ***	12.5(↑) ***	-	12.5 ***
PA-3	50 ***	25 ***	12.5(↑) **	12.5(↑) ***	100 *	100 ***	6.25(↑) *	1.6(↑) *	12.5(↑) *	12.5(↑) ***	-	25 ***
PA-4	-	-	-	6.25(↑) *	-	-	-	0.2(↑) **	-	-	-	-