Mechanism of *in vitro* cytotoxicity of antimicrobial peptides in combination with stabilizing excipients

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B.Pharm

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meraki (μεράκι)

(n.) the soul, creativity, or love put into something; the essence of yourself that is put into your work

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Bye Felicia

List of Abbreviations

3D	Three dimensional
AMP	Antimicrobial peptide
ATP	Adenosine triphosphate
Caco2	Human colorectal adenocarcinoma cell line
CDDEP	Centre of Disease Dynamics, Economics and Policy
CPP	Cell penetrating peptide
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species
FBS	Foetal bovine serum
FDA	Food and Drug Administration
GI	Gastrointestinal
GRAS	Generally Regarded As Safe
HCl	Hydrochloric acid
HepG2	Human hepatocellular liver carcinoma cell line
hLF1-11	Human-derived Lactoferrin 1-11
HREC	Health Research Ethics Committee
IM	Intramuscular
IC_{50}	Half maximal inhibitory concentration
ISO	International Organisation for Standardisation
IV	Intravenous

LDH	Lactate dehydrogenase
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mV	Millivolts
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide-hydrogen
nM	Nanomolar
NASEM	National Academies of Sciences, Engineering and Medicine
NEAA	Non-essential amino acids
OECD	Organisation for Economic Co-Operation and development
Pen/Strep	Penicillin/streptomycin
PBS	Phosphate buffered saline
P/L	Peptide/ lipid
RNA	Ribonucleic acid
SC	Subcutaneous
SFM	Serum free medium
VRE	Vancomycin-resistant *Enterococcus*
WHO	World Health Organization
µM	Micromolar

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Abstract

Emerging antibiotic resistance poses a critical public health threat, particularly the alarming increase of resistant bacteria commonly associated with gastrointestinal (GI) infections. Antimicrobial peptides (AMPs) are a diverse class of peptides produced by many organisms as a first line defence mechanism against microbial threats and show promising potential as alternatives to conventional antibiotics. This class includes melittin and mastoparan, well studied cationic α-helical toxins isolated from bee and wasp venom respectively. Nisin Z, on the other hand, is also an AMP and classified as a cationic bacteriocin produced by bacterial strains of *Lactococcus lactis.* Previous research on the antibacterial effects of these peptides against GI pathogens, such as *Staphylococcus aureus* and *Escherichia coli,* support the therapeutic application as pharmaceuticals. However, clinical advancement of antimicrobial peptides is limited by the associated toxicity towards mammalian cells and the lack of sufficient data on this cytotoxicity. Furthermore, most peptide formulations include excipients to enhance absorption or increase the stability of the peptide and these excipients also have the risk of interacting with the peptide in such a way as to affect the cytotoxicity thereof.

Therefore, the aim of this study was to investigate and characterise the mechanisms of *in vitro* cytotoxicity of two venom peptides, melittin and mastoparan, and the bacteriocin peptide, nisin Z, toward the human hepatocellular liver carcinoma cell line (HepG2) and human epithelial colorectal adenocarcinoma cell line (Caco-2). In addition, this study aims to evaluate and describe the varying cytotoxicity of AMPs in combination with peptide stabilising excipients (L-glutamic acid, chitosan and polysorbate 80) when compared to individual peptide toxicity. Cytotoxicity was investigated and determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and the lactate dehydrogenase (LDH) assay. Neutral red staining was additionally employed to visually illustrate the varying cytotoxic effect of combination treatments compared to viable cells.

It was determined that treatments with melittin:excipient combinations resulted in lower cytotoxicity towards HepG2 and Caco-2 cells relative to melittin alone treatment of 1 µM. Caco-2 cells treated with mastoparan:L-glutamic acid combinations resulted in higher cytotoxicity when compared to both individual mastoparan and L-glutamic acid treatments with 61.80±4.97%, 54.02±5.79% and 53.89±6.65% at 40 µM: 0.75 mg/ml, 40 µM: 1.5 mg/ml and 40 µM: 3 mg/ml respectively. Chitosan in combination with mastoparan similarly displayed cytotoxicity towards Caco-2 cells with respective values of 54.41±3.95%, 57.17±4.28% and 55.71±7.18% at 40 µM: 5 mg/ml, 40 µM: 10 mg/ml and 40 µM: 20 mg/ml treatments. Nisin Z in combination with polysorbate 80 displayed high cytotoxicity in both HepG2 and Caco2 cells. The cytotoxicity was determined as 76.14±2.15%, 72.78±6.08% and 59.14±11.07% at

370 µM: 2 mg/ml, 370 µM: 4 mg/ml and 370 µM: 8 mg/ml, respectively towards HepG2 cells and 72.90±6.70%, 80.49±3.92% and 87.73±3.03% for 370 µM: 2 mg/ml, 370 µM: 4 mg/ml and 370 µM: 8 mg/ml, respectively towards Caco-2 cells. The LDH assay suggested that melittin and mastoparan induce necrotic cell death in HepG2 and Caco-2 cells. Mastoparan in combination with L-glutamic acid and chitosan is furthermore suggested to cause necrosis in Caco-2 cells, whereas nisin Z:polysorbate 80 combinations induced cell death possibly by means of apoptosis in both cell lines.

It was concluded that peptide stabilising excipients in combination with melittin decreases the individual cytotoxicity of melittin. L-glutamic acid and chitosan, individually, in combination with mastoparan induced a higher cytotoxic effect than mastoparan alone towards Caco-2 cells. Finally, polysorbate 80 in combination with nisin Z was the most cytotoxic combination that displayed high cell death in both cell lines. Determining the cytotoxicity and additionally the antibacterial effect of AMPs in combination with peptide stabilising excipients can impact the clinical advancement and application of these novel antibiotics in the treatment of threatening GI infections.

Key words: Antimicrobial peptides, melittin, mastoparan, nisin Z, L-glutamic acid, chitosan, polysorbate 80, cytotoxicity, necrosis, apoptosis

Chapter 1

Introduction and aim of study

1.1 Introduction and aim of study

Antibiotics are seen as the pillars of modern medicine. However, emerging, increasing resistance to standard and last resort antibiotics are posing critical public health threats. Among Gram-positive pathogens, a global pandemic of gastrointestinal (GI) infections caused by resistant *Staphylococcus aureus* and *Enterococcus* species currently present the most urgent threat (Ventola, 2015:280). The decline in antibiotic research and development over the past few decades clearly highlight the need for new antibacterial agents or novel alternatives (Steckbeck *et al*., 2014:11). Antimicrobial peptides (AMPs) are a diverse class of naturally occurring molecules that function as the first line of defence against microbial threats in many organisms. Numerous studies have shown these AMPs to be promising and respectable alternatives to conventional antibiotics (Bahar & Ren, 2013:1543; Baltzer & Brown, 2011:229; Marr *et al*., 2006:468; Parisien *et al*., 2008:1). Potent antibacterial activity of numerous AMPs have been tested and proven towards various GI infection-causing pathogens and include various strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and various *Shigella* and *Salmonella* species (Ebbensgaard *et al*., 2015).

AMPs are a diverse class of molecules generally defined as oligopeptides consisting of no more than 50 amino acid residues with a net positive charge at physiological pH and possess high amphipathic properties (Steckbeck *et al*., 2014:12). Natural AMPs are produced in both prokaryotic bacteria and eukaryotic organisms, including protozoa, fungi, plants, insects, vertebrates and humans. As a result of various cells producing AMPs, these peptides are found in numerous sources such as epithelial cells and tissues of various organs, including the skin and even in the venom of insects (Bahar & Ren, 2013:1544). In addition, they are furthermore referred to as host defence peptides for their involvement as regulators and effectors of the innate immune system of higher organisms. Generally, AMPs can be characterised by their predominant secondary structures with cationic α-helical and β-sheet structures being the most studied and thoroughly characterised (Baltzer & Brown, 2011:229).

Where conventional antibiotics target specific cellular activities (e.g., deoxyribonucleic acid [DNA] or protein synthesis), most AMPs initially target the highly charged lipopolysaccharide within the cell membrane, which is universal in all microorganisms (Bahar & Ren, 2013:1545). The mechanisms of action of these membrane-active AMPs briefly include: the carpet like model (induces a detergent-like effect), the aggregate channel model (formation of unstructured aggregates in the membrane leading to pore formation), toroidal pore model (induces an inward transmembrane fold in the membrane) and the barrel-stave model (aggregation of a barrel-like ring in the membrane, forming an aqueous pore) (Bradshaw, 2003:234). Although direct cell membrane interaction is required for the antimicrobial activity of

several AMPs, many also have intracellular targets. Intracellular activity of AMPs can inhibit DNA, cell wall or protein synthesis, inhibit protease of microbes, inhibit ribonucleic acid (RNA) polymerase or activate autolysin proteins inside the target cell additionally to the membrane active mechanisms (Bahar & Ren, 2013:1550; Parisien *et al*., 2008:6).

Owing to the above mentioned mechanisms, cationic AMPs exert a broad-spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, viruses, protozoa and fungi (Fjell *et al*., 2012:37; Marr *et al.,* 2006:468). In contrast, AMPs also have the ability to interact with host cells that result in cytolytic and haemolytic activity contributing to their cytotoxicity and limiting their therapeutic potential. Where interaction with unicellular bacteria causes the death of the organism itself, interaction with mammalian cells mostly leads to necrotic cell death. Although apoptotic cell death has similarly been proven, it mostly occurs in cancer cells (Aoki & Ueda, 2013:1060; Gaspar *et al*., 2013).

Many venom and bacteriocin peptides from this class of cationic AMPs show promising antibacterial activity to specifically combat infectious agents in the GI tract (Hassan *et al*., 2012:729). Melittin is the principal toxin in the venom of the European honey bee, *Apis mellifera*. It is a small linear cationic peptide with a net charge of +6 at physiological pH and possesses amphipathic properties. Melittin is composed of a known 26 amino acid sequence (Table 1.1) with an α-helical conformation. This peptide is highly membrane active and exerts promising anti-Gram-positive, anti-Gram-negative, antiviral, antifungal, antiparasitic and antitumor effects (Gajski *et al*., 2016:57; Raghuraman & Chattopadhyay, 2007:190). Melittin also shows favourable antibacterial effects against specific GI pathogens and include *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Salmonella* species (Ebbensgaard *et al*., 2015).

Mastoparan is a toxic component in the venom of the Korean Yellow Jacket social wasp - *Vespula lewisii*. It is also a cationic peptide with a net charge of +4 at physiological pH and comprises an α-helical structural configuration. Mastoparan is composed of 14 amino acid residues (Table 1.1) and possesses additional cell penetrating properties. This peptide exhibits effective antimicrobial activity against various Gram-positive and Gram-negative bacteria, cancer cells, fungi, protozoa and viruses (Irazazabal *et al*., 2016:2704; Moreno & Giralt, 2015:1137). The antibacterial effects of mastoparan have been studied and proven against various infectious agents in the GI tract, including *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Li *et al.,* 2000:205).

Nisin Z is a bacteriocin peptide produced by various strains of *Lactococcus lactis*, a non-pathogenic lactic acid bacterium. It is classified as a Type A (I) cationic lantibiotic and comprises 34 amino acid residues (Table 1.1). At physiological pH, nisin Z has a net positive

charge of +3 and is predominantly configured to β-sheet structures. While nisin Z only has antibacterial activity against Gram-positive species, it also exerts promising antitumor and anti-inflammatory effects (El-Jastimi & Lafleur, 1997:157; Shin *et al*., 2015:1450). Among GI pathogens, nisin Z exhibits antibacterial effects against *Enterococcus faecalis*, *Escherichia coli* and *Shigella* and *Salmonella* species (Maher & McClean, 2006:1291; Tong *et al*., 2014).

Table 1.1: Amino acid sequence of antimicrobial peptides (see Addendum A for amino acid abbreviations)

AMPs	Length	Amino acid sequence
Melittin	26	GIGAVLKVLTTGLPALISWIKRKRQQ
Mastoparan	14	INLKALAALAKKIL
Nisin Z	34	ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK

Although all three peptides display promising therapeutic potential against GI infectious pathogens; mastoparan, and especially melittin, show cytolytic and haemolytic activity toward eukaryotic cells. In contrast, nisin Z does not demonstrate any cytotoxicity towards a variety of mammalian cells, including red blood cells (Kindrachuk *et al.,* 2012:319). It is essential to understand how these AMPs activate various pathways to cause cell death and thereby determine the acute, sub-acute and chronic effects of these therapeutics. As cell death may be caused by apoptosis or necrosis, further investigation and clarification of these mechanisms of AMP toxicity on mammalian cell lines are important for the clinical advancement of oral or intravenous (IV) administered AMP drugs (Cummings *et al*., 2012:12.8.1).

The antimicrobial activity of AMPs relies on the insertion of the peptide into the target membrane in such a way to induce the formation of a transmembrane pore. Cell death will occur as a result of destabilisation and disruption of the membrane. AMPs mostly cause necrotic cell death, although apoptosis has been demonstrated. Necrosis in mammalian cells is mainly characterised by the loss of membrane integrity, cell lysis, leakage of the cytoplasmic cell contents and cell death. In contrast, during apoptotic death the cell membrane remains intact with very little release of the intercellular contents. Cells that undergo apoptosis eventually undergo secondary necrosis with loss of membrane integrity resulting in cell lysis (Fjell *et al*., 2012:38; Laverty & Gilmore, 2014).

Although the antimicrobial activity of AMPs has extensively been studied and characterised on various organisms and bacterial strains, there still remains a lack of sufficient data on the cytotoxicity effects thereof. The majority of the cytotoxicity studies that have been done mainly focus on the haemolytic activity towards red blood cells and do not investigate or characterise the necrotic cell death in mammalian cells. It is critical to determine and assess how AMPs interact with various mammalian cells, especially GI epithelial cells to be able to evaluate their

cytotoxicity and therapeutic potential. Necrosis in GI epithelial cells can be determined by the lactate dehydrogenase (LDH) assay, which determines the percentage cytotoxicity as a function of the effect that the peptide has on the plasma membrane integrity of the cells and subsequent lactate dehydrogenase release, if membrane damage occurred. In contrast, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay measures mitochondrial function and integrity to indirectly determine cytotoxicity, which can be indicative of apoptosis or necrosis in GI epithelial cells (Maher & McClean, 2006:1290).

AMPs are required to be specially formulated in order to be implemented as acceptable therapeutic alternatives for antibiotics. Currently, clinically used antibiotics are mostly administered orally or by means of IV injection for the treatment of GI infections. However, these two routes of administration pose some challenges for peptide based drugs such as AMPs. Pharmaceutical excipients are often added to peptide formulations to perform a specific function and aid in formulation limitations. Excipients in peptide formulations may assist in stabilising the peptide molecule or act as an absorption enhancer (Banga, 2015:219).

Many excipients can be included in peptide formulations, however, L-glutamic acid, chitosan and polysorbate 80 were included in this study. L-glutamic acid is an amino acid excipient and frequently used as a stabiliser in peptide formulations. It is anionic and lowers the pH of the solution resulting in increased peptide solubility. Chitosan is a natural polysaccharide and is often included as an absorption enhancer or viscosity enhancer. It is a cationic molecule which is used in peptide formulations to prevent aggregation. Polysorbate 80, on the other hand, is used as a surfactant in peptide formulations. It is non-ionic and prevents aggregation (Challener, 2015:s37; Kamerzell *et al*., 2011:1123).

It is possible, however, for an excipient to interact with the peptide, cause aggregation and affect the cytotoxic activity thereof by increasing or decreasing the effect. Many studies conducted on the cytotoxicity profile of AMP candidates often characterised the cytotoxicity of the peptide alone and not in combination with formulation additives. For improved implementation of potential drug candidates additional preclinical testing is necessary for specific drug-excipient combinations since the independent safety profile of either drug or excipient does not determine the overall safety profile of the formulation (Andrade *et al*., 2011:163; Kamerzell *et al*., 2011:1122). The cytotoxicity of these excipients in combination with the selected AMPs have not been studied or characterised in mammalian cells. HepG2, a human hepatocellular liver carcinoma cell line, and Caco-2, a human colorectal adenocarcinoma cell line, were chosen as crude *in vitro* representation of small intestinal- and liver cells.

Aim and objectives

The aim of this study was to investigate and characterise the mechanism of cytotoxicity of the AMPs, melittin, mastoparan and nisin Z alone, and in combination with peptide stabilising excipients, L-glutamic acid, chitosan and polysorbate 80 on mammalian cells.

The objectives of the study were to:

- Determine and characterise the cytotoxicity of venom peptides, melittin and mastoparan (cytotoxic from literature) on HepG2 and Caco2 cells by means of the MTT assay.
- Compare the cytotoxicity thereof with nisin Z (not cytotoxic from literature) on HepG2 and Caco2 cells by means of the MTT assay.
- Compare the mechanisms of the three peptides by means of mitochondrial function (MTT assay) versus membrane damage and LDH leakage (LDH assay).
- Determine and describe the varying effect on the cytotoxicity of the different peptide stabilising excipients when in combination with AMPs on HepG2 and Caco2 cells.

Ethics

An ethic application was submitted to the Health Research Ethics Committee (HREC) of the North-West University for *in vitro* cytotoxicity experiments done on HepG2 and Caco2 cells. The study and all experimental procedures were approved under Pharmacen.

Structure of dissertation

This dissertation begins with an introductory chapter, Chapter 1, which provides the background and justification for the research project along with the aim and objectives of the study. It is followed by the relevant literature overview in Chapter 2 and focuses on antimicrobial peptides as novel antibiotic therapeutics and formulation thereof, associated cytotoxicity of venom and bacteriocin peptides on mammalian cells and stabilising peptide excipients as formulation additives. In Chapter 3, the scientific methods used to determine *in vitro* cytotoxicity are described. The results and statistical analysis obtained from the *in vitro* experiments are illustrated in various graphs and are discussed in Chapter 4. Finally, Chapter 5 draws the final conclusion that summarises the results obtained in this study and offers recommendations for future research.

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Chapter 2

Literature study

2.1 The emergence of antibiotics and then resistance

Before antibiotics, in the early 1900's and during World War I, many people died from simple cuts, burns and giving birth, while infections like pneumonia, tuberculosis and syphilis caused some of the major fatalities. Soldiers would receive amputations after serious wounding in the field just to prevent wound infections that would ultimately cause death if left untreated. Upon the discovery of antibiotics, a type of antimicrobial drug which is used to prevent or treat bacterial infections, these miracle drugs was used to treat most fatal diseases, fight lethal infections and lower the death toll. Other diseases were addressed to such an extreme that some even became extinct, such as polio, yellow fewer and diphtheria with only some exceptions and outbreaks over the recent years (Aminov, 2010).

The first antibiotic, penicillin, was discovered in the late 1920's by Alexander Fleming. This finding led to the discovery of many novel antibiotic classes between 1950 and 1970 (Aminov, 2010; Davies & Davies, 2010:41). This era is still referred to as the "golden era of antibiotic discovery"; and antimicrobials have become the greatest discovery of the twentieth century (Aminov, 2010; Coates & Bergstrom, 2013:1079). However, after this period funding into antibiotic research and development in the pharmaceutical industry were on the decline. This resulted in no new discovery of other antibiotic classes available on the market to this present day (Coates & Bergstrom, 2013:1079). Heading into the new millennium in 2000, 15 of the 18 largest pharmaceutical companies abandoned the antibiotic field in the United States alone (Ventola, 2015:279).

Penicillin which was discovered in 1928, only became therapeutically available for use in 1943. Already in 1940, this antibiotic showed antibiotic resistance in the laboratory and once this antibiotic was widely used, resistant strains became prevalent in 1945 (Aminov, 2010; Davies & Davies, 2010:419). Figure 2.1 illustrates the year when some antibiotics were therapeutically introduced and the year when resistance was identified. During this period of emerging antibiotic resistant pathogens, a strategy was initiated which included research into possible modification of existing antibiotics to be more effective and have less sensitivity toward resistance mechanisms (Aminov, 2010). However, resistance to these heavy modified antibiotics arose shortly after they became therapeutically available.

Unfortunately, antibiotics have lost their effectiveness over the years due to the increase in resistant microbial strains (Lee, Hall *et al.,* 2016:25). Antibiotic resistance is now one of the critical health threats the world is facing in the 21st century, where first line and last resort antibiotics are failing because of this emerging phenomenon (WHO, 2014:69; Steckbeck *et al.*, 2014:11). Both the World Health Organisation (WHO) (2014:69) and The Centre of Disease Dynamics, Economics & Policy's (CDDEP) (2015:26) annual reports on antibiotic resistance

state that the irrational misuse and overuse of antibiotics in the health sector, and also in agriculture, are accelerating the natural progression of antibiotic resistance. Statistics show that between 2000 and 2010, antibiotic consumption in the health sector alone increased by 36% (Van Boeckel *et al.*, 2014:745), although research done by the Review on Antimicrobial Resistance Commission (2016:11) shows that antibiotic resistance is responsible for 700 000 deaths per year worldwide.

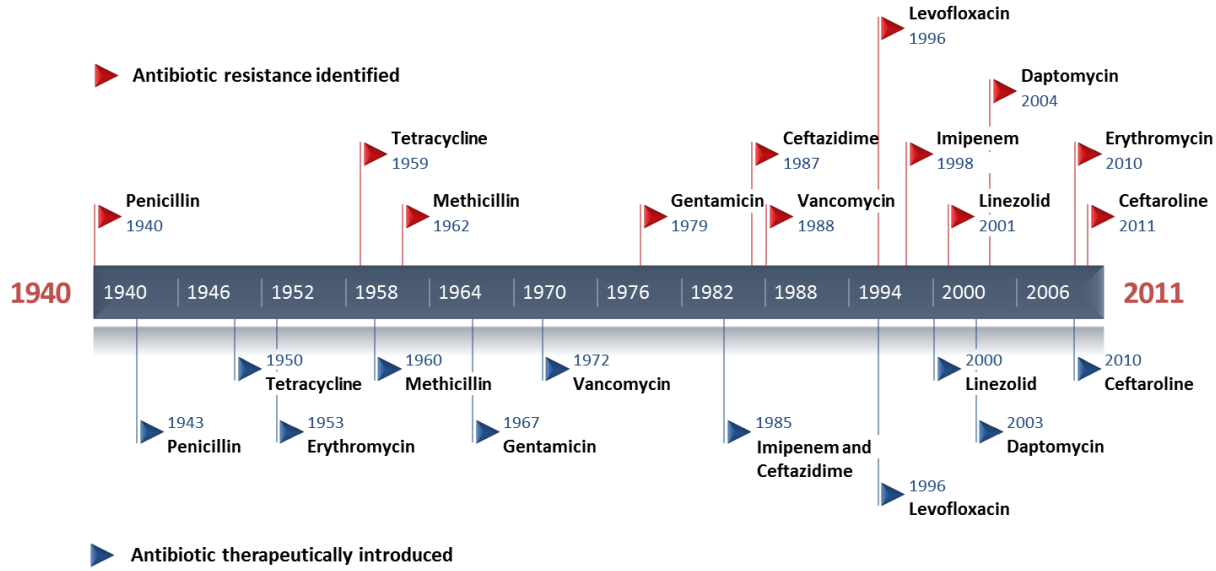


Figure 2.1: Timeline of when specific antibiotics were therapeutically introduced and when resistance was identified (adapted from Davies & Davies, 2010:419; Ventola, 2015:277)

Among resistant bacteria strains, Gram-positive *Staphylococcus aureus* and *Enterococcus* species currently pose a global pandemic threat. These pathogens are commonly associated with gastrointestinal (GI) infections of which more than 200 million cases are reported per year alone in the United States of America. In addition, worldwide statistics show that up to six million children die yearly due to GI infections (Ventola *et al.*, 2015:280). Pathogens responsible for GI infection have been prioritised by the WHO for urgent research and development into novel therapeutics; and include *Staphylococcus aureus, Helicobacter pylori* and *Salmonella* and *Shigella* species (WHO, 2014:13)

As a result, intensive clinical and non-clinical research are now being invested in by numerous companies and organisations to identify new and non-conventional antibiotic therapies due to the rising resistance against the limited number available antibiotics which possess similar modes of action over the same activity spectrum (Mahlapuu *et al.*, 2016). Further research specifically addressing alternative treatments for GI infections is of utmost importance as it exhibits concerning degrees of antibiotic resistance (Kim *et al.*, 2017:101).

2.1.1 Alternatives to conventional antibiotics

The attention of research on conventional antibiotics has shifted the last few years to potential alternative treatment options to address the emerging antibiotic resistance. As conventional antibiotics are mostly administered orally and/or intravenously (IV), the focus of new studies remains on alternative therapy options via these routes. Many new adverse approaches are being explored, including monoclonal antibody-based products, immune modulating biologicals, bacteriophage therapy with gene-editing enzymes and predatory bacteria, to name a few (Aminov, 2010; Da Cunha *et al*., 2017:235; Lin *et al*., 2017:165; Reardon, 2015:403; Wright & Brown, 2013:1086).

However, extensive research has proven antimicrobial peptides (AMPs) as respectable and promising alternative candidates for conventional antibiotics (Bahar & Ren, 2013:1544; Baltzer & Brown, 2011:229; Marr *et al.,* 2006:468; Parisien *et al.*, 2008:9). Pharmaceutical companies are researching and developing AMPs for the treatment of various GI infections as studies have proven antimicrobial activity against vancomycin-resistant *Enterococci* (VRE)*, Escherichia coli, Clostridium difficile, Helicobacter pylori* and *Bacillus* species (Maher & McClean, 2006:1290).

AMPs are a diverse class of naturally occurring molecules that function as a first line of defence against microbial threats in organisms (Bahar & Ren, 2013:1544; Parisien *et al.*, 2008:6; Steckbeck *et al*., 2014:11). They are virtually found in all organisms and display remarkable structural and functional diversity (Mahlapuu *et al*., 2016). Therapeutic use of AMPs is further supported by their diverse potential pharmacological applications, immunomodulatory properties and various advantages over conventional antibiotics. These advantages briefly include their remarkable broad-spectrum of activity, high potency, rapid speed of action and low propensity for bacterial resistance development (Baltzer & Brown, 2011:229; Marr *et al*., 2006:468).

2.2 Antimicrobial peptides

The discovery of AMPs date back to the 1940's and up to date more than 5 000 AMPs have been discovered from natural sources or have been synthetically produced (Bahar & Ren, 2013:1544; Lee, Hall *et al*., 2016:25). AMPs are best described as gene-encoded, ribosomally synthesised oligopeptides (Li *et al.,* 2012:208). They are a diverse class of molecules that can generally be defined as small peptides composed of 50 or less amino acid residues, which have a net positive charge at physiological pH and contain around 50% hydrophobic amino acids (Baltzer & Brown, 2011:229; Guilhelmelli *et al*., 2013:353). Figure 2.2 illustrates the

distribution of AMPs as a function of sequence length, net charge and hydrophobic content, as well as the distribution of amino acid residues in AMPs.

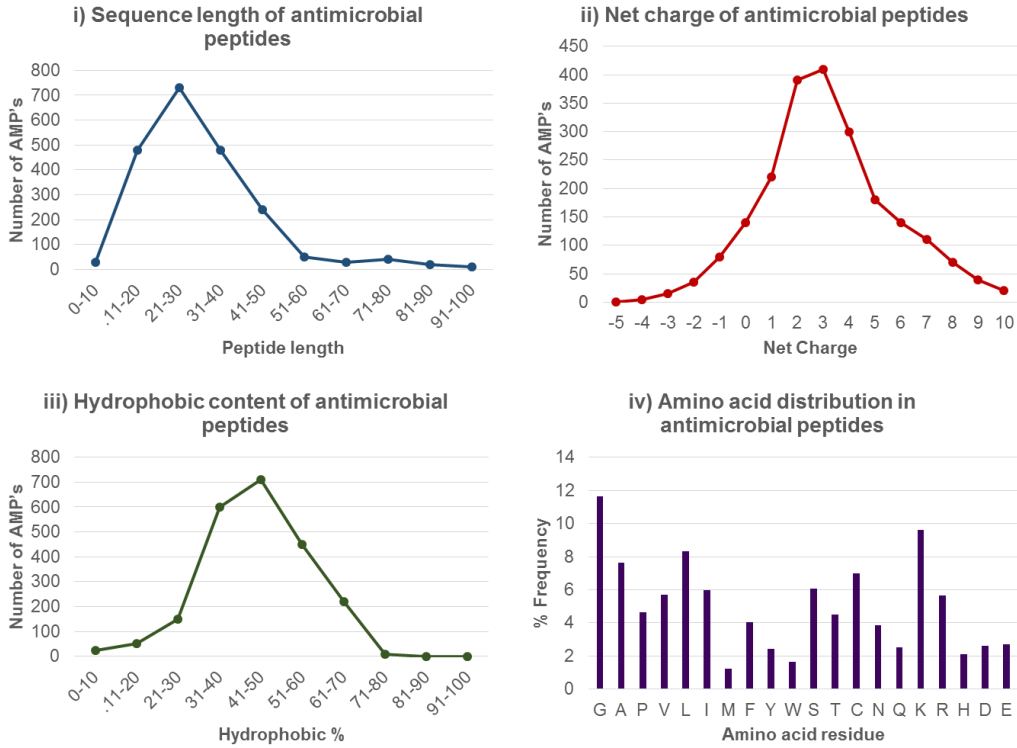


Figure 2.2: Distribution of antimicrobial peptides as a function of i) sequence length, ii) net charge and iii) hydrophobic content and iv) distribution of amino acid residues in antimicrobial peptides registered to the Antimicrobial Peptide Database (adapted from Wang, 2013:733; Wang *et al.,* 2016:D1087)

These peptides are produced by all living organisms, from prokaryotic bacteria to eukaryotic fungi, plants, insects, vertebrates and humans. They have been isolated from various sources, including the shells, venom or haemolymph of insects, epithelial cells throughout mammalian intestines or skin and the flowers, seeds or roots of plants, to name a few (Li *et al.,* 2012:208; Giuliani *et al.,* 2007:2). Living organisms produce AMPs as a nonspecific defence mechanism. Bacteria produce AMPs in defence, and to kill other bacteria when competing for the same ecological niche (Mahlapuu *et al*., 2016). In higher multicellular organisms, AMPs play an important role in the innate immune system where they act as a defence against invading pathogenic microbes (Cézard *et al.,* 2011:926; Fjell *et al.*, 2012:37). For this reason they are also referred to as host defence peptides and demonstrate potential as novel therapeutic agents contrary to conventional antibiotics (Baltzer & Brown, 2011:229; Fjell *et al.*, 2012:37). These peptides possess a broad-spectrum of activity against a range of bacteria, enveloped viruses, fungi and unicellular protozoa (Baltzer & Brown, 2011:229; Marr *et al.,* 2006:468). However, the unique spectrum of each AMP is determined by their specific amino acid sequence and structural conformation (Guilhelmelli *et al*., 2013:353). Furthermore, some AMPs

may also be classified as cell penetrating peptides (CPPs) due to their cell penetrating ability to translocate through cell membranes and internalise without any damage to the membrane. It is important to note that not all CPP have antimicrobial activity and are mostly therapeutically used to transport and deliver a variety of bioactive molecules into living cells (Guidotti *et al*., 2017:407; Splith & Neundorf, 2011:393).

2.2.1 Structure

The class of AMPs can be characterised in many different groups, according to their origin (natural or synthetic), size, amino acid sequence, spectrum of action (functionality), hydrophobicity, net charge or mechanism of action (Andersson *et al*., 2016:45; Li *et al.,* 2012:207). Despite the diversity, all AMPs share a mutual three-dimensional arrangement - an amphipathic conformation in aqueous solution, or the ability to fold into this amphipathic conformation after direct interaction with the target microbial cell membrane (Baltzer & Brown, 2011:229; Cézard *et al.,* 2011:926). The amphipathic molecule is called the secondary structure of an AMP and consists of positively charged hydrophilic amino acid residues on the one side and hydrophobic amino acid residues on the opposite side (either divided in the length or breadth of the structure). This arrangement allows the positively charged residues to strongly interact with negatively charged microbial cell membranes while the hydrophobic amino acid residues facilitate the permeation into the lipid phase of the membrane (Bahar & Ren, 2013:1545; Baltzer & Brown, 2011:229).

AMPs are therefore scientifically categorised based on these predominant secondary structures. Four groups of AMPs, based on their well-defined secondary structure, have been proposed: i) α-helical peptides, ii) β-sheet peptides, iii) loop peptides, and iv) extended structures (Steckbeck *et al*., 2014:12). Figure 2.3 shows an example of each of these four secondary structure groups and its three dimensional (3D) structure. Though cationic AMPs make up the vast majority of this whole class, anionic AMPs can also be categorised in its own sub-group according to structure, but because they hold weak antimicrobial activity they are thus excluded from this research (Guilhelmelli *et al.,* 2013:353). Among these groups, α-helix and β-sheet structures are more common and also most studied (Bahar & Ren, 2013:1544; Baltzer & Brown, 2011:229). This study will include cationic α-helical and β-sheet peptides.

The α-helical peptides form the largest subgroup of AMPs and are highly positively charged with helical structures. They are often unstructured in aqueous solution and fold into their secondary structure upon binding with the microbial membrane. This α-helical conformation allows them to be either adsorbed onto the membrane or directly inserted into it. Peptides with α-helical rotations include magainins, melittin and mastoparan (Andersson *et al*., 2016:45; Cézard *et al.,* 2011:926; Guilhelmelli *et al.,* 2013:353).

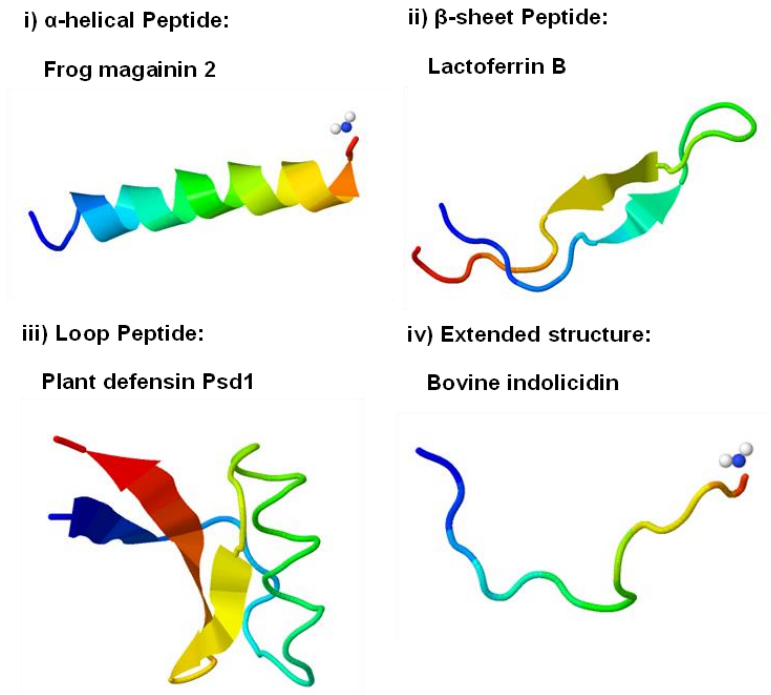


Figure 2.3: Examples from each of the four secondary structure groups showing the three dimensional conformation (adapted from Berman *et al.,* 2000:235; Wang *et al*., 2016:D1087). i) α- helical, ii) β-sheet, iii) loop and iv) extended secondary peptide structure groups

The β-sheet peptides are composed of at least two β-strands that are stabilised by two to five disulphide bridges. They hold a cyclic conformation that is already structured in aqueous solutions. Binding to the microbial membrane only stabilises the β-sheet conformation further. Examples of peptides with this secondary structure include human alpha defensins and the lantibiotic, nisin (Baltzer & Brown, 2011:229; Cézard *et al*., 2011:928).

2.2.2 Mode of action

Research has proven that the mode of action and active microbial spectrum of AMPs directly correlate with their specific structural properties. These properties govern the AMPs interaction with the target bacteria and include their amino acid sequence, molecular size, cationic nature, secondary structure, hydrophobicity and amphipathicity (Baltzer & Brown, 2011:230). In addition, the molecular properties and lipid membrane composition of the target bacteria also play a role in the interaction (Guilhelmelli *et al*., 2013:354). AMPs have a higher affinity to interact with negatively charged bacterial membranes, which renders the antibacterial effect more selective towards bacteria (prokaryotes) than mammalian cells (eukaryotes). However, at higher concentrations than needed for antibacterial activity, this activity becomes cytotoxic to mammalian or host cells, leading to cell death (Aoki & Ueda, 2013:1066; Laverty & Gilmore, 2014).

A basic model for mechanism of action has been proposed, namely: i) AMP attraction to target membrane, ii) AMP interaction and attachment onto target membrane and iii) insertion of AMP into target membrane or translocation through membrane via self-promoted uptake (Cézard *et al.,* 2011:928; Fjell *et al*., 2012:38). Direct antibacterial activity of AMPs is dependent on membrane interaction (Fjell *et al*., 2012:38).

Although all AMPs interact with the target membrane, studies suggest that these peptides be divided into two classes when addressing specific modes of action on bacteria and mammalian cells: a) membrane disruptive peptides and b) non-membrane disruptive peptides (intracellular targets) (Giuliani *et al.,* 2007:4). Mechanisms of membrane disruption can again be characterised by four models, i.e.: the barrel stave model, carpet like model, toroidal pore model and the aggregate channel model. These models of mechanism of action of AMPs are illustrated in Figure 2.4.

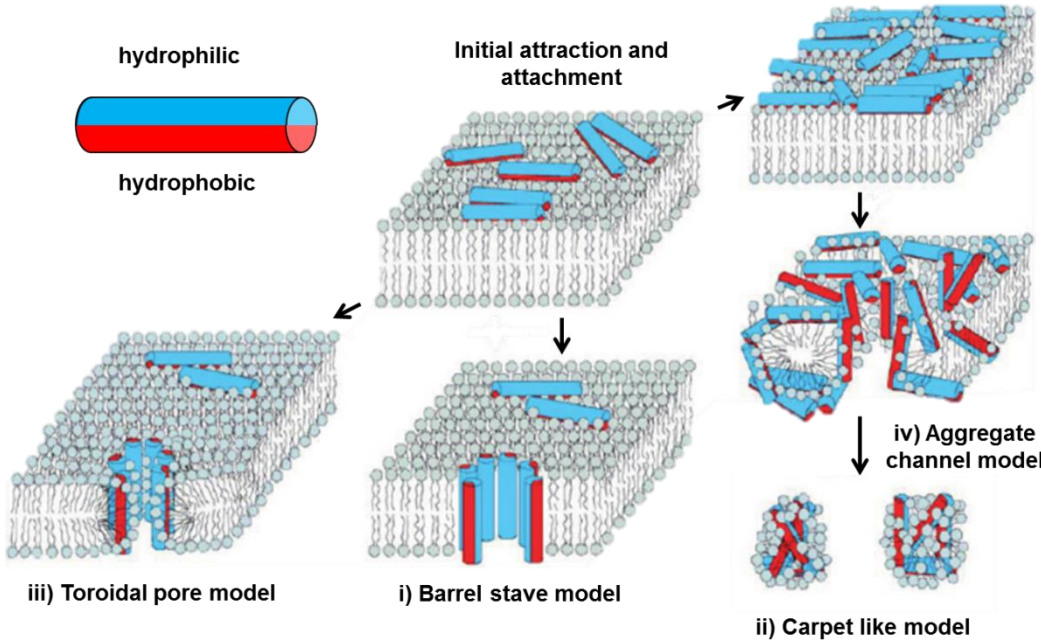


Figure 2.4: Proposed models of membrane disruptive action mechanisms of antimicrobial peptides (adapted from Salditt *et al.,* 2006:1484). i) Barrel stave model, ii) carpet like model, iii) toroidal pore model and iv) aggregate channel model

Firstly, the barrel stave model includes the accumulation of various individual amphipathic peptides, also referred to as *staves,* which bind to the target membrane. After binding, the peptides assume an orientation that allows the hydrophobic residues to bind with the lipid membrane, while the hydrophilic residues orientate inward toward each other. This *barrel-like* arrangement inserts itself perpendicularly into the membrane and allows the formation of an aqueous channel or transmembrane pore. The barrel stave model will cause cell death as a result of disturbance of membrane function due to lipid redistribution, loss of polarisation,

activation of hydrolases or leakage of cellular contents (Giuliani *et al.,* 2007:7; Li *et al*., 2012:212).

With the carpet like model, the AMPs bind to the membrane similar to the barrel stave model, but also cover the membrane in a detergent-like fashion. Once a concentration threshold is reached, membrane patches are formed. These patches are referred to as AMP-coated vesicles where the hydrophobic sides of the membrane face inward. The membrane ruptures and disintegrates; and it leads to complete cell death (Guilhelmelli *et al.,* 2013:354; Li *et al.,* 2012:211).

The toroidal pore model is the third proposed model and is very similar to the barrel stave model. The AMPs aggregate on the target membrane and continuously insert themselves perpendicularly into the membrane so that the participating lipids obtain a positive curve inwards. From this a transmembrane pore forms with an inward curved pore structure that includes AMPs as well as lipids from the membrane itself. Toroidal pore formation leads to membrane disruption and allows leakage of macromolecules such as liposomes and other internal cells. This model differs from the barrel stave model with regards to the pore structure lining and that the AMPs remain permanently bound to the lipid moieties of the membrane (Bahar & Ren, 2013:1549; Cézard *et al.,* 2011:930).

Finally, the aggregate channel model is a highly unstructured mode of action and very similar to the carpet like model, with the exception of not forming vesicles. AMPs bind parallel to the membrane surface by displacing lipid structures in the membrane. After binding, AMPs insert itself into the membrane and cluster to form unstructured aggregates, which lead to the formation of pores. This model leads to the destabilisation of the membrane as well as ion and macromolecule leakage (Giuliani *et al.,* 2007:9; Li *et al.,* 2012:211).

AMPs can additionally or complementary interact with intercellular targets and bind to ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and proteins to inhibit DNA, RNA, protein or cell wall synthesis (Cézard *et* al., 2011:930; Li *et al.,* 2012:211). Furthermore, AMPs can accumulate in the cytoplasm and interfere with the cytoplasm membrane formation or inhibit enzyme activity and nucleic acid synthesis. Studies have in addition proven AMPs to induce cell death by apoptosis or necrosis, however, this is highly cell type dependent (Guilhelmelli *et al*., 2013:356).

In unicellular organisms, such as bacteria, treatment of, or exposure to AMP therapeutics leads to the death of the organism itself. However, when multicellular mammalian cells are exposed to AMP therapeutics at higher concentrations than needed for its antibacterial effect, it results

in necrotic cell death of the mammalian cells. The AMP drug is thereby classified as a toxin. AMPs may furthermore lead to apoptosis in cancer cells (Oyinloye *et al*., 2015:153).

It is essential to understand how AMPs activate various pathways to cause cell death and thereby determine the acute, sub-acute and chronic effects of these therapeutics. As cell death may be caused by apoptosis or necrosis, further investigation and clarification of these mechanisms of AMP toxicity on mammalian cell lines are important for the clinical advancement of oral or IV administered AMP drugs (Cummings *et al*., 2012:12.8.1).

Table 2.1: Distinct and shared morphological and biochemical features of apoptosis and necrosis (adapted from Cummings *et al*., 2012:12.8.3; Kroemer *et al*., 2009:6)

Apoptosis	Necrosis
Distinct features	
ATP-dependent - decreased or maintained ATP levels	ATP depletion – loss of ATP concentrations
Activation of caspases	Caspases are not activated
Pyknosis – cell shrinkage and decrease in cellular and nuclear volume	Oncosis – cell swelling and increase in cellular volume
Organelles retain integrity	Organelle swelling and loss of integrity
Fragmentation and formation of apoptotic bodies	Vacuolation of the cytoplasm
Chromatin condensation	No chromatin condensation
Maintenance of membrane integrity	Loss of membrane integrity and membrane rupture
Maintenance of plasma membrane Ca^{2+} gradient	Loss of plasma membrane Ca^{2+} gradient
Exposed phosphatidylserine externally	Degraded phosphatidylserine remains internal or released
No release of intercellular contents	Release of intercellular contents
No inflammation follows	Induces inflammatory reactions/response
Shared features	
DNA degradation and nuclear condensation	
Membrane permeability transition	

Apoptosis can be defined as an active, programmed process that regulates cellular death. It is a cell intrinsic mechanism not only employed upon cell damage or stress, but is also activated during normal cell development and morphogenesis (Nikoletopoulou *et al*., 2013:3448). In contrast, necrosis is a passive form of cell death and the consequence of ATP depletion to a level that is unsuited for cell survival. Cells become necrotic in response to acute or overwhelming trauma, physical damage or exposure to toxins (Edinger & Thompson, 2004:664). The morphological characteristics of necrotic cells include the vacuolation of the

cytoplasm, an increase of cell volume, swelling of organelles, rupture of the plasma membrane and cell lysis resulting in the leakage of cytoplasmic cell contents.

Contrary to apoptosis, the nucleus remains largely intact and only distends. Cell death due to necrosis is always followed by inflammatory reactions as a result of the selective release of factors such as HMBG1 and HDGF from the necrotic cells that evoke the inflammatory response. (Laverty & Gilmore, 2014; Nikoletopoulou *et al*., 2013:3449). After apoptosis, apoptotic bodies will ultimately also undergo secondary necrosis in the absence of phagocytic cells as they lose membrane integrity and their metabolic pathways shut down (Fink & Cookson, 2005:1910). Table 2.1 differentiates between apoptosis and necrosis based on several distinct morphological and biochemical features.

2.2.3 Selectivity of antibacterial activity

Selectivity of antibacterial activity and the ability to distinguish between bacterial cells and mammalian cells are two very important features of AMPs to guarantee their potential therapeutic use as novel antibiotics. There are three factors that contribute to the selectivity of the antibacterial activity of AMPs and include the cationic property and lipid charge, transmembrane potential and the presence of cholesterol (Marr *et al*., 2006:470). Figure 2.5 illustrates the difference in selectivity of AMPs towards bacterial cells and mammalian or host cells. Research done by Matsuzaki (2009:1688) stresses the fact that selectivity of AMPs is expressed in the presence of both mammalian and bacterial cells and that AMPs are potentially toxic to mammalian cells in the absence of bacteria.

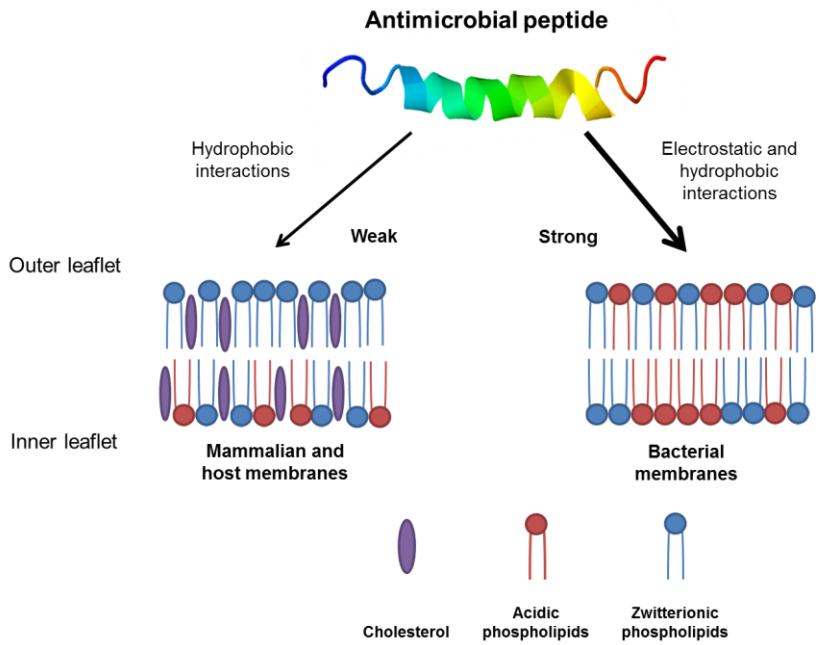


Figure 2.5: Selectivity of antimicrobial peptides towards mammalian, host and bacterial cells

Although AMPs are both cationic and amphipathic, the cationic properties of these peptides contribute more to cell selectivity. This cationic charge causes a higher affinity to more negatively charged molecules (Matsuzaki, 2009:1689). The cell membranes of bacteria are heavily negatively charged containing acidic phospholipid head groups such as phosphatidylglycerol, cardiolipin and phosphatidylserine. This organisation of negatively charged molecules in the lipid bilayer of bacterial membranes renders bacteria much more vulnerable to AMP activity (Baltzer & Brown, 2011:230). Cell walls of Gram-negative bacteria contain lipopolysaccharides, whereas Gram-positive bacteria contain teichoic acids and lipoteichoic acids. These anionic molecules present in the cell walls also contribute to the overall negative charge (Matsuzaki, 2009:1689). In contrast, mammalian cells contain predominantly neutral zwitterionic phospholipids such as phosphatidylethanolamine, phosphatidylcholine and ingomyelin in the outer leaflet of the plasma membrane. Mammalian cell membranes additionally consist of acidic phospholipids, but these molecules are usually sequestered in the inner leaflet of the plasma membrane. This composition of the membrane makes them much less susceptible to the same antibacterial activity (Baltzer & Brown, 2011:230; Giuliani *et al*., 2007:5).

The outer leaflet and inner leaflet of a membrane contribute to a cell's inside negative transmembrane potential. It is proposed that this inside negative transmembrane potential facilitates the insertion of positively charged peptides into the membrane and is responsible for the permeabilisation of the membrane (Giuliani *et al*., 2007:5; Matsuzaki, 2009:1689). In comparison, the inside negative transmembrane potential of bacterial cells is stronger, ranging from -130 to -150 millivolts (mV), compared to that of mammalian cells, with values between -90 to -110 mV, resulting in a greater susceptibility of bacterial cells towards AMPs (Mahlapuu *et al*., 2016; Matsuzaki, 2009:1689).

The widely distributed presence of cholesterol in mammalian cell membranes directly protects these cells against AMP activity. Cholesterol can reduce the antibacterial activity of AMPs either by direct interaction with the peptide and neutralising it or through stabilising the lipid bilayer. Again, bacterial cells are more vulnerable due to the absence of cholesterol (Giuliani *et al*., 2007:5).

2.2.4 Pharmacological and therapeutic potential

Many factors and features of AMPs present them promising candidates as novel antibiotics and other diverse potential therapeutics. These fast acting peptides have a broad-spectrum of antimicrobial activity against bacteria which can extend to fungi, viruses and parasites as well. AMPs also have a low propensity for resistance development; and at similar concentrations of conventional antibiotics, AMPs have the ability to kill multi-drug resistant bacteria (Marr *et al.,*

2006:468). Since most AMPs target the bacterial cell membrane, they are potential candidates against persister cells and biofilm formation (Bahar & Ren, 2013:1556). Furthermore, they exhibit synergistic effects when combined with classical antibiotics (Baltzer & Brown, 2011:233). In GI infections, many AMP candidates have shown remarkable antibacterial effects against various strains of VRE, *Staphylococcus aureus, Helicobacter pylori, Escherichia coli, Micrococcus luteus* and *Clostridium difficile* (Maher & McClean, 2006:1290; Tong *et al*., 2014).

AMPs therapeutic potential goes further than just their antimicrobial effects. They are in addition active modulators of the innate host immune defence system, promote wound healing and have the ability to neutralise endotoxins (Giuliani *et al.*, 2007:10). Studies have further shown the therapeutic potential of cell penetrating peptides against cancer and the production of tumour cell death by certain cationic AMPs (Carmona-Ribeiro & Carrasco, 2014:18042). Moreover, AMPs with cell penetrating properties have demonstrated the ability to act as vector molecules to transport therapeutic molecules across cell and tissue barriers. These molecules briefly include proteins, peptides, macromolecules, nanoparticles and nucleic acids (Guidotti *et al*., 2017:417; Splith & Neundorf, 2011:389). AMPs can be utilised to coat medical devices as a bio-disinfectant in immunocompromised patients to prevent the adherence of micro-organisms and biofilm formation (Li *et al.,* 2012:213). Some studies suggest using AMPs as a natural bio-preservative as an alternative to chemical preservatives to prevent food spoilage and contamination by pathogens, and enhance shelf-life (Rai *et al*., 2016:3392).

Even though these peptides have all these advantages over conventional antibiotics and possess many attractive properties, they also have limitations. These briefly include the high cost implications of large scale production and purification, development of resistance and the possible resistance towards the human immune system, unwanted toxicity towards eukaryotic cells, low peptide stability *in vivo* and susceptibility to proteolytic degradation, as well as the possibility of allergy development (Cézard *et al*., 2011:933; Baltzer & Brown, 2011:232; Kim *et al*., 2013:122; Otvos & Wade, 2014). Topical applications of AMP therapeutics have generally been focussed on to address some of these limitations (Giuliani *et al*., 2007:16).

2.2.5 Current FDA approved antimicrobial peptide drugs

Pexiganan, also referred to as MSI-78, is a peptide derived from magainin-2 provided by the company Magainin Pharmaceuticals. Magainins are linear peptides isolated from the skin of the African frog, *Xenopus laevis.* Pexiganan was one of the first generation AMPs developed in the early 90's which made it to clinical trials. During Phase III trials Pexiganan proved to be an effective treatment for diabetic foot ulcers with no notable toxic side effects, but unfortunately was rejected by the Food and Drug Administration (FDA) as it did not provide an improved

performance above conventional treatment (Cézard *et al*., 2011:933; Steckbeck *et al*., 2014:12).

Since then Gramicidin S and Polymyxin B, both ingredients in topical creams and solutions, are the only AMPs that are approved by the FDA to date. Gramicidin S is a derivative of gramicidin produced by the Gram-positive bacterium *Bacillus brevis*; and Polymyxin B is isolated from various strains of *Bacillus polymyxa* bacteria. These drugs are, however, limited to topical application due to their nephrotoxicity and neurotoxicity tendencies associated with IV administration (Baltzer & Brown, 2011:232). Additionally, there are only a couple of AMPs currently in clinical development for the treatment of various bacterial pathogens or as immunomodulatory agents. The majority of these AMPs consists of analogues of natural AMPs or modified derivatives thereof; and are intended for topical use only (Andersson *et al*., 2016:52; Steckbeck *et al*., 2014:13).

Human-derived Lactoferrin 1-11 (hLF1-11) is the only AMP drug currently in clinical development intended for IV administration and aims to treat life threatening infections that follow stem cell transplantation. Aside from hLf1-11, OP-145 derived from human cathelicidin LL-37, is also an exception as it is formulated as eardrops to treat chronic bacterial infections (Fjell *et al*., 2012:47). AMPs that are presently in clinical trials are displayed in Table 2.2. Although the commercialisation interest in therapeutic AMP drugs is continuously increasing, many pharmaceutical companies still face formulation issues that limit the clinical utility thereof. The lack of oral and IV AMP formulations currently in development together with the growing emergence of antibiotic resistance clearly highlights the need for research on orally and IV administered AMP drug dosage forms.

Table 2.2: Antimicrobial peptides currently in clinical trials (adapted from Andersson *et al*., 2016:53; Fjell *et al*., 2012:47; Mahlapuu *et al*., 2016)

Peptide	Amino acid sequence	AMP source & description	Application		Trial phase	Company
			Administration	Indication		
Pexiganan acetate (MSI-78)	GIGKFLKKAKKFGKAFVKILKK	Synthetic analogue of magainin 2 derived from frog skin	Topical cream	Diabetic foot ulcer and infections	Phase III	Dipexium Pharmaceuticals. Inc.
hLF1-11	GRRRRSVQWCA	Human-derived Lactoferrin 1-11	Intravenous	Neutropenic stem cell transplant patients	I / II	AM-Pharma B.V.
OP-145	IGKEFKRIVERIKRFLRELVRPLR	Synthetic 24-merpeptide derived from LL-37	Ear drops	Chronic bacterial ear infection	I/II	OctoPlus Inc.
Omiganan (MBI-226)	ILRWPWWPWRRK	Synthetic cationic peptide derived from indolicidin	Topical cream	Topical antiseptic, prevention of catheter infections	III	Mallinckrodt
Omiganan (CLS001)	ILRWPWWPWRRK	Synthetic cationic peptide derived from indolicidin	Topical cream	Severe acne and rosacea	II / III	Cutanea Life Sciences, Inc.
Iseganan (IB-367)	RGGLCYCRGRFCVCVGR	Synthetic 17-mer peptide derived from protegrin 1	Mouth wash	Oral mucositis	III	Ardea Biosciences
C16G2	TFFRLFNRSFTQALGKGGGKNL RIIRKGIHIIKKY	Synthetic specifically targeted AMP	Mouth wash	Prevent tooth decay caused by *Strep. mutans*	II	C3 Jian Inc.
Lytixar (LTX-109)	RWR	Synthetic antimicrobial peptidomimetic	Topical cream	Uncomplicated Gram-positive skin infections	II	Lytix Biopharma AS

2.3 Melittin, Mastoparan and Nisin Z as therapeutic antimicrobial peptides

Between the immense diversity of naturally occurring and synthetic AMPs; and the rising interest in AMP research, cationic amphipathic peptides remain the most studied and characterised of all. In addition, AMP candidates with cationic properties also show significantly enhanced potential as novel antibiotic therapeutics, as it allows for stronger interaction and improved antimicrobial action between the peptide drug and target bacteria (Baltzer & Brown, 2011:229). Melittin and mastoparan are cationic AMPs with α-helical secondary structures. Both are derived from the venom of eukaryotic organisms and were included in this study. Melittin shows promising antibacterial effects in a low nanomolar (nM) range in comparison to conventional antibiotics, whereas mastoparan is included for its cell penetrating properties. The lantibiotic, nisin Z is a cationic bacteriocin (prokaryotic origin) with a predominantly β-sheet secondary structure and is included in this study in contrast to the venom peptides.

2.3.1 Melittin

Melittin is the principal toxin in the venom of the European honey bee, *Apis mellifera* (Raghuraman & Chattopadhyay, 2007:190). This is a very well-studied peptide that constitutes approximately 50% of dry bee venom (Gajski *et al*., 2016:56). Melittin is a small linear basic peptide that possesses amphipathic characteristics due to its specific arrangement of amino acid residues. This peptide is composed of a known 26 amino acid sequence (GIGAVLKVLTTGLPALISWIKRKRQQ) (see Addendum A for amino acid abbreviations) which at physiological pH has a net charge of +6 and is predominantly hydrophobic and cationic (Gajski & Garaj-Vrhovac, 2013:699; Raghuraman & Chattopadhyay, 2007:190). Melittin is well known for its haemolytic effects and broad-antimicrobial activities due to its amphipathic property and membrane activity (Asthana *et al*., 2004:55042).

The amphipathic property of melittin makes it highly soluble in water (>250 mg/ml) where it exists as a monomer with random coil conformations at low peptide concentrations and neutral pH. At basic pH, increased peptide concentration or addition of salt, the monomer transforms to a tetramer structure with a pronounced helical conformation and hydrophobic inner surface (Asthana *et al*., 2004:55042; Jamasbi *et al*., 2016:41). When interacting with membranes or in the presence of lipid micelles, melittin also transforms into a bent α-helical rod conformation in which non-polar, hydrophobic and neutral amino acids are situated at the N-terminus (residues 1–20) and hydrophilic and basic amino acids are located at the C-terminus (residues 21–26) (Jamasbi *et al*., 2016:41). Figure 2.6 illustrates the 3D α-helical structure of melittin as well as the amino acid sequence with amphipathic characteristics.

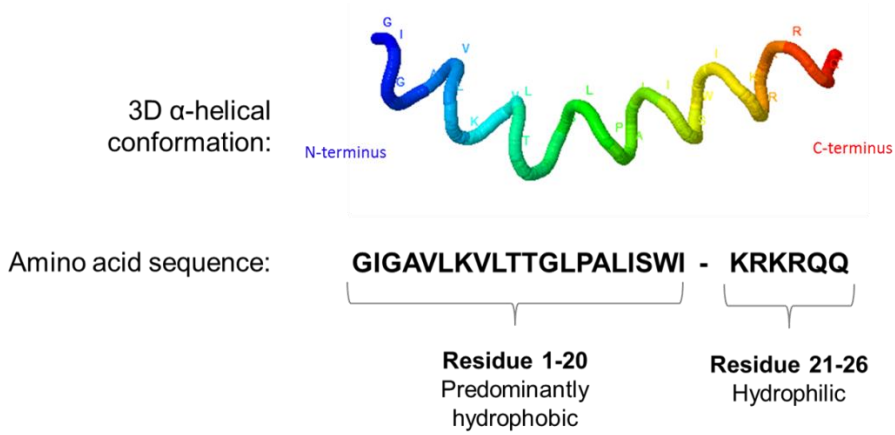


Figure 2.6: The three dimensional α-helical conformation and amino acid sequence with amphipathic characteristics of melittin (adapted from Berman *et* al., 2000:235; Wang *et al*., 2016:D1087)

This peptide additionally acts as a natural detergent with high surface and membrane tension and has the ability to interact, insert itself and disrupt both natural and synthetic membranes by forming tetramer aggregates as stable pores. When melittin is inserted into the lipid bilayer, the orientation of the peptide is determined by the peptide/lipid (P/L) ratio which affects the membrane disruptive activity. At low P/L ratio (~1 mol %), the helical segments of melittin are parallel aligned to the bilayer which is not disruptive to the membrane. At high P/L ratio (> 4 mol %), melittin reorients from a parallel alignment to a transmembrane conformation and directly inserts itself in the bilayer which leads to pore formation (Jamasbi *et al*., 2016:41). These induced pores cause ion leakage and disorder in the lipid bilayer membrane structure which lead to cell lysis and provides melittin with cytolytic properties.

Membrane interaction studies propose the barrel stave model and carpet like model for melittin's interaction with zwitterionic lipid bilayers and negatively charged lipid vesicles, respectively (Asthana *et al*., 2004:55042). However, recent research proposed that melittin also interacts with membranes through the toroidal pore mechanism to cause cell death (Lee, Hall *et al*., 2016:29). Membrane disorder and disruption caused by melittin can furthermore attribute to changes in membrane potential, aggregation of membrane proteins and induction of hormone secretion (Gajski & Garaj-Vrhovac, 2013:699; Raghuraman & Chattopadhyay, 2007:190). Cell membrane disruption depends on various factors which include the geometry and thickness of the membrane, polar head group charge and the overall membrane composition (Jamasbi *et al*., 2016:43).

Additionally, it can stimulate G-protein enzymes, protein kinase C, adenylate cyclase and phospholipases. The cytolytic actions affect all lipid membranes that come in contact with melittin and causes both apoptotic and necrotic cell death differing from cell type to cell type (Gajski & Garaj-Vrhovac, 2013:100). The lytic properties also affect vesicles, where the degree

of vesicle leakage is dependent on the lipid composition thereof (Jamasbi *et al*., 2016:40). The negative aspect of these wide-spectrum lytic properties is that they also extend to human membranes, red blood cells and other endogenous cells (Gajski *et al*., 2016:57). Nonetheless, owing to all of the properties of melittin, it not only has therapeutic potential, but is similarly a promising candidate as a novel antimicrobial drug.

Research has established that melittin possesses a broad-spectrum of action and exerts antibacterial, antiviral, antifungal and antiparasitic effects (Gajski *et al*., 2016:56; Wang *et al.*, 2016:D1087). This cationic peptide further acts as an anti-inflammatory drug and *in vivo* and *in vitro* testing have delivered positive results where it can potentially treat atherosclerosis and rheumatoid arthritis. Many studies report melittin's ability to inhibit tumour cell growth and its ability to induce apoptosis or necrosis, thereby signifying the potential use of bee venom as complementary or alternative treatment for cancer. Melittin is considered for the coating of medical devices and vaccination against infectious pathogens as immunologic adjuvants (Gajski *et al.,* 2016:57; Gajski & Garaj-Vrhovac, 2013:700; Moreno & Giralt, 2015:1133). Additionally, this peptide's whole and partial amino acid sequence have been incorporated in designs of synthetic proteins with the idea that these synthetic proteins will mimic melittin's properties and effects (Lee *et al*., 2013:14243).

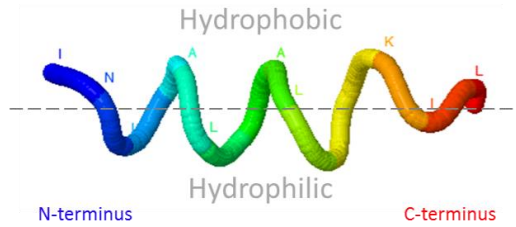
Although melittin is the most studied bee venom peptide, the clinical and therapeutic development still remains in the preclinical phase. Despite the therapeutic efficacy and broad microbial spectrum of melittin, the use thereof is majorly limited due to its cytotoxicity, high cost of production and instability that causes poor *in vivo* bioavailability (Moreno & Giralt, 2015:1130).

2.3.2 Mastoparan

Mastoparan, from the Korean yellow jacket wasp *Vespula lewisii,* is the most studied peptide in wasp venom. This compound is a tetradeca-peptide which is described as a polypeptide that consists of 14 amino acids with the sequence: INLKALAALAKKIL (see Addendum A for amino acid abbreviations) (Yamamoto *et al*., 2014:3934). In aqueous solutions, mastoparan remains mostly unstructured and at physiological pH has a net positive charge of 4 (Lee, Baek *et al*., 2016). The amino acid chain is rich in hydrophobic and basic hydrophilic residues which have the ability to adapt to an amphipathic α-helical structure in lipid environments or when binding to membranes (De Azevedo *et al*., 2015:113; Irazazabal *et al*., 2016:2704). The amphipathic property of mastoparan not only plays an important part in its membrane-interaction, penetration and cytotoxicity, but is also the key factor in the peptide's therapeutic potential (Irazazabal *et al*., 2016:2705; Katsu *et al*., 1990:185).

Studies have proven mastoparan to possess a strong affinity to phospholipid bilayer membranes and upon interaction with these membranes; this peptide takes on the α-helical conformation. When mastoparan forms this α-helical structure the positively charged hydrophilic residues all lie on the one side of the helix, whereas the negatively charged hydrophobic residues occupy the other side. The positive side chain is responsible for the interaction and penetration of the peptide into the membrane which classifies mastoparan as a cell penetrating peptide (Katsu *et al*., 1990:185; Lee, Baek *et al*., 2016). Studies propose the peptide-membrane interaction follows the carpet model mechanism (Nakao *et al*., 2011:491). Figure 2.7 illustrates the 3D α-helical conformation of mastoparan with amphipathic properties as well as the amino acid sequence thereof.

3D α-helical conformation:



Amino acid sequence: **INLKALAALAKKIL**

Figure 2.7: The three dimensional α-helical conformation of mastoparan with amphipathic properties and amino acid sequence (adapted from Berman *et al*., 2000:235; Wang *et al*., 2016:D1087)

Upon interaction, mastoparan can either insert itself into the lipid bilayer and cause membrane destabilisation, which leads to cell lysis or interact directly with intracellular G-proteins on the cytoplasmic face of the membrane. The activation of the G-proteins will cause perturbation of transmembrane signalling pathways, stimulate phospholipases A, C and D, mobilise mitochondrial Ca^{2+}, and lead to cell death by necrosis and/or apoptosis (Moreno & Giralt, 2015:1136). Additionally, G-proteins control exocytosis where activating these proteins triggers the secretion of molecules which affects cell viability. These secretory molecules vary between different cell types and include the secretion of histamine from mast cells resulting in an inflammatory response, catescholamines from chromaffin cells, serotonin from platelets, prolactin from the anterior pituitary gland and even insulin from pancreatic β-cells (Lee, Baek *et al*., 2016; Nakao *et al*., 2011:490). Mastoparan affects the membranes of erythrocytes resulting in haemolysis and contributing to its cytotoxicity (Lee, Baek *et al*., 2016).

Among the wide variety of biological effects that mastoparan exhibits, it is well known to induce mitochondrial permeability transition. This phenomenon occurs when the permeability of the inner membrane of the mitochondria, the energy conversion organelle in all eukaryote cells, is noticeably increased. It results in the formation of a pore which is also referred to as the

permeability transition pore. The pore allows the mitochondria to swell and rupture due to the loss of membrane potential. When the mitochondrion ruptures, it releases various proteins to trigger subsequent stages of apoptosis or necrosis in the cell (De Azevedo *et al*., 2015:118; Yamamoto *et al*., 2014:3934). In cancer cells the mitochondria are the target during therapy for their role in apoptosis induction when their membranes become permeabilised. Mastoparan's cytotoxicity and ability to induce mitochondrial permeability transitioning in cancer cells render it a promising antitumor agent. Recent studies demonstrated that mastoparan caused increased mitochondrial permeability and disrupted membrane potential in melanoma cells which resulted in the release of pro-apoptotic proteins and led to apoptosis (De Azevedo *et al*., 2015:118).

As an amphipathic CPP mastoparan exhibits effective antimicrobial activity against various Gram-positive and Gram-negative bacteria, cancer cells, fungi, protozoa and viruses (Wang *et al*., 2016:D1087). Due to mastoparan's broad-spectrum of activity, it has great potential to become a novel antimicrobial agent or even a new compound in biotechnological applications in biomedicine (Lee, Baek *et al*., 2016). Mastoparan is being considered as an additive to use in combination with antibiotics to combat multi-, extended- or pan-resistant bacteria in clinical practice (Moreno & Giralt, 2015:1137). Yet, this peptide alone has also been reported to have activity against both colistin-susceptible and colistin-resistant *Acinetobacter baumannii*, from the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) group bacteria (Vila-Farrés *et al*., 2015:35). As an antiviral agent, mastoparan demonstrated broad-spectrum activity against five different families of enveloped viruses, directly disrupting their lipid envelope structures (Moreno & Giralt, 2015:1137). Furthermore, mastoparan can potentially be used as a pharmaceutical application agent to deliver macromolecules into cells. The capacity of mastoparan to cross plasma membranes and even the blood-brain barrier led to a study that addressed the transport of doxorubicin, a drug used in cancer treatment of the central nervous system. Both *in vitro* and *in vivo* experiments showed efficient translocation of the doxorubicin encapsulated transferrin modified liposomes over the blood-brain barrier (Moreno & Giralt, 2015:1138).

Some of the drawbacks in developing mastoparan as a therapeutic agent include their low stability and susceptibility to peptidases in the human body, haemolytic properties and also their selective disability to distinguish between mammalian and target cells, (Vila-Farrés *et al.*, 2015:35). However, various studies do report some selectivity observed between cancer cells and non-tumorigenic cells, as well as between mammalian cells and bacterial cells (De Azevedo *et al*., 2015:118; Nakao *et al*., 2011:491).

2.3.3 Nisin Z

Nisin Z differs from melittin and mastoparan by not being a toxin, but is classified as a Type A (I) lantibiotic. This specific type of lantibiotic is a class of AMPs which consists of linear peptides that contain uncommon post-translationally modified amino acids, such as lanthionine or methyllanthionine in heterocyclic ring structures. While they are profoundly different in structure in comparison to other natural cationic host defence peptides, the class lantibiotics share similar physio-chemical- and analogous immunomodulatory properties to previously described host defence peptides. Additionally, both classes share the ability to interact with negatively charged biological membranes. Lantibiotics are ribosomally synthesised polypeptides produced by Gram-positive bacteria as antimicrobial molecules to defend them against other Gram-positive species and thus are also classified as bacteriocins. Over the years the interest in lantibiotics have intensely grown owing to their distinctive biochemistry, genetic regulation, range of biological functions and the potential of engineering unique protein structures (Islam *et al.*, 2012:1528; Kindrachuk *et al*., 2012:316; Mulders *et al*., 1991:581).

Nisin is a heat-stable peptide produced by numerous strains of *Lactococcus lactis*, a non-pathogenic lactic acid bacterium. In 1969 it received its *Generally Regarded as Safe* (GRAS) status which was approved by the WHO and later in 1988 the US Federal FDA approved its use as a safe food additive (Kindrachuk *et al*., 2012:316; Lewies *et al*., 2017:245). While nisin has widely been used as a natural food preservative for more than 40 years, it also shows promising antimicrobial activity against Gram-positive bacteria with high potency, low cellular cytotoxicity at antimicrobial concentrations and limited ability to cultivate antimicrobial resistance (De Vos *et* al., 1993:213; Kindrachuk *et al*., 2012:316). Numerous naturally occurring variants of nisin have been identified from a wide range of taxonomically distinct organisms and isolated from various ecological environments. In addition, bioengineered variants of nisin have been developed in an attempt to enhance peptide properties for several biological applications (Shin *et al*., 2015:1450). From the natural variants, nisin A and nisin Z are the most studied among the group and are structurally similar, except for the substitution of a single amino acid. At position 27, histidine is found in nisin A, where it is replaced with asparagine in nisin Z (Mulders *et al*., 1991:584). Though both variants have similar antimicrobial activities, nisin Z produces larger zones of inhibition in agar diffusion assays together with improved solubility and diffusion properties at pH levels above 6 and therefore was chosen to be investigated further in this study (De Vos *et al*., 1993:216).

Nisin Z is an elongated cationic peptide with an atomic mass of 3.5 kDa and the capacity to adopt amphipathic structures. It comprises 34 amino acid residues with the sequence: ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK (see Addendum A for amino acid

abbreviations). At physiological pH this peptide has a net positive charge of +3 which ensures an excellent stability and solubility profile up to 100 µg/ml (De Vos *et al*., 1993:216; Wang *et al*., 2016:D1087; Wiedemann *et al*., 2001:1772). The N-terminal domain (amino acid residues 1-19) is predominantly hydrophobic and involves the first three ring structures, A–C. Whereas the C-terminal domain (amino acid residues 20–34) is made up of the remaining two ring structures, D and E, forming the amphipathic structure with a segregation of hydrophilic and hydrophobic sides (Lins *et al*., 1999:115). Figure 2.8 clarifies the primary structure of nisin Z before and after post-translational modifications, and the formation of the five ring structures (A–E), whereas Figure 2.9 illustrates the 3D conformation of nisin Z including the amino acid sequence and amphipathic properties.

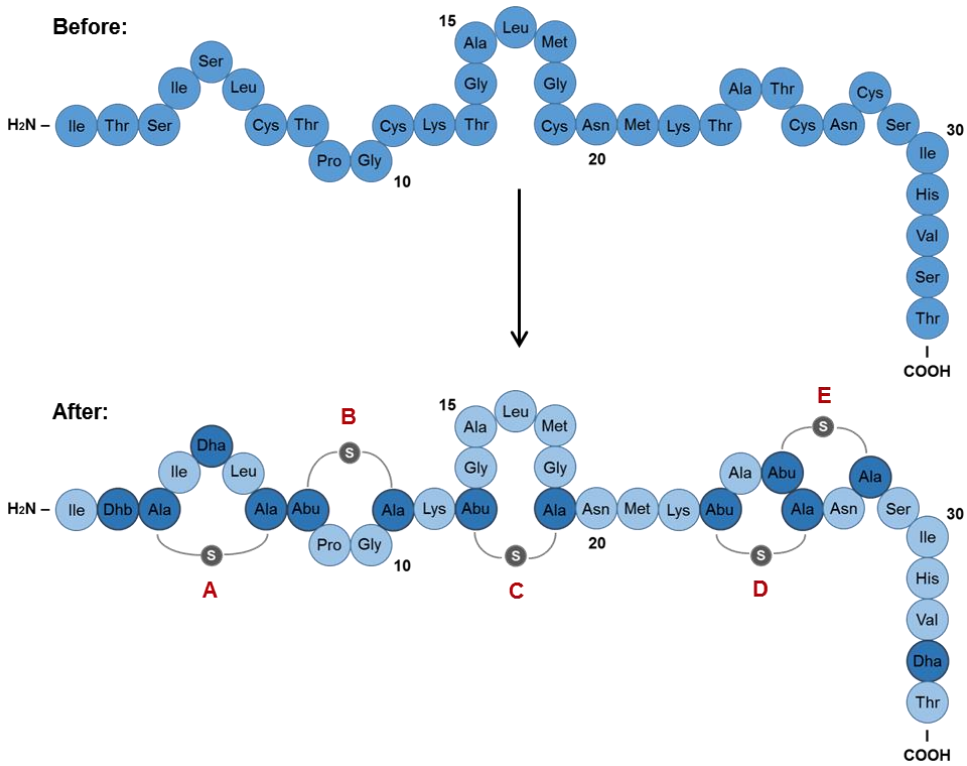


Figure 2.8: The primary structure of nisin Z before and after post translational modification and the formation of five ring structures (A-E) (adapted from Lins *et al*., 1999:112; Van Kraaij *et al*., 2000:903)

The antimicrobial activity of nisin Z is dependent on maintaining the amphipathic configuration formed by the lanthionine bridges and while this antimicrobial activity of nisin Z has extensively been studied, the exact mechanism behind this complex pore formation is still unknown (Islam *et al*., 2012:1528; Kindrachuk *et al*., 2012:325). Upon dissolution in water, nisin Z adapts to form α-helical, but predominantly β-sheet structures. Further investigation into membrane interaction showed that nisin Z forms higher proportions of β-sheet conformations than the free form thereof in water and therefore is classified as a β-sheet peptide (El-Jastimi & Lafleur, 1997:157).

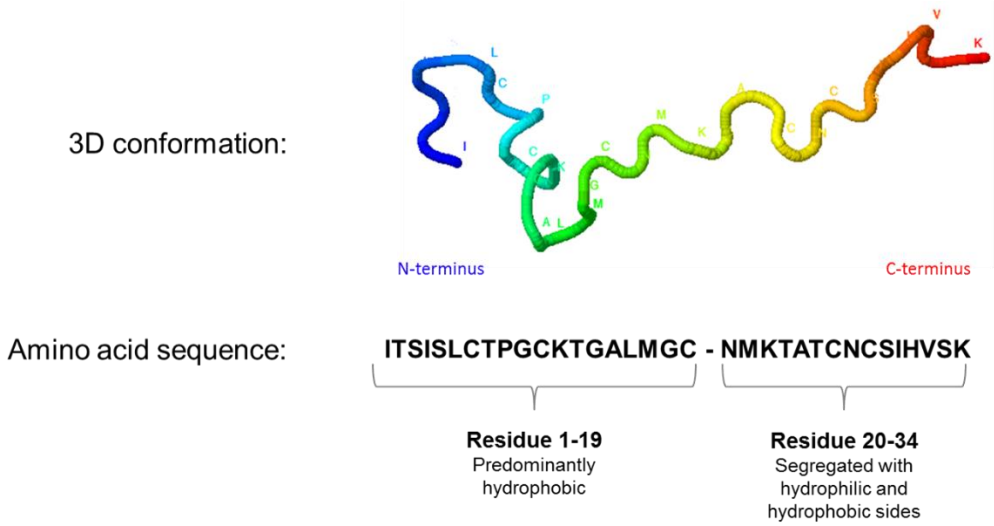


Figure 2.9: The three dimensional conformation of nisin Z with amino acid sequence and amphipathic properties (adapted from Berman *et al*., 2000:235; Wang *et al*., 2016:D1087)

Several studies on membrane interaction revealed that the initial interaction is governed by the C-terminus of nisin Z as this moiety contains the larger positive charge. This adsorption to the membrane leads to aggregation of several nisin Z molecules which adopt a transmembrane configuration. In contrast, the insertion of nisin Z into the lipid bilayer is solely mediated by the predominant hydrophobic N-terminus. The peptide molecule is inserted parallel to the lipid interface with the N-terminus pulled more deeply into the membrane. The amphipathic property of the C-terminus causes the hydrophobic residues thereof to be immersed in the membrane while hydrophilic residues maintain a domain at the interface on the outside of the membrane. This transmembrane configuration, where the C-terminal domain stretches from one side of the bilayer to the other, leads to the formation of a pore. Nisin Z thereby disrupts membrane integrity and induces rapid efflux of ions and cytoplasmic solutes resulting in membrane depolarisation and instant termination of all cellular processes causing cell death (Islam *et al*., 2012:1528; Lins *et al*., 1999:115; Wiedemann *et al*., 2001:1777).

In addition to the above mechanism, nisin Z has a high affinity for binding to lipid II molecules resulting in pore formation. Lipid II is a membrane bound cell wall enzyme involved in peptidoglycan synthesis found in bacteria and serves as a docking molecule for nisin Z. Studies have reported increased potency of antimicrobial activity of nisin Z when lipid II is available for binding. These studies further revealed that in the absence of lipid II, pore formation only occurs at high concentrations (micromolar range) in optimal conditions where the membrane consists of more than 60% anionic lipids. In contrast, when lipid II is present, nisin Z can exert dual action antimicrobial effects at nanomolar concentrations which include

an effective combination of pore formation and inhibition of peptidoglycan synthesis (Islam *et al*., 2012:1529; Wiedemann *et al*., 2001:1777). Thus, nisin Z activity against other microorganisms that do not contain lipid II in their membranes, such as yeast and fungi, are lower in comparison to that against bacteria where the lipid II molecule is present. Further studies discovered the low toxicity of nisin Z in animal model studies as a result of the high affinity binding to this bacterial docking molecule (Wiedemann *et al*., 2001:1778).

Besides the direct antimicrobial activity, nisin Z has additional multifunctional immunomodulatory properties. Several studies have demonstrated that nisin Z beneficially modulates host immunity both *in vivo* and *ex vivo*. Some of these immune responses include: the ability to stimulate neutrophil extracellular trap production through activating human neutrophils, up-regulate multiple pathways related to growth factor receptors, induce chemokine production, inhibit LPS-induced pro-inflammatory responses, as well as increase CD4 and CD8 T-lymphocytes while decreasing the level of B-lymphocytes (Kindrachuk *et al*., 2012:325; Shin *et al*., 2015:1457).

Although nisin Z's spectrum of action extends mostly to Gram-positive bacteria, studies have established that combinations of nisin Z and conventional antibiotics can not only enhance their current activity, but can also extend their activity to be effective against Gram-negative pathogens (Kuwano *et al*., 2005:399; Lewies *et al*., 2017:245). Additionally, studies report nisin Z's effectiveness against antibiotic resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and VRE strains (Dolser & Gerceker, 2011:514; Piper *et al*., 2009:548). Not only is nisin Z a promising novel antibiotic agent with immunomodulatory properties, but researchers have discovered and demonstrated wide biomedical applications beyond its antimicrobial activities. A study established that nisin Z can reduce and prevent biofilm formation caused by bacteria build up on medical devices or damaged tissue (Okuda *et al*., 2013:5574). Electrospun nanofibre wound dressing containing nisin Z showed reduced *Staphylococcus aureus* colonisation and accelerated wound healing in a murine excisional skin infection model (Heunis *et al*., 2013:3932). Moreover, this lantibiotic exhibits antitumor and cytotoxic effects on cancer cells. Data from a study done on head and neck squamous cell carcinoma showed that nisin Z promoted cell apoptosis, suppressed cancerous cell proliferation, inhibited angiogenesis, while *in vivo* it inhibited tumorigenesis and prolonged survival (Kamarajan *et al*., 2015; Shin *et al*., 2015:1457).

Based on all the above mentioned properties, nisin Z is indeed a promising candidate for further investigation as a possible novel antibiotic agent. Moreover, numerous studies have delivered promising laboratory and clinical results while minimal to no clinically critical resistance has developed or been reported (Shin *et al*., 2015:1459). Table 2.3 includes a brief

summary of the physical, chemical and antimicrobial properties of the AMPs melittin, mastoparan and nisin Z.

Table 2.3: Summary of the physical, chemical and antimicrobial properties of melittin, mastoparan and nisin Z

	Melittin	Mastoparan	Nisin Z
Classification of AMP	Toxin	Toxin	Lantibiotic, type A (I) bacteriocin
Source	European Honeybee venom, *Apis mellifera* (Eukaryotic)	Korean yellow jacket wasp venom, *Vespula lewisii* (Eukaryotic)	Gram-positive bacteria, *Lactococcus lactis* (Prokaryotic)
Amino acid sequence	GIGAVLKVLTTGLPALISWIKRKRQQ	INLKALAALAKKIL	ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK
Length	26	14	34
Secondary structure	α-helical structure with amphipathic properties	α-helical structure with amphipathic properties	B-sheet structure with well-defined lanthionine containing rings and amphipathic properties
Net charge (physiological pH)	+6	+4	+3
Molecular weight	2846,46 g/mol	1478,91 g/mol	3354,09 g/mol
Solubility	Water soluble	Water soluble	Low solubility at physiological pH. Soluble in 0.01 M HCl
Main antimicrobial mechanism of action	Transmembrane pore formation	Membrane disruptive detergent-like mechanism and mitochondrial permeability transition	Lipid II-mediated pore formation
Spectrum of antimicrobial activity	Gram-positive and Gram-negative bacteria, viruses and fungi	Gram-positive and Gram-negative bacteria, viruses, fungi, protozoa and yeast	Gram-positive bacteria
Other peptide activities	Anti-inflammatory, anticancer, cytolytic, heamolytic	Anticancer, cytolytic, heamolytic, cell penetrating properties	Anti-inflammatory, anticancer, food preservative properties
Antibacterial against gastro-intestinal pathogens	*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis* and *Salmonella* species	*Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*	*Enterococcus faecalis, Escherichia coli* and *Shigella* and *Salmonella* species

Though studies have suggested and proven the potential treatment of various GI infections with AMPs through antimicrobial studies on multiple organisms and bacterial strains, it is still critical to investigate the interaction of these peptides with mammalian GI epithelial cells. Table 2.4 displays the minimum inhibitory concentration (MIC) of melittin, mastoparan and nisin Z against bacterial pathogens commonly associated with GI infections. Ampicillin and vancomycin are antibiotics clinically used to treat these infections and are also included as a reference.

Table 2.4: Minimum inhibitory concentration of melittin, mastoparan, nisin Z, ampicillin and vancomycin against various bacterial pathogens commonly associated with gastrointestinal infections (Ebbensgaard *et al*., 2015; Irazazabal *et al*., 2016:2702; Lewies *et al*., 2017:249; Li *et al*., 2000:205; Tong *et al.,* 2014)

Organism	Minimum inhibitory concentration (µg/ml)				
	Melittin	Mastoparan	Nisin Z	Ampicillin	Vancomycin
Staphylococcus aureus	4,17	3,12	10	1,04	1-2
Escherichia coli	5	6,25	16,67	2,5	400
Enterococcus faecalis	2-8	2-32	1000	4	Resistant
***Salmonella* species**	32-64	123	9	<1	Resistant

Although research into the cytotoxicity towards mammalian cells is limited, Table 2.5 illustrates previous studies performed on the *in vitro* cytotoxic activity of melittin, mastoparan and nisin Z against various tumour and non-tumour cell lines.

2.4 Formulation of antimicrobial peptides

AMPs require special formulation to be administered as an acceptable therapeutic drug. The formulation of such a product is critically dependant on the physiochemical properties of the drug as well as the route of administration. Currently, antibiotics that are clinically used to treat GI infections are mostly administered orally or by means of the intravenous (IV) route. However, these two routes of administration pose significant challenges for peptide based drugs such as AMPs.

Table 2.5: Cytotoxicity of melittin, mastoparan and nisin Z against various non-tumour and tumour cell lines (De Azevedo *et al*., 2015:115; Kaur & Kaur, 2015; Rady *et al*., 2017:20)

Cell line	Origin	IC_{50} (µM)
Melittin		
HepG2	Human liver hepatocellular carcinoma	1,2
U251	Human malignant glioblastoma tumour	3,0
H460	Human lung carcinoma	1,7
Su8686	Human pancreatic carcinoma	1,9
LA-N-1 NB	Human neuroblastoma, bone marrow metastatis	1,2
HaCat	Human keratinocyte	2,6
HT29	Human colon adenocarcinoma	1,2
Caco-2	Human epithelial colorectal adenocarcinoma	1,8
Jurkat	Human leukemic T cell lymphoblast	1,0
Mastoparan		
A2058	Human metastatic melanoma	140
MCF-7	Human breast adenocarcinoma	432,5
MDA-MB-231	Human breast adenocarcinoma	251.5
SiHa	Human cervical carcinoma	172,1
SK-BR3	Human breast adenocarcinoma	320,3
U87	Human glioblastoma astrocytoma	311,7
Jurkat	Human leukemic T cell lymphoblast	77,9
Nisin Z		
MCF-7	Human breast adenocarcinoma	105,5
HepG2	Human liver hepatocellular carcinoma	112,3
Jurkat	Human leukemic T cell lymphoblast	225,0
HT29	Human colon adenocarcinoma	89,9
Caco-2	Human epithelial colorectal adenocarcinoma	115

2.4.1 Routes of Administration

The oral route is the most convenient route for administrating drugs with the highest degree of patient compliance (Banga, 2015:233). It further has the advantage of being a self-administered route which is uncomplicated, pain free, non-invasive, cost effective, and it also supports a variety of dosage forms (Verma *et al*., 2010:54). After a drug is administered orally, it is absorbed through or between the GI epithelial cells and reaches the systemic blood circulation, where it is then circulated to different parts of the body and to the target site to have a systemic effect. For the administered drug to exert its pharmacological effect, the drug molecule must remain intact during and after intestinal absorption. However, the protective

mechanisms of the GI-tract, which include various biochemical and physical barriers, counteract the absorption of orally administered drugs and compromise the molecule structure leading to low drug bioavailability (Hamman *et al*., 2005:166; Verma *et al*., 2010:54). These mechanisms thus pose a challenge or limit the use of the oral route to administer drugs such as peptide based drugs that are susceptible to the physical and biochemical barriers. AMP drug formulations demonstrate low oral bioavailability as a result of pre-systemic enzymatic degradation and poor penetration of the intestinal epithelial cells (Mahlapuu *et al*., 2016). As a consequence of the challenges and limits of orally administered peptide drugs, therapeutic peptide formulations are currently administered almost exclusively by the parenteral route (Banga, 2015:219).

While parenteral delivery consists of three mayor routes, namely intravenous (IV), intramuscular (IM) and subcutaneous (SC), IV administration remains the method of choice for systemic delivery of peptide drugs. Once a drug is administered via injection or infusion IV, it is directly inserted into the blood and is immediately available systemically with a quick onset of action. By this route absorption is bypassed which is beneficial for drug molecules that are susceptible to the physical and biochemical barriers of the GI-tract (Ratnaparkhi *et al*., 2011:32; Verma *et al*., 2010:57). However, IV administration of AMP drug formulations are limited by their short half-life as a result of enzymatic degradation in the blood plasma and removal of the drug molecule from circulation by the liver and kidneys (Mahlapuu *et al*., 2016). In addition, many patients abstain from IV treatment as it is more painful, shows a higher risk of infection, mostly needs to be done under the supervision of a healthcare professional, and has a higher risk of forming air embolisms or blood clots (Verma *et al*., 2010:57).

2.4.2 Stability of AMP therapeutics

AMP therapeutics are complex molecules comprised of several amino acid residues with defined primary and secondary structures. Principal protein or peptide stability is the result of a spontaneous, physical process called folding, where amino acids inside the unfolded, linear polypeptide chain (primary structure) interact with each other to cause a fold in the chain to produce a three dimensional secondary structure. This conformational structure, also called the native conformation, is biologically active, marginally more stable than the unfolded state, and is essential for the therapeutic activity of the peptide drug (Banga, 2015:73; Gokarn *et al.,* 2006:291). Looking at the term stability in the context of protein or peptide structure, it can be defined as the tendency to maintain the native conformation (Jacob *et al*, 2006:155).

Proteins can degrade through physical processes namely, denaturation, adsorption, aggregation and precipitation. Denaturation is a process where there is a disruption in a protein’s secondary structure which results in loss of the native structure and its original

characteristics. This process involves unfolding of the protein in a sharp transition in structure from the native conformation to the unfolded state, where only a small disruption in the folded structure can lead to the unfolding of the entire molecule. Stress factors that cause destabilisation initiating denaturation include extreme pH levels, thermal stress, ultra violet light and exposure to denaturing agents and chemicals (Banga, 2015:74; Jacob *et al*., 2006:155).

The amphipathic characteristics of protein molecules are largely responsible for their surface-active properties which result in their adsorption at liquid-solid, liquid-air and liquid-liquid interfaces. Adsorption is the consequence of temperature, pH or agitation induced stresses. Though the principal is very simple, this process can significantly impact the stability and potency of a protein drug and also act as the initiating step for aggregation (Gokarn *et al.,* 2006:293; Jacob *et al.,* 2006:161). According to various studies, the most frequently manufacture and formulating problem, when facing protein instability, is protein aggregation. Protein molecules can undergo an irreversible reaction called self-association, to form higher-order multimers. These higher-order multimers, called aggregates, can either be soluble or insoluble. Whereas aggregation occurs due to a wide variety of intrinsic and extrinsic factors, the most common mechanism involved in protein aggregation includes the denaturation of the protein followed by non-covalent association at hydrophobic interfaces. This physical instability may either lead to the loss of therapeutic activity when the aggregate is formed, or the aggregate may exert its therapeutic activity, but lose it upon dissociation if soluble. Nonetheless, the formation of an insoluble aggregate which is still therapeutically active, threatens to cause blockage to small veins, membranes, tubing and pumps, while the larger aggregated protein may also be more immunogenic. Precipitation is the macroscopic result of aggregation (Banga, 2015:75; Gokarn *et al.,* 2006293).

Additionally, protein molecules are subjected to a variety of chemical degradation reactions which include hydrolysis, deamination, oxidation, beta elimination and disulphide exchange. Protein hydrolysis is a degradation pathway where peptide bonds are broken within the protein molecule resulting in smaller amino acid chains (Banga, 2015:85). Deamination is a non-enzymatic hydrolytic reaction which is generally catalysed by acid, base, heat and ionic strength. This reaction involves the direct hydrolysis of the side-chain amide groups to form free carboxylic acid, disrupting the native state of the protein and leaving it susceptible to proteases and denaturation (Jacob *et al.,* 2006:157).

The most general degradation pathway of proteins and peptides, is oxidation. Protein oxidation involves a covalent modification to the protein structure and can be induced by temperature, pH, light, formulation impurities and trace amounts of metal ions. Amino acid residues that are susceptible to oxidation include methionine, cysteine, tryptophan, tyrosine and histidine

(Banga, 2015:85; Gokarn *et al*., 2006:294). Beta elimination involves parallel cleavage and intramolecular cross-linking reactions, where thermal stress will cause the destruction of disulphide bonds within a protein. Cysteine, lysine, phenylalanine, serine and threonine residues, under alkaline pH conditions, are susceptible to this process (Gokarn *et al.,* 2006:294). A peptide or protein with more than one disulphide bridge between two cysteine residues or unpaired cysteine residues may undergo disulphide exchange. The cysteine residues react in a way to modify old disulphide bridges or to form new disulphide bridges. This process results in proteins with non-native conformations and incorrect disulphide linkages (Banga 2015:87).

Peptide and protein drugs, for example AMP therapeutics, comprise several amino acid residues. For these drugs, there are numerous reactive sites that are susceptible to chemical degradation, which in return can catalyse physical degradation and visa-versa. The successful formulation of peptide and protein drugs depends on ensuring chemical and physical stability throughout manufacturing, distribution and during storage which is also crucial for ensuring efficacy and safety of the drug.

2.5 Excipients with stabilising properties

Stabilising peptide drug formulations against various chemical and physical stresses during manufacturing, shipping and storage still remain the principal challenge in developing safe and effective protein therapeutics. Excipients are additives that may be included in peptide formulations to enhance the stability of the final drug product and protect against stresses as mentioned in Section 2.4.1. Generally, excipients can be classified by their functional role in the formulation or the mechanism by which they stabilise proteins (Gokarn *et al*., 2006:295). For the purpose of the study, only the excipients that are going to be investigated will be mentioned and the stabilising mechanism thereof stated.

2.5.1 L-glutamic acid

L-glutamic acid is a non-essential amino acid normally used as an excipient in liquid protein formulations to increase stability. This water soluble amino acid is negatively charged in its anionic form at physiological pH and has the ability to establish an acidic environment as it lowers the pH in saturated solutions. The main purpose of amino acid additives is to reduce surface adsorption, inhibit protein aggregation and thereby increase stability (Banga, 2015:114; Forney-Stevens *et al*., 2016:697). Although amino acids can stabilise proteins through a variety of mechanisms, the specific mechanism in a specific formulation remains unclear. This class of additives has the ability to increase the ionic strength of the solution while decreasing the electrostatic interactions between protein drug moieties (Challener, 2015:s37). In addition,

amino acids have anti-oxidant properties and its buffer capacity to further protect and stabilise proteins (Kamerzell *et al*., 2011:1122). More specific, L-glutamic acid is used in formulations to increase the solubility of proteins and prevent drug precipitation. Other mechanisms may include the indirect chelation of metal ions to reduce or prevent aggregation, direct binding to the protein or stabilising proteins due to preferential exclusion (Banga, 2015:114; Chi, 2012:261).

2.5.2 Chitosan

Chitosan is a natural polysaccharide that consists of copolymers of glucosamine and N-acetyl glucosamine which is produced by partial deacetylation of chitin, the primary component of cell walls in fungi and exoskeletons of arthropods. The term chitosan is generally used to describe a series of chitosan polymers rather than a specific molecule as these polymers differ in molecular weight (50 kDa to 2000 kDa), viscosity and degree of deacetylation (40–98%). The physico-chemical properties further define chitosan as a cationic polyamine with a high charge density and the ability to adhere to negatively charged surfaces (Singla & Chawla, 2001:1048). Chitosan exhibits several beneficial biological properties that all contribute to it being a promising additive in protein formulations; these properties briefly include its biocompatibility, biodegradability, mucoadhesive characteristics, low toxicity and low antigenic potential (Andrade *et al*., 2011:158). Studies have also reported chitosan to possess analgesic, antitumor, anti-oxidant and antimicrobial activities (Aranaz *et al*., 2009:207; Fernandez-Saiz *et al*., 2009:917). In protein formulations, cationic polymers enhance overall stability by means of preventing protein aggregation either through enhancing the protein assembly, chelating metal ions, suppressing protein oxidation or causing preferential exclusion of the excipient itself (Challener, 2015:s37; Chi, 2012:260).

2.5.3 Polysorbate 80

Polysorbate 80 is a non-ionic surfactant with amphipathic properties that consists of heterogeneous molecular weight mixtures of fatty acid esters of polyoxyethylene sorbitan. This surfactant is also known as Tween 80 and is one of the most frequently used surfactant excipients in protein formulations (Kamerzell *et al.,* 2011:1123). The principal stabilising mechanism of surfactants aims to prevent surface and stress induced aggregation by competing with proteins on various surfaces and interfaces. By out-competing proteins, they prevent adsorption, protein denaturation as a result of adsorption and the subsequent aggregation. In addition, polysorbates may act as ‘chaperone’ molecules by promoting protein folding and refolding to prevent aggregation (Chi, 2012:260). Although polysorbate 80 is frequently used as a surfactant in various pharmaceutical products, these additives are susceptible to oxidative degradation, where the degradation products thereof may negatively

influence protein stability in drug formulations and induce or accelerate protein degradation (Gokarn *et al*., 2006:301). Table 2.6 includes a brief summary of the three stabilising excipients, L-glutamic acid, chitosan and polysorbate 80; as well as their physical, chemical and peptide stabilising properties.

Table 2.6: Summary of the physical, chemical and peptide stabilising properties of L-glutamic acid, chitosan and polysorbate 80

	L-Glutamic Acid	Chitosan	Polysorbate 80
Source	Non-essential amino acid	Chitin	Heterogeneous mixture of polyoxyethylene sorbitan
Molecular formula	$C_5H_9NO_4$	$(C_6H_{11}NO_4)n$	$C_{24}H_{44}O_6$
Molecular weight	147,13 g/mol	50 000 – 190 000 g/mol	428,6 g/mol
Solubility	Water soluble, 8,64 g/l at 25℃	Water insoluble, soluble in diluted acid solutions	Water soluble, 0,1 ml/ml
Net charge of solution	Anionic solution	Cationic solution	Non-ionic solution
Classification as excipient	Amino acid	Polysaccharide	Surfactant
Peptide stabilising mechanism/s	• Reduce surface adsorption • Inhibit peptide aggregation • Increase ionic strength of solution • Buffer capacity • Anti-oxidant properties • Preferential exclusion	• Prevent peptide aggregation • Enhance peptide assembly • Chelate metal ions • Suppress peptide oxidation • Preferential exclusion	• Prevent surface and stress induced aggregation by out-competing peptide on surfaces and interfaces • Prevent adsorption and peptide denaturation • Promote peptide folding and refolding

Even though many excipients used in drug formulations are listed as GRAS in the inactive ingredient list approved by the FDA, they still have the ability to affect the stability, efficacy and immunogenicity of the drug molecule itself. Regardless of the specific stabilising mechanism of an excipient, each protein formulation and specific drug-excipient interaction are extremely unique as a result of their individual physical and chemical properties. (Banga, 2015:111; Gokarn *et al*., 2006:292). It is important for excipients to exhibit biocompatible interactions with both the protein drug as well as host organism, however, these additives may additionally exert other toxicological or biological activities that affect the overall safety profile of the drug formulation. It is important to note the high concentrations of excipients are often used in the final peptide formulations to prevent aggregation. Hence, additional preclinical testing is

necessary for specific drug-excipient combinations since the independent safety profile of either drug or excipient does not determine the overall safety profile of the formulation (Andrade *et al*., 2011:163; Kamerzell *et al*., 2011:1122).

2.6 References

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Chapter 3

Methods

3.1 Introduction

During drug development and preclinical testing of orally administered drugs, there are five critical drug-like properties that need to be investigated, namely: absorption, distribution, metabolism, elimination and toxicity. For intravenous (IV) administered drugs absorption is avoided as drug molecules are delivered directly into the bloodstream. Between 2006 and 2010, more than one-quarter of the drug candidates that entered clinical development and testing failed due to unexpected drug toxicity and safety issues (O'Brien, 2014:4). Determining the toxicity profile of potential new drug candidates during development and preclinical testing will enhance the probability of clinical success (Li, 2005:179).

Laboratory mammals have been extensively used as *in vivo* models to study drug toxicity. However, this approach presented many drawbacks over the years, which briefly include: assays are expensive and many species-specific; large amounts of the drug candidate are needed; poor comparability to human responses as animal models frequently do not reflect human toxicity due to functional differences; it is a low to moderate-throughput model, and the ethical dilemma of testing on living animals (NASEM, 2015:60).

Cytotoxicity testing is an *in vitro* cell-based approach that measures different parameters that are involved in the progression of cell death and proliferation. The term 'cytotoxicity' refers to the potential of a substance to alter basal cellular functions and cell behaviour in such a way that it results in cell death. Compared to animal models, this cell-based approach is easier to perform and reproduce, the tests are less expensive, experimental conditions are easier to maintain and control, ethically it is less ambiguous and is a high-throughput analysis model (Cummings *et al*., 2012:12.8.1; Mahto *et al*., 2012:87).

Gastrointestinal (GI) models are tissue-engineered models that emulate the GI environment and are used in cytotoxicity testing of drug candidates. In this study, two Caucasian human cell lines, HepG2 and Caco-2, were employed as representative models to assess the interaction of various AMP drug formulations with intestinal epithelial cells to evaluate their cytotoxic properties. Both HepG2, a hepatocellular liver carcinoma cell line, and Caco-2, a colorectal adenocarcinoma cell line, are adherent cell lines with epithelial morphology and functional characteristics similar to *in vivo* human conditions. Caco-2 cultures are extensively used in toxicity studies as they form a polarised enterocyte-like monolayer that imitates the intestinal barrier. In contrast, HepG2 cells represent an *in vitro* liver toxicity model used to characterise the hepatotoxicity of a drug (Orbach *et al*., 2017:1900).

Using more than one human intestinal cell line together with multiple cytotoxicity assays have been reported to possess a predictive value of whether a particular substance retains a

hazardous effect towards the clinical use thereof in humans (Hurley *et al*., 2016:76). In this study cell cultivation and cytotoxicity evaluations were done according to the International Organisation for Standardisation (ISO) (2009:5) guidelines as well as the guidelines specified by the Organisation for Economic Co-operation and Development (OECD) (2010).

3.2 Experimental design

The experimental approach of this study was divided between the three AMP drug candidates that were selected. The specific drug candidate alone and in combination with various concentrations of three individual stabilising excipients were investigated to determine the initial and altered cytotoxicity. The cytotoxicity studies were conducted using two mammalian cell lines and all combinations of treated cells were stained and photographed. Figure 3.1 illustrates a detailed representation of the experimental design of the study.

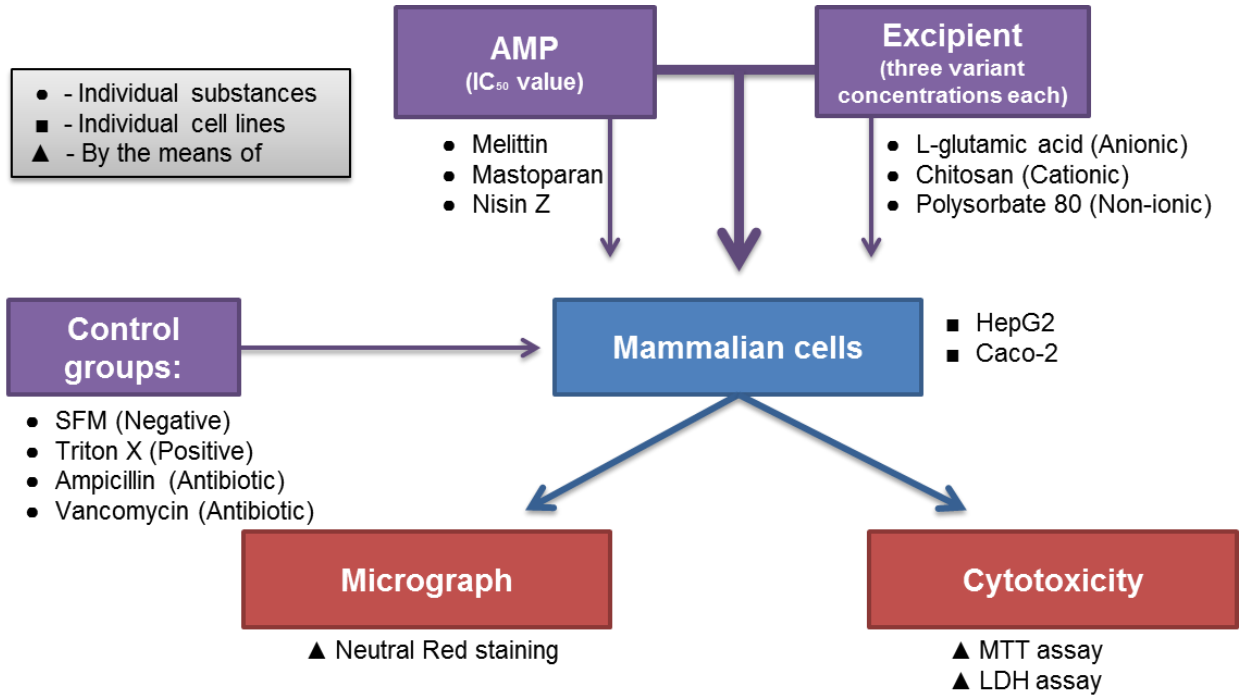

Figure 3.1: Detailed graphical representation of the experimental design

3.3 Mammalian cell cultures

3.3.1 Cell culturing

An ethics application was submitted and approved by the Health Research Ethics Committee (HREC) of the North-West University for *in vitro* cytotoxicity experiments done on HepG2 and Caco2 cells. HepG2 and Caco-2 cell lines were purchased from the American Type Culture Collection (ATCC) (catalogue number HB-8065 and CL-101 respectively). Cells of both cell lines were separately grown and subsequently cultured in 75 cm^2 cell culture flasks (Whitehead

Scientific, catalogue number 708003). Cell passages 24 to 37 were used for the HepG2 cell line and cell passages 54 to 62 for the Caco-2 cell line.

Both HepG2 and Caco-2 cells were maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Separations, catalogue number SH30243.FS) and fortified with additional foetal bovine serum (FBS) to a final concentration of 10% (Thermo Fisher Scientific, catalogue number 10499044). The growth medium was further supplemented with 1% non-essential amino acids (NEAA) (Lonza, catalogue number BE13-114E) and 1% penicillin/streptomycin (Pen/Strep) (Lonza, catalogue number DE17-602E). The cell cultures were maintained and incubated under standard culture conditions for mammalian cells, which entails a 5% CO_2 atmosphere at 37°C, using a Forma Steri-Cycle CO_2 Incubator (Thermo Scientific, Labotec, Midrand, SA). The culture medium was renewed twice a week or when necessary under aseptic conditions in a horizontal laminar air flow cabinet.

3.3.2 Sub-culturing cells

Prior to growth media exchange and sub-culturing, cells were inspected by the means of a light microscope (Axiovert 25, Carl Zeiss Microscopy, New York, USA) to determine the percentage confluency. Upon ±80% confluency, both HepG2 and Caco-2 cells were sub-cultured by means of trypsination. The growth medium was carefully decanted and the cell layer rinsed once with 10 ml phosphate buffered saline (PBS) (Sigma-Aldrich, catalogue number P4417). To this end, 3 ml of a 1% trypsin/EDTA solution (Lonza, catalogue number BE02-007E) was added to the flask to detach the cells from both the flask surface and from each other. After the trypsination solution was added, the HepG2 and Caco-2 cells were incubated for 3 and 5 min, respectively. Following incubation, 6–8 ml of growth medium was added to the flask to neutralise the trypsination effect. The cell suspension was agitated to loosen cells and gently pipetted to render a homogenous suspension. Appropriate aliquots of cell suspension were transferred to new cell culture flasks containing fresh growth medium and returned to the incubator.

3.3.3 Seeding cells into 96-well plates

The HepG2 and Caco-2 cells were seeded into 96-well plates. A cell suspension was obtained by means of trypsination as described in Section 3.3.2. The complete cell suspension was transferred to a 15 ml tube and centrifuged for 4 min at 400 xg (Hermle Z300, LASEC, Cape Town, SA). The medium was carefully decanted not to disturb the cell pellet that formed on the bottom of the tube. The cell pellet was re-suspended with 1 ml serum free growth media (SFM), which consisted of DMEM and supplemented with 1% NEAA and 1% Pen/Strep. Cells in suspension were counted using an automated cell counter (Invitrogen Countess, Thermo

Fisher Scientific). A volume of 50 µL cell suspension and 50 µL 0.4% Trypan Blue (Sigma Aldrich, catalogue number RNBC 9848) were mixed in a 2.5 ml Eppendorf tube using a micro-pipet. The cell counting mixture was triturated to render a homogenous mixture and 10 µL of the mixture was extracted and carefully expelled onto both counting chambers on the slide. Afterwards, the cell suspension was diluted to achieve 2.5 x 10^4 cells/well for HepG2 cells and 4.0 x 10^4 cells/well for Caco-2 cells in 100 µL.

Seeding of cells into 96-well plates occurred under strict sterile conditions in the horizontal laminar air flow cabinet. A total volume of 100 µL of the final cell suspension was pipetted into each well to be treated and left to adhere overnight before treatment for cytotoxicity studies commenced.

3.4 Preparation of antimicrobial drug candidates, excipients and control groups

3.4.1 Melittin

A 0.8 mg/ml stock solution of melittin (>97% synthetic) (Sigma Aldrich, catalogue number M4171) was prepared in PBS with a final concentration of 280.8 µM aliquot in 45 µL stock samples in 0.1 ml Eppendorf tubes and stored at -20°C. For each experiment melittin was freshly prepared; the stock solution was thawed at 24°C, which was established as room temperature and diluted to the desired concentration with SFM. A clear transparent solution was obtained with no precipitation in PBS stock solution and further dilution in SFM also rendered a clear red solution with no precipitation.

3.4.2 Mastoparan

An 1 mg/ml stock solution of mastoparan (>97% synthetic) (Sigma Aldrich, catalogue number M5280) was prepared in PBS with a final concentration of 676 µM aliquot in 80 µL stock samples in 0.1 ml Eppendorf tubes and stored at -20°C. For each experiment mastoparan was freshly prepared; the stock solution was thawed at room temperature and diluted to the desired concentration with SFM. A clear transparent solution was obtained with no precipitation in PBS stock solution and further dilution in SFM also rendered a clear red solution with no precipitation.

3.4.3 Nisin Z

Nisin Z®, + Ultrapure nisin Z (>95% HPLC) was a kind donation from Handary (Brussels, Belgium). A 20 mg/ml stock solution of nisin Z was prepared in 0.01 M HCl with a final concentration of 5 962.90 µM aliquot in 40 µL stock samples in 0.1 ml Eppendorf tubes and stored at -20°C. For each experiment nisin Z was freshly prepared; the stock solution was

thawed at room temperature and diluted to the desired concentration with SFM. A clear solution was obtained with no precipitation in 0.01 M hydrochloric acid (HCl) stock solution. Further dilution however in SFM caused some yellow precipitation to form in a red-pink solution.

3.4.4 Excipients

During optimisation studies, each excipient solution was freshly prepared prior to the experiment. L-glutamic acid (Sigma Aldrich, catalogue number G8415) stock solutions were prepared in SFM at 0.75 mg/ml, 1.5 mg/ml, 2.25 mg/ml, 3 mg/ml, 3.75 mg/ml and 4.5 mg/ml. L-glutamic acid was fully dissolved in SFM and a clear yellow-orange solution was obtained as a result of a decrease in pH of the solution. Chitosan (low molecular weight) (Sigma Aldrich, catalogue number 448869) stock solutions were prepared in SFM at 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml and 20 mg/ml. Chitosan did not dissolve in the SFM and remained in the solution as undissolved flakes. Polysorbate 80 (Sigma Aldrich, catalogue number P4780) stock solutions were prepared in SFM at 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml. The viscous liquid form of polysorbate 80 dissolved completely in SFM and a clear red solution was obtained.

For the final cytotoxicity studies, excipient stock solutions were freshly prepared prior to each experiment. The SFM used in these preparations were incubated overnight at 37°C. Final excipient solutions were prepared for each toxicity assay as 0.75 mg/ml, 1.5 mg/ml and 3 mg/ml for L-glutamic acid; 5 mg/ml, 1.5 mg/ml and 20 mg/ml for chitosan; and 2 mg/ml, 4 mg/ml and 8 mg/ml for polysorbate 80.

3.4.5 Control groups

3.4.5.1 Negative control group for toxicity studies

A negative control group is material that does not produce a cytotoxic effect and is included to demonstrate the background response of the cells (ISO, 2009:2). For both cytotoxicity studies SFM was chosen as a negative control. SFM was prepared by supplementing DMEM with 1% NEAA and 1% Pen/Strep.

3.4.5.2 Positive control group for toxicity studies

A positive control group provides a reproducible cytotoxic response and is included to demonstrate an appropriate test system response (ISO, 2009:1). For both cytotoxicity studies Triton X-100 (Sigma Aldrich, catalogue number T8787) was chosen as a positive control. A

volume of 40 µL of Triton X was dissolved in 10 ml SFM and the solution was stored at 4°C until utilised.

3.4.5.3 Antibiotic control groups for toxicity studies

Two antibiotic control groups were included in this study as a parameter to compare the cytotoxicity of the drug:excipient combinations to clinically used antibiotics. For both cytotoxicity studies, vancomycin (Sigma Aldrich, catalogue number 75423) and ampicillin (Sigma Aldrich, catalogue number A5354) were used as they are clinically utilised in the treatment of various GI infections (Kim *et al*., 2017:93). Vancomycin is normally used to treat GI infections caused by vancomycin susceptible *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, vancomycin susceptible *Enterococcus* species and *Clostridium difficile* strains. However, its antibacterial spectrum is limited to Gram-positive pathogens only. Ampicillin, on the other hand, has antibacterial activity against Gram-positive and Gram-negative bacteria including *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Salmonella* species, which are also GI infection causing agents (Ebbensgaard *et al.,* 2015; Tong *et al*., 2014).

Previous *in vitro* studies done on the antibacterial effect of vancomycin and ampicillin, on the above mentioned pathogens that commonly cause GI infections, were used to determine the minimum inhibitory concentration (MIC) to be used as control group in cytotoxicity assays (Jones, 2006:s21; Kim *et al*., 2015:464). An 1 mg/ml stock solution of vancomycin was prepared in sterile water and further diluted during each experiment with SFM to obtain a final solution of 32 µg/ml. A 10 mg/ml stock solution of ampicillin was prepared in sterile water and further diluted during each experiment with SFM to obtain a final solution of 20 µg/ml. Both antibiotics, ampicillin and vancomycin were fully dissolved in the final solution in SFM with no precipitation; and both rendered clear red solutions.

3.5 Optimisation studies

The MTT assay, described in Section 3.6.1 was used with each AMP drug and stabilising excipient individually to plot a dose-response curve. A dose-response curve describes the relationship between increasing concentrations of the material being tested and the change in response as a result of increased concentrations. This was used to determine the half maximal inhibitory concentration (IC_{50}) of the drug candidates and the excipient concentration range that sustained cell viability above 80%. The IC_{50} of the drug is defined as the concentration AMP required to reduce the absorbance of the formazan product by 50% and thus indicating 50% cell death (Maher & McClean, 2006:1290).

Melittin was used as a model peptide for the AMP drug candidates to determine the exposure time for the cytotoxicity assays as it is the most cytotoxic between the three peptides (Hurley *et al.*, 2016:77). A concentration range of 0.625 µM to 20 µM was tested over 4, 8 and 24 hours (h). An exposure time of 6 h was later also tested as most clinical antibiotics are prescribed to be taken every 6 h.

The concentration range of each peptide that was investigated was determined with reference to previous literature published on the *in vitro* cytotoxicity profiles (Hurley *et al*., 2016:78; Lewies *et al.,* 2017:246; Vila-Farrés *et al.,* 2015:36). The MTT assay was repeated on melittin (concentration range 0.5 µM–6 µM), mastoparan (concentration range 20 µM–80 µM) and nisin Z (concentration range 150 µM–425 µM) over a 6 h exposure time to determine the IC_{50} values. For the excipients, the MTT assay was used to determine a concentration range that maintained and exceeded 80% cell viability in order that the possible excipient toxicity did not affect the final cytotoxicity of drug:excipient combinations. This included L-glutamic acid (0.75 mg/ml, 1.5 mg/ml, 2.25 mg/ml, 3 mg/ml, 3.75 mg/ml and 4.5 mg/ml), chitosan (2.5 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml and 20 mg/ml) and polysorbate 80 (2.5 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml).

3.6 Cytotoxicity assays

Cytotoxicity evaluations were done according to the guidelines specified by both the International Organisation for Standardisation (ISO, 2009:5) as well as the Organisation for Economic Co-operation and Development (OECD, 2010).

For the cytotoxicity studies, HepG2 and Caco-2 cells were seeded into 96-well plates at 2.5×10^4 cells per well and 4.0×10^4 cells per well, respectively and as described in Section 3.3.3. For the LDH assay, 96-well black plates were used. After cell seeding, the plates were left overnight for cells to attach and reach approximately 90% confluency before treatment began. For each well to be treated, the growth media was carefully removed beforehand. Each treatment consisted of an individual AMP drug alone, and in combination with three different concentrations of the three selected stabilising excipients over an exposure time of 6 h (Table 3.1). For each test the relevant controls were included. All experiments were at least performed in triplicate and independently repeated.

Table 3.1 Different treatment combinations of antimicrobial drugs (IC_{50} values) with three different concentrations of each protein stabilising excipient. Values were determined during experimental optimisation

	L- glutamic acid µM / mg/ml	Chitosan µM / mg/ml	Polysorbate 80 µM / mg/ml
Melittin	**1:0** 1:0.75 1:1.5 1:3 **0:3**	**1:0** 1:5 1:10 1:20 **0:20**	**1:0** 1:2 1:4 1:8 **0:8**
Mastoparan	**40:0** 40:0.75 40:1.5 40:3 **0:3**	**40:0** 40:5 40:10 40:20 **0:20**	**40:0** 40:2 40:4 40:8 **0:8**
Nisin Z	**370:0** 370:0.75 370:1.5 370:3 **0:3**	**370:0** 370:5 370:10 370:20 **0:20**	**370:0** 370:2 370:4 370:8 **0:8**

3.6.1 Colorimetric tetrazolium dye assay

The MTT assay was used to detect *in vitro* cell viability and proliferation and therefore indirectly measured cytotoxicity. This method was developed by Mosmann (1983:56) to be used as a quantitative colorimetric assay. The principle of the method is based on the reduction of yellow tetrazolium salt (MTT substrate) by the mitochondrial dehydrogenase enzymes of living cells to produce crystalline purple formazan as product (Figure 3.2) (Fotakis & Timbrell, 2006:172). This formazan product accumulates as insoluble precipitate inside living cells and can be dissolved by DMSO to render a solution of which the absorbance is read to determine cell viability. Cells that have died do not have the ability to convert MTT substrate into purple formazan. Thus, the quantity of formazan that are produced as product is presumably directly proportional to the number of living cells (Maher & 257 McClean, 2006:1290).

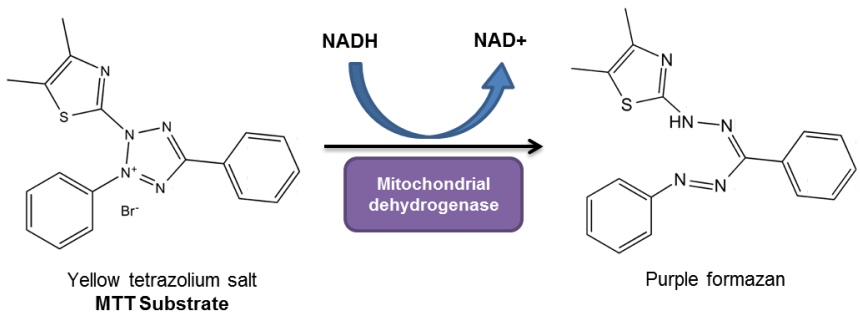

Figure 3.2: The mitochondrial reduction of yellow tetrazolium salt to in-soluble purple formazan (adapted from Aula *et al*., 2015:47839)

After cell seeding, overnight incubation time (time for cells to adhere and reach ~90% confluency) and 6 h treatment exposure as described in Section 3.6; growth medium was carefully removed and cells were rinsed once with 100 µL PBS. A 5 mg/ml MTT solution was prepared by diluting 5 mg of MTT substrate (Sigma Aldrich, catalogue number M5655) in 1 ml PBS which was further diluted in a 1:10 ratio in SFM. The cells were treated with 100 µL of diluted MTT solution and incubated for 4 h at 37°C. After incubation, the MTT solution was carefully removed and replaced with 100 µL DMSO to dissolve the formazan crystalline product and incubated for another 1 h at 37°C. DMSO served as a blank. Cell viability was determined using a microplate reader (SpectraMax® Paradigm™ Molecular devices, USA) measuring the absorbance at a wavelength of 560 nm and background at a wavelength of 630 nm. Cell viability is expressed as a percentage relative to the untreated control, which was set at 100% viable using Equation 3.1, where the Δ absorbance of the treatment, blank and control was calculated by subtracting the absorbance from 560 nm from the absorbance from 630 nm, respectively.

$$\text{Cell viability (\%)} = \frac{\Delta \text{ treatment} - \Delta \text{blank}}{\Delta \text{control} - \Delta \text{blank}} \times 100 \qquad \textbf{Eq (3.1)}$$

3.6.2 Lactate dehydrogenase assay

CytoTox-ONE™ Homogeneous Membrane Integrity assay (Promega, catalogue number G7891) is a fluorometric method that was used to determine cell membrane integrity and determine the number of nonviable cells. Cell membrane integrity is often a parameter that is used to define cell viability. When cell membrane damage occurs, it results in the loss of intracellular lactate dehydrogenase (LDH) into extracellular medium and the LDH leakage serves as an indicator for cell death (Fotakis & Timbrell, 2006:171). This assay was developed by Decker and Lohmann-Matthes (1988:63) and is based on the reduction of nicotinamide adenine dinucleotide (NAD) by LDH to the reduced product of nicotinamide adenine dinucleotide-hydrogen (NADH). The CytoTox-ONE™ assay measures LDH release in the conversion of resazurin into fluorescent resorufin by the means of NADH (Figure 3.3).

The instructions of the CytoTox-ONE™ Homogeneous Membrane Integrity assay kit were followed. After cell seeding, overnight incubation time (time for cells to adhere and reach ~90% confluency) and 6 h treatment exposure as described in Section 3.6, the plate was removed from the incubator and left to equilibrate at room temperature (approximately 20–30 min). During equilibration, 2 µL of Lysis Solution was added to the positive control wells to serve as the maximum LDH release control. A volume of 50 µL CytoTox-ONE™ Reagent was added to each well and gently mixed for 30 seconds (s), after which the plate was incubated for 10 min at room temperature. Each well was subsequently treated with 25 µL Stop Solution in the same

order of addition that was used for adding the CytoTox-ONE™ Reagent and gently mixed for 10 s. The fluorescence was recorded at an excitation wavelength of 560 nm and an emission wavelength of 590 nm using a microplate reader (SpectraMax® Paradigm™ Molecular devices, USA).

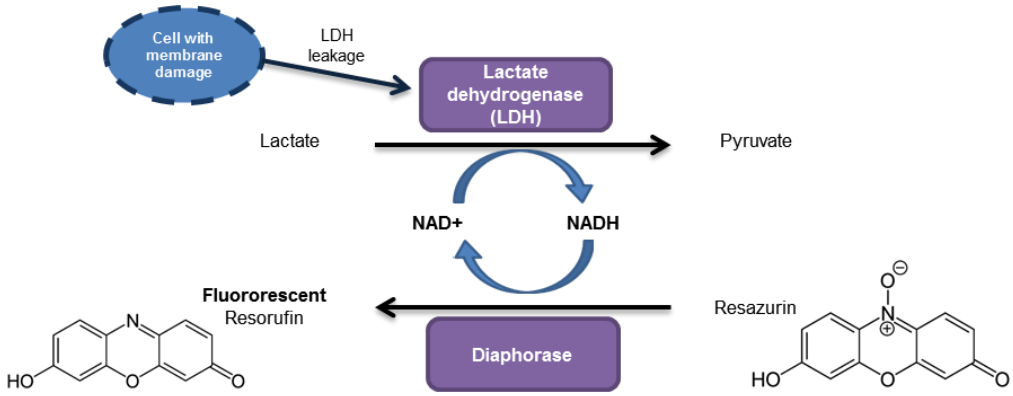

Figure 3.3: LDH release from damaged cell membranes promotes the reduction of NAD to produce NADH, which converts resazurin to fluorescent resorufin (adapted from Aula *et al*., 2015:47840)

Results are expressed as a percentage relative to the untreated control and the maximum LDH release control, which was set as having 0% and 100% LDH release, respectively, using Equation 3.2. The average culture medium fluorescence was subtracted from each value to exclude the background from the medium.

$$\text{LDH release (\%)} = \frac{\text{Fl. treatment-Fl. control}}{\text{Fl. max LDH release-Fl. control}} \times 100 \qquad \textbf{Eq (3.2)}$$

3.6.3 Neutral Red Staining

Neutral red dye (Sigma Aldrich, catalogue number N2889) was used in this study to visually illustrate cell viability as previously described by Wentzel *et al.* (2017:29). In principle, viable cells have the ability to incorporate and bind this weakly cationic red dye to anionic and/or phosphate groups of the lysosomal matrix, where nonviable cells will not take up the dye. Thus, the neutral red dye accumulates in the liposomes of viable cells which can be visualised under a light microscope (Repetto *et al.*, 2008:1125).

After cell seeding, overnight incubation time (time for cells to adhere and reach ~90% confluency) and 6 h treatment exposure as described in Section 3.6, growth medium was carefully removed and cells were rinsed twice with 100 µL PBS. Cells were treated with 10 µl of a 0.33% (v/v) Neutral Red Reagent (Sigma Aldrich, catalogue number 2889) solution diluted in 90 µL PBS and incubated for 2 h at 37°C. After incubation, the neutral red solution was removed and replaced with 100 µL Neutral Red Assay Fixative (Sigma Aldrich, catalogue number N4270). The cells were fixated for 60 s and rinsed twice with 100 µL PBS. Cells were

photographed in 100 µL PBS using a Nikon Eclipse TS100 microscope equipped with a Nikon TV Lens C-0.35X camera and IC Capture Version 2.3 capturing software (The Imaging Source Europe GmbH).

3.7 Data analysis

All experiments were done in triplicate and independently repeated. The optimisation and cytotoxicity data were analysed using GEN5 Software. The results were analysed and processed using GraphPad Prism™ Version 5 (GraphPad 363 Software Inc., Dan Diego, CA, USA). Dose-response curves were plotted and fitted by non-linear regression. Curves were compared statistically by means of the extra sum of squares test, Hill slope and regression coefficient. These curves were used to determine the IC_{50} values of AMP drug candidates and determine the cell viability range of the protein stabilising excipients. Column bar graphs were plotted as the mean value of all the data points within a treatment and error bars were displayed as standard deviation. Data is represented as mean ± standard deviation. One way analysis of variance (ANOVA) was performed to determine if there were any statistically significant differences between the mean values of combination treatments when compared to the AMP alone treatment. Dunnett's multiple comparison post-hoc tests were used to analyse non-parametric data. Differences between the data of the different methods and techniques were deemed statistically significant if $p \leq 0.05$.

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Chapter 4

Results and discussions

4.1 Introduction

The potential therapeutic use of antimicrobial peptides (AMPs), more in particular venom and bacteriocin peptides as novel antibiotics to treat threatening gastrointestinal (GI) infections is severely hindered by the lack of studies done on the cytolytic activities thereof (Hassan *et al*., 2012:729). Although the antimicrobial activities of these peptides have extensively been studied and characterised on various organisms and bacterial strains, further investigation of the cytotoxic properties towards mammalian cell lines is required for clinical advancement (Li, 2005:183). As peptide drugs that are administered orally or by means of intravenous (IV) injection face some formulation limitations, it is necessary for these peptides to be specially formulated to ensure therapeutic success (Banga, 2015:219). Pharmaceutical excipients are additives in peptide formulations to aid in formulation limitations and additionally stabilise the peptide drug, however, also they have the risk of interacting with the peptide in such a way as to affect the cytotoxicity thereof (Andrade *et al*., 2011:163; Chaudhari & Patil, 2012:27). The majority of cytotoxic studies that have been conducted on AMPs primarily focus on the haemolytic activity, while the limited research available on the cytolytic activity does not bring formulation additives into account when evaluating the overall cytotoxic property (Kamerzell *et al*., 2011:1122; Mahlapuu *et al*., 2016).

For this study, venom peptides, melittin and mastoparan; and the bacteriocin peptide nisin Z were used in combination with different concentrations of peptide stabilising excipients (including L-glutamic acid, chitosan and polysorbate 80) to investigate and characterise the cytotoxicity towards mammalian GI cells. HepG2 and Caco-2 cell lines were utilised to assess the interaction between AMP formulations and intestinal epithelial cells, as they have similar epithelial morphology and functional characteristics as the *in vivo* human GI environment (Orbach *et al*., 2017:1900). Cytotoxicity was investigated and determined utilising the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate mitochondrial function and integrity, as well as the lactate dehydrogenase (LDH) assay, which measures LDH release as a result of plasma membrane damage. Where the MTT assay is indicative of both necrotic and apoptotic cell death, the LDH assay determines necrotic cell death (Maher & McClean, 2006:1290). In principle, viable cells have the ability to incorporate and bind neutral red dye to the lysosome, whereas nonviable cells will not take up the dye (Repetto *et al*., 2008:1125). Therefore, neutral red staining was additionally used to visually illustrate the varying cytotoxic effects of combination treatments compared to viable cells. All cytotoxic experiments were performed according to the International Organization for Standardization (ISO) (2009:5) guidelines as well as the guidelines specified by the Organization for Economic Co-operation and Development (OECD) (2010).

4.2 Drug exposure time optimisation

The MTT assay was used to determine the drug exposure time to be utilised during the final cytotoxicity experiments. Melittin was used as the model peptide between the three AMPs as it is the most cytotoxic according to literature (Hurley *et al*., 2016:77). A concentration series of melittin (0,625 µM – 20 µM) were performed on the HepG2 cell line using the MTT assay. The endpoint of cytotoxicity was measured after 4, 8 and 24 h exposure. Dose-response curves were plotted of the results obtained and are depicted in Figure 4.1. Curves were fitted by non-linear regression and curves were compared statistically by means of the extra sum of squares test.

It was observed that an increase in concentration caused a decrease in the percentage cell viability after all three time exposures. The cytotoxic effect of different concentrations of melittin after 4 and 8 h of treatment delivers the same dose-response curves with only minor differences. After 24 h exposure to 0.625 µM, 1.25 µM and 2.5 µM melittin, there is an increase in the percentage cell viability compared to 4 and 8 h exposure to the same concentrations. This could be attributed to cell regeneration and proliferation after initial toxicity. The highest concentration tested, 20 µM, decreased the percentage cell viability to less than 10% after 4, 8 and 24h exposure. The half maximal inhibitory concentration (IC_{50}) were determined using GraphPad Prism and values were reported as mean (95% confidence interval). IC_{50} values were determined as 2.11 µM (1.90-2.23), 2.06 µM (1.15-2.97) and 3.23 µM (1.56-4.90) for 4, 8 and 24 h exposure, respectively.

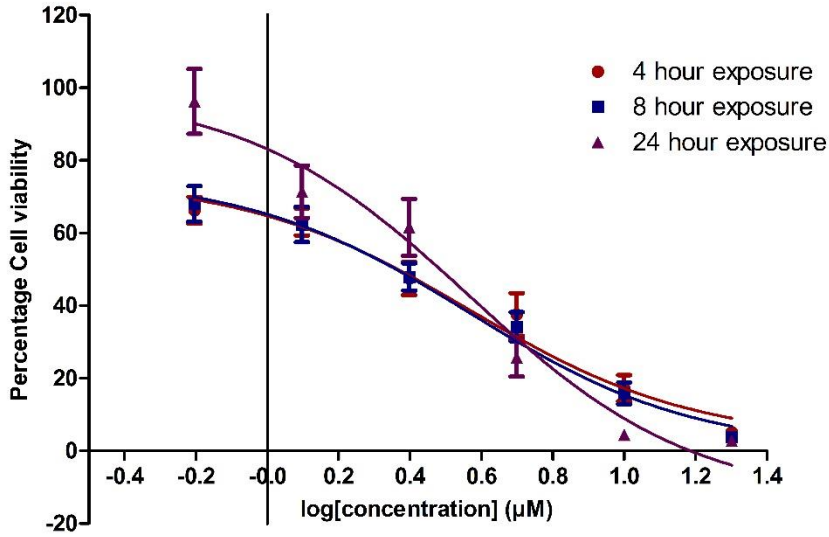

Figure 4.1: Dose-response curves of melittin cytotoxicity as measured with the MTT assay towards HepG2 cells after 4, 8 and 24h drug exposure. Data represented as mean±SD (n=6)

The steepness of the dose-response curves was measured by their Hill coefficients and represented as Hill coefficient±SD. Many inhibitory dose-response curves have a standard slope of -1.0, where Hill coefficients more negative than -1.0 is an indication of a steeper inhibitory dose-response curve. With focus on this study, a compound with a steeper curve than the standard slope indicates a greater decrease in cell viability with increasing concentration compared to the standard. Hill coefficients for the dose-response curves of 4, 8 and 24 h exposure were determined as -1.14±0.66, -1.15±0.58, and -1.59±0.65 respectively. Although the hill coefficient is similar after 4 and 8 h exposure and higher after 24 h, there is only a slight increase in the inhibition (thus decrease in cell viability) in comparison to a standard slope. The regression of each slope was additionally determined as R^2, the coefficient of determination. It is a statistical measure to determine how close the regression line represents the obtained data, where an R^2 value of 1 indicates that the regression line perfectly represents the data. The regression of the different exposure times was determined as 0.84, 0.87 and 0.85 for 4, 8 and 24 h respectively, meaning that the regression lines for the exposure times represent the data almost perfectly at an average of 85%.

After this optimisation experiment it was concluded that the most optimum time exposure would either be 4 or 8 h exposure as both time series delivered similar dose-response curves. It is further concluded that the cytotoxicity induced by melittin treatment shows concentration-dependent mechanism of cytotoxicity instead of time-dependant. Moreover, exposure of 24 h allowed cells to regenerate and proliferate at lower concentrations, which would contradict the cytotoxic effect in the final experiments, while higher concentrations resulted in lower percentage cell viability in contrast to the IC_{50} value obtained. It was therefore decided to continue all other optimisation experiments and final cytotoxicity studies at an exposure time of 6 h. The time-dependant bactericidal action of both antibiotic controls, vancomycin and ampicillin, further justified the 6 h time exposure that was used as 6 h dosage intervals are most frequently used in the clinical setting for these antibiotics (Levison & Levison, 2009:803).

4.3 Inhibitory concentration determination of antimicrobial peptides

A concentration series for melittin, mastoparan and nisin Z were individually performed using the MTT assay on HepG2 cells after 6 h of treatment to determine the IC_{50} value of each AMP and therefore the concentration to be used in the final cytotoxicity experiments. Dose-response curves were plotted of the results obtained and are depicted in Figure 4.2. Curves were fitted by non-linear regression and curves were compared statistically by means of the extra sum of squares test. IC_{50} values were statistically determined using Graphpad Prism.

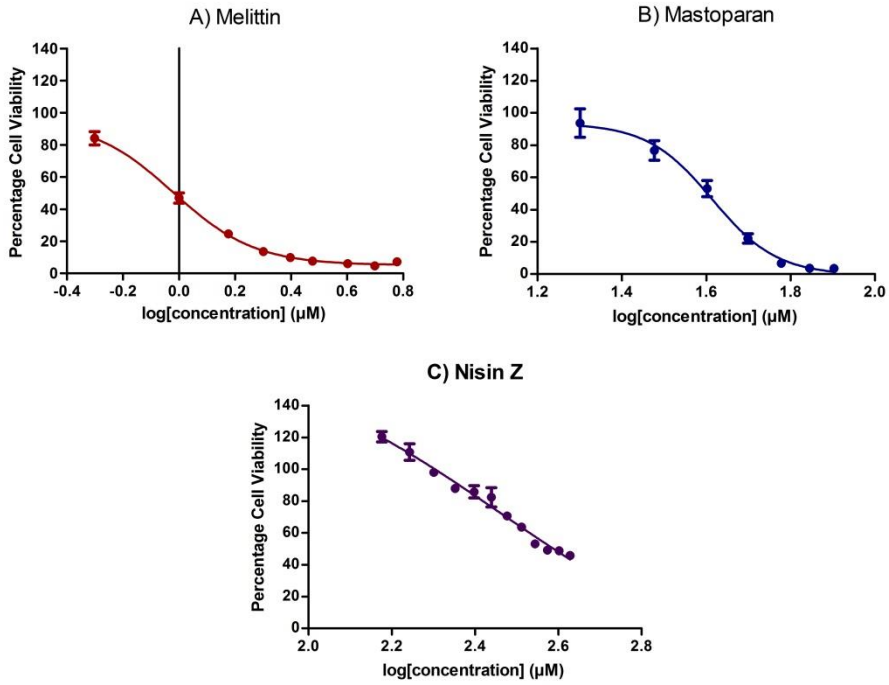

Figure 4.2: Dose-response curves of melittin (A), mastoparan (B) and nisin Z (C) cytotoxicity as measured by the MTT assay towards HepG2 cells after 6h drug exposure. Data represented as mean±SD (n=6)

The concentration range of melittin that was investigated included 0.5 µM, 1 µM, 1.5 µM, 2 µM, 2.5 µM, 3 µM, 4 µM, 5 µM and 6 µM. The concentration series for mastoparan and nisin Z ranged from 20 µM to 80 µM with intervals of 10 µM and 150 µM to 425 µM with intervals of 25 µM respectively. For all three peptides it was observed that the higher the concentration, the more cytotoxic it became towards the HepG2 cells which resulted in a decrease in percentage cell viability when the concentration increased. The IC_{50} values were statistically determined and used as 1 µM (0.84-1.08) for melittin, 40 µM (37.94-44.68) for mastoparan and 370 µM (259.0-496.50) for nisin Z. Previous cytotoxic studies done on HepG2 cells indicated IC_{50} values of 1.26 µM (Sharkawi *et al*., 2015:3327) and 1.4 µM (Rady *et al*.,2017:20) for melittin, 50 µM for mastoparan (Hurley *et al*., 2016:83) and 105.46 µM (Paiva *et al*., 2012:2854) and 225 µM (Kaur & Kaur, 2015) for Nisin Z. The steepness of the dose-response curves was measured by their Hill coefficients and determined as -2.98±0.39, -5.66±1.29 and -1.46±2.06 for melittin, mastoparan and nisin Z. In comparison to a standard slope of -1, nisin Z cause less change in cell viability than melittin and mastoparan with increasing concentrations. From the Hill coefficient of mastoparan it is concluded that it has a more potent effect on the cell viability with increasing concentrations. The coefficient of determination of the respective regression lines were also analysed and calculated as 0.97 for melittin and 0.91 for mastoparan and nisin Z. Therefore, it is concluded that the regression line displayed in Figure 4.2 perfectly approximates the data.

4.4 Cell-viable concentration range of excipients

Excipients can affect and alter the cytotoxicity of drug molecules when they are used in combination (Chaudhari & Patil, 2012:27). As this study focussed mainly on the cytotoxicity of the selected AMPs and the varying effect that excipients may exert when in combination, it is important that the excipient does not exhibit its own cytotoxicity and that the final cytotoxicity, if any, of the combination to be tested is a result of synergism and not of additive toxicity. Literature available on the cytotoxicity of these excipients towards GI epithelial cells is limited as most excipients are approved as safe by the Food and Drug Administration (FDA). Therefore, the cytotoxicity of the individual peptide stabilising excipients was investigated at 6h exposure to determine the concentration range that sustained >80% cell viability. A concentration series of L-glutamic acid, chitosan and polysorbate 80 was performed using the MTT assay to determine the cytotoxicity towards HepG2 cells and are depicted in Figure 4.3.

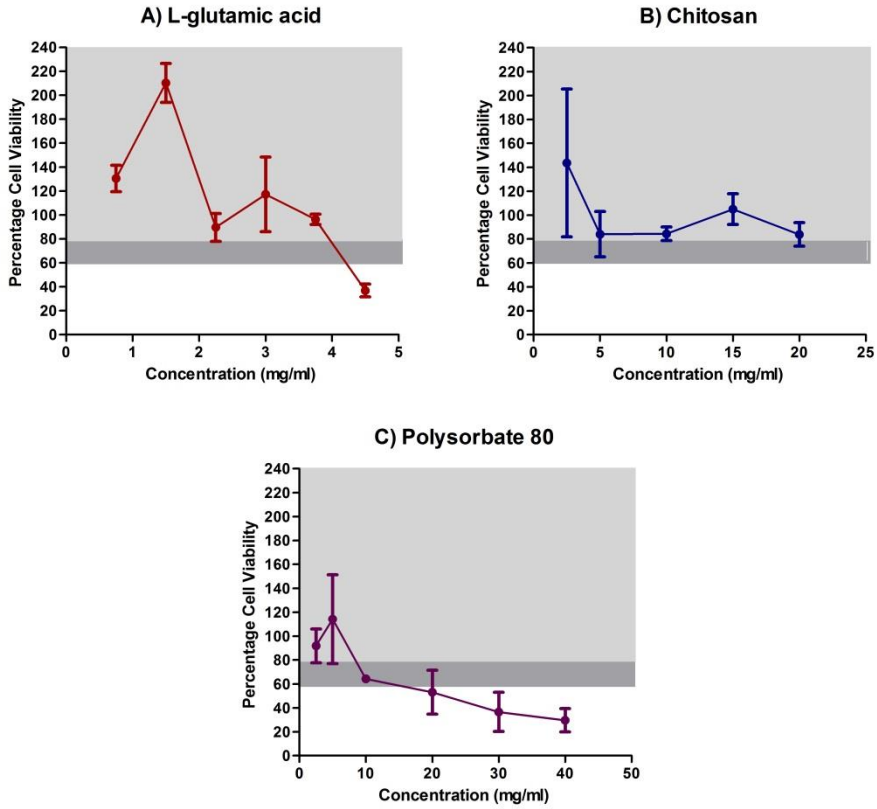

Figure 4.3: Viability of HepG2 cells treated with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C) for 6h measured by the MTT assay. Data represented as mean±SD (n=6)

The light grey areas depicted in Figure 4.3 displays the established criteria set in this study for the excipients to sustain cell viability between 80-100% or above. However, measured cell viability between 60-80% is still acceptable and is presented by the darker grey areas. Since

there is a lack of data on the cytotoxicity of these excipients, the criteria set will be handled as the control to establish the concentration range to be used.

From the 0.75 mg/ml, 1.5 mg/ml, 2.25 mg/ml, 3 mg/ml, 3.75 mg/ml and 4.5 mg/ml solutions of L-glutamic acid that was investigated, all concentrations under 3.75 mg/ml was able to sustain a percentage cell viability higher than 80%. In Figure 4.3 A all the previously mentioned concentrations are seen to occur in the light grey area set as criteria. Percentage cell viability at 4.5 mg/ml was observed as 36.96±5.31% which falls outside the established criteria. The concentration series for chitosan included 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml and 20 mg/ml and resulted in percentage cell viability observed as 143.70±61.79%, 84.15±18.91%, 84.47±5.71%, 105.01±12.90% and 83.86±9.86% respectively. The data from all concentrations of chitosan that were investigated are plotted in the light grey area with only two standard deviations occurring in the dark grey area as depicted in Figure 4.3 B. The 2.5 mg/ml and 5 mg/ml solutions of polysorbate 80 were the only two specifically tested concentrations to sustain a percentage cell viability above 80% with values observed as 91.93±14.11% and 114.23±37.21% respectively and are depicted in the light grey area as seen in Figure 4.3 C. For polysorbate 80 the concentration series also included 10 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml, but these solutions were too toxic towards the HepG2 cells and as observed in Figure 4.3 C were unable to sustain a percentage cell viability above 80%.

Three concentrations of each excipient were selected based on the above results to be used in the final cytotoxicity experiments. These values were selected that each value was double the strength of the previous value while still being able to sustain a percentage cell viability above 80% (occurring in the light grey area on the respective line graphs). In conclusion of the above results the concentrations series were determined as 0.75 mg/ml, 1.5 mg/ml and 3 mg/ml for L-glutamic acid, 5 mg/ml, 10 mg/ml and 20 mg/ml for chitosan and 2 mg/ml, 4 mg/ml and 8 mg/ml for polysorbate 80.

4.5 Antibiotic control reference

The antibiotic control groups were included in this study as cytotoxic reference of clinically used antibiotics to which the final combination formulations that were tested could be compared to. Ampicillin and vancomycin are antibiotics used in many GI infections caused by bacterial pathogens. Numerous studies have compared the antibacterial effect of melittin, mastoparan and nisin Z to both ampicillin and vancomycin towards *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Salmonella* species, as described in Section 2.3.3 (Ebbensgaard *et al*., 2015; Irazazabal *et al*., 2016:2702; Lewies *et al*., 2017:249; Li *et al*., 2000:205; Tong *et al*., 2014). Solutions of 20 µg/ml and 32 µg/ml ampicillin and vancomycin were used as these are minimum inhibitory concentrations (MIC) against GI infection

pathogens (Jones, 2006:s21; Kim *et al*., 2015:464). The cytotoxicity of both antibiotics after 6 h treatment was determined towards HepG2 cells and Caco-2 using the MTT and LDH assay (Figure 4.4).

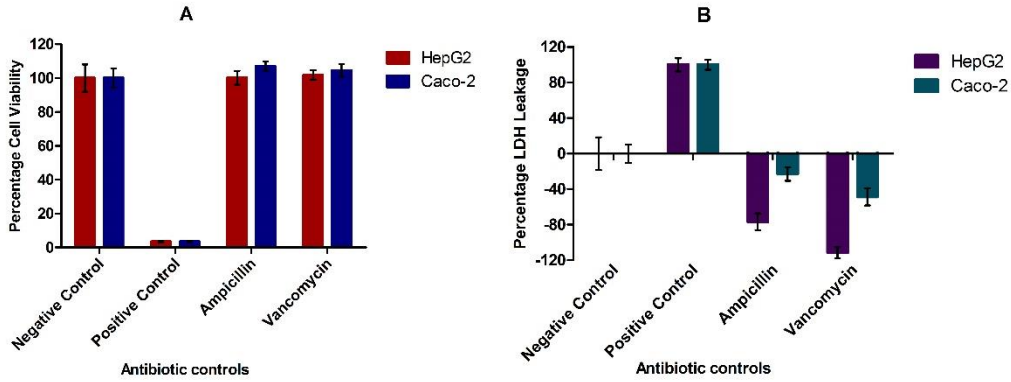

Figure 4.4: HepG2 and Caco-2 cells treated with ampicillin and vancomycin for 6h to determine the cell viability using the MTT assay (A) and LDH release using the LDH assay (B). Data represented as mean±SD (n=6)

The negative control consisted of cells maintained in serum free media (SFM) and cells were treated with Triton X to serve as the positive control in both assays. It was observed that both antibiotics had percentage cell viabilities comparable to the negative control for both cell lines in both assays. In Figure 4.4 A, the MTT assay was used to evaluate the cytotoxicity towards both cells. Percentage cell viability was observed for ampicillin as 100.21±4.02% and 106.98±2.95% and for vancomycin as 101.91±2.74% and 104.49±3.85% towards HepG2 and Caco-2 cells respectively. Data obtained using the LDH assay is depicted in Figure 4.4 B. This assay indicated that ampicillin and vancomycin did not cause LDH leakage, which is a result of membrane damage. It was therefore confirmed that these MIC values for both ampicillin and vancomycin did not exhibit cytotoxicity towards the HepG2 or the Caco-2 cells.

4.6 Cytotoxicity

The potential of melittin, mastoparan and nisin Z as novel antibiotics used in GI infections was investigated based on their cytotoxicity towards HepG2 and Caco-2 cells. The use of these AMPs for their antibacterial effects toward bacterial pathogens commonly associated with GI infections is widely supported by many studies, however the data on cytotoxic effects toward mammalian cells remain insufficient (Asthana *et al*., 2004:55044; Irazazabal *et al*., 2016:2702; Tong *et al*., 2014). In this study, the cytotoxicity was measured using the MTT assay, which is indicative of necrotic and apoptotic cell death, to evaluate mitochondrial function and integrity; and the LDH assay, which determines necrotic cell death by indicating LDH release as a result of plasma membrane damage. For the MTT assay, cell viability was expressed as a percentage relative to the negative control, which was set at 100%. Results obtained from the

LDH assay were expressed as a percentage relative to the negative control and the maximum LDH release control (positive control), which was set at 0% and 100% LDH release, respectively. Results from the LDH assay are representative of dead cells and although this assay is indicative of necrotic cell death, additional assays would need to be done to justify results. For both assays, the negative control consisted of cells maintained in SFM and Triton X was used as the positive control with added Lysis solution during the LDH assay. Any combination treatments that resulted in significant cytotoxicity in the cell were additionally stained using neutral red dye. Light microscope micrographs were taken to visually illustrate the cytotoxic effects of combination treatments in comparison to drug alone treatments and the negative control.

All the experiments were at least performed in triplicate and independently repeated. All three AMPs were investigated in combination with three concentrations of each excipient. Cytotoxicity was determined after 6 h exposure to the relevant combination treatment. The IC_{50} values for the AMPs were determined and used as fixed concentrations as 1 µM for melittin, 40 µM for mastoparan and 370 µM for nisin Z. The concentration series of the excipients were determined as 0.75 mg/ml, 1.5 mg/ml and 3 mg/ml for L-glutamic acid; 5 mg/ml, 10 mg/ml and 20 mg/ml for chitosan; and 2 mg/ml, 4 mg/ml and 8 mg/ml for polysorbate 80. For additional control groups for each combination to be evaluated, the AMPs and the highest concentration of excipient were also individually investigated and included in this study. GraphPad Prism was used to process and analyse the results obtained from both cytotoxicity assays. Column bar graphs were plotted as the mean value of all the data points within a treatment and error bars were displayed as standard deviation. Data are represented as mean ± standard deviation. One way analysis of variance (ANOVA) was performed to determine if there were any statistically significant differences between the mean values of combination treatments when compared to the AMP alone treatment. Dunnett's multiple comparison post-hoc tests were used to analyse non-parametric data. Statistical significant differences are represented by * if $p \leq 0.05$. See Addendum B for the full ANOVA reports of each treatment. Furthermore, HepG2 and Caco-2 cells were stained using neutral red dye after 6 h exposure to all treatments, however, only the combinations which showed significant cytotoxicity compared to the negative control and individual treatments are included.

4.6.1 Melittin

Melittin is a cationic venom peptide that was included in this study for its antibacterial effect against various GI infection causing pathogens, including *Staphylococcus aureus, Escherichia coli* and *Enterococcus faecalis* (Ebbensgaard *et al*., 2015). The cytotoxic effect of melittin when in combination with L-glutamic acid (anionic excipient), chitosan (cationic excipient) and

polysorbate 80 (non-ionic excipient) towards GI cells was investigated, which is depicted in Figures 4.5 – 4.8.

4.6.1.1 MTT assay

In Figure 4.5, it is observed that 6 h HepG2 cell treatment with 1 µM melittin alone resulted in a 53.78±14.04% percentage cell viability compared to the negative control. L-glutamic acid and chitosan, when tested alone, were able to sustain percentage cell viability at 89.26±5.10% and 79.28±1.51% respectively. However, polysorbate 80 was only able to sustain percentage cell viability at 62.81±5.19%, which was lower than the optimisation experiments discussed in Section 4.4, but still acceptable according to the established criteria.

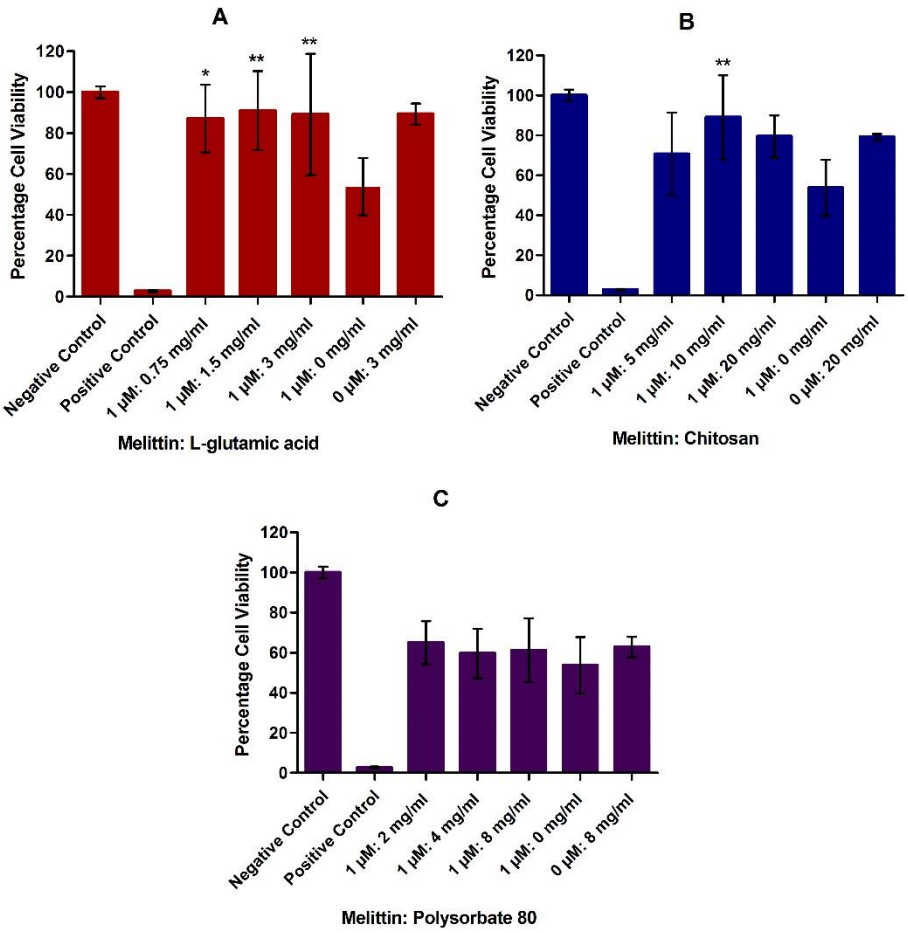

Figure 4.5: Viability of HepG2 cells treated with different melittin:excipient combinations after 6h exposure measured by the MTT assay. Cell treatments consisted of 1 µM melittin in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

The MTT assay on HepG2 cells indicated that the treatment of melittin in combination with all three individual excipients resulted in no additional or significant cytotoxicity, but rather resulted

in increased percentage cell viability in comparison to individual treatments. Melittin in combination with all three concentrations of L-glutamic acid increased the cell viability significantly ($p<0.0001$) relative to melittin alone.

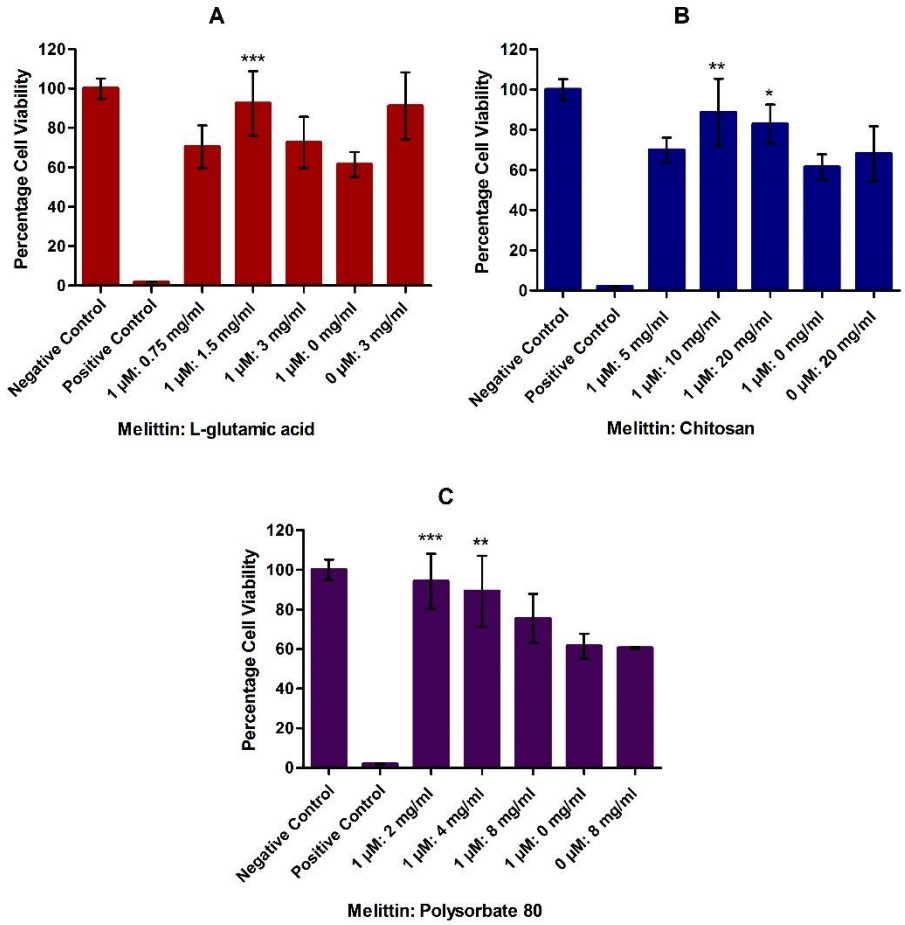

Figure 4.6: Viability of Caco-2 cells treated with different melittin:excipient combinations after 6h exposure measured by the MTT assay. Cell treatments consisted of 1 µM melittin in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

The same treatment was repeated on Caco-2 cells and is depicted in Figure 4.6. From the results obtained, melittin treatment alone resulted in 61.48±6.29% percentage cell viability compared to the negative control. It is furthermore observed that individual treatments of L-glutamic acid, chitosan and polysorbate 80 portrayed similar cell viabilities in comparison with HepG2 cells.

Caco-2 cells likewise showed an increased percentage cell viability after the treatment of melittin in combination with the excipients. The only significant change between the two cell lines is the notable increased Caco-2 cell viability of melittin in combination with polysorbate 80 in Figure 4.6 C compared to the same treatment on HepG2 cells ($p<0.0001$).

Decreased cytotoxicity of melittin when in combination with the peptide stabilising excipients can be attributed to drug-excipient interactions, especially with the molecule charge differences of cationic melittin in combination with anionic L-glutamic acid, cationic chitosan and non-ionic polysorbate 80. Furthermore, studies have found that stabilising the secondary structure of AMPs could similarly affect the antimicrobial activity thereof and decrease both antibacterial and cytolytic activity (Fjell *et al*., 2012:47). However, a study done by Laverty & Gilmore (2014) found that the incorporation of L-enantiomeric amino acid residues, such as L-glutamic acid, into formulations that contain lytic AMPs with α-helical conformations, such as melittin, resulted in decreased lytic activity towards eukaryotic cells whilst preserving their antibacterial properties. Further research on this subject is thus recommended.

4.6.1.2 LDH assay

The LDH assay more specifically determines whether cytotoxicity observed as altered cell viability in the MTT assay is due to membrane damage, which is a morphological feature of necrotic cell death.

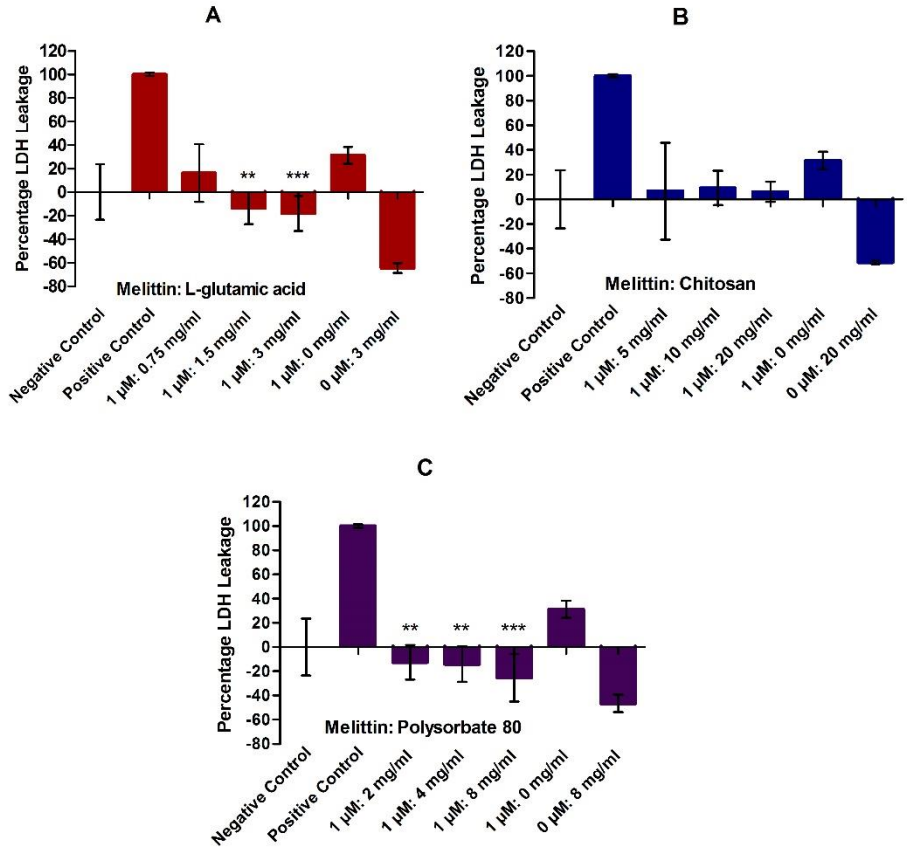

Figure 4.7: LDH release from HepG2 cells treated with different melittin:excipient combinations after 6h exposure measured by the LDH assay. Cell treatments consisted of 1 µM melittin in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

This assay is able to detect low levels of plasma membrane damage and is not harmful to viable cells. In conclusion, this assay is able to measure LDH leakage from damaged plasma membranes even though percentage cell viability is high for the specific treatment according to the MTT assay. Results obtained from cytotoxic experiments done on HepG2 and Caco-2 cells using the LDH assay are depicted in Figure 4.7 and Figure 4.8, respectively. Although no remarkable cytotoxicity was detected by the MTT assay for melittin:excipient combinations towards both cell lines, some membrane damage and subsequent LDH leakage were measured.

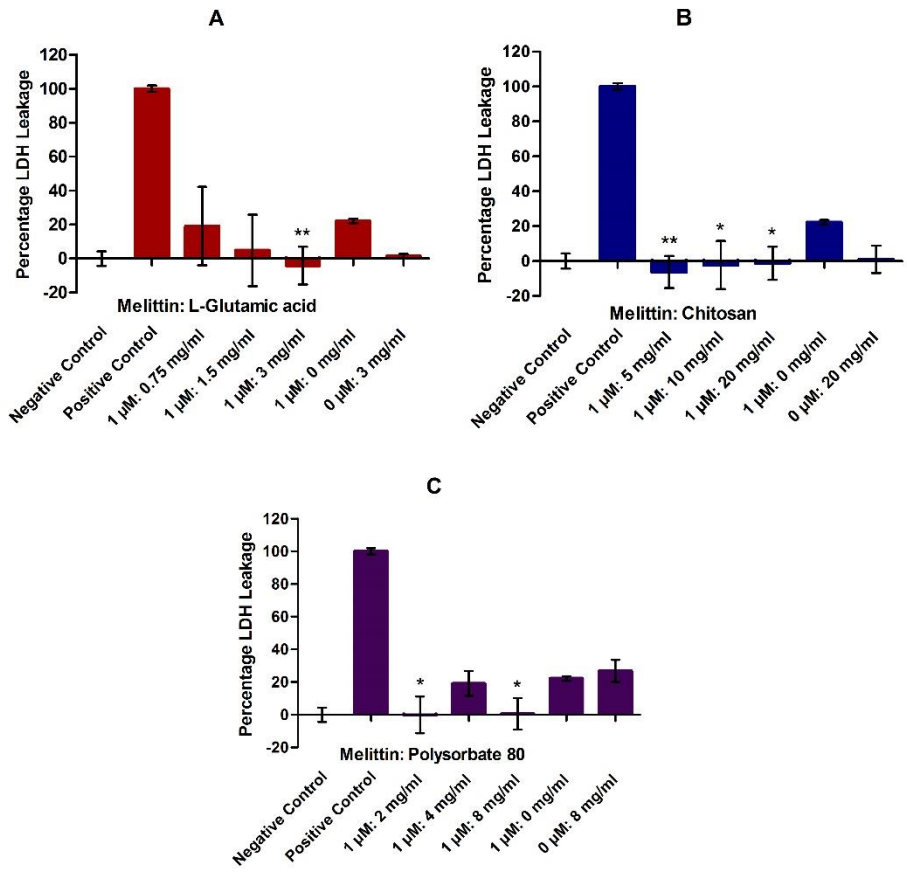

Figure 4.8: LDH release from Caco-2 cells treated with different melittin:excipient combinations after 6h exposure measured by the LDH assay. Cell treatments consisted of 1 µM melittin in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

In Figure 4.7, melittin treatment alone on HepG2 cells resulted in 31.30±7.07% LDH leakage when compared to the negative control. It is additionally observed that L-glutamic acid, chitosan and polysorbate 80 alone did not cause any percentage LDH leakage after 6 h of exposure in comparison to the negative control.

Figure 4.7 A depicts melittin in combination with L-glutamic acid. The only combination where membrane damage was detected was 1 µM: 0.75 mg/ml with measured 16.14±24.55% LDH leakage. All three combinations of melittin and chitosan resulted in insignificant LDH leakage of less than 10% as depicted in Figure 4.7 B. Additionally, melittin:polysorbate 80 combinations did not result in any measured LDH leakage even though the results were significant relative to melittin alone ($p<0.0001$) (Figure 4.7 C). It is suggested that any membrane damage that was observed on HepG2 cells can mostly be attributed to the cytolytic effects of melittin as excipient alone treatments do not result in measured LDH leakage.

Melittin treatments were repeated on Caco-2 cells using the LDH assay with minimal LDH leakage measured (Figure 4.8). Caco-2 cells displayed a 22.16±1.38% LDH leakage after 6 h of melittin treatment as compared to the negative control. Where L-glutamic acid and chitosan did not display membrane damage, treatment with polysorbate 80 alone resulted in 26.74±6.77% LDH leakage as seen in Figure 4.8 C.

The combination of melittin and L-glutamic acid displays decreased LDH leakage as the concentration of excipient increases. This could be attributed to the increasing negative charge of the excipient that it somehow protects against cell membrane damage caused by the cationic melittin molecules. Chitosan in combination with melittin resulted in no measured LDH leakage in comparison to the negative control group, but statistically differs at all three concentrations ($p<0.0004$). Although both melittin and polysorbate 80 respectively caused similar LDH leakage, the combination thereof did not result in significantly added LDH leakage. On the contrary, only 1 µM: 4 mg/ml resulted in LDH leakage of 17.08±7.60% as seen in Figure 4.8 C. In conclusion, the results of the LDH assay on both cell lines after melittin alone treatment could be indicative of necrotic cell death.

4.6.2 Mastoparan

The second cationic venom peptide included in this study was mastoparan, which was incorporated for its cell penetrating properties as well as its antibacterial effect against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* (Li *et al*., 2000:205). The cytotoxic effect of mastoparan when in combination with L-glutamic acid (anionic excipient), chitosan (cationic excipient) and polysorbate 80 (non-ionic excipient) towards mammalian GI cells was investigated, which is depicted in Figures 4.9 – 4.12.

4.6.2.1 MTT assay

Figure 4.9 presents the cytotoxicity of mastoparan and combination treatments with excipients towards HepG2 cells measured by the MTT assay after 6 h exposure. In comparison to the negative control, mastoparan alone treatment (40 µM) resulted in 24.95±0.98% cell viability.

Additionally, excipient alone treatments sustained high cell viabilities at 83.13±2.94% for L-glutamic acid, 93.57±0.89% for chitosan and 79.62±3.02% for polysorbate 80.

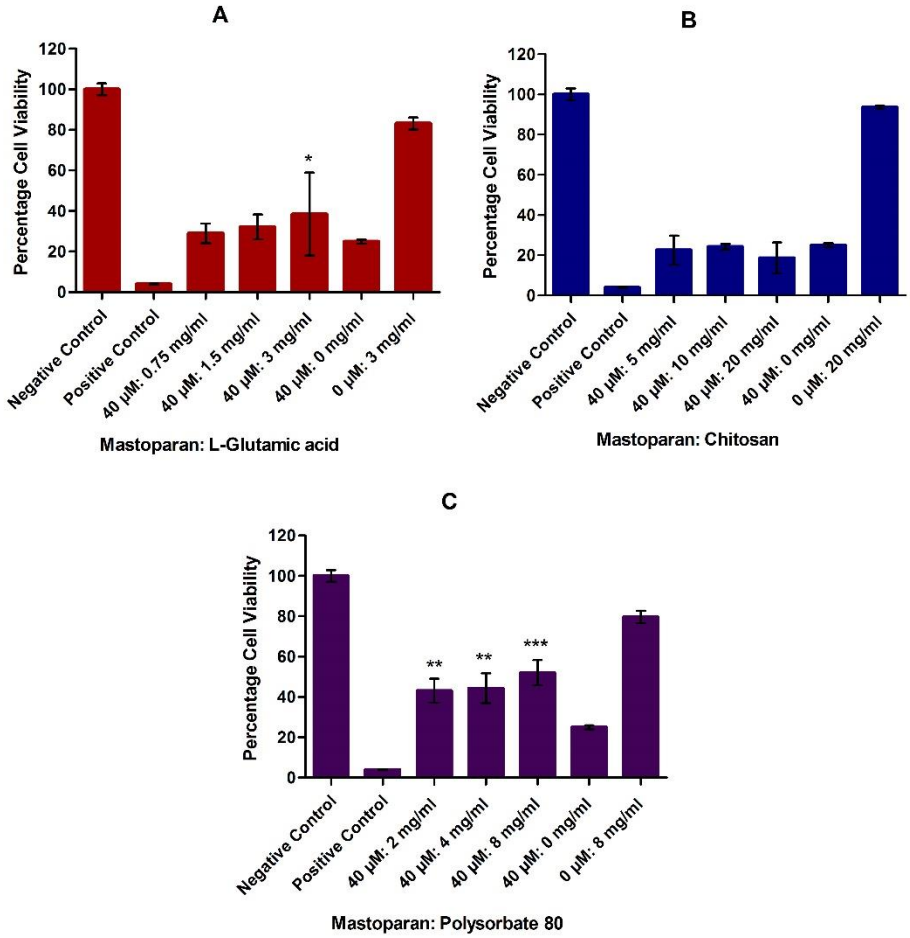

Figure 4.9: Viability of HepG2 cells treated with different mastoparan:excipient combinations after 6h exposure measured by the MTT assay. Cell treatments consisted of 40 µM mastoparan in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

When mastoparan in combination with L-glutamic acid was tested, increased cell viability was observed compared to mastoparan alone treatment as seen in Figure 4.9 A. When relating these results to those of the melittin:L-glutamic acid combinations, it can be seen that with melittin:L-glutamic acid the cell viability is considerably higher than the melittin treatment alone and resulted in similar percentage cell viability as L-glutamic acid alone treatment. However, mastoparan in combination with L-glutamic acid resulted in a slight increased percentage cell viability compared to mastoparan alone treatment and a notable lower cell viability than L-glutamic acid alone. Thus, the cytotoxicity of the drug:excipient combination with mastoparan is relatively similar to the drug alone effect compared to the excipient alone effect.

Similar cytotoxicity is observed in Figure 4.9 B for mastoparan:chitosan combination treatments relative to mastoparan alone. The same phenomenon appears here, as described above, when comparing the percentage cell viability of mastoparan combination treatments to the related melittin combination treatments. The overall cell viability of mastoparan combinations ensued in similar percentage cell viability compared to mastoparan alone and chitosan alone, whereas the percentage cell viability of melittin:chitosan combinations is more similar to the chitosan alone treatment than tot the melittin alone treatment. Although research done by Laverty & Gilmore (2014) proved that L-glutamic acid was able to decrease the cytolytic effects of α-helical AMPs, the comparable cytotoxicity effects of mastoparan in combination with L-glutamic acid and chitosan could be attributed to mastoparan cell penetrating properties (Moreno & Giralt, 2015:1138).

Mastoparan:polysorbate 80 treatments demonstrated a percentage cell viability between the mastoparan alone and polysorbate 80 alone treatment. No cytotoxicity is observed although the results differs significantly ($p<0.0001$) as depicted in Figure 4.9 C.

The same treatment was repeated on Caco-2 cells with more significant cytotoxic effects observed with mastoparan:excipient combinations compared to HepG2 cells ($p<0.0001$) (Figure 4.10). Mastoparan alone treatment caused a percentage cell viability of 69.07±9.8% compared to the negative control. Although this percentage cell viability differs from the cytotoxicity observed towards HepG2 cells, excipient alone treatments resulted in similar cell viability percentages compared to related HepG2 treatments.

Mastoparan:L-glutamic acid combinations are depicted in Figure 4.10 A. Caco-2 cells treated with this combination showed a significant decrease in percentage cell viability when compared to both individual mastoparan and L-glutamic acid treatments, with 38.20±4.97%, 45.98±5.79% and 46.11±6.65% at 40 µM: 0.75 mg/ml, 40 µM: 1.5 mg/ml and 40 µM: 3 mg/ml, respectively ($p<0.0001$). This is the first combination treatment where all three combinations resulted in a more cytotoxic effect compared to the individual drug and excipient treatments.

In Figure 4.10 B mastoparan:chitosan combinations show a decrease in percentage cell viability in comparison to individual treatments of mastoparan and chitosan. Thus, these combinations similarly resulted in a more significant cytotoxic effect towards the Caco-2 cells ($p<0.0001$). Percentage cell viability is presented as 45.59±3.95%, 42.83±4.28% and 44.29±7.18% for 40 µM: 5 mg/ml, 40 µM: 10 mg/ml and 40 µM: 20 mg/ml treatments, respectively.

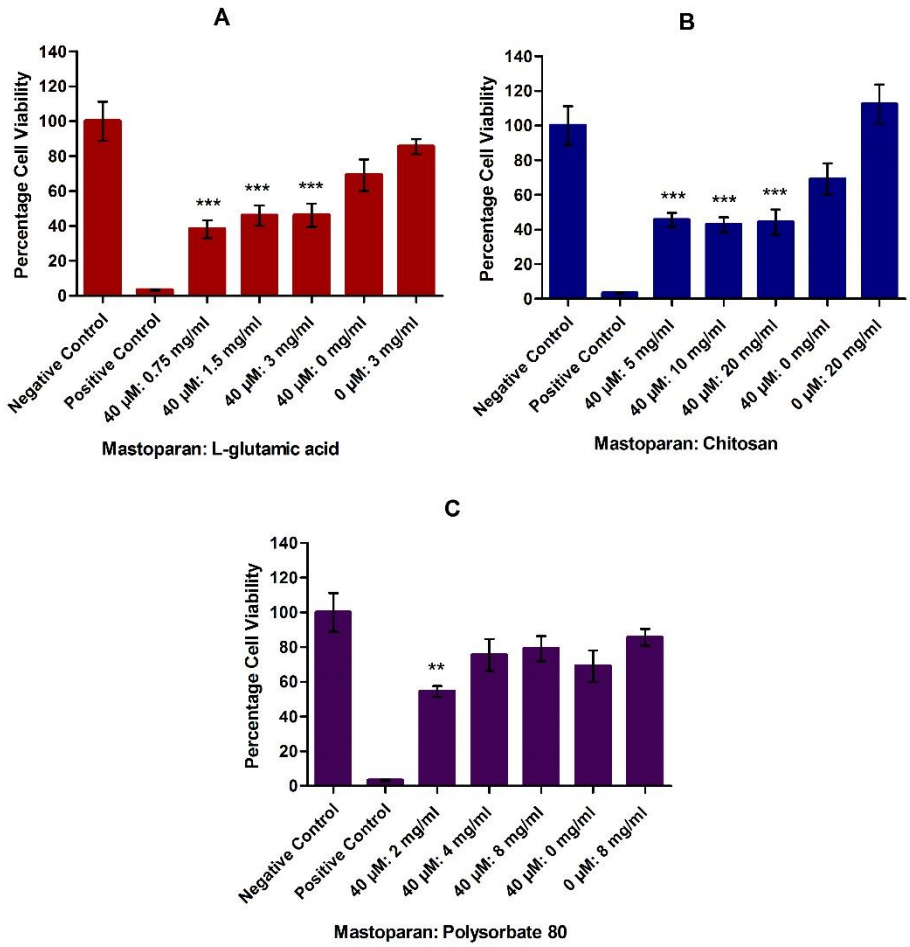

Figure 4.10: Viability of Caco-2 cells treated with different mastoparan:excipient combinations after 6h exposure measured by the MTT assay. Cell treatments consisted of 40 µM mastoparan in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

Combinations of mastoparan and polysorbate 80 again ensured a percentage cell viability between mastoparan alone and polysorbate 80 alone treatment. However, the combination of 40 µM: 2 mg/ml resulted in a decreased percentage cell viability compared to mastoparan alone treatment.

Although mastoparan alone treatment towards HepG2 cells caused a lower percentage cell viability comparatively towards Caco-2 cells, combination treatments with mastoparan:L-glutamic acid and mastoparan:chitosan showed more cytotoxic effects in Caco-2 cells than in HepG2 cells.

4.6.2.2 LDH assay

The cytotoxicity of mastoparan combination treatments towards HepG2 and Caco-2 cells measured by the LDH assay are depicted in Figure 4.11 and Figure 4.12, respectively.

HepG2 cells exposure to 40 µM mastoparan resulted in 27.02±18.41% LDH leakage compared to the negative control. Excipient alone treatments did not cause membrane damage as represented by the negative LDH leakage depicted in Figure 4.11 for the respective excipients.

The combination treatments of mastoparan and L-glutamic acid showed increased LDH leakage compared to mastoparan alone with 40 µM: 0.75 mg/ml causing the most membrane damage with resulted 51.69±18.59% LDH leakage.

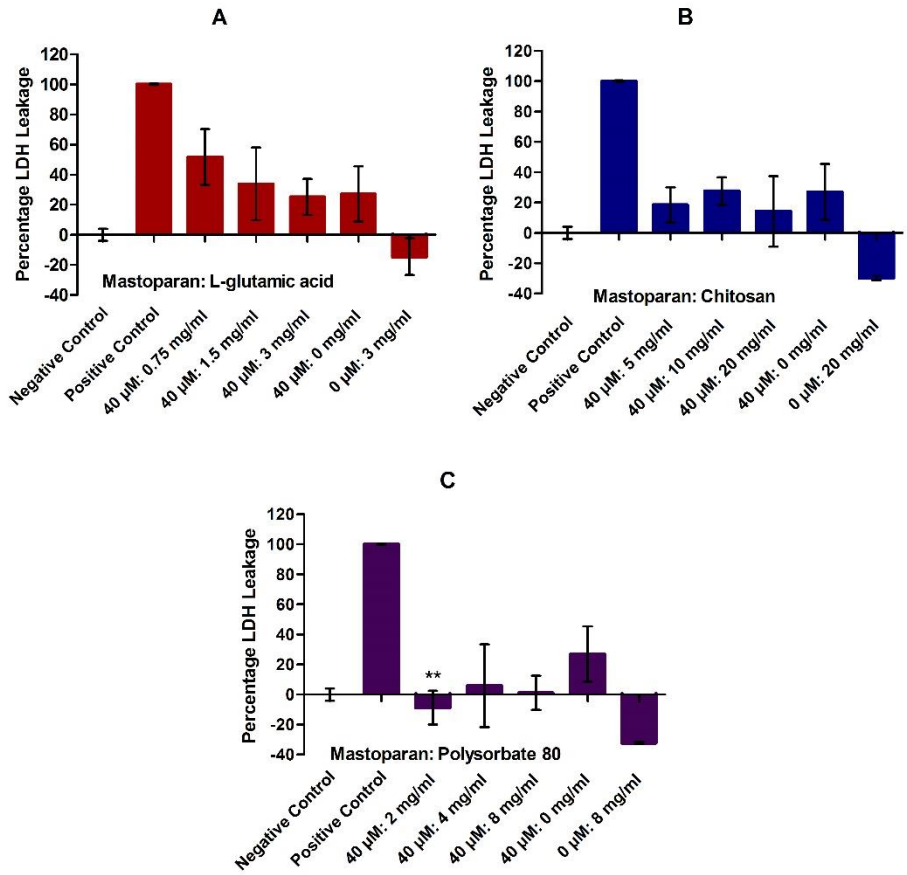

Figure 4.11: LDH release from HepG2 cells treated with different mastoparan:excipient combinations after 6h exposure measured by the LDH assay. Cell treatments consisted of 40 µM mastoparan in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

Figure 4.11 B depicts similar LDH leakage caused by the three melittin:chitosan combinations when compared to LDH leakage caused by mastoparan alone. LDH leakage was measured as 18.48±11.61%, 27.60±8.99% and 14.15±23.13% for 40 µM: 5 mg/ml, 40 µM: 10 mg/ml and 40 µM: 20 mg/ml treatments, respectively.

Only one combination of 40 µM: 4 mg/ml mastoparan:polysorbate 80 caused low levels of LDH leakage relatively to mastoparan alone and the positive control.

Mastoparan treatments were repeated on Caco-2 cells using the LDH assay (Figure 4.12). Membrane damage with subsequent LDH leakage was measured at 49.03±10.33% for 40 µM mastoparan treatment. The excipient alone treatments did not result in LDH leakage, similar to the results obtained towards the HepG2 cells.

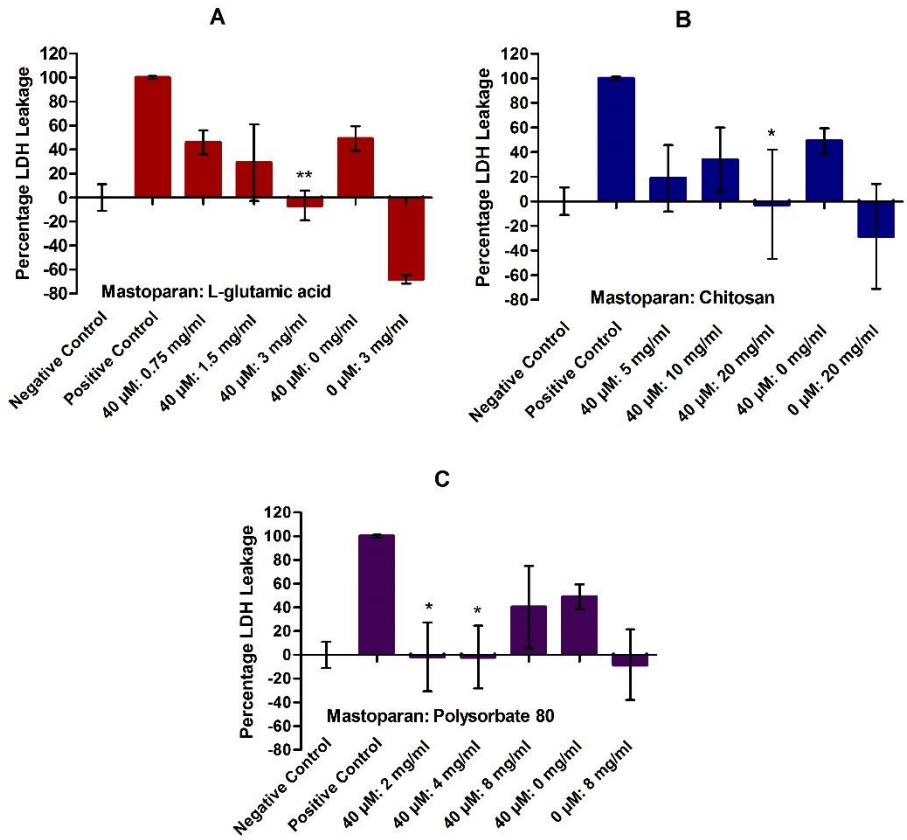

Figure 4.12: LDH release from Caco-2 cells treated with different mastoparan:excipient combinations after 6h exposure measured by the LDH assay. Cell treatments consisted of 40 µM mastoparan in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

Mastoparan in combination with L-glutamic acid did cause membrane damage and LDH leakage at 46.05±9.89% and 29.03±31.96% for 40 µM: 0.75 mg/ml and 40 µM: 1.5 mg/ml treatments, correspondingly. The highest concentration combination did not result in any LDH leakage. The results depicted in Figure 4.12 A follows the same trend in Caco-2 cells as observed towards HepG2 cells, where an increase in the concentration of the excipient caused a decrease in the resulted LDH leakage. This again can be attributed to the increased stabilising effect the excipient exhibits towards the peptide drug resulting in loss of antimicrobial activity and subsequent cytotoxicity (Fjell *et al*., 2012:47).

In Figure 4.12 B, increase an in LDH leakage is observed from 40 µM: 5 mg/ml to 40 µM: 10 mg/ml mastoparan:chitosan treatment. Owing to a high standard deviation of

44.36% for 40 µM: 20 mg/ml treatment, no remarks on the results can accurately be made. The large deviation in these results can be attributed to the insolubility of chitosan which resulted in an inaccurate amount being treated during the experiment.

LDH levels are observed at 40.26±10.33% for 40 µM: 8 mg/ml mastoparan:polysorbate 80 treatment comparable to mastoparan alone treatment as seen in Figure 4.12 C. It is concluded that significant membrane damaged caused in HepG2 and Caco-2 cells after mastoparan alone, mastoparan:L-glutamic acid and mastoparan:chitosan treatments could be the result of necrotic cell death that these treatments induced. To fully and precisely assess the occurrence of necrosis, further assays are needed.

4.6.2.3 Neutral Red Micrographs

Mastoparan in combination with L-glutamic acid and chitosan, respectively, resulted in the highest cytotoxicity towards Caco-2 cells in comparison to mastoparan alone treatment. The varying cytotoxic effect was visually portrayed by staining viable cells with neutral red dye as depicted in Figure 4.13. In principle, non-viable cells do not have the ability to be stained by this dye.

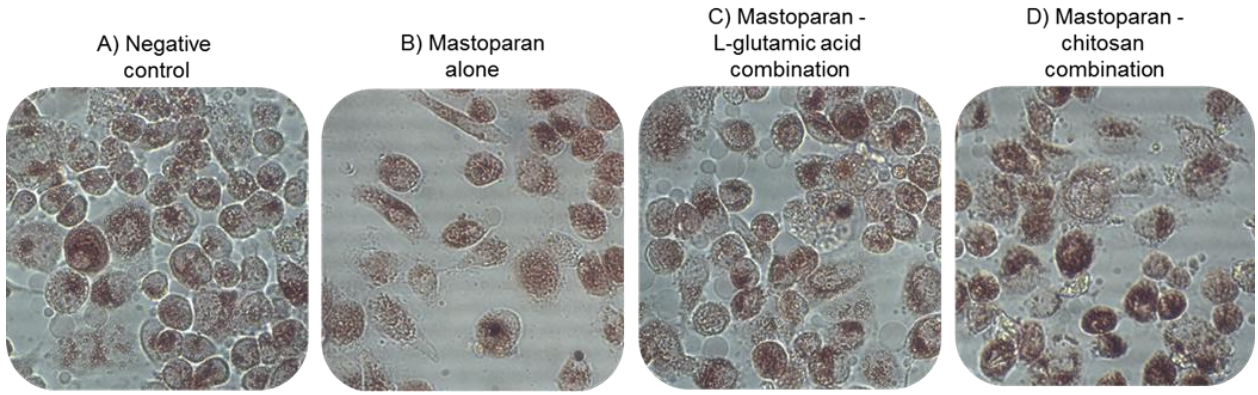

Figure 4.13: Light microscope images (40x magnification) of neutral red stained Caco-2 cells after 6 h exposure to SFM (A), mastoparan (B), mastoparan:L-glutamic acid (C) and mastoparan:chitosan (D)

The negative control (Figure 4.13 A) illustrates viable Caco-2 cells with normal morphology under standard culture conditions. Viable Caco-2 cells present with well-defined cell membranes that are spherical in shape and tend to grow in clusters. Cells treated with 40 µM mastoparan, depicted in Figure 4.13 B, shows a reduced density of cells and morphological changes in cell shape when compared to the negative control. These cells do not cluster, present with undefined cell membranes and are either spherical or rectangular in shape. In Figure 4.13 C, after mastoparan:L-glutamic acid treatment, a combination of intact, well defined cells, cells presenting with undefined membranes and empty vesicles are observed. Cell density is more comparable to the cell density of the negative control. L-glutamic acid and

chitosan excipients are seen as crystal-like shapes observed in Figure 4.13 C and Figure 4.13 D, respectively. Cells treated with mastoparan in combination with chitosan resulted in lower cell density and changed morphological cell shape.

4.6.3 Nisin Z

Nisin Z is a cationic bacteriocin peptide and included in this study for its antibacterial effect against various infectious agents in the GI tract, including *Escherichia coli*, *Enterococcus faecalis* and *Shigella* and *Salmonella* species (Maher & McClean, 2006:1291; Tong *et al*., 2014). The cytotoxic effect of nisin Z when in combination with L-glutamic acid (anionic excipient), chitosan (cationic excipient) and polysorbate 80 (non-ionic excipient) towards mammalian GI cells was investigated, which is displayed in Figures 4.14 – 4.17.

4.6.3.1 MTT assay

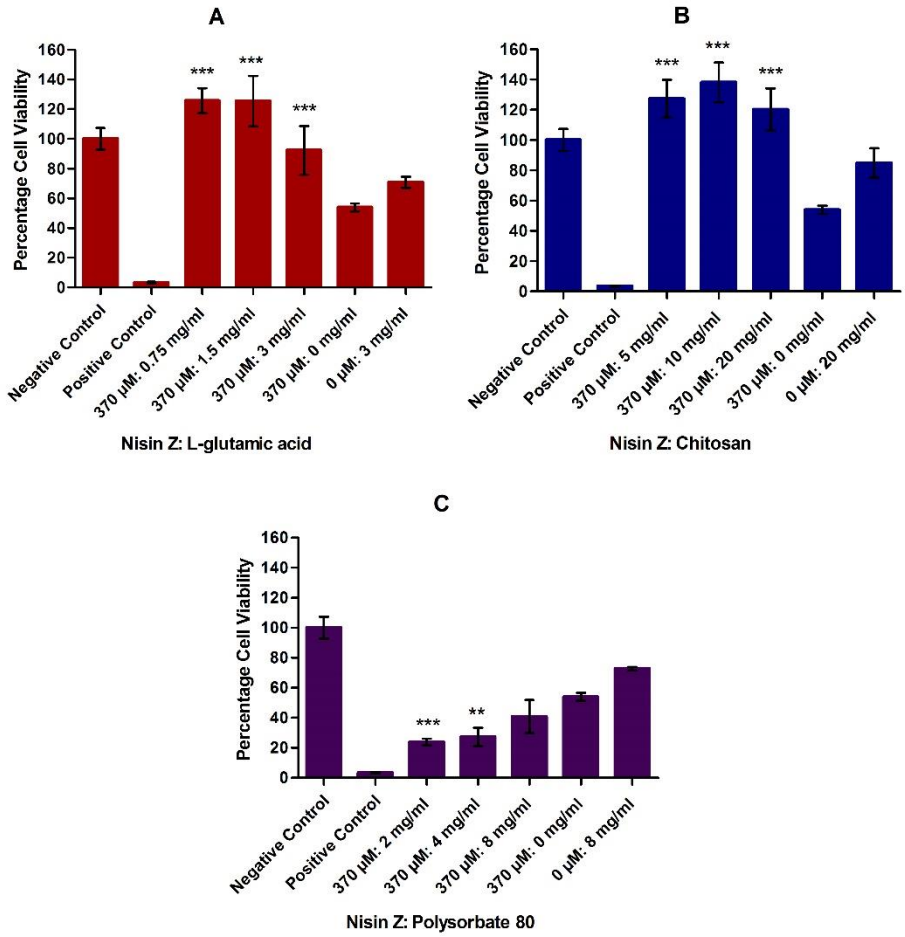

Figure 4.14: Viability of HepG2 cells treated with different nisin Z:excipient combinations after 6h exposure measured by the MTT assay. Cell treatments consisted of 370 µM nisin Z in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

HepG2 cells treated with 370 µM nisin Z resulted in 53.93±2.73% cell viability in relation to the negative control, as depicted in Figure 4.14. Individual treatments of L-glutamic acid, chitosan and polysorbate 80 were able to maintain cell viability at 70.72±3.71%, 84.83±9.74% and 72.84±1.15% after 6 h exposure, respectively.

The MTT assay on HepG2 cells indicated that the treatment of nisin Z in combination with L-glutamic acid and chitosan resulted in no additional or significant cytotoxicity, but rather resulted in increased percentage cell viability in comparison to the relevant individual treatments. These combinations statistically differ significantly from the nisin Z treatment ($p<0.0001$).

Polysorbate 80, when in combination with nisin Z, however, was able to produce a cytotoxic effect greater than both treatments alone, which caused a decrease in cell viability, as observed in Figure 4.14 C. Cell viability is indicated as 23.86±2.15%, 27.22±6.08% and 40.86±11.07% for 370 µM: 2 mg/ml, 370 µM: 4 mg/ml and 370 µM: 8 mg/ml treatments, individually. It is observed that higher concentrations polysorbate 80 in combination with nisin Z, produced a higher percentage cell viability and thus lower cytotoxicity. It seems that although polysorbate 80 has the ability to potentiate the cytotoxic effects of nisin Z, the higher concentration of this excipient then decreases the same cytotoxic effect. Morphological features of the cell line can also play a role here (Lee *et al*., 2013:14246).

The treatments of nisin Z in combination with the three excipients were repeated on Caco-2 cells and are depicted in Figure 4.15. In contrast to melittin and mastoparan treatments, where combination treatments have a similar or reduced effect on the cell viability of Caco-2 cells in comparison with HepG2 cells, nisin Z combination treatments overall have a higher decrease in percentage cell viability of Caco-2 cells compared to the same treatments towards HepG2 cells.

Percentage cell viability of Caco-2 cells were 51.08±9.67% after 6 h treatment with 370 µM nisin Z when compared to the negative control. Individual treatments of L-glutamic acid, chitosan and polysorbate 80 were able to maintain cell viability at 91.19±16.94%, 68.08±13.70% and 60.50±0.43% after 6 h exposure, separately.

The combination treatments of nisin Z:L-glutamic acid caused a similar percentage cell viability compared to nisin Z treatment alone in spite of the high cell viability L-glutamic acid alone was able to cause. In Figure 4.15 B, it is observed that nisin Z:chitosan combinations also resulted in similar percentage cell viability compared to nisin Z alone treatment as well as that of nisin Z:L-glutamic acid combinations. The cytotoxic effect towards the cells could be attributed to nisin Z's effect alone on the cells.

As seen on the HepG2 cells, the combination treatments of nisin Z:polysorbate 80 did produce significant cytotoxic effects larger than both treatments alone and ensued in decreased Caco-2 cell viability, as appreciated in Figure 4.15 C ($p<0.0001$). Cell viability is indicated as 27.10±6.70%, 19.51±3.92% and 12.27±3.03% for 370 µM: 2 mg/ml, 370 µM: 4 mg/ml and 370 µM: 8 mg/ml treatments respectively. In contrast to the finding towards HepG2 cells, higher concentrations polysorbate 80 in combination with nisin Z in Caco-2 cells resulted in lower percentage cell viability and thus higher cytotoxicity. A study performed by Natrajan & Sheldon (2000:1193) concluded that the chelating effects of polysorbate 80 cause membrane destabilisation and enhanced the antibacterial effect of nisin Z when in combination. This could attribute to the greater cytotoxicity of the combination.

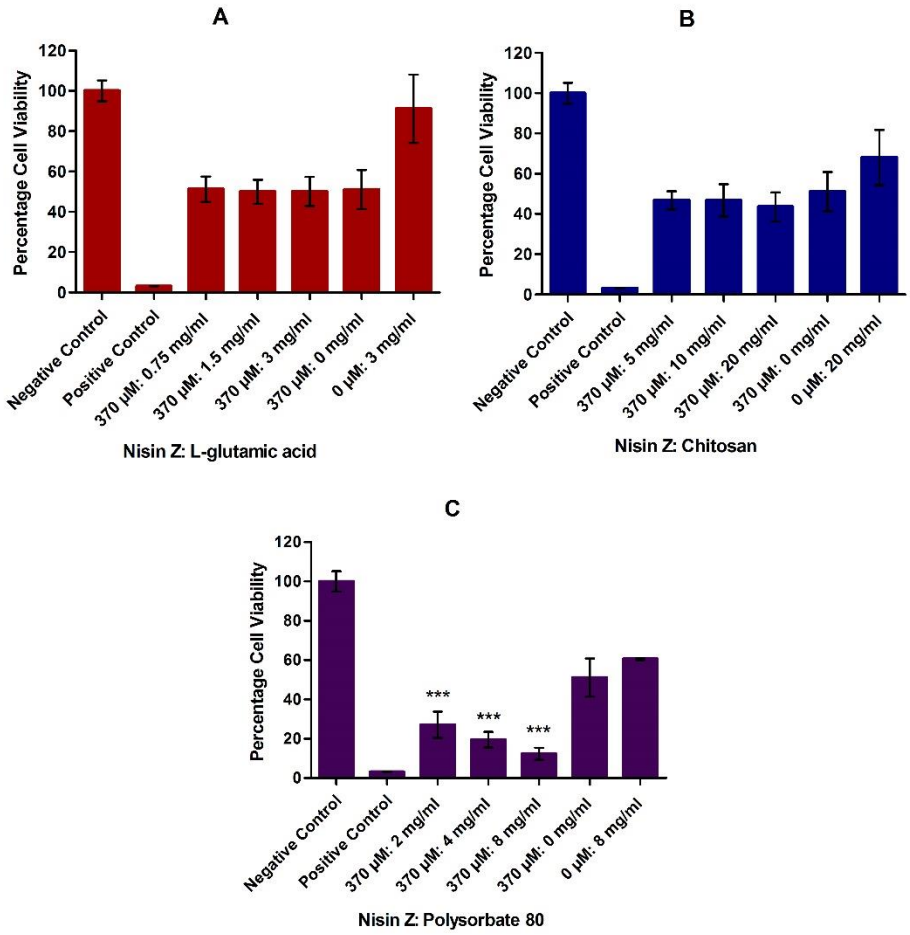

Figure 4.15: Viability of Caco-2 cells treated with different nisin Z:excipient combinations after 6h exposure measured by the MTT assay. Cell treatments consisted of 370 µM nisin Z in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

4.6.3.2 LDH assay

As depicted in Figure 4.16, HepG2 cells individually treated with nisin Z, L-glutamic acid, chitosan and polysorbate 80, alone, produced no LDH leakage, an indication that no membrane damage occurred due to treatment in comparison to the negative control. In addition, no LDH leakage was measured with treatments of nisin Z in combination with L-glutamic acid and chitosan, correspondingly.

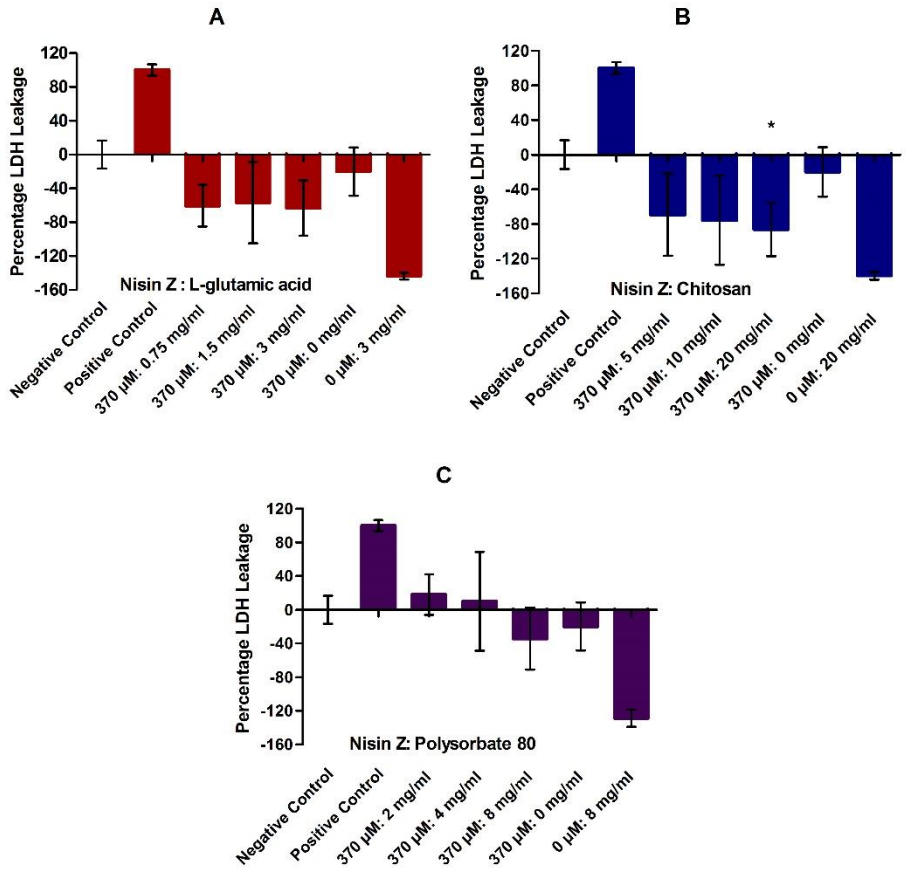

Figure 4.16: LDH release from HepG2 cells treated with different nisin Z:excipient combinations after 6h exposure measured by the LDH assay. Cell treatments consisted of 370 µM nisin Z in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

Two combinations of nisin Z:polysorbate 80 did ,however, cause membrane damage with the subsequent LDH leakage measured at 17.91±24.07 and 9.93±58.85% for 370 µM: 2 mg/ml and 370 µM: 4 mg/ml respectively. This data correlates with the results obtained from the MTT assay of the corresponding treatments on HepG2 cells, where increased concentrations of polysorbate 80 in combination with nisin Z led to an increase in percentage cell viability and thus a decrease in cytotoxic effect towards cells. It is evident from the LDH data that increasing

the concentration of polysorbate 80 decreases the cytotoxic effects, resulting in reduced membrane damage and decreased LDH leakage from cells.

The treatments of nisin Z in combination with the excipients were repeated on Caco-2 cells. From Figure 4.17, which illustrates the results from the LDH assay, it is observed that no alone treatment or combination treatment resulted in any measured LDH leakage relative to the negative control. The polysorbate 80 alone treatment was the only treatment that produced a percentage LDH leakage of 6.69±17.29. From the literature it is concluded that nisin Z does not exert cytotoxic effects towards mammalian cells, however no LDH leakage measured by the assay could be indicative of apoptotic cell death induced by nisin Z rather than necrosis which this assay determines (Shin *et al*., 2015:1453).

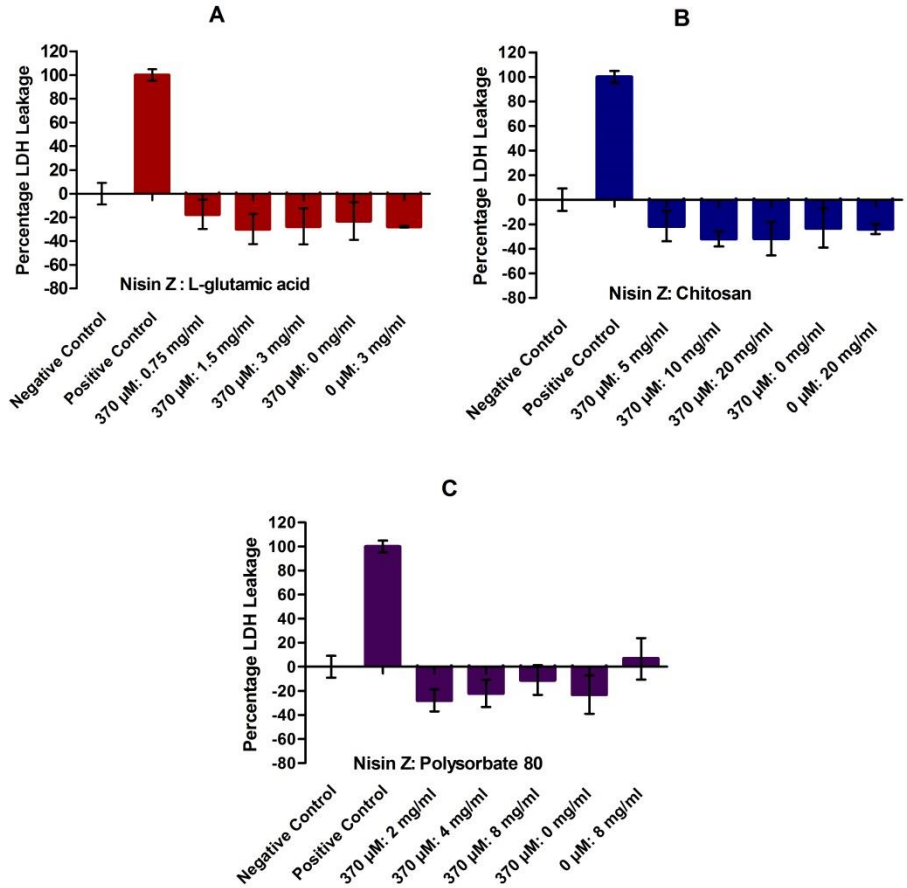

Figure 4.17: LDH release from Caco-2 cells treated with different nisin Z:excipient combinations after 6h exposure measured by the LDH assay. Cell treatments consisted of 370 µM nisin Z in combination with different concentrations of L-glutamic acid (A), chitosan (B) and polysorbate 80 (C). Data represented as mean±SD (n=6)

4.6.3.3 Neutral Red Micrographs

The highest cytotoxicity towards HepG2 and Caco-2 cells were observed with nisin Z in combination with polysorbate 80 in comparison to nisin Z treatment alone. The varying cytotoxic effect was visually presented by staining viable cells with neutral red dye as shown in Figure 4.18 and Figure 4.19. In principle, non-viable cells do not have the ability to be stained by this dye.

The negative control (Figure 4.18 A) illustrates viable HepG2 cells with normal morphology under standard culture conditions. These viable HepG2 cells have well-defined cell membranes that are polygonal (epithelial-like) in shape and tend to grow closely together, almost enfolding parts of other cells. Treatment of nisin Z (Figure 4.18 B) resulted in changed cell shape and undefined cell membranes in comparison to the negative control. The nuclei of some cells can furthermore be observed. Figure 4.18 C elucidates HepG2 cells after nisin Z:polysorbate 80 treatment. As seen in the micrograph, individual cells cannot be defined and traces of polysorbate 80 can be identified as crystal-like shapes that are displayed in colour.

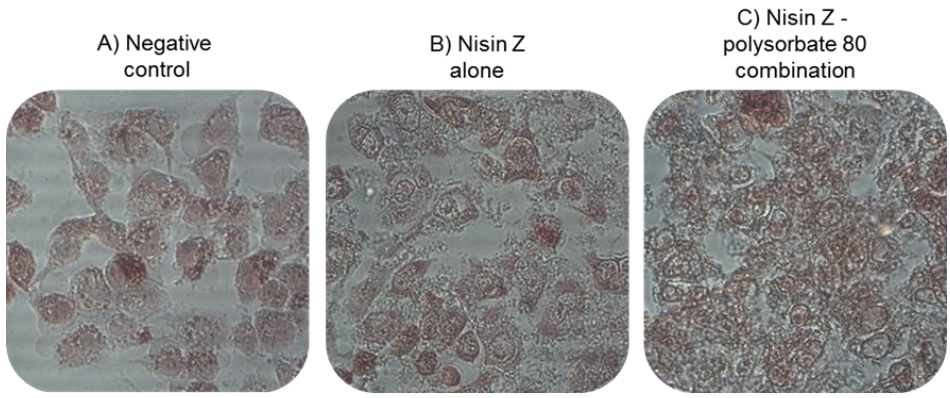

Figure 4.18: Light microscope images (40x magnification) of neutral red stained HepG2 cells after 6 h exposure to SFM (A), nisin Z (B), nisin Z:polysorbate 80 (C)

The negative control (Figure 4.19 A) illustrates viable Caco-2 cells with normal morphology under standard culture conditions. These cells possess well-defined cell membranes which are spherical in shape and tend to grow in clusters. Post treatment with 370 µM nisin Z alone (Figure 4.19 B), it is observed that the cell membranes between cells are less defined, but overall cell morphology is similar to the negative control. In Figure 4.9 C cells were treated with nisin Z in combination with polysorbate 80. More nonviable cells are detected here in comparison to the same treatment towards HepG2 cells. Individual Caco-2 cells cannot be distinguished from each other and due to the lack of red dye compared to the negative control; it can be assumed this treatment is highly cytotoxic to the cells. Polysorbate 80 is identified as crystal-like structures depicted in colour and are evident in the micrograph.

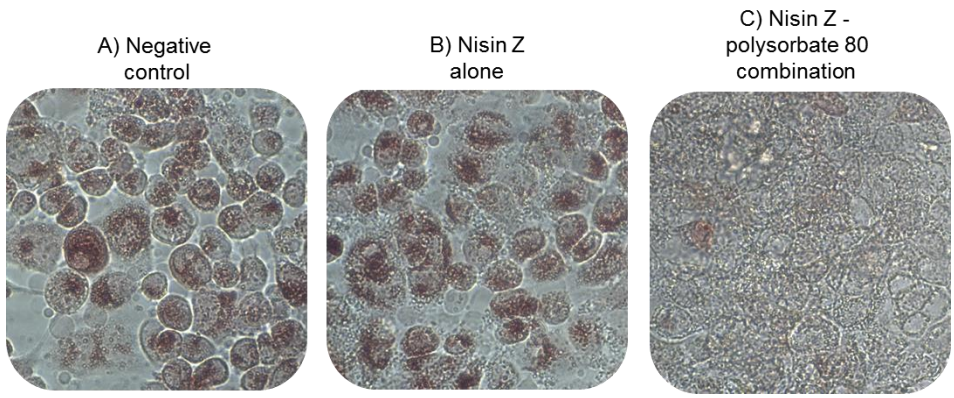

Figure 4.19: Light microscope images (40x magnification) of neutral red stained Caco-2 cells after 6 h exposure to SFM (A), nisin Z (B), nisin Z:polysorbate 80 (C)

4.7 Summary and conclusion

The aim and objectives of this study were to determine and characterise the cytotoxicity of venom and bacteriocin peptides and describe the varying effect in cytotoxicity when these peptides were used in combination with peptide stabilising excipients. To assess this cytotoxicity study as whole, final conclusions will be summarised as comparisons with the focus on deviations and tendencies between cell lines, cytotoxicity assays, AMPs, excipients and combination treatments thereof. Responses of HepG2 and Caco-2 cells towards the cytotoxicity of each treatments utilising the MTT and LDH assay, is summarised in Table 4.1.

It is concluded that Caco-2 cells, in comparison to HepG2 cells, were more resilient towards treatments of the AMPs alone, as seen in the varying IC_{50} values, and towards the various combination treatments tested. This was also observed in a study performed by Jacobs (2015). Treatments of nisin Z alone and in combination with excipients, however, did result in higher toxicity in Caco-2 cells compared to the HepG2 cells. This may be attributed to the different action mechanisms of venom and bacteriocin peptides, or more specifically, to an unknown interaction between nisin Z and Caco-2 cells. In general, the MTT assay showed that smaller concentrations of venom peptides were required to inhibit the half maximal cell viability than the bacteriocin peptide concentration needed to evoke the same response. This is prevalent in the IC_{50} values determined on HepG2 cells. A study done by Lewies *et al*., (2017:250) also displayed a more cytotoxic effect of melittin towards HaCat cells in comparison to the cytotoxicity of nisin Z.

Additionally, the venom peptides, melittin and mastoparan, caused more membrane damage and subsequent LDH leakage than the bacteriocin peptide, nisin Z, as determined by the LDH assay. In conclusion, melittin and mastoparan exerted more potent cytolytic effects towards mammalian cells and as they caused cell membrane damage. Cell death may likely be attributed to necrosis, but additional cytotoxicity assays are needed to correctly identify necrotic

cell death. Previous studies on the cytotoxicity of melittin and mastoparan towards Caco-2 cells also concluded necrosis for both peptides as mechanism of toxicity (Chaisakul *et al.,* 2016:1576; Gajski & Garaj-Vrhovac, 2013:700). In contrast, nisin Z demonstrated cytotoxic effects towards both cell lines, but did not induce cell membrane damage and thus apoptotic cell death is suggested. Another study also found apoptosis induced by nisin Z in HepG2 cells (Paiva *et al*., 2012:2856).

As stated, melittin and mastoparan are both venom peptides, but individual treatments resulted in different cytotoxic effects when tested in combination with excipients. Both cytotoxicity assays done on both cell lines after melittin combination treatments, resulted in decreased cytotoxicity in comparison to melittin alone treatment with no significant results. Mastoparan in combination with L-glutamic acid and chitosan respectively resulted in higher cytotoxicity towards Caco-2 cells when compared to mastoparan alone treatments as measured by the MTT assay. The LDH assay revealed greater membrane damage and LDH leakage after treatments of mastoparan:L-glutamic acid on HepG2 cells; and mastoparan:L-glutamic acid and mastoparan:chitosan treatments on Caco-2 cells, in comparison to mastoparan alone treatments towards the relevant cell line. The difference in observed cytotoxicity between the two venom peptides can possibly be attributed to the additional cell penetrating properties of mastoparan (Splith & Neundorf, 2011:388).

Nisin Z treatments on Caco-2 cells show more promising cytotoxic effects compared to HepG2 cells, with the exception of HepG2 cells treated with nisin Z in combination with polysorbate 80, which resulted in higher cytotoxicity compared to nisin Z alone treatment. The MTT assay performed on Caco-2 cells displayed cytotoxicity of nisin Z:L-glutamic acid and nisin Z:chitosan treatments equal to that of nisin Z alone treatment, thus no change in cytotoxic effect occurred. A significant increase in cytotoxicity was however demonstrated by nisin Z:polysorbate 80 treatment under the same conditions. The only LDH leakage observed with nisin Z was the combination of nisin Z with polysorbate towards HepG2 cells. From all these combinations tested with nisin Z, the combination with polysorbate 80 shows significant toxicity and could be attributed to the chelating effects of the excipient and possible synergism between nisin Z and polysorbate 80 (Natrajan & Sheldon, 2000:1193).

Table 4.1: Overall summary of the results obtained during the cytotoxicity assays towards HepG2 and Caco-2 cells. Treatments were considered (+) with a red box if they produced a cytotoxic effect at any dose tested of the relevant treatment. If no cytotoxic effect occurred within the ranges of the combination treatment, the response was assigned (-) with a green box. Treatments that resulted in overall cytotoxicity similar to the peptide in combination were allocated with a (=) and a blue box

Combination treatment	Range	MTT assay		LDH assay	
		HepG2	Caco-2	HepG2	Caco-2
Antibiotic reference (µg/ml)					
Ampicillin	20	-	-	-	-
Vancomycin	32	-	-	-	-
Antimicrobial peptides (µM)					
Melittin	1	+	+	+	+
Mastoparan	40	+	+	+	+
Nisin Z	370	+	+	-	-
Excipients (mg/ml)					
L-glutamic acid	3	-	-	-	-
Chitosan	20	-	-	-	-
Polysorbate 80	8	-	-	-	+
Combination treatments (µM: mg/ml)					
Melittin: L-glutamic acid	1: 0.75-3	-	-	+	+
Melittin: Chitosan	1: 5-20	-	-	+	-
Melittin: Polysorbate 80	1: 2-8	-	-	-	+
Mastoparan: L-glutamic acid	40: 0.75-3	-	+	+	+
Mastoparan: Chitosan	40: 5-20	=	+	+	+
Mastoparan: Polysorbate 80	40: 2-8	-	+	+	+
Nisin Z: L-glutamic acid	370: 0.75-3	-	=	-	-
Nisin Z: Chitosan	370: 5-20	-	=	-	-
Nisin Z: Polysorbate 80	370: 2-8	+	+	+	-

+	Cytotoxic	-	Not cytotoxic
=	Similar cytotoxicity		

In comparison to the conventional antibiotics, ampicillin and vancomycin, melittin, mastoparan and nisin Z resulted in higher toxicity in both cell lines. The cytotoxicity of these alone treatments to the respective AMPs hinders the clinical application thereof as pharmaceuticals. Although the combinations of AMPs with peptide stabilising excipients resulted in the respective cytotoxicity as reported above, the varying effect of the excipients on the antibacterial effect is unknown and has to be determined before a comprehensive comparison and conclusion can be made of their therapeutic potential above conventional antibiotics.

The small concentration range that was used for the excipients is in retrospect seen as a limitation of this study. Between three pre-determined concentrations, there could not be established whether a concentration-dependent effect was evident or not. With larger and wider data sets an accurate pattern could have been established. Another hurdle was determining necrotic cell death. Although the LDH assay is indicative of necrosis, the data obtained from this assay and the MTT assay is insufficient to attribute cell death specifically to necrosis if LDH leakage was evident. For this additional cytotoxicity assays are recommended.

Indirectly, this study also aimed to determine the effect of excipients with different net charge towards the cationic peptide molecule and also the varying effect on the cytotoxicity. The excipients were intentionally chosen as L-glutamic acid with anionic charge, chitosan with cationic charge and non-ionic polysorbate 80. Taking all the results from this study into account, it is evident that there is no universal effect between specifically charged excipients and the cationic peptide. In conclusion, any interaction between an excipient and peptide molecule in a formulation, is specific to the formulation. Furthermore, the ability of excipients to increase the cytotoxicity of AMPs toward cells can be attributed to various interactions, including individual additive toxicity, interactions between the moieties with resulted aggregation to cause added toxicity, structural modification of the peptide primary or secondary structure resulting in more potent cytolytic effects, or potential synergistic toxic effects between the peptide and excipient, to briefly name some possibilities. This study, however, does not aim to determine the specific interaction mechanism behind increased cytotoxicity of combination treatments as it is more complex and requires more resources and extensive research into various possible structural and chemical environmental changes.

4.8 References

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Chapter 5

Conclusion and future prospects

5.1 Conclusion and future prospects

The critical threat of emerging antibiotic resistance clearly highlights the need for antimicrobial peptides (AMP) to be clinically introduced as respectable alternatives to conventional antibiotics (Mahlapuu *et al*., 2016; WHO, 2014:69). Before AMPs can therapeutically be used as novel treatments against fatal and resistant gastrointestinal (GI) infections, the safety issues that limit its potential, with regards to its cytotoxic effects towards mammalian cells, must be addressed (Astashkina *et al*., 2012:83; Hassan *et al*., 2012:730). Previous studies have proven potent antibacterial effects of AMPs towards various bacterial strains commonly associated with GI infections in comparison to current protocol antibiotics, as described in Section 2.3.3, but fail to characterise the toxicity thereof (Ebbensgaard *et al*., 2015; Irazazabal *et al*., 2016:2702; Tong *et al*., 2014). The few cytotoxicity studies performed to date mainly focus on the haemolytic activity of AMPs, while there is still a lack in data that is able to describe and characterise the interactions and cytotoxic effects toward GI cells (Mahlapuu *et al*., 2016).

This study aimed to investigate and comparatively characterise the mechanisms of *in vitro* cytotoxicity of two venom peptides, melittin and mastoparan, and a bacteriocin peptide, nisin Z, towards HepG2 and Caco-2 cells. In addition, this study evaluated and described the varying cytotoxicity of AMPs in combination with peptide stabilising excipients (L-glutamic acid, chitosan and polysorbate 80) compared to peptide alone toxicity.

Final conclusions

Experiments determining the initial cytotoxicity of the three AMPs revealed that the IC_{50} values obtained for melittin and mastoparan were considerably lower than the concentration needed for nisin Z to inhibit half the maximal cell viability. It is concluded that venom peptides exhibit stronger cytotoxic effects towards mammalian cells in comparison to bacteriocin peptides. A study performed by Lewies *et al.,* (2017:250) furthermore displayed a more cytotoxic effect of melittin towards HaCat cells compared to the cytotoxicity of nisin Z. It is also evident from the LDH assay performed on HepG2 and Caco-2 cells that melittin and mastoparan caused cell damage and subsequent cell death which could be indicative of necrotic cell death. Previous studies on the cytotoxicity of these peptides toward Caco-2 cells concluded necrosis as the mechanism of toxicity (Chaisakul *et al*., 2016:1576; Gajski & Garaj-Vrhovac, 2013:700). Although cytotoxicity of nisin Z was demonstrated by the MTT assay, the same concentration did not result in membrane damage and cell death may, therefore, be possibly be ascribed to apoptosis in both cell lines. Paiva *et al*., (2012:2856) also found nisin Z to induce apoptosis in HepG2 cells.

This study is the first to mainly investigate the varying cytotoxic effects of AMPs in combination with excipients when compared to individual peptide cytotoxicity. Although melittin combination treatments towards both cell lines did not result in higher toxicity compared to melittin alone treatment, it is still significant to report decreased cytotoxicity as the effect on antibacterial activity of this combination is unknown. On the other hand, mastoparan, which is also a venom peptide, was able to exert higher cytotoxicity towards Caco-2 cells when in combination with L-glutamic acid and chitosan compared to mastoparan alone. Membrane damage occurred in both cell lines after treatments of the above mentioned combinations and therefore it can be suggested that mastoparan:L-glutamic acid and mastoparan:chitosan possibly induced necrotic cell death in HepG2 and Caco-2 cells. It is proposed that the cell penetrating properties of mastoparan plays a crucial role in the cytotoxicity of mastoparan in combination with peptide stabilising excipients as melittin, also a cationic α-helical venom peptide, did not exhibit the same results when in combination (Moreno & Giralt, 2015:1138). Generally, HepG2 cells were more susceptible towards the cytotoxic effects of AMP alone and combination treatments in comparison to Caco-2 cells. This was also seen in a study performed by Jacobs (2015). However, Caco-2 cells treated with nisin Z alone and in combination, resulted in lower cell viability compared to HepG2 cells after the same treatment. Nisin Z in combination with polysorbate 80 demonstrated high cytotoxicity in both HepG2 and Caco-2 cell lines with suggested apoptotic cell death as they did not induce membrane damage.

In conclusion, the aims and objectives of this study were achieved as I was able to characterise the mechanisms of cytotoxicity of melittin, mastoparan and nisin Z towards mammalian cells and express the greater cytotoxicity of venom peptides in comparison to bacteriocin peptides. The study was also able to determine and evaluate the increased cytotoxicity of nisin Z in combination with polysorbate 80 as well as mastoparan in combination with L-glutamic acid and chitosan respectively.

Future prospects

The following changes and additional studies are recommended for future research as a follow-up for this study in order to improve the clinical success and application of AMPs as alternatives to antibiotic treatment in GI infections caused by bacterial pathogens:

- The results obtained from cytotoxicity experiments on AMP combinations should be compared to additional antibacterial experiments to determine the varying effect of excipient addition on the potency of the antibacterial effect and it should be compared to effects of AMP alone treatment.

- The potential of mastoparan's cell penetrating properties and the potential as a drug carrier should be investigated. This could be useful in drug delivery to resistant cells such as cancer.
- The membrane interaction of nisin Z remains unclear in literature; therefore further investigation ought to be conducted on the interaction between nisin Z and Caco-2 cells, as Caco-2 cells displayed higher susceptibility towards this peptide above the more potent venom peptides.
- Additional cytotoxicity studies have to be performed with combinations of nisin Z and polysorbate 80 that include a wider concentration series of both; and additionally the antibacterial effects of the combination should be determined.
- For accurately determining cell death as a result of necrosis or apoptosis, it is recommended that additional assays be performed in combination with the MTT and the LDH assay.
- When addressing combinations of drugs and excipients to be used in treatments, interaction studies must further be done to evaluate the increasing, decreasing, additive or possible synergistic effect.

5.2 References

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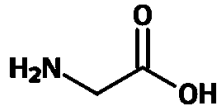
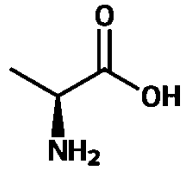
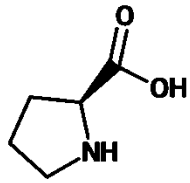
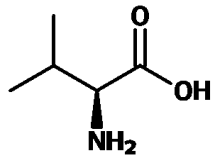
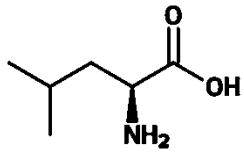
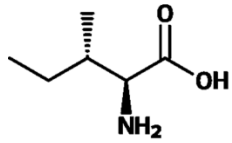
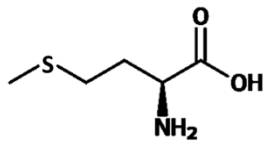
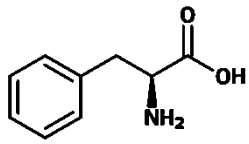
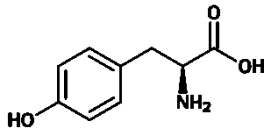
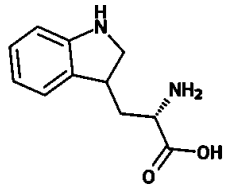
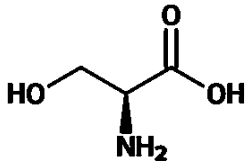
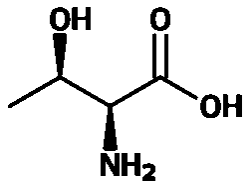
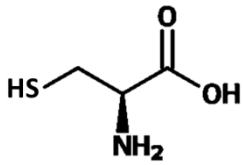
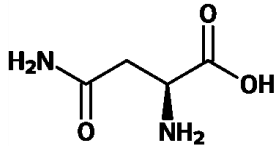
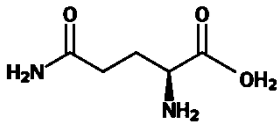
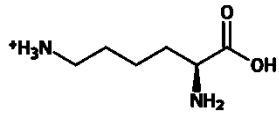
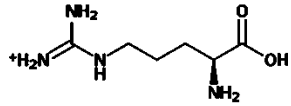
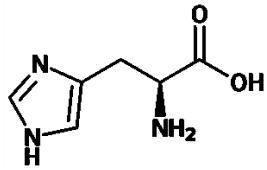
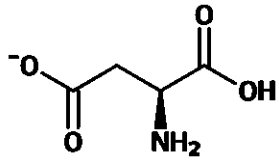
Addendum A

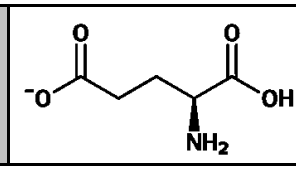
Amino acid abbreviations and properties

Amino acid abbreviations and properties of twenty common amino acids

Table A.1: Chemical properties and abbreviations of the twenty common amino acids found in proteins and peptides

Name and molecular formula:	Chemical structure:	Abbreviation: Three letter	Single letter	Molecular weight: (g/mol)	Essential/ Non-essential
Non-polar, aliphatic R-Group					
Glycine $C_2H_5NO_2$		Gly	G	75	Non-essential
Alanine $C_3H_7NO_2$		Ala	A	89	Non-essential
Proline $C_2H_9NO_2$		Pro	P	115	Non-essential
Valine $C_5H_{11}NO_2$		Val	V	117	Essential
Leucine $C_6H_{13}NO_2$		Leu	L	131	Essential
Isoleucine $C_6H_{13}NO_2$		Ile	I	131	Essential
Methionine $C_5H_{11}NO_2S$		Met	M	149	Essential
Aromatic R-Group					
Phenyl-alanine $C_9H_{11}NO_2$		Phe	F	165	Essential
Tyrosine $C_9H_{11}NO_3$		Tyr	Y	181	Non-essential

Tryptophan $C_{11}H_{12}N_2O_2$		Trp	W	204	Essential
Polar, uncharged R-Group					
Serine $C_3H_7NO_3$		Ser	S	105	Non-essential
Threonine $C_4H_9NO_3$		Thr	T	119	Essential
Cysteine $C_3H_7NO_2S$		Cys	C	121	Non-essential
Asparagine $C_4H_8N_2O_3$		Asn	N	132	Non-essential
Glutamine $C_5H_9NO_4$		Gln	Q	146	Non-essential
Positively charged R-Group (Basic amino acids)					
Lysine $C_6H_{14}N_20_2$		Lys	K	146	Essential
Arginine $C_6H_{14}N_4O_2$		Arg	R	174	Non-essential
Histidine $C_6H_9N_3O_2$		His	H	155	Essential
Negatively charged R-Group (Acidic amino acids)					
Aspartate $C_4H_7NO_4$		Asp	D	133	Non-essential

Glutamate $C_5H_9NO_4$		Glu	E	147	Non-essential

Addendum B

Analysis of variance (ANOVA) reports

Determining statistical significant differences

The analysis of variance (ANOVA) was performed to determine if there were any statistically significant differences between the mean values of combination treatments when compared to the AMP alone treatment. Dunnett post-hoc tests were used to analyse non-parametric data. Differences between the data of the different methods and techniques were deemed statistically significant if $p \leq 0.05$. The reports of the ANOVA performed on data obtained from the cytotoxicity assays are depicted in Figures B.1-B.12. Abbreviations for combinations were used and are reported as: 0.75 mg/ml L-glutamic acid (L1), 1.5 mg/ml L-glutamic acid (L2), 3 mg/ml L-glutamic acid (L3), 5 mg/ml chitosan (C1), 10 mg/ml chitosan (C2), 20 mg/ml chitosan (C3), 2 mg/ml polysorbate 80 (P1), 4 mg/ml polysorbate 80 (P2) and 8 mg/ml polysorbate 80 (P3) relative to the AMP molecule tested.

Table Analyzed	**Melittin 6h MTT HepG2**				
One-way analysis of variance					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	4,886				
R squared	0,4705				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	21,69				
P value	0,0168				
P value summary	*				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	14360	10	1436		
Residual (within columns)	16170	55	294,0		
Total	30530	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Melittin alone vs L1	-33,29	3,363	Yes	*	-61.10 to -5.474
Melittin alone vs L2	-37,19	3,757	Yes	**	-65.00 to -9.375
Melittin alone vs L3	-35,36	3,572	Yes	**	-63.17 to -7.544
Melittin alone vs C1	-16,87	1,704	No	ns	-44.68 to 10.95
Melittin alone vs C2	-35,24	3,560	Yes	**	-63.05 to -7.424
Melittin alone vs C3	-25,62	2,589	No	ns	-53.44 to 2.189
Melittin alone vs P1	-11,32	1,144	No	ns	-39.14 to 16.49
Melittin alone vs P2	-5,823	0,5882	No	ns	-33.64 to 21.99
Melittin alone vs P3	-7,492	0,7569	No	ns	-35.31 to 20.32
Melittin alone vs Negative control	-46,22	4,669	Yes	***	-74.03 to -18.41

Figure B.1: Analysis of variance report for cytotoxicity data obtained from the MTT assay after melittin combination treatments on HepG2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Melittin 6h MTT Caco-2				
One-way analysis of variance					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	5,872				
R squared	0,5164				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	14,49				
P value	0,1519				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	9064	10	906,4		
Residual (within columns)	8489	55	154,3		
Total	17550	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Melittin alone vs L1	-8,954	1,248	No	ns	-29.11 to 11.20
Melittin alone vs L2	-30,97	4,318	Yes	***	-51.13 to -10.82
Melittin alone vs L3	-11,06	1,543	No	ns	-31.22 to 9.090
Melittin alone vs C1	-8,426	1,175	No	ns	-28.58 to 11.73
Melittin alone vs C2	-27,20	3,793	Yes	**	-47.36 to -7.049
Melittin alone vs C3	-21,31	2,970	Yes	*	-41.46 to -1.152
Melittin alone vs P1	-32,71	4,561	Yes	***	-52.87 to -12.56
Melittin alone vs P2	-27,67	3,857	Yes	**	-47.82 to -7.515
Melittin alone vs P3	-13,87	1,934	No	ns	-34.03 to 6.281
Melittin alone vs Negative control	-38,52	5,370	Yes	***	-58.67 to -18.36

Figure B.2: Analysis of variance report for cytotoxicity data obtained from the MTT assay after melittin combination treatments on Caco2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Melittin 6h LDH HepG2				
One-way analysis of variance					
P value	0,0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	4,525				
R squared	0,4514				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	22,06				
P value	0,0148				
P value summary	*				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	17380	10	1738		
Residual (within columns)	21120	55	384,0		
Total	38490	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs L1	15,17	1,341	No	ns	-16.62 to 46.96
Control vs L2	44,93	3,972	Yes	**	13.15 to 76.72
Control vs L3	49,48	4,373	Yes	***	17.69 to 81.26
Control vs C1	24,63	2,177	No	ns	-7.156 to 56.42
Control vs C2	22,23	1,965	No	ns	-9.563 to 54.01
Control vs C3	25,22	2,229	No	ns	-6.569 to 57.01
Control vs P1	43,81	3,872	Yes	**	12.02 to 75.60
Control vs P2	45,35	4,009	Yes	**	13.56 to 77.14
Control vs P3	56,62	5,005	Yes	***	24.83 to 88.41
Control vs Negative control	31,30	2,767	No	ns	-0.4846 to 63.09

Figure B.3: Analysis of variance report for cytotoxicity data obtained from the LDHassay after melittin combination treatments on HepG2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Melittin 6h LDH Caco-2				
One-way analysis of variance					
P value	0,0004				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	4,004				
R squared	0,4213				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	34,76				
P value	0,0001				
P value summary	***				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	6395	10	639,5		
Residual (within columns)	8786	55	159,7		
Total	15180	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Melittin alone vs L1	3,027	0,4148	No	ns	-17.48 to 23.53
Melittin alone vs L2	17,37	2,381	No	ns	-3.129 to 37.88
Melittin alone vs L3	26,21	3,592	Yes	**	5.706 to 46.71
Melittin alone vs C1	28,47	3,902	Yes	**	7.968 to 48.97
Melittin alone vs C2	24,47	3,354	Yes	*	3.972 to 44.98
Melittin alone vs C3	23,40	3,206	Yes	*	2.892 to 43.90
Melittin alone vs P1	22,36	3,064	Yes	*	1.855 to 42.86
Melittin alone vs P2	3,082	0,4223	No	ns	-17.42 to 23.58
Melittin alone vs P3	21,67	2,970	Yes	*	1.166 to 42.17
Melittin alone vs Negative control	22,16	3,037	Yes	*	1.655 to 42.66

Figure B.4: Analysis of variance report for cytotoxicity data obtained from the LDH assay after melittin combination treatments on Caco-2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Mastoparan 6h MTT HepG2				
One-way analysis of variance					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	46,75				
R squared	0,8947				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	52,86				
P value	P<0.0001				
P value summary	***				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	31090	10	3109		
Residual (within columns)	3658	55	66,51		
Total	34750	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Mastoparan alone vs L1	-4,017	0,8531	No	ns	-17.25 to 9.213
Mastoparan alone vs L2	-7,188	1,527	No	ns	-20.42 to 6.042
Mastoparan alone vs L3	-13,55	2,878	Yes	*	-26.78 to -0.3219
Mastoparan alone vs C1	2,368	0,5029	No	ns	-10.86 to 15.60
Mastoparan alone vs C2	0,6765	0,1437	No	ns	-12.55 to 13.91
Mastoparan alone vs C3	6,258	1,329	No	ns	-6.972 to 19.49
Mastoparan alone vs P1	-18,14	3,853	Yes	**	-31.37 to -4.910
Mastoparan alone vs P2	-19,34	4,108	Yes	**	-32.57 to -6.115
Mastoparan alone vs P3	-27,15	5,765	Yes	***	-40.38 to -13.92
Mastoparan alone vs Negative Control	-75,05	15,94	Yes	***	-88.28 to -61.82

Figure B.5: Analysis of variance report for cytotoxicity data obtained from the MTT assay after mastoparan combination treatments on HepG2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Mastoparan 6h MTT Caco-2				
One-way analysis of variance					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	47,62				
R squared	0,8965				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	13,26				
P value	0,2093				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	23400	10	2340		
Residual (within columns)	2702	55	49,13		
Total	26100	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Mastoparan alone vs L1	30,87	7,628	Yes	***	19.50 to 42.24
Mastoparan alone vs L2	23,10	5,707	Yes	***	11.73 to 34.47
Mastoparan alone vs L3	22,96	5,673	Yes	***	11.59 to 34.33
Mastoparan alone vs C1	23,48	5,803	Yes	***	12.11 to 34.86
Mastoparan alone vs C2	26,24	6,484	Yes	***	14.87 to 37.61
Mastoparan alone vs C3	24,78	6,123	Yes	***	13.41 to 36.15
Mastoparan alone vs P1	14,55	3,596	Yes	**	3.182 to 25.92
Mastoparan alone vs P2	-6,395	1,580	No	ns	-17.77 to 4.977
Mastoparan alone vs P3	-10,06	2,486	No	ns	-21.43 to 1.311
Mastoparan alone vs Negative Control	-30,93	7,642	Yes	***	-42.30 to -19.55

Figure B.6: Analysis of variance report for cytotoxicity data obtained from the MTT assay after mastoparan combination treatments on Caco-2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Mastoparan 6h LDH HepG2				
One-way analysis of variance					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	6,413				
R squared	0,5383				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	21,72				
P value	0,0166				
P value summary	*				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	18550	10	1855		
Residual (within columns)	15910	55	289,3		
Total	34460	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Mastoparan alone vs L1	-24,67	2,512	No	ns	-52.26 to 2.921
Mastoparan alone vs L2	-6,774	0,6898	No	ns	-34.37 to 20.82
Mastoparan alone vs L3	1,919	0,1954	No	ns	-25.67 to 29.51
Mastoparan alone vs C1	8,538	0,8695	No	ns	-19.05 to 36.13
Mastoparan alone vs C2	-0,5873	0,05980	No	ns	-28.18 to 27.01
Mastoparan alone vs C3	12,87	1,311	No	ns	-14.72 to 40.46
Mastoparan alone vs P1	35,76	3,641	Yes	**	8.163 to 63.35
Mastoparan alone vs P2	21,13	2,152	No	ns	-6.463 to 48.72
Mastoparan alone vs P3	25,85	2,633	No	ns	-1.740 to 53.45
Mastoparan alone vs Negative Control	27,02	2,751	No	ns	-0.5760 to 54.61

Figure B.7: Analysis of variance report for cytotoxicity data obtained from the LDH assay after mastoparan combination treatments on HepG2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Mastoparan 6h LDH Caco-2				
One-way analysis of variance					
P value	0,0003				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	4,099				
R squared	0,4270				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	22,59				
P value	0,0124				
P value summary	*				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	28380	10	2838		
Residual (within columns)	38080	55	692,4		
Total	66460	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Mastoparan alone vs L1	2,986	0,1966	No	ns	-39.70 to 45.67
Mastoparan alone vs L2	20,01	1,317	No	ns	-22.68 to 62.69
Mastoparan alone vs L3	55,63	3,661	Yes	**	12.94 to 98.31
Mastoparan alone vs C1	30,35	1,998	No	ns	-12.34 to 73.04
Mastoparan alone vs C2	15,44	1,017	No	ns	-27.24 to 58.13
Mastoparan alone vs C3	51,55	3,394	Yes	*	8.868 to 94.24
Mastoparan alone vs P1	50,65	3,334	Yes	*	7.963 to 93.34
Mastoparan alone vs P2	50,75	3,341	Yes	*	8.066 to 93.44
Mastoparan alone vs P3	8,775	0,5776	No	ns	-33.91 to 51.46
Mastoparan alone vs Negative Control	49,03	3,228	Yes	*	6.347 to 91.72

Figure B.8: Analysis of variance report for cytotoxicity data obtained from the LDH assay after mastoparan combination treatments on Caco-2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Nisin Z 6h MTT HepG2				
One-way analysis of variance					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	92,77				
R squared	0,9440				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	28,91				
P value	0,0013				
P value summary	**				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	115700	10	11570		
Residual (within columns)	6858	55	124,7		
Total	122500	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Nisin Z alone vs L1	-71,78	11,13	Yes	***	-89.89 to -53.66
Nisin Z alone vs L2	-71,51	11,09	Yes	***	-89.63 to -53.40
Nisin Z alone vs L3	-38,39	5,955	Yes	***	-56.50 to -20.27
Nisin Z alone vs C1	-73,38	11,38	Yes	***	-91.50 to -55.27
Nisin Z alone vs C2	-84,27	13,07	Yes	***	-102.4 to -66.15
Nisin Z alone vs C3	-66,31	10,29	Yes	***	-84.42 to -48.19
Nisin Z alone vs P1	30,07	4,664	Yes	***	11.96 to 48.18
Nisin Z alone vs P2	26,71	4,143	Yes	**	8.597 to 44.83
Nisin Z alone vs P3	13,07	2,028	No	ns	-5.042 to 31.19
Nisin Z alone vs Negative Control	-46,07	7,145	Yes	***	-64.18 to -27.95

Figure B.9: Analysis of variance report for cytotoxicity data obtained from the MTT assay after nisin Z combination treatments on HepG2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Nisin Z 6h MTT Caco-2				
One-way analysis of variance					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	75,98				
R squared	0,9325				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	9,287				
P value	0,5051				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	31200	10	3120		
Residual (within columns)	2259	55	41,07		
Total	33460	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Nisin Z alone vs L1	-0,1706	0,04612	No	ns	-10.57 to 10.23
Nisin Z alone vs L2	1,070	0,2893	No	ns	-9.326 to 11.47
Nisin Z alone vs L3	0,9617	0,2599	No	ns	-9.434 to 11.36
Nisin Z alone vs C1	4,328	1,170	No	ns	-6.068 to 14.72
Nisin Z alone vs C2	4,297	1,161	No	ns	-6.099 to 14.69
Nisin Z alone vs C3	7,507	2,029	No	ns	-2.889 to 17.90
Nisin Z alone vs P1	23,98	6,481	Yes	***	13.58 to 34.38
Nisin Z alone vs P2	31,57	8,531	Yes	***	21.17 to 41.96
Nisin Z alone vs P3	38,81	10,49	Yes	***	28.41 to 49.20
Nisin Z alone vs Negative Control	-48,92	13,22	Yes	***	-59.32 to -38.53

Figure B.10: Analysis of variance report for cytotoxicity data obtained from the MTT assay after nisin Z combination treatments on Caco-2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Nisin Z LDH HepG2				
One-way analysis of variance					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	5,414				
R squared	0,4960				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	12,53				
P value	0,2513				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	80300	10	8030		
Residual (within columns)	81580	55	1483		
Total	161900	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Nisin Z alone vs L1	40,61	1,826	No	ns	-21.87 to 103.1
Nisin Z alone vs L2	37,04	1,666	No	ns	-25.44 to 99.52
Nisin Z alone vs L3	43,26	1,946	No	ns	-19.22 to 105.7
Nisin Z alone vs C1	49,30	2,217	No	ns	-13.18 to 111.8
Nisin Z alone vs C2	55,59	2,500	No	ns	-6.885 to 118.1
Nisin Z alone vs C3	66,31	2,982	Yes	*	3.828 to 128.8
Nisin Z alone vs P1	-37,84	1,702	No	ns	-100.3 to 24.64
Nisin Z alone vs P2	-29,86	1,343	No	ns	-92.34 to 32.62
Nisin Z alone vs P3	14,28	0,6423	No	ns	-48.20 to 76.76
Nisin Z alone vs Negative Control	-19,92	0,8960	No	ns	-82.40 to 42.55

Figure B.11: Analysis of variance report for cytotoxicity data obtained from the LDH assay after nisin Z combination treatments on HepG2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone

Table Analyzed	Nisin Z 6h LDH Caco-2				
One-way analysis of variance					
P value	0,0006				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	11				
F	3,798				
R squared	0,4085				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	5,616				
P value	0,8464				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	5622	10	562,2		
Residual (within columns)	8142	55	148,0		
Total	13760	65			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Nisin Z alone vs L1	-5,461	0,7775	No	ns	-25.20 to 14.28
Nisin Z alone vs L2	6,857	0,9762	No	ns	-12.88 to 26.59
Nisin Z alone vs L3	4,612	0,6566	No	ns	-15.13 to 24.35
Nisin Z alone vs C1	-1,564	0,2226	No	ns	-21.30 to 18.17
Nisin Z alone vs C2	8,756	1,246	No	ns	-10.98 to 28.49
Nisin Z alone vs C3	8,585	1,222	No	ns	-11.15 to 28.32
Nisin Z alone vs P1	4,828	0,6873	No	ns	-14.91 to 24.56
Nisin Z alone vs P2	-1,060	0,1509	No	ns	-20.80 to 18.68
Nisin Z alone vs P3	-11,86	1,688	No	ns	-31.59 to 7.880
Nisin Z alone vs Negative Control	-23,00	3,274	Yes	*	-42.73 to -3.260

Figure B.12: Analysis of variance report for cytotoxicity data obtained from the LDH assay after nisin Z combination treatments on Caco-2 cells. Dunnett post-hoc tests compared combination treatments to melittin alone