In vitro evaluation of the cytotoxic, antibacterial and antioxidant properties of selected chitosan derivatives and melittin

G Jacobs

22119027

(B.Pharm)

Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Pharmaceutics at the Potchefstroom Campus of the North-West University

Supervisor: Prof LH du Plessis

Co-Supervisor: Dr JF Wentzel

November 2015

Acknowledgements

I would like to thank the following people who gave me the necessary support, time and assistance to complete this dissertation:

First I would like to thank my Heavenly Father who gave me the strength to finish when I felt like giving up.

My supervisor, Prof. Lissinda H. du Plessis, for all the help and guidance throughout this study.

My co- supervisor, Dr. Jaco F. Wentzel, for all the assistance and helping me complete this study.

Prof. Sandy van Vuuren from the University of the Witwatersrand, thank you for all your help with the antimicrobial work.

To my parents, Gawie and Elri, thank you for giving me the opportunity to study at a university, to undertake a Master's degree and all your unfailing love and support.

To my fiancé, Stefan, thank you for all the motivation, encouragement and for always being there.

To the rest of my family and friends, thank you for all the encouragement and support.

To the North-West University Potchefstroom, thank you for the opportunity to complete my Master's degree.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

µg – Microgram

µM - Micromolar

AMP – Antimicrobial peptide

ATCC - American Type Culture Collection

C – Celsius

Caco-2 - Human epithelial colorectal adenocarcinoma cells

CS – Chitosan

Da – Degree of acetylation

DCMC - Dicarboxymethyl chitosan

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DPPH - 1, 1- Diphenyl- 2- picrylhydrazyl

FBS - Foetal bovine serum

FIC - Fractional Inhibitory Concentration

FITC - Fluorescein isothiocyanate

FL1 - Green fluorescent

FL2 - Red fluorescent

FSC - Forward light scatter

g – Gram

HepG2 – Human hepatocellular liver carcinoma cell line

HUVEC - Human umbilical vein endothelial cells

INT - Iodonitrotetrazolium violet

L – Litre

LDH - Lactate dehydrogenase

M – Molar

MBC - Minimum Bactericidal Concentration

MFI - Mean fluorescence intensity

mg – Milligram

mg/ml – Milligram per millilitre

MIC - Minimum Inhibitory Concentration

mM – Millimolar

MTT - 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

MW – Molecular weight

NaCl – Sodium chloride

NEAA - Non-essential amino acids

PBS - Phosphate- buffered saline

PI - Propidium iodide

ROS - Reactive Oxygen Species

SSC - Side light scatter

TEC – Triethyl chitosan

TEO – Chitosan oligomeres

TMC – Trimethyl chitosan

WHO – World Health Organization

Σ – Sum of

List of Equations

Abstract

Title: *In vitro* evaluation of the cytotoxic, antibacterial and antioxidant properties of selected chitosan derivatives and melittin

New excipients to improve the oral absorption of drugs are developing significantly. Polymers are used to enhance drug absorption through the gastrointestinal tract. Chitosan is a biocompatible polymer that is capable of opening tight junctions in membranes and therefore widely used as an absorption enhancer. This polymer is, however, insoluble under normal physiological conditions and a wide range of more soluble chitosan derivatives was developed for the delivery of compounds in the more alkaline environment of the intestines. Furthermore, the ability of chitosan to act as a functional excipient is advantageous in terms of antioxidant- and antimicrobial activity. Chitosan, in combination with melittin, a cationic peptide component of bee venom, has been shown to have synergistic absorption effects *in vitro*. Therefore the aim and objectives of this study was to characterise chitosan derivatives and to evaluate their antioxidant- and antimicrobial activity and determine the cytotoxic effects. *In vitro* evaluation was performed on human hepatocellular liver carcinoma cell line (HepG2 cells) and human epithelial colorectal adenocarcinoma cell line (Caco-2 cells) and antimicrobial activity was determined on four bacterial strains.

The antioxidant activity of four different chitosan derivatives namely, trimethyl chitosan (TMC), triethyl chitosan (TEC), dicarboxymethyl chitosan (DCMC) and chitosan oligomers (TEO) was determined using the 1, 1- Diphenyl- 2- picrylhydrazyl (DPPH) assay. Additionally, the antimicrobial and *in vitro* cytotoxicity was determined of the four derivatives, melittin and a combination thereof. By using the disc and well diffusion assays and also the minimum inhibitory concentration (MIC) assay, the antimicrobial activity was determined. The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT), lactate dehydrogenase (LDH) assays and flow cytometry were employed to determine the cytotoxicity.

It was determined that TEC displayed antioxidant activity with 25.37 ± 4.00%, 43.98 ± 6.67% and 47.66 ± 4.13% at 10 mg/ml, 25 mg/ml and 50 mg/ml, respectively, whereas the rest of the derivatives indicated as reactive oxygen species (ROS). Only TMC and melittin indicated antimicrobial activity against *Staphylococcus aureus* (*S. aureus*) and

Escherichia coli (*E. coli*) at a concentration of 1.56 mg/ml for TMC and 0.0625 mg/ml and 0.03125 mg/ml for melittin respectively. TMC also displayed the most cytotoxicity in HepG2 cells with 27.35 ± 1.48%, 24.56 ± 1.19%, 19.66 ± 2.05% and 17.70 ± 1.54% at 5 mg/ml, 10 mg/ml, 25 mg/ml and 50 mg/ml, respectively and in Caco-2 cells 33.90 ± 3.40%, 32.43 ± 3.17%, 34.95 ± 4.34% and 26.82 ± 3.32% at 5 mg/ml, 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. Other than TMC, DCMC indicated decreased cell viability at high concentrations with 27.08 ± 3.15% at 50 mg/ml in HepG2 cells and 34.45 ± 9.46% at 50 mg/ml in Caco-2 cells. Flow cytometry also indicated that the mechanism by which TMC decrease cell viability in HepG2 cells is through apoptosis and in Caco-2 cell necrosis.

It was concluded that TEC has antioxidant activity and TMC, melittin and a combination thereof has antimicrobial activity against selected bacterial strains, which might be an important contribution in the healing of wounds with skin infections. TMC indicated the most cytotoxicity in both cell lines and from previous results, it can be said that the molecular weight (MW) and degree of quaternisation (DQ) influences the functional properties and effect on cell viability of chitosan derivatives.

Keywords: Polymers, absorption enhancer, chitosan, antioxidant activity, antimicrobial activity, cytotoxicity, flow cytometry

Uittreksel

Titel: *In vitro* evaluering van die sitotoksiese, antibakteriële en antioksidant eienskappe van geselekteerde chitosan derivate en melittin

Nuwe hulpstowwe om orale absorpsie van geneesmiddels te bevorder word daagliks ontwikkel. Polimere word gebruik om geneesmiddel absorpsie te bevorder deur die spysverteringskanaal. Kitosaan is 'n polimeer wat in staat is om hegte aansluitings oop te maak en sodoende algemeen gebruik as absorpsie bevorderaar. Kitosaan is egter onoplosbaar onder normale fisiologiese toestande en 'n reeks oplosbare derivate is ontwikkel vir die aflewering van geneesmiddels in meer neutrale en basiese omgewings van die dunderm. Verder, die vermoë van kitosaan om as 'n funksionele hulpstof te reageer is voordelig in terme van antioksidant en antimikrobiese aktiwiteit. Kitosaan in kombinasie met melitien, 'n kationiese peptied en die hoof komponent van bye gif, het gewys dat dit 'n *in vitro* sinergistiese absorpsie effekte het. Dus die doel van die studie was om kitosaan derivate te karakteriseer en om die antioksidant en antimikrobiese aktiwiteit en sitotoksisiteit te bepaal. *In vitro* eksperimente is gedoen op menslike lewer kanker sellyn (HepG2) en menslike epiteel kolorektale adenokarsinoma selle (Caco-2) en die antimikrobiese aktiwiteit is bepaal op vier bakteriële variante.

Die antioksidant aktiwiteit van die vier kitosaan derivate naamlik, trimetiel chitosan (TMC), trietiel chitosan (TEC), dikarboksiemetiel chitosan (DCMC) en chitosan oligomere (TEO) is bepaal met behulp van die antioksidant metode (DPPH). Die antimikrobiese aktiwiteit en *in vitro* sitotoksisiteit is bepaal van die vier derivate, melitien en 'n kombinasie daarvan. Deur gebruik te maak van die skyfie diffusie, putjie diffusie en minimum inhiberende konsentrasie metode, is die antimikrobiese aktiwiteit bepaal. Die lewensvatbaarheid (MTT), laktaat dehidrogenase (LDH) en vloeisitometrie metodes is gebruik om die sitotoksisiteit te bepaal.

Dit is bevind dat TEC antioksidant aktiwiteit getoon het met 25.37 ± 4.00%, 43.98 ± 6.67% en 47.66 ± 4.13% by 10 mg/ml, 25 mg/ml en 50 mg/ml onderskeidelik. Die res van die derivate het geen antioksidant aktiwiteit getoon nie en eerder as 'n pro-oksidant opgetree wat lei tot 'n reaktiewe suurstof spesie (ROS). Net TMC en melitien het antimikrobiese aktiwiteit getoon teen *Staphylococcus aureus* en *Escherichia coli* met

ŉ konsentrasie van 1.56 mg/ml vir TMC en 0.0625 mg/ml en 0.03125 mg/ml vir melitien onderskeidelik. TMC het ook die meeste sitotoksisiteit getoon in HepG2 selle met 27.35 ± 1.48%, 24.56 ± 1.19%, 19.66 ± 2.05% en 17.70 ± 1.54% by 5 mg/ml, 10 mg/ml, 25 mg/ml en 50 mg/ml onderskeidelik en vir Caco-2 selle 33.90 ± 3.40%, 32.43 ± 3.17%, 34.95 ± 4.34% en 26.82 ± 3.32% by 5 mg/ml, 10 mg/ml, 25 mg/ml en 50 mg/ml onderskeidelik. Behalwe vir TMC, het DCMC verlaagde sel lewensvatbaarheid by hoë konsentrasies met 27.08 ± 3.15% by 50 mg/ml in HepG2 selle en 34.45 ± 9.46% by 50 mg/ml in Caco-2 selle. Vloeisitometrie het ook gewys dat die meganisme waarmee TMC verlaagde sel lewensvatbaarheid het in HepG2 selle is as gevolg van apoptose en in Caco-2 selle nekrose.

Die gevolgtrekking van hierdie ondersoeke is dat TEC antioksidant aktiwiteit het en TMC, melitien en ŉ kombinasie daarvan het antimikrobiese aktiwiteit teen sommige van die bakteriële stamme wat belangrik kan wees in die genesing van wonde wat geïnfekteerd is. TMC het die meeste sitotoksisiteit aangedui in albei sellyne en as vorige studies in ag geneem word, kan dit gesê word dat die molekulêre gewigte (MW) en graad van kwaternisering (DQ) van die derivate die funksionele eienskappe en effek op sel lewensvatbaarheid beïnvloed.

Sleutelwoorde: Polimere, absorpsie bevorderaar, kitosaan, antioksidant aktiwiteit, antimikrobiese aktiwiteit, sitotoksisiteit, vloeisitometrie

CHAPTER 1

INTRODUCTION AND AIM OF STUDY

Introduction

The oral route of drug administration remains the most beneficial route of drug administration when considering good patient compliance. The gastrointestinal tract is the reason for limited absorption of peptide drugs and drugs with poor water solubility. This causes reduced bioavailability of these drugs (Aungst, 1993, Hamman *et al*., 2005). Therefore the search for new excipients to incorporate in dosage forms to improve drug delivery and bioavailability is continuing (Aleeva *et al*., 2009). Polymers are used in drug delivery systems to improve the delivery of drugs through the gastrointestinal tract by enhancing absorption. When a polymer exhibit additional functions apart from a filler, binder or lubricant, it can be described as a functional excipient (Liu *et al*., 2015). It is therefore important that an excipient needs to be characterised before using it in a dosage form to determine possible pharmacological toxic effects and interactions with these drugs.

Chitosan, a natural cationic polymer obtained from chitin, is known for its use as an absorption enhancer by opening tight junctions to enhance the absorption of drugs through the paracellular pathway (Borchard and Junginger, 2001, Kotzé *et al*., 1998, Thanou *et al*., 2001). It is suspected to have wound healing properties and antioxidant and antimicrobial activity as well. The development of chitosan derivatives became necessary because chitosan is insoluble in neutral and basic environments in the intestine (Li *et al*., 1992, Mourya and Inamdar, 2009). Derivatives were also synthesised to develop possible functional properties such as antioxidant and antimicrobial activity which could be useful in the pharmaceutical industry. Derivatives exhibiting antimicrobial activity would also be advantageous in wound healing for the treatment of skin infections (Archana *et al*., 2013). The synthesis of chitosan derivatives entails the reductive methylation of chitosan by repeating the process to produce TMC, TEC, DCMC and TEO (Domard *et al*., 1986). Other derivatives can be produced by various other chemical synthesis procedures.

It has been shown that chitosan with different DQ have different effects on absorption (Enslin, 2005, Snyman *et al*., 2003, Venter, 2005b), enzyme inhibition (Oberholzer, 2009) and gene transfection ability (Venter, 2005a). Different MW and DQ also influence the toxicity (Fischer *et al*., 2003, Schipper *et al*., 1996). It is known that chitosan and one of its derivatives namely TMC, has antioxidant- and antimicrobial

activity against selected bacteria. In spite of this, the cytotoxicity of TMC is poorly characterised and is not known of some of the other derivatives and therefore it is important to characterise the polymers.

Antioxidants are substances which prevent or delays oxidative damage to cells and are necessary to prevent diseases such as Alzheimer's disease, arthritis and to delay the ageing process. Studies have shown that chitosan and some of its derivatives has antioxidant activity which have advantageous applications especially in the food industry (Alexandrova *et al*., 1999, Darmadji and Izumimoto, 1994, Kamil *et al*., 2002). Compounds with antimicrobial activity are advantageous when considering antimicrobial resistance of compounds. The increasing resistance of both Gram-positive and Gram- negative bacteria can lead to deaths around the world (Cornaglia, 2009). Therefore the need exists to discover new compounds to overcome antimicrobial resistance (Clark, 1996). Chitosan and some of its derivatives have been shown to exhibit antimicrobial activity against selected bacterial strains (Chen *et al*., 1998, Rhoades and Roller, 2000). The antimicrobial activity of chitosan could make it a valuable alternative for antibiotics.

Therefore the overall aim of this study was to characterise chitosan derivatives by determining their antioxidant- and antimicrobial activity and characterise their cytotoxic effects. This entailed an *in vitro* study on human hepatocellular liver carcinoma cell line and human epithelial colorectal adenocarcinoma cells. These cell lines were used as these are the sites the polymers will most likely be in contact with after oral administration.

Problem statement

The development of functional excipients is important to modify the release or absorption of a drug in the body. Absorption enhancers are functional excipients which are incorporated in formulations to improve the absorption and therefore increase the bioavailability of the drug (Aungst, 2000).

Before compounds can be used as functional excipients they have to be characterised to understand the functionality of the compound. Problems that are associated with excipients or absorption enhancers include cytotoxicity that may compromise the safety of the compound (Pifferi and Restani, 2003). On the other hand, excipients exhibiting antimicrobial and antioxidant activities may be advantageous. Therefore, in this study we investigated the cytotoxic-, antimicrobial- and antioxidant activities of absorption enhancers, including the chosen chitosan derivatives, namely: TMC, DCMC, TEC and TEO. Melittin, an antimicrobial peptide (AMP) with absorption enhancing effects was included in the study to determine synergistic cytotoxic, antibacterial or antioxidant activities.

Aim and Objectives

The aim of this study was to characterise and investigate the antioxidant- and antimicrobial activity and the cytotoxicity of chitosan derivatives, TMC, DCMC, TEC and TEO in combination with the AMP, melittin.

The objectives of the study were:

- To determine the antioxidant activity of different chitosan derivatives.
- To determine the effect of different chitosan derivatives, melittin and combinations thereof on the antimicrobial activity against Gram- positive bacteria namely, *S. aureus* and *Staphylococcus epidermidis* (*S. epidermidis*) and Gram- negative bacteria namely, *E. coli* and *Pseudomonas aeruginosa* (*P. aeruginosa*).
- To determine the effect of different chitosan derivatives, melittin and combinations thereof on the cytotoxicity of HepG2 and Caco-2 cells.

Structure of dissertation

Chapter 1 gives a brief background of the study along with a problem statement and aim and objectives of the study. Chapter 2 will focus on the relevant literature regarding polymers, absorption in the gastrointestinal tract, absorption enhancers,

chitosan and its derivatives, applications thereof, melittin and the antioxidant- and antimicrobial activity of chitosan derivatives. In chapter 3 the synthesis of chitosan derivatives, the experimental design and the methods used to achieve the aim and objectives in this study is described. Chapter 4 illustrates the results that were found regarding antioxidant-, antimicrobial activity and cytotoxicity in this study along with a discussion thereof. Chapter 5 is the conclusion and summarises the results that were found in this study along with possible future prospects and recommendations. This study is unique as the author is unaware of studies using a combination of two absorption enhancers and their cytotoxicity profiles. This study will help identify if chitosan derivatives is non- toxic as the literature states, if the combination of two absorption enhancers will increase the absorption enhancing effects and the antioxidant and antimicrobial activity of chitosan derivatives.

The Harvard referencing style is used in this dissertation in accordance with the North- West University. The references in the text are sorted alphabetically, then chronologically. Multiple references from the same author in the same year are identified by the letters ‘a’, ‘b’, ‘c’, etc., placed after the year of publication. Journal names are written out.

References

ALEEVA, G. N., ZHURAVLEVA, M. V. & KHAFIZ'YANOVA, R. K. 2009. The role of excipients in determining the pharmaceutical and therapeutic properties of medicinal agents. *Pharmaceutical Chemistry Journal,* 43**,** 230-234.

ALEXANDROVA, V. A., OBUKHOVA, G. V., DOMNINA, N. S. & TOPCHIEV, D. A. 1999. Modification of chitosan for construction of efficient antioxidant biodegradable macromolecular systems. *Macromolecular Symposia,* 144**,** 413-422.

ARCHANA, D., DUTTA, J. & DUTTA, P. K. 2013. Evaluation of chitosan nano dressing for wound healing: Characterization, *in vitro* and *in vivo* studies. *International Journal of Biological Macromolecules,* 57**,** 193-203.

AUNGST, B. J. 1993. Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism. *Journal of Pharmaceutical Sciences,* 82**,** 979-87.

AUNGST, B. J. 2000. Intestinal permeation enhancers. *Journal of Pharmaceutical Sciences,* 89**,** 429-42.

BORCHARD, G. & JUNGINGER, H. E. 2001. Modern drug delivery applications of chitosan. *Advanced Drug Delivery Reviews,* 52**,** 103.

CHEN, C. S., LIAU, W. Y. & TSAI, G. J. 1998. Antibacterial effects of N-sulfonated and N-sulfobenzoyl chitosan and application to oyster preservation. *Journal of Food Protection,* 61**,** 1124-8.

CLARK, A. M. 1996. Natural products as a resource for new drugs. *Pharmaceutical Research,* 13**,** 1133-44.

CORNAGLIA, G. 2009. Fighting infections due to multidrug-resistant Gram-positive pathogens. *Clinical Microbiology and Infection,* 15**,** 209-11.

DARMADJI, P. & IZUMIMOTO, M. 1994. Effect of chitosan in meat preservation. *Meat Science,* 38**,** 243-254.

DOMARD, A., RINAUDO, M. & TERRASSIN, C. 1986. New method for the quaternization of chitosan. *International Journal of Biological Macromolecules,* 8**,** 105-107.

ENSLIN, G. M. A. 2005. *In Vitro Evaluation of the Absorption Enhancement Properties of Chitosans, Monocaprin and Melittin*, Tshwane University of Technology.

FISCHER, D., LI, Y., AHLEMEYER, B., KRIEGLSTEIN, J. & KISSEL, T. 2003. *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials,* 24**,** 1121-1131.

HAMMAN, J. H., ENSLIN, G. M. & KOTZE, A. F. 2005. Oral delivery of peptide drugs: barriers and developments. *BioDrugs,* 19**,** 165-77.

KAMIL, J. Y., JEON, Y.-J. & SHAHIDI, F. 2002. Antioxidative activity of chitosans of different viscosity in cooked comminuted flesh of herring (*Clupea harengus*). *Food Chemistry,* 79**,** 69-77.

KOTZÉ, A. F., LUEßEN, H. L., DE LEEUW, B. J., DE BOER, B. G., COOS VERHOEF, J. & JUNGINGER, H. E. 1998. Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). *Journal of Controlled Release,* 51**,** 35-46.

LI, Q., DUNN, E., GRANDMAISON, E. & GOOSEN, M. 1992. Applications and properties of chitosan. *Journal of Bioactive and Compatible Polymers,* 7**,** 370-397.

LIU, H., TAYLOR, L. S. & EDGAR, K. J. 2015. The role of polymers in oral bioavailability enhancement. *Polymer*.

MOURYA, V. K. & INAMDAR, N. N. 2009. Trimethyl chitosan and its applications in drug delivery. *Journal of Materials Science: Materials in Medicine,* 20**,** 1057-79.

OBERHOLZER, I. D. 2009. *Peroral and nasal delivery of insulin with Pheroid™ technology.* North-West University.

PIFFERI, G. & RESTANI, P. 2003. The safety of pharmaceutical excipients. *Il Farmaco,* 58**,** 541-550.

RHOADES, J. & ROLLER, S. 2000. Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Applied and Environmental Microbiology,* 66**,** 80-6.

SCHIPPER, N. M., VÅRUM, K. & ARTURSSON, P. 1996. Chitosans as Absorption Enhancers for Poorly Absorbable Drugs. 1: Influence of Molecular Weight and Degree of Acetylation on Drug Transport Across Human Intestinal Epithelial (Caco-2) Cells. *Pharmaceutical Research,* 13**,** 1686-1692.

SNYMAN, D., HAMMAN, J. H. & KOTZE, A. F. 2003. Evaluation of the mucoadhesive properties of N-trimethyl chitosan chloride. *Drug Development and Industrial Pharmacy,* 29**,** 61-9.

THANOU, M., VERHOEF, J. C. & JUNGINGER, H. E. 2001. Oral drug absorption enhancement by chitosan and its derivatives. *Advanced Drug Delivery Reviews,* 52**,** 117-126.

VENTER, C. 2005a. *Chitosan and quaternised chitosan polymers as gene transfection agents.* North-West University.

VENTER, J. P. 2005b. *Design and evaluation of chitosan and N-trimethyl chitosan chloride microspheres for intestinal drug delivery.* North-West University.

CHAPTER 2

LITERATURE STUDY

2.1. Introduction

There is an on-going search for new excipients to include in various dosage forms through every possible route such as oral (tablets, capsules etc.), dermal, parenteral, nasal, rectal, ophthalmic preparations and on the oral mucosa (Mansour *et al*., 2010). Excipients are used along with the active ingredient to assist in effective drug delivery. These components can act as a lubricant, coating or filling agent or solubilising agent etc. When an excipient can perform various other functions along with their basic function, they can be classified as a functional excipient (Aleeva *et al*., 2009). It is also important to characterise excipients before using it in a dosage form to examine their possible interactions. The polymers used in this study were characterised in terms of its antioxidant-, antimicrobial activity and cytotoxicity.

2.2. Polymers

Polymers have been used in the biomedical and pharmaceutical industry to improve drug delivery systems. Pharmaceutical polymers are used to improve drug delivery through the gastrointestinal tract (Liu *et al*., 2015). Cationic polymers are polymers with a positive charge such as chitosan. Some of these polymers have advantageous properties for instance wound healing properties, antioxidant and antimicrobial activity and absorption enhancers which make them a functional excipient. Absorption enhancers (see section **2.4**) are used to improve the absorption of drugs for various routes although the oral route remains the most common route of administration and is generally researched to improve absorption of drugs (Borchard *et al*., 1996).

2.3. Gastrointestinal absorption of drugs

The oral route of drug administration is considered more advantageous regarding patient compliance. The efficiency of this route of administration is sometimes limited due to poor oral bioavailability of selected drugs. This is especially true in the case of compounds with poor water solubility as well as peptide based drugs. These compounds have low bioavailability when orally administrated due to their degradation by digestive enzymes and the acidic stomach environment (Aungst, 1993, Bayat *et al*., 2008, Hamman *et al*., 2005, Owens *et al*., 2003). This decrease in bioavailability can be attributed to the extended deliverance route of oral drugs, where they must first

be absorbed in the gastrointestinal tract, metabolised by the liver and then absorbed in the systemic circulation (Benet *et al*., 1996). Hence, the active ingredient is required to reach the cell membrane of the target cell, where it will be absorbed whether it is dermal, mucosal or intestinal absorption (Aungst, 2012). The gastrointestinal tract is the main area where absorption takes place in the human. However, only a portion of the substance is absorbed due to the gastrointestinal tract containing certain barriers (DeSesso and Jacobson, 2001, Yeh *et al*., 1995). There are several factors that influence the absorption of drugs from the gastrointestinal tract namely, the environment of the gastrointestinal tract, physiochemical properties of the drug (e.g. solubility) and physiological factors (e.g. pH, gastric emptying) (Dahan *et al*., 2009). The delivering of drugs to the colon is a possible solution to overcome the degradation of substances owing to the fact that the colon has low enzymatic activity and is also more susceptible to absorption enhancers (Yang *et al*., 2002, Yeh *et al*., 1995).

2.4. Absorption enhancers

According to the World Health Organization (WHO) a pharmaceutical excipient or an absorption enhancer is defined as a substance which is included in the drug delivery system or dosage form other than the active ingredient of the drug product. This can lead to improved stability, bioavailability and effectiveness (WHO, 1999). Absorption enhancers are successful as excipients in dosage forms where the drug is poorly absorbed and is used to maximise the bioavailability of the drug (Enslin, 2005, Lin *et al*., 2011, Thanou *et al*., 2001b) to the desired therapeutic level of the drug (Maher *et al.*, 2007). It can also be employed to overcome any shortcomings regarding membrane permeability (Hamman and Steenekamp, 2012) and to enhance the transport of drugs across the gastrointestinal mucosal epithelial (van Hoogdalem *et al*., 1989, Zhou, 1994). Various types of absorption enhancers have been investigated to improve the oral absorption of biopharmaceuticals, including lipids (Kalepu *et al*., 2013), polymers (Nakamura *et al*., 2008) and cell penetrating peptides (Fonseca *et al*., 2009). The term cell penetrating peptide and AMP is in some cases used interchangeably and the peptides have in most cases both cell penetrating potential as well as antimicrobial activity.

Drugs are transported across intestinal epithelial and absorbed into the systemic circulation through the transcellular pathway or the paracellular pathway. The transcellular pathway involves the transport of small molecules and lipophilic drugs across the plasma membrane whereas the paracellular pathway makes use of tight junctions to transport large molecules and hydrophilic drugs (Gonzalez-Mariscal *et al*., 2008). The mechanism by which absorption enhancers improve absorption is by acting either on the tight junctions, mucous layer or membrane components (Lehr *et al*., 1993, Murakami *et al*., 1982, Tengamnuay and Mitra, 1990). Polymers, as absorption enhancers, functions by preventing metabolic activity and also enhancing the permeability of membranes by opening tight junctions (**Figure 2.1)** (Aungst, 2012, Borchard *et al*., 1996, Lueβen *et al*., 1996).

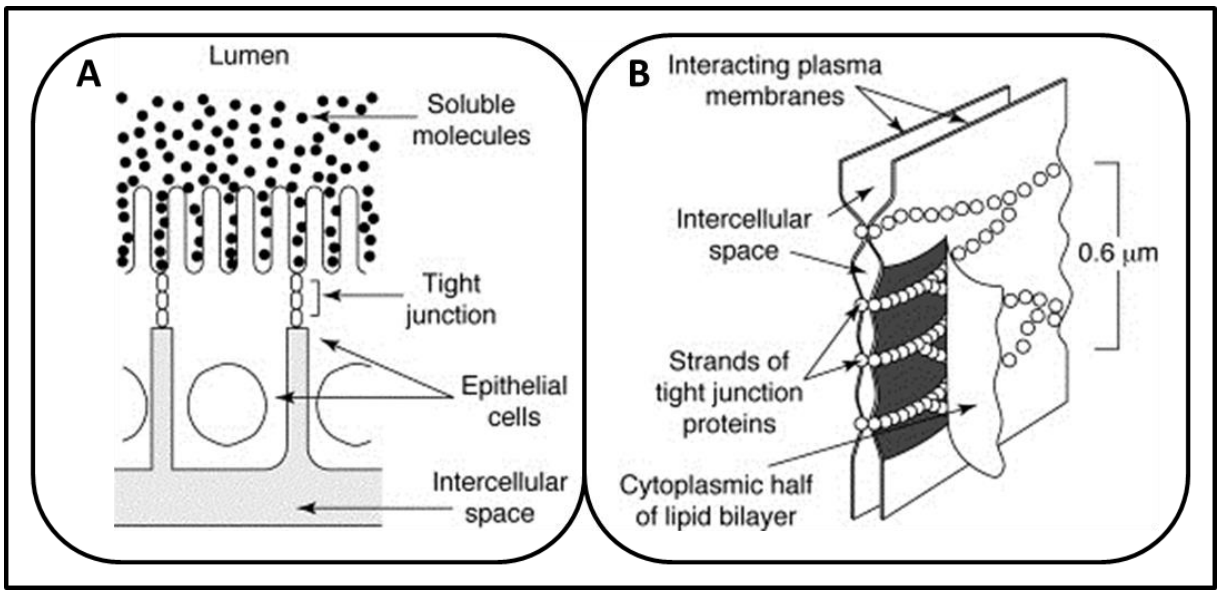


Figure 2.1: Schematic representation of tight junctions which hold epithelial cells together (A) and an enlargement of tight junctions viewed from the side indicating the size of the tight junction and intercellular space (B) (Junginger and Verhoef, 1998, Magos, 1991).

Absorption enhancers must adhere to certain properties such as, an immediate response, therapeutic plasma drug levels must be reached, the effect must be reversible, should not display systemic or toxic effects and should not cause damage to membranes (Fix, 1996, Junginger and Verhoef, 1998). It is also important to characterise an excipient before using it in a dosage form.

There are various types of compounds which are used as absorption enhancers to promote the absorption of drugs (Aungst, 1993, Hamman *et al*., 2005, Hinchcliffe and Illum, 1999). In **Table 2.1** a summary of the compounds applicable to absorption of drugs in the intestines is given.

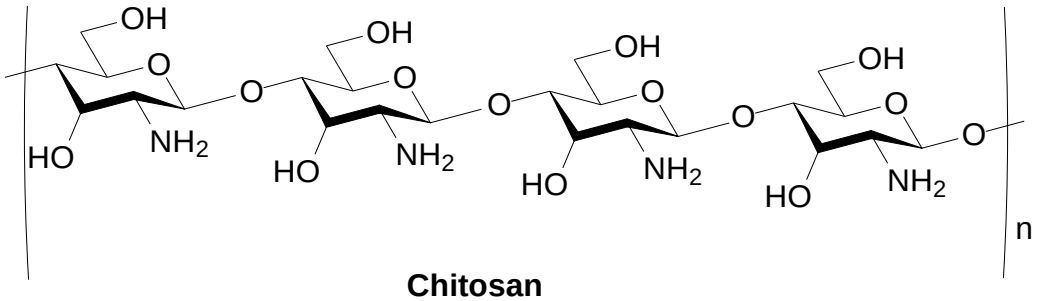
Table 2.1: A summary of compounds used to improve intestinal absorption of drugs as well as their mechanism of action (adapted from Aungst, 1993, Hamman *et al.*, 2005, Hinchcliffe and Illum, 1999).

Absorption enhancer	**Example**	**Mechanism of action**
Surfactants	Sodium lauryl sulphate, sodium dodecyl sulphate	Membrane damage
Bile salts	Sodium taurocholate	Opening of tight junctions, Inhibition of enzymes, Disruption of membranes
Enzyme inhibitors	Bestatin	Inhibition of enzymes
Cationic polymers	Chitosan N-trimethyl chitosan chloride	Opening of tight junctions (ionic interaction with cell membrane and mucoadhesion)
Toxins and venom extracts	Melittin (bee venom)	Opening of tight junctions

When absorption enhancers are used in combination with another excipient or in combination with an antimicrobial compound, it may show a synergistic effect due to their different mechanisms of absorption enhancement (Enslin *et al*., 2008). Melittin has potential as an absorption enhancer although the mechanism by which it exhibits the absorption enhancing effect is uncertain (Liu *et al*., 1999). Both chitosan and melittin are cationic polymers and improve absorption by enhancing paracellular permeability. The use of absorption enhancers in combination from different classes has hardly been studied and the ideal absorption enhancer remains a problem to improve the absorption of drugs which is poorly absorbed (Enslin, 2005).

2.5. Chitosan

Chitin was first discovered in mushrooms by a French professor, Henri Braconnot, in 1811 (Brück *et al*., 2010, Khoushab and Yamabhai, 2010, Mati-Baouche *et al*., 2014, Thirunavukkarasu and Shanmugam, 2009). Nowadays it is accepted that chitin is the main component of the exoskeletons of crustaceans and can also be found in cell walls of yeast and fungi (Rinaudo, 2006). As a long-chain polymer, chitin can undergo N-deacetylation to produce chitosan and its derivatives (Mourya and Inamdar, 2009). Chitosan is a polymer that is derived from chitin through alkaline hydrolysis also known as the deacetylation of chitin. Chitosan as a pharmaceutical excipient has several advantages such as low toxicity, biodegradability, biocompatibility, antimicrobial activity and mucoadhesive properties (Al-Qadi *et al*., 2012). These advantages make chitosan a favourable absorption enhancer in drug delivery. The proposed mechanism by which chitosan can act as an absorption enhancer is by interacting with tight junctions whereas the tight junctions open and lead to the absorption of macromolecular drugs through paracellular permeation (Borchard and Junginger, 2001, Kotzé *et al*., 1998, Thanou *et al*., 2001b). Chitosan (**Figure 2.2**) is a cationic polymer with a potential in drug delivery systems (Ilium, 1998). This positive charge of chitosan is responsible for its various properties and applications in the industry.



Chitosan

Figure 2.2: Schematic illustration of the chemical structure of chitosan. Adapted from (Chen *et al*., 2013)

Chitosan has the following molecular structure $(GlcN)_n$ + $(GNAc)_m$ $(C_6H_{11}N_1O_4)n$ + $(C_8H_{13}\ N_1O_5)m$. Chitosan is a polysaccharide composed of GlcN and GlcNAc linked with **β** (1 → 4)-glycosidic linkages. Chitosan is obtained by removing die acetyl groups (CH_3-CO) from chitin. This process, known as deacetylation, releases the amine groups (NH) and results in chitosan with cationic characteristics.

Applications

Chitosan and its derivatives have various functional properties making it possible to use them in various applications. This includes the use in biomedicine (Larsson *et al*., 2013, Rinaudo, 2006) where chitosan is used in cosmetics as a film- forming agent (Baldrick, 2010, Dodane and Vilivalam, 1998, Jayakumar *et al*., 2010b, van der Merwe *et al*., 2004b), as well as in tissue engineering (Jayakumar *et al*., 2010a). Chitosan is also used in the food industry and can promote weight loss (Thanou *et al*., 2001a) and wound healing in surgery (Dodane and Vilivalam, 1998, Soares *et al.*, 2014). The wound healing effect of chitosan is advantageous because it can deliver a drug to the wound which helps in the process of healing (Dash *et al*., 2011). This wound healing effect and antimicrobial activity of chitosan can contribute in treating microbial skin infections and further research is required (Archana *et al*., 2013).

Chitosan can be used in the agriculture and horticultural industry. Chitosan has a bactericidal or bacteriostatic effect which helps in controlling postharvest fungal diseases (Bautista-Baños *et al*., 2006, Rabea *et al*., 2003). It can also increase the crop yield and delay ripening of fruits by forming a semi- permeable barrier on the surface of the fruit which leads to extended storage life of products (Cheah *et al*., 1997, El Ghaouth *et al*., 1992, Rabea *et al*., 2003). This coating of chitosan can assist in sustainable agriculture. Chitosan is also effective in water filtration, where it can remove any particulates, dissolved substances and unwanted metal ions (also known as the chelation of metal ions) by forming an intermolecular hydrogen bond (Renault *et al*., 2009, Zeng *et al*., 2008). Another field where chitosan can be applied is in wine making where it immobilises enzymes to heighten the aroma of wines, musts and fruit juices (Spagna *et al*., 1998, Spagna *et al*., 2001, Zappino *et al*., 2015). It also has a high affinity to phenolic compounds which leads to good anti- browning action in wines. The reason for the browning effect in wines is due to the presence of phenolic compounds and causes economic damage (Spagna *et al*., 1996). Chitosan has various pharmaceutical applications such as controlled drug release using several routes of administration and has been used to produce tablets, beads, liposomes etc. (Sieval *et al*., 1998). Due to chitosan being cationic, it is able to open tight junctions on the negatively charged cell membranes and thus increasing the mucosal absorption (Hamman *et al*., 2003). This is noteworthy for the transport of large hydrophilic drugs

like peptides (Kotze *et al.*, 1999a, Thanou *et al*., 2000). Due to chitosan which is cationic, many pharmaceutical applications can be applied such as antioxidant activity (Guo *et al*., 2006, Jarmila and Vavrikova, 2011), antimicrobial activity (de Britto *et al*., 2011, Sadeghi *et al*., 2008, Wiarachai *et al*., 2012), gene transfection (Venter, 2005a) and absorption enhancing effects (Enslin, 2005, Snyman *et al*., 2003, Venter, 2005b). According to most literature, chitosan is non- toxic although the toxicity of polymers depend on their MW and DQ (Fischer *et al*., 2003). Some studies have shown that chitosan can be cytotoxic depending on the concentration and salt used although more research is required (Carreño-Gómez and Duncan, 1997).

2.6. Chitosan derivatives

Due to the ability of chitosan to open tight junctions in membranes it can therefore increase the bioavailability of a drug, functioning as an absorption enhancer. This potentially makes chitosan important in the formulation of biopharmaceuticals (Antunes *et al*., 2012). However, it is insoluble in water and will only become soluble in a more acidic environment where the pH is below 5.6 (Mourya and Inamdar, 2009). Chitosan derivatives have been formulated to overcome this problem of chitosan's solubility (Na *et al*., 2013, van der Merwe *et al*., 2004b) and also to determine their functional properties such as antioxidant and antimicrobial activity which will be discussed in section **2.8** and **2.9** respectively.

Various derivatives of chitosan have been synthesised including trimethyl chitosan (TMC) (Sadeghi *et al*., 2008), triethyl chitosan (TEC) (Bayat *et al*., 2008), dicarboxymethyl chitosan (DCMC) (Muzzarelli *et al*., 1998) and chitosan oligomers (TEO) (Sun *et al*., 2007).

As described in section **2.5**, chitosan is obtained by the N-deacetylation of chitin. Chitosan and TMC consists out of glucosamine units (Freepons, 1991) and is known as a polymer. The synthesis of chitosan derivative, TMC include the reductive methylation of chitosan by adding three methyl groups as described in section **2.6.1**. TEC is obtained by adding three ethyl groups to chitosan and is described in section **2.6.2**. DCMC is the derivative of chitosan which contains a negative charge and has two carboxymethyl groups added to its structure. Chitosan oligomers are the same as

chitosan except with shorter chains of only 2-20 repeating units which makes this polymer water soluble. Thus TEO is included in this study because of its water solubility (Yamada *et al*., 2005).

The various derivatives have not only increased water solubility, but also several other functional properties, making them suitable excipients in numerous dosage forms and for countless applications. They can be classified as multifunctional polymers, where a substance has two or more functions in a formulation (Rios, 2006). Recent studies indicate that chitosan and its derivatives have antibacterial effects against both Gram-negative and Gram- positive bacteria (Jayakumar *et al*., 2010b, Sadeghi *et al.*, 2008) and also antioxidant activity (Alexandrova *et al*., 1999).

2.6.1. TMC

The synthesis of TMC involves the reductive methylation of chitosan of the amino groups using a reaction with methyl iodide on the C-2 position of chitosan (Domard *et al*., 1986), whereas TMC with different DQ can be acquired by repeating the step (Hamman *et al*., 2002). The synthesis of TMC can be seen in **Figure 2.3**.

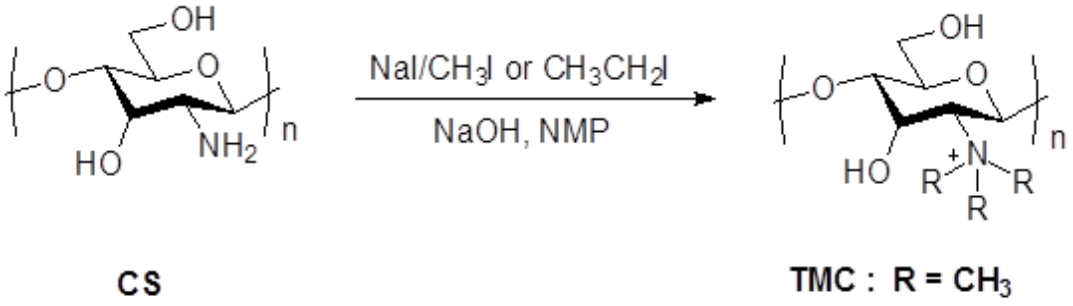


Figure 2.3: Schematic illustration of the synthesis of chitosan derivative, Trimethyl chitosan (TMC). Adapted from (Chen *et al*., 2013)

The DQ is the amount of positive charges available for interactions on a molecule and is an indication of the charge density of the polymer (Hamman *et al*., 2003, Thanou *et*

al., 2000). The degree of deacetylation (DD) involves the alkaline N- deacetylation of chitin to chitosan (**Figure 2.4**). The compound becomes more water soluble because of the free amino (-NH_2) groups that is produced during protonation (Dung *et al.*, 1994, Sieval *et al*., 1998). This influences the solubility and physical properties of chitosan. Also a higher DQ accounts for better mucoadhesivity of TMC due to an increased positive charge to interact with the negatively charged cell membrane and cause the opening of tight junctions (Hamman *et al*., 2003, Kotze *et al*., 1999a).

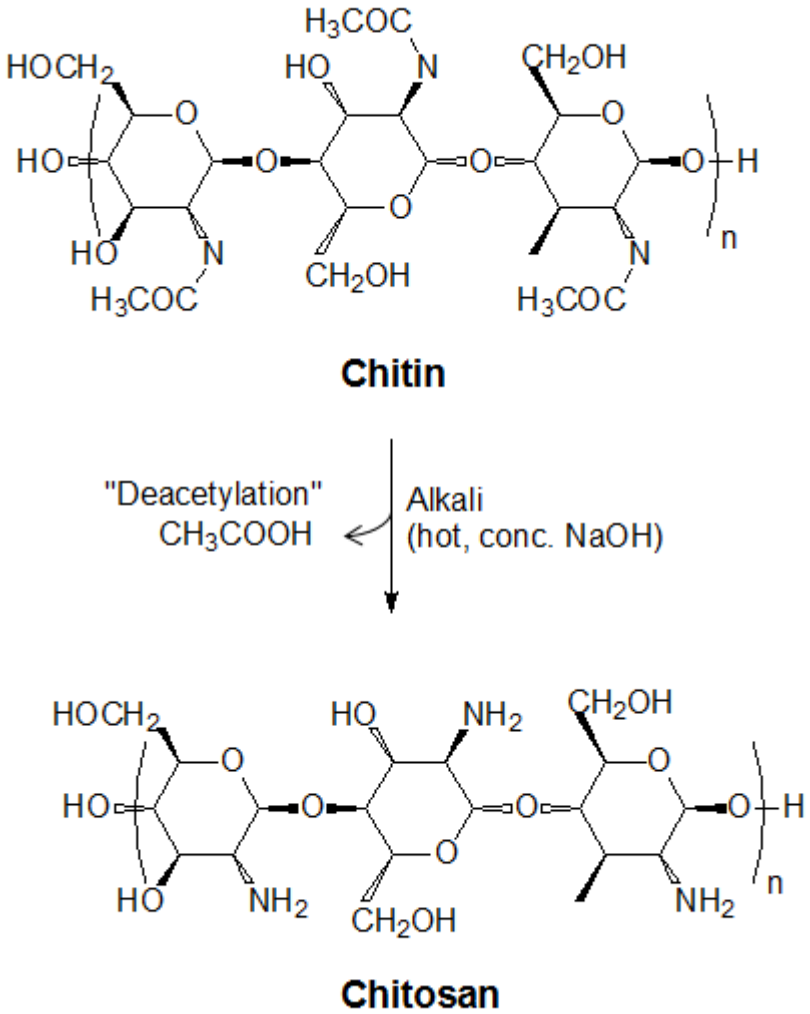


Figure 2.4: Schematic illustration of the deacetylation of chitin to produce chitosan. Adapted from (Raafat and Sahl, 2009)

TMC has absorption enhancing effects by opening the tight junctions of epithelial cells through the paracellular pathway and has the best absorption enhancing effects at high DQ (Kotze *et al*., 1999b, van der Merwe *et al*., 2004b). A high DQ, which is the number of positive charges available for interactions to take place on a molecule, will result in improved absorption enhancing properties. This can be explained by the positive charges of TMC with high DQ where it interacts with the cell membrane which has a negative charge (Thanou *et al*., 2000). Like chitosan, TMC binds to cell membranes to cause paracellular permeability. TMC has several advantages over chitosan which include the use in neutral and basic environments where chitosan is ineffective. Thus it can be said that TMC is soluble over a wide range of pH. This can aid with the delivery of hydrophilic drugs such as protein and peptide drugs (van der Merwe *et al*., 2004b).

TMC has various applications in the pharmaceutical field (Mourya and Inamdar, 2009) where the cationic nature of this polymer has led to investigating gene and vaccine delivery through oral, buccal, nasal and colonic routes (Amidi *et al*., 2006, van der Merwe *et al*., 2004a). Another advantageous application of TMC is that it induces humoral immunity which is important to prevent infectious diseases (Keijzer *et al*., 2011) and has antimicrobial activity against *S. aureus* and *E. coli* (Geng *et al*., 2013). TMC also has antioxidant activity at low concentrations where it exhibit radical scavenging activity (Ozhan *et al*., 2012). According to Amidi *et al.* (2006), TMC showed less toxicity when used in the form of nanoparticles (Amidi *et al*., 2006). Although the toxicity of TMC has been determined when used in nanoparticle form, the *in vivo* and *in vitro* toxicity thereof has not been established and needs further investigating.

2.6.2. TEC

TEC has three ethyl groups (**Figure 2.5**) to replace the protons on the C-2 position on chitosan and is involved in making TEC more water soluble. It enhances absorption through tight junctions and therefore acts as an absorption enhancer to enhance the absorption of hydrophilic compounds in the epithelial of the colon (Mukhopadhyay *et al*., 2012). The incorporation of TEC in formulations can be useful to increase the

product's mucoadhesive properties for absorption at a specific place e.g. the gastrointestinal tract (Atyabi *et al*., 2007).

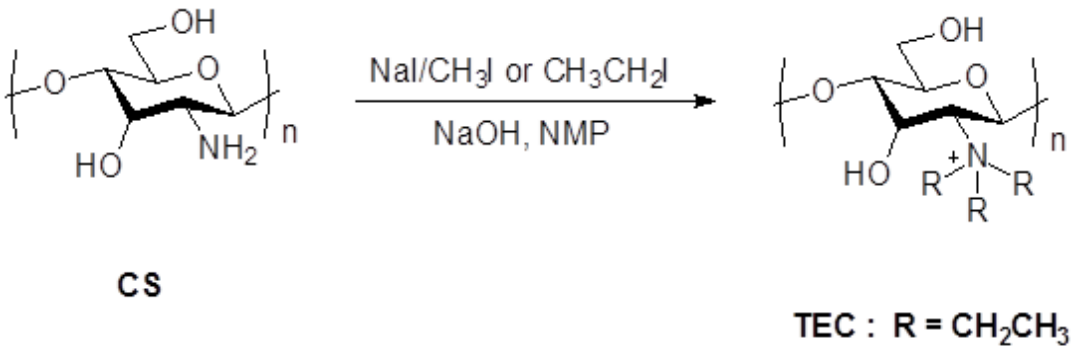
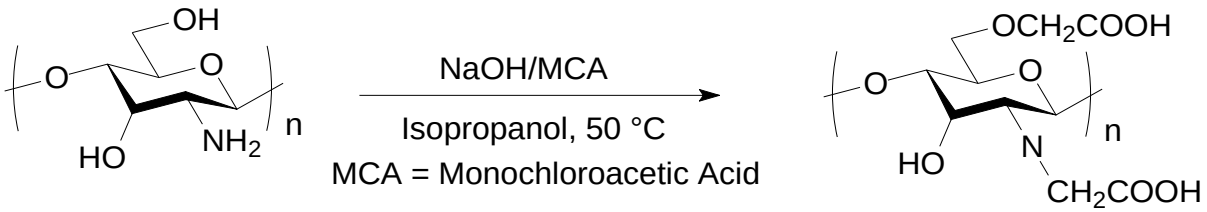


Figure 2.5: Schematic illustration of the synthesis of chitosan derivative, Triethyl chitosan (TEC). Adapted from (Chen *et al*., 2013)

2.6.3. DCMC

DCMC has two dicarboxymethyl groups in its structure (**Figure 2.6**) which contributes to its use in the industry. DCMC is used in tissue engineering where it is applied to treat bone lesions and improve bone mineralisation as well as osteogenesis (Jayakumar *et al*., 2010b). Muzzarelli *et al.,* (1998) confirmed that DCMC is involved with the reconstruction of bone tissue by chelating calcium and magnesium. They found that bone defects in sheep can be healed by DCMC (Muzzarelli *et al*., 1998). Chitosan and its derivative (DCMC) are generally used as enzyme inhibitors. Enzyme inhibitors decrease the proteolytic enzymes in the gastrointestinal tract to improve the bioavailability of a drug (Shah *et al*., 2004). This property of being an enzyme inhibitor is especially advantageous in the use of hypertension medication where it can act as an angiotensin- converting enzyme inhibitor. Higher ACE inhibition is acquired at high degrees of deacetylation (Park *et al*., 2003). Unfortunately, because DCMC is negatively charged it has no antioxidant (Sun *et al*., 2008) and antimicrobial activity (Tantala *et al*., 2012).



CS **N, O-Carboxymethyl CS**

Figure 2.6: Schematic illustration of the synthesis of N, O-Carboxymethyl chitosan. (Chen *et al*., 2013)

2.6.4. TEO/ Chitosan oligomers

Oligomers are molecules which consist of a few monomer units linked to each other, in contrast to a polymer which the amount of monomers is unlimited. Triethyl chitosan oligomer (TEO) are prepared from the degradation of chitosan and shows antioxidant activity as well as antimicrobial activity (No *et al*., 2002, Sun *et al*., 2007). These effects can be attributed to the DQ and MW. Low MW and high DQ increase the scavenging effect and therefore the antioxidant activity. A low MW will increase the antimicrobial activity against Gram- negative bacteria and a high MW will increase the antimicrobial activity of Gram- positive bacteria. Also, high DQ at neutral conditions will increase the antimicrobial activity.

2.7. Melittin

Melittin, as a principal component of honey bee (*Apis mellifera*) venom (apitoxin), is a protein which consists of 26 amino acid residues in a single chain (Bazzo *et al*., 1988, Kreil, 1973, Terwilliger and Eisenberg, 1982). The amino acid sequence of melittin is shown in **Figure 2.7**.

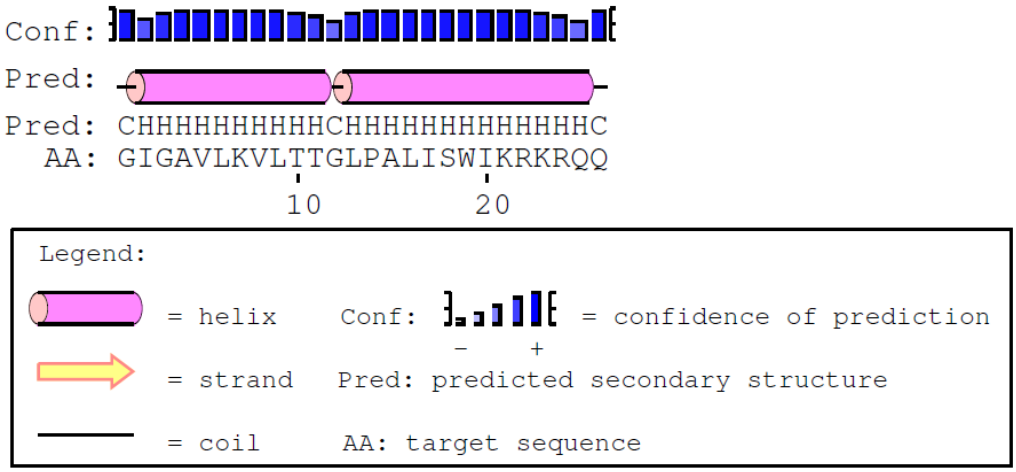


Figure 2.7: Predicted amino acid secondary structure of melittin. Secondary protein structures predicted by the PSIPRED online protein sequence analysis workbench (http://bioinf.cs.ucl.ac.uk/psipred/) (Habermann and Jentsch, 1967)

Honey bee venom is composed out of a mixture of peptides, enzymes and amines, of which melittin is the main lethal component (Ferreira *et al*., 2010). The purpose of bee venom is to defend the colony against other insects and organisms by inducing pain and inflammation and even death (Van Vaerenbergh *et al*., 2014). Melittin is released through biosynthesis from promelittin to produce the active form melittin, which has haemolytic activity (Sciani *et al*., 2010). Melittin is also an example of an antimicrobial peptide (AMP) and has antibacterial, antiviral, as well as anti- inflammatory action in cells. Melittin has also been shown to induce apoptosis in tumour cells and can inhibit cell growth (Zhang *et al*., 2014). It has potent antimicrobial activity, especially against Gram-positive bacteria (Al-Ani *et al*., 2015, Alia *et al*., 2013, Falco *et al*., 2013). The mechanism by which melittin acts as an AMP, is through the permeabilisation of cell membranes where melittin causes pore formation as well as the disruption of phospholipid bilayers which then causes lysis of cells where it also disrupts the plasma membrane in bacterial cells (Adade *et al*., 2013, Bazzo *et al*., 1988, Terwilliger and Eisenberg, 1982). It is also an effective absorption enhancer depending on the concentration of melittin used (Liu *et al*., 1999). Studies have shown that melittin acts as an absorption enhancer at concentrations below 2.42 µM, whereas it exhibits cytotoxic effects at higher concentrations (Liu *et al*., 1999, Maher and McClean, 2008). Melittin has, however, been proven to be cytotoxic; especially to cancer cells (Gajski

and Garaj-Vrhovac, 2013). In a study done by Enslin *et al.,* (2008), different types of absorption enhancers were used in combination to determine if the absorption enhancing effect will be potentiated and if the absorption enhancement effect will increase at low concentrations when absorption enhancers are used in combination. Previous research has indicated that TMC (with DQ of 48 and 64%), dicarboxymethyl chitosan oligosaccharide (DCMCO) and chitosan lactate oligomer at concentrations of 0.25 and 0.5% w/v together with monocaprin and melittin at concentrations of 1.3 and 2 mM and 1 and 1.5 µM respectively, have been successful absorption enhancers. Monocarpin (a monoglyceride of a fatty acid) and melittin resulted that absorption enhancers in low concentrations causes higher drug absorption compared with individual absorption enhancers (Enslin *et al*., 2008).

2.8. Antioxidant activity

Free radicals are molecules which consists out of an unpaired electron which is highly unstable and reactive (Cheeseman and Slater, 1993). Free radicals are produced in cells during normal aerobic metabolism, producing reactive oxygen species (ROS) (Choi *et al*., 2002, Lobo *et al*., 2010). This can lead to cellular damage and cause diseases such as cancer (Kinnula and Crapo, 2004), neurological diseases (Alzheimer's) (Sas *et al*., 2007), cardiovascular diseases (Singh and Jialal, 2006), inflammatory diseases (arthritis, vasculitis) (Sreejayan and Rao, 1996) and the ageing process (Harman, 1956, Rahman, 2007). A mechanism to prevent damage to cells and these diseases, antioxidants are used. Halliwell (2007) defined an antioxidant as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell, 2007).

It has been reported that chitosan and some of its derivatives has antioxidant properties (Alexandrova *et al*., 1999) due to the hydroxyl and amino groups (Huang *et al*., 2005). Yin *et al.,* (2002) stated that by decreasing the MW of chitosan and its derivatives, the antioxidant activity and scavenging activity increases (Yin *et al*., 2002). The ability of chitosan to have antioxidant activity is advantageous in the use as food additives to delay lipid oxidation in meat and seafood products (Darmadji and Izumimoto, 1994, Kamil *et al*., 2002)

2.9. Antimicrobial activity

Antimicrobial compounds are important as alternative treatment to antibiotics due to antibiotic resistance (Swartz, 2000). Antimicrobial resistance is defined by the World Health Organization (WHO) as a microorganism which is resistant to an antimicrobial drug where the treatment was once effective and the microorganism can tolerate the treatment of the drug against it (WHO, 2015). There are increasing resistance against antibiotics for both Gram- positive and Gram- negative bacteria and also in bacteria which were once susceptible (Cornaglia, 2009). This can lead to global deaths of individuals because of the failing of medical treatment (WHO, 2015). Thus the need exist to discover and develop new compounds to use against bacterial infections (Clark, 1996).

Chitosan has been shown to have antimicrobial activity against both Gram- positive (e.g. *S. aureus*) and Gram- negative (e.g. *E. coli, P. aeruginosa*) bacteria and also antifungal activity (Chen *et al*., 1998, Rhoades and Roller, 2000). It has been reported that the antimicrobial activity of chitosan depends on the MW (Jeon *et al*., 2001). The mechanism by which chitosan exerts antimicrobial activity is unsure but it is suggested that the polycationic structure of chitosan interacts with the anionic components (proteins) on the surface of Gram- negative bacteria (Nikaido, 1996). For Gram-positive bacteria, chitosan causes lysis of cells and thereafter death by interfering with the cell membrane charges on the surface of the cell (Ye *et al*., 2004). The antimicrobial activity is advantageous as an alternative for antibiotics to overcome antimicrobial resistance.

2.10. Cytotoxicity

In vitro cytotoxicity is the first step in risk assessment toxicology and biocompatibility studies. Cytotoxicity, also known as cell viability, is the establishing of dead cells after treatment. Cytotoxicity can be caused by various aspects which include apoptosis and necrosis. Apoptosis is known as programmed cell death and occurs during the maintenance of tissue homeostasis to sustain cell populations as well as embryonic development (Kerr *et al*., 1972). Necrosis is the accidental and/or abnormal cell death due to environmental disturbances (Fink and Cookson, 2005) or cell injury (Vermes *et*

al., 2000). In toxicology it is important to characterise the mode of cell death, as it can lead to different effects *in vivo*.

2.11. Conclusion

Although the oral route of drug administration is the most advantageous, its efficiency is limited in drugs with poor water solubility and peptide drugs. Excipients are included in dosage forms to overcome these limitations. Multifunctional polymers are advantageous to use because of their additional functions such as antioxidant- and antimicrobial activity. Chitosan, a natural polymer, is believed to enhance absorption of drugs and is non- toxic. Due to its solubility issues derivatives were synthesised to overcome this problem and also to discover other functional properties. Melittin is a known absorption enhancer and exhibits antimicrobial activity. The possible use of combining two absorption enhancers, for example chitosan derivatives and melittin, can significantly increase the absorption of drugs in the small intestine.

However, there is a lack of research on the cytotoxicity and the need exists to characterise the derivatives and melittin before they can be used in dosage forms.

2.12. References

ADADE, C. M., OLIVEIRA, I. R., PAIS, J. A. & SOUTO-PADRON, T. 2013. Melittin peptide kills Trypanosoma cruzi parasites by inducing different cell death pathways. *Toxicon,* 69**,** 227-39.

AL-ANI, I., ZIMMERMANN, S., REICHLING, J. & WINK, M. 2015. Pharmacological synergism of bee venom and melittin with antibiotics and plant secondary metabolites against multi-drug resistant microbial pathogens. *Phytomedicine,* 22**,** 245-255.

AL-QADI, S., GRENHA, A. & REMUÑÁN-LÓPEZ, C. 2012. Chitosan-Based Systems for Mucosal Delivery of Biopharmaceuticals. *Chitosan-Based Systems for Biopharmaceuticals.* John Wiley & Sons, Ltd.

ALEEVA, G. N., ZHURAVLEVA, M. V. & KHAFIZ'YANOVA, R. K. 2009. The role of excipients in determining the pharmaceutical and therapeutic properties of medicinal agents (Review). *Pharmaceutical Chemistry Journal,* 43**,** 230-234.

ALEXANDROVA, V. A., OBUKHOVA, G. V., DOMNINA, N. S. & TOPCHIEV, D. A. 1999. Modification of chitosan for construction of efficient antioxidant biodegradable macromolecular systems. *Macromolecular Symposia,* 144**,** 413-422.

ALIA, O., LAILA, M. & AL-DAOUDEANTONIUS 2013. Antimicrobial Effect of Melittin Isolated from Syrian Honeybee (*Apismellifera)* Venom and its Wound Healing Potential. *International Journal of Pharmaceutical Sciences Review and Research,* 21**,** 318-324.

AMIDI, M., ROMEIJN, S. G., BORCHARD, G., JUNGINGER, H. E., HENNINK, W. E. & JISKOOT, W. 2006. Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. *Journal of Control Release,* 111**,** 107-16.

ANTUNES, F., ANDRADE, F. & SARMENTO, B. 2012. Chitosan-Based Nanoparticulates for Oral Delivery of Biopharmaceuticals. *Chitosan-Based Systems for Biopharmaceuticals.* John Wiley & Sons, Ltd.

ARCHANA, D., DUTTA, J. & DUTTA, P. K. 2013. Evaluation of chitosan nano dressing for wound healing: Characterization, in vitro and in vivo studies. *International Journal of Biological Macromolecules,* 57**,** 193-203.

ATYABI, F., MAJZOOB, S., DORKOOSH, F., SAYYAH, M. & PONCHEL, G. 2007. The impact of trimethyl chitosan on in vitro mucoadhesive properties of pectinate beads along different sections of gastrointestinal tract. *Drug Development and Industrial Pharmacy,* 33**,** 291-300.

AUNGST, B. J. 1993. Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism. *Journal of Pharmaceutical Sciences,* 82**,** 979-87.

AUNGST, B. J. 2012. Absorption enhancers: applications and advances. *Journal of the American Association of Pharmaceutical Scientists,* 14**,** 10-8.

BALDRICK, P. 2010. The safety of chitosan as a pharmaceutical excipient. *Regulatory Toxicology and Pharmacology,* 56**,** 290-299.

BAUTISTA-BAÑOS, S., HERNÁNDEZ-LAUZARDO, A. N., VELÁZQUEZ-DEL VALLE, M. G., HERNÁNDEZ-LÓPEZ, M., AIT BARKA, E., BOSQUEZ-MOLINA, E. & WILSON, C. L. 2006. Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. *Crop Protection,* 25**,** 108-118.

BAYAT, A., DORKOOSH, F. A., DEHPOUR, A. R., MOEZI, L., LARIJANI, B., JUNGINGER, H. E. & RAFIEE-TEHRANI, M. 2008. Nanoparticles of quaternized chitosan derivatives as a carrier for colon delivery of insulin: *Ex vivo* and *in vivo* studies. *International Journal of Pharmaceutics,* 356**,** 259-266.

BAZZO, R., TAPPIN, M. J., PASTORE, A., HARVEY, T. S., CARVER, J. A. & CAMPBELL, I. D. 1988. The structure of melittin. *European Journal of Biochemistry,* 173**,** 139-146.

BENET, L. Z., KROETZ, D., SHEINER, L., HARDMAN, J. & LIMBIRD, L. 1996. Pharmacokinetics: the dynamics of drug absorption, distribution, metabolism, and elimination. *Goodman and Gilman's the Pharmacological Basis of Therapeutics***,** 3-27.

BORCHARD, G. & JUNGINGER, H. E. 2001. Modern drug delivery applications of chitosan. *Advanced Drug Delivery Reviews,* 52**,** 103.

BORCHARD, G., LUEBEN, H. L., DE BOER, A. G., VERHOEF, J. C., LEHR, C.-M. & JUNGINGER, H. E. 1996. The potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption. III: Effects of chitosan-glutamate and carbomer on epithelial tight junctions *in vitro*. *Journal of Controlled Release,* 39**,** 131-138.

BRÜCK, W. M., SLATER, J. W. & CARNEY, B. F. 2010. 2 Chitin and Chitosan from Marine Organisms. *Chitin, chitosan, oligosaccharides and their derivatives: Biological activities and applications***,** 11.

CARREÑO-GÓMEZ, B. & DUNCAN, R. 1997. Evaluation of the biological properties of soluble chitosan and chitosan microspheres. *International Journal of Pharmaceutics,* 148**,** 231-240.

CHEAH, L. H., PAGE, B. B. C. & SHEPHERD, R. 1997. Chitosan coating for inhibition of sclerotinia rot of carrots. *New Zealand Journal of Crop and Horticultural Science,* 25**,** 89-92.

CHEESEMAN, K. H. & SLATER, T. F. 1993. An introduction to free radical biochemistry. *British Medical Bulletin,* 49**,** 481-93.

CHEN, C. S., LIAU, W. Y. & TSAI, G. J. 1998. Antibacterial effects of N-sulfonated and N-sulfobenzoyl chitosan and application to oyster preservation. *Journal of Food Protection,* 61**,** 1124-8.

CHEN, M. C., MI, F. L., LIAO, Z. X., HSIAO, C. W., SONAJE, K., CHUNG, M. F., HSU, L. W. & SUNG, H. W. 2013. Recent advances in chitosan-based nanoparticles for oral delivery of macromolecules. *Advanced Drug Delivery Reviews,* 65**,** 865-79.

CHOI, C. W., KIM, S. C., HWANG, S. S., CHOI, B. K., AHN, H. J., LEE, M. Y., PARK, S. H. & KIM, S. K. 2002. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science,* 163**,** 1161-1168.

CLARK, A. M. 1996. Natural products as a resource for new drugs. *Pharmaceutical Research,* 13**,** 1133-44.

CORNAGLIA, G. 2009. Fighting infections due to multidrug-resistant Gram-positive pathogens. *Clinical Microbiology and Infection,* 15**,** 209-11.

DAHAN, A., MILLER, J. & AMIDON, G. 2009. Prediction of Solubility and Permeability Class Membership: Provisional BCS Classification of the World's Top Oral Drugs. *Journal of the American Association of Pharmaceutical Scientists,* 11**,** 740-746.

DARMADJI, P. & IZUMIMOTO, M. 1994. Effect of chitosan in meat preservation. *Meat Science,* 38**,** 243-254.

DASH, M., CHIELLINI, F., OTTENBRITE, R. M. & CHIELLINI, E. 2011. Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science,* 36**,** 981-1014.

DE BRITTO, D., CELI GOY, R., CAMPANA FILHO, S. P. & ASSIS, O. B. 2011. Quaternary salts of chitosan: history, antimicrobial features, and prospects. *International Journal of Carbohydrate Chemistry,* 2011.

DESESSO, J. M. & JACOBSON, C. F. 2001. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology,* 39**,** 209-228.

DODANE, V. & VILIVALAM, V. D. 1998. Pharmaceutical applications of chitosan. *Pharmaceutical Science and Technology Today,* 1**,** 246-253.

DOMARD, A., RINAUDO, M. & TERRASSIN, C. 1986. New method for the quaternization of chitosan. *International Journal of Biological Macromolecules,* 8**,** 105-107.

DUNG, P. L., MILAS, M., RINAUDO, M. & DESBRIÈRES, J. 1994. Water soluble derivatives obtained by controlled chemical modifications of chitosan. *Carbohydrate Polymers,* 24**,** 209-214.

EL GHAOUTH, A., PONNAMPALAM, R., CASTAIGNE, F. & ARUL, J. 1992. Chitosan coating to extend the storage life of tomatoes. *HortScience,* 27**,** 1016-1018.

ENSLIN, G. M., HAMMAN, J. H. & KOTZE, A. F. 2008. Intestinal drug absorption enhancers: synergistic effects of combinations. *Drug Development and Industrial Pharmacy,* 34**,** 1343-9.

ENSLIN, G. M. A. 2005. *In Vitro Evaluation of the Absorption Enhancement Properties of Chitosans, Monocaprin and Melittin*, Tshwane University of Technology.

FALCO, A., BARRAJÓN-CATALÁN, E., MENÉNDEZ-GUTIÉRREZ, M. P., COLL, J., MICOL, V. & ESTEPA, A. 2013. Melittin-loaded immunoliposomes against viral surface proteins, a new approach to antiviral therapy. *Antiviral Research,* 97**,** 218-221.

FERREIRA JUNIOR, R. S., SCIANI, J. M., MARQUES-PORTO, R., JUNIOR, A. L., ORSI, R. D. O., BARRAVIERA, B. & PIMENTA, D. C. 2010. Africanized honey bee (Apis mellifera) venom profiling: Seasonal variation of melittin and phospholipase A2 levels. *Toxicon,* 56**,** 355-362.

FINK, S. L. & COOKSON, B. T. 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infection and Immunity,* 73**,** 1907-1916.

FISCHER, D., LI, Y., AHLEMEYER, B., KRIEGLSTEIN, J. & KISSEL, T. 2003. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials,* 24**,** 1121-1131.

FIX, J. A. 1996. Strategies for delivery of peptides utilizing absorption-enhancing agents. *Journal of Pharmaceutical Sciences,* 85**,** 1282-5.

FONSECA, S. B., PEREIRA, M. P. & KELLEY, S. O. 2009. Recent advances in the use of cell-penetrating peptides for medical and biological applications. *Advanced Drug Delivery Reviews,* 61**,** 953-964.

FREEPONS, D. Chitosan, does it have a place in agriculture? Proceedings of the Plant Growth Regulator Society of America (USA), 1991.

GAJSKI, G. & GARAJ-VRHOVAC, V. 2013. Melittin: a lytic peptide with anticancer properties. *Environmental Toxicology and Pharmacology,* 36**,** 697-705.

GENG, X., YANG, R., HUANG, J., ZHANG, X. & WANG, X. 2013. Evaluation antibacterial activity of quaternary-based chitin/chitosan derivatives in vitro. *Journal of Food Science,* 78**,** M90-7.

GONZALEZ-MARISCAL, L., HERNANDEZ, S. & VEGA, J. 2008. Inventions designed to enhance drug delivery across epithelial and endothelial cells through the paracellular pathway. *Recent Patents on Drug Delivery and Formulation,* 2**,** 45-76.

GUO, Z., LIU, H., CHEN, X., JI, X. & LI, P. 2006. Hydroxyl radicals scavenging activity of N-substituted chitosan and quaternized chitosan. *Bioorganic and Medicinal Chemistry Letters,* 16**,** 6348-6350.

HABERMANN, E. & JENTSCH, J. 1967. [Sequence analysis of melittin from tryptic and peptic degradation products]. *Hoppe Seylers Z Physiol Chem,* 348**,** 37-50.

HALLIWELL, B. 2007. Biochemistry of oxidative stress. *Biochemical Society Transactions,* 35**,** 1147-50.

HAMMAN, J. & STEENEKAMP, J. 2012. Excipients with specialized functions for effective drug delivery. *Expert Opinion on Drug Delivery,* 9**,** 219-30.

HAMMAN, J. H., ENSLIN, G. M. & KOTZE, A. F. 2005. Oral delivery of peptide drugs: barriers and developments. *BioDrugs,* 19**,** 165-77.

HAMMAN, J. H., SCHULTZ, C. M. & KOTZE, A. F. 2003. N-trimethyl chitosan chloride: optimum degree of quaternization for drug absorption enhancement across epithelial cells. *Drug Development and Industrial Pharmacy,* 29**,** 161-72.

HAMMAN, J. H., STANDER, M. & KOTZÉ, A. F. 2002. Effect of the degree of quaternisation of N-trimethyl chitosan chloride on absorption enhancement: in vivo evaluation in rat nasal epithelia. *International Journal of Pharmaceutics,* 232**,** 235-242.

HARMAN, D. 1956. Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology,* 11**,** 298-300.

HINCHCLIFFE, M. & ILLUM, L. 1999. Intranasal insulin delivery and therapy. *Advanced drug delivery reviews,* 35**,** 199-234.

HUANG, R., MENDIS, E. & KIM, S. K. 2005. Factors affecting the free radical scavenging behavior of chitosan sulfate. *International Journal of Biological Macromolecules,* 36**,** 120-127.

ILIUM, L. 1998. Chitosan and Its Use as a Pharmaceutical Excipient. *Pharmaceutical Research,* 15**,** 1326-1331.

JARMILA, V. & VAVRIKOVA, E. 2011. Chitosan derivatives with antimicrobial, antitumour and antioxidant activities--a review. *Current Pharmaceutical Design,* 17**,** 3596-607.

JAYAKUMAR, R., PRABAHARAN, M., NAIR, S. V. & TAMURA, H. 2010a. Novel chitin and chitosan nanofibers in biomedical applications. *Biotechnology Advances,* 28**,** 142-150.

JAYAKUMAR, R., PRABAHARAN, M., NAIR, S. V., TOKURA, S., TAMURA, H. & SELVAMURUGAN, N. 2010b. Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications. *Progress in Materials Science,* 55**,** 675-709.

JEON, Y.-J., PARK, P.-J. & KIM, S.-K. 2001. Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydrate Polymers,* 44**,** 71-76.

JUNGINGER, H. E. & VERHOEF, J. C. 1998. Macromolecules as safe penetration enhancers for hydrophilic drugs—a fiction? *Pharmaceutical Science and Technology Today,* 1**,** 370-376.

KALEPU, S., MANTHINA, M. & PADAVALA, V. 2013. Oral lipid-based drug delivery systems – an overview. *Acta Pharmaceutica Sinica B,* 3**,** 361-372.

KAMIL, J. Y., JEON, Y.-J. & SHAHIDI, F. 2002. Antioxidative activity of chitosans of different viscosity in cooked comminuted flesh of herring (*Clupea harengus*). *Food Chemistry,* 79**,** 69-77.

KEIJZER, C., SLUTTER, B., VAN DER ZEE, R., JISKOOT, W., VAN EDEN, W. & BROERE, F. 2011. PLGA, PLGA-TMC and TMC-TPP nanoparticles differentially modulate the outcome of nasal vaccination by inducing tolerance or enhancing humoral immunity. *PLoS One,* 6**,** e26684.

KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer,* 26**,** 239-57.

KHOUSHAB, F. & YAMABHAI, M. 2010. Chitin Research Revisited. *Marine Drugs,* 8**,** 1988-2012.

KINNULA, V. L. & CRAPO, J. D. 2004. Superoxide dismutases in malignant cells and human tumors. *Free Radical Biology and Medicine,* 36**,** 718-44.

KOTZÉ, A. F., LUEßEN, H. L., DE LEEUW, B. J., DE BOER, B. G., COOS VERHOEF, J. & JUNGINGER, H. E. 1998. Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). *Journal of Controlled Release,* 51**,** 35-46.

KOTZE, A. F., THANOU, M. M., LUEBETAEN, H. L., DE BOER, A. G., VERHOEF, J. C. & JUNGINGER, H. E. 1999a. Enhancement of paracellular drug transport with highly quaternized N-trimethyl chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2). *Journal of Pharmaceutical Sciences,* 88**,** 253-7.

KOTZE, A. F., THANOU, M. M., LUESSEN, H. L., DE BOER, B. G., VERHOEF, J. C. & JUNGINGER, H. E. 1999b. Effect of the degree of quaternization of N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). *European Journal of Pharmaceutics and Biopharmaceutics,* 47**,** 269-74.

KREIL, G. 1973. Structure of melittin isolated from two species of honey bees. *Federation of European Biochemical Societies Letters,* 33**,** 241-244.

LARSSON, M., HUANG, W.-C., HSIAO, M.-H., WANG, Y.-J., NYDÉN, M., CHIOU, S.-H. & LIU, D.-M. 2013. Biomedical applications and colloidal properties of amphiphilically modified chitosan hybrids. *Progress in Polymer Science,* 38**,** 1307-1328.

LEHR, C. M., BODDÉ, H. E., BOUWSTRA, J. A. & JUNGINGER, H. E. 1993. A surface energy analysis of mucoadhesion II. Prediction of mucoadhesive performance by spreading coefficients. *European Journal of Pharmaceutical Sciences,* 1**,** 19-30.

LIN, Y., FUJIMORI, T., KAWAGUCHI, N., TSUJIMOTO, Y., NISHIMI, M., DONG, Z., KATSUMI, H., SAKANE, T. & YAMAMOTO, A. 2011. Polyamidoamine dendrimers as novel potential absorption enhancers for improving the small intestinal absorption of poorly absorbable drugs in rats. *Journal of Controlled Release,* 149**,** 21-28.

LIU, H., TAYLOR, L. S. & EDGAR, K. J. 2015. The role of polymers in oral bioavailability enhancement; a review. *Polymer*.

LIU, P., DAVIS, P., LIU, H. & KRISHNAN, T. R. 1999. Evaluation of cytotoxicity and absorption enhancing effects of melittin – a novel absorption enhancer. *European Journal of Pharmaceutics and Biopharmaceutics,* 48**,** 85-87.

LOBO, V., PATIL, A., PHATAK, A. & CHANDRA, N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews,* 4**,** 118-26.

LUEBEN, H. L., BOHNER, V., PÉRARD, D., LANGGUTH, P., VERHOEF, J. C., DE BOER, A. G., MERKLE, H. P. & JUNGINGER, H. E. 1996. Mucoadhesive polymers in peroral peptide drug delivery. V. Effect of poly(acrylates) on the enzymatic degradation of peptide drugs by intestinal brush border membrane vesicles. *International Journal of Pharmaceutics,* 141**,** 39-52.

MAGOS, L. 1991. C. G. Wilson and N. Washington. Physiological pharmacokinetics: Biological barriers to drug absorption. Ellis Horwood. *Journal of Applied Toxicology,* 11**,** 155-156.

MAHER, S., FEIGHERY, L., BRAYDEN, D. J. & MCCLEAN, S. 2007. Melittin as a permeability enhancer II: in vitro investigations in human mucus secreting intestinal monolayers and rat colonic mucosae. *Pharmaceutical Research,* 24**,** 1346-56.

MAHER, S. & MCCLEAN, S. 2008. Melittin exhibits necrotic cytotoxicity in gastrointestinal cells which is attenuated by cholesterol. *Biochemical Pharmacology,* 75**,** 1104-1114.

MANSOUR, H. M., SOHN, M., AL-GHANANEEM, A. & DELUCA, P. P. 2010. Materials for Pharmaceutical Dosage Forms: Molecular Pharmaceutics and Controlled Release Drug Delivery Aspects. *International Journal of Molecular Sciences,* 11**,** 3298-3322.

MATI-BAOUCHE, N., ELCHINGER, P.-H., DE BAYNAST, H., PIERRE, G., DELATTRE, C. & MICHAUD, P. 2014. Chitosan as an adhesive. *European Polymer Journal,* 60**,** 198-212.

MOURYA, V. K. & INAMDAR, N. N. 2009. Trimethyl chitosan and its applications in drug delivery. *Journal of Materials and Science: Materials and Medicine,* 20**,** 1057-79.

MUKHOPADHYAY, P., MISHRA, R., RANA, D. & KUNDU, P. P. 2012. Strategies for effective oral insulin delivery with modified chitosan nanoparticles: A review. *Progress in Polymer Science,* 37**,** 1457-1475.

MURAKAMI, T., YATA, N., TAMAUCHI, H. & KAMADA, A. 1982. Studies of Absorption Promoters for Rectal Delivery Preparations. II. A Possible Mechanism of Promoting Efficacy of Enamine Derivatives in Rectal Absorption. *Chemical and Pharmaceutical Bulletin,* 30**,** 659-665.

MUZZARELLI, R. A. A., RAMOS, V., STANIC, V., BRUNO, D., MATTIOLI-BELMONTE, M., TOSI, G. & GIARDINO, R. 1998. Osteogenesis promoted by calcium phosphate N,N-dicarboxymethyl chitosan. *Carbohydrate Polymers,* 36**,** 267-276.

NA, L., WANG, J., WANG, L. & MAO, S. 2013. A novel permeation enhancer: N-succinyl chitosan on the intranasal absorption of isosorbide dinitrate in rats. *European Journal of Pharmaceutical Sciences,* 48**,** 301-306.

NAKAMURA, K., MORISHITA, M., EHARA, J., ONUKI, Y., YAMAGATA, T., KAMEI, N., LOWMAN, A. M., PEPPAS, N. A. & TAKAYMA, K. 2008. Key functions in polymer carriers for intestinal absorption of insulin. *International Journal of Pharmaceutics,* 354**,** 135-142.

NIKAIDO, H. 1996. Outer membrane. *Escherichia coli and Salmonella: Cellular and molecular biology* 1**,** 29-47.

NO, H. K., YOUNG PARK, N., HO LEE, S. & MEYERS, S. P. 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology,* 74**,** 65-72.

OWENS, D. R., ZINMAN, B. & BOLLI, G. 2003. Alternative routes of insulin delivery. *Diabetic Medicine,* 20**,** 886-98.

OZHAN, A., MORIMURA, S. & KIDA, K. 2012. Physiological activities of chitosan and N-trimethyl chitosan chloride in U937 and 3T3-L1 cells. *Polymers for Advanced Technologies,* 23**,** 228-235.

PARK, P. J., JE, J. Y. & KIM, S. K. 2003. Angiotensin I converting enzyme (ACE) inhibitory activity of hetero-chitooligosaccharides prepared from partially different deacetylated chitosans. *Journal of Agricultural and Food Chemistry,* 51**,** 4930-4.

RAAFAT, D. & SAHL, H. G. 2009. Chitosan and its antimicrobial potential–a critical literature survey. *Microbial biotechnology,* 2**,** 186-201.

RABEA, E. I., BADAWY, M. E. T., STEVENS, C. V., SMAGGHE, G. & STEURBAUT, W. 2003. Chitosan as Antimicrobial Agent: Applications and Mode of Action. *Biomacromolecules,* 4**,** 1457-1465.

RAHMAN, K. 2007. Studies on free radicals, antioxidants, and co-factors. *Clinical Interventions in Aging,* 2**,** 219-236.

RENAULT, F., SANCEY, B., BADOT, P. M. & CRINI, G. 2009. Chitosan for coagulation/flocculation processes – An eco-friendly approach. *European Polymer Journal,* 45**,** 1337-1348.

RHOADES, J. & ROLLER, S. 2000. Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Applied and Environmental Microbiology,* 66**,** 80-6.

RINAUDO, M. 2006. Chitin and chitosan: Properties and applications. *Progress in Polymer Science,* 31**,** 603-632.

RIOS, M. 2006. Debating excipient functionality. *Pharmaceutical Technology,* 30**,** 50.

SADEGHI, A. M. M., DORKOOSH, F. A., AVADI, M. R., SAADAT, P., RAFIEE-TEHRANI, M. & JUNGINGER, H. E. 2008. Preparation, characterization and antibacterial activities of chitosan, N-trimethyl chitosan (TMC) and N-diethylmethyl chitosan (DEMC) nanoparticles loaded with insulin using both the ionotropic gelation and polyelectrolyte complexation methods. *International Journal of Pharmaceutics,* 355**,** 299-306.

SAS, K., ROBOTKA, H., TOLDI, J. & VECSEI, L. 2007. Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. *Journal of the Neurological Sciences,* 257**,** 221-39.

SCIANI, J. M., MARQUES-PORTO, R., LOURENÇO JUNIOR, A., ORSI, R. D. O., JUNIOR, R. S. F., BARRAVIERA, B. & PIMENTA, D. C. 2010. Identification of a novel melittin isoform from Africanized Apis mellifera venom. *Peptides,* 31**,** 1473-1479.

SHAH, R. B., PALAMAKULA, A. & KHAN, M. A. 2004. Cytotoxicity evaluation of enzyme inhibitors and absorption enhancers in Caco-2 cells for oral delivery of salmon calcitonin. *Journal of Pharmaceutical Sciences,* 93**,** 1070-1082.

SIEVAL, A. B., THANOU, M., KOTZE´, A. F., VERHOEF, J. C., BRUSSEE, J. & JUNGINGER, H. E. 1998. Preparation and NMR characterization of highly substitutedN-trimethyl chitosan chloride. *Carbohydrate Polymers,* 36**,** 157-165.

SINGH, U. & JIALAL, I. 2006. Oxidative stress and atherosclerosis. *Pathophysiology,* 13**,** 129-42.

SNYMAN, D., HAMMAN, J. H. & KOTZE, A. F. 2003. Evaluation of the mucoadhesive properties of N-trimethyl chitosan chloride. *Drug Development and Industrial Pharmacy,* 29**,** 61-9.

SOARES, P. A. G., BOURBON, A. I., VICENTE, A. A., ANDRADE, C. A. S., BARROS JR, W., CORREIA, M. T. S., PESSOA JR, A. & CARNEIRO-DA-CUNHA, M. G. 2014. Development and characterization of hydrogels based on natural polysaccharides: Policaju and chitosan. *Materials Science and Engineering: C,* 42**,** 219-226.

SPAGNA, G., ANDREANI, F., SALATELLI, E., ROMAGNOLI, D., CASARINI, D. & PIFFERI, P. G. 1998. Immobilization of the glycosidases: α-l-arabinofuranosidase and β-d-glucopyranosidase from Aspergillus niger on a chitosan derivative to increase the aroma of wine. Part II. *Enzyme and Microbial Technology,* 23**,** 413-421.

SPAGNA, G., BARBAGALLO, R. N., CASARINI, D. & PIFFERI, P. G. 2001. A novel chitosan derivative to immobilize α-L-rhamnopyranosidase from Aspergillus niger for application in beverage technologies. *Enzyme and Microbial Technology,* 28**,** 427-438.

SPAGNA, G., PIFFERI, P. G., RANGONI, C., MATTIVI, F., NICOLINI, G. & PALMONARI, R. 1996. The stabilization of white wines by adsorption of phenolic compounds on chitin and chitosan. *Food Research International,* 29**,** 241-248.

SREEJAYAN, N. & RAO, M. N. 1996. Free radical scavenging activity of curcuminoids. *Arzneimittelforschung,* 46**,** 169-71.

SUN, T., YAO, Q., ZHOU, D. & MAO, F. 2008. Antioxidant activity of N-carboxymethyl chitosan oligosaccharides. *Bioorganic and Medicinal Chemistry Letters,* 18**,** 5774-5776.

SUN, T., ZHOU, D., XIE, J. & MAO, F. 2007. Preparation of chitosan oligomers and their antioxidant activity. *European Food Research and Technology,* 225**,** 451-456.

SWARTZ, M. N. 2000. Impact of antimicrobial agents and chemotherapy from 1972 to 1998. *Antimicrobial agents and chemotherapy,* 44**,** 2009-2016.

TANTALA, J., THONGNGAM, M., RACHTANAPUN, P. & RACHTANAPUN, C. 2012. Antimicrobial activity of chitosan and carboxymethyl chitosan from different types and sources of chitosan. *Italian Journal of Food Science,* 24**,** 97.

TENGAMNUAY, P. & MITRA, A. 1990. Bile Salt–Fatty Acid Mixed Micelles as Nasal Absorption Promoters of Peptides. I. Effects of Ionic Strength, Adjuvant Composition, and Lipid Structure on the Nasal Absorption of [D-Arg2]Kyotorphin. *Pharmaceutical Research,* 7**,** 127-133.

TERWILLIGER, T. & EISENBERG, D. 1982. The structure of melittin. II. Interpretation of the structure. *Journal of Biological Chemistry,* 257**,** 6016-6022.

THANOU, M., VERHOEF, J. C. & JUNGINGER, H. E. 2001a. Chitosan and its derivatives as intestinal absorption enhancers. *Advanced Drug Delivery Reviews,* 50, Supplement 1**,** S91-S101.

THANOU, M., VERHOEF, J. C. & JUNGINGER, H. E. 2001b. Oral drug absorption enhancement by chitosan and its derivatives. *Advanced Drug Delivery Reviews,* 52**,** 117-126.

THANOU, M. M., KOTZÉ, A. F., SCHARRINGHAUSEN, T., LUEßEN, H. L., DE BOER, A. G., VERHOEF, J. C. & JUNGINGER, H. E. 2000. Effect of degree of quaternization of N-trimethyl chitosan chloride for enhanced transport of

hydrophilic compounds across intestinal Caco-2 cell monolayers. *Journal of Controlled Release,* 64**,** 15-25.

THIRUNAVUKKARASU, N. & SHANMUGAM, A. 2009. Extraction of chitin and chitosan from mud crab Scylla tranquebarica (Fabricius, 1798). *International Journal on Applied Bioengineering,* 4**,** 31-33.

VAN DER MERWE, S. M., VERHOEF, J. C., KOTZÉ, A. F. & JUNGINGER, H. E. 2004a. N-Trimethyl chitosan chloride as absorption enhancer in oral peptide drug delivery. Development and characterization of minitablet and granule formulations. *European Journal of Pharmaceutics and Biopharmaceutics,* 57**,** 85-91.

VAN DER MERWE, S. M., VERHOEF, J. C., VERHEIJDEN, J. H. M., KOTZÉ, A. F. & JUNGINGER, H. E. 2004b. Trimethylated chitosan as polymeric absorption enhancer for improved peroral delivery of peptide drugs. *European Journal of Pharmaceutics and Biopharmaceutics,* 58**,** 225-235.

VAN HOOGDALEM, E. J., DE BOER, A. G. & BREIMER, D. D. 1989. Intestinal drug absorption enhancement: an overview. *Pharmacology and Therapeutics,* 44**,** 407-443.

VAN VAERENBERGH, M., DEBYSER, G., DEVREESE, B. & DE GRAAF, D. C. 2014. Exploring the hidden honeybee (Apis mellifera) venom proteome by integrating a combinatorial peptide ligand library approach with FTMS. *Journal of Proteomics,* 99**,** 169-178.

VENTER, C. 2005a. *Chitosan and quaternised chitosan polymers as gene transfection agents.* North-West University.

VENTER, J. P. 2005b. *Design and evaluation of chitosan and N-trimethyl chitosan chloride microspheres for intestinal drug delivery.* North-West University.

VERMES, I., HAANEN, C. & REUTELINGSPERGER, C. 2000. Flow cytometry of apoptotic cell death. *Journal of Immunological Methods,* 243**,** 167-190.

WHO. 2015. *Antimicrobial Resistance Fact sheet No194 April 2015* [Online]. Available: http://www.who.int/mediacentre/factsheets/fs194/en/ [Accessed 21 October 2015].

WIARACHAI, O., THONGCHUL, N., KIATKAMJORNWONG, S. & HOVEN, V. P. 2012. Surface-quaternized chitosan particles as an alternative and effective organic antibacterial material. *Colloids and Surfaces B: Biointerfaces,* 92**,** 121-129.

YAMADA, K., ODOMI, M., OKADA, N., FUJITA, T. & YAMAMOTO, A. 2005. Chitosan oligomers as potential and safe absorption enhancers for improving the pulmonary absorption of interferon-α in rats. *Journal of Pharmaceutical Sciences,* 94**,** 2432-2440.

YANG, L., CHU, J. S. & FIX, J. A. 2002. Colon-specific drug delivery: new approaches and in vitro/in vivo evaluation. *International Journal of Pharmaceutics,* 235**,** 1-15.

YE, L., HE, L., GAO, T. & LI, L. 2004. Studies on the antibiotic activity and its stability ofchitosan. *Journal of Beijing Union University (Natural Sciences),* 18**,** 79-82.

YEH, P.-Y., BERENSON, M. M., SAMOWITZ, W. S., KOPEČKOVÁ, P. & KOPECEK, J. 1995. Site-specific drug delivery and penetration enhancement in the gastrointestinal tract. *Journal of Controlled Release,* 36**,** 109-124.

YIN, X.-Q., LIN, Q., ZHANG, Q. & YANG, L.-C. 2002. Scavenging Activity of Chitosan and Its Metal Complexes. *Chinese Journal of Applied Chemistry,* 19**,** 325-328.

ZAPPINO, M., CACCIOTTI, I., BENUCCI, I., NANNI, F., LIBURDI, K., VALENTINI, F. & ESTI, M. 2015. Bromelain immobilization on microbial and animal source

chitosan films, plasticized with glycerol, for application in wine-like medium: Microstructural, mechanical and catalytic characterisations. *Food Hydrocolloids,* 45**,** 41-47.

ZENG, D., WU, J. & KENNEDY, J. F. 2008. Application of a chitosan flocculant to water treatment. *Carbohydrate Polymers,* 71**,** 135-139.

ZHANG, H., ZHAO, B., HUANG, C., MENG, X. M., BIAN, E. B. & LI, J. 2014. Melittin restores PTEN expression by down-regulating HDAC2 in human hepatocelluar carcinoma HepG2 cells. *PLoS One,* 9**,** e95520.

ZHOU, X. H. 1994. Overcoming enzymatic and absorption barriers to non-parenterally administered protein and peptide drugs. *Journal of controlled release,* 29**,** 239-252.

CHAPTER 3

METHODS

3.1. Introduction

Previous studies have indicated that chitosan with various degrees of quaternisation have different absorption enhancing effects (Enslin, 2005, Snyman *et al*., 2003, Venter, 2005b), gene transfection ability (Venter, 2005a), enzyme inhibition (Oberholzer, 2009) and toxicity (Van der Merwe, 2000, Venter, 2005b) where low toxicity is expected of chitosan at a high degree of acetylation and high molecular weights (Schipper *et al*., 1996). Gene transfection ability entails the deliberate introducing of nucleic acids into a specific cell to cure a disease or improve symptoms (Recillas-Targa, 2006). The ability of chitosan to inhibit certain enzymes may promote its use as an absorption enhancer. This property can increase the bioavailability of specific drugs and improve absorption. In this chapter, the methodology will be described where the antimicrobial activity, cytotoxicity and antioxidant activity of the four chitosan derivatives (TMC, TEC, DCMC and TEO) and melittin were investigated. The antimicrobial activity of the chitosan derivatives and melittin were tested on two Gram- positive and Gram- negative bacterial strains by employing the disc diffusion, well diffusion, MIC and minimum bactericidal concentration (MBC) methods. The MTT assay, LDH assay and flow cytometric assays were utilized to examine the cytotoxic effect of these derivatives. Finally, the DPPH assay was used to determine the antioxidant activity of the chitosan derivatives.

3.2. Synthesis of chitosan derivatives

Four chitosan derivatives were used namely, TMC, TEC, DCMC and TEO (Hamman *et al*., 2002, Jonker *et al*., 2002). TMC were synthesised as described by Domard, (1986), Sieval, (1998), Hamman, (2001), Snyman, (2003) and van Heerden, (2014) from Chitoclear® high MW supplied by Primex, Finland. The chitosan has a MW of 263700 Da and a degree of acetylation (DA) of 97%.

The synthesis of TMC involves the mixture of chitosan (2 g), sodium iodide (4.8 g), 11 ml of a sodium hydroxide solution (15% w/v) and 11.5 ml iodomethane in 80 ml N-methylpyrrolidone. This mixture was stirred on a water bath for 45 minutes at 60ºC. The product was precipitated from the solution with ethanol and isolated by centrifugation. The final products were dissolved in 40 ml of NaCl (Sodium chloride)

solution (5% w/v) to exchange the iodide- ion with a chloride- ion. Finally it was dissolved in 40 ml water and precipitated from solution with ethanol whereas it was dried at 40ºC in a vacuum oven (Domard *et al*., 1986, Hamman and Kotze, 2001, Sieval *et al*., 1998).

TEC were synthesised as described by Avadi *et al.* (2003), from Chitoclear® medium molecular weight, also supplied by Primex, Finland. The chitosan has a MW of 77670 Da and a DA of 93%. TEC was synthesised using the same method as TMC as described by Domard, (1986), Hamman, (2001) and Sieval, (1998) with some alterations. Chitosan (200 mg) was dissolved in N-methylpyrrolidone (8 ml) and stirred magnetically at room temperature for 4 hours. Sodium hydroxide (1.2 ml), 480 mg sodium iodide and 3 ml ethyl iodide was added to the solution. The mixture was heated under stirring for 6 hours at 60ºC. The product was precipitated from solution using acetone and separated by centrifugation. The product was then dissolved in 4 ml of NaCl solution (10% w/v) whereas it was precipitated with 200 ml acetone, centrifuged and dried (Avadi *et al*., 2003).

DCMC was synthesised from Chitoclear® high MW, similar to TMC. DCMC was prepared from chitosan by suspending 30 g of chitosan in 3 l water. To dissolve chitosan, glacial acetic acid (27 g) was added and then stirred for 20 minutes whereas glyoxylic acid was added. Sodium borohydride (90 g) was then delivered in 2.5 L water using a peristaltic pump. The solution was then dialysed against water for 36 hours whereas the solution was then freeze- dried and sterilised (Dung *et al*., 1994).

TEO was synthesised from chitosan oligosaccharide with a MW of 135000 Da and a DA of 79% as described by Oberholzer, (2009) and Venter, (2005).

All the synthesised polymers were characterised by 1H-NMR analysis, Infrared analysis and the DQ and MW calculated as described by Venter, (2005), Oberholzer, (2009), Snyman, (2003) and Hamman, (2001).

3.3. Preparation of Melittin

A 1 mg/ml stock solution of >97% synthetic melittin (acquired from Sigma Aldrich, Catalogue number: M4171) was prepared in Phosphate- buffered saline (PBS) and serial dilutions were made ranging from 0.5 µM to 10 µM.

3.4. Experimental design

The experimental approach for this study was divided into three main branches, namely (1) antimicrobial studies, (2) cytotoxicity studies and (3) antioxidant activity studies (**Figure 3.1**). Chitosan derivatives and melittin and also a combination thereof were investigated to determine the antimicrobial activity and cytotoxicity. The antimicrobial studies were conducted on four bacterial strains namely, *E. coli, P. aeruginosa, S. aureus* and *S. epidermidis.* The cytotoxicity studies were investigated using mammalian the cell lines, human hepatocellular liver carcinoma cell line and human epithelial colorectal adenocarcinoma cells. The antioxidant activity of chitosan derivatives was also determined.

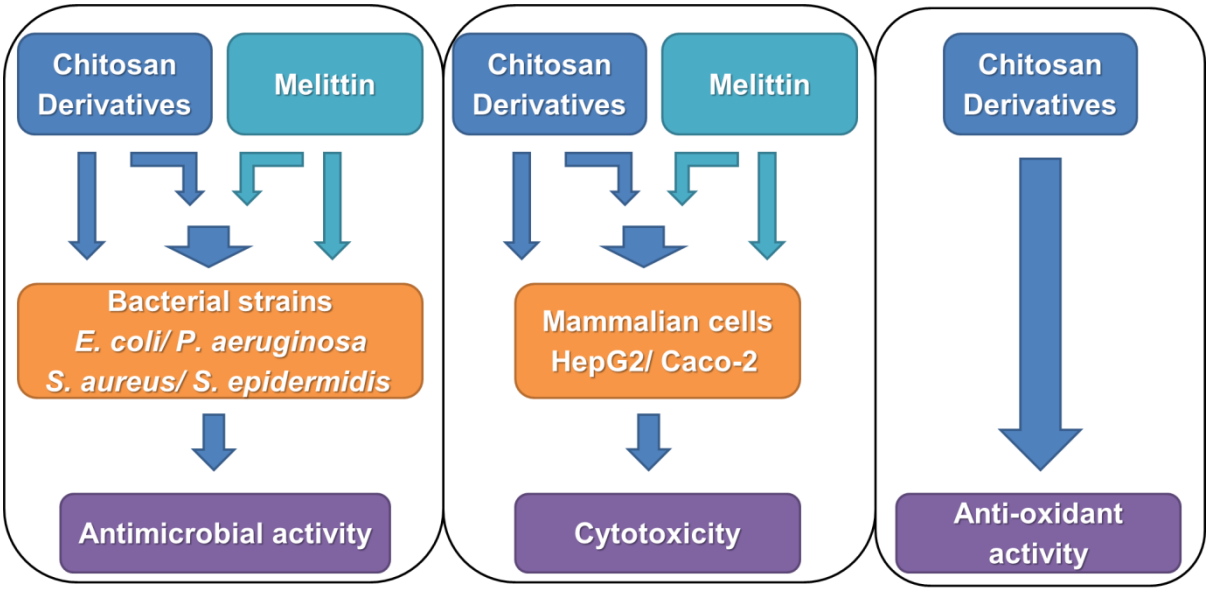


Figure 3.1: Experimental design of the studies on chitosan derivatives and melittin to determine the antimicrobial activity, cytotoxicity and antioxidant activity thereof

3.5. Antioxidant activity of the derivatives: DPPH radical scavenging assay

In normal aerobic metabolism, free radical reactions take place and produce ROS. These ROS can cause damage to biological molecules such as deoxyribonucleic acid

(DNA) and proteins. This can lead to several diseases such as diabetes and cancer as well as playing a significant part in ageing and cancer. Antioxidants play an important role in counteracting ROS by reducing oxidative stress and therefore damage to biological molecules is reduced (Choi *et al*., 2002). The DPPH radical scavenging assay will be used to determine if chitosan and its derivatives possess any antioxidant activity. DPPH (acquired from Sigma Aldrich, with catalogue number: D9132) is a stable radical where it appears as a deep violet colour in solution with ethanol at an absorption band of 520 nm. When the DPPH solution is mixed with the substance for testing antioxidant activity and a pale violet colour is produced, the substance depicts antioxidant activity. This assay is since it is the standard assay to use when determining antioxidant activity.

Chitosan derivatives (5 mg; 10 mg; 25 mg; 50 mg) were dissolved in 1 ml PBS and 50 µl of the derivatives were added to each allocated well (96- well plate). A 0.1 mM solution of DPPH was made in absolute ethanol and 50 µl of this solution were added to the wells containing the derivatives. Trolox (0.2 M) was used as the positive control and 50 µl was added to the allocated wells. DPPH (50 µl) was also added to the Trolox. The negative control consisted out of 50 µl PBS and 50 µl DPPH. The well was shaken and left to stand for 20 minutes at room temperature. A blank of absolute ethanol (100 µl) was included and the absorbance was measured at 490 nm. The radical scavenging activity was measured using the following equation (Xing *et al*., 2005):

Equation 3.1:

$$\text{Scavenging effect (\%)} = 1 - (A_{samples}/A_{control}) \times 100$$

Where A_{sample} is the absorbance of the sample used and $A_{control}$ absorbance of the DPPH solution.

3.6. Antimicrobial activity

3.6.1. Bacterial strain preparation

The antimicrobial activity of the chitosan derivatives and melittin were studied on two gram- positive and gram- negative bacterial strains. All bacterial strains were acquired from the American Type Culture Collection (ATCC) and kindly provided by Prof Sandy van Vuuren (Department of Pharmacy and Pharmacology, University of the Witwatersrand). For Gram- positive strains, *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12223) were used and for Gram- negative, *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 27853). These bacterial strains were selected on the basis of their prevalence to cause skin infections. Skin infections are relevant because chitosan derivatives are used in the treatment of wounds and have potential in the treating skin infections. Bacterial strains were cultured in tryptone soya broth (Oxoid CM0129) for 18-24 hours at 37ºC.

3.6.2. Disc diffusion assay

In an attempt to determine the antimicrobial activity of the chitosan derivatives and melittin, the antimicrobial disc diffusion assays were performed on two bacterial strains *S. aureus* and *E. coli.* The disc diffusion assay (Kirby–Bauer assay) tests whether bacteria are affected by specific substances. In this test, disks containing the substance of interest are introduced on an agar plate pre-inoculated with bacteria. If the tested substance inhibits the bacteria from growing, there will be an area around the disk where the bacteria have not spread to. This is also known as the zone of inhibition. Mueller Hinton agar plates were used and inoculated with the relevant bacterial strains. The discs were saturated with different concentrations of the chitosan derivatives (5 mg; 10 mg; 25 mg; 50 mg) and melittin (0.5 µM) and placed onto the agar surface. Ciprofloxacin discs (1 µg) were used for positive antimicrobial controls. PBS was used as the negative control. The plates were incubated at 37ºC for ~24 hours. After incubation, the plates were examined and the zone of inhibition measured.

3.6.3. Well Diffusion assay

To complement the disc diffusion assay, the well diffusion assay was also performed. This assay is similar to the disk diffusion assay but utilizes larger volumes of the tested substance in pre-casted wells instead of disks. Agar was poured into assay plates where it was left to cool down at room temperature until the agar solidified. The agar plates were inoculated with the selected bacteria (*S. aureus* and *E. coli*). Wells were created in the plates using a glass Pasteur pipette and 80 µl of the samples were introduced into the wells. The samples namely, TMC, TEC, DCMC and TEC (5 mg; 10 mg; 25 mg; 50 mg) were dissolved in 1 ml PBS. Different concentrations of melittin was also tested (0.5 µM; 1.5 µM and 5 µM). The antimicrobial control used was Ciprofloxacin (0.01 mg/ml) and the negative control PBS. The plates were placed at 4 ºC for 60 minutes to set and then incubated at 37ºC for ~24 hours. After incubation, the zones of inhibition were measured to determine microbial growth.

3.6.4. Minimum Inhibitory Concentration (MIC)

The MIC is the lowest concentration of a drug that inhibits growth of an organism and is routinely used to determine the antimicrobial activity of substances (Andrews, 2001). This assay is used to determine both the concentration of substance and which substance that must be used against a specific bacterial strain.

Cultures was inoculated in tryptone soya broth and incubated for 24 hours. Samples of chitosan derivatives and melittin were prepared where the starting concentration of the derivatives was 50 mg/ml (dissolved in 1 ml PBS) and melittin was 1 mg/ml. Ciprofloxacin was used as the antimicrobial control at a concentration of 0.01 mg/ml. Plates (96- well) were prepared aseptically where 100 µl of sterile Mueller Hinton broth were placed in the wells. Samples (100 µl) were placed into the first row of the wells and serially diluted. The bacterial inoculum (1 µl) was transferred into 100 ml of sterile broth for all four bacterial strains (100 µl) respectively and each well was inoculated. Following inoculation, the plate was sealed with a sterile adhesive sealer and incubated at 37ºC for 24 hours. A 0.4 mg/ml iodonitrotetrazolium violet (INT) solution (40 µl) was added to each well after incubation. The 96- well plates were examined

after 6 hours to visually determine a colour change (purple- pink) in the presence of microbial growth. The end point MIC value was taken as the lowest concentration of sample that resulted in the inhibition of growth, which can be seen as the absence of the purple- pink colour of the indicator.

3.6.5. Minimum Bactericidal Concentration (MBC)

The MBC is defined as the lowest concentration of an antimicrobial substance necessary to neutralize or kill a particular bacteria (Andrews, 2001, Yilmaz, 2012).

After the MIC was completed, a sample from wells showing no growth were streaked on sterile tryptone soya agar plates to check bacterial growth. The plates were incubated at 37ºC for 24 hours. After incubation, bacterial growth was visually examined.

3.6.6. Interactive combination studies

The interaction of TMC and melittin on the bacterial strains were also investigated. After MIC values for each combination were determined, the fractional inhibitory concentration index was used to determine the interaction of the samples on the selected bacteria. The sum of the fractional inhibitory concentration (ΣFIC) was used to calculate each combination using the following equation (Mabona *et al*., 2013):

Equation 3.2:

$$FIC(i) = \frac{\text{MIC of Melittin in combination with TMC}}{\text{MIC of Melittin independantly}}$$

Equation 3.3:

$$FIC(ii) = \frac{\text{MIC of TMC in combination with Melittin}}{\text{MIC of TMC independantly}}$$

The sum of the FIC index is thus calculated as (**Equation 3.4**):

ΣFICI = FIC (*i*) + FIC (*ii*).

The interactions were classified as shown in **Table 3.1** (van Vuuren and Viljoen, 2011).

Table 3.1: Fractional inhibitory concentrations and their predicted interaction

ΣFIC value	**Interaction**
≤ 0.5	Synergy
>0.5 – 1.0	Additive
> 1.0 – ≤4.0	Non- interactive
> 4.00	Antagonistic

Using the values obtained from the above equation, the interaction of TMC and melittin was either classified as Synergy, Additive, Non- interactive or Antagonistic.

3.7. Cytotoxicity

3.7.1. Mammalian cell cultures

Cell lines were purchased from ATCC. Human hepatocellular liver carcinoma cell line (HepG2; catalogue number HB-8065) and human epithelial colorectal adenocarcinoma cells (Caco-2; catalogue number HB-8065) were utilized in this study. HepG2 cells are routinely used as the standard cell line for cytotoxicity experiments and it has several advantages, including a rapid growth rate and is well characterised. Caco-2 cells, which are intestinal cells, were used because of the reported absorption enhancement functions of chitosan and its derivatives in intestinal cells (Enslin *et al*., 2008).

Mammalian cells were propagated in cell culture flasks (T75 cm^2) at 37ºC and in a humidified 5% CO_2 atmosphere. HepG2 as well as Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Seperations, Catalogue number: SH30243.FS) and fortified with foetal bovine serum (FBS) (Lonza, Catalogue number: DE14-801FI) to a final concentration of 10%. The growth media were supplemented with 1% non-essential amino acids (NEAA) (Lonza, Catalogue number: 13-114E) as well as 1% antibiotics (penicillin and ampicillin) (Lonza, Catalogue number: 17-602E). The medium were renewed twice a week, or as necessary. Cells were sub-cultured when ±80% confluency was reached. To this end, culture medium was carefully decanted and the cell layer gently washed with 1x PBS (Sigma, Catalogue number: P4417-100Tab) to remove all traces of serum that contains trypsin inhibitor. A small volume of trypsin-EDTA (Lonza, Catalogue number: CC-5012) solution (between 2.0 to 3.0 ml) was added to the flask to detach cells from the bottom of the flask and separated them from each other. When passaging with trypsin, HepG2 cells were incubated for 3 minutes while Caco-2 cells were incubated for 5 minutes. Growth medium were added (6.0 to 8.0 ml) and cells aspirated by gently pipetting. Appropriate aliquots of the cell suspension were added to the new culture vessels. The same procedure was being followed for experimental analysis, except the cells were added to 96- well plates for the analysis.

3.7.2. Cell proliferation studies

The MTT Cell Proliferation Assay (ATCC, Catalogue number: 30-1010K) was used to determine the optimal amount of cells necessary for experiments for each cell line because of the difference in cell lines and laboratory environment.

Cells were harvested and resuspended at 1 x 10^6 cells per ml. Serial dilutions were prepared of cells in culture medium (DMEM) from 1 x 10^6 to 1 x 10^3 cells per ml. A 100 µl of the dilutions were plated out in triplicate into the wells of a 96- well plate. Three control wells of serum free medium were included to provide the blanks for the absorbance readings. The cells were incubated under appropriate conditions for the cell line for between 6 to 48 hours (for most cell types, 12 hours is sufficient). A volume of 10 µl of the MTT reagent (5 mg/ml) was added to each well including the controls. The plate was incubated for a further 4 hours. The presence of the purple precipitate

was observed periodically under a microscope. When the purple precipitate was clearly visible, a 100 µl of detergent reagent namely dimethylsulfoxide (DMSO) was added to all wells including the controls, while swirling gently. The plate was left in the dark for 1 hour at room temperature. The absorbance was then measured in each well at 570 nm in a 96- well plate reader. The average values from the triplicate readings were determined and subtracted from the average value for the blank. The absorbance was plotted against number of cells/ml. The number of cells used in an assay was chosen within the linear portion of the plot and yield an absorbance of between 0.75 – 1.25 (see **Annexure 3**).

3.8. Cytotoxicity Assays

3.8.1. MTT assay

The MTT assay is used to measure the *in vitro* cell viability and proliferation of cells. In principle, yellow tetrazolium MTT is reduced by mitochondrial dehydrogenase enzymes to purple formazan crystals in the cell which is non- water soluble. The formazan crystals can be dissolved by DMSO where the amount of formazan product can be determined and is an indication of the number of living cells (Mosmann, 1983). This assay was used because it is a fast way of detecting living cells and has a high level of accuracy. It is also the standard assay to determine cell viability.

HepG2 and Caco-2 cells were seeded into 96- well plates. For HepG2, 2.5 x 10^4 cells were seeded per well and Caco-2, 2.0 x 10^4 cells. The plates were incubated overnight for cells to attach. After cells reached ~90% confluency, they were carefully washed with 1 volume of PBS (±0.5 ml). The cells were then treated with different samples. The treatments consisted of a concentration series of chitosan derivatives (5 mg; 10 mg; 25 mg; 50 mg) and melittin (0.5 µM; 1 µM; 1.5 µM; 3 µM; 5 µM) and were tested on both cell lines. Combination studies of the chitosan derivatives (25 mg) and melittin (1 µl) were also performed. All experiments included positive- (Triton X-100) and negative controls (Serum free media). A treatment period of 4 hours were utilized and afterwards washed with PBS to remove traces of the substances. A 5 mg/ml stock solution of MTT was prepared by diluting 5 mg of MTT powder in 1 ml PBS. 10 µl of

the stock solution and 90 µl serum free media were added to each well. The plates were incubated for 2 hours at 37ºC. After incubation, all the media were carefully removed with a pipette and 100 µl DMSO were added to each well and incubated for 1 hour. DMSO also served as a blank. The optical density was measured at a wavelength of 560 nm and the background at 630 nm with a plate reader. All experiments were at least performed in triplicate and independently repeated.

3.8.2. LDH assay

The CytoTox-ONE™ Homogeneous Membrane Integrity assay (Promega) is used to estimate the number of non- viable cells in multiwell plates where the release of LDH from cells with damaged membranes is measured through fluorescence. LDH, which is present in all mammalian cells, is released into the extracellular spaces of cells when the plasma membrane is damaged (Chan *et al*., 2013). Therefore the measurement of released LDH is an indication of extensive cell membrane damage. This assay was used because it is a rapid method that is widely used to determine cell viability by indicating cellular membrane integrity.

HepG2 and Caco-2 cells were seeded into 96- well plates as described earlier (section **3.7.1**). After treatment, the plates were removed from the incubator and equilibrated to room temperature (approximately 20–30 minutes). A lysis solution was used to generate a maximum LDH release control where 2 µl of the kit Lysis Solution were added to the positive control wells. CytoTox-ONE™ Reagent (100 µl) were added to each well and mixed for 30 seconds. The plate was incubated for 10 minutes at 22ºC. Stop Solution (Promega) were added (50 µl) to each well in the same order of addition that was used for adding the CytoTox-ONE™ Reagent. The plate was gently mixed for 10 seconds and the fluorescence recorded at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. All experiments were at least performed in triplicate and independently repeated. The treatments included in the experiments were chitosan derivatives and melittin where a concentration series of chitosan derivatives (5 mg; 10 mg; 25 mg; 50 mg) and melittin (0.5 µM; 1 µM; 1.5 µM; 3 µM; 5 µM) were tested on both cell lines and also combination studies of the derivatives (25 mg) and melittin (1 µl) were done.

3.8.3. Flow cytometry

Apoptosis, also known as programmed cell death, occurs during the maintenance of tissue homeostasis to sustain cell populations as well as embryonic development. Apoptosis is characterized by morphologic features which include cellular shrinkage, loss of plasma membrane, condensation of the cytoplasm and nucleus and DNA fragmentation (Kanduc *et al*., 2002, Van Cruchten and Van Den Broeck, 2002). Necrosis is where accidental and/or abnormal cell death occurs due to environmental disturbances (Fink and Cookson, 2005) or cell injury (Vermes *et al.*, 2000). To determine whether a substance causes cell death through apoptosis or necrosis, the FITC Annexin V Apoptosis Detection Kit I from BD Biosciences (Catalogue number: 556547) were used to examine the cell death pattern after treatment. This assay was used because it is a reliable method to study a large amount of cells in order to distinguish between apoptosis and necrosis.

Fluorescence of single cells was measured by a FACSVerse™ (BD Bioscience) bench top flow cytometer equipped with blue (488 nm) and red (640 nm) lasers. Events were acquired on BD FACSuite^{TM} software, version 1 (Becton & Dickson, Mountain view, CA, USA). Annexin V FITC apoptosis detection kit (BD Biosciences) was used for the detection of apoptosis using flow cytometry. The assay was performed according to the manufacturer's instructions with minor alterations. In brief, cells were seeded in a 96-well plate and grown to about 90% confluence. The cells were exposed to different chitosan derivatives and melittin as described earlier. After 24 hours, each well's cells were washed twice with 200 µl of PBS and suspended in 1X binding buffer at a concentration of approximately 1 x 10^6 cells/ml. 100 µL of this solution was then transferred to 12 x 75 mm flow tubes (BD Biosciences) and 5 µL of FITC Annexin V and 5 µL of propidium iodide (PI) was added. Samples were then briefly vortexed and incubated in the dark at room temperature for 20 min. After incubation, 100 µL 1X binding buffer was added to each tube and then analysed using the BD FACSVerse System and BD FACSuite Software. For a positive apoptosis control, HepG2 cells were treated with 1mM staurosporine (Sigma-Aldrich) for 4 hours.

Amplification of signals were carried out at logarithmic scale and measurement of events plotted on forward light scatter (FSC), side light scatter (SSC), green fluorescent (FL1) and red fluorescent (FL2). A gating strategy was used to distinguish the fluorescently labelled cell population from unstained populations. A total of 10 000 events as defined by gates, were counted. Positive as well as stained and unstained negative controls were included in each analysis. The FACSVerse were calibrated using FACSuite™ CS&T research beads. Data was processed with FCS Express V4 software (De Novo Software, CA, USA). Gates were set on the dot plot FSC and SSC during analysis. The geometric means of fluorescence for all the parameters were calculated from the respective histograms or two parameter fluorescence dotplots. Fluorescence results were expressed in arbitrary units as mean fluorescence intensity (MFI) and cell counts were expressed as percentage. All experiments were at least performed in triplicate and independently repeated. Refer to **Figure 3.2-3.4** for detailed explanation of the above mentioned. It can be concluded that dead cells have a decreased FSC and increased SSC.

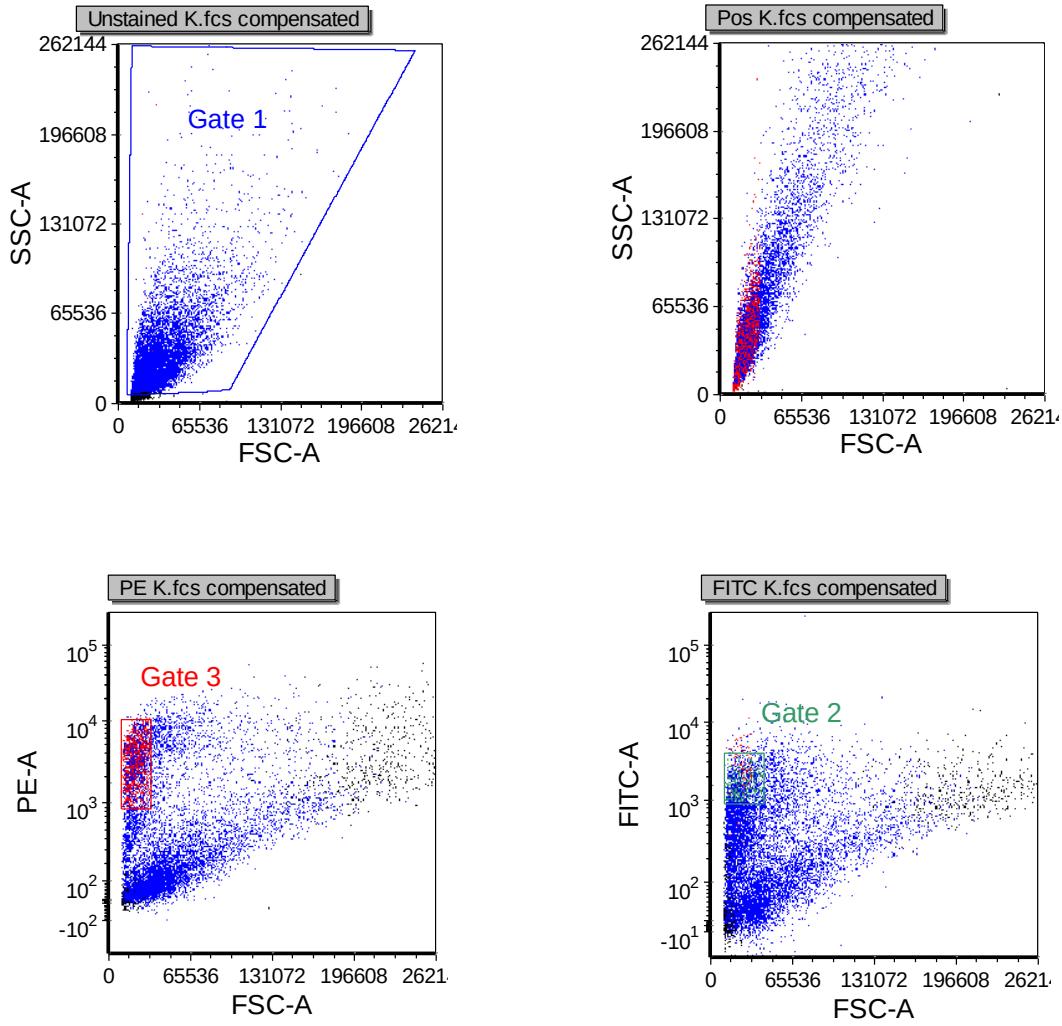


Figure 3.2: The unstained control contains only untreated, unstained cells to determine the scatter profile of the cells. The positive control is included in the gating strategy to determine the scatter profile of the maximal response. The FITC and PI controls include untreated, but stained cells to determine the background fluorescence of each of the dyes separately.

In the gating strategy, the first gate, Gate 1 in blue is set on the live cells detected in the cell (untreated) control in the Forward scatter (FSC)/ Side scatter area (SSC) plot. All of these blue cells are included in the rest of the analysis. The difference in the FSC/ SSC area is clearly different when the positive control is compared to the control. Next a gate, Gate 2 in green, is set around the FITC background cells. This is usually a small percentage of cells ~15% that is excluded from the analysis.

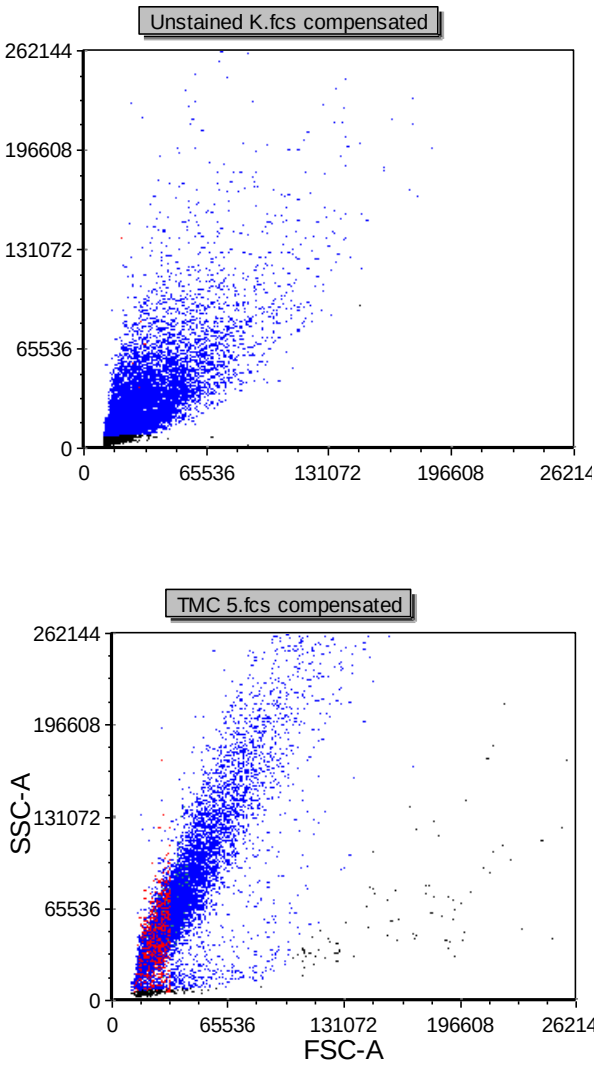
Another gate, Gate 3 in red is set around the PI background fluorescent cells and is also excluded from the analysis.

Legend: FSC an indicator of the size of cells

SSC an indicator of the complexity or granularity of cells

FITC represents the Annexin V conjugated with FITC

PE represents the propidium iodide



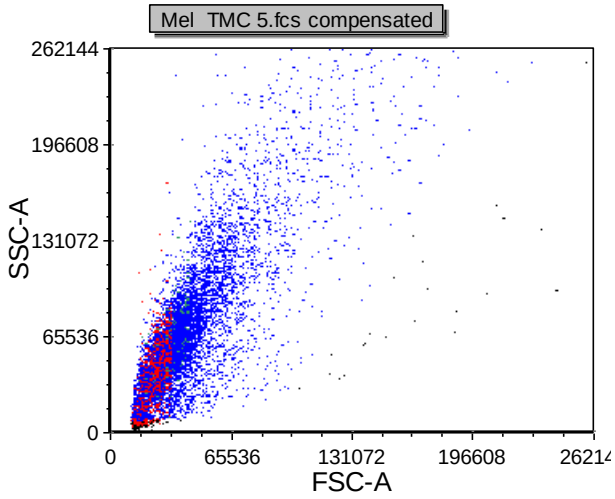
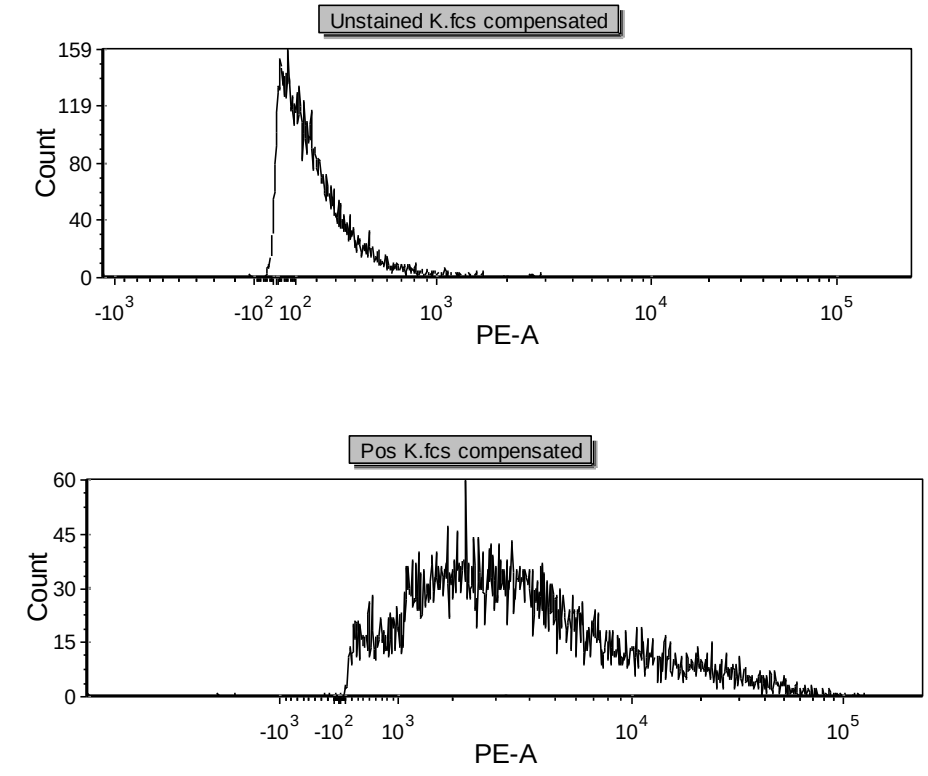


Figure 3.3: In this figure the difference in scatter profile of HepG2 cells is clearly evident between the untreated control, the TMC 5 mg/ml and the combination of TMC with melittin. A scatter profile of cells with decreased FSC and increased SSC is indicative of cells undergoing apoptosis.



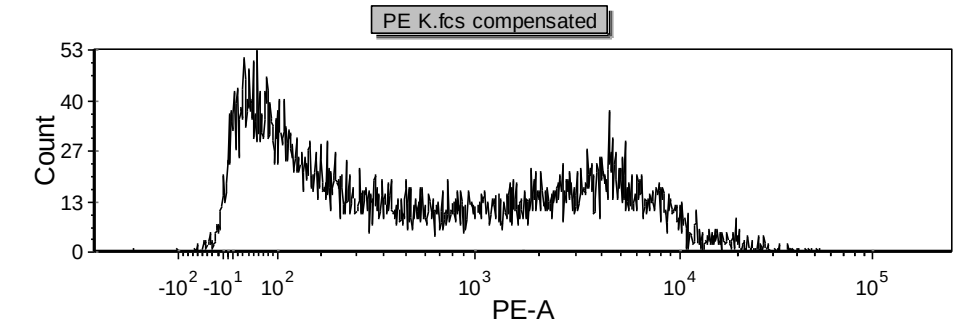


Figure 3.4: Histograms are included in the analysis to give an indication of the intensity of the fluorescence. In these plots the relatively low background fluorescence is clearly visible in the propidium iodide control. Similar plots were used for the Annexin V-FITC.

3.9. Statistical evaluation

All experiments were done in triplicate and independently repeated at least two times. Colorimetric assay data was analysed using GEN5 Software. The results were then processed using GraphPad Prism 5. GraphPad Prism 5 was also used to draw column and line graphs. Outliers were removed using the modified Thompson tau method (Cimbala, 2011). The mean value of all the data points was calculated and contains a standard deviation as calculated by **equation 3.5**. Results in graphs are expressed as mean±standard deviation.

Equation 3.5:

$$\sigma = \sqrt{\sum_{i=1}^{N} p_i (x_i - \mu)^2}, \quad \text{where} \quad \mu = \sum_{i=1}^{N} p_i x_i.$$

P-values were determined with the Kruskal–Wallis test. This test is a non-parametric method for testing whether samples originate from the same distribution and can be used for comparing two or more independent samples of equal or different sample sizes.

3.10. References

ANDREWS, J. M. 2001. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy,* 48**,** 5-16.

AVADI, M., ZOHURIAAN-MEHR, M., YOUNESSI, P., AMINI, M., TEHRANI, M. R. & SHAFIEE, A. 2003. Optimized synthesis and characterization of N-triethyl chitosan. *Journal of Bioactive and Compatible Polymers,* 18**,** 469-479.

CHAN, F. K.-M., MORIWAKI, K. & DE ROSA, M. J. 2013. Detection of Necrosis by Release of Lactate Dehydrogenase (LDH) Activity. *Methods in Molecular Biology,* 979**,** 65-70.

CHOI, C. W., KIM, S. C., HWANG, S. S., CHOI, B. K., AHN, H. J., LEE, M. Y., PARK, S. H. & KIM, S. K. 2002. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science,* 163**,** 1161-1168.

CIMBALA, J. M. 2011. *Outliers [Online]* [Online]. Available: https://www.mne.psu.edu/me345/Lectures/outliers.pdf [Accessed 2 November 2015].

DOMARD, A., RINAUDO, M. & TERRASSIN, C. 1986. New method for the quaternization of chitosan. *International Journal of Biological Macromolecules,* 8**,** 105-107.

DUNG, P. L., MILAS, M., RINAUDO, M. & DESBRIÈRES, J. 1994. Water soluble derivatives obtained by controlled chemical modifications of chitosan. *Carbohydrate Polymers,* 24**,** 209-214.

ENSLIN, G. M., HAMMAN, J. H. & KOTZE, A. F. 2008. Intestinal drug absorption enhancers: synergistic effects of combinations. *Drug Development and Industrial Pharmacy,* 34**,** 1343-9.

ENSLIN, G. M. A. 2005. *In Vitro Evaluation of the Absorption Enhancement Properties of Chitosans, Monocaprin and Melittin*, Tshwane University of Technology.

FINK, S. L. & COOKSON, B. T. 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infection and immunity,* 73**,** 1907-1916.

HAMMAN, J. H. & KOTZE, A. F. 2001. Effect of the type of base and number of reaction steps on the degree of quaternization and molecular weight of N-trimethyl chitosan chloride. *Drug Development and Industrial Pharmacy,* 27**,** 373-80.

HAMMAN, J. H., STANDER, M. & KOTZÉ, A. F. 2002. Effect of the degree of quaternisation of N-trimethyl chitosan chloride on absorption enhancement: in vivo evaluation in rat nasal epithelia. *International Journal of Pharmaceutics,* 232**,** 235-242.

JONKER, C., HAMMAN, J. H. & KOTZÉ, A. F. 2002. Intestinal paracellular permeation enhancement with quaternised chitosan: *in vitro* evaluation. *International Journal of Pharmaceutics,* 238**,** 205-213.

KANDUC, D., MITTELMAN, A., SERPICO, R., SINIGAGLIA, E., SINHA, A. A., NATALE, C., SANTACROCE, R., DI CORCIA, M. G., LUCCHESE, A., DINI, L., PANI, P., SANTACROCE, S., SIMONE, S., BUCCI, R. & FARBER, E. 2002. Cell death: apoptosis versus necrosis. *International Journal of Oncology,* 21**,** 165-70.

MABONA, U., VILJOEN, A., SHIKANGA, E., MARSTON, A. & VAN VUUREN, S. 2013. Antimicrobial activity of southern African medicinal plants with dermatological relevance: From an ethnopharmacological screening approach, to combination studies and the isolation of a bioactive compound. *Journal of Ethnopharmacology,* 148**,** 45-55.

MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods,* 65**,** 55-63.

OBERHOLZER, I. D. 2009. *Peroral and nasal delivery of insulin with PheroidTM technology.* North-West University.

RECILLAS-TARGA, F. 2006. Multiple strategies for gene transfer, expression, knockdown, and chromatin influence in mammalian cell lines and transgenic animals. *Molecular Biotechnology,* 34**,** 337-54.

SCHIPPER, N. M., VÅRUM, K. & ARTURSSON, P. 1996. Chitosans as Absorption Enhancers for Poorly Absorbable Drugs. 1: Influence of Molecular Weight and Degree of Acetylation on Drug Transport Across Human Intestinal Epithelial (Caco-2) Cells. *Pharmaceutical Research,* 13**,** 1686-1692.

SIEVAL, A. B., THANOU, M., KOTZE´, A. F., VERHOEF, J. C., BRUSSEE, J. & JUNGINGER, H. E. 1998. Preparation and NMR characterization of highly substitutedN-trimethyl chitosan chloride. *Carbohydrate Polymers,* 36**,** 157-165.

SNYMAN, D., HAMMAN, J. H. & KOTZE, A. F. 2003. Evaluation of the mucoadhesive properties of N-trimethyl chitosan chloride. *Drug Development and Industrial Pharmacy,* 29**,** 61-9.

VAN CRUCHTEN, S. & VAN DEN BROECK, W. 2002. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anatomia, Histologia Embryologia,* 31**,** 214-23.

VAN DER MERWE, S. M. 2000. *In vitro evaluation of the toxicity profile of N-trimethyl chitosan chloride.*

VAN VUUREN, S. & VILJOEN, A. 2011. Plant-based antimicrobial studies--methods and approaches to study the interaction between natural products. *Planta Medica,* 77**,** 1168-82.

VENTER, C. 2005. *Chitosan and quaternised chitosan polymers as gene transfection agents.* North-West University.

VENTER, J. P. 2005. *Design and evaluation of chitosan and N-trimethyl chitosan chloride microspheres for intestinal drug delivery.* North-West University.

VERMES, I., HAANEN, C. & REUTELINGSPERGER, C. 2000. Flow cytometry of apoptotic cell death. *Journal of Immunological Methods,* 243**,** 167-190.

XING, R., YU, H., LIU, S., ZHANG, W., ZHANG, Q., LI, Z. & LI, P. 2005. Antioxidant activity of differently regioselective chitosan sulfates in vitro. *Bioorganic and Medicinal Chemistry,* 13**,** 1387-1392.

YILMAZ, M. T. 2012. Minimum inhibitory and minimum bactericidal concentrations of boron compounds against several bacterial strains. *Turkish Journal of Medical Sciences,* 42**,** 1423-1429.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Introduction

In the drug delivery field, there is continued research into novel excipients. Although most excipients are classified as inactive ingredients in dosage forms, there are also great advantages in using multifunctional excipients. Natural polymers are good examples of multifunctional excipients having apart from their basic function, additional properties including stimuli sensitive, mucoadhesion, enzyme inhibition, intestinal epithelium absorption enhancement, efflux pump inhibition, increased buffer capacity, taste-masking ability, pharmacological activity and the ability to form conjugates or interact with enzymes responsible for drug metabolism. Chitosan as a natural polymer is one of the best studied multifunctional excipients, but has certain limitations as discussed in Chapter 2. Derivatives of chitosan are therefore synthesised not only to improve the solubility of chitosan at physiological pH, but also to expand on the number of multifunctional polymers that can be used in dosage forms. Chitosan and one of its derivatives namely TMC, have been shown to have antioxidant activity as well as antimicrobial activity against selected bacteria. TEC, DCMC and TEO have proven absorption enhancing, enzyme inhibition and antioxidant activities respectively. However the cytotoxicity of these derivatives is not known and it is therefore important to further characterise the polymers.

This chapter describes the possible antioxidant activity as well as the antimicrobial properties and cytotoxicity of four chitosan derivatives, melittin and combinations of them.

4.2. Chemical characterisation of chitosan derivatives

The different chitosan derivatives (**Table 4.1**) were selected based on the chemical structures, DQ and MW as determined by other studies (Enslin, 2005, Snyman *et al*., 2003, Van der Merwe, 2000).

Table 4.1 Properties of quaternised chitosan derivatives synthesised and chemically characterised previously and further investigated in this study (adapted from Hamman, (2001) and Snyman, (2003)).

Chitosan derivative	Degree of quaternisation (%)	Molecular weight (g/mole)
TMC	65.50	168 000
TEC	26.71	82 810
TEO	27.90	9000

TMC with high degrees of quaternisation have the most pronounced effect on absorption enhancement of compounds in Caco-2 cells (Kotze *et al*., 1999, Thanou *et al*., 2000). The degree of quaternisation and molecular weight of the derivatives was also an important factor in gene transfection (Venter, 2005) and antimicrobial activity (No *et al*., 2002, Zheng and Zhu, 2003). The TMC used in this study had a DQ of 65.50% and relatively high MW. The synthesised TEC had a lower degree of quaternisation and molecular weight compared to TMC. This could be attributed to the smaller molecular size and higher reactivity if iodomethane, compared to iodoethane used during the manufacturing (Hamman and Kotze, 2001, Snyman *et al*., 2003). The low molecular weight TEO was chosen based on results from previous studies where low molecular weight chitosan oligomers had increased antioxidant activity compared to chitosan (Sun *et al*., 2007, Zhao *et al*., 2013). DCMC was utilised in this study because of its negative charge compared to the quaternised derivatives with positive charge (Geisberger *et al*., 2013). The DCMC used in this study had a degree of substitution of 90% (Oberholzer, 2009).

4.3. Antioxidant activity

The antioxidant activity of different concentrations of chitosan derivatives was determined using the DPPH assay. The antioxidant activity was determined because of the known antioxidant activity of chitosan, TMC and chitosan oligomers, whereas no literature is available on the antioxidant activity of the rest of the chitosan derivatives used in this study. The concentrations of the derivatives tested were 5 mg/ml; 10 mg/ml; 25 mg/ml and 50 mg/ml. Trolox, which is a derivative of vitamin E and exhibits excellent free radical scavenging properties, was used as the positive control at a concentration of 0.2 M.

The DPPH assay was employed to determine the scavenging effect of the derivatives and the results is depicted in **Figure 4.1-4.4**. The scavenging ability of a compound indicates if it has antioxidant properties. Antioxidants exert their effects by scavenging the reactive oxygen species, therefore if a compound displayed a scavenging effect, it has antioxidant activity (Halliwell *et al*., 1992). According to literature, chitosan and its derivatives with a low MW and high DQ (high positive charge density) has increased scavenging effect and therefore increased antioxidant activity (Guo *et al*., 2006, Jarmila and Vavrikova, 2011). According to Zhao *et al.,* (2013), when the concentration of chitosan and chitosan oligomer increases, the scavenging effect also increases.

4.3.1. TMC

The scavenging effect of TMC used in this study is depicted in **Figure 4.1**. Compared to trolox (which was assumed to be a 100% effective scavenger for this study), TMC had no scavenging effect. There was a concentration dependent decrease in the scavenging percentage. All TMC concentrations differed statistically significantly from trolox. This lack of antioxidant potential could possibly be attributed to TMC acting as a pro-oxidant. It was hypothesised that substances with lower scavenging effects may have a pro-oxidant effect by generating ROS in physiological buffers (Roy *et al*., 2010). These results differ from the results found by Guo *et al.,* (2006). They found that TMC with high DQ >80.5% exhibited scavenging effect. Although the TMC used in this study has a high

DQ, the high MW can also influence the scavenging effect and may be the reason for the low scavenging observed.

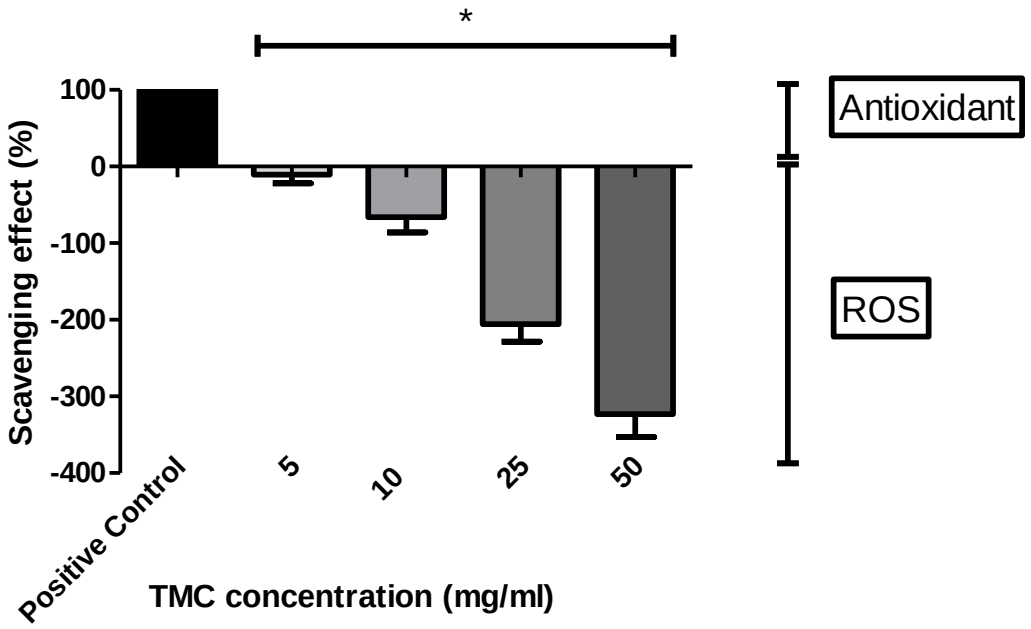


Figure 4.1: Scavenging effect (%) representing the antioxidant activity of TMC at different concentrations (5 mg/ml; 10 mg/ml; 25 mg/ml and 50 mg/ml) with trolox as the positive control which is normalised to have a 100% scavenging effect. Statistical significant differences is indicated with * ($p \leq 0.05$; n=3). The bars in the negative direction are indicative of TMC acting as a reactive oxygen species (ROS).

4.3.2. TEC

The scavenging effect of TEC is portrayed in **Figure 4.2**. Compared to trolox, TEC displayed a concentration dependant increase in the scavenging percentage with 25.37 ± 4.00%, 43.98 ± 6.67% and 47.66 ± 4.14% at 10 mg/ml, 25 mg/ml and 50 mg/ml of TEC respectively. This differed statistically significantly from the positive control. Accordingly, as the concentration of TEC increases, the scavenging effect increases. The lowest concentration of TEC, 5 mg/ml has no scavenging effect and acted as a ROS. These results are in accordance with the literature where it states that when the concentration of chitosan derivatives increases, the scavenging effect also increases (Zhao *et al*., 2013). Additionally, the TEC used has a low MW which can attribute to the scavenging

effect it displayed, although the DQ is lower than necessary for the scavenging effect based on studies done on other chitosan derivatives. This is the first study on TEC to determine the antioxidant activity to my knowledge.

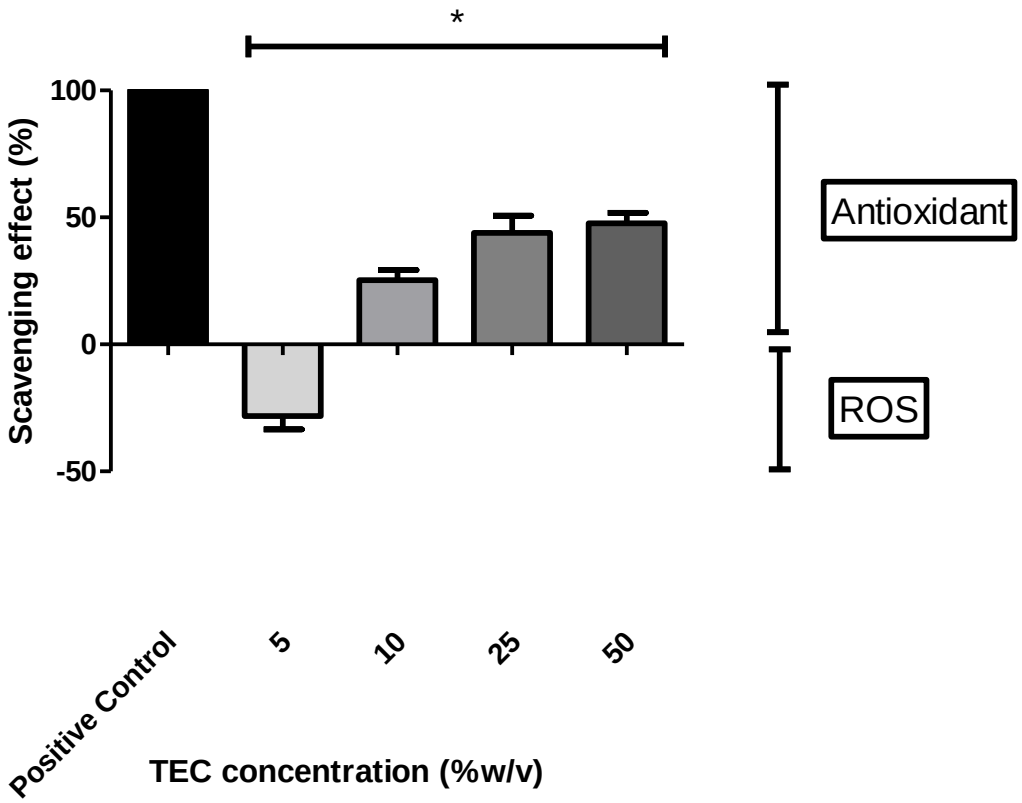


Figure 4.2: Scavenging effect (%) representing the antioxidant activity of TEC at different concentrations (5 mg/ml; 10 mg/ml; 25 mg/ml and 50 mg/ml) with trolox as the positive control which is normalised to have a 100% scavenging effect. Statistical significant differences is indicated with * ($p \leq 0.05$; n=3). The bars in the negative direction are indicative of TEC acting as a reactive oxygen species (ROS).

4.3.3. DCMC

The scavenging effect of DCMC is illustrated in **Figure 4.3**. DCMC showed no scavenging effect when compared to trolox. A concentration dependant increase in ROS could be observed. All the concentrations of DCMC differed significantly from trolox. DCMC seems to act as a ROS instead of having any antioxidant activity. Since DCMC

is the derivative which has a negative charge instead of a positive charge, it can contribute to DCMC showing no scavenging effect. This is because of the positive charge which is necessary for antioxidant activity. According to Sun *et al.,* (2008), chitosan derivatives with a high DA, will have a decreased scavenging effect. DCMC has a DA of 90% which may account for the lack of scavenging effect. Similar results were found by Sun *et al.,* (2008) where carboxymethyl chitosan oligosaccharide was used and they also found no scavenging effect.

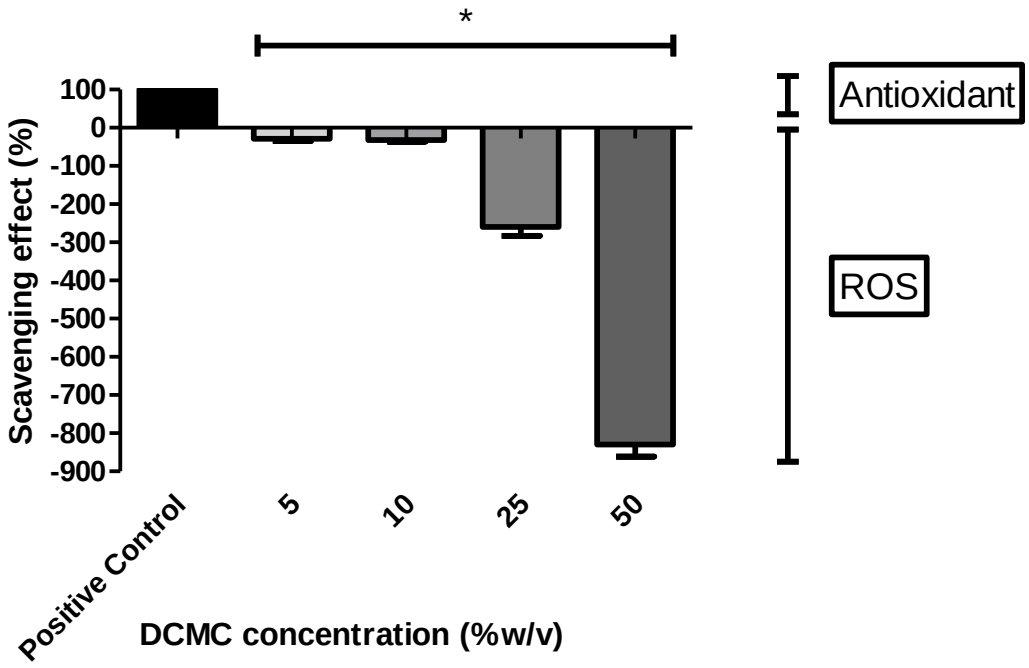


Figure 4.3: Scavenging effect (%) representing the antioxidant activity of DCMC at different concentrations (5 mg/ml; 10 mg/ml; 25 mg/ml and 50 mg/ml) with trolox as the positive control which is normalised to have a 100% scavenging effect. Statistical significant differences is indicated with * ($p \leq 0.05$; n=3). The bars in the negative direction are indicative of DCMC acting as a reactive oxygen species (ROS).

4.3.4. TEO

The scavenging effect of TEO is represented in **Figure 4.4**. When compared to trolox, TEO had no scavenging effect at high concentrations. The lowest concentration of TEO, 5 mg/ml, exhibited a small percentage scavenging effect with 28.05±7.194% and differs to what Zhao *et al.,* (2013) observed. TEO at a concentration of 25 mg/ml differed

statistically significantly from trolox. This lack of effect can be explained by TEO possibly acting as a ROS. Owing to TEO having a low DQ, the lack of scavenging effect can be explained by this although TEO has a low MW. Results found by Zhao *et al.,* (2013) differ from the results found in this study. They found that chitosan oligosaccharide (5000 Da) has a high scavenging effect. This can be ascribed to the preparation conditions that differed where they dissolved the derivative in acetic acid instead of sodium chloride in this study. It is also important to note that the study by Zhao *et al*., (2013) used low concentrations of the chitosan oligomer (0.2-2 mg/ml), whereas in this study higher concentrations of TEO (5-25 mg/ml) was used. This is also the first study to specifically investigate TEO that chemically differs from chitosan oligomer.

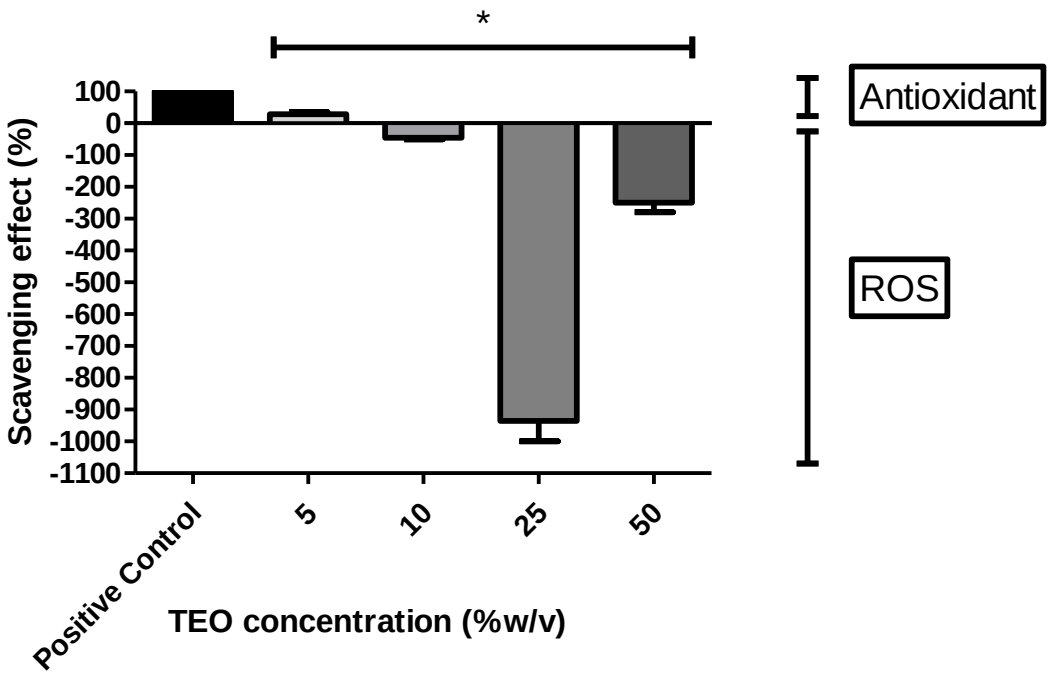


Figure 4.4: Scavenging effect (%) representing the antioxidant activity of TEO at different concentrations (5 mg/ml; 10 mg/ml; 25 mg/ml and 50 mg/ml) with trolox as the positive control which is normalised to have a 100% scavenging effect. Statistical significant differences is indicated with * ($p \leq 0.05$; n=3). The bars in the negative direction are indicative of TEO acting as a reactive oxygen species (ROS).

4.4. Antimicrobial activity

The antimicrobial activity of chitosan derivatives was determined using the disc diffusion assay, well diffusion assay, MIC and MBC assays. The antimicrobial activity of the chitosan derivatives was determined because of the known antimicrobial activity of chitosan and TMC (de Britto *et al*., 2011, Sadeghi *et al*., 2008, Wiarachai *et al*., 2012). Studies performed on carboxymethyl chitosan (Tantala *et al*., 2012) and chitosan oligomers indicated that this derivative has weak antimicrobial activity when compared to chitosan (No *et al*., 2002). According to my knowledge the rest of the derivatives have not been investigated for antimicrobial activity yet. Based on previous studies, the concentrations of the derivatives tested were 5 mg/ml; 10 mg/ml; 25 mg/ml and 50 mg/ml using the disc and well diffusion assays. The concentration used for the MIC and MBC assays were 50 mg/ml.

According to literature, the MW and DQ of chitosan and its derivatives influence the antimicrobial activity. Chitosan with a low MW will increase the antimicrobial activity against Gram- negative bacteria where a high MW will increase the antimicrobial activity against Gram- positive bacteria (No *et al*., 2002, Zheng and Zhu, 2003). Also, a high DQ at acidic conditions will decrease the antimicrobial activity whereas a high DQ at neutral conditions increase the antimicrobial activity (Rúnarsson *et al*., 2007, Xu *et al*., 2010). The high DQ means that the derivative has a high positive charge density (cationic). Cell membranes of bacteria are anionic, although Gram- negative bacteria have a higher negative charge than Gram- positive bacteria. When chitosan derivatives interact with the bacteria cell membrane, it leads to cell damage, leakage of intracellular components and eventually death (Chung *et al*., 2004, Yang *et al*., 2000).

It is important to assess the antimicrobial properties of chitosan derivatives due to their possible use in wound healing and food preservatives. Four bacterial strains were selected on the basis of their prevalence to cause skin infections and included Gram-positive strains, *S. aureus* and *S. epidermidis* and for Gram- negative, *E. coli* and *P. aeruginosa.* *S. aureus* is a coccal bacterium which is found on the skin and a common cause for skin infections such as impetigo, cellulitis, abscesses and pimples. *S. epidermidis* is a gram-positive cocci bacterium which is part of the normal human flora. Patients with compromised immune systems are more susceptible to infections caused

by this bacterium and are the reason for postoperative wound infections. *E. coli* is an anaerobic bacillus bacterium and responsible for most food poisoning which is the source of gastroenteritis and diarrhoea. *P. aeruginosa* is also a bacillus bacterium which is aerobic and patients with wound infections and burns are at high risk for this bacterium. These bacteria were used because of their role on the skin and *E. coli* is the model bacteria for experiments to be done.

The antimicrobial activity of chitosan derivatives, melittin and a combination of them was investigated on two bacterial strains using the disc diffusion and the well diffusion assay. These assays yielded no concrete results and were deemed not sensitive enough for the measurement of antimicrobial activity of chitosan derivatives (results not shown, see **Annexure 1**). Therefore the minimum inhibitory concentration (MIC) assay was performed to determine the antimicrobial activity (if derivatives inhibit growth of the bacteria) of different concentrations of chitosan derivatives and displayed in **Table 4.2 and 4.3**. The colours in the table are indicative of the effect of the samples used on the selected bacterial strains. The positive control is in blue to indicate the control; the green colours are the samples which were effective in showing antimicrobial activity against the bacteria and the samples in red was not effective in exhibiting antimicrobial activity.

4.4.1. TMC

Using the MIC assay, it was found that TMC has antimicrobial activity against the Gram-positive bacteria *S. aureus* at a concentration of 1.56 mg/ml and against *S. epidermidis* at a concentration of 4.69 mg/ml. The concentration of antimicrobial activity against *S. epidermidis* is slightly higher than for *S. aureus.* For the Gram- negative bacteria, TMC has antimicrobial activity against *E. coli* at a concentration of 1.56 mg/ml. TMC revealed no observable antimicrobial activity against *P. aeruginosa* at the highest concentration tested (12.5 mg/ml) (see **Figure 4.5** for summary). This antimicrobial activity may be attributed to TMC having a high DQ in a neutral environment. The high MW of TMC can probably explain the antimicrobial activity against the Gram- positive bacteria (*S. aureus* and *S. epidermidis*) although TMC showed antimicrobial activity against *E. coli* which is a Gram- negative bacterium. The high MW and DQ of TMC contribute to the antimicrobial

activity which is similar to what literature states of TMC. These results are similar to what is found in other studies done on TMC. These studies showed that TMC has antimicrobial activity against *S. aureus* although the TMC had different MW and DQ (Sadeghi *et al*., 2008, Wiarachai *et al*., 2012).

4.4.2. TEC

At the concentrations tested for TEC (12.5 mg/ml), no apparent antimicrobial activity was detected against any of the Gram- positive or Gram- negative strains tested. Regarding the fact that the TEC used has a low DQ and relatively high MW, the results that show it has no antimicrobial activity can be explained by these properties. To my knowledge the antimicrobial activity of TEC has not been previously determined.

4.4.3. DCMC

DCMC at a maximum concentration of 12.5 mg/ml showed no antimicrobial activity against any of the Gram- positive or Gram- negative strains tested. Due to DCMC being negatively charged, this can possibly be the reason for no antimicrobial activity shown. The only other studies performed to my knowledge, was done on carboxymethyl chitosan which showed no antimicrobial activity according to Tantala *et al.,* (2012).

4.4.4. TEO

The concentrations tested for TEO, up to 12.5 mg/ml, no evident antimicrobial activity was observed on any of the bacterial strains tested. The TEO used has a low DQ and MW which can be the cause of TEO presenting no antimicrobial activity. The results obtained corresponds to other studies done on chitosan oligomers where they also found that chitosan oligomers has weak or no antimicrobial activity against *S. aureus, E. coli* and *P. aeruginosa* (No *et al*., 2002).

4.4.5. Melittin

Melittin has antimicrobial activity against *S. aureus* at a concentration of 0.0625 mg/ml and against *E. coli* at a concentration of 0.03125 mg/ml. At the highest concentration tested of melittin namely, 0.25 mg/ml, no noticeable antimicrobial activity was detected against *S. epidermidis* and *P. aeruginosa* (see **Figure 4.5** for summary). Melittin is a known antimicrobial peptide which has known antimicrobial activity especially against Gram- positive bacteria (Al-Ani *et al*., 2015, Falco *et al*., 2013).

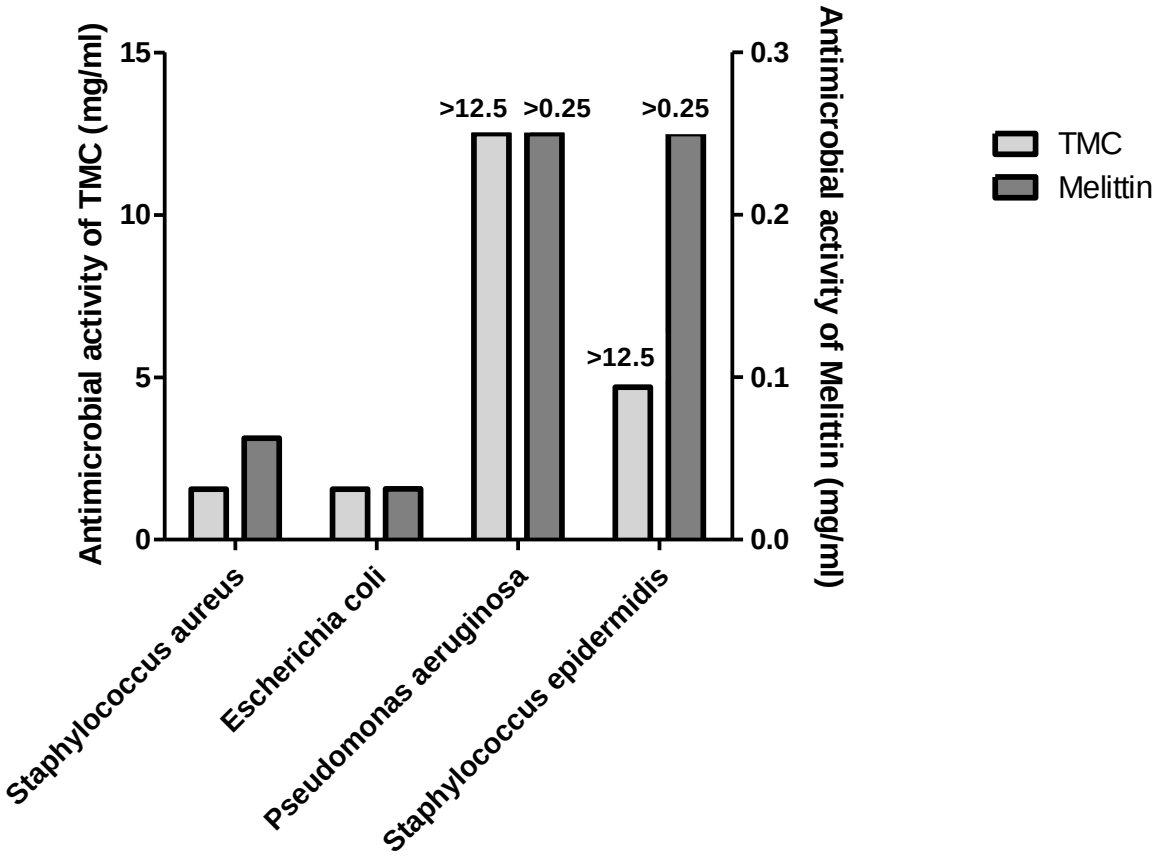


Figure 4.5: The antimicrobial activity of TMC and melittin on selected bacteria where it resulted in antimicrobial activity against *S. aureus* and *E. coli* with p≤0.05; n=3.

4.4.6. Combinations

After MIC values for each combination were determined, the fractional inhibitory concentration index (FIC) was used to determine the interaction of the samples on the

selected bacteria. The values obtained for TMC and melittin on selected bacterial strains, were 1.9 for *S. aureus* and 2.8 for *E. coli* (see **Annexure 2**). Using the FIC index, it was determined that the interaction on *S. aureus* and *E. coli* were non- interactive at the concentrations tested. The concentrations tested on *S. epidermidis* and *P. aeruginosa* was not effective. The antimicrobial activity of chitosan derivatives in combination with melittin has not to my knowledge been determined yet.

Table 4.2: MIC values of the chitosan derivatives and melittin tested on Gram- positive bacteria.

Sample	Minimum inhibitory concentration (MIC) (mg/ml)	
	Gram- positive bacteria	
	Staphylococcus aureus	*Staphylococcus epidermidis*
	ATCC #: 25923	ATCC #: 12223
Ciprofloxacin (Positive control)	0,000625	0,000078
TMC	1,56	4,69
TEC	>12.5	>12.5
DCMC	>12.5	>12.5
TEO	>12.5	>12.5
Melittin	0,0625	>0.25

TMC & Melittin	1.9	-

Table 4.3: MIC values of the chitosan derivatives and melittin tested on Gram- negative bacteria.

Sample	Minimum inhibitory concentration (MIC) (mg/ml)	
	Gram- negative bacteria	
	Escherichia coli	*Pseudomonas aeruginosa*
	ATCC #: 8739	ATCC #: 27853
Ciprofloxacin (Positive control)	0,000625	0,000625
TMC	1,56	>12.5
TEC	>12.5	>12.5
DCMC	>12.5	>12.5
TEO	>12.5	>12.5
Melittin	0,03125	>0.25
TMC & Melittin	2.8	-

To summarise, TMC and melittin have antimicrobial activity against both *S. aureus* and *E. coli*, whereas the rest of the derivatives showed no antimicrobial activity at the concentrations tested against any of the four bacterial strains. The combination of TMC and melittin showed non- interactive antimicrobial activity against *S. aureus* and *E. coli*, and were not effective against *S. epidermidis* and *P. aeruginosa* at the concentrations tested.

4.5. Cytotoxicity

4.5.1. Optimal cell count

The MTT Cell Proliferation Assay was used to determine the optimal amount of cells necessary for the performance of cytotoxicity experiments. It was found that for HepG2 cells 50 000 cells is optimal and for Caco-2 cells 20 000 cells (see **Annexure 3**).

4.5.2. Time series of different concentrations of chitosan derivatives

The time series of chitosan derivatives (5 mg/ml) is depicted in **Figure 4.6-4.9**. The cell viability of HepG2 cells treated with TMC over a time series is illustrated in **Figure 4.6.** It can be seen that after a few hours the cell viability decreases. After treating the cells with TMC for four hours, the cell viability decreases rapidly when compared to the control with 17.05 ± 12.37% after 24 hours. In **Figure 4.7** the cytotoxicity of TEC can be seen over a time series. A decrease in cell viability can also be observed in cells treated with TEC, but it is far less pronounced than that observed in cells treated with TMC with 73.24 ± 27.18% after 24 hours. The cell viability of the DCMC treated cells also decreased after two hours of treatment as seen in **Figure 4.8** and after 24 hours 23.96 ± 19.20% statistically differed from the negative control. TEO caused the cell viability to decrease only after four hours of treatment (**Figure 4.9**). When compared to the rest of the derivatives, TEO least affected the cell viability over a 24 hour period with 74.30 ± 23.00%. Therefore it was decided to continue all the cytotoxicity studies at an incubation period of 4 hours.

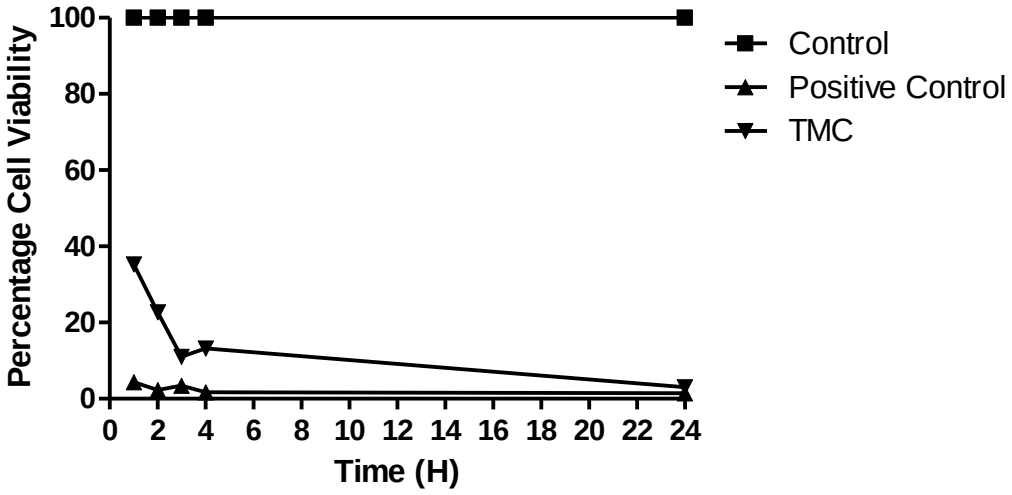


Figure 4.6: Cell viability of HepG2 cells treated with TMC for different time intervals using the MTT assay. Triton X-100 was used as a positive control and the cell control was set as 100% viable. n=3.

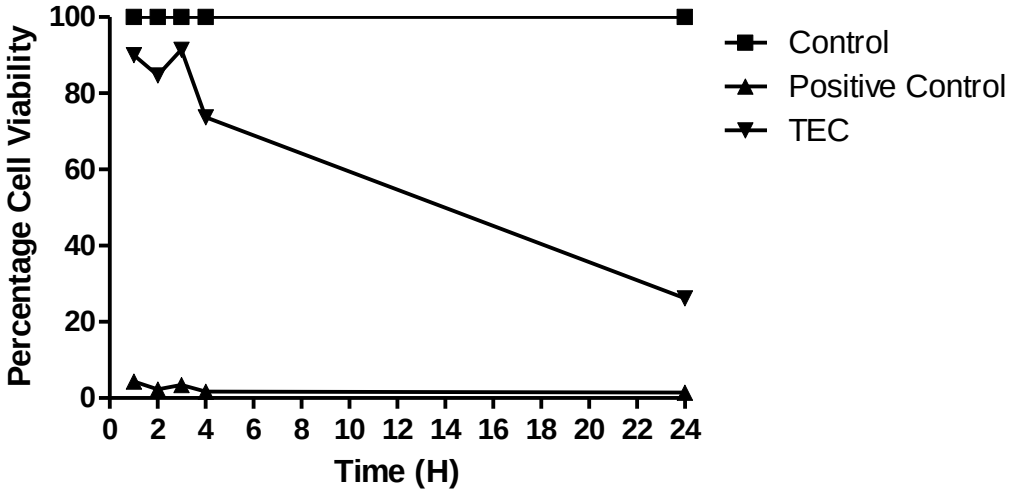


Figure 4.7: Cell viability of HepG2 cells treated with TEC for different time intervals using the MTT assay. Triton X-100 was used as a positive control and the cell control was set as 100% viable. n=3.

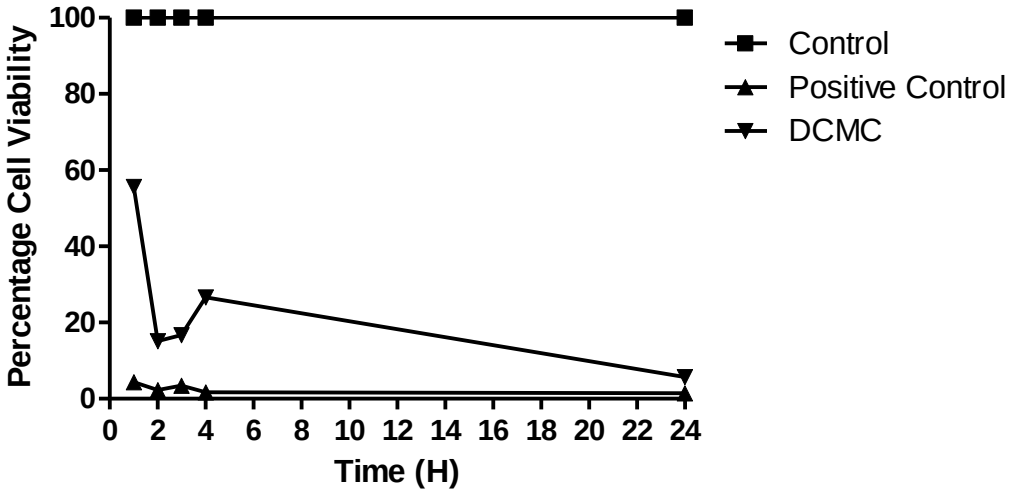


Figure 4.8: Cell viability of HepG2 cells treated with DCMC for different time intervals using the MTT assay. Triton X-100 was used as a positive control and the cell control was set as 100% viable. n=3.

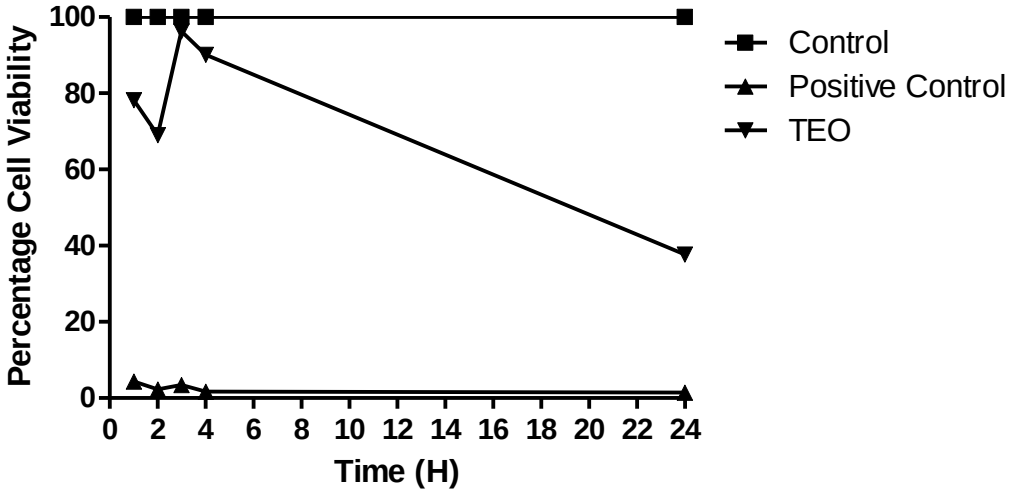


Figure 4.9: Cell viability of HepG2 cells treated with TEO for different time intervals using the MTT assay. Triton X-100 was used as a positive control and the cell control was set as 100% viable. n=3.

4.5.3. Cytotoxic concentration series of chitosan derivatives

A concentration series of chitosan derivatives were performed on both HepG2 and Caco-2 cell lines using the MTT and LDH assays. The MTT and LDH measure different endpoints of cytotoxicity. This is important to characterise the mode of cell death. This was done to assess the cytotoxicity of the derivatives of four different concentrations which is depicted in **Figure 4.10-4.15**. The concentrations that were used were 5 mg/ml; 10 mg/ml; 25 mg/ml, 50 mg/ml and 75 mg/ml. Triton X-100 was used as the positive control and the negative control consisted of cells maintained in serum free media.

Based on a study by Fischer *et al.,* (2003), they determined that polymers with a high MW will have increased cytotoxicity. Also, polymers with high cationic charges will increase the cytotoxicity (Fischer *et al*., 2003). Therefore it can be presumed that chitosan and its derivatives with high MW and high DQ will have a negative effect on cytotoxicity.

According to my knowledge, no cytotoxicity experiments of chitosan derivatives were done on HepG2 and Caco-2 cells. There was however a study done on the cytotoxicity of polymers on L929 mouse fibroblasts by Fischer *et al.,* (2002) and Enslin *et al.,* (2008) investigated the absorption enhancing abilities of TMC on Caco-2 cells.

4.5.3.1. HepG2 cells

The cytotoxicity of the concentration series of chitosan derivatives on HepG2 cells indicated that most derivatives had little cytotoxic effects at low concentrations with the exception of TMC which is cytotoxic at all concentrations tested (**Figure 4.10**). Lower cell viability could be observed at higher concentrations of most derivatives.

The MTT assay indicated that all TMC concentrations tested (5 mg/ml; 10 mg/ml; 25 mg/ml, 50 mg/ml and 75 mg/ml) had an overwhelming negative impact on HepG2 cell viability (**Figure 4.10 A**). It was also observed that compared to the negative control, TMC displayed a concentration dependent decrease in cell viability with 27.35 ± 1.48%, 24.56 ± 1.19%, 19.66 ± 2.05% and 17.70 ± 1.54% at 5 mg/ml, 10 mg/ml, 25 mg/ml and 50 mg/ml of TMC respectively. The cytotoxicity of TMC may be explained by the high MW and DQ of this derivative and is similar to what Fischer *et al.* (2002) found.

HepG2 cells treated with TEC showed much higher cell viability compared to TMC treated cells and a concentration dependant increase in toxicity could also be observed (**Figure 4.10 B**). The results of TEC having less cytotoxic effects on HepG2 cells can be associated with the low DQ and relatively low MW.

The MTT assay indicated that HepG2 cells treated with DCMC had high cell viability and toxicity is also concentration dependant (**Figure 4.10 C**). As the concentration increased, the cell viability decreased which also indicated a concentration dependent decrease in cell viability with 27.08 ± 3.15% at 50 mg/ml of DCMC. There is a drastically difference in cell viability from a concentration of 25 mg/ml which is still viable, to a concentration of 50 mg/ml which is not viable. Considering DCMC is negatively charged it can affect the cytotoxicity and is therefore theoretically less cytotoxic than TMC which is positively charged with a high DQ.

HepG2 cells treated with TEO showed higher cell viability at the concentrations tested and is similar to what TEC displayed (**Figure 4.10 D**). TEO also showed a concentration dependant increase in toxicity. The reason for this can also be explained by the low DQ and low MW of TEO. The high concentrations of TEO show a decrease in cell viability can possibly be ascribed by TEO being insoluble at high concentrations.

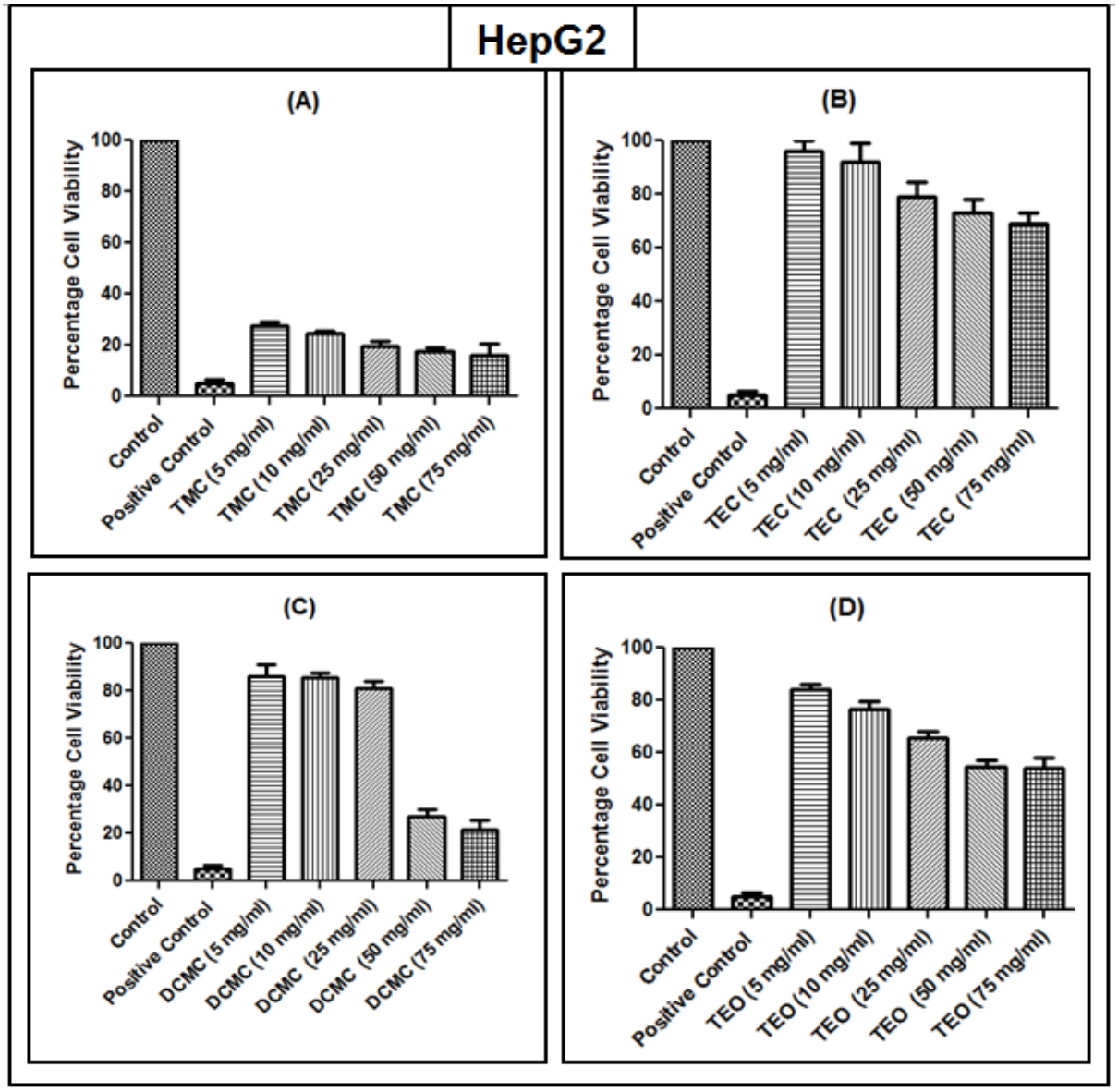


Figure 4.10: HepG2 cells treated with different concentrations (5-75 mg/ml) of chitosan derivatives to determine the percentage cell viability using the MTT assay. The control (cells in serum free media) is normalised to have 100% cell viability with mean±SD and n=3. A) TMC; B) TEC; C) DCMC and D) TEO.

To determine if low cell viability observed in TMC treated cells are due to membrane damage, the LDH assay was used. For all the concentrations tested for TMC, it is clear from the results that high levels of LDH leaked from HepG2 cells into the cell media which indicate substantial cell membrane damage (**Figure 4.11**). The increased LDH leakage as the concentration of TMC increased can be compared to the MTT assay which also indicated a concentration dependant increase in toxicity. The increased LDH leakage

with an increase in concentration differed statistically significant from the negative control with 99.08 ± 1.59% at 50 mg/ml of TMC.

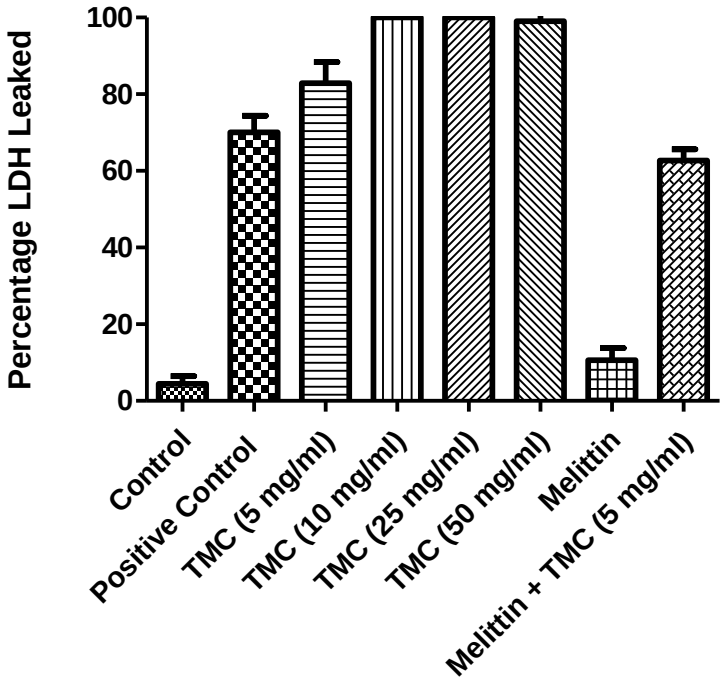


Figure 4.11: HepG2 cells treated with different concentrations of TMC (5 mg/ml; 10 mg/ml; 25 mg/ml and 50 mg/ml) using the LDH assay. The control (cells in serum free media) is normalised to have 100% cell viability with mean±SD. n=3.

Using a light microscope, cellular morphology was visually investigated after exposure to the different chitosan derivatives (**Figure 4.12**). The negative control (**Figure 4.12 a**) indicates how the HepG2 cells appear under normal cell culture conditions. The cellular morphology changed when treated with the different derivatives in comparison to the untreated cells. TMC (**Figure 4.12 b**) and TEC (**Figure 4.12 c**) indicated that the cells are squarer in shape and less spherical cells occurred. DCMC (**Figure 4.12 d**) and TEO (**Figure 4.12 e**) is more spherical shaped. It is clear from these results that most chitosan derivatives have some influence on HepG2 cellular morphology. This is interesting and should be further investigated to determine if these morphology changes have any long term cytotoxic effects. Although cellular morphology is not a direct indication of cell viability, it is clear that chitosan derivatives can affect the cell and induce phenotypical changes.

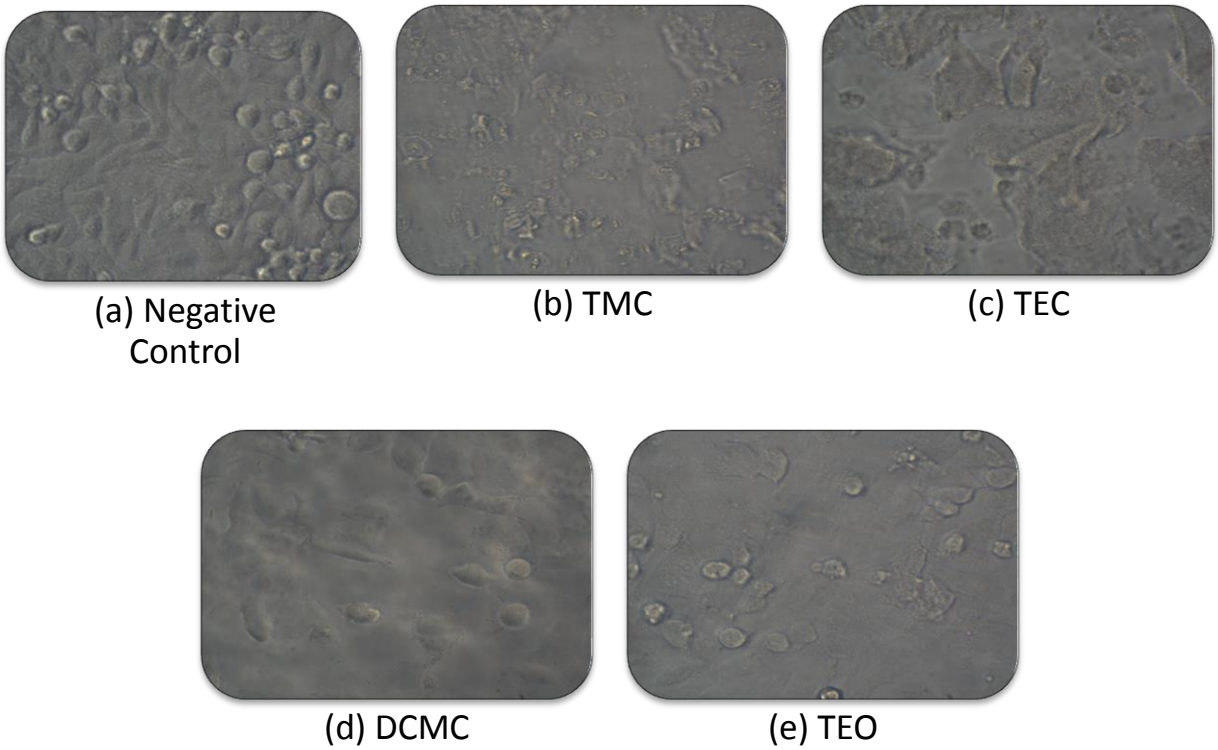


Figure 4.12: Microscopic image of (a) Untreated HepG2 cells; (b) HepG2 cells treated with TMC (5 mg/ml) where the morphological changes is evident; (c) HepG2 cells treated with TEC (5 mg/ml); (d) HepG2 cells treated with DCMC (5 mg/ml); (e) HepG2 cells treated with TEO (5 mg/ml).

4.5.3.2. Caco-2 cells

The cytotoxicity of chitosan derivatives was also determined on the intestinal Caco-2 cell line using the MTT assay. The results are expressed as a percentage of the negative control, which is assumed to be 100% viable (**Figure 4.13**).

Cells treated with different concentrations of TMC again showed the lowest cell viability (**Figure 4.13 A**). Again TMC indicated a concentration dependent decrease in cell viability when compared to the negative control with 33.90 ± 3.40%, 32.43 ± 3.17%, 34.95 ± 4.34% and 26.82 ± 3.32% at 5 mg/ml, 10 mg/ml, 25 mg/ml and 50 mg/ml of TMC

respectively. The high MW and DQ may influence the low cell viability of TMC which is also observed in the HepG2 cells.

Caco-2 cells treated with TEC showed no difference in cytotoxicity when compared to the control (**Figure 4.13 B**). TEC has a low DQ and also relatively low MW in comparison to TMC which causes the high cell viability.

DCMC only indicated toxicity at the highest concentration (50 mg/ml) tested with 34.45 ± 9.46% at 50 mg/ml (**Figure 4.13 C**). Again the fact that DCMC is negatively charged influence the effect on the cytotoxicity on Caco-2 cells as well. At a concentration of 50 mg/ml the decrease in cell viability can possibly be attributed to DCMC being insoluble at high concentrations.

TEO showed no cytotoxicity at any concentration of these derivatives on Caco-2 cells (**Figure 4.13 D**). The TEO used has a low DQ and also the lowest MW of the derivatives used which can explain the high cell viability.

In general, Caco-2 cells seem to be more tolerable to most chitosan derivatives tested compared to HepG2 cells. It is important to note that many of the studies using TMC with various MW and DQ for absorption enhancement used Caco-2 cells. They also investigated the toxicity of these TMC using the MTT method. In most of the cases low cell toxicity was found, proving TMC a safe absorption enhancer.

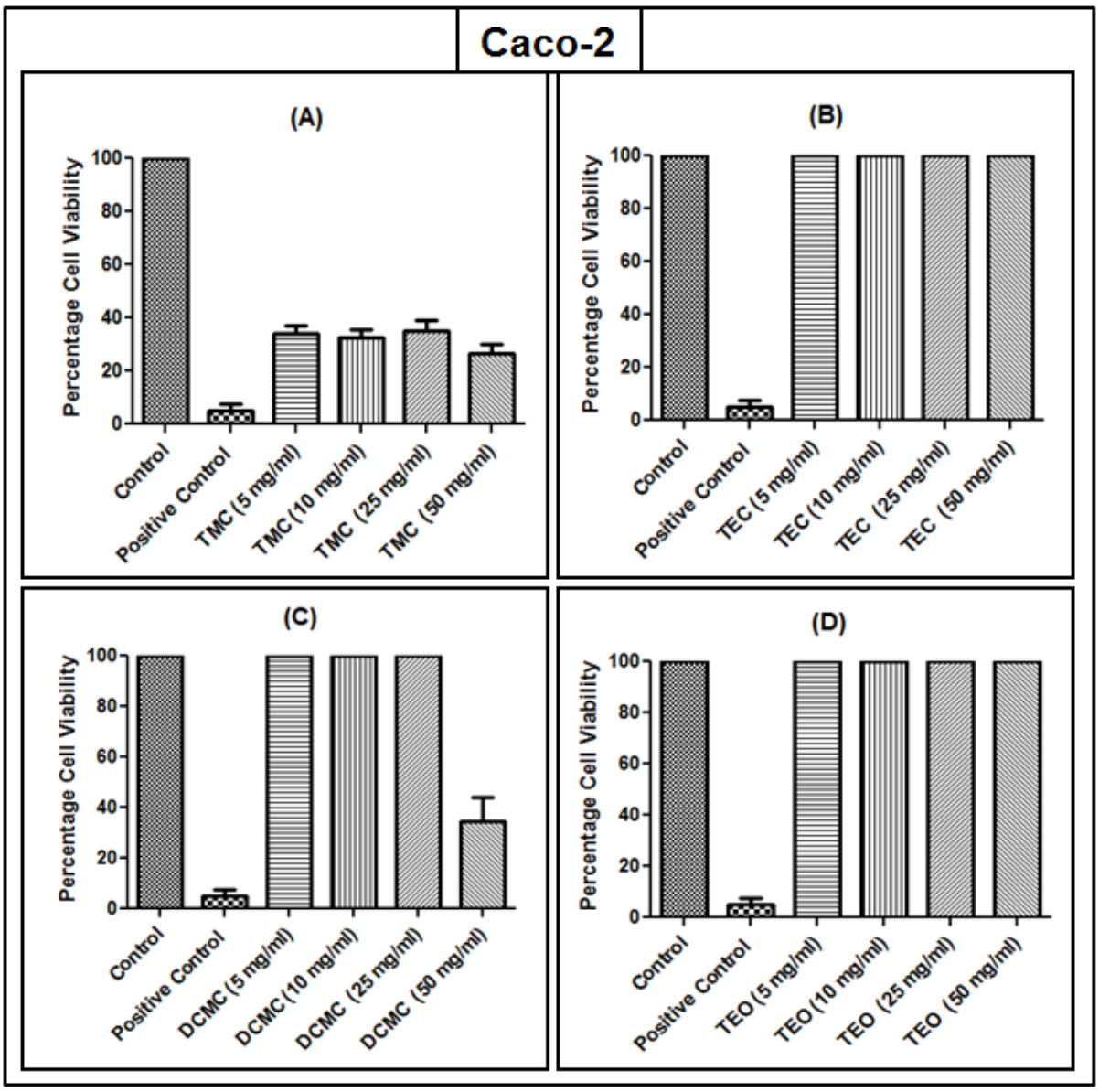


Figure 4.13: Caco-2 cells treated with different concentrations of chitosan derivatives to determine the percentage cell viability using the MTT assay. The control (cells in serum free media) is assumed to have 100% cell viability with mean±SD and n=3. A) TMC; B) TEC; C) DCMC and D) TEO.

The effect of TMC on Caco-2 cell membrane integrity was also investigated with the LDH assay. All the concentrations tested of TMC causes extensive cell membrane damage and seem to be concentration dependent (**Figure 4.14**). This concentration dependant decrease in cell viability correlates with the results found with the MTT assay and differs statistically from the negative control with 72.68 ± 4.75% at 50 mg/ml of TMC. These results are a good indication that TMC causes cell death through damaging the cellular membranes.

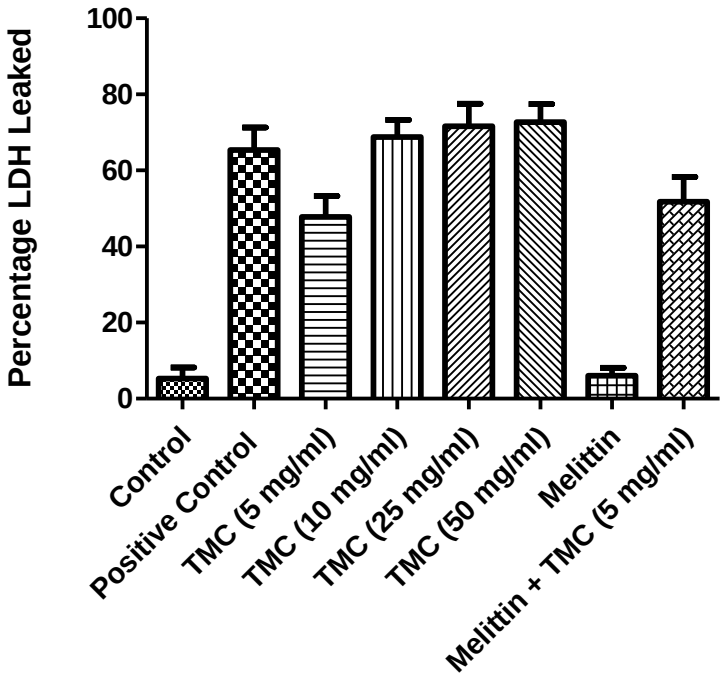


Figure 4.14: Caco-2 cells treated with different concentrations of TMC (5 mg/ml; 10 mg/ml; 25 mg/ml and 50 mg/ml) using the LDH assay. The control (cells in serum free media) is assumed to have 100% cell viability with mean±SD. n=3.

The derivatives effect on cellular morphology was also investigated for Caco-2 cells where morphological changes were apparent (**Figure 4.15**). The negative control indicate the appearance of Caco-2 cells under normal cell culture condition (**Figure 4.15 a**). TMC (**Figure 4.15 b**) and TEC (**Figure 4.15 c**) indicated that the cells are squarer in shape and more irregular. DCMC (**Figure 4.15 d**) and TEO (**Figure 4.15 e**) is more circular in shape and forms clumps in comparison to the untreated cells. The cells look like it is elevated and convex.

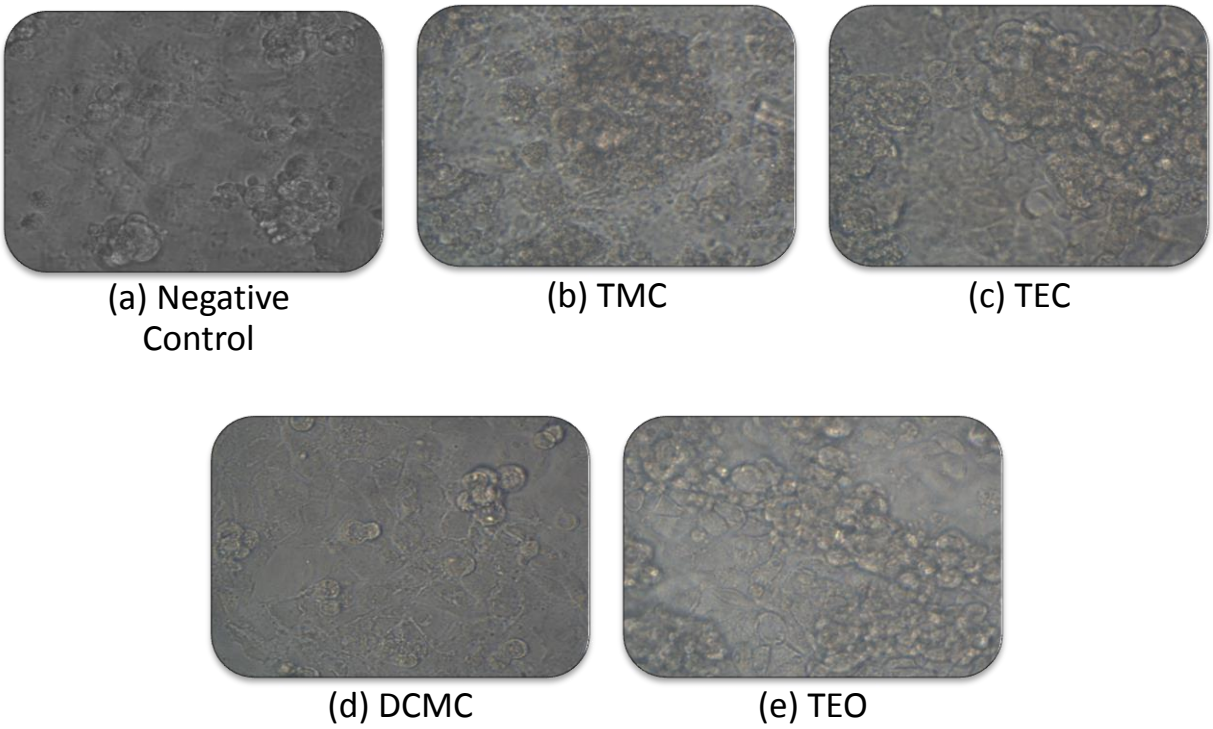


Figure 4.15: Microscopic image of (a) Untreated Caco-2 cells; (b) Caco-2 cells treated with TMC (5 mg/ml); (c) Caco-2 cells treated with TEC (5 mg/ml); (d) Caco-2 cells treated with DCMC (5 mg/ml); (e) Caco-2 cells treated with TEO (5 mg/ml).

4.5.4. Cytotoxic concentration series of melittin

A concentration series of melittin was performed using the MTT assay on HepG2 cells (**Figure 4.16**) and also the LDH assay on HepG2 (**Figure 4.17**) and Caco-2 cells (**Figure 4.18**). The concentration series of melittin used were 0.62 µM; 1.25 µM; 2.5 µM; 5 µM and 10 µM. The purpose of this is to determine the cytotoxicity of melittin in these concentrations on the specific cell lines.

It was observed that the higher the concentration of melittin, the more cytotoxic the concentration is to both cell lines. For both the MTT and LDH assays, the percentage cell viability of both HepG2 and Caco-2 cells decreased when the concentration of melittin increased. It is also clear from the LDH assay that melittin causes considerable cell

membrane damage which is concentration dependant, as have been observed in previous studies with 52.54 ± 38.68% for HepG2 cells and 48.49 ± 38.18% for Caco-2 cells which differed statistically from the control (Maher *et al*., 2010, Maher and McClean, 2008). Studies have shown that melittin is used in cancer therapy and from these results it can be speculated that melittin is indeed a probable treatment against cancer although further research is required (Jo *et al*., 2012, Liu *et al*., 2002).

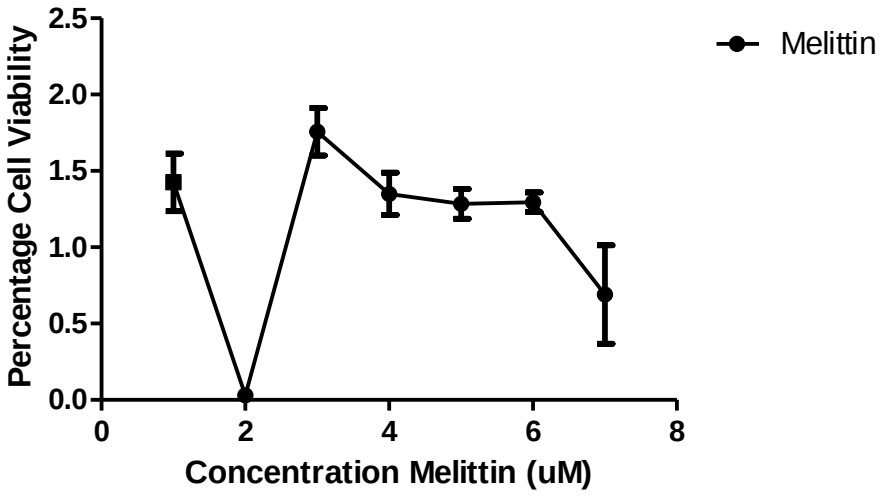


Figure 4.16: Cell viability of HepG2 cells treated with different concentrations of melittin (MTT). Triton X-100 was used as a positive control and the cell control was set as 100% viable. n=3.

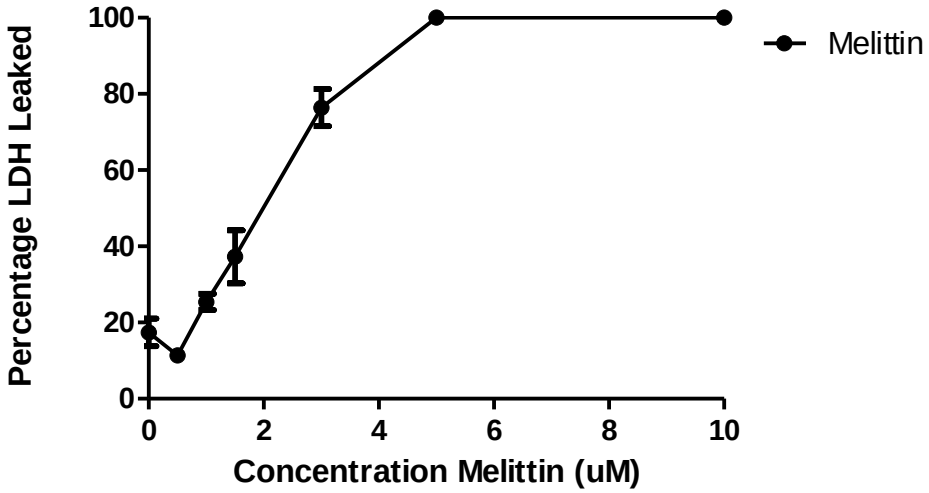


Figure 4.17: Amount of LDH leakage from HepG2 cells treated with melittin at different concentrations. Triton X-100 was used as a positive control and the cell control was set as 100% viable with mean±SD. n=3.

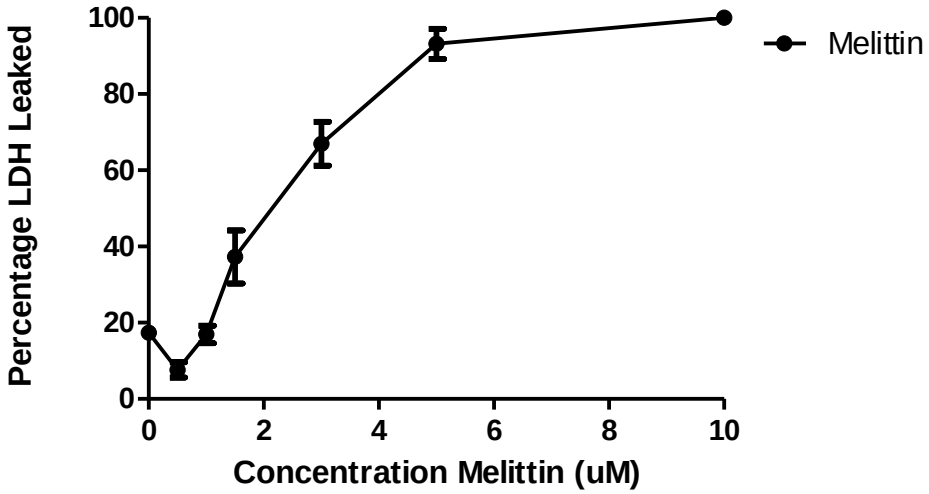


Figure 4.18: Amount of LDH leaked from Caco-2 cells treated with melittin at different concentrations (LDH). Triton X-100 was used as a positive control and the cell control was set as 100% viable with mean±SD. n=3.

4.5.5. Chitosan derivatives in combination with melittin

It has been shown that chitosan derivatives in combination with melittin enhance absorption in intestinal cells (Enslin *et al*., 2008). As part of this study, the cytotoxicity of chitosan derivatives in combination with melittin were investigated on HepG2 and Caco-2 cell lines using the MTT and LDH assays. The concentrations of the derivatives that were used were 25 mg/ml and for melittin 1.25 µM.

The combination of the derivatives and melittin resulted in decreased cell viability after treating HepG2 cells in comparison to individual treatment (**Figure 4.19**). The combination of TMC and melittin differed statistically from the negative control at 19.93 ± 7.26%. Thus the combination treatment appears to have a cytotoxic effect on HepG2 cells compared to cells only treated with melittin.

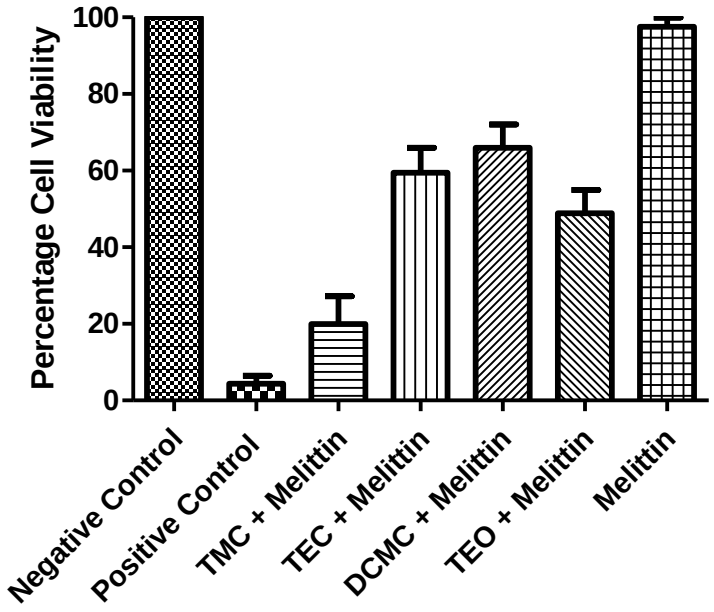


Figure 4.19: HepG2 cells treated with a combination of chitosan derivatives and melittin (MTT) with Triton X-100 as the positive control. The control (cells in serum free media) is assumed to have 100% cell viability with mean±SD. n=3.

In contrast to the HepG2 cells, the Caco-2 cell viability was not affected by the combination of derivatives and melittin (**Figure 4.20**). Only the combination of TMC and

melittin showed decreased cell viability when compared with the negative control at 34.01 ± 5.61%. This may be due to membrane differences between the two cell lines and the presence of tight junctions in intestinal cells.

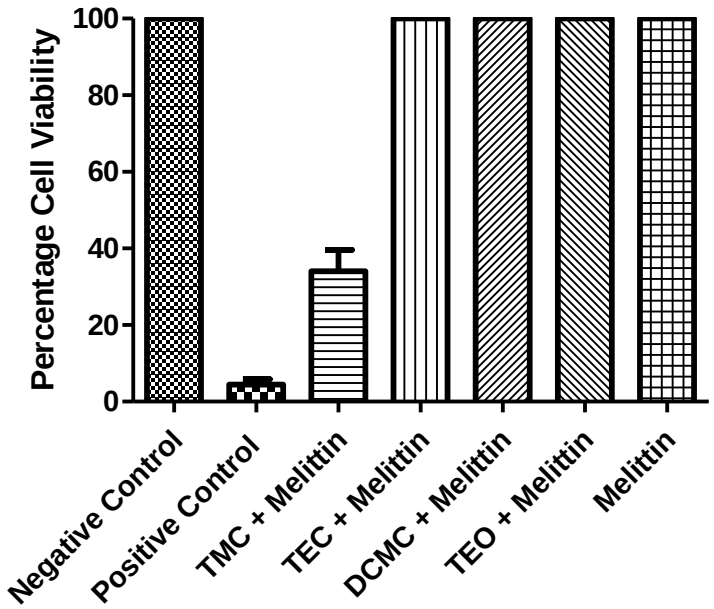


Figure 4.20: Caco-2 cells treated with a combination of chitosan derivatives and melittin (MTT) with Triton X-100 as the positive control. The control (cells in serum free media) is assumed to have 100% cell viability with mean±SD. n=3.

4.5.6. Flow cytometry

Flow cytometry was used to establish if the cell death caused by TMC is apoptotic or necrotic in origin. Apoptosis is programmed cell death while necrosis is accidental cell death due to trauma or injury to the cell. It is important to distinguish between these two cell deaths pathways if the mechanism of cytotoxicity of TMC is to be understood.

Cultured HepG2 and Caoc-2 cells were treated with a concentration series of TMC (5 mg/ml; 10 mg/ml; 25 mg/ml, 50 mg/ml) and analysed with the FITC Annexin V Apoptosis Detection kit using flow cytometry. Melittin and a combination of melittin and TMC (5 mg/ml) were also assessed. Triton-X 100 was used as the positive control and cells in serum free media served as the negative control.

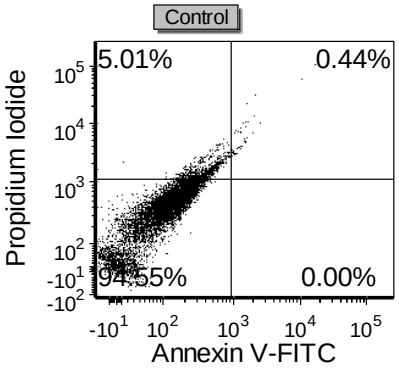
The flow cytometric representative dotplots of HepG2 cells is depicted in **Figure 4.21**. The Annexin V-FITC is plotted on the x-axis and an increase in fluorescence indicates increased Annexin V detection, a process of apoptosis. The PI is plotted on the y-axis and an increase in fluorescence indicates increased DNA binding due to permeable cell membranes, a process of necrosis. In **Figure 4.21 A**, 94.55% of the untreated cells (negative control) are displayed in the lower left quadrant which indicates the cells are viable. HepG2 cells treated with Triton X-100 (positive control) indicate that 95.46% of the cells are undergoing late apoptosis or necrosis and is indicated in the right upper quadrant (**Figure 4.21 B**). This is expected from Triton X-100, as it causes extensive membrane damage. For TMC 5 mg/ml (**Figure 4.21 C**), most of the cells (71.00%) are undergoing late apoptosis whereas 18.83% of the cells are in early apoptosis which can be seen in the lower right quadrant. TMC 10 mg/ml (**Figure 4.21 D**) displayed an amount of 49.16% cells undergoing late apoptosis or necrosis and 37.89% of cells undergoing early apoptosis. For TMC at a concentration of 25 mg/ml (**Figure 4.21 E**), 46.33% of cells are in late apoptosis or necrosis and 53.31% in early apoptosis. TMC at a concentration of 50 mg/ml (**Figure 4.21 F**) indicated that 40.84% of cells are undergoing necrosis or late apoptosis whereas 41.07% are in early apoptosis. This effect of TMC largely correlates with the MTT and LDH assays which indicate that cells treated with TMC is less viable than untreated cells. It can be deduced from the flow cytometry results that TMC induces apoptosis in HepG2. Additionally, a large population of cells seem to be undergoing late apoptosis or necrosis after 4 hours exposed to TMC. The FITC Annexin V Apoptosis Detection kit does not discriminate between late apoptosis and necrosis and this finding should be further investigated.

HepG2 cells treated with melittin (**Figure 4.21 G**), showed that 45.76% of cells are in early apoptosis and 44.52% in late apoptosis or necrosis. It is interesting to note that in contrast to these results, the MTT and LDH assays indicated that HepG2 cells treated with 1.25 µM melittin were still viable.

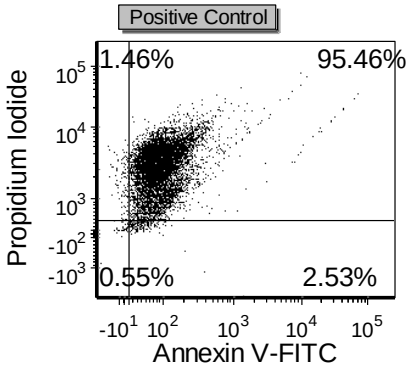
In apoptotic cells, the membrane phospholipid, phosphatidylserine, is translocated from the inner to the outer surface of the plasma membrane, thus exposing it to the external cellular environment. Annexin V can then bind to this translocated phospholipid and is interpreted by the software as early apoptosis. It should be kept in mind that this translocation is reversible and does not necessarily lead to cell death. Although flow

cytometry indicates that a large population of cells are undergoing “early” apoptosis after treatment with melittin, this not necessarily results in programmed cell death. The only study using flow cytometry to determine melittin’s cytotoxicity to my knowledge was by Černe *et al.,* (2013), where they used HUVEC cells (Human umbilical vein endothelial cells) and determined that melittin causes cell death at high concentrations.

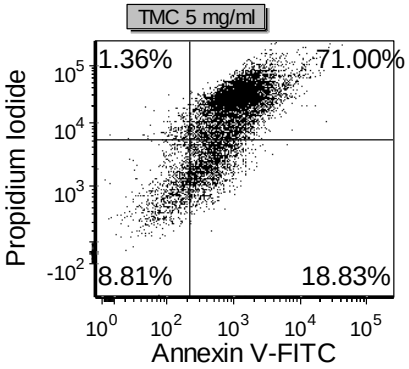
The combination of TMC (5 mg/ml) and melittin are shown in **Figure 4.21 H.** Most of the cells (83.03%) are undergoing late apoptosis or necrosis and only a small amount of 13.96% are in early apoptosis. This effect also correlates with the results of the MTT and LDH assay. The possible reason for this is that melittin acts as a potentiating compound. When in combination with another compound, melittin decreases the cell viability which was also found in the MTT and LDH assays.



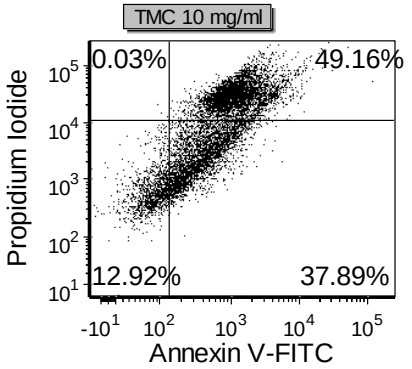
(A)



(B)



(C)



(D)

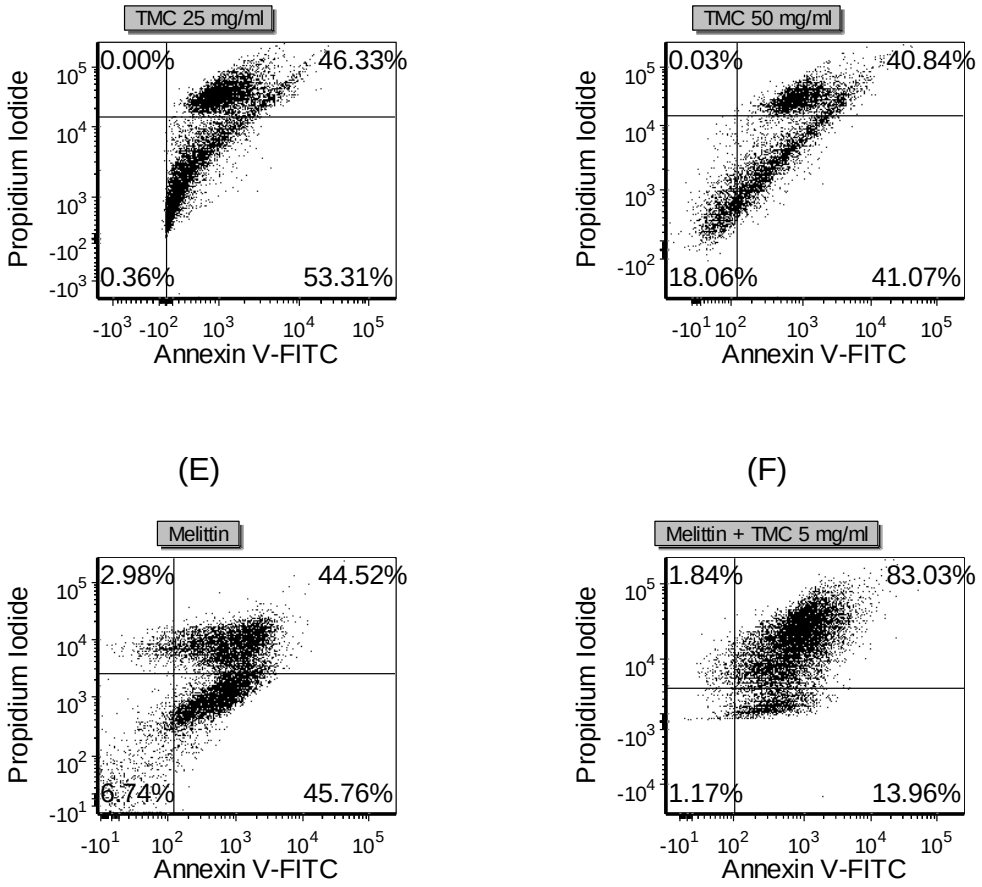


Figure 4.21: Flow cytometric representative dotplots of HepG2 cells analysed for Annexin V binding. Gating of 10 000 cells was performed with regards to propidium iodide and Annexin V-FITC fluorescence. Background labelling of living cells is found in the lower left quadrant, whereas apoptotic cells are shown in the lower right quadrant, stained with Annexin V only. Necrotic cells labelled by both PI and Annexin V-FITC are found in the upper right quadrant. Data are presented on a bi-exponential scale that allows accurate visualisation of population with low or background fluorescence. A) Untreated HepG2 cells; B) Cells treated with Triton X-100; C) HepG2 cells treated with TMC (5 mg/ml); D) HepG2 cells treated with TMC (10 mg/ml); E) HepG2 cells treated with TMC (25 mg/ml); F) HepG2 cells treated with TMC (50 mg/ml); G) HepG2 cells treated with melittin; H) HepG2 cells treated with a combination of melittin and TMC (5 mg/ml).

To summarise, the effects of TMC and melittin on HepG2 cells is depicted in **Figure 4.22**. It can be seen that TMC is inclined to cause late apoptosis or necrosis especially at a concentration of 50 mg/ml. The combination of TMC and melittin indicate more late apoptosis or necrosis.

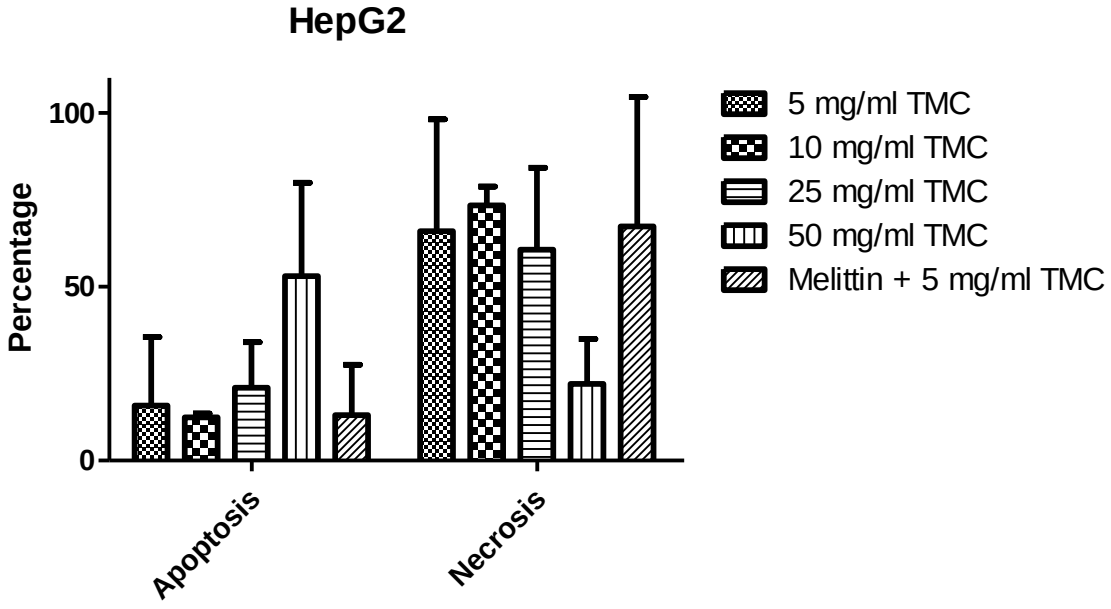


Figure 4.22: Summary of the percentage apoptotic and necrotic cells of experiment on HepG2 cells.

In **Figure 4.23** and **Figure 4.24** the fluorescence intensity of the various treatments on HepG2 cells is displayed. When compared to Caco-2 cells, it can be seen that HepG2 is more sensitive when considering the fluorescence intensity.

All 4 treatments on the HepG2 cells resulted in increased fluorescence intensity compared to the control. These results suggest that all of the treatments elicited some amount of Annexin V-FITC binding (**Figure 4.23**). Propidium iodide is cell permeable and excluded from live cells. It binds to DNA and is a good indicator of DNA content. Necrotic cells are defined by a lack of integrity of cell membrane, whereas in apoptotic cells the cell membranes stay intact. At a late stage in the apoptotic process, enzymes known as endonucleases, break down linkages between nucleosomes. This results in small DNA fragments in a process known as DNA fragmentation. This is easily observably PI- histograms. In **Figure 4.24** the amount of DNA stained by PI in HepG2

cells can be observed for the different treatments. The control is shown as the black histogram. A bi- fluorescent histogram can be observed for melittin, TMC (10 mg/ml) and TMC (50 mg/ml). This indicates that a portion of the cells is still undergoing apoptosis, where a large part is already dead.

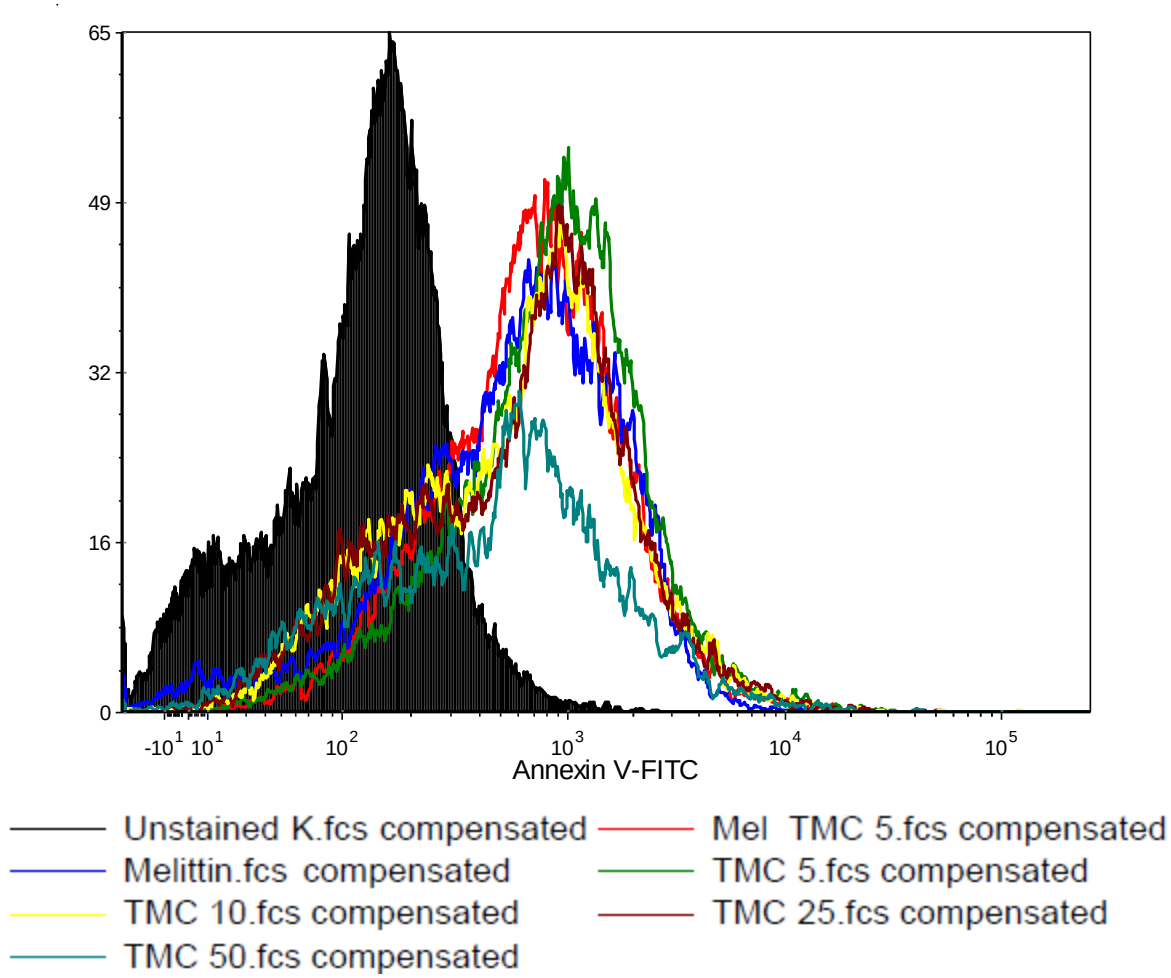


Figure 4.23: Overlay histogram indicating the intensity of Annexin V-FITC staining of HepG2 cells with permeable cell membranes.

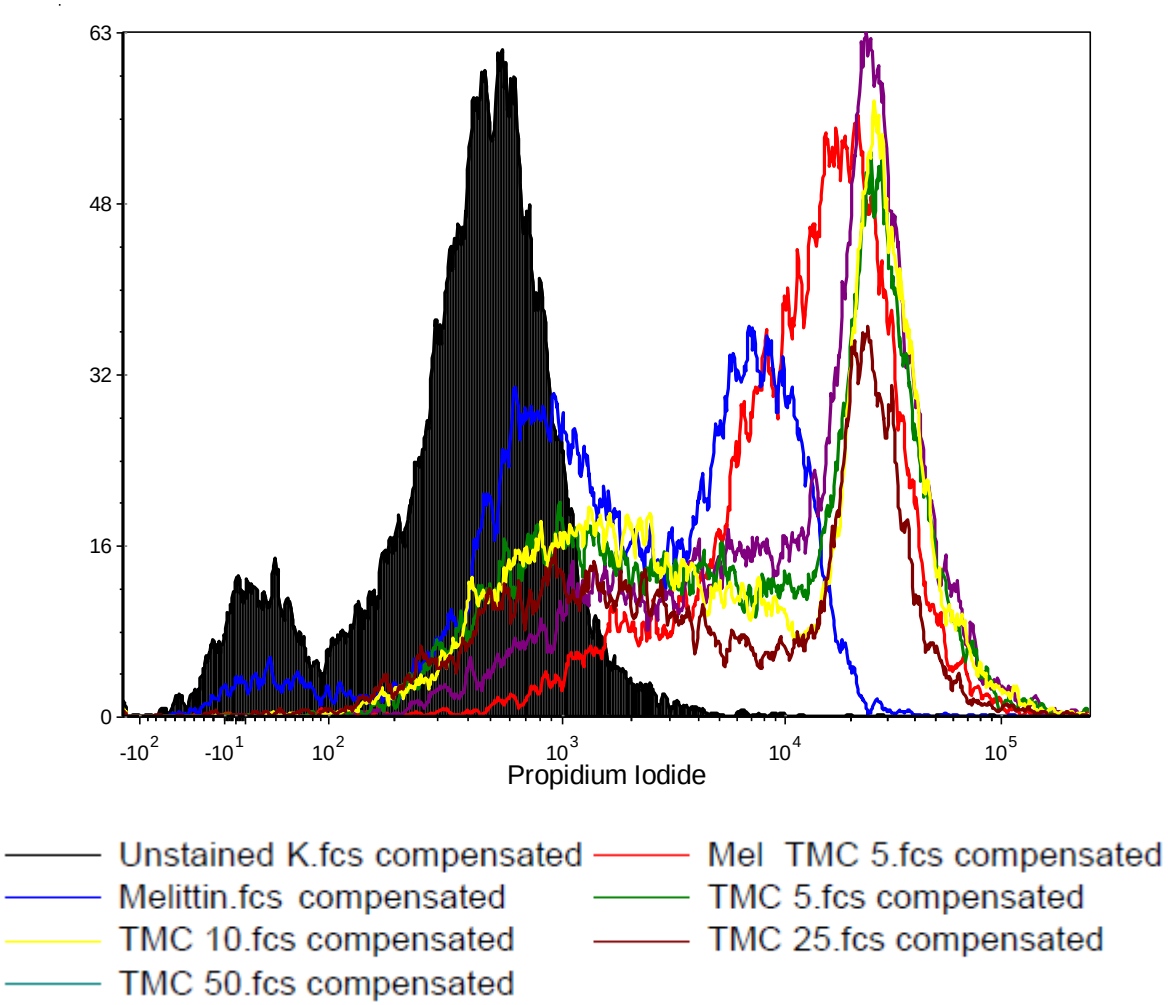


Figure 4.24: Overlay histogram indicating the intensity of propidium iodide staining of HepG2 cells with permeable cell membranes. Bi- fluorescent histogram indicating various levels of DNA staining.

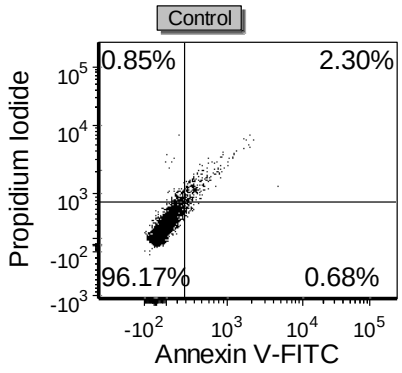
The flow cytometric representative dotplots of Caco-2 cells is depicted in **Figure 4.25**. In **Figure 4.25 A**, 96.17% of the untreated cells (negative control) are displayed in the lower left quadrant which indicates the cells are viable. Caco-2 cells treated with Triton X-100 (positive control) indicate that 93.55% of the cells are undergoing late apoptosis or necrosis and is indicated in the right upper quadrant (**Figure 4.25 B**). For TMC 5 mg/ml (**Figure 4.25 C**), 69.98% of the cells are undergoing late apoptosis or necrosis and only 2.46% early apoptosis. TMC 10 mg/ml (**Figure 4.25 D**) displayed an amount of 76.34% cells undergoing late apoptosis or necrosis and 7.44% of cells undergoing early apoptosis. For TMC at a concentration of 25 mg/ml (**Figure 4.25 E**), 75.79% of cells are in late apoptosis or necrosis and 10.77% in early apoptosis. TMC at a concentration of 50 mg/ml (**Figure 4.25 F**) indicated that 72.33% of cells are undergoing necrosis or late

apoptosis whereas 11.70% are in early apoptosis. This illustrated that as the concentration of TMC increased, the cells became less viable. This effect of TMC correlates with the MTT and LDH assays which indicate that cells treated with TMC is less viable than untreated cells. Due to the cells which appeared mostly in the upper right quadrant and only somewhat in the lower right quadrant, it can be deducted that Caco-2 cells treated with TMC undergoes necrosis. This is maybe due to TMC being more specific to tight junctions in Caco-2 cells than HepG2 cells.

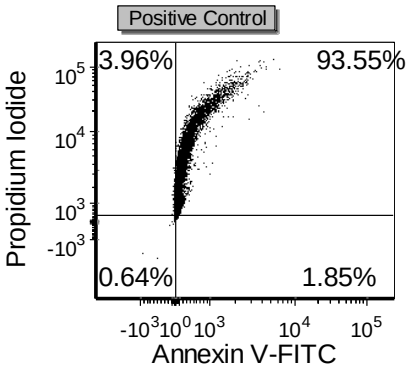
Caco-2 cells treated with melittin (**Figure 4.25 G**), showed that 29.60% of the cells are still viable, 20.65% of cells are in early apoptosis and 45.20% in late apoptosis or necrosis. These result that half of the cells treated with melittin is still viable and half of the cells are not viable, can be compared to the MTT and LDH assay where some of the cells are still viable depending on the concentration of melittin. The only study using flow cytometry to determine melittin's cytotoxicity to my knowledge was by Černe *et al.,* (2013), where they used HUVEC cells and determined that melittin causes cell death at high concentrations.

The combination of TMC (5 mg/ml) and melittin are shown in **Figure 4.25 H.** Most of the Caco-2 cells (77.11%) are undergoing late apoptosis or necrosis and only a small amount of 10.41% are in early apoptosis. A small amount of the cells (7.61%) are still viable. This effect also correlates with the results of the MTT and LDH assay. The possible reason for this is that melittin acts as a potentiating compound. When in combination with another compound, melittin decreases the cell viability which was also found in the MTT and LDH assays.

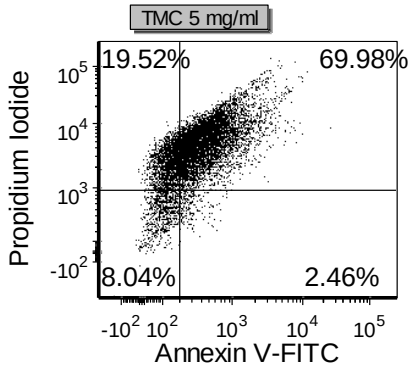
These results can be interpreted that Caco-2 cells most probably causes necrosis because of all the cells in most of the treatments, appeared in the right upper quadrant to indicate necrosis. The minimum cells appeared in the lower right quadrant which can also contribute to the conclusion of necrosis as mechanism of decreased cell viability. To my knowledge, this is the first flow cytometry study done on the mechanism of cell death on HepG2 and Caco2 cells.


Control
0.85%
2.30%
96.17%
0.68%
Propidium Iodide
Annexin V-FITC

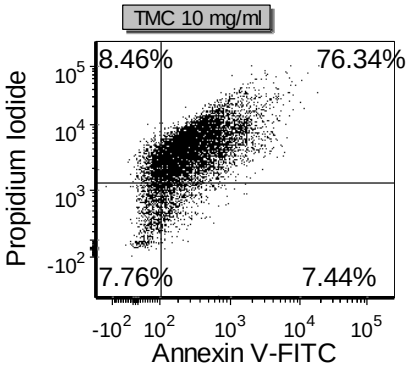
(A)


Positive Control
3.96%
93.55%
0.64%
1.85%
Propidium Iodide
Annexin V-FITC

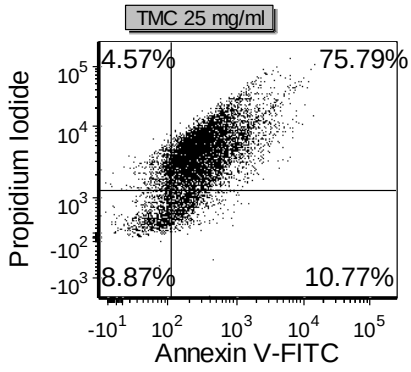
(B)


TMC 5 mg/ml
19.52%
69.98%
8.04%
2.46%
Propidium Iodide
Annexin V-FITC

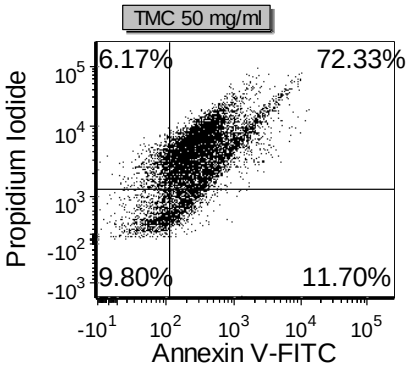
(C)


TMC 10 mg/ml
8.46%
76.34%
7.76%
7.44%
Propidium Iodide
Annexin V-FITC

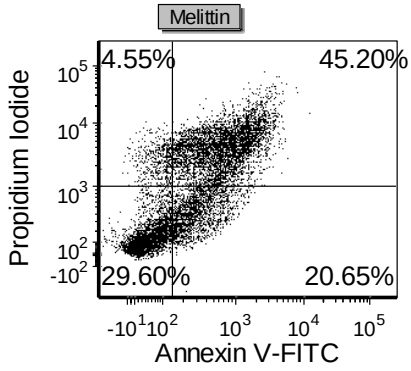
(D)


TMC 25 mg/ml
4.57%
75.79%
8.87%
10.77%
Propidium Iodide
Annexin V-FITC

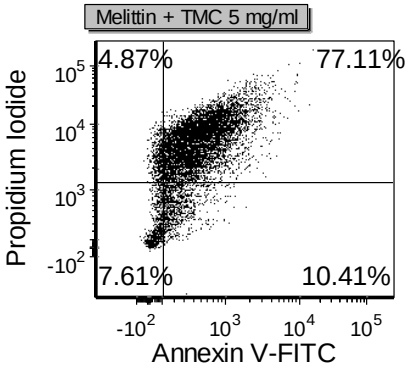
(E)


TMC 50 mg/ml
6.17%
72.33%
9.80%
11.70%
Propidium Iodide
Annexin V-FITC

(F)


Melittin
4.55%
45.20%
29.60%
20.65%
Propidium Iodide
Annexin V-FITC

(G)


Melittin + TMC 5 mg/ml
4.87%
77.11%
7.61%
10.41%
Propidium Iodide
Annexin V-FITC

(H)

Figure 4.25: Flow cytometric representative dotplots of Caco-2 cells analysed for Annexin V binding. Gating of 10 000 cells was performed with regards to propidium iodide and Annexin V-FITC fluorescence. Background labelling of living cells is found in the lower left quadrant, whereas apoptotic cells are shown in the lower right quadrant, stained with Annexin V only. Necrotic cells labelled by both PI and Annexin V-FITC are found in the upper right quadrant. Data are presented on a bi-exponential scale that allows accurate visualisation of population with low or background fluorescence. A) Untreated Caco-2 cells; B) Cells treated with Triton X-100; C) Caco-2 cells treated with TMC (5 mg/ml); D) Caco-2 cells treated with TMC (10 mg/ml); E) Caco-2 cells treated with TMC (25 mg/ml); F) Caco-2 cells treated with TMC (50 mg/ml); G) Caco-2 cells treated with melittin; H) Caco-2 cells treated with a combination of melittin and TMC (5 mg/ml).

In **Figure 4.26** a summary of the effect of TMC and melittin on Caco-2 cells is illustrated. It can be seen that Caco-2 cells are more likely to undergo necrosis than apoptosis. The percentage necrosis is higher than apoptosis when compared to the percentage on HepG2 cells.

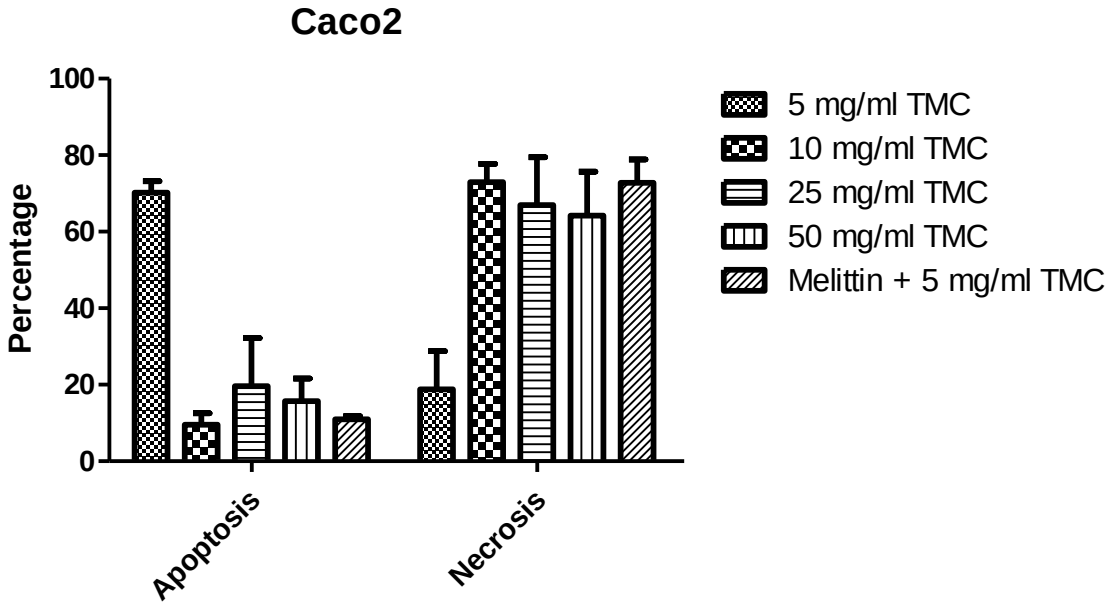


Figure 4.26: Summary of the percentage apoptotic and necrotic cells of experiment on Caco-2 cells.

In **Figure 4.27** and **Figure 4.28** fluorescence intensity of the various treatments on Caco-2 cells is displayed. It appears that Caco-2 cells show lower fluorescence intensity than HepG2 cells. This indicates decreased sensitivity of the Caco-2 cells to the treatments. Compared to HepG2 cells in **Figure 4.23** the Annexin V-FITC fluorescence was much lower indicating lower levels of apoptosis. The bi- fluorescence histogram of the PI is also absent from **Figure 4.28** and the level of DNA fluorescence much lower.

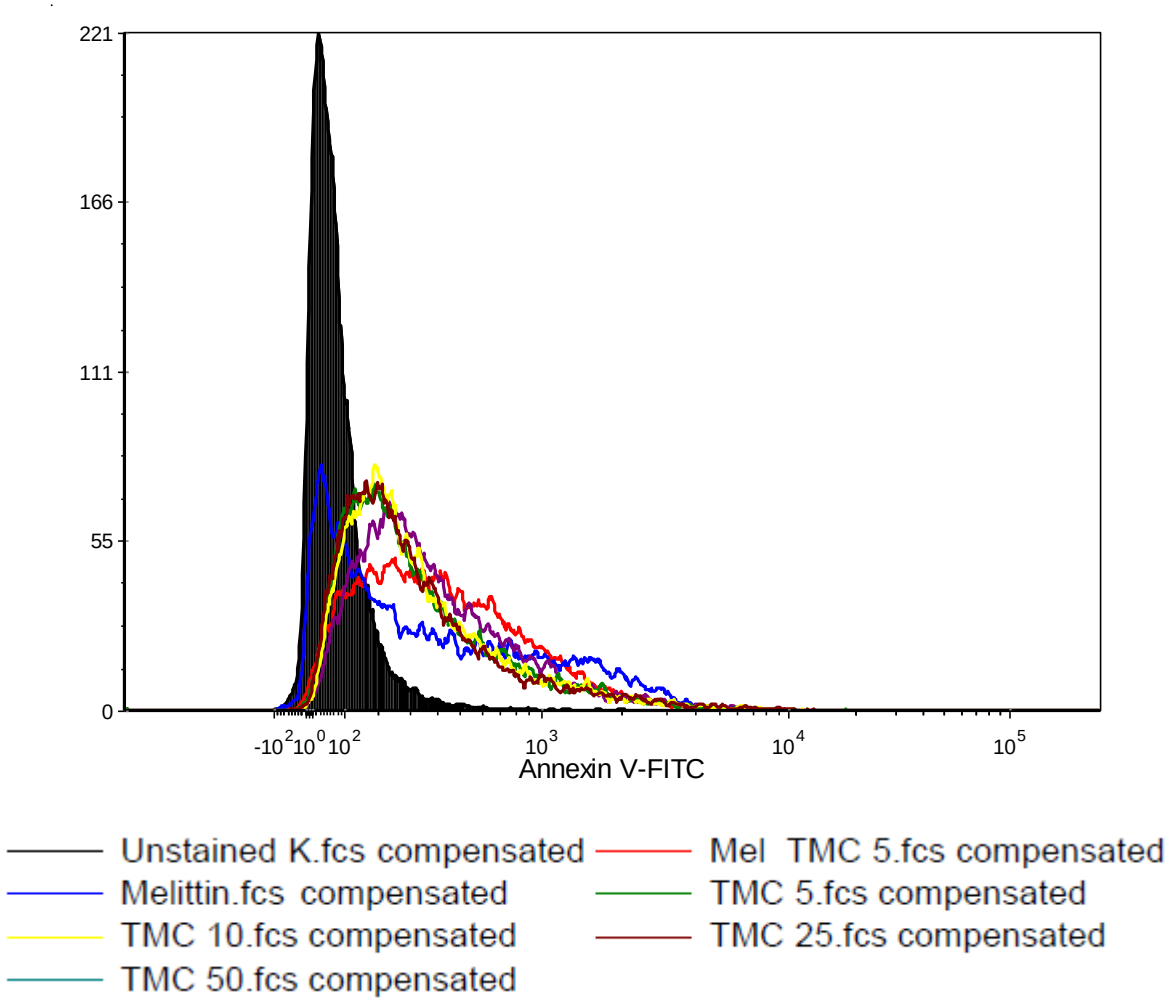


Figure 4.27: Overlay histogram indicating the intensity of Annexin V-FITC staining of Caco-2 cells with permeable cell membranes.

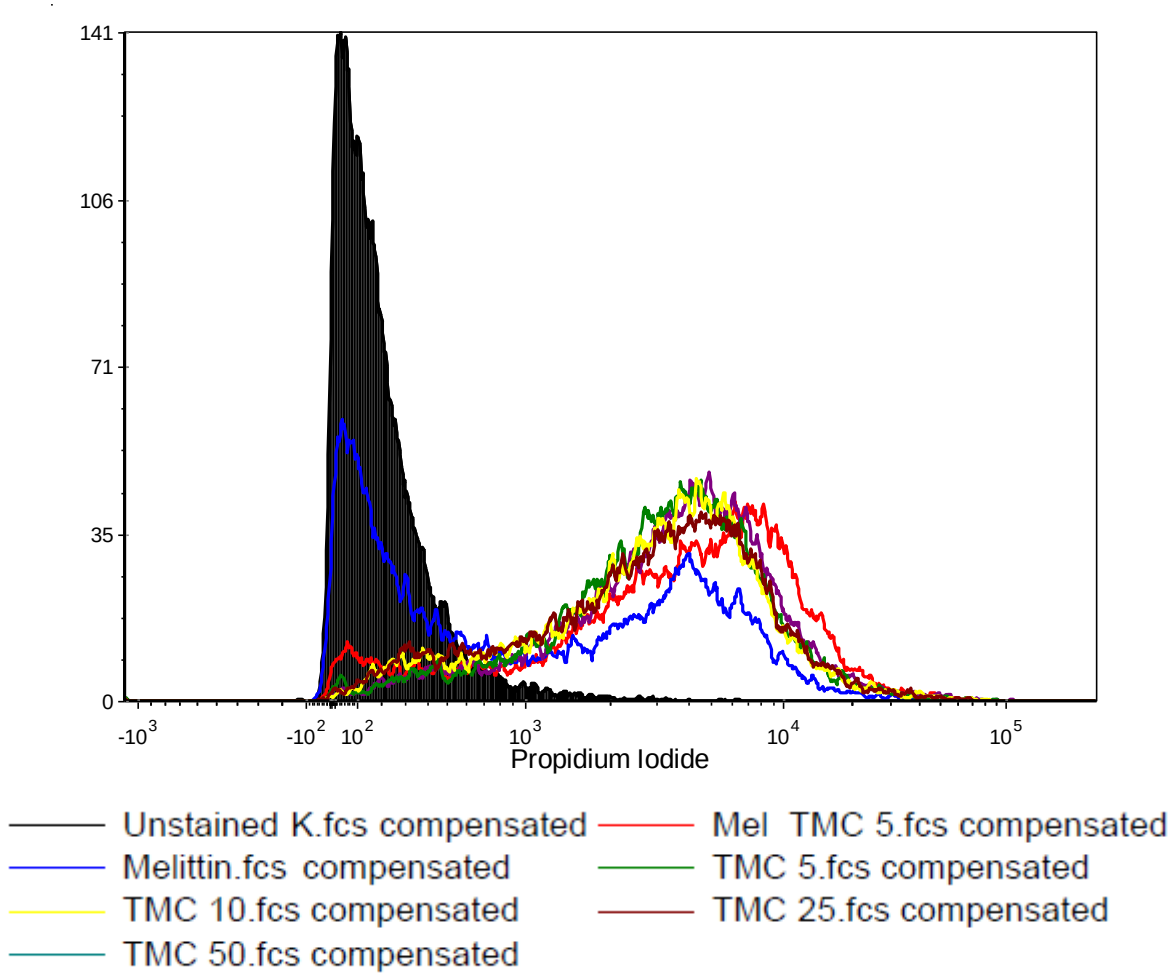


Figure 4.28: Overlay histogram indicating the intensity of propidium iodide staining of Caco-2 cells with permeable cell membranes. Bi- fluorescent histogram indicating various levels of DNA staining.

4.6. Conclusion

Since the aim and objectives of this study were to characterise chitosan derivatives and to evaluate their antioxidant- and antimicrobial activity and determine the cytotoxic effects assays were completed to determine these effects. For the antioxidant activity, chitosan derivative namely TEC, indicated the most scavenging effect of all the derivatives. TMC, melittin and a combination of the two displayed antimicrobial activity at the concentrations tested against two of the bacterial strains used namely, *S. aureus* and *E.coli.* TMC exhibited the highest cytotoxic effects on both HepG2 and Caco-2 cells and it was confirmed by using the flow cytometry technique that it was caused by apoptosis and necrosis for HepG2 and Caco-2 cells respectively.

4.7. References

AL-ANI, I., ZIMMERMANN, S., REICHLING, J. & WINK, M. 2015. Pharmacological synergism of bee venom and melittin with antibiotics and plant secondary metabolites against multi-drug resistant microbial pathogens. *Phytomedicine,* 22**,** 245-255.

CHUNG, Y. C., SU, Y. P., CHEN, C. C., JIA, G., WANG, H. L., WU, J. C. & LIN, J. G. 2004. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. *Acta Pharmacologica Sinica,* 25**,** 932-6.

DE BRITTO, D., CELI GOY, R., CAMPANA FILHO, S. P. & ASSIS, O. B. 2011. Quaternary salts of chitosan: history, antimicrobial features, and prospects. *International Journal of Carbohydrate Chemistry,* 2011.

ENSLIN, G. M., HAMMAN, J. H. & KOTZE, A. F. 2008. Intestinal drug absorption enhancers: synergistic effects of combinations. *Drug Development and Industrial Pharmacy,* 34**,** 1343-9.

ENSLIN, G. M. A. 2005. *In Vitro Evaluation of the Absorption Enhancement Properties of Chitosans, Monocaprin and Melittin*, Tshwane University of Technology.

FALCO, A., BARRAJÓN-CATALÁN, E., MENÉNDEZ-GUTIÉRREZ, M. P., COLL, J., MICOL, V. & ESTEPA, A. 2013. Melittin-loaded immunoliposomes against viral surface proteins, a new approach to antiviral therapy. *Antiviral Research,* 97**,** 218-221.

FISCHER, D., LI, Y., AHLEMEYER, B., KRIEGLSTEIN, J. & KISSEL, T. 2003. *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials,* 24**,** 1121-1131.

GEISBERGER, G., GYENGE, E. B., MAAKE, C. & PATZKE, G. R. 2013. Trimethyl and carboxymethyl chitosan carriers for bio-active polymer–inorganic nanocomposites. *Carbohydrate Polymers,* 91**,** 58-67.

GUO, Z., LIU, H., CHEN, X., JI, X. & LI, P. 2006. Hydroxyl radicals scavenging activity of N-substituted chitosan and quaternized chitosan. *Bioorganic and Medicinal Chemistry Letters,* 16**,** 6348-6350.

HALLIWELL, B., GUTTERIDGE, J. M. C. & CROSS, C. E. 1992. Free radicals, antioxidants, and human disease: Where are we now? *The Journal of Laboratory and Clinical Medicine,* 119**,** 598-620.

HAMMAN, J. H. & KOTZE, A. F. 2001. Effect of the type of base and number of reaction steps on the degree of quaternization and molecular weight of N-trimethyl chitosan chloride. *Drug Development and Industrial Pharmacy,* 27**,** 373-80.

JARMILA, V. & VAVRIKOVA, E. 2011. Chitosan derivatives with antimicrobial, antitumour and antioxidant activities. *Current Pharmaceutical Design,* 17**,** 3596-607.

JO, M., PARK, M. H., KOLLIPARA, P. S., AN, B. J., SONG, H. S., HAN, S. B., KIM, J. H., SONG, M. J. & HONG, J. T. 2012. Anti-cancer effect of bee venom toxin and melittin in ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway. *Toxicology and Applied Pharmacology,* 258**,** 72-81.

KOTZE, A. F., THANOU, M. M., LUESSEN, H. L., DE BOER, B. G., VERHOEF, J. C. & JUNGINGER, H. E. 1999. Effect of the degree of quaternization of N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). *European Journal of Pharmaceutics and Biopharmaceutics,* 47**,** 269-74.

LIU, X., CHEN, D., XIE, L. & ZHANG, R. 2002. Effect of honey bee venom on proliferation of K1735M2 mouse melanoma cells *in-vitro* and growth of murine B16 melanomas *in-vivo*. *Journal of Pharmacy and Pharmacology,* 54**,** 1083-1089.

MAHER, S., DEVOCELLE, M., RYAN, S., MCCLEAN, S. & BRAYDEN, D. J. 2010. Impact of amino acid replacements on *in vitro* permeation enhancement and cytotoxicity of the intestinal absorption promoter, melittin. *International Journal of Pharmaceutics,* 387**,** 154-160.

MAHER, S. & MCCLEAN, S. 2008. Melittin exhibits necrotic cytotoxicity in gastrointestinal cells which is attenuated by cholesterol. *Biochemical Pharmacology,* 75**,** 1104-1114.

NO, H. K., YOUNG PARK, N., HO LEE, S. & MEYERS, S. P. 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology,* 74**,** 65-72.

OBERHOLZER, I. D. 2009. *Peroral and nasal delivery of insulin with Pheroid™ technology/Ian D. Oberholzer.* North-West University.

ROY, M. K., KOIDE, M., RAO, T. P., OKUBO, T., OGASAWARA, Y. & JUNEJA, L. R. 2010. ORAC and DPPH assay comparison to assess antioxidant capacity of tea infusions: relationship between total polyphenol and individual catechin content. *International Journal of Food Sciences and Nutrition,* 61**,** 109-24.

RÚNARSSON, Ö. V., HOLAPPA, J., NEVALAINEN, T., HJÁLMARSDÓTTIR, M., JÄRVINEN, T., LOFTSSON, T., EINARSSON, J. M., JÓNSDÓTTIR, S., VALDIMARSDÓTTIR, M. & MÁSSON, M. 2007. Antibacterial activity of methylated chitosan and chitooligomer derivatives: Synthesis and structure activity relationships. *European Polymer Journal,* 43**,** 2660-2671.

SADEGHI, A. M. M., DORKOOSH, F. A., AVADI, M. R., SAADAT, P., RAFIEE-TEHRANI, M. & JUNGINGER, H. E. 2008. Preparation, characterization and antibacterial activities of chitosan, N-trimethyl chitosan (TMC) and N-diethylmethyl chitosan (DEMC) nanoparticles loaded with insulin using both the ionotropic gelation and polyelectrolyte complexation methods. *International Journal of Pharmaceutics,* 355**,** 299-306.

SNYMAN, D., HAMMAN, J. H. & KOTZE, A. F. 2003. Evaluation of the mucoadhesive properties of N-trimethyl chitosan chloride. *Drug Development and Industrial Pharmacy,* 29**,** 61-9.

SUN, T., ZHOU, D., MAO, F. & ZHU, Y. 2007. Preparation of low-molecular-weight carboxymethyl chitosan and their superoxide anion scavenging activity. *European Polymer Journal,* 43**,** 652-656.

TANTALA, J., THONGNGAM, M., RACHTANAPUN, P. & RACHTANAPUN, C. 2012. Antimicrobial activity of chitosan and carboxymethyl chitosan from different types and sources of chitosan. *Italian Journal of Food Science,* 24**,** 97.

THANOU, M. M., KOTZÉ, A. F., SCHARRINGHAUSEN, T., LUEßEN, H. L., DE BOER, A. G., VERHOEF, J. C. & JUNGINGER, H. E. 2000. Effect of degree of quaternization of N-trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal Caco-2 cell monolayers. *Journal of Controlled Release,* 64**,** 15-25.

VAN DER MERWE, S. M. 2000. *In vitro evaluation of the toxicity profile of N-trimethyl chitosan chloride.* North- West University.

VENTER, C. 2005. *Chitosan and quaternised chitosan polymers as gene transfection agents.* North-West University.

WIARACHAI, O., THONGCHUL, N., KIATKAMJORNWONG, S. & HOVEN, V. P. 2012. Surface-quaternized chitosan particles as an alternative and effective organic antibacterial material. *Colloids and Surfaces B: Biointerfaces,* 92**,** 121-129.

XU, T., XIN, M., LI, M., HUANG, H. & ZHOU, S. 2010. Synthesis, characteristic and antibacterial activity of N,N,N-trimethyl chitosan and its carboxymethyl derivatives. *Carbohydrate Polymers,* 81**,** 931-936.

YANG, D.-Z., LIU, X.-F., LI, Z., XU, H.-Y., GUAN, Y.-L. & YAO, K.-D. 2000. On the factors influencing the antibacterial activity of chitosan. *Chinese Journal of applied chemistry,* 17**,** 602-605.

ZHAO, D., WANG, J., TAN, L., SUN, C. & DONG, J. 2013. Synthesis of N-furoyl chitosan and chito-oligosaccharides and evaluation of their antioxidant activity *in vitro*. *International Journal of Biological Macromolecules,* 59**,** 391-5.

ZHENG, L.-Y. & ZHU, J.-F. 2003. Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydrate Polymers,* 54**,** 527-530.

CHAPTER 5

CONCLUSION AND FUTURE PROSPECTS

5.1. Conclusion and Future prospects

The aim of this study was to determine the antioxidant- and antimicrobial activity and cytotoxicity of chitosan derivatives and melittin. Although chitosan and many of its derivatives has been studied and characterised, this is the first study that uses the chosen derivatives (TMC, TEC, DCMC and TEO), with their respective MW and DQ. A study done by Enslin *et al.,* (2008) also used these derivatives to determine their absorption enhancing effects whereas this study characterised the derivatives in terms of their cytotoxicity and functional properties.

The antioxidant activity of different concentrations of chitosan derivatives were studied by using the DPPH radical scavenging assay. Chitosan and some of its derivatives have known antioxidant activity except for the derivatives used in this study. Only the chitosan derivative TEC presented antioxidant activity which is concentration dependent. The rest of the derivatives showed no antioxidant activity and similar to Roy *et al.,* (2010), it was hypothesised that they acted as pro-oxidants leading to ROS (Roy *et al*., 2010).

To evaluate the antimicrobial activity of chitosan derivatives at different concentrations, melittin and a combination of the two, the MIC assay was employed. The antimicrobial activity was determined because chitosan and TMC has been known to exhibit antimicrobial activity which could be useful for treating infections related to selected bacteria. The antimicrobial activity of the rest of the derivatives used in this study is still unknown. The results of these experiments found that of all the derivatives, only the TMC used in this study had antimicrobial activity at the concentrations tested against *S. aureus, S. epidermidis* and *E. coli.* Melittin, which is a known antimicrobial peptide, also indicated antimicrobial activity against *S. aureus* and *E. coli.* The combination of TMC and melittin showed antimicrobial activity against *S. epidermidids* and *E. coli*. We speculated that this effect is synergistic due to previous studies that found TMC and melittin exhibits synergistic absorption enhancing effects but after investigation it was proposed that this interaction is known as a non-interactive interaction.

Experiments determining the cytotoxicity of chitosan derivatives and melittin revealed that the TMC used in this study is the most cytotoxic derivative at all the concentrations

tested whereas the rest of the derivatives showed cytotoxicity at high concentrations tested on both HepG2- and Caco-2 cells. It can be concluded that the cytotoxicity is concentration dependent. Based on the results obtained from the LDH assay, it could be seen that TMC causes extensive cell membrane damage to both cell lines used. Melittin also induced considerable cell membrane damage at high concentrations. The combination of TMC and melittin resulted in decreased cell viability in HepG2 cells whereas the cell viability of Caco-2 cells was not affected. This could be due to Caco-2 cells having tight junctions and the derivatives having more specificity towards the tight junctions than the cell membranes. It is suggested that this effect of the combination of these two absorption enhancers have a potentiating effect on HepG2 cells in terms of cytotoxicity and needs to be further investigated as a possible anticancer treatment. Using the flow cytometry technique, it was determined that the mechanism by which chitosan derivatives and melittin induce cell membrane damage of HepG2 cells is largely through late apoptosis. Caco-2 cells undergo necrosis when treated with chitosan derivatives and melittin, although the cell death is not as pronounced as in the HepG2 cells.

To establish the effect of chitosan derivatives and melittin the cell viability is necessary to determine the safety of these compounds before using it in dosage forms. Previous studies indicated that polymers with high MW and DQ will have increased cytotoxicity and can be compared to the current study where the TMC used has a high MW and DQ (Fischer *et al*., 2003). Enslin *et al.,* (2008) indicated that the combination of TMC and melittin displayed a synergistic effect whereas the current study indicated a potentiating effect with regards to the cytotoxicity (Enslin *et al*., 2008). The antioxidant activity of TMC and TEO differs to what was found in other studies although the MW and DQ of these derivatives used are different than Guo *et al.,* (2006) and Zhao *et al.,* (2013). For DCMC the results found were similar to what Sun *et al.,* (2008) found (Guo *et al*., 2006, Sun *et al*., 2008, Zhao *et al*., 2013). TEC is the only derivative in this study where no other studies were done to determine antioxidant activity (de Britto *et al*., 2011, Sadeghi *et al*., 2008, Wiarachai *et al*., 2012). It was shown by other studies that TMC exhibits antimicrobial activity whereas carboxymethyl chitosan (Tantala *et al*., 2012) and TEO has weak antimicrobial activity (No *et al*., 2002). According to the experimental results, it can be concluded that the antioxidant- and antimicrobial activity and the cytotoxicity is dependent on the MW and DQ.

Although the present investigation is the first one which describes the antioxidant- and antimicrobial activity of some of chitosan derivatives and also the cytotoxicity thereof, more research is required before these derivatives can be used in the pharmaceutical industry. It was observed that the mechanism by which chitosan derivatives cause cell damage to HepG2 cells, namely apoptosis, may indicate a possible cancer treatment but further research is necessary. As a result of melittin causing considerable cell membrane damage by apoptosis, the use of melittin in cancer treatment is a future prospect and should be further investigated in developing a potential drug to treat this disease. Previous studies have also indicated the potential use of melittin in cancer where the tumour killing ability was enhanced of some chemotherapeutic drugs (Jo *et al*., 2012). *In vivo* experiments should also be completed to confirm the mechanism of cell death. Before melittin can be used as a potential drug, further studies are necessary but these results propose a good future for this compound.

In conclusion, the aims and objectives of this study were achieved and I was able to show that TEC has antioxidant activity, TMC and melittin has antimicrobial activity against selected bacterial strains and TMC is the most cytotoxic at the concentrations tested and specific MW and DQ.

For future studies I will recommend that a wider concentration series of chitosan derivatives and melittin be used to determine if the cytotoxicity is indeed concentration dependent and cytotoxic at higher concentrations. Other AMP's instead of melittin may also present promising results. Different concentrations of the combination treatments could possibly have different results than this study and also the use of different concentrations of both compounds for example chitosan derivatives in high concentrations and an AMP in lower concentrations. I would suggest that it be further characterised in terms of cell death and mode of cell death to propose a mechanism of action for the combination of TMC and melittin. A broader spectrum of bacterial strains may also be included to determine if the derivatives has possible antimicrobial activity against other bacterial strains even though they were ineffective against the selected bacterial strains used in this study. Also additional interaction studies should be done to determine if synergism can be obtained. Furthermore, the antimicrobial activity of the derivative TMC may be a possible treatment against bacterial skin

infections and further research to combine its antimicrobial activity along with its wound healing properties would be ideal. Also, I would suggest determining the *in vitro* antioxidant activity of chitosan derivatives for their future use as antioxidants.

5.2. References

DE BRITTO, D., CELI GOY, R., CAMPANA FILHO, S. P. & ASSIS, O. B. 2011. Quaternary salts of chitosan: history, antimicrobial features, and prospects. *International Journal of Carbohydrate Chemistry,* 2011.

ENSLIN, G. M., HAMMAN, J. H. & KOTZE, A. F. 2008. Intestinal drug absorption enhancers: synergistic effects of combinations. *Drug Development and Industrial Pharmacy,* 34**,** 1343-9.

FISCHER, D., LI, Y., AHLEMEYER, B., KRIEGLSTEIN, J. & KISSEL, T. 2003. *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials,* 24**,** 1121-1131.

GUO, Z., LIU, H., CHEN, X., JI, X. & LI, P. 2006. Hydroxyl radicals scavenging activity of N-substituted chitosan and quaternized chitosan. *Bioorganic and Medicinal Chemistry Letters,* 16**,** 6348-6350.

JO, M., PARK, M. H., KOLLIPARA, P. S., AN, B. J., SONG, H. S., HAN, S. B., KIM, J. H., SONG, M. J. & HONG, J. T. 2012. Anti-cancer effect of bee venom toxin and melittin in ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway. *Toxicology and Applied Pharmacology,* 258**,** 72-81.

NO, H. K., YOUNG PARK, N., HO LEE, S. & MEYERS, S. P. 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology,* 74**,** 65-72.

ROY, M. K., KOIDE, M., RAO, T. P., OKUBO, T., OGASAWARA, Y. & JUNEJA, L. R. 2010. ORAC and DPPH assay comparison to assess antioxidant capacity of tea infusions: relationship between total polyphenol and individual catechin content. *International Journal of Food Science and Nutrition,* 61**,** 109-24.

SADEGHI, A. M. M., DORKOOSH, F. A., AVADI, M. R., SAADAT, P., RAFIEE-TEHRANI, M. & JUNGINGER, H. E. 2008. Preparation, characterization and antibacterial activities of chitosan, N-trimethyl chitosan (TMC) and N-diethylmethyl chitosan (DEMC) nanoparticles loaded with insulin using both the ionotropic gelation and polyelectrolyte complexation methods. *International Journal of Pharmaceutics,* 355**,** 299-306.

SUN, T., YAO, Q., ZHOU, D. & MAO, F. 2008. Antioxidant activity of N-carboxymethyl chitosan oligosaccharides. *Bioorganic and Medicinal Chemistry Letters,* 18**,** 5774-5776.

TANTALA, J., THONGNGAM, M., RACHTANAPUN, P. & RACHTANAPUN, C. 2012. Antimicrobial activity of chitosan and carboxymethyl chitosan from different types and sources of chitosan. *Italian Journal of Food Science,* 24**,** 97.

WIARACHAI, O., THONGCHUL, N., KIATKAMJORNWONG, S. & HOVEN, V. P. 2012. Surface-quaternized chitosan particles as an alternative and effective organic antibacterial material. *Colloids and Surfaces B: Biointerfaces,* 92**,** 121-129.

ZHAO, D., WANG, J., TAN, L., SUN, C. & DONG, J. 2013. Synthesis of N-furoyl chitosan and chito-oligosaccharides and evaluation of their antioxidant activity *in vitro*. *International Journal of Biological Macromolecules,* 59**,** 391-5.

Annexure 1

Determining the antimicrobial activity of chitosan derivatives, melittin and a combination of the two on bacterial strains

The disc diffusion and well diffusion assays were performed (as described in chapter 3) on two bacterial strains namely, *Staphylococcus aureus* and *Escherichia coli.*

A. Disc diffusion

Figure A.1 and **Figure A.2** indicate that no antimicrobial activity was exhibited by chitosan derivatives and yielded no concrete results. As can be seen on the agar, no inhibition of the growth of the bacterial strains could be seen around the circular opening where the derivatives were placed. This assay was deemed not sensitive enough and the well diffusion assay was performed.

Figure A.1: Disc diffusion assay before treatment with chitosan derivatives (TMC, TEC, DCMC and TEO).

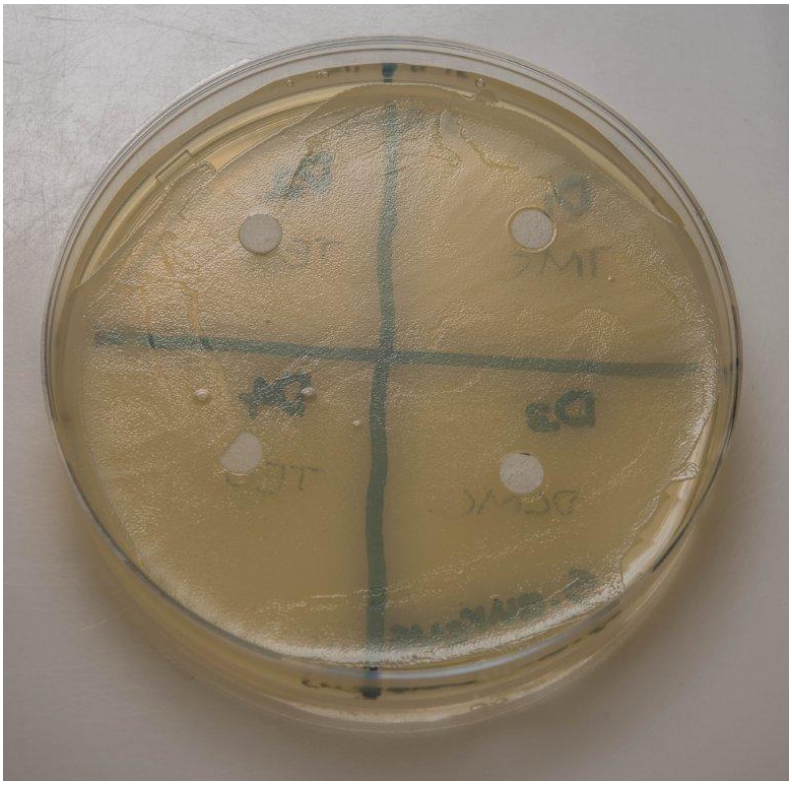


Figure A.2: Disc diffusion assay after treatment of chitosan derivatives (TMC, TEC, DCMC and TEO).

B. Well diffusion

The well diffusion assay was then performed on the same bacterial strains as the disc diffusion assay. It can be visually observed that the positive control is effective in inhibiting growth of the selected bacterial strain whereas chitosan derivatives showed no inhibition of growth around the circular opening (**Figure B.1**).

Figure B.1: Well diffusion after treating the selected bacteria with the positive control, negative control and chitosan derivatives.

Annexure 2

A. Calculation of the FIC index

For *S. aureus* (calculation 1):

$$\text{FIC (i)} = \frac{\text{MIC of Melittin in combination with TMC}}{\text{MIC of Melittin independantly}}$$

$$= \frac{0.04}{0.09}$$

$$= 0.4$$

$$\text{FIC (ii)} = \frac{\text{MIC of TMC in combination with Melittin}}{\text{MIC of TMC independantly}}$$

$$= \frac{2.34}{1.56}$$

$$= 1.5$$

The sum of the FIC index is thus calculated as:

$$\Sigma\text{FICI} = \text{FIC (i)} + \text{FIC (ii)}$$

$$= 0.4 + 1.5$$

$$= 1.9$$

For *E. coli* (calculation 2):

$$\text{FIC (i)} = \frac{\text{MIC of Melittin in combination with TMC}}{\text{MIC of Melittin independantly}}$$

$$= \frac{0.04}{0.03}$$

$$= 1.33$$

$$\text{FIC (ii)} = \frac{\text{MIC of TMC in combination with Melittin}}{\text{MIC of TMC independantly}}$$

$$= \frac{2.34}{1.56}$$

$$= 1.5$$

The sum of the FIC index is thus calculated as:

$\Sigma FICI = FIC\ (i) + FIC\ (ii)$

$= 1.3 + 1.5$

$= 2.8$

Annexure 3

A. Determining the optimal cell count

As described in chapter 3, the optimal cell count is necessary to determine the amount of cells used in an experiment. The amount of cells to be used for HepG2 and Caco-2 cells is illustrated in **Figure C.1** and **Figure C.2** respectively.

1. HepG2 cells

The number of cells to use must yield an absorbance of 0.75 – 1.25 (refer to **Figure C.1**) where it can be read from the graph as 50 000 cells.

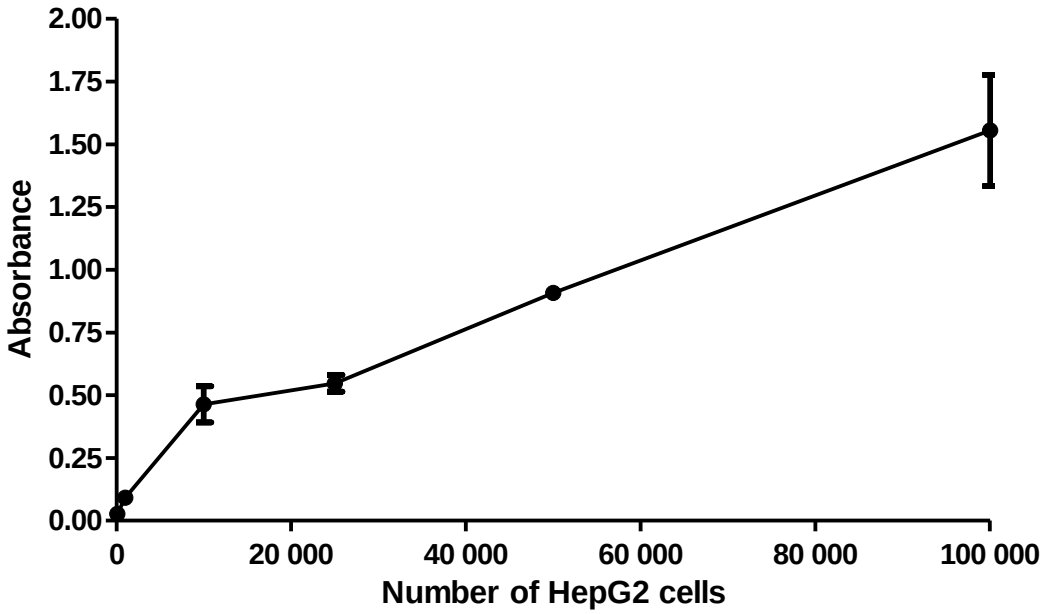


Figure C.1: The amount of HepG2 cells necessary for cytotoxicity experiments.

2. Caco-2 cells

The amount of cells necessary for cytotoxicity experiments on Caco-2 cells were 20 000 (100 µl) (refer to **Figure C.2**). For Caco-2 cells, the same assay was used and it was determined that 20 000 cells or 100 µl of cell suspension is required for experiments. The cell concentrations determined here was applied to all consequent experiments.

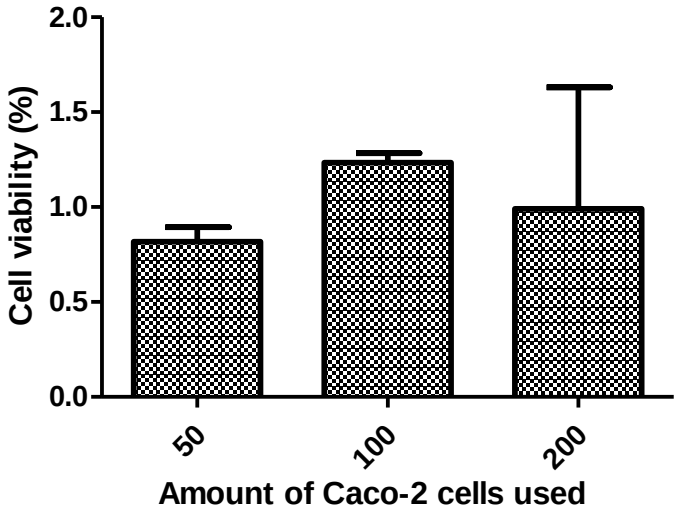


Figure C.2: Amount of Caco-2 cells necessary for cytotoxicity experiments.

Annexure 4

Annexure 4 includes a review article submitted to the Molecules Journal and published on the 24 August 2015. The article was written in cooperation with colleagues.

Molecules **2015**, *20*, 1-x manuscripts; doi:10.3390/molecules200x0000x

OPEN ACCESS

molecules

ISSN 1420-3049

www.mdpi.com/journal/molecules

Review

The Potential Use of Natural and Structural Analogues of Antimicrobial Peptides in the Fight against Neglected Tropical Diseases

Lewies Angélique, Wentzel Johannes Frederik, Jacobs Garmi and Du Plessis Lissinda Hester *

Centre of Excellence for Pharmaceutical Sciences (PHARMACEN), North-West University, Potchefstroom 2520, South Africa; E-Mails: 20577966@nwu.ac.za (L.A.); 20134045@nwu.ac.za (W.J.F.); 22119027@nwu.ac.za (J.G.)

***** Authors to whom correspondence should be addressed; E-Mail: Lissinda.DuPlessis@nwu.ac.za; Tel.:+27-018-299-4246; Fax: +27-087-231-5432.

Academic Editor: Thomas J. Schmidt

Received: 13 July 2015 / Accepted: 10 August 2015/ Published:

Abstract: Recently, research into the development of new antimicrobial agents has been driven by the increase in resistance to traditional antibiotics and Emerging Infectious Dis.. Antimicrobial peptides (AMPs) are promising candidates as alternatives to current antibiotics in the treatment and prevention of microbial infections. AMPs are produced by all known living species, displaying direct antimicrobial killing activity and playing an important role in innate immunity. To date, more than 2000 AMPs have been discovered and many of these exhibit broad-spectrum antibacterial, antiviral and anti-parasitic activity. Negl.Trop. Dis. (NTDs) are caused by a variety of pathogens and are particularly wide-spread in low-income and developing regions of the world. Alternative, cost effective treatments are desperately needed to effectively battle these medically diverse diseases. AMPs have been shown to be effective against a variety of NTDs, including African trypanosomes, leishmaniosis and Chagas disease, trachoma and leprosy. In this review, the potential of selected AMPs to successfully treat a variety of NTD infections will be critically evaluated.

Keywords: Antimicrobial peptides (AMPs); Negl.Trop. Dis. (NTDs); malaria; parasites; antibacterial; antiviral; parasitic worms; innate immunity

1. Introduction

At the beginning of the new millennium, the international community, led by the United Nations, committed themselves to the Millennium Development Goals in order to drastically reduce extreme poverty and combat disease amongst the poorest populations of the world. Now, at the end of the 15 year period of this ambitious action plan, we can look back and evaluate the successes and shortcomings of this development framework. The sixth goal of this framework is to combat HIV/AIDS, malaria and other diseases. This goal has brought much-needed attention to neglected diseases, leading to the establishment of the Global Network for Negl.Trop. Dis. Control and a World Health Organisation (WHO) department, specifically tasked to address neglected tropical disease-related issues. Negl.Trop. Dis. (NTDs) are a diverse group of 17 disabling conditions, mostly affecting the world's poorest populations [1]. These diseases affect more than 1.4 billion people around the world, causing more than half a million deaths annually [1,2]. Although these diseases distress a considerable portion of the global population, they may be considered "neglected" due to the dire lack of effective treatments and funding [3]. Despite all the positive progress from the Millennium Goals framework, NTDs still cause massive global suffering in millions of people. New treatment strategies are desperately needed to ease the burden caused by these tropical diseases.

The emerging and increasing resistance to antibiotics has become a threat to global public health and is driving novel research into the development of new antimicrobial agents. AMPs are promising candidates as alternatives to current antibiotics in the treatment and prevention of microbial infections [4–6]. Although there are a number of AMPs in clinical development [4,7,8], only a few have been successfully applied commercially. Perhaps the best known is the lantibiotic nisin (APD ID: AP00205) produced by the gram-positive bacterium *Lactococcus lactis*. This AMP exhibits antimicrobial activity against many gram-positive bacteria, including food-borne pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes*, is not toxic to animals and was approved by the WHO in 1969 and the US Federal Food and Drug Administration (FDA) in 1988 for the use as a food preservative [9]. AMPs not only have broad-spectrum antibacterial activity, but also display antiviral and anti-parasitic activity [10] and therefore have potential for use in the treatment of a wide variety of NTDs. On the other hand, some of these microbes, for example the filarial worm *Onchocerca volvulus* [11], could prove to be a source of novel AMPs with therapeutic potential against microbial infections.

The aim of this review is to critically evaluate the potential of selected AMPs (both natural and structural analogues of natural AMPs) to successfully treat a variety of NTDs and malaria. NTDs that are covered include those caused by bacteria (leprosy/Hansen disease and trachoma), protozoa (chagas disease, human African trypanosomiasis and leishmaniosis), helminths (taeniasis and onchocerciasis) and viruses (dengue viral disease and rabies).

2. Antimicrobial Peptides

Antimicrobial peptides (AMPs) are considered natural antibiotics and are produced by all known living species, ranging from bacteria, fungi, and plants to invertebrates, non-mammalian vertebrates and

mammals. To date, more than 2000 AMPs from various sources with a broad range of activities (ranging from antimicrobial activity to anticancer, spermicidal, chemotactic or antiviral activity) have been listed on APD2 [12], a database largely dedicated to natural AMPs [13,14]. For the scope of this review, only AMPs that are ribosomally synthesised [15] will be included in order to distinguish them from classical natural peptide antibiotics, such as vancomycin, which are assembled by non-ribosomal peptide synthetases [16]. Non-ribosomally synthesised AMPs can however be found in lower organisms such as bacteria, for example baceridin (APD ID: AP02372) which has recently been isolated from a plant-associated *Bacillus* strain [17,18]. Gene-encoded, ribosomally synthesised AMPs are evolutionary conserved components of the innate immune system, which serve as a first line of defence against microbial infections [19,20] and are therefore also referred to as host defence peptides. Foregoing the adaptive immune response (humoral and cell mediated immunity), the innate immune system can be mobilized against a variety of microorganisms, even if the host is encountering these microbes for the first time. After the initial infection, the interferon (IFN) response can be triggered by the recognition of foreign gene segments (this includes genomic dsRNA/DNA, mRNA and replication intermediates) or proteins by pattern recognition receptors (PRR). PRRs are divided into two families: (1) the cytoplasmic pathogen detectors which include the RNA sensitive retinoic acid-induced gene (RIG-I) and melanoma differentiation associated gene 5 (MDA5), and (2) the trans-membrane toll-like receptors (TLR) [21–23]. The recognition of alien genomic material by these PRRs triggers a complex cascade of cellular events which finally concludes with the deployment of immune regulatory proteins. Bacteria and fungi are also known to utilize cytolytic and antimicrobial peptides to obtain a competitive advantage over other micro-organisms in their habitat [24]. These peptides also play an important role in the innate immune system of most plants and animals [25].

1.1.2.1. Classification of AMPs

The selectivity and antimicrobial potency of AMPs towards microbes are determined by structural parameters including net charge and structural conformation [26,27]. Although structurally based classifications are generally used to divide AMPs into subgroups, AMPs may also be classified according to their net charge, which is determined by their amino acid composition.

2.5.1. 2.1.1. Classification of AMPs According to Net Charge

Based on the net charge, AMPs can be divided into anionic AMPs (AAMPs) and cationic AMPs (CAMPs). AAMPs are rich in aspartic and glutamic acid and consist of 5 to 70 amino acids with a net negative charge of −1 to −7 [28,29]. CAMPs are the most abundant form of AMP found in nature and are also the most thoroughly studied for therapeutic use. Hence, this review will primarily focus on the use of CAMPs for the treatment of selected NTDs. For a comprehensive overview of AAMPs, see Harris *et al.* [28]. CAMPs (from here on referred to simply as AMPs) typically consist of 12 to 100 amino acids with a net positive charge of +2 to +9 due to the excess of basic amino acids (arginine, lysine and/or histidine) compared to acidic amino acids [10].

2.5.2. 2.1.2. Classification According to Secondary Structure

Despite being small in size, AMPs can be divided into three major structural classes (Figure 1) based on their secondary structure [27,30–32]:

i. **α-helical AMPs**: These peptides are unstructured linear peptides free of cysteine residues that fold into α-helixes upon contact with membranes. They consist of approximately 50 % hydrophobic residues, favouring an amphiphilic conformation upon interaction with membranes, which enables them to permeabilise microbial membranes. Some of these peptides are not strictly α-helical and may possess an internal kink and/or a flexible unstructured segment at the N- and/or C terminus. For example, melittin (APD ID: 00146) (Figure 1A) from bee venom and the human cathelicidin LL-37 (APD ID: AP00310) (Figure 1B).

ii. **Linear/extended AMPs**: These peptides are linear without cysteine residues and contain a high proportion of proline, arginine, glycine, tryptophan and histidine. Some of these AMPs form extended coils. Examples include indolicidin (APD ID: AP00150) (Figure 1C) from bovine leukocytes.

iii. **β-sheet AMPs**: These peptides contain six to eight cysteine residues, forming two or more disulphide bonds, resulting in a stabilized β-sheet structure. For example α and β defensins such as human neutrophil peptide-1 (HNP-1, APD ID: AP00176) (Figure 1D) from mammals.

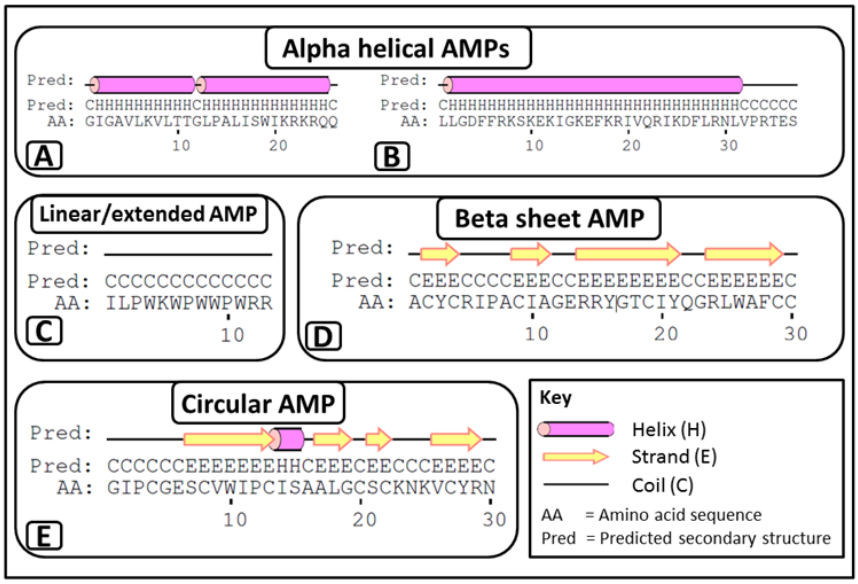


Figure 1. Secondary structures of the three major AMP classes. Examples of α-helical AMPs include (**A**) melittin, two α-helixes joined by a hinge between residues 11–12, and (**B**) LL-37, α-helix with an unstructured segment at the C-terminal; (**C**) indolicidin, which can be classified as an extended coil; and (**D**) human neutrophil peptide 1, which is an example of a β-sheet. In addition to the three major classes, circular AMPs including (**E**) circulin A, consisting of both α-helix and β-sheet structures, are also found in nature. Secondary structures were generated with the PSIPRED protein prediction server [33]. Disulphide bonds are not shown.

AMPs that do not typically fit into the previous three secondary structural classes are classified as circular AMPs [34]. The plant-derived circulin A (APD ID: AP00274) (Figure 1E) from the *Chassalia parviflora* species can be categorised into this group. Circulin A consists of a combined α-helix and β-sheet structure [35] and forms the so-called cyclic cysteine knot [36]. Though they display β-sheet-like structures, Θ defensins can also be classified into this group, as they form cyclic octadecamers, which consist of two antiparallel β-sheets linked by three disulphide bonds [37].

The major forms of AMPs found in mammals are cathelicidins and defensins, although they are also found in non-mammals such as birds [38,39], fish [40,41], reptiles [42,43] and even plants [44] and insects [45]. Cathelicidins and defensins have been extensively reviewed elsewhere [46–50] and will therefore not be discussed in detail in this review.

1.2.2.2. Target Organisms and Mode of Action of AMPs

AMPs have broad-spectrum antibacterial, antiviral, antifungal and anti-parasitic activities. There are also AMPs that display anti-protist activity. A single AMP can have a single microbial target or have multiple microbial targets simultaneously, *i.e.* displaying broad spectrum antibacterial activity, as well as being antifungal, anti-parasitic and antiviral (Table 1) [13,14].

Table 1. Antimicrobial peptides which display antibacterial (gram positive and negative) as well as antiviral, antifungal and anti-parasitic activity [13,14].

AMP	APD ID	Source	Sequence	Structure
Magainin 2	AP00144	African clawed frog *(Xenopus laevis)*	GIGKFLHSAKKFGKAFVGEIMNS	α-helix
Melittin	AP00146	Honey bee (*Apis mellifera)* (also *A. florea, A. cerana*)	GIGAVLKVLTTGLPALISWIKRKRQQ	α-helix
Dermaseptin-S1	AP00157	Sauvages leaf frog (*Phyllomedusa sauvagii*)	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	α-helix
HNP-1	AP00176	Human (*Homo sapiens*)	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	β-sheet
LL-37	AP00310	Human (*Homo sapiens)* Chimpanzee (*Pan troglodytes*)	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	α-helix
BMAP-27	AP00366	Domestic cattle (*Bos taurus*)	GRFKRFRKKFKKLFKKLSPVIPLLHLG	α-helix

2.5.3. 2.2.1. Perturbation of the Microbial Membranes by AMPs

Biophysical properties such as the secondary structure, net charge and hydrophobicity influence the interaction of AMPs with membranes as determined via experimentation with model and biological membranes [10,51,52]. Although, investigations of membrane mediated microbial activities of AMPs

focuses mainly on bacteria [25,53], AMPs have also been found to display direct killing action against parasites [54], fungi [55] and enveloped viruses [56] through direct membrane or capsid interactions (Figure 2). The mechanism of interaction will differ depending on the microbial organism and the AMP under investigation, but generally the initial attraction between an AMP and the microbial membrane occur through electrostatic interactions. The cationic property of AMPs allow them to target negatively charged microbial membranes opposed to neutral zwitterionic phospholipid containing bilayer membranes of mammalian cells. The higher levels of cholesterol in mammalian cells can further be used as basis for AMPs to distinguish between microbial and mammalian cell membranes [53]. Although in most cases there seems to be no specific receptors for peptide binding, in the case of viruses, AMPs are thought to disrupt the viral capsid or interfere with host entry by binding to the viral glycoproteins [57]. Following binding of AMPs to cell membranes, cell death can occur by disrupting membrane integrity, resulting in the leakage of the cytoplasm, depolarization and osmotic imbalance, swelling of the cells and, finally, cell lysis [54,58,59].

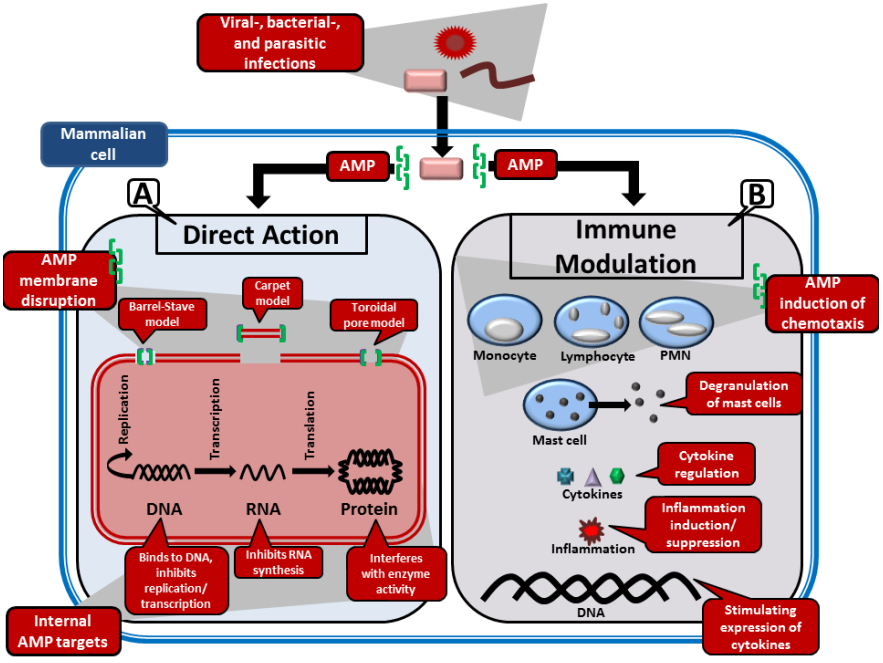


Figure 2. Main antimicrobial mechanisms of AMPs during the infection of a mammalian host cell. (**A**) The direct microbial action of AMPs involves the binding of these peptides to the microbial membrane/capsid, resulting in the infectious agent's neutralization. There are three models proposed for this action: (i) the barrel-stave model, where the AMPs embed themselves into the membrane to form pores; (ii) the carpet model, which proposes that small portions of the membrane be removed by AMPs; and (iii) the toroidal model, which is similar to the barrel-stave model, but with the exception that the AMPs are permanently bound to the phospholipids in the membrane. Internal functions of AMPs may include DNA replication/translation inhibition, transcription inhibition and enzyme inhibition. (**B**) Many AMPs are also involved in immunoregulation. Some of these regulatory functions may include angiogenesis, cell proliferation, cytokine regulation, chemotaxis of certain leukocyte classes (including monocytes, lymphocytes and polymorphonuclear leukocytes or PMN),

degranulation of mast cells, stimulation of phagocytosis and even modulation of gene expression.

Three main permeabilization models exist:

i. **Barrel-stave model**: This model is used to describe the formation of pores in the lipid membrane. This is achieved through the amphipathic peptides that insert in a perpendicular orientation to the membrane and align so that the hydrophilic side chains point inward and form the transmembrane pore, while the hydrophobic side chains face outward and interact with the lipid bilayer [10,60]. This was the first model to be proposed as the mechanism of peptide induced pores [61]. AMPs that this model typically applies to are those that promote loss of electrochemical potential at low, well-defined peptide:phospholipid ratios [54]. The only AMP for which there is clear evidence of the barrel-stave mechanism is the non-ribosomally synthesised alamethicin (APD ID: AP02197), produced by the fungi *Trichoderma viride* [62].

ii. **Toroidal model**: In this model, pores, consisting of peptides intercalated with lipids, are formed in the lipid membrane [63]. Amphipathic peptides accumulate parallel to the membrane surface, where their partial insertion causes the outer (but not the inner) leaflet to expand. The peptides re-orientate perpendicularly to the bilayer once a critical threshold is reached, thereby relieving tension. The bilayer undergoes a positive curvature and its thickness is modified, which creates transient pores consisting of both peptide and lipid molecules [54].

iii. **Carpet model**: Partial membrane solubilisation in a detergent-like manner occurs with this model [54]. The amphipathic peptides accumulate parallel to the membrane via electrostatic interaction with anionic phospholipids—covering the membrane in a carpet-like fashion. When a threshold concentration is reached, detergent-like activity causes the formation of micelles and membrane pores [10].

It should be noted that although *in vitro* experiments have determined that some AMPs, for example magainin 2 (APD ID: AP00144) derived from the skin of the African clawed frog (*Xenopus laevis)*, display cell selectivity towards microbial organisms without significant cytotoxicity towards mammalian cells [64,65], most AMPs should be considered as displaying "partial selectivity". It is suggested that AMPs only display cell selectivity when placed in an actual situation where mammalian host cells are confronted with microbial organisms and that AMPs could potentially be toxic towards mammalian cells in the absence of microbial organisms. This fact is thought to be underestimated by current *in vitro* experimental procedures with regard to the volume of cells used for mammalian haemolysis (cytotoxic) *vs.* bacterial assays which does not give an accurate representation of the cytotoxic *vs.* the minimum inhibitory concentrations [26].

2.5.4. 2.2.2. Interaction of AMPs with Internal Targets

Although the main mechanism by which AMPs appear to directly kill microbial organisms is through membrane permeabilization, some AMPs do not damage microbial membranes but still manage to kill or inhibit the growth of these organisms. Alternatively, some AMPs may cause disruption of the

microbial membrane without leading to inhibition or death of the microbial organisms [66,67]. AMPs are therefore also believed to display direct killing action through the interaction with anionic intracellular targets such as DNA and RNA (Figure 2) [25,68,69]. These AMPs still have to gain access to their internal targets through the cell membrane and are believed to translocate over the cell membrane of the microbial organism without causing significant damage to the membrane, although the precise mechanism is still not fully understood, as highlighted by Nicolas [68]. AMPs are thought to exert their intracellular activity through inhibition of nucleic acid and protein synthesis, inhibition of enzymatic activity, and the inhibition of cell wall synthesis and cytoplasmic membrane septum formation [25,69].

2.5.5. 2.2.3. Modulation of the Immune System by AMPs

Although the primary focus has been to identify AMPs with broad-spectrum antimicrobial activity through *in vitro* testing, many *in vivo* occurring AMPs have little direct antimicrobial activity [70]. The direct killing activities of some of these AMPs by either membrane perturbation or interaction with intracellular targets at physiological concentrations are antagonized by salt conditions, monovalent and divalent ions and serum [71,72]. Conversely, many of these peptides seem to be more involved in immunoregulation by acting as effectors of innate immune system. Some of these regulatory functions may include angiogenesis, cell proliferation, cytokine regulation, chemotaxis of certain leukocyte classes, degranulation of mast cells, stimulation of phagocytosis and even modulation of gene expression [70,73,74]. For example, human LL-37 has a pro-inflammatory action by up-regulation of specific chemokines (MCP-1 and IL-8) and binding to chemokine receptors such as IL-8RB, CCR, and CXCR-4 [75]. LL-37 receptors have been found in many different cell types, including monocytes, T-helper cells, mast cells, and epithelial cells, which suggest that certain AMPs can directly interact with host cells to induce immune responses [76,77]. However, it has also been shown that the induction of LL-37 has an *in vitro* anti-inflammatory function by inhibiting tumour necrosis factor α (TNF-α) production in macrophages [78]. These types of AMPs can counteract pro-inflammatory responses by preventing lipopolysaccharides (LPS) from inducing TNF-α production, thus suppressing the expression of LPS regulated genes in macrophages [75]. Apart from this indirect suppression, the LL-37 peptides can also induce specific anti-inflammatory genes by directly influencing macrophage gene expression, preventing an overwhelming immune response which could lead to fatal sepsis or endotoxemia [75]. Consequently, it seems that certain AMPs serve a dual, and seemingly counterproductive, immunoregulatory function by stimulating both pro-inflammatory and anti-inflammatory responses. This dualistic action may be a symmetric feedback mechanism to battle infection while simultaneously managing the septic levels by supressing the expression of certain cytokines. It is becoming clear that AMPs are a vital component of the germ line encoded innate immune system.

1.3.2.3. Therapeutic Potential and Obstacles Associated with AMPs

Many AMPs are promising candidates for drug development. The major advantages these peptides offer compared to conventional antibiotics are that they have a broader spectrum of antimicrobial action [10]; they exert their activity at micromolar concentrations [5,79] and they have a limited probability of

inducing pathogen resistance [80]. Although some AMPs, such as melittin, have low therapeutic potential because of their characteristic cytolytic activity, these natural AMPs can be used as templates to create peptide analogues with increased therapeutic efficacy. Structural analogues of melittin have proven to display similar or higher levels of antimicrobial activity with reduced toxicity [81–83]. Hybrid peptide formulations could also improve the antimicrobial activity and reduce toxicity [84,85]. AMPs also play a role in modulating the immunity (both the innate and adaptive immunity in higher organisms) and using these naturally occurring AMPs as templates to design novel drug candidates for the selective up-regulation of the innate immunity and suppression of pro-inflammatory cytokine response could prove to be effective anti-infective therapies [86]. One of the major limitations for the use of AMPs is that they are peptide based, which makes systemic use difficult, due to bioavailability issues. Therefore, most AMPs in clinical development are being considered for topical application [4,8]. However, the use of nano- and microparticles has been shown to be beneficial for the oral delivery of insulin [87,88]. In cancer therapy, the use of nanoparticles, containing melittin, has a higher affinity towards diseased cells over healthy host cells [89]. A study by Piras *et al.* showed that the use of nanoparticles loaded with temporin B (APD ID: AP00095), an AMP from the European common frog, *Rana temporaria,* increased the antibacterial activity and reduced cytotoxicity against mammalian cells [90]. The use of AMPs encapsulated in nanoparticles should therefore be further investigated regarding its potential use in the treatment of antimicrobial infections. AMPs can also be considered for multi-drug treatment regimens, as synergistic interactions were observed when antibiotic and AMP combinations were used against methicillin-resistant *Staphylococcus aureus* (MRSA) [91]. Finally, AMPs have a broader range of activity, as conventional antibiotics cannot be used for the treatment of viruses and are less effective against most parasites, while several AMPs have been shown to display antiviral and anti-parasitic activities [37,54,57].

3. Negl.Trop. Dis.

According to the World Health Organization (WHO), Negl.Trop. Dis. (NTDs) are a diverse group of 17 disabling, though neglected, conditions mostly affecting the world's poorest populations [1]. These diseases distress more than 1.4 billion people of low-income countries in Asia, Latin America and sub-Saharan Africa, causing more than 500,000 deaths annually [1,2]. In Africa alone, communicable diseases account for more than 70% of the total disease burden and deaths, with NTDs making up at least a fifth of this burden [92]. While these illnesses affect a considerable portion of the population, they may be considered "neglected" due to the dire lack of effective treatments and the fact that they are largely overlooked by funders and governing bodies (less than 5% of global health funding is allocated to alleviating the NTD burden) [3]. Although NTDs exist in the funding shadows of diseases like tuberculosis and HIV/AIDS, neglected diseases are noticeably contributing to the severity of the HIV pandemic by increasing the risk of horizontal and vertical HIV transmission [76,93,94]. While not all NTDs result in the loss of life, their morbidity and the accompanying economic loss are immense. NTDs can result in deformities and permanent damage, stunted growth, mental abnormalities and loss of eyesight and hence a lifetime of disability. The economic burden of NTDs are expressed as disability adjusted life years (DALYs), where each DALY unit refers to a healthy year lost due to premature death or disability [95]. The burden of NTDs amounts to staggering 46–57 million DALYs lost each year,

second only to HIV/AIDS, with malaria and tuberculosis only coming in at third and fourth place, respectively [96].

The 17 NTD infections are caused by a wide variety of organisms including vector-borne protozoa (*Trypanosoma* and leishmaniosis), parasitic worms (helminths including *Taenia solium, Echinococcus granulosus, Echinococcus multilocularis, Wuchereria bancrofti, Opisthorchis* spp. *Fasciola* spp. and *Paragonimus* spp., *Onchocerca volvulus* and *Schistosoma*), bacteria (*Mycobacterium ulcerans, Mycobacterium leprae, Treponema pallidum, Treponema carateum* and *Chlamydia trachomatis*) and viruses (*Flavivirus* and *Lyssavirus*) [1]. Due to the initial lack of funding and the diverse nature of NTDs, research, diagnosis, prevention and treatment strategies are challenging. In late 2000, the international community committed itself to drastically reduce poverty and combat disease amongst poor populations of the world, known as the Millennium Development Goals. This action plan has brought much needed attention to the neglected diseases, leading to the establishment of the Global Network for Neglected Tropical Disease Control and a WHO department specifically tasked to address NTD related issues [3]. Despite all this positive progress, NTDs still cause massive global suffering in millions of people. Novel, cost-effective, rationally designed treatments are desperately needed to ease the burden of NTDs.

1.4.3.1. Neglected Bacterial Infections

The discovery of penicillin, the world's first antibiotic, by Alexander Fleming in 1928 was a major milestone in the treatment of bacterial infections. However, an ever-increasing rise in antibiotic resistance amongst bacterial species has led research into the development of new therapeutic agents for bacterial infections [97]. AMPs are promising candidates in this regard [4,5,98]. As mentioned earlier, AMPs neutralize bacteria through direct killing actions which involve membrane disruption and interaction with intracellular targets [67]. NTDs caused by bacterial infections include leprosy and trachoma. Both these diseases have debilitating consequences that remain even after the disease is cured, contributing to the socio-economic burden of these diseases. Current treatment regimens involve the use of antibiotics, and although these prove to be effective the occurrence of antibiotic resistance could affect therapeutic outcomes in the future. This section will focus on the potential use of AMPs for the treatment of leprosy and trachoma.

2.5.6. 3.1.1. AMPs against Mycobacterium Infections and Hansen's Disease

The mycobacterium genus includes pathogens that are known to cause serious infections in both humans and animals. Over 50 different mycobacterium species are known to be involved in human disease and most notably include Mycobacterium tuberculosis (causative agent of tuberculosis), M. leprae (leprosy) and M. ulcerans (Buruli ulcer) [6]. Mycobacteria are intracellular micro-organisms capable of infecting and replicating in host macrophages [99]. Due to the complexity and high lipid content of the mycobacterial membrane and emergence of multi-drug resistant strains, it is notoriously hard to effectively treat these infections [100]. Contributing to mycobacterium infections are the HIV/AIDS pandemic and the wide spread use of immuno-suppressive drugs [6]. Alternatives treatment strategies to conventional antibiotics may prove beneficial against this diverse genus of diseases.

Over the past two decades, numerous antimicrobial peptides, including LL-37, have been identified that offer direct and/or indirect antimicrobial activity against mycobacteria [101]. A large portion of the LL-37 peptide consists of hydrophobic amino acids, enabling this AMP to bind and disrupt the cell membrane of certain micro-organisms [46,102]. It has also been shown that during a mycobacterium infection, LL-37 is able to directly inhibit the host cell's response by interacting with TLRs [103]. This AMP also plays a crucial role in the innate immune response to mycobacterium infections in several cell types [101]. In summary, the AMP, LL-37, has the capacity to fortify specific host innate immune responses, in addition to directly eliminating mycobacteria and/or inhibit their growth. AMPs like these may enable the development of novel and innovative antibiotics based on natural host antimicrobial peptides. Several AMPs play a central role in immune activation and inflammation regulation, possibly providing a unique foundation for the development of novel therapeutic agents.

"*And if the priest sees that the scab has indeed spread on the skin, then the priest shall pronounce him unclean. It is leprosy*." Leviticus 13:8.

References to leprosy (Hansen disease)-like symptoms date back millennia and can be found in ancient Egyptian writings and those of Hippocrates [104]. This chronic human disease is caused by the bacteria *M. leprae* and can have potentially debilitating neurological effects [105]. Although the implementation of global multi-drug therapy programs has considerably reduced the burden of leprosy, areas like Africa, Latin America and India still report more than 200,000 cases annually [106]. Until recently, the origins of leprosy could only be speculated on, with written records found in India (600 BC), China (500 BC) and even the armies of Alexander the Great likely referencing this disease between 327–325 BC [107]. Today, through comparative genomic studies, it has been deduced that leprosy spread from Africa to India, Europe and the Americas, mainly along colonisation, migration and slave trade routes [104]. There are four distinct and regionally specific *M. leprae* strains found globally, with strain 1 mainly found in Asia, East Africa and the Pacific; strain 2 is less predominant and is only found in regions of Malawi, Ethiopia, Nepal and New Caledonia; strain 3 is found in America, Europe and North Africa; and strain 4 is mainly predominant in the Caribbean and West Africa [104]. *M. leprae* has a genome of 3,268,203 base pairs (bp) that encodes for 1604 proteins [108], a genome that is considerably smaller than that of *M. tuberculosis* [109].

Gerhard Henrik Armauer Hansen (1841–1912) dedicated his entire career to studying leprosy and identified *M. leprae* as the causative agent of this disease in 1873, which is also historically the first human-disease-causing bacterium to be identified [110]. Leprosy is a curable disease when treated with multi-drug therapy. Although it estimated that 95% of modern people have a natural immunity to leprosy, studies indicate that a small portion of the population is susceptible to this disease due to a defect in cell-mediated immunity [111]. In the 1940s, the sulfone drug, Promin (sodium glucosulfone) was introduced as the first effective treatment for leprosy and was replaced with dapsone (diaminodiphenyl sulfone) in the 1950s [112]. Nowadays, treatment consists of rifampicin and clofazimine once monthly while a dosage dapsone daily are used for treatment of patients with multi-bacillary infections over a period of 1 year [113]. Disadvantages of this modern treatment regimen include the long treatment periods and the looming prospect of antimicrobial resistance. Another major drawback, apart from understanding the complex clinical presentation of leprosy, is the lack of an appropriate experimental model.

AMPs appear to play an important role in the immune response to leprosy. The innate immune recognition of *M. leprae* seems to be mediated by the toll-like receptor 2 (TLR2) and mutations/polymorphisms in *TLR2* can affect host susceptibility to leprosy [114]. The cytokines interleukin (IL) 10 and 15 are also known to regulate macrophage functions in lesions [115]. The AMP, human β-defensin 3 (hBD3) (APD ID: AP00283), is also up-regulated during a leprosy type 1 reaction [106]. The induction of vitamin D dependant antimicrobial peptides LL-37, is one of the antimicrobial mechanisms employed by the host cell following phagocytosis [115]. In leprosy, LL-37 levels are relatively low due to the *M. leprae* mediated inhibition of the gene encoding for LL-37 (*CAMP*) [116]. This inhibition occurs through the up-regulation of the miRNA, has-mir-21, by *M. leprae*. Suppressing the translation of this miRNA, may lead to an improved innate immune response to *M. leprae*, enhancing the antimicrobial activity of LL-37. Another AMP involved in this disease is hepcidin (APD ID: AP00193). This defensin has a dual role as a hormone and antimicrobial peptide and plays a key part in hypoferremia of inflammation which increases the microbial resistance of host cells [117]. Hepcidin seems to be mainly up-regulated in multi-bacillary lesions, where it binds directly to the iron exporter efflux protein, ferroportin, resulting in its degradation [118]. By doing this, the host cell is limiting the circulating iron available to extracellular bacteria. Additionally, Stat3 is phosphorylated, which stimulates a downstream cytokine induced inflammatory response. Due to natural and artificial evolutionary pressures, pathogens, including *M. leprae*, are becoming antibiotic-resistant at alarming rates [119]. In order to combat the further emergence and spread of antibiotic resistance, new and innovative treatments strategies for microbial infections must be found. Pathogens are much less likely to successfully adapt their iron metabolic pathways to use alternative molecules after iron sequestration by AMPs.

2.5.7. 3.1.2. AMPs against Trachoma

Trachoma is one of the oldest recorded diseases of mankind, and although it has been eradicated from the developed world, it is still prevalent in poverty-stricken, underdeveloped countries. Areas where trachoma is endemic are usually overcrowded, lacking in proper public health care facilities, sanitation and access to clean water [120]. The disease is most prevalent in Asia, Africa, the Middle East, Australia and Central and South America [121]. Trachoma is the leading cause of infectious blindness globally and is also referred to as the leading preventable cause of blindness worldwide [122]. Currently it is estimated that in the 51 endemic countries, trachoma is responsible for the visual impairment of 1.8 million people, of whom 500,000 are irreversibly blind, and approximately 232 million people living in trachoma-endemic areas are at risk of infection [121].

This infection is caused by the gram-negative obligate intracellular bacterium *Chlamydia trachomatis*. It has no known animal reservoir and infection is transmitted to the eyes through physical contact with the discharge from the nose and eyes of infected people or flies (*Musca sorbens*), acting as passive vectors [122]. The life cycle of *C. trachomatis* is unique compared to other bacteria due to its parasite-like dependence on a eukaryotic host cell to complete its replication cycle [123]. During this bacteria's life cycle, it adopts two distinct forms, the small extracellular elementary body (EB) and the larger intracellular reticulate body (RB). The EB is metabolically inactive and infectious, whereas the RB is metabolically active and represents the replicating form of *C.*

trachomatis. The EB attaches to and enters host epithelial cells, transform into an RB, and exists inside inclusion bodies inside the host cell cytoplasm. The RBs use energy-rich metabolic intermediates from the host and undergo binary fission. Consequently, following the amplification of RBs, inclusions begin to take up a great part of the host cytoplasm and start to fuse. RBs differentiate back into EBs and are released through the lysis of host cells, infecting other cells [120,124]. The intracellular inclusions make it difficult for the immune system to eliminate the pathogen [120]. Two *C. trachomatis* biovars exist; trachoma to which serovars A-K are assigned and *Lymphogranuloma venereum* to which serovars L1, L2, L2a and L3 are assigned [125]. Serovars A, B, Ba and C in the trachoma biovar cause trachoma and serovars D-K are responsible for sexually transmitted genital tract infections [124]. Sexually transmitted *C. trachomatis* infections may be implicated in ocular trachoma infections in children [126].

The SAFE program for the treatment and management of trachoma has been established by the WHO [127], where the acronym SAFE represents: **S**urgery for trachomatous trichiasis, **A**ntibiotics for active disease, **F**acial cleanliness to reduce transmission and **E**nvironmental improvements. Traditionally, antibiotic treatment consists of the use of 1% topical tetracycline ointment on both eyes twice daily for six weeks or a single oral dose of azithromycin. The treatment with azithromycin is favoured, as it has the potential to be used for mass community based treatment in endemic regions and displays higher patient compliance and effectiveness [128,129]. Much progress is being made in achieving the goal set by the WHO to eliminate blinding trachoma globally by 2020, with the disease being eradicated in some countries [121]. However, due to persistent infections and therefore antibiotic treatment, antibiotic resistance may become an issue. Therefore, alternative treatment strategies need to be investigated.

AMPs have been investigated for the use of the treatment of *C. trachomatis* infections. A study performed by Donati *et al*. showed that *in vitro* SMAP-29 (APD ID: AP00155), an α-helical cathelicidin from sheep (*Ovis aries*), was able to reduce the inclusion number for 10 strains of *C. trachomatis* (including serovars A, D, E, H and I). The integrity of EBs was also compromised [130]. Although, the direct therapeutic use of SMAP-29 is problematic because of its cytotoxic and hemolytic activity towards mammalian cells, this study indicates that SMAP-29 could be a useful compound in the development of anti-chlamydial drugs using this natural peptide as a template and making analogues with higher specificity and lower toxicity [131]. Another approach, which may be considered instead of the direct application of these peptides, is AMP gene therapy [132]. This approach uses recombinant plasmid vectors to express AMPs, which may potentially be cytotoxic to healthy host cells, in a controlled manner in infected cells. Studies performed by Lazarev *et al*. [133–135] demonstrated the potential use of AMP gene therapy for the treatment of *C. trachomatis*-infected cells. An *in vitro* study by Lazarev and colleagues showed that the introduction of recombinant plasmid vectors expressing the melittin gene, under the strict control of an inducible promoter, showed inhibition of *C. trachomatis* in infected cells. The mechanism by which the bacterial growth was inhibited was suggested to be through direct cytotoxic effect of melittin on the bacteria. It was also shown that the transmembrane potential of the transfected cells was lowered, which could disrupt the adhesion of the bacteria to the cell and hinder the normal process of intracellular development [133]. In a subsequent *in vivo* study by Lazarev *et al*., melittin was produced in the genital tract of mice infected with *C. trachomatis*, using the plasmid vector system. The majority of the melittin-protected mice were free of the pathogen after 27 days [135]. In a later *in vitro* study performed by the same group, the anti-chlamydial activity of the recombinant plasmid vector

encoding cyto-insectotoxin 1a (CIT 1a, APD ID: AP02163), a linear cytolytic AMP found in the venom of the central Asian ant spider, *Lachesana tarabaevi*, was investigated [134]. This study concluded that CIT 1a was an attractive candidate for targeting intracellular pathogens, as *C. trachomatis* infection was inhibited in the early stages of its life cycle, with a higher efficacy than had previously been reported for melittin [133] and with a negligible effect on cell viability. The effect of the direct expression of melittin in infected cells on cell viability was, however, not reported for the previous two studies. Based on these results, CIT 1a can be considered a potential agent for gene therapy for *C. trachomatis* infections.

1.5.3.2. Neglected Protozoan Infections

All protozoa are unicellular eukaryotic organisms with a trophozoite stage and a resistant cyst form. Medically important phyla include apicomplexa, such as *Plasmodium* and *Toxoplasma*, sarcomastigophora such as *Trypanosoma* and ciliophora such as *Trichomonas*. The pathogenicity of protozoa is not well understood, but in general they have fewer pathogenic mechanisms than bacteria. They have the distinct advantage of being able to avoid host defences by several mechanisms. Transmission of protozoa to humans occurs through the faecal-oral route or through a vector [136,137]. Neglected diseases caused by protozoa include chagas disease, human African trypanosomiasis and leishmaniosis. Most of these protozoan diseases have enormous impact on human health and cause great economic and social burdens [138]. The typical protozoan infections are appealing targets for AMPs, as the molecules can be targeted to stages in the invertebrate vector or the vertebrate host. There are excellent reviews on the possibility of using AMPs as new chemotherapeutic options for malaria, chagas disease and sleeping sickness, as well as leishmaniosis [34,139]. This section will focus on the potential use of AMPs for the treatment of the neglected diseases chagas disease, sleeping sickness, leishmaniosis and malaria.

2.5.8. 3.2.1. AMPs against Chagas Disease

Chagas disease, or American trypanosomiasis, is caused by the parasite *Trypanosoma cruzi*. The disease was initially confined to South America, but 2010 epidemiological data include North-America, Europe, Australia and Japan as sites of *T. cruzi* infection [136,137,140]. The parasite is spread by the triatomine bug (part of the diverse Reduviidae family), which infects the human host when feeding, and it is also spread by travellers, organ transplants, blood transfusions, contaminated soil, food or water and by mother to infant transmission. The parasite has a complex life cycle including a trypomastigote in human blood and the epimastigote in the vector [140]. The clinical presentation occurs in two phases: an acute phase that lasts about two months after initial infection, and a chronic phase that can last for years. The acute infection is often misdiagnosed or unrecognized. However, treatment is most effective when initiated during the acute phase. From the intermediate phase, which is characterised by patients with chronic infection without clinical symptoms, 20%–30% of patients progress to a more serious chronic stage with chronic inflammation of the heart or digestive muscles [141]. There are only two drugs available for the treatment of chagas disease, benznidazole and nifurtimox. Both of these drugs are highly effective if administrated during the acute phase, but the efficacy declines with

prolonged infection. Additionally, these drugs are toxic with negative side effects and are not effective in the chronic stage of infection [138,142,143]. There is a dire need for alternatives and AMPs from natural compounds seem to be an attractive option.

Several studies have reported promising effects of crude venom extracts containing AMPs from various sources against protozoan parasites. The crude venom of *Apes mellifera* was shown to decrease the viability and alter the ultrastructure of all *T. cruzi* developmental forms. This venom was also shown to be selective towards the parasite with no toxicity in mammalian cells at the tested concentrations [144]. In a follow up study by the same group, the AMP melittin from the venom of *A. mellifer*a was used to prove that the peptide induced morphological alterations in the different developmental forms of the parasite, which could be characterized as apoptosis and autophagy. They also provided evidence that melittin could be used in concentrations up to 1 µg/mL to treat infected host cells [145]. Another group investigated the efficacy of the antimicrobial peptides apidaecin 14 and melittin from *A. mellifera*, penaeidin (APD ID: AP00392) from the black tiger shrimp (*Penaeus monodon*), cecropin A (APD ID: AP00139) from the silk moth *Hyalophora cecropia*, moricin (APD ID: AP00147) from the silk worm *Bombyx mori* and magainin 2 produced by the African clawed frog (*Xenopus laevis*). Cecropin A, magainin 2, apidaecin and melittin were able to kill *T. cruzi*. These peptides were also investigated in combinations for synergistic effects, and in each case the addition of a second AMP increased toxicity [146]. Previously synthetic analogues of magainin 2, namely magainin B, G and H, were evaluated against *T. cruzi*. Only magainin B and G were effective against the parasite [147]. Synthetic derivatives of cecropin B were active against *T. cruzi*, where Shiva-1 the peptide with only 40% homology was most effective at killing promastigotes [148].

Dermaseptins and phylloseptins are cutaneous secretions of the tree frog *Phyllomedusa* genus. Dermasiptin-01 (APD ID: AP01389) from *Phyllomedusa oreades* showed significant lytic activity against *T. cruzi* with minimal activity against erythrocytes [149]. Dermaseptins form *Phyllomedusa nordestina* was investigated by another group. Dermaseptin 1 and 4 and phylloseptin-7 and 8 were effective against *T. cruzi* at micromolar concentrations. Dermaseptin 1 and 4 and phylloseptin 8 were also non-toxic to macrophages [150]. AMPs from various aquatic animals were also evaluated against *T. cruzi* promastigotes. Only tachyplesin I (APD ID: AP00214) from the horseshoe crab *Tachypleus tridentatus* was able to kill *T. cruzi* epi- and promastigotes at micromolar concentrations. Tachyplesin I was, however, hemolytic at high concentrations [151].

2.5.9. 3.2.2. AMP against Human African Trypanosomiasis

Human African trypanosomiasis is a parasitic disease mainly caused by two subspecies, *Trypanosoma brucei gambiense* and *T. brucei rhodesiense,* and causes chronic sleeping sickness in sub-Saharan African countries [136]. The clinical presentation of the disease is complex, making diagnostics challenging and treatment difficult [137]. The life cycle is similar to chagas disease with a trypomastigote phase in the human reservoir; however the epimastigote exists in the tsetse fly vector. Due to parenteral administration and severe toxicity associated with current chemotherapy options increasing incidence of treatment failure is observed [152]. The drugs used in the treatment depend on the infecting species as well as the stage of infection. Pentamidine and suramin is used in the first stage, and although effective, suramin has severe side effects. Second stage treatment includes IV melarsoprol,

eflornithine and a combination of nifurtimox and eflornithine [153,154]. In comparison with the other parasitic infections, relatively few AMPs have been identified or evaluated for efficacy against *T. brucei*.

Temporin-SHd (APD ID: AP02118) from the North-African frog *Pelophylax saharicus* had significant growth inhibitory activity against *T. bruce*i and *T. cruzi* [155]. Mammalian AMPs including α-defensins, β-defensins and cathelicidins have proved successful against *T. brucei*. *In vivo* administration of cathelicidins to mice infected with *T. brucei* significantly reduced parasitemia [156]. In another study the bovine cathelicidin BMAP-27 (APD ID: AP00366) from *Bos taurus* (bovine) and its derivative BMAP-18 inhibited both life cycles of *T. brucei* at low micromolar concentrations. BMAP-18 induced apoptosis-like cell death, but necrosis was induced at higher concentrations [157].

2.5.10. 3.2.3. AMPs against Leishmaniosis

Leishmaniosis species causes serious disease in humans. The typical infection is cutaneous, although visceral (kala-azar) and mucocutaneous leishmaniasis also exist. Visceral leishmaniasis caused by *Leishmania donovani* is fatal if left untreated. Cutaneous leishmaniasis caused by *L. major*, *L. tropica*, *L. mexicana* and *L. panamensis* frequently occurs every 3 to 18 months and leaves disfiguring scars [136,158]. Leishmania have a simple life cycle with only an amastigote in mammalian cells and promastigote in the insect (sand fly) [137]. The current chemotherapy available includes pentavalent antimonials sodium stibogluconate and meglumine antimoniate, amphotericin B and its lipid formulation AmBisome® (amphotericin B) and pentamidine. Antimonials remain effective for some forms of leishmaniasis, but the drugs' usefulness is limited by the required parenteral administration for 28 days and the emergence of significant resistance. The use of pentamidine is limited due to its toxicity [159]. Several AMPs have been evaluated against *Leishmania* and some promising candidates have been identified.

Temporins, isolated from the European common frog, *Rana temporaria*, represent natural peptides with high anti-leishmanial activity. One of the first studies to prove the anti-leishmanial activity of temporins investigated temporin A (APD ID: AP00094) and B (APD ID: AP00095) against *L. donovani* promastigotes and *L. pifanoi* amastigotes. They found that both temporin A and B were active against the insect and mammalian stages at 15–25 μM concentrations [160]. Chadbourne *et al.* investigated the potential use of temporins against *L. mexicana* insect stage promastigotes and the mammalian stage amastigotes. The temporins investigated included temporins A, B and 1Sa. These AMPs showed no significant activity against *L. mexicana* amastigotes, but significant anti-leishmanial activity was observed against promastigotes with temporin A [161]. Temporin-1Sa (APD ID: AP00898) isolated from the North-African frog, *Pelophylax (Rana) saharica*, had significant activity against the promastigote and amastigote stages of *L. infantum* at a concentration that was not harmful to macrophages [162]. In another study temporin-Shd had similar activity against several species of Leishmania promastigotes and amastigotes [155]. Temporins A, B, 1Sa, F (APD ID: AP00098) and L (APD ID: AP00101) were effective against *L. mexicana* promastigotes, but the amastigotes were resistant to all temporins tested. The resistance was attributed to the lack of proteophosphoglycan and anionic charge in the membrane of the amastigotes [163]. The proposed mechanisms of action whereby they function include rapid disruption of plasma membrane potential and decrease in intracellular ATP levels [160].

Dermaseptin S4 (APD ID: AP00160) from the South-American Sauvages leaf frog (*Phyllomedusa sauvagii*) and its synthetic analogues induced potent lysis for *L. major* promastigotes. Positive or negative mono-substitutions in the synthetic peptides did not significantly affect the antileishmanial activity [51]. Dermaseptin 01 was shown to be active against *L. infantum* promastigotes by membrane damage and flagella alterations [164]. Dermaseptin from *Phyllomedusa hypochondrialis* (DShypo 01) was evaluated against *L. amazonensis*. It proved more effective at killing promastigotes than dermaseptin 01, and was not toxic to white blood cells or erythrocytes [165].

Other AMPs from various organisms active against *Leishmania* include gomesin (APD ID: AP00191) from the tarantula spider *Acanthoscurria gomesiana*, indolicidin and plant derived thionins. Gomesin decreased the viability of *L. amazonensis* promastigotes with little activity against human erythrocytes [166]. Indolicidin and two peptides derived from seminalplasmin exhibited significant anti-leishmanial activity. Additional to the membrane effects these peptides were also able to induce autophagy in *L. donovani* [167]. Different isoforms of thionins from common wheat (*Triticum aestivum*) were tested against *L. donovani* promastigotes. These thionins proved effective in the low micromolar range and membrane permeability was found to be an essential step in the lethal mechanism [168].

Also, synthetic cecropin A, andropin and dermaseptin were found to be active against *L. major* and *L. panamensis*. These three synthetic peptides have higher therapeutic potential were also highly selective with low toxicity against human erythrocytes and dendritic cells [169]. Pexiganan (Locilex®), a synthetic magainin-based lysine-rich peptide, which is undergoing phase 3 clinical trials for diabetic foot ulcers [4], induces apoptosis in Leishmania promastigotes. Activity is favourable if the strain of Leishmania is surface protease-deficient [170,171]. However, an arginine-rich variant of pexiganan proved to be protease resistant and displayed enhanced activity against wild type Leishmania *in vitro* [172]. These studies provide valuable insights into the use of AMPs against parasitic infections

2.5.11. 3.2.4. AMPs against Malaria

Malaria is an infectious disease caused by parasites of the *Plasmodium* genus. This life-threatening disease is responsible for 219 million new cases and 660,000 deaths annually, making it one of the deadliest modern infections [173]. These parasites are primarily hosted by female *Anopheles* mosquitoes, which act as vectors transmitting the protozoan organisms to humans when feeding. There are four known species that infect humans: *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae*, though *P. falciparum* causes the majority of malaria infections [136,173]. The parasite has a complex life cycle with an asexual cycle in the human host and a sexual cycle in the mosquito. The asexual cycle includes ring and trophozoite stages in red blood cells, which are targets for chemotherapy. The sexual stage in the mosquito includes gametocytes and sporozoites, which are transmitted to humans [137]. Clinically, the disease presents initially with flu-like symptoms, which may be difficult to recognise as malaria. If left untreated, within the first 24 h *P. falciparum* malaria may progress to a more severe illness. Chemotherapy, when initiated early, is successful, but increasing resistance to current treatment options is proving problematic [174]. Additionally, many antimalarial drugs are toxic in high doses, though Pheroid vesicles have been shown to reduce toxicity [175]. Various AMPs have been investigated as a possible new class of antimicrobial drugs. A very thorough review on this topic was recently published [34], though only selected examples will be highlighted in this section.

The hemolytic dermaseptin S4 was shown to have antimalarial activity against *P. falciparum* (FCR3 strain). The peptide causes significant lysis of the ring and trophozoite stages of the parasite [176]. Natural peptides melittin and transportan 10 (TP10), mastoparan X (APD ID: AP02355) and anoplin (APD ID: AP00447) from the *Vespula lewisii* wasp, were effective against *Plasmodium* sporogenic stages and were considered to block transmission [177]. Synthetic analogues of cecropin B, SB-37 and shiva-1 were effective at limiting the growth of different stages of *P. falciparum* [148].

Gomesin was evaluated against *P. falciparum* and *P. berghei*. This AMP inhibited chloroquine-sensitive (3D7) and chloroquine-resistant (W2) parasites, but only at micromolar levels, compared to nanomolar levels of artesunate. Gomesin was more effective against *P. berghei* mature gametocytes and also proved successful as a transmission blocking agent [178]. These examples of AMPs all act against different life cycles of the parasite, with different mechanisms of action. At this stage the most promising candidates in AMPs for malaria seems to be those that are involved in transmission-blocking. However, this is still a developing field and many of the AMPs active against other parasites have not been evaluated in malaria models.

1.6.3.3. Neglected Helminth-Related Infections

Helminths, also known as parasitic worms [179], are a group of evolutionary unrelated organisms that can be divided into two major groups, namely the Platyhelminthes (flatworms) and Nematoda (roundworms) [180,181]. Worldwide, more than 2 billion people are infected with one or more of these parasitic worms, including the hookworm, *Necator americanus;* the roundworm, *Ascaris lumbricoides*; and *Ancylostoma duodenale*; and the whipworm, *Trichuris trichiura* [182]. Although these parasitic infections primarily affect underdeveloped populations with insufficient sanitation, housing, water supplies and primary health care systems [183,184], they are also widespread in developed countries [185].

Presently, the treatments for helminth infection are parasite-specific and involve regular administration of anti-parasitic drugs. Although these treatments (including benzimidazoles for hookworm infections and praziquantel for schistosomiasis infections) are currently effective, parasite re-infections are still a problem and helminths will most likely develop resistance to these drugs [186]. Consequently, investigations into novel helminth-specific treatments should seriously be considered. There are currently eight helminth infections identified by the WHO as NTDs. This section will focus on the potential use of AMPs for the treatment of taeniasis, cysticercosis and onchocerciasis.

2.5.12. 3.3.1. Possible AMPs against Taeniasis and Cysticercosis

Taeniasis is a parasitic intestinal infection caused by two species of adult tapeworms, namely *Taenia solium* (pork tapeworm) and *Taenia saginata* (beef tapeworm). This parasitic disease is contracted when humans ingest raw or underprepared beef (infected with *T. saginata)* or pork (*T. solium*). The accidental consumption of *T. solium* eggs, through contaminated food or water, can lead to cysticercosis [187]. The eggs move into the digestive tract, where they hatch and develop into larvae (cysticerci). These larvae can then enter the circulation and invade host tissue and organs, such as muscles, eyes, skin and the central nervous system. If the cysticerci spread to the brain, neurocysticercosis may develop, which can be a fatal condition [188]. Symptoms include blindness,

headache, dementia, meningitis and epilepsy. Current treatment of taeniasis includes praziquantel or niclosamide [189]. Treatment of neurocysticercosis includes long courses with praziquantel or albendazole, and also supporting therapy with anti-epileptic drugs and corticosteroids [190]. There are, however, drawbacks involved when using these drugs. Praziquantel can induce epileptic seizures in patients with neurocysticercosis, whereas niclosamide is only effective against adult intestinal tapeworms [187,191].

Temporin A and iseganan IB-367 (a protegrin-1 (APD ID: AP00195) derivative that belongs to the cathelicidins family) have anti-parasitic effects against *T. crassiceps* [192], a parasite which has a close relationship with *T. solium.* Iseganan IB-367 functions by disrupting cell membranes through the induction of pores. Both these peptides reduce the parasitic load and damage the tegumentary surface of the cysticerci. In a study by Landa *et al.*, temporin A reduced the parasitic load by 50% and iseganan IB-367 by about 25%. These peptides also induced morphological changes *in vitro* in the cysticerci [193]. AMPs have therefore been shown to be effective in destroying cysticerci and damaging the wall of the cysticerci, which is critical for the death of the cestode. These, or structurally similar peptides, should be investigated as alternatives to current anthelminthic treatment to bolster the treatment of taeniasis and cysticercosis.

2.5.13. 3.3.2. AMPs from Onchocerciasis

Onchocerciasis, also known as "river blindness", is caused by the filarial worm *Onchocerca volvulus* [194], the second most common cause of blindness from infection after trachoma [195]. The disease is transmitted by the bites of infected blackflies (*Simulium* species) which breed in fast flowing streams and rivers, predominantly in central Africa [196]. The adult worms produce microfilariae in the human body where it migrates to the eyes, skin and other organs. Symptoms develop as a result of the microfilariae moving around in the body and inducing inflammatory responses. Severe itching, skin lesions and nodules under the skin are some of the symptoms that infected people develop. Eye lesions can also develop, causing visual impairment ultimately leading to permanent blindness. The recommended treatment for this disease includes ivermectin once yearly for 10 to 15 years [194]. Repeated doses of ivermectin over several years are required for the elimination of an onchocerciasis infection to halt the transmission of the parasite in the long term [197].

Human neutrophil peptide 1–3 (HNP1–3) was identified in *O. volvulus* worms extracts [198]. Human neutrophil peptides are α-defensins that belong to the family of cationic trisulfide-comprising antimicrobial peptides [199]. HNP1-3 has been shown to mediate the macrophage response to micro-organisms by stimulating the release of IFN-γ and TNF-α [200]. Defensins can neutralize a target micro-organism by binding and permeabilizing its membrane. Although human neutrophil peptides can still bind to the surface of *O. volvulus*, this parasitic worm may have developed a limited resistance to these AMPs through co-evolution. This may explain why human neutrophil peptides were isolated with *O. volvulus* worm extracts, as the peptides are able to bind to the surface of the worm but not cause permeabilization and death. Identifying and/or investigating natural AMP homologs of human neutrophil peptides may yield alternative treatments for onchocerciasis.

In another study, Eberle *et al.* identified at least three excretory/secretory products (including galectin) of *O. ochengi* and *O. volvulus* with significant antibacterial activity against *E. coli* [11]. The

excretory/secretory peptides of many parasites are similar to host defence peptides and, as mentioned earlier, host defence peptides have been shown to have antibacterial, antifungal, anti-parasitic and antiviral activities [201]. The peptides excreted/secreted by *O. ochengi* and *O. volvulus* present a promising pool of potential novel AMPs and further investigation into these peptides is needed [11].

1.7.3.4. Antiviral Peptides and Their Potential Use to Treat Viral Diseases and Possible Application to Viral Negl.Trop. Dis.

Viral diseases are one of the leading causes of morbidity and mortality globally, especially in lower income countries. Adding to this problem, antiviral treatments are expensive to develop and are usually only effective against a single virus [202]. The control of viral diseases has always been a challenge, due to their genetic diversity, short and effective replication cycles, diverse transmission means, and adaptability, as well as the wide variety of hosts. In their history, the US Food and Drug Administration (FDA) has only approved about 60 antiviral drugs for seven viruses (cytomegalovirus, herpes simplex virus, hepatitis B and C viruses, human immunodeficiency virus-1 (HIV-1), influenza, and varicella-zoster virus) and almost half of the approved antiviral drugs are for HIV-1 [203]. Despite this, a considerable amount of peptides have been shown to be effective against wide range of viral infections [204–208]. There seem to be two main antiviral mechanisms of AMPs as reviewed in Klotman and Chang [57]. The first mechanism is similar to the antibacterial activity of AMPs and involves direct disruption of viral envelopes or interaction with internal viral targets, while the second is thought to be an indirect antiviral action by stimulating specific innate immune mechanisms of the infected host cell.

When the first antiviral AMP (HNP-1) was discovered in 1986, it was believed that these peptides only had a direct effect on enveloped viruses while overlooking non-enveloped viruses [209]. HNP-1 was reported to have a direct inhibitory effect on the enveloped viruses such as herpes simplex virus-1 and 2, influenza virus and vesicular stomatitis [209]. It is hypothesised that the method of direct inactivation is the disruption of the viral capsid or preventing host entry by binding to the viral glycoproteins [57], but the exact mechanism is not clear and requires further investigation. AMPs (including HNP-3 and human β-defensin-3) have no detectable direct effect on the non-enveloped rhino-, echo- and reoviruses [209,210]. However, it has been shown that the AMPs HNP-1 and β-defensin-3 can stimulate infected host cells to subdue the viral replication of non-enveloped viruses after virion entry [211]. Instead of blocking the binding of the virus to the host cell and subsequent endocytosis, antiviral peptides may obstruct the release of the virion from the endosomes. Still, further studies are needed to elucidate the mechanisms by which AMPs can suppress non-enveloped viral replication.

In a study done by Carriel-Gomes *et al.*, they evaluated the *in vitro* antiviral activity of nine AMPs against the human adenovirus (respiratory strain), type 1 herpes simplex virus and rotavirus (SA11 strain) [212]. Although several AMPs tested showed promising antiviral activity, most peptides were cytotoxic at their active concentrations. However, modifying the peptide structures of these AMPs may reduce their cytotoxicity and make them attractive antiviral treatments. In order for the cell to mount an effective antiviral offensive, most viral infections lead to the stimulation of a complex cascade of host cell signalling pathways (including Jak-STAT- and Toll pathways), ultimately resulting in the expression of IFN-stimulated genes (ISGs), which is able to directly inhibit viral replication [213–215]. The secretion

of cytokines belonging to the interferon (IFN) family (IFN type I and III in particular), play an important role in this innate immune response by activating the expression of IFN-stimulated genes (ISGs) [216,217]. Many AMPs have been shown to exhibit antiviral activity during several phases of viral pathogenesis [218] and several AMPs play a central role in immune activation and inflammation regulation [77,219], possibly providing a unique foundation for the development of novel therapeutic agents. This section will focus on the potential use of AMPs for the treatment of dengue viral disease and rabies.

2.5.14. 3.4.1. AMPs against Dengue Viral Disease

Dengue viral disease is a mosquito (*Aedes aegypti*)-borne pathogen predominant in tropical and sub-tropical regions, putting more than 3.6 billion humans at risk of infection [220,221]. This disease is caused by one or more of five serotypes of the dengue virus (DEN-1 to -5) belonging to the genus *Flavivirus* [222,223]. Genetically, at least four of the five dengue virus serotypes seem to share a common ancestor in sub-human primates from central and east Africa [224]. The modern dengue virus is an enveloped, single positive-stranded RNA virus with a genome of ~11,000 bp that codes for three structural proteins and seven non-structural proteins [225]. Phylogenetic analysis indicated that dengue viruses have exceptionally high mutation rates (as high as 1 nucleotide mutation per life cycle), which is most likely due to the lack of proofreading ability in their RNA-dependant RNA-polymerase [226,227]. Only the female mosquitos act as vectors for the dengue virus and they are infectious throughout their entire lifespan [228]. Alarmingly, there has been a rapid ecological expansion of the virus's vector, *A. aegypti*, over the past few decades, dramatically increasing the risk of human infection [221]. Dengue virus infects approximately 500 million people across 124 countries annually. The resulting infection causes nearly 21,000 deaths and an economic burden of more than US$950 million each year [220,229,230]. The majority of mortalities are caused when dengue viral disease progresses to severe dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) [220]. Due to the five diverse dengue virus serotypes, high mutation rate and lack of a working animal model, no effective vaccine exists for this virus [231]. Additionally, disease prevention is impractical due to difficult vector control, and clinical treatment is limited to secondary shock prevention care. There is a dire need for the development of alternative anti-dengue viral treatments.

As an alternative approach, Rothan and colleagues investigated the potential of the AMP latarcin (Ltc-1; APD ID: AP01010) from the venom of the ant spider *Lachesana tarabaevi* to inhibit the replication of dengue viruses in cultured Vero cells [232,233]. In dengue virus, Rothan *et al.* showed that the Ltc-1 peptide binds to the non-structural protein 3 (NS3) which may inhibit substrate binding to the NS3 active site, hindering the formation of the NS2B co-factor active site [233]. For the release of the mature dengue viral structural and non-structural proteins, the NS2B-NS3pro complex must first cleave the dengue viral poly-protein at specific sites [234,235]. Consequently, the Ltc-1 inhibition of the NS2B-NS3pro complex can halt virus replication by hindering the post-translational function of the dengue viral poly-protein [233,236].

In another study, two synthetic AMPs designed to target the domain III of the dengue DENV-2 E protein significantly inhibited virus entry in to LLC-MK2 cultured cells [237]. Efficient entry of the virus particle into the host cell is crucial to the success of any infection. In the case of the dengue

virus, attachment to the host cell surface receptors and subsequent viral–cell membrane fusion is mediated by the virus's E protein [238]. The E protein consists of three domains comprising domain I (the central structural domain), domain II (the dimerization domain) and domain III (the receptor-binding domain) [239]. Apart from cell entry, the E protein is also the chief target for protective antibodies against the dengue virus, and there are several conserved regions between the dengue serotypes [238]. The two synthetic AMPs designed by Alhoot and colleagues targeted a short amino acid sequence in the lateral loop of domain III in strain 2 of the dengue virus. This may reduce the viral load during early infection and buy time for an effective immune response which can reduce the severity of infection. This inhibitory function of these synthetic AMPs against dengue virus can provide a basis for identifying similar natural AMPs to develop new therapeutic strategies against dengue infections.

2.5.15. 3.4.2. AMPs against Rabies

Rabies has an exceptionally high fatality rate for an infectious disease (fatality rate of virtually 100% after symptoms appear) and is responsible for an estimated 55,000 deaths annually [240]. However, due to underreporting, deaths may be as much as 100 times higher [241]. This zoonotic disease has been plaguing mankind for more than 5000 years and has a very unique and wide range of hosts for a viral disease, as it can infect almost all warm-bodied animals [242,243]. Rabies mainly affects the poor, rural communities of developing regions and is 100% vaccine preventable. The causative agent of rabies is the enveloped, negative-sensed, single-stranded RNA Lyssavirus (Lyssa, Greek goddess of madness and frenzy) from the *Rhabdoviridae* family [244]. The Lyssavirus genome consists of approximately 12,000 bp and encodes for 5 viral proteins. The virus is found in the saliva of infected animals and is usually transmitted through their bites. The rabies virus can supress the host's innate immune response by subduing the type I interferon response [245]. After the initial wound entry, Lyssavirus uses the central nervous system to invade the host's neurons, where it replicates and spreads [246]. Symptoms of the classical rabies infection commonly include hydrophobia, muscle spasms, extreme aggression (erratically attacking objects or other humans) and terror [242,247]. Nowadays, rabies infections are primarily controlled by limiting animal infections. In the United States, if a human is exposed to a rabies-infected animal, a prophylaxis containing rabies immunoglobulin together with an inactivated rabies virus vaccine is administered [248]. With the approval of the WHO, viral vector vaccines and DNA vaccines have been introduced in developing countries to reduce vaccination costs [249,250]. Pre-exposure rabies vaccines exist, but are not widely used and mainly reserved for individuals that have a high risk of infection, including researchers and veterinarians due to their high costs [251].

In a study by Real *et al.*, short, natural peptides that target rabies viruses were evaluated for their antiviral potential [252]. They proposed an antiviral drug discovery strategy based on the mimicry of natural peptides such as the lebocin 1 and 2 (APD ID: AP00359) from silkworms (*Bombyx mori*) and μ-conotoxin from cone snails (*Conus geographus*). A large amount of peptides were screened for their binding affinity to the phosphoprotein of the rabies virus. The viral phosphoprotein plays an important role in the transcription-replication complex and reduces non-specific RNA binding by acting as a chaperone for the nucleoprotein [254,255]. Only selected peptides with high binding affinity were then evaluated for their antiviral potential against rabies viruses. After an *ex vivo* inhibition of viral replication assay, they identified four structurally diverse peptides (C2, C6, C8 and P16) that exhibited strong rabies

virus inhibitory properties [252–253]. Three of the four AMPs (C6, C8 and P16) had an algorithmically predicted α-helical conformation, while the AMP with highest antiviral activity, C2, seemed not to possess a helical-like structure. The central role that the viral phosphoprotein plays in the transcription-replication complex makes this protein an attractive target for antiviral treatments. Real *et al.* showed that AMPs based on natural peptides can be used to drastically inhibit the replication of rabies viruses. Their work also offers an alternative strategy for identifying novel antiviral peptides, with the objective to develop new types of antiviral treatments. Besides this study, very little published work is available on the antiviral treatments for rabies. There have, however, been some AMPs identified for other viruses in the *Rhabdovirus* family, including casein and α_{s2}-casein, which can drastically inhibit the replication of the infectious haematopoietic necrosis virus in salmonid fish [256]. Another study also found that human α-defensin-1 has antiviral activity against the haemorrhagic septicaemia virus [257]. All these findings suggest that AMPs are a viable antiviral treatment option for viruses in the *Rhabdovirus* family, and more efforts should be made to identify and investigate these peptides as possible rabies treatments.

4. Conclusions

NTDs are a diverse group of 17 disabling, though neglected, conditions affecting a fifth of the world's population, resulting in more than 500,000 deaths annually. These infections, prioritized by the WHO, are caused by a wide variety of organisms including vector-borne protozoa, parasitic worms, bacteria and viruses. Due to the diverse nature of NTDs and the initial lack of funding, research, diagnosis, prevention and treatment strategies are challenging. The Millennium Development Framework is a United Nations-led coalition for the alleviation of poverty in the developing regions of the world by 2015. One of the goals of this framework is to combat HIV/AIDS, malaria and Negl.Trop. Dis.. Although this goal has brought much-needed attention to neglected diseases, NTDs still cause massive global suffering in millions of people. Innovative, cost-effective, rationally designed treatments are desperately needed to ease the burden of NTDs.

Due to the ever-increasing rise in antibiotic resistance, there is a dire need for the development of alternative treatment strategies. AMPs, produced by all known living species, may be considered natural antibiotics due to their central role in the innate immune system, providing the first line of defence against microbial infections. These peptides have a broad range of activity against bacteria, parasites and viruses. Numerous studies have focused on the potential use of AMPs for the treatment of NTDs, as summarised in Table 2. Many of these AMPs have shown to be promising candidates for the development of therapeutic agents in the battle against NTD infections. However, many obstacles still need to be overcome, including the cytotoxic effects of some peptides and challenges regarding bio-availability. Structural analogues of natural AMPs and hybrid peptide formulations have shown promising results in lowering cytotoxicity and should therefore be further investigated for the possible treatment of NTDs. Additionally, bio-availability issues may be overcome by utilizing nano- or mircoparticle formulations which can also increase specificity and decrease toxicity associated with some AMPs. Apart from the direct application of AMPs, they may be considered for multi-drug treatment regimens as synergistic interactions have been observed when used in combination with conventional antibiotics. Due to this synergism and different mechanisms of action between AMPs and

antibiotics, the probability of inducing pathogen resistance to antibiotics can be drastically reduced. AMPs that are currently in clinical trials for the treatment of other conditions should be investigated as potential candidates for the treatment of NTDs. These AMPs might prove to be effective against NTDs; for example, pexiganan, which is undergoing phase 3 clinical trials for diabetic foot ulcers, served as template for the development of an arginine-rich variant that displays potent anti-leishmanial activity.

Currently, the greatest obstacle for the use of AMPs, which especially affects its use in the treatment of NTDs, is the cost of the large-scale synthesis of these peptides. Additionally, for large-scale application, isolation from natural sources is also not a viable option. Progress has been made in developing DNA recombinant methods to successfully synthesise and purify AMPs for therapeutic application in a cost effective manner [258–260], but the commercial feasibility of these methods still need to be evaluated. Also, because ribosomally synthesised AMPs are expressed by single genes, they may be considered for use in gene therapy for introduction directly into infected tissue. This could significantly reduce the cost associated with the large-scale production and purification of AMPs.

In conclusion, AMPs are effective against a variety of infectious diseases including NTDs. AMPs offer innovative treatment possibilities as they can be used as single anti-microbial agents and in combination with conventional antibiotics, as well as immunomodulating agents. Future research should focus on addressing the issues related to toxicity and challenges associated with mass manufacturing.

Table 2. Summary of selected AMPs that are associated with or display activity against neglected tropical dis eases.

Type	Infection (causative agent)	AMP	Source	Notes	Ref
Bacterial	**Leprosy** *Mycobacterium leprae*	Human β-defensin 3	*Homo sapiens* (Human)	Up-regulated during a leprosy type 1 infections	[106]
		LL-37	*Homo sapiens* (Human) *Pan troglodytes* (Chimpanzee)	*M. leprae* inhibits *CAMP* - the gene encoding for LL-37	[116]
		Hepcidin	*Homo sapiens* (Human)	Involved in the degradation of ferroportin in multibacillary lesions	[118]
	Trachoma *Chlamydia trachomatis*	SMAP-29	*Ovis aries* (Sheep)	Reduced inclusion number at concentration of 10 µg/mL. Compromised integrity of extracellular elementary body	[130]
		Melittin	*Apis mellifera* (Honey bee)	Direct cytotoxic effect on *C. trachomatis*. Hindering normal process of intracellular development by lowering the transdermal potential and disrupting the adhesion of the bacteria to the cell	[133]
		Cyto-insectotoxin 1a	*Lachesana tarabaevi* (Central Asian ant spider)	Inhibit *C. trachomatis* infection at an early stage, with higher efficacy than melittin and negligible effect on cell viability	[134]
Parasites	**Chagas disease** (*Trypanosoma cruzi)*	Melittin	*A. mellifera* (Honey bee)	Induced morphological alterations in the different developmental forms of the parasite, which could be characterized as apoptosis and autophagy.	[145]
		Dermasiptin-01	*Phyllomedusa oreades* (Tree frog)	Lytic activity	[149]

Table 2. *Cont.*

Type	Infection (causative agent)	AMP	Source	Notes	Ref
Parasites	**Chagas disease** (*Trypanosoma cruzi)*	Tachyplesin I	*Tachypleus tridentatus* (Horseshoe crab)	Able to kill *T. cruzi* epimastigote and promastigotes at micromolar concentrations.	[151]
	Human African Trypanosomiasis (*Trypanosoma brucei)*	Temporin-SHd	*Pelophylax saharica* (Sahara frog)	Growth inhibitory activity against *T. brucei* and *T. cruzi*	[155]
		BMAP-27	*Bos Taurus* (Cattle)	Inhibited both life cycles of both *T. brucei* at low micromolar concentrations	[157]
	Leishmaniasis (*L. donovani, L major, L. tropica, L. Mexicana* and *L. panamensis*)	Temporin A and B	*Rana temporaria* (European common frog)	Active against insect and mammalian stages at 15-25μM concentrations. Rapid disruption of plasma membrane potential and decrease in intracellular ATP levels	[160]
		Dermaseptin S4	*Phyllomedusa sauvagii*, (South America Sauvages leaf frog)	Potent lysis for *L. major* promastigotes.	[51]
		Dermasiptin-01	*Phyllomedusa oreades* (tree frog)	Active against *L. infantum* promastigotes by membrane damage and flagella alterations	[164]
		Gomesin	*Acanthoscurria gomesiana* (Tarantula spider)	Decreased the viability of *L. amazonensis* promastigotes with little activity against human erythrocytes	[166]
		Indolicidin	Bovine leukocytes	Membrane effects and able to induce autophagy in *L. donovani*	[167]

Table 2. *Cont.*

Type	Infection (causative agent)	AMP	Source	Notes	Ref
Parasites	**Leishmaniasis** (*L. donovani, L major, L. tropica, L. Mexicana* and *L. panamensis*)	Thionins	*Triticum aestivum* (Wheat)	Proved effective against *L. donovani* promastigotes in the low micromolar range, membrane permeability	[168]
Helminths	**Onchocerciasis (River blindness)** (*Onchocerca volvulus*)	HNP1-3	*Homo sapiens* (Human)	HNP1–3 can bind to *Onchocerca volvulus* surfaces and is known to mediate the macrophage response against other micro-organisms by the release of IFN-γ and TNF-α	[200]
		Galectin Peroxidoxin-2 ALT-1 (all peptides are AMP precursors)	*Onchocerca ochengi* (Black fly) *Onchocerca volvulus* (Roundworm)	Excretory/secretory products of *Onchocerca volvulus* with significant antibacterial activity against *E. coli* The peptides Galectin, Peroxidoxin-2 and ALT-1 are all AMP precursors	[11]
Viruses	**Dengue viral disease** (family *Flaviviridae*)	Latarcin	*Lachesana tarabaevi* (Ant spider)	Able to inhibit the replication of dengue viruses in *in vitro* cultured cells	[232,233]
	Rabies (*Lyssavirus*)	C2, C6, C8 and P16	Synthetic	Synthetic AMPs based on naturally according peptides C2, C6, C8 and P16 peptides exhibited strong rabies virus inhibitory properties	[252]

Acknowledgement

The authors would like to extend their gratitude to Annemarie Wentzel for assisting with language editing of the manuscript. Part of this work was funded by the National Research Foundation (NRF) of South Africa.

Author contributions

All the authors were responsible for critical review of the manuscript. All of the authors were responsible for the writing of certain sections of the manuscript according to their field of expertise.

Conflicts of interest

Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF does not accept any liability in regard thereto.

The authors report no conflict of interest.

References

1. WHO. Neglected Tropical Diseases. Available online: http://www.who.int/neglected_diseases/diseases/en/ (accessed on 18 August 2015).
2. Hotez, P.J.; Molyneuz, D.H.; Fenwick, A.; Ottesen, E.; Ehrlich Sachs, S.; Sachs, J.D. Incorporating a rapid-impact package for Negl.Trop. Dis. with programs for HIV/AIDS, tuberculosis, maleria. *PLOS Med.* **2006**, *3*, doi:10.1371/journal.pmed.0030102.
3. Feasey, N.; Wansbrough-Jones, M.; Mabey, D.C.; Solomon, A.W. Neglected tropical diseases. *Br. Med. Bull.* **2010**, *93*, 179–200.
4. Fox, J.L. Antimicrobial peptides stage a comeback. *Nat. Biotechnol.* **2013**, *31*, 379–382.
5. Hancock, R.E.; Lehrer, R. Cationic peptides: A new source of antibiotics. *Trends Biotechnol.* **1998**, *16*, 82–88.
6. Wagner, D.; Young, L.S. Nontuberculous mycobacterial infections: A clinical review. *Infection* **2004**, *32*, 257–270.
7. Gordon, Y.J.; Romanowski, E.G.; McDermott, A.M. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr.Res.* **2005**, *30*, 505–515.
8. Hancock, R.E.; Sahl, H.G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006**, *24*, 1551–1557.
9. Jones, E.; Salin, V.; Williams, G.W. Nisin and the market for commercial bacteriocins. TAMRC Consumer and Product Research Report No. *CP-01-05*.

2005. Avaliable online: http://ageconsearch.umn.edu/bitstream/90779/2/CP%2001%2005%20Nisin%20Report.pdf (accessed on 18 August 2015).
10. Jenssen, H.; Hamill, P.; Hancock, R.E. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* **2006**, *19*, 491–511.
11. Eberle, R.; Brattig, N.W.; Trusch, M.; Schlüter, H.; Achukwi, M.D.; Eisenbarth, A.; Renz, A.; Liebau, E.; Perbandt, M.; Betzel, C. Isolation, identification and functional profile of excretory-secretory peptides from Onchocerca ochengi. *Acta Tropica* **2015**, *142*, 156–166.
12. Wang, G.; Li, X.; Wang, Z. The Antimicrobial Peptide Database. Available online: http://aps.unmc.edu/AP/main.php (Accesed on: 22 June 2015).
13. Wang, G.; Li, X.; Wang, Z. APD2: The updated antimicrobial peptide database and its application in peptide design. *Nucleic Acids Res.* **2009**, *37*, D933–D937.
14. Wang, Z.; Wang, G. APD: The Antimicrobial Peptide Database. *Nucleic Acids Res.* **2004**, *32*, D590–D592.
15. Papagianni, M. Ribosomally synthesized peptides with antimicrobial properties: Biosynthesis, structure, function, and applications. *Biotechnol. Adv.* **2003**, *21*, 465–499.
16. Felnagle, E.A.; Jackson, E.E.; Chan, Y.A.; Podevels, A.M.; Berti, A.D.; McMahon, M.D.; Thomas, M.G. Nonribosomal peptide synthetases involved in the production of medically relevant natural products. *Mol. Pharmaceutics* **2008**, *5*, 191–211.
17. Niggemann, J.; Bozko, P.; Bruns, N.; Wodtke, A.; Gieseler, M.T.; Thomas, K.; Jahns, C.; Nimtz, M.; Reupke, I.; Bruser, T.; *et al*. Baceridin, a cyclic hexapeptide from an epiphytic bacillus strain, inhibits the proteasome. *Chem. Bio. Chem.***2014**, *15*, 1021–1029.
18. Wang, G.; Mishra, B.; Lau, K.; Lushnikova, T.; Golla, R.; Wang, X. Antimicrobial peptides in 2014. *Pharmaceuticals* **2015**, *8*, 123–150.
19. Midorikawa, K.; Ouhara, K.; Komatsuzawa, H.; Kawai, T.; Yamada, S.; Fujiwara, T.; Yamazaki, K.; Sayama, K.; Taubman, M.A.; Kurihara, H.; *et al*. Staphylococcus aureus susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. *Infection Immunity* **2003**, *71*, 3730–3739.
20. Hancock, R.E.; Diamond, G. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol.* **2000**, *8*, 402–410.
21. Akira, S.; Uematsu, S.; Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **2006**, *124*, 783–801.
22. Jensen, S.; Thomsen, A.R. Sensing of RNA viruses: A review of innate immune receptors involved in recognizing RNA virus invasion. *J. Virol.* **2012**, *86*, 2900–2910.

23. Loo, Y.M.; Fornek, J.; Crochet, N.; Bajwa, G.; Perwitasari, O.; Martinez-Sobrido, L.; Akira, S.; Gill, M.A.; Garcia-Sastre, A.; Katze, M. G.; *et al.* Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J. Virol.* **2008**, *82*, 335–345.
24. Cotter, P.D.; Hill, C.; Ross, R.P. Bacteriocins: Developing innate immunity for food. *Nat. Rev. Microbiol.* **2005**, *3*, 777–788.
25. Brogden, K.A. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* **2005**, *3*, 238–250.
26. Matsuzaki, K. Control of cell selectivity of antimicrobial peptides. *Biochim. Biophys. Acta* **2009**, *1788*, 1687–1692.
27. Takahashi, D.; Shukla, S.K.; Prakash, O.; Zhang, G. Structural determinants of host defense peptides for antimicrobial activity and target cell selectivity. *Biochimie* **2010**, *92*, 1236–1241.
28. Harris, F.; Dennison, S.R.; Phoenix, D.A. Anionic antimicrobial peptides from eukaryotic organisms. *Curr. Protein Pept. Sci.* **2009**, *10*, 585–606.
29. Laverty, G.; Gorman, S.P.; Gilmore, B.F. The potential of antimicrobial peptides as biocides. *Int. J. Mol. Sci.* **2011**, *12*, 6566–6596.
30. Brogden, K.A.; Ackermann, M.; McCray, P.B., Jr.; Tack, B.F. Antimicrobial peptides in animals and their role in host defences. *Int. J. Antimicrob. Agents* **2003**, *22*, 465–478.
31. Huang, Y.; Huang, J.; Chen, Y. Alpha-helical cationic antimicrobial peptides: Relationships of structure and function. *Protein & cell* **2010**, *1*, 143–152.
32. Boman, H.G. Gene-encoded peptide antibiotics and the concept of innate immunity: An update review. *Scand. J. Immunol.* **1998**, *48*, 15–25.
33. Buchan, D.W.A.; Minneci, F.; Nugent, T.C.O.; Bryson, K.; Jones, D.T. Scalable web services for the PSIPRED Protein Analysis Workbench. *Nucleic Acids Res.* **2013**, *41*, 340–348.
34. Vale, N.; Aguiar, L.; Gomes, P. Antimicrobial peptides: A new class of antimalarial drugs? *Front. Pharmacol.* **2014**, *5*, doi:10.3389/fphar.2014.00275.
35. Fujikawa, K.; Suketa, Y.; Hayashi, K.; Suzuki, T. Chemical structure of circulin A. *Experientia* **1965**, *21*, 307–308.
36. Craik, D.J.; Daly, N.L.; Bond, T.; Waine, C. Plant cyclotides: A unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. *J. Mol. Biol.* **1999**, *294*, 1327–1336.
37. Lehrer, R.I.; Cole, A.M.; Selsted, M.E. Theta-Defensins: Cyclic peptides with endless potential. *J. Biol. Chem.* **2012**, *287*, 27014–27019.
38. van Dijk, A.; Molhoek, E.M.; Bikker, F.J.; Yu, P.L.; Veldhuizen, E.J.; Haagsman, H.P. Avian cathelicidins: Paradigms for the development of anti-infectives. *Veterinary Microbiol.* **2011**, *153*, 27–36.
39. Ma, D.; Zhou, C.; Zhang, M.; Han, Z.; Shao, Y.; Liu, S. Functional analysis and induction of four novel goose (Anser cygnoides) avian beta-defensins in

response to salmonella enteritidis infection. *Comp. Immunol. Microbiol. Infectious Dis.* **2012**, *35*, 197–207.

40. Scocchi, M.; Pallavicini, A.; Salgaro, R.; Bociek, K.; Gennaro, R. The salmonid cathelicidins:
A gene family with highly varied C-terminal antimicrobial domains. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* **2009**, *152*, 376–381.
41. Casadei, E.; Wang, T.; Zou, J.; Gonzalez Vecino, J.L.; Wadsworth, S.; Secombes, C.J. Characterization of three novel beta-defensin antimicrobial peptides in rainbow trout (Oncorhynchus mykiss). *Mol. Immunol.* **2009**, *46*, 3358–3366.
42. Alibardi, L. Immunocytochemical detection of beta-defensins and cathelicidins in the secretory granules of the tongue in the lizard Anolis carolinensis. *Acta histochemica* **2015**, *117*, 223–227.
43. Zhao, H.; Gan, T. X.; Liu, X. D.; Jin, Y.; Lee, W. H.; Shen, J. H.; Zhang, Y. Identification and characterization of novel reptile cathelicidins from elapid snakes. *Peptides* **2008**, *29*, 1685–1691.
44. Lin, K.F.; Lee, T.R.; Tsai, P.H.; Hsu, M.P.; Chen, C.S.; Lyu, P.C. Structure-based protein engineering for alpha-amylase inhibitory activity of plant defensin. *Proteins* **2007**, *68*, 530–540.
45. Cornet, B.; Bonmatin, J.M.; Hetru, C.; Hoffmann, J.A.; Ptak, M.; Vovelle, F. Refined
three-dimensional solution structure of insect defensin A. *Structure* **1995**, *3*, 435–448.
46. Ramanathan, B.; Davis, E.G.; Ross, C.R.; Blecha, F. Cathelicidins: Microbicidal activity, mechanisms of action, and roles in innate immunity. *Microbes Infection* **2002**, *4*, 361–372.
47. Ganz, T. Defensins: Antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* **2003**, *3*, 710–720.
48. Suarez-Carmona, M.; Hubert, P.; Delvenne, P.; Herfs, M. Defensins: “Simple” antimicrobial peptides or broad-spectrum molecules? *Cytokine Growth Factor Rev.* **2015**, *26*, 361–370.
49. Vandamme, D.; Landuyt, B.; Luyten, W.; Schoofs, L. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cellular Immunol.* **2012**, *280*, 22–35.
50. Zanetti, M.; Gennaro, R.; Romeo, D. Cathelicidins: A novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Letters* **1995**, *374*, 1–5.
51. Feder, R.; Dagan, A.; Mor, A. Structure-activity relationship study of antimicrobial dermaseptin S4 showing the consequences of peptide oligomerization on selective cytotoxicity. *J. Biol. Chem.* **2000**, *275*, 4230–4238.

52. Unger, T.; Oren, Z.; Shai, Y. The effect of cyclization of magainin 2 and melittin analogues on structure, function, and model membrane interactions: Implication to their mode of action. *Biochemistry* **2001**, *40*, 6388–6397.
53. Yeaman, M.R.; Yount, N.Y. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* **2003**, *55*, 27–55.
54. Torrent, M.; Pulido, D.; Rivas, L.; Andreu, D. Antimicrobial peptide action on parasites. *Curr. Drug Targets* **2012**, *13*, 1138–1147.
55. Park, C.; Lee, D.G. Fungicidal effect of antimicrobial peptide arenicin-1. *Biochim. Biophys. Acta* **2009**, *1788*, 1790–1796.
56. Dean, R.E.; O'Brien, L.M.; Thwaite, J.E.; Fox, M.A.; Atkins, H.; Ulaeto, D.O. A carpet-based mechanism for direct antimicrobial peptide activity against vaccinia virus membranes. *Peptides* **2010**, *31*, 1966–1972.
57. Klotman, M.E.; Chang, T.L. Defensins in innate antiviral immunity. *Nat. Rev. Immunol.* **2006**, *6*, 447–456.
58. Xiong, Y.Q.; Mukhopadhyay, K.; Yeaman, M.R.; Adler-Moore, J.; Bayer, A.S. Functional interrelationships between cell membrane and cell wall in antimicrobial peptide-mediated killing of Staphylococcus aureus. *Antimicrob. Agents Chemother.* **2005**, *49*, 3114–3121.
59. Gee, M.L.; Burton, M.; Grevis-James, A.; Hossain, M.A.; McArthur, S.; Palombo, E.A.;
Wade, J.D.; Clayton, A.H. Imaging the action of antimicrobial peptides on living bacterial cells. *Sci. Rep.* **2013**, *3*, 1557–1563.
60. Powers, J.P.; Hancock, R.E. The relationship between peptide structure and antibacterial activity. *Peptides* **2003**, *24*, 1681–1691.
61. Baumann, G.; Mueller, P. A molecular model of membrane excitability. *J. Supramolecular Struct.* **1974**, *2*, 538–557.
62. Yang, L.; Harroun, T.A.; Weiss, T.M.; Ding, L.; Huang, H.W. Barrel-stave model or toroidal model? A case study on melittin pores. *Biophys. J.* **2001**, *81*, 1475–1485.
63. Carnicelli, V.; Lizzi, A.R.; Ponzi, A.; Amicosante, G.; Bozzi, A.; Di Giulio, A. Interaction between antimicrobial peptides (AMPs) and their primary target, the biomembranes. In *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education*; Méndez-Vilas, A., Ed.; Formatex Research Center: Badajoz, Spain, 2013; Volume 2, pp 1123–1134.
64. Matsuzaki, K.; Sugishita, K.; Harada, M.; Fujii, N.; Miyajima, K. Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. *Biochim. Biophys. Acta* **1997**, *1327*, 119–130.
65. Pandey, B.K.; Srivastava, S.; Singh, M.; Ghosh, J.K. Inducing toxicity by introducing

a leucine-zipper-like motif in frog antimicrobial peptide, magainin 2. *Biochem. J.* **2011**, *436*, 609–620.
66. Koo, S.P.; Bayer, A.S.; Yeaman, M.R. Diversity in antistaphylococcal mechanisms among membrane-targeting antimicrobial peptides. *Infection Immunity* **2001**, *69*, 4916–4922.
67. Podda, E.; Benincasa, M.; Pacor, S.; Micali, F.; Mattiuzzo, M.; Gennaro, R.; Scocchi, M. Dual mode of action of Bac7, a proline-rich antibacterial peptide. *Biochim. Biophys. Acta* **2006**, *1760*, 1732–1740.
68. Nicolas, P. Multifunctional host defense peptides: Intracellular-targeting antimicrobial peptides. *FEBS J.* **2009**, *276*, 6483–6496.
69. Otvos, L., Jr. Antibacterial peptides and proteins with multiple cellular targets. *J. Pept. Sci. An Off. Publ. Europ. Pept. Soc.* **2005**, *11*, 697–706.
70. Bowdish, D.M.; Davidson, D.J.; Hancock, R.E. A re-evaluation of the role of host defence peptides in mammalian immunity. *Curr. Protein Pept. Sci.* **2005**, *6*, 35–51.
71. Wu, G.; Ding, J.; Li, H.; Li, L.; Zhao, R.; Shen, Z.; Fan, X.; Xi, T. Effects of cations and pH on antimicrobial activity of thanatin and *s*-thanatin against Escherichia coli ATCC25922 and B. subtilis ATCC 21332. *Curr. Microbiol.* **2008**, *57*, 552–557.
72. Johansson, J.; Gudmundsson, G.H.; Rottenberg, M.E.; Berndt, K.D.; Agerberth, B. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J. Biol. Chem.* **1998**, *273*, 3718–2374.
73. Niyonsaba, F.; Hirata, M.; Ogawa, H.; Nagaoka, I. Epithelial cell-derived antibacterial peptides human beta-defensins and cathelicidin: Multifunctional activities on mast cells. *Curr. Drug Targets. Inflammation Allergy* **2003**, *2*, 224–231.
74. Oppenheim, J.J.; Biragyn, A.; Kwak, L.W.; Yang, D. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *Annals Rheumatic Dis.* **2003**, *62*, ii17–ii21.
75. Scott, M.G.; Davidson, D.J.; Gold, M.R.; Bowdish, D.; Hancock, R.E. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J. Immunol.* **2002**, *169*, 3883–3891.
76. Tjabringa, G.S.; Aarbiou, J.; Ninaber, D.K.; Drijfhout, J.W.; Sorensen, O.E.; Borregaard, N.; Rabe, K.F.; Hiemstra, P.S. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. *J. Immunol.* **2003**, *171*, 6690–6696.
77. Yang, D.; Biragyn, A.; Hoover, D.M.; Lubkowski, J.; Oppenheim, J.J. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annual Rev. Immunol.* **2004**, *22*, 181–215.
78. Gough, M.; Hancock, R.E.; Kelly, N.M. Antiendotoxin activity of cationic peptide antimicrobial agents. *Infection Immunity* **1996**, *64*, 4922–4927.

79. Bucki, R.; Pastore, J.J.; Randhawa, P.; Vegners, R.; Weiner, D.J.; Janmey, P.A. Antibacterial activities of rhodamine B-conjugated gelsolin-derived peptides compared to those of the antimicrobial peptides cathelicidin LL37, magainin II, and melittin. *Antimicrob. Agents Chemother.* **2004**, *48*, 1526–1533.
80. Marr, A.K.; Gooderham, W.J.; Hancock, R.E. Antibacterial peptides for therapeutic use: Obstacles and realistic outlook. *Curr. Opin. Pharmacol.* **2006**, *6*, 468–472.
81. Sun, X.; Chen, S.; Li, S.; Yan, H.; Fan, Y.; Mi, H. Deletion of two C-terminal Gln residues of 12-26-residue fragment of melittin improves its antimicrobial activity. *Peptides* **2005**, *26*, 369–375.
82. Blondelle, S.E.; Houghten, R.A. Hemolytic and antimicrobial activities of the twenty-four individual omission analogues of melittin. *Biochemistry* **1991**, *30*, 4671–4678.
83. Asthana, N.; Yadav, S.P.; Ghosh, J.K. Dissection of antibacterial and toxic activity of melittin: A leucine zipper motif plays a crucial role in determining its hemolytic activity but not antibacterial activity. *J. Biol. Chem.* **2004**, *279*, 55042–55050.
84. Wu, R.; Wang, Q.; Zheng, Z.; Zhao, L.; Shang, Y.; Wei, X.; Liao, X.; Zhang, R. Design, characterization and expression of a novel hybrid peptides melittin (1-13)-LL37 (17-30). *Mol. Biology Rep.* **2014**, *41*, 4163–4169.
85. Brogden, N.K.; Brogden, K.A. Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals? *Int. J. Antimicrob. Agents* **2011**, *38*, 217–225.
86. Finlay, B.B.; Hancock, R.E. Can innate immunity be enhanced to treat microbial infections? *Nat. Rev. Microbiol.* **2004**, *2*, 497–504.
87. Marais, E.; Hamman, J.; Plessis, L.; Lemmer, R.; Steenekamp, J. Eudragit(R) L100/N-trimethylchitosan chloride microspheres for oral insulin delivery. *Molecules* **2013**, *18*, 6734–6747.
88. Sarmento, B.; Ribeiro, A.; Veiga, F.; Ferreira, D.; Neufeld, R. Oral bioavailability of insulin contained in polysaccharide nanoparticles. *Biomacromolecules* **2007**, *8*, 3054–3060.
89. Huang, C.; Jin, H.; Qian, Y.; Qi, S.; Luo, H.; Luo, Q.; Zhang, Z. Hybrid melittin cytolytic Peptide-driven ultrasmall lipid nanoparticles block melanoma growth *in vivo*. *ACS Nano* **2013**, *7*, 5791–5800.
90. Piras, A.M.; Maisetta, G.; Sandreschi, S.; Gazzarri, M.; Bartoli, C.; Grassi, L.; Esin, S.; Chiellini, F.; Batoni, G. Chitosan nanoparticles loaded with the antimicrobial peptide temporin B exert a long-term antibacterial activity in vitro against clinical isolates of Staphylococcus epidermidis. *Front. Microbiol.* **2015**, *6*, doi: 10.3389/fmicb.2015.00372.

91. Mataraci, E.; Dosler, S. *In vitro* activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin-resistant Staphylococcus aureus biofilms. *Antimicrob. Agents Chemother.* **2012**, *56*, 6366–6371.
92. Engels, D.; Savioli, L. Reconsidering the underestimated burden caused by Negl.Trop. Dis.. *Trends Parasitol.* **2006**, *22*, 363–366.
93. Gallagher, M.; Malhotra, I.; Mungai, P.L.; Wamachi, A.N.; Kioko, J.M.; Ouma, J.H.; Muchiri, E.; King, C.L. The effects of maternal helminth and malaria infections on mother-to-child HIV transmission. *Aids* **2005**, *19*, 1849–1855.
94. Hotez, P.J.; Molyneux, D.H.; Fenwick, A.; Kumaresan, J.; Sachs, S.E.; Sachs, J.D.; Savioli, L. Control of Negl.Trop. Dis. *N. Engl. J. Med.* **2007**, *357*, 1018–1027.
95. Hotez, P.J.; Fenwick, A.; Savioli, L.; Molyneux, D.H. Rescuing the bottom billion through control of Negl.Trop. Dis. *Lancet* **2009**, *373*, 1570–1575.
96. Norris, J.; Adelman, C.; Spantchak, Y.; Marano, K. *Social and Economic Impact Review on Neglected Tropical Diseases*; Hudson Institute's Center for Science in Public Policy: Washington, DC, USA, 2012; 1–26.
97. WHO. Antimicrobial Resistance: Fact sheet N° 194. Available online: http://www.who.int/
mediacentre/factsheets/fs194/en/ (accessed on 18 August 2015).
98. Zucca, M.; Savoia, D. The post-antibiotic era: Promising developments in the therapy of infectious diseases. *Int. J. Biomed. Sci.: IJBS* **2010**, *6*, 77–86.
99. Barrow, W.W. Processing of mycobacterial lipids and effects on host responsiveness. *Front. Biosci.:A J. Virtual Lib.* **1997**, *2*, d387–d400.
100. Barrow, W.W. Treatment of mycobacterial infections. *Rev. Scientifique Tech.* **2001**, *20*, 55–70.
101. Mendez-Samperio, P. The human cathelicidin hCAP18/LL-37: A multifunctional peptide involved in mycobacterial infections. *Peptides* **2010**, *31*, 1791–1798.
102. Turner, J.; Cho, Y.; Dinh, N.N.; Waring, A.J.; Lehrer, R.I. Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob. Agents Chemother.* **1998**, *42*, 2206–2214.
103. Gilliet, M.; Lande, R. Antimicrobial peptides and self-DNA in autoimmune skin inflammation. *Curr. Opin. Immunol.* **2008**, *20*, 401–407.
104. Monot, M.; Honore, N.; Garnier, T.; Araoz, R.; Coppee, J.Y.; Lacroix, C.; Sow, S.; Spencer, J.S.; Truman, R.W.; Williams, D.L.; *et al*. On the origin of leprosy. *Science* **2005**, *308*, 1040–1042.
105. WHO Leprosy Fact sheet N° 101. Available online:http://www.who.int/mediacentre/factsheets/
fs101/en/(accessed on 18 August 2015).
106. Cogen, A.L.; Walker, S.L.; Roberts, C.H.; Hagge, D.A.; Neupane, K.D.; Khadge, S.;
Lockwood, D.N. Human beta-defensin 3 is up-regulated in cutaneous leprosy type 1 reactions. *PLoS Negl.Trop. Dis.* **2012**, *6*, e1869.

107. Bennett, B.H.; Parker, D.L.; Robson, M. Leprosy: Steps along the journey of eradication. *Public Health Rep.* **2008**, *123*, 198–205.
108. Cole, S.T.; Eiglmeier, K.; Parkhill, J.; James, K.D.; Thomson, N.R.; Wheeler, P.R.; Honore, N.; Garnier, T.; Churcher, C.; Harris, D.; *et al*. Massive gene decay in the leprosy bacillus. *Nature* **2001**, *409*, 1007–1011.
109. Cole, S.T.; Brosch, R.; Parkhill, J.; Garnier, T.; Churcher, C.; Harris, D.; Gordon, S.V.; Eiglmeier, K.; Gas, S.; Barry, C.E., 3rd; *et al*. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* **1998**, *393*, 537–544.
110. Irgens, L.M. The discovery of the leprosy bacillus. Tidsskrift for den Norske Laegeforening: Tidsskrift for Praktisk Medicin, Ny Raekke **2002**, 122, 708–709.
111. de Messias-Reason, I.; Kremsner, P.G.; Kun, J.F. Functional haplotypes that produce normal ficolin-2 levels protect against clinical leprosy. *J. Infectious Dis.***2009**, *199*, 801–804.
112. Rees, R.J.; Pearson, J.M.; Waters, M.F. Experimental and clinical studies on rifampicin in treatment of leprosy. *Br. Med. J.* **1970**, *1*, 89–92.
113. Pinheiro, R.O.; de Souza Salles, J.; Sarno, E.N.; Sampaio, E.P. Mycobacterium leprae-host-cell interactions and genetic determinants in leprosy: An overview. *Future Microbiol.* **2011**, *6*, 217–230.
114. Bochud, P.Y.; Hawn, T.R.; Siddiqui, M.R.; Saunderson, P.; Britton, S.; Abraham, I.; Argaw, A.T.; Janer, M.; Zhao, L.P.; Kaplan, G.; *et al*. Toll-like receptor 2 (TLR2) polymorphisms are associated with reversal reaction in leprosy. *J. Infectious Dis.* **2008**, *197*, 253–261.
115. Liu, P.T.; Stenger, S.; Li, H.; Wenzel, L.; Tan, B.H.; Krutzik, S.R.; Ochoa, M.T.; Schauber, J.; Wu, K.; Meinken, C.; *et al*. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* **2006**, *311*, 1770–1773.
116. Liu, P.T.; Wheelwright, M.; Teles, R.; Komisopoulou, E.; Edfeldt, K.; Ferguson, B.;
Mehta, M.D.; Vazirnia, A.; Rea, T.H.; Sarno, E.N.; *et al*. MicroRNA-21 targets the vitamin
D-dependent antimicrobial pathway in leprosy. *Nat. Med.* **2012**, *18*, 267–273.
117. Nemeth, E.; Rivera, S.; Gabayan, V.; Keller, C.; Taudorf, S.; Pedersen, B.K.; Ganz, T. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J. Clin. Invest.* **2004**, *113*, 1271–1276.
118. Johnson, E.E.; Wessling-Resnick, M. Iron metabolism and the innate immune response to infection. *Microbes Infection* **2012**, *14*, 207–216.
119. Rodriguez-Rojas, A.; Rodriguez-Beltran, J.; Couce, A.; Blazquez, J. Antibiotics and antibiotic resistance: A bitter fight against evolution. *Int. J. Med. Microbiol.* **2013**, *303*, 293–297.

120. Bechtle, M.; Chen, S.; Efferth, T. Neglected diseases caused by bacterial infections. *Curr. Med. Chem.* **2010**, *17*, 42–60.
121. WHO. Trachoma: Fact sheet N°382. Available online: http://www.who.int/mediacentre/factsheets/fs382/en/ (accessed on 18 August 2015).
122. Courtright, P.; West, S.K. Contribution of sex-linked biology and gender roles to disparities with trachoma. *Emerging Infectious Dis.* **2004**, *10*, 2012–2016.
123. Solomon, A.W.; Peeling, R.W.; Foster, A.; Mabey, D.C. Diagnosis and assessment of trachoma. *Clin.Microbiol.Rev.* **2004**, *17*, 982–1011.
124. Mabey, D.C.; Solomon, A.W.; Foster, A. Trachoma. *Lancet* **2003**, *362*, 223–239.
125. Burton, M.J. Trachoma: An overview. *Br.med.bull.* **2007**, *84*, 99–116.
126. Potroz, M.G.; Cho, N.J. Natural products for the treatment of trachoma and Chlamydia trachomatis. *Molecules* **2015**, *20*, 4180–4203.
127. WHO. Report of the 2nd Global scientific meeting on trachoma. Avaliable online: http://www.who.int/blindness/2nd%20GLOBAL%20SCIENTIFIC%20MEETING.pdf (accessed on 18 August 2015).
128. Solomon, A.W.; Holland, M.J.; Alexander, N.D.; Massae, P.A.; Aguirre, A.; Natividad-Sancho, A.; Molina, S.; Safari, S.; Shao, J.F.; Courtright, P.; *et al*. Mass treatment with single-dose azithromycin for trachoma. *N. Engl. J. Med.* **2004**, *351*, 1962–1971.
129. Evans, J.R.; Solomon, A.W. Antibiotics for trachoma. *Cochrane Database Syst. Rev.* **2011**, doi:10.1002/14651858.CD001860.pub3.
130. Donati, M.; Di Leo, K.; Benincasa, M.; Cavrini, F.; Accardo, S.; Moroni, A.; Gennaro, R.; Cevenini, R. Activity of cathelicidin peptides against Chlamydia spp. *Antimicrob. Agents Chemother.* **2005**, *49*, 1201–1202.
131. Dawson, R.M.; Liu, C.Q. Cathelicidin peptide SMAP-29: Comprehensive review of its properties and potential as a novel class of antibiotics. *Drug Dev. Res.* **2009**, *70*, 481–498.
132. Huang, G.T.; Zhang, H.B.; Kim, D.; Liu, L.; Ganz, T. A model for antimicrobial gene therapy: Demonstration of human beta-defensin 2 antimicrobial activities *in vivo*. *Hum. Gene Therapy* **2002**, *13*, 2017–2025.
133. Lazarev, V.N.; Parfenova, T.M.; Gularyan, S.K.; Misyurina, O.Y.; Akopian, T.A.; Govorun, V.M. Induced expression of melittin, an antimicrobial peptide, inhibits infection by Chlamydia trachomatis and Mycoplasma hominis in a HeLa cell line. *Int. J. Antimicrob. Agents* **2002**, *19*, 133–137.
134. Lazarev, V.N.; Shkarupeta, M.M.; Polina, N.F.; Kostrjukova, E.S.; Vassilevski, A.A.;
Kozlov, S.A.; Grishin, E.V.; Govorun, V.M. Antimicrobial peptide from spider venom inhibits Chlamydia trachomatis infection at an early stage. *Arch. Microbiol.* **2013**, *195*, 173–179.
135. Lazarev, V.N.; Shkarupeta, M.M.; Titova, G.A.; Kostrjukova, E.S.; Akopian, T.A.; Govorun, V.M. Effect of induced expression of an antimicrobial peptide melittin

on Chlamydia trachomatis and Mycoplasma hominis infections *in vivo*. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 946–950.
136. Cox, F.E.G. Parasitic protozoa. In *Modern Parasitology: A Textbook of Parasitology*. 2nd ed.; Cox, F.E.G., Ed.; Blackwell Science Ltd: Oxford, UK, 1996; p 276.
137. Spicer, J.W. *Clinical Bacteriology, Mycology and Parasitology*. Harcourt Publishers Ltd: London, UK, 2000, p221.
138. Andrews, K.T.; Fisher, G.; Skinner-Adams, T.S. Drug repurposing and human parasitic protozoan diseases. *Int J Parasitol Drugs Drug Resist* **2014**, *4*, 95–111.
139. McGwire, B.S.; Kulkarni, M.M. Interactions of antimicrobial peptides with Leishmania and trypanosomes and their functional role in host parasitism. *Exp. Parasitology* **2010**, *126*, 397–405.
140. W.H.O. Chagas disease (American Trypanosomiasis). *Fact Sheet No 340* March 2015. Avaliable online: http://www.who.int/chagas/en/ (accessed on 18 August 2015).
141. Bern, C.; Montgomery, S.P.; Herwaldt, B.L.; Rassi, A., Jr.; Marin-Neto, J.A.; Dantas, R.O.; Maguire, J.H.; Acquatella, H.; Morillo, C.; Kirchhoff, L.V.; *et al*. Evaluation and treatment of chagas disease in the United States: A systematic review. *JAMA* **2007**, *298*, 2171–2181.
142. Soy, D.; Aldasoro, E.; Guerrero, L.; Posada, E.; Serret, N.; Mejia, T.; Urbina, J.A.; Gascon, J. Population pharmacokinetics of benznidazole in adult patients with chagas disease. *Antimicrob. Agents Chemother.* **2015**, *59*, 3342–3349.
143. Urbina, J.A. Recent clinical trials for the etiological treatment of chronic chagas disease: Advances, challenges and perspectives. *J. Eukaryot. Microbiol.* **2015**, *62*, 149–156.
144. Adade, C.M.; Chagas, G.S.; Souto-Padron, T. Apis mellifera venom induces different cell death pathways in Trypanosoma cruzi. *Parasitology* **2012**, *139*, 1444–1461.
145. Adade, C.M.; Oliveira, I.R.; Pais, J.A.; Souto-Padron, T. Melittin peptide kills Trypanosoma cruzi parasites by inducing different cell death pathways. *Toxicon* **2013**, *69*, 227–239.
146. Fieck, A.; Hurwitz, I.; Kang, A.S.; Durvasula, R. Trypanosoma cruzi: Synergistic cytotoxicity of multiple amphipathic anti-microbial peptides to T. cruzi and potential bacterial hosts. *Exp. Parasitol* **2010**, *125*, 342–347.
147. Huang, C.M.; Chen, H.C.; Zierdt, C.H. Magainin analogs effective against pathogenic protozoa. *Antimicrob. Agents Chemother.* **1990**, *34*, 1824–1826.
148. Jaynes, J.M.; Burton, C.A.; Barr, S.B.; Jeffers, G.W.; Julian, G.R.; White, K.L.; Enright, F.M.; Klei, T.R.; Laine, R.A. *In vitro* cytocidal effect of novel lytic peptides on Plasmodium falciparum and Trypanosoma cruzi. *FASEB J.* **1988**, *2*, 2878–2883.

149. Brand, G.D.; Leite, J.R.; Silva, L.P.; Albuquerque, S.; Prates, M.V.; Azevedo, R.B.; Carregaro, V.; Silva, J.S.; Sa, V.C.; Brandao, R.A.; *et al*. Dermaseptins from Phyllomedusa oreades and Phyllomedusa distincta. Anti-Trypanosoma cruzi activity without cytotoxicity to mammalian cells. *J. Biol. Chem.* **2002**, *277*, 49332–49340.
150. Pinto, E.G.; Pimenta, D.C.; Antoniazzi, M.M.; Jared, C.; Tempone, A.G. Antimicrobial peptides isolated from Phyllomedusa nordestina (Amphibia) alter the permeability of plasma membrane of Leishmania and Trypanosoma cruzi. *Exp Parasitol* **2013**, *135*, 655–660.
151. Lofgren, S.E.; Miletti, L.C.; Steindel, M.; Bachere, E.; Barracco, M.A. Trypanocidal and leishmanicidal activities of different antimicrobial peptides (AMPs) isolated from aquatic animals. *Exp. Parasitol* **2008**, *118*, 197–202.
152. Barrett, M.P.; Boykin, D.W.; Brun, R.; Tidwell, R.R. Human African trypanosomiasis: Pharmacololgical re-engagement with a neglected disease. *Br. J. Pharmacol* **2007**, *152*, 1155–1171.
153. W.H.O. Trypanosomiasis, human African (sleeping sickness). *Fact sheet No 259* May 2015. Avaliable online: http://www.who.int/mediacentre/factsheets/fs259/en/ (accessed on 18 August 2015).
154. Brun, R.; Blum, J.; Chappuis, F.; Burri, C. Human African trypanosomiasis. *Lancet* **2010**, *375*, 148–159.
155. Abbassi, F.; Raja, Z.; Oury, B.; Gazanion, E.; Piesse, C.; Sereno, D.; Nicolas, P.; Foulon, T.; Ladram, A. Antibacterial and leishmanicidal activities of temporin-SHd, a 17-residue long membrane-damaging peptide. *Biochimie* **2013**, *95*, 388–399.
156. McGwire, B.S.; Olson, C.L.; Tack, B.F.; Engman, D.M. Killing of African trypanosomes by antimicrobial peptides. *J. Infectious Dis.***2003**, *188*, 146–152.
157. Haines, L.R.; Thomas, J.M.; Jackson, A.M.; Eyford, B.A.; Razavi, M.; Watson, C.N.; Gowen, B.; Hancock, R.E.; Pearson, T.W. Killing of trypanosomatid parasites by a modified bovine host defense peptide, BMAP-18. *PLoS Negl.Trop. Dis.* **2009**, *3*, e373.
158. (WHO), W. H. O. Leishmaniasis *Fact sheet No375* February 2015. Avaliable online: http://www.who.int/mediacentre/factsheets/fs375/en/ (accessed on 18 August 2015).
159. de Menezes, J.P.; Guedes, C.E.; Petersen, A.L.; Fraga, D.B.; Veras, P.S. Advances in Development of New Treatment for Leishmaniasis. *Biomed Res Int* **2015**, *2015*, doi:org/10.1155/2015/815023.
160. Mangoni, M.L.; Saugar, J.M.; Dellisanti, M.; Barra, D.; Simmaco, M.; Rivas, L. Temporins, small antimicrobial peptides with leishmanicidal activity. *J. Biol. Chem.* **2005**, *280*, 984–990.

161. Chadbourne, F.L.; Raleigh, C.; Ali, H.Z.; Denny, P.W.; Cobb, S.L. Studies on the antileishmanial properties of the antimicrobial peptides temporin A, B and 1Sa. *J. Pept. Sci. An Off. Publ. Europ. Pept. Soc.* **2011**, *17*, 751–755.
162. Abbassi, F.; Oury, B.; Blasco, T.; Sereno, D.; Bolbach, G.; Nicolas, P.; Hani, K.; Amiche, M.; Ladram, A. Isolation, characterization and molecular cloning of new temporins from the skin of the North African ranid Pelophylax saharica. *Peptides* **2008**, *29*, 1526–1533.
163. Eggimann, G.A.; Sweeney, K.; Bolt, H.L.; Rozatian, N.; Cobb, S.L.; Denny, P.W. The role of phosphoglycans in the susceptibility of Leishmania mexicana to the temporin family of anti-microbial peptides. *Molecules* **2015**, *20*, 2775–2785.
164. Eaton, P.; Bittencourt, C.R.; Costa Silva, V.; Veras, L.M.; Costa, C.H.; Feio, M.J.; Leite, J.R. Anti-leishmanial activity of the antimicrobial peptide DRS 01 observed in Leishmania infantum (syn. Leishmania chagasi) cells. *Nanomedicine* **2014**, *10*, 483–490.
165. Brand, G.D.; Leite, J.R.; de Sa Mandel, S.M.; Mesquita, D.A.; Silva, L.P.; Prates, M.V.;
Barbosa, E.A.; Vinecky, F.; Martins, G.R.; Galasso, J.H.; *et al*. Novel dermaseptins from Phyllomedusa hypochondrialis (Amphibia). *Biochem. Biophys. Res. Commun.* **2006**, *347*, 739–746.
166. Silva, P.I., Jr.; Daffre, S.; Bulet, P. Isolation and characterization of gomesin, an 18-residue cysteine-rich defense peptide from the spider Acanthoscurria gomesiana hemocytes with sequence similarities to horseshoe crab antimicrobial peptides of the tachyplesin family. *J. Biol. Chem.* **2000**, *275*, 33464–33470.
167. Bera, A.; Singh, S.; Nagaraj, R.; Vaidya, T. Induction of autophagic cell death in Leishmania donovani by antimicrobial peptides. *Mol Biochem Parasitol* **2003**, *127*, 23–35.
168. Berrocal-Lobo, M.; Molina, A.; Rodriguez-Palenzuela, P.; Garcia-Olmedo, F.; Rivas, L. Leishmania donovani: Thionins, plant antimicrobial peptides with leishmanicidal activity. *Exp Parasitol* **2009**, *122*, 247–249.
169. Perez-Cordero, J.J.; Lozano, J.M.; Cortes, J.; Delgado, G. Leishmanicidal activity of synthetic antimicrobial peptides in an infection model with human dendritic cells. *Peptides* **2011**, *32*, 683–690.
170. Kulkarni, M.M.; McMaster, W.R.; Kamysz, E.; Kamysz, W.; Engman, D.M.; McGwire, B.S. The major surface-metalloprotease of the parasitic protozoan, Leishmania, protects against antimicrobial peptide-induced apoptotic killing. *Mol. Microbiol.* **2006**, *62*, 1484–1497.
171. Kulkarni, M.M.; McMaster, W.R.; Kamysz, W.; McGwire, B.S. Antimicrobial peptide-induced apoptotic death of leishmania results from calcium-de pend ent, caspase-independent mitochondrial toxicity. *J. Biol. Chem.* **2009**, *284*, 15496–15504.

172. Kulkarni, M.M.; Karafova, A.; Kamysz, W.; McGwire, B.S. Design of protease-resistant pexiganan enhances antileishmanial activity. *Parasitology Res.* **2014**, *113*, 1971–1976.
173. W.H.O. Malaria. *Fact Sheet No 94* April 2015. Avaliable online: http://www.who.int/
mediacentre/factsheets/fs094/en/ (accessed on 18 August 2015).
174. WHO. Guidelines for the Treatment of Malaria. Avaliable online: http://apps.who.int/
iris/bitstream/10665/162441/1/9789241549127_eng.pdf (accessed on 18 August 2015).
175. Du Plessis, L.H.; van Niekerk, A.C.; Maritz, M.M.; Kotze, A.F. *In vitro* activity of Pheroid vesicles containing antibiotics against Plasmodium falciparum. *J. Antibiot.* **2012**, *65*, 609–614.
176. Krugliak, M.; Feder, R.; Zolotarev, V.Y.; Gaidukov, L.; Dagan, A.; Ginsburg, H.; Mor, A. Antimalarial activities of dermaseptin S4 derivatives. *Antimicrob. Agents Chemother.* **2000**, *44*, 2442–2451.
177. Carter, V.; Underhill, A.; Baber, I.; Sylla, L.; Baby, M.; Larget-Thiery, I.; Zettor, A.; Bourgouin, C.; Langel, U.; Faye, I.; *et al*. Killer bee molecules: Antimicrobial peptides as effector molecules to target sporogonic stages of Plasmodium. *PLoS Pathog* **2013**, *9*, e1003790.
178. Moreira, C.K.; Rodrigues, F.G.; Ghosh, A.; Varotti Fde, P.; Miranda, A.; Daffre, S.;
Jacobs-Lorena, M.; Moreira, L.A. Effect of the antimicrobial peptide gomesin against different life stages of Plasmodium spp. *Exp Parasitol* **2007**, *116*, 346–353.
179. Parker, G.A.; Ball, M.A.; Chubb, J.C. Why do larval helminths avoid the gut of intermediate hosts? *J. Theor. Biol.* **2009**, *260*, 460–473.
180. Halton, D.W. Microscopy and the helminth parasite. *Micron* **2004**, *35*, 361–390.
181. Hotez, P.J.; Brindley, P.J.; Bethony, J.M.; King, C.H.; Pearce, E.J.; Jacobson, J. Helminth infections: The great Negl.Trop. Dis. *J. Clin. Invest.* **2008**, *118*, 1311–1321.
182. Awasthi, S.; Bundy, D.A.; Savioli, L. Helminthic infections. *Br. Med. J.* **2003**, 327, 431–433.
183. Crompton, D.W.T. How Much Human Helminthiasis Is There in the World? *J. Parasitology* **1999**, *85*, 397–403.
184. Stephenson, L.S.; Latham, M.C.; Ottesen, E.A. Malnutrition and parasitic helminth infections. *Parasitology* **2000**, *121*, S23–S38.
185. WHO. *World Heatlh Report 1999*; World Health Organization: Geneva, Switzerland, 1999.

186. Anthony, R.M.; Rutitzky, L.I.; Urban, J.F. Jr.; Stadecker, M.J.; Gause, W.C. Protective immune mechanisms in helminth infection. *Nat. Rev. Immunol.* **2007**, *7*, 975–987.
187. Li, T.; Ito, A.; Chen, X.; Long, C.; Okamoto, M.; Raoul, F.; Giraudoux, P.; Yanagida, T.; Nakao, M.; Sako, Y.; *et al*. Usefulness of pumpkin seeds combined with areca nut extract in community-based treatment of human taeniasis in northwest Sichuan Province, China. *Acta Tropica* **2012**, *124*, 152–157.
188. Flisser, A.; Avila, G.; Maravilla, P.; Mendlovic, F.; Leon-Cabrera, S.; Cruz-Rivera, M.; Garza, A.; Gomez, B.; Aguilar, L.; Teran, N.; *et al*. Taenia solium: Current understanding of laboratory animal models of taeniosis. *Parasitology* **2010**, *137*, 347–357.
189. Bustos, J.A.; Rodriguez, S.; Jimenez, J.A.; Moyano, L.M.; Castillo, Y.; Ayvar, V.; Allan, J.C.; Craig, P.S.; Gonzalez, A.E.; Gilman, R.H.; *et al*. Detection of Taenia solium Taeniasis Coproantigen Is an Early Indicator of Treatment Failure for Taeniasis. *Clin. Vaccine Immunol.: CVI* **2012**, *19*, 570–573.
190. WHO Taeniasis/ cysticercosis: Fact Sheet N°376. Available online: http://www.who.int/ mediacentre/factsheets/fs376/en/#(accessed on 23 June 2015).
191. García, H.H.; Gonzalez, A.E.; Evans, C.A.W.; Gilman, R.H.; for the Cysticercosis Working Group in Peru. Taenia solium cysticercosis. *Lancet* **2003**, *362*, 547–556.
192. Kościuczuk, E.M.; Lisowski, P.; Jarczak, J.; Strzałkowska, N.; Jóźwik, A.; Horbańczuk, J.; Krzyżewski, J.; Zwierzchowski, L.; Bagnicka, E. Cathelicidins: Family of antimicrobial peptides. A review. *Mol. Biology Rep.* **2012**, *39*, 10957–10970.
193. Landa, A.; Jiménez, L.; Willms, K.; Jiménez-García, L.F.; Lara-Martínez, R.; Robert, L.; Cirioni, O.; Barańska–Rybak, W.; Kamysz, W. Antimicrobial peptides (Temporin A and Iseganan IB-367): Effect on the cysticerci of Taenia crassiceps. *Mol. Biochem. Parasitology* **2009**, *164*, 126–130.
194. WHO Onchocerciasis: Fact Sheet N°374. Available online: http://www.who.int/ mediacentre/factsheets/fs374/en/#(accessed on 23 June 2015).
195. CDC Parasites—Onchocerciasis (also known as River Blindness). Available online: http://www.cdc.gov/parasites/onchocerciasis/ (accessed on 23 June 2015).
196. Noma, M.; Nwoke, B.E.; Nutall, I.; Tambala, P.A.; Enyong, P.; Namsenmo, A.; Remme, J.; Amazigo, U.V.; Kale, O.O.; Seketeli, A. Rapid epidemiological mapping of onchocerciasis (REMO): Its application by the African Programme for Onchocerciasis Control (APOC). *Ann. Trop. Med. Parasitology* **2002**, *96*, S29–S39.
197. Tekle, A.H.; Elhassan, E.; Isiyaku, S.; Amazigo, U.V.; Bush, S.; Noma, M.; Cousens, S.; Abiose, A.; Remme, J. H. Impact of long-term treatment of onchocerciasis with

ivermectin in Kaduna State, Nigeria: First evidence of the potential for elimination in the operational area of the African Programme for Onchocerciasis Control. *Parasit Vectors* **2012**, *5*, doi:10.1186/1756-3305-5-28.
198. Marti, T.; Erttmann, K.D.; Gallin, M.Y. Host-Parasite Interaction in Human Onchocerciasis: Identification and Sequence Analysis of a Novel Human Calgranulin. *Biochem. Biophys. Res. Commun.* **1996**, *221*, 454–458.
199. Ganz, T.; Selsted, M.E.; Szklarek, D.; Harwig, S.S.; Daher, K.; Bainton, D.F.; Lehrer, R.I. Defensins. Natural peptide antibiotics of human neutrophils. *J. Clin. Invest.* **1985**, *76*, 1427–1435.
200. Soehnlein, O.; Kai-Larsen, Y.; Frithiof, R.; Sorensen, O.E.; Kenne, E.; Scharffetter-Kochanek, K.; Eriksson, E.E.; Herwald, H.; Agerberth, B.; Lindbom, L. Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages. *J. Clin. Invest.* **2008**, *118*, 3491–3502.
201. Mookherjee, N.; Hancock, R.E. Cationic host defence peptides: Innate immune regulatory peptides as a novel approach for treating infections. *Cell. Mol. Life Sci.: CMLS* **2007**, *64*, 922–933.
202. Gwyer Findlay, E.; Currie, S.M.; Davidson, D.J. Cationic host defence peptides: Potential as antiviral therapeutics. *BioDrugs: Clin. Immunotherapeutics, Biopharm. Gene Therapy* **2013**, *27*, 479–493.
203. De Clercq, E. Antiviral drugs: Current state of the art. *J. Clin. Virol.* **2001**, *22*, 73–89.
204. Albiol Matanic, V.C.; Castilla, V. Antiviral activity of antimicrobial cationic peptides against Junin virus and herpes simplex virus. *Int. J. Antimicrob. Agents* **2004**, *23*, 382–389.
205. Orsi, N. The antimicrobial activity of lactoferrin: Current status and perspectives. *Biometals* **2004**, *17*, 189–196.
206. Sun, L.; Finnegan, C.M.; Kish-Catalone, T.; Blumenthal, R.; Garzino-Demo, P.; La Terra Maggiore, G.M.; Berrone, S.; Kleinman, C.; Wu, Z.; Abdelwahab, S.; *et al*. Human beta-defensins suppress human immunodeficiency virus infection: Potential role in mucosal protection. *J. Virol.* **2005**, *79*, 14318–14329.
207. van der Strate, B.W.; Beljaars, L.; Molema, G.; Harmsen, M.C.; Meijer, D.K. Antiviral activities of lactoferrin. *Antiviral Res.* **2001**, *52*, 225–239.
208. Yasin, B.; Pang, M.; Turner, J.S.; Cho, Y.; Dinh, N.N.; Waring, A.J.; Lehrer, R.I.; Wagar, E.A. Evaluation of the inactivation of infectious Herpes simplex virus by host-defense peptides. *Eur. J. Clin. Microbiol. Infectious Dis.* **2000**, *19*, 187–194.
209. Daher, K.A.; Selsted, M.E.; Lehrer, R.I. Direct inactivation of viruses by human granulocyte defensins. *J. Virol.* **1986**, *60*, 1068–1074.
210. Duits, L.A.; Nibbering, P.H.; van Strijen, E.; Vos, J.B.; Mannesse-Lazeroms, S.P.; van Sterkenburg, M.A.; Hiemstra, P.S. Rhinovirus increases human beta-

defensin-2 and -3 mRNA expression in cultured bronchial epithelial cells. *FEMS Immunol. Med. Microbiol.* **2003**, *38*, 59–64.

211. Buck, C.B.; Day, P.M.; Thompson, C.D.; Lubkowski, J.; Lu, W.; Lowy, D.R.; Schiller, J.T. Human alpha-defensins block papillomavirus infection. *Proc. Natl. Acad. Sci. USA.* **2006**, *103*, 1516–1521.
212. Carriel-Gomes, M.C.; Kratz, J.M.; Barracco, M.A.; Bachere, E.; Barardi, C.R.; Simoes, C.M. *In vitro* antiviral activity of antimicrobial peptides against herpes simplex virus 1, adenovirus, and rotavirus. *Memorias do Instituto Oswaldo Cruz* **2007**, *102*, 469–472.
213. Edinger, A.L.; Thompson, C.B. Death by design: Apoptosis, necrosis and autophagy. *Curr. Opin. Cell Biol.* **2004**, *16*, 663–669.
214. Levy, D.E.; Marie, I.J.; Durbin, J.E. Induction and function of type I and III interferon in response to viral infection. *Curr. Opin. Cell Biol.* **2011**, *1*, 476–486.
215. Stetson, D.B.; Medzhitov, R. Type I interferons in host defense. *Immunity* **2006**, 25, 373–381.
216. Randall, R.E.; Goodbourn, S. Interferons and viruses: An interplay between induction, signalling, antiviral responses and virus countermeasures. *J. Gen. Virol.* **2008**, *89*, doi: 10.1099/vir.0.83391-0.
217. Takeuchi, O.; Akira, S. Innate immunity to virus infection. *Immunol. Rev.* **2009**, *227*, 75–86.
218. Salvatore, M.; Garcia-Sastre, A.; Ruchala, P.; Lehrer, R.I.; Chang, T.; Klotman, M.E. Alpha-Defensin inhibits influenza virus replication by cell-mediated mechanism(s). *J. Infectious Dis.***2007**, *196*, 835–843.
219. Koczulla, A.R.; Bals, R. Antimicrobial peptides: Current status and therapeutic potential. *Drugs* **2003**, *63*, 389–406.
220. Beatty, M.E.; Stone, A.; Fitzsimons, D.W.; Hanna, J.N.; Lam, S.K.; Vong, S.; Guzman, M.G.; Mendez-Galvan, J.F.; Halstead, S.B.; Letson, G.W.; *et al*. Americas Dengue Prevention Boards Surveillance Working, G., Best practices in dengue surveillance: A report from the Asia-Pacific and Americas Dengue Prevention Boards. *PLoS Negl.Trop. Dis.* **2010**, *4*, e890.
221. Halstead, S.B. Dengue virus-mosquito interactions. *Ann. Rev. Entomol.* **2008**, *53*, 273–291.
222. Halstead, S.B. Pathogenesis of dengue: Challenges to molecular biology. *Science* **1988**, *239*, 476–481.
223. Normile, D. Tropical medicine. Surprising new dengue virus throws a spanner in disease control efforts. *Science* **2013**, *342*, doi: 10.1126/science.342.6157.415.
224. Vasilakis, N.; Holmes, E.C.; Fokam, E.B.; Faye, O.; Diallo, M.; Sall, A.A.; Weaver, S.C. Evolutionary processes among sylvatic dengue type 2 viruses. *J. Virol.* **2007**, *81*, 9591–9595.

225. Rodenhuis-Zybert, I.A.; Wilschut, J.; Smit, J.M. Dengue virus life cycle: Viral and host factors modulating infectivity. *Cell. Mol. Life Sci.: CMLS* **2010**, *67*, 2773–2786.
226. Steinhauer, D.A.; Domingo, E.; Holland, J.J. Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. *Gene* **1992**, *122*, 281–288.
227. Wittke, V.; Robb, T.E.; Thu, H.M.; Nisalak, A.; Nimmannitya, S.; Kalayanrooj, S.; Vaughn, D.W.; Endy, T.P.; Holmes, E.C.; Aaskov, J.G. Extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period. *Virology* **2002**, *301*, 148–156.
228. Siler, J.F.; Hall, M.W.; Kitchens, A.P. Dengue: Its History, Epidemiology, Mechanism of Transmission, Etiology, Clinical Manifestations, Immunity, and Prevention. Philipp. *J. Sci.* **1926**, *29*, 1-308.
229. Bhatt, S.; Gething, P.W.; Brady, O.J.; Messina, J.P.; Farlow, A.W.; Moyes, C.L.; Drake, J.M.; Brownstein, J.S.; Hoen, A.G.; Sankoh, O.; *et al*. The global distribution and burden of dengue. *Nature* **2013**, *496*, 504–507.
230. Shepard, D.S.; Undurraga, E.A.; Halasa, Y.A. Economic and disease burden of dengue in Southeast Asia. *PLoS Negl.Trop. Dis.* **2013**, *7*, e2055.
231. Rothman, A.L. Immunity to dengue virus: A tale of original antigenic sin and tropical cytokine storms. *Nat. Rev. Immunol.* **2011**, *11*, 532–543.
232. Kozlov, S.A.; Vassilevski, A.A.; Feofanov, A.V.; Surovoy, A.Y.; Karpunin, D.V.; Grishin, E.V. Latarcins, antimicrobial and cytolytic peptides from the venom of the spider Lachesana tarabaevi (Zodariidae) that exemplify biomolecular diversity. *J. Biol. Chem.* **2006**, *281*, 20983–20992.
233. Rothan, H.A.; Bahrani, H.; Rahman, N.A.; Yusof, R. Identification of natural antimicrobial agents to treat dengue infection: *In vitro* analysis of latarcin peptide activity against dengue virus. *BMC Microbiol.* **2014**, *14*, doi:10.1186/1471-2180-14-140.
234. Lindenbach, B.D.; Rice, C.M. Molecular biology of flaviviruses. *Adv. Virus Res.* **2003**, *59*, 23–61.
235. Perera, R.; Kuhn, R.J. Structural proteomics of dengue virus. *Curr. Opin. Microbiol.* **2008**, *11*, 369–377.
236. Noble, C.G.; Seh, C.C.; Chao, A.T.; Shi, P.Y. Ligand-bound structures of the dengue virus protease reveal the active conformation. *J. Virol.* **2012**, *86*, 438–446.
237. Alhoot, M.A.; Rathinam, A.K.; Wang, S.M.; Manikam, R.; Sekaran, S.D. Inhibition of dengue virus entry into target cells using synthetic antiviral peptides. *Int. J. Med. Sci.* **2013**, *10*, 719–729.
238. Chen, Y.; Maguire, T.; Marks, R.M. Demonstration of binding of dengue virus envelope protein to target cells. *J. Virol.* **1996**, *70*, 8765–8772.

239. Modis, Y.; Ogata, S.; Clements, D.; Harrison, S.C. Structure of the dengue virus envelope protein after membrane fusion. *Nature* **2004**, *427*, 313–319.
240. Wallace, R.M.; Gilbert, A.; Slate, D.; Chipman, R.; Singh, A.; Cassie, W.; Blanton, J.D. Right place, wrong species: A 20-year review of rabies virus cross species transmission among terrestrial mammals in the United States. *PloS one* **2014**, *9*, e107539.
241. Cleaveland, S.; Fevre, E.M.; Kaare, M.; Coleman, P.G. Estimating human rabies mortality in the United Republic of Tanzania from dog bite injuries. *Bull. World Health Organ.* **2002**, *80*, 304–310.
242. Schnell, M.J.; McGettigan, J.P.; Wirblich, C.; Papaneri, A. The cell biology of rabies virus: Using stealth to reach the brain. *Nat. Rev. Microbiol.* **2010**, *8*, 51–61.
243. WHO. Rabies Fact Sheet N°99. Available online: http://www.who.int/mediacentre/ factsheets/fs099/en/(accessed on 18 August 2015).
244. Lyles, D.S.; Rupprecht, C.E. Rhabdoviridae. *Fields' Virology*; Fields, B.N., Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E., Eds.; Williams and Wilkins: Philadelphia, PA, USA, 2007; Volume 4, pp 1364–1408.
245. Brzozka, K.; Finke, S.; Conzelmann, K.K. Identification of the rabies virus alpha/beta interferon antagonist: Phosphoprotein P interferes with phosphorylation of interferon regulatory factor 3. *J. Virol.* **2005**, *79*, 7673–7681.
246. Kelly, R.M.; Strick, P.L. Rabies as a transneuronal tracer of circuits in the central nervous system. *J. Neurosci. Methods* **2000**, *103*, 63–71.
247. Warrell, D.A. The clinical picture of rabies in man. *Trans. R. Soc. Trop. Med. Hyg.* **1976**, *70*, 188–195.
248. Manning, S.E.; Rupprecht, C.E.; Fishbein, D.; Hanlon, C.A.; Lumlertdacha, B.; Guerra, M.; Meltzer, M.I.; Dhankhar, P.; Vaidya, S.A.; Jenkins, S.R.; *et al*. Advisory Committee on Immunization Practices Centers for Disease, C.; Prevention, Human rabies prevention—United States, 2008: Recommendations of the Advisory Committee on Immunization Practices. *MMWR. Recomm. Rep.* **2008**, *57*, 1–28.
249. Cenna, J.; Tan, G.S.; Papaneri, A.B.; Dietzschold, B.; Schnell, M.J.; McGettigan, J.P. Immune modulating effect by a phosphoprotein-deleted rabies virus vaccine vector expressing two copies of the rabies virus glycoprotein gene. *Vaccine* **2008**, *26*, 6405–6414.
250. Lodmell, D.L.; Parnell, M.J.; Bailey, J.R.; Ewalt, L.C.; Hanlon, C.A. Rabies DNA vaccination of non-human primates: Post-exposure studies using gene gun methodology that accelerates induction of neutralizing antibody and enhances neutralizing antibody titers. *Vaccine* **2002**, *20*, 2221–2228.

251. Dodet, B.; Africa Rabies Expert, B.; Adjogoua, E.V.; Aguemon, A.R.; Amadou, O.H.;
Atipo, A.L.; Baba, B.A.; Ada, S.B.; Boumandouki, P.; Bourhy, H.; *et al*. Fighting rabies in Africa: The Africa Rabies Expert Bureau (AfroREB). *Vaccine* **2008**, *26*, 6295–6298.
252. Real, E.; Rain, J.C.; Battaglia, V.; Jallet, C.; Perrin, P.; Tordo, N.; Chrisment, P.; D'Alayer, J.; Legrain, P.; Jacob, Y. Antiviral drug discovery strategy using combinatorial libraries of structurally constrained peptides. *J. Virol.* **2004**, *78*, 7410–7417.
253. A Database of Antiviral Peptides: Available online: http://crdd.osdd.net/servers/avpdb/
browse.php?by=Rabies%20virus&TYPE=Virus (accessed on 21 August 2015).
254. Jacob, Y.; Real, E.; Tordo, N. Functional interaction map of lyssavirus phosphoprotein: Identification of the minimal transcription domains. *J. Virol.* **2001**, *75*, 9613–9622.
255. Wojczyk, B.S.; Stwora-Wojczyk, M.; Shakin-Eshleman, S.; Wunner, W.H.; Spitalnik, S.L. The role of site-specific N-glycosylation in secretion of soluble forms of rabies virus glycoprotein. *Glycobiology* **1998**, *8*, 121–130.
256. Rodriguez Saint-Jean, S.; De las Heras, A.; Carrillo, W.; Recio, I.; Ortiz-Delgado, J.B.; Ramos, M.; Gomez-Ruiz, J.A.; Sarasquete, C.; Perez-Prieto, S.I., Antiviral activity of casein and alphas2 casein hydrolysates against the infectious haematopoietic necrosis virus, a rhabdovirus from salmonid fish. *J. Fish Dis.* **2013**, *36*, 467–481.
257. Falco, A.; Mas, V.; Tafalla, C.; Perez, L.; Coll, J.M.; Estepa, A. Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): Inactivation of virus particles and induction of a type I interferon-related response. *Antiviral Res.* **2007**, *76*, 111–123.
258. Ali, M.P.; Yoshimatsu, K.; Suzuki, T.; Kato, T.; Park, E.Y. Expression and purification of
cyto-insectotoxin (Cit1a) using silkworm larvae targeting for an antimicrobial therapeutic agent. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 6973–6982.
259. Bommarius, B.; Jenssen, H.; Elliott, M.; Kindrachuk, J.; Pasupuleti, M.; Gieren, H.; Jaeger, K.E.; Hancock, R.E.; Kalman, D. Cost-effective expression and purification of antimicrobial and host defense peptides in Escherichia coli. *Peptides* **2010**, *31*, 1957–1965.
260. Li, Y. Recombinant production of antimicrobial peptides in Escherichia coli: A review. *Protein Expression Purif.* **2011**, *80,* 260–267.