The study of cationic amphiphilic peptides with anti-cancer selective toxicity

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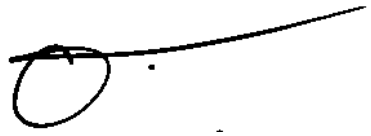
Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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Abstract

The exposure of organisms to environmental stresses and pathogens results in rapid activation of a range of defensive pathways that act as part of the innate immune system. The most common innate immunity response is the activation of cationic amphiphilic peptides in response to microbial infection. Moreover, cationic amphiphilic peptides possess desirable attributes for the pharmaceutical development of cancer-selective drugs. They selectively and rapidly kill cancer cells without killing normal mammalian cells and have a broad spectrum of mechanisms of action. The aim of this exploratory study was to screen for cationic amphiphilic peptides with anti-proliferative activity that is induced by genotoxicity. GeneFishing® technology, 2-D gel analysis and bioassays were used to identify and analyse molecules induced in response to genotoxic stress in an embryonic cell line originating from the dung beetle *Euoniticellus intermedius*. Bioassay results revealed that the cell line has constitutive expression of probable cationic amphiphilic proteins that are further induced by camptothecin treatment. GeneFishing® and 2-D gel analysis showed changes in gene expression at both transcriptional and translational levels, respectively. Overall, the study failed to identify the involvement or induction of cationic amphiphilic peptides in response to genotoxic stress. However, gene expression analyses revealed changes in the expression of classes of proteins involved in stress response, oxidative phosphorylation, mitochondrial maintenance, protein translation, cytoskeletal proteins and immunophilins. The results show that the cell line constitutively expresses probable cationic amphiphilic peptides which are further induced by camptothecin.

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List of abbreviations

ABP-CM4	antibacterial peptide-CM4
CAPs	Cationic amphiphilic peptides
DNA	Deoxyribonucleic acid
ERK	extracellular signal-regulated kinase
FPRL	formyl peptide-like receptor
hBD	human β-defensin
HEK-293	human embryonic kidney cell line
HeLa	epithelial carcinoma cell lines
HIV	human immunodeficiency virus
HT-1080	fibrosarcoma cell line
RNA	Ribonucleic acid
TFE	2,2,2-trifluoroethanol
MAPK	mitogen-activated protein kinase
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
PBMCs	mononuclear cells

Chapter 1: Introduction

1.1 Introduction

The exposure of organisms to environmental stresses, toxins and pathogens results in rapid activation of a range of energy efficient defensive pathways that act as part of the innate immune system. The most common innate immunity response is the activation of peptides in response to microbial infection. This response is conserved from microorganisms to humans (Papo and Shai, 2003). These peptides are widely synthesised in areas of the organism that are regularly exposed to pathogens. For instance, dermicidin, a natural antibiotic is secreted with sweat and found on the surface of human skin (Paulmann et al., 2012). Most antimicrobial peptides are amphipathic and positively charged and are widely known as cationic amphiphilic peptides (CAPs) (Hancock, 2001). The above properties contribute to their ability to interact with and rapidly disrupt the negatively charged phospholipid membranes of microorganisms (Hancock, 2001; Leuschner and Hansel, 2004). Consequently, CAPs have antibiotic activity against a range of pathogenic bacteria, viruses, fungi and protozoa. Furthermore, they have selective anti-cancer toxicity (Lehmann et al., 2006).

1.2 Cationic amphiphilic peptides overview

CAPs range between 5–40 amino acids in length and are in most cases membrane active (Hoskin and Ramamoorthy, 2008; Hancock, 2001). Moreover, they can be classified into peptides that have toxicity against bacteria and viruses but not against mammalian cells and fungi; and those with toxicity against bacteria, viruses, fungi and mammalian cells (Papo and Shai, 2003). CAPs play a significant role in the innate immune system of a range of plants and animal species by acting as natural antibiotics against bacterial, enveloped viruses and fungal pathogens (Papo et al., 2003). They are also capable of acting synergistically with each other or other host immune molecules. For instance, magainin II and peptide PLGa have been shown to have synergistic toxicity in frogs, while lysozyme showed synergistic antibacterial toxicity with a range of conventional antibiotics (Hancock, 2001). Furthermore, they have the capacity to act as immune modulators for adaptive immunity in vertebrates (Hoskin and Ramamoorthy, 2008; Giuliani et al., 2007). For instance, human α- and β-defensins have been established to exclusively chemo attract different groups of T lymphocytes and immature dendritic cells (Wu et al., 2003). CAPs have been proposed to be

involved in infection clearance and wound healing by acting as chemokines or by inducing chemokine production (Giuliani et al., 2007). Interestingly CAPs have cancer-selective toxicity linked to the electrostatic interactions between their net positive charge and the highly negative charge on cancer cells (Hoskin and Ramamoorthy, 2008). They are also capable of inducing apoptosis through interaction with the mitochondrion (Papo et al., 2003). Thus CAPs are potential candidates for anticancer drugs development since they have been reported to possess a novel mode of action, less cytotoxicity to normal cells and have a low likelihood of resistance development (Hsu et al., 2011). CAPs are synthesised in a range of cells such as epidermal, epithelial cells and neutrophils (Scot et al., 2008; Lohner and Prossnigg, 2009).

1.3 Structure of CAPs

CAPs are divided into four major secondary structural classes; α-helices, β-sheets connected by atleast one disulphide bridge, extended polyproline-like helices and loop structures (Ntwasa, 2012; Powers and Hancock, 2003) (Figure 1). The formation of secondary structures enables peptides residues to aggregate into an amphipathic structure that enables solubility in phospholipid membranes (Ntwasa, 2012).

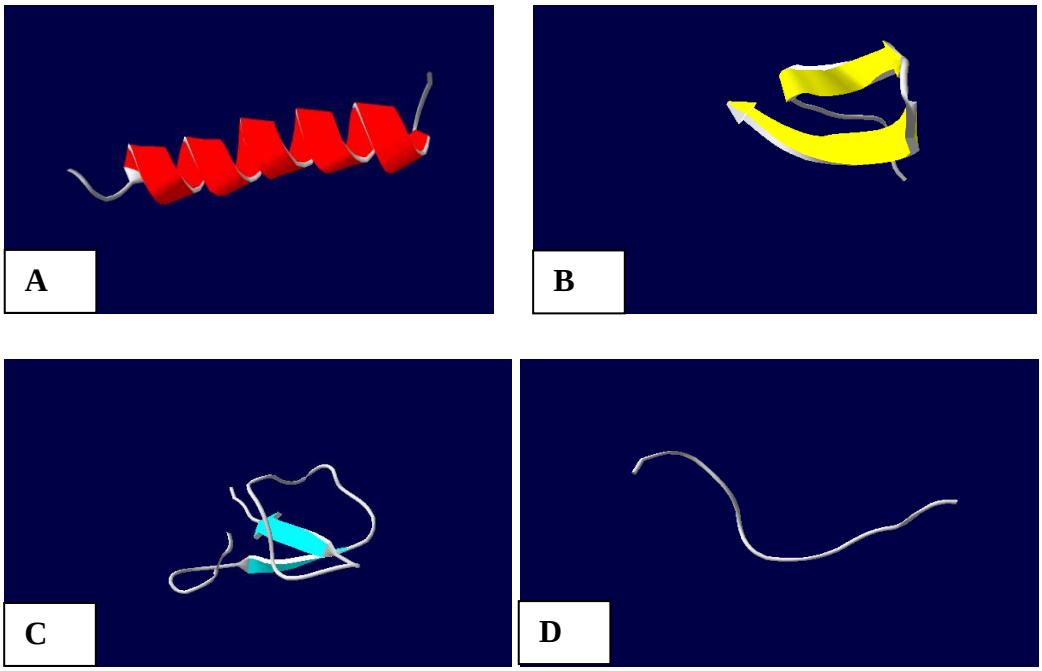


Figure 1: Structures and examples of peptides representing the four major classes of CAPs: (A) α-helical, magainin 2 (PDB ID: 2MAG); (B) β-sheet, lactoferricin B (PDB ID: 1LFC); (C) Loop, thanatin (PDB ID: 8TFU); (D) Extended, indolucidin (PDB ID: 1G89). The 2-D images were generated with swis PDB viewer.

1.4 Cationic amphiphilic peptides structural-functional relationships

Despite a variety of sequences and structures, CAPs share common features such as biochemical properties, antimicrobial and anticancer activities (Hoskin and Ramamoorthy, 2008).

They are linear peptides, generally unstructured in solution, amphipathic and positively charged due to a high content of basic lysine and arginine residues. As previously mentioned these properties result in the rapid disintegration of negatively charged phospholipid membranes (Hancock, 2001; Leuschner and Hansel, 2004). The overall positive charge of CAPs is believed to be crucial in facilitating electrostatic interaction between the peptide and anionic lipopolysaccharides of the membrane during the initial binding (Powers and Hancock, 2003). Furthermore, they are capable of integrating into the phospholipid membrane due to their amphiphilicity, resulting in rapid disruption of the membrane (Paulmann et al., 2012). In addition, the induction of α-helices, β-sheets, extended polyproline-like helices and loop secondary structures in a hydrophobic environment enhances the amphipathicity of CAPs (Hoskin and Ramamoorthy, 2008; Powers and Hancock, 2003). The carpet model, barrel-stave model, toroidal-pore worm model and detergent-like membrane lytic mechanism have been used to describe the mode of action of CAPs' membrane disruption. The membrane disruption property of CAPs has been established to be dependent on a range of physicochemical properties such as the amino acid sequence, net charge, amphipathicity and structural folding in membranes (Hoskin and Ramamoorthy, 2008; Hsu et al., 2011).

1.5 Mechanisms of action of CAPs

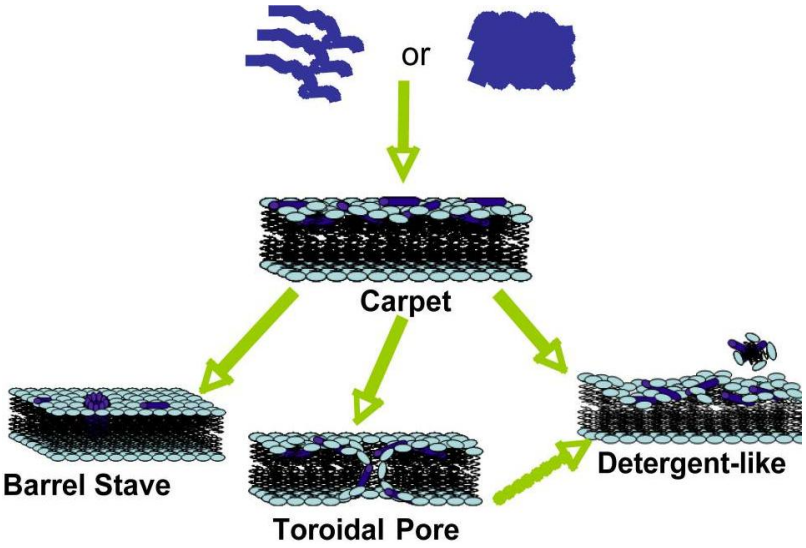
1.5.1 Overview

The membrane-selective toxicity of CAPs has been linked to the electrostatic interactions between their net positive charge and the negative charge of membranes, secondary structure conformation and the amphipathic nature of the peptides (Jang et al., 2011; Lohner and Prossnigg, 2009).

The mechanisms of cell necrosis are localised on the cell membrane and involve either pore formation or disruption of membrane integrity (Maher and Mclean, 2004). Disruption of the membrane integrity is a result of alterations in the phospholipid bilayer architecture and thus

leads to the leakage of cytoplasmic contents (Chen et al., 2010). Interestingly the peptides are able to discriminate between different lipid components of cell membranes which ultimately enhance selectivity (Lohner and Prossnigg, 2009). Furthermore, electrostatic interactions facilitate the adsorption of the peptide to the membrane which is followed by conformational changes of the unstructured peptide into a more amphipathic molecule with a defined secondary structure. The active peptide interacts with the hydrophobic core leading to membrane disruption and/or membrane potential disturbance (Drechsler and Andre, 2011; Lohner and Prossnigg, 2009). Javadpour et al., (1999) showed that a leucine/alanine, 21-mer peptide had an increased propensity to form helical conformations in amphipathic environments and ultimately increased toxicity against immortalised mouse fibroblast cells, compared to 14-mer peptides and glycine rich 21-mer peptides. The above study demonstrates the significance of the secondary structure conformation and amphipathicity in membrane disruption (Drechsler and Andre, 2011; Javadpour et al., 1999). Moreover, displacement of divalent ions such as Mg^{2+} and Ca^{2+} from membrane surfaces destabilises the membrane and facilitates binding regions for the peptides (Giuliani et al., 2007)

Several models have been proposed to explain the membrane-localised selective activity of CAPs (Figure 2). Membrane lysis is a result of peptide aggregation on the membrane and formation of ion-channel pores. Furthermore, membrane disintegration leads to depolarisation of the membrane potential (Papo and Shai, 2003).



(Hoskins and Ramamoorthy, 2008)

Figure 2: Models for CAPs membrane permeabilisation. Depending on the mechanism, peptides can either oligomerise before interacting with the membrane or oligomerise in the membrane.

1.5.2 The carpet model

The carpet model proposes that peptides accumulate and bind on the surface of the phospholipid membrane and then integrate into the membrane after reaching a given threshold level. The permeation varies depending on the type of peptide, with mechanisms such as detergent-like disintegration and channel aggregate formation. The interaction between the membrane and peptides is predetermined by hydrophobic charges (Hancock, 2001; Papo and Shai, 2003). The disturbance of membrane integrity results in the leakage of cytoplasmic contents, disturbance of the membrane potential and consequently the disintegration of the membrane. Furthermore, the rapid depolarisation of the target cells leads to rapid death (Powers and Hancock, 2003).

In the detergent-like mechanism, peptides spread on the surface of the membrane in an orientation parallel to the phospholipid bilayer head region. The peptides aggregate to a threshold concentration giving the peptide aggregate a highly amphipathic character that enables the peptides to act like a detergent thereby breaking the phospholipid membrane into micelles or bicelles-like small fragments (Hoskin and Ramamoorthy, 2008).

1.5.3 The toroidal-pore model

The toroidal-pore mechanism involves the orientation of peptides parallel to the phospholipid bilayer surface before they are eventually located in proximity to the head region of the phospholipid bilayer. The latter positioning facilitates the interaction between the hydrophilic side of the helix with the hydrophilic lipid head groups and the water phase outside bilayer, while the hydrophobic phase of the helix is embedded in the hydrophobic region of the lipid layer. Local aggregation of the peptides on the membrane surface increases until a threshold level is reached; and this increases the potential of toroidal-pore formation (Hoskin and Ramamoorthy, 2008).

1.5.4 The barrel starve model

Peptides aggregate in the membrane by aligning parallel to the phospholipids ultimately forming an ion-channel pore (Hoskin and Ramamoorthy, 2008). The membrane bound peptides are capable of recognising each other and then oligomerise to form the ion-channel pore. Moreover, the amphipathicity and secondary structure conformation of the peptides

play a significant role in pore formation. Peptides that use this mechanism are capable of interacting strongly with both zwitterionic and negatively-charged membranes and are consequently non-selective (Giuliani et al., 2007; Papo and Shai, 2003).

1.5.5 The aggregate or channel-forming model

The aggregate model proposes that membrane permeabilisation is dependent on the oligemerisation of the peptides within the membrane without a fixed stoichemistry. The oligemerisation is concentration and voltage dependant, and ultimately follows a sigmoidal curve. Electrostatic interactions have been established to play a significant role in the initial interaction (Ntwasa, 2012).

1.6 CAPs interaction with subcellular targets

Although the primary target of CAPs toxicity is the cell membrane, they have been described to translocate across the membrane and cause mitochondria morphological distortions and inhibit energised mitochondria in fungi. Some peptides have been proposed to result in the blockage of virus-cell fusion and the activity of HIV long terminal repeats. In some instance, peptides are translocated into the cytoplasm where they have enzyme targets (Hancock, 2001). An antibacterial peptide, ABP-CM4 has been reported to have significant antifungal activity through the disruption of cell membrane architecture, the cytoskeleton, and interaction with the mitochondrion and deoxyribonucleic acid (DNA) (Chen et al., 2010). CAPs have also been reported to interact and interfere with the synthesis of DNA and ribonucleic acid (RNA) in bacteria (Powers and Hancock, 2003). Furthermore, nisin A and gallidermin from *Lactococcus lactis* and *Streptococcus gallinarium*, respectively, have been reported to inhibit peptidoglycan synthesis (Maher and McClean, 2006).

1.7 The role of CAPs in apoptosis

Some CAPs have been reported to induce apoptosis through interaction with the host mitochondrion (Maher and McClean, 2006). This is directly linked to their ability to translocate across the lipid bilayer into the cytoplasm and other organelles (Terrone et al., 2003).

It has been established that CAPs translocate across the lipid bilayer via a gradient and composition dependant mechanism with minimal disruption to the integrity and permeability of the membrane (Terrone et al., 2003). The CAP, $(KLAKLAK)_2$ has been demonstrated to disrupt plasma and mitochondrial membranes, and subsequently induce caspase-independent and caspase-dependent cell death, respectively. The α-helical peptide nanostructure has been established to be highly stable, membrane permeable and capable of inducing caspase-independent and Bax/Bak-independent apoptosis in breast cancer cells with minimal potency to normal cells (Standley et al., 2010). Human antimicrobial peptide, cathelicidin LL-37 has been reported to induce apoptosis *in vitro* in human lung epithelial cell line A549. The peptide induced caspase-dependant cell apoptosis in a dose-dependent manner. Furthermore disruption of cell membrane integrity and cell death was limited to tumour cells only (Lau et al., 2010).

1.8 The expression of CAPs in response to stress and roles in immunity

1.8.1 CAPs synthesis and modification

CAPs are principally synthesized in the skin, eyes and lungs since these tissues are significantly exposed to pathogens (Papo and Shai, 2006). They are either synthesised and stored in these specialised cells and released as a result of infection or their synthesis and secretion is triggered by infection (Martin et al., 1995). Human β-defensin-1 is constitutively expressed in intestinal epithelial cells while the expression of β-defensin-3 is induced as a result of wounding or injury (Yeung et al., 2011).

Mammals synthesize and secrete CAPs in the skin, mucosal surfaces and neutrophils and these facilitate local response to infection (Scot et al., 2008; Yeung et al., 2011). For instance, dermicidin is a human antibacterial peptide with a broad spectrum of toxicity, which is synthesised in the eccrine sweat glands, secreted into sweat and is transported to the skin surface during sweating. Dermcidin is constitutively expressed as a full length 110 amino acid peptide with an N-terminal 19 amino acid signal peptide. The peptide is further differentially processed into C-terminal peptides of 47 amino acid residues or less. The peptide has been reported to have *in vitro* toxicity against pathogens such as, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Candida albicans.* Human cathelicidin LL-37 and human β-defensin (hBD-2) are expressed in neutrophils and keratinocytes, respectively, in response to injury and inflammation (Reig, et al., 2004), while human β-

defensin-1 (hBD-1) is constitutively expressed in the epithelial cells of the intestines (Yeung et al., 2011).

In amphibians, such as the South African clawed frog, *Xenopus laevis*, the antimicrobial peptide magainin II is synthesized and secreted in the granular skin glands and gastrointestinal tract (granular cells) (Lehmann et al., 2006; Lohner and Prossnigg, 2009). Both magainin I and II have been reported to be synthesised and stored in granular skin glands in frogs and consequently released to the skin surface in response to skin injury or exposure to pathogens or toxins. Furthermore, isolation of magainin cDNA has led to the conclusion that both magainin I and II are encoded by a single precursor (Reilly et al., 1994).

CAPs are synthesized as protein precursors with a generally conserved precursor region, highly diverse biologically active region and an endoplasmic reticulum targeting signalling peptide (Martin, et al., 1995; Patrzykat and Douglas, 2003). Moreover, CAPs are single gene-encoded peptides (Patrzykat and Douglas, 2003), and can be either ribosomally or non-ribosomally synthesized (Scot et al., 2008).

Cathelicidin-associated antimicrobial peptides are ribosomally synthesised peptides with a conserved precursor region of approximately 100 amino acid residues, and with homology to cysteine protease inhibitor, cathelicidin. Moreover, the highly diverse C-terminal cationic antimicrobial domain is cleaved off during formation of the mature antimicrobial peptide known as cathelicidin (Nissen-Meyer and Nes, 1997). Post-translational modifications of CAPs include the formation of disulphide bonds, C-terminal amidation, N-terminal pyroglutamic acid formation and, in a few cases, glycosylation. These modifications contribute to the stability and activity of some CAPs. Furthermore, multiple isoforms of a peptide can be derived by N-terminal truncation. CAPs may also be cleavage products of larger molecules, such as histones and ribosomal proteins (Patrzykat and Douglas, 2003).

1.8.2 Signalling pathways involved in CAPs response and action

Injury, inflammation and microbial pathogenicity determinants induce the expression of CAPs as a first line of defence (Leuschner and Hansel, 2004; Yeung et al., 2011). Furthermore, local CAP synthesis can be increased rapidly through degranulation of phagocytes or via Toll receptor-mediated pathways (Patrzykat and Douglas, 2003).

Wounding and exposure to pathogen-associated molecular patterns such as lipopolysaccharides trigger innate immune pathways that induce the synthesis and secretion of CAPs. Injury or exposure to bacterial endotoxins induces the production and secretion into blood of a range of antibacterial peptides from insect blood cells and fat cells. This immune response resembles the mammalian immune response and acute-phase response since gene expression is regulated by a kB-related *cis*-regulatory motif and by an NF-kB-related transcription factor (Nissen-Meyer and Nes, 1997).

Bacterial endotoxins, lipopolysaccharides, lipoteichoic acids and unmethylated CpG DNA are capable of interacting with Toll-like receptors triggering a range of host signalling pathways. Such an interaction has been linked to the up-regulation of apoptosis inducing factors (tumour necrosis factors), pro-inflammatory and anti-inflammatory (interleukin 6) cytokines, and chemokine-like inflammatory proteins. CAPs are capable of suppressing the up-regulation of these pathways and prevent endotoxaemia by binding directly to the endotoxins or receptors (Hancock, 2001). For instance, the peptide ABP-CM4 from the Chinese silk worm, *Bombyx mori* has been demonstrated to be capable of preventing lipopolysaccharides from binding to CD14+ and consequently preventing the production of cytokines and nitric oxide (Chen et al., 2010). Furthermore, the immunomodulation of human peptide, cathelicidin LL-37's is mediated by inducing the phosphorylation of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase ½ (ERK ½) and p38 kinase. This peptide is also able to stimulate innate immunity effector cells through a formyl peptide-like receptor 1 (FPRL-1). FPRL-1 is a pertussis toxin sensitive, G protein-coupled receptor that has been reported to be the chemotactic receptor on neutrophils, T-cells subsets and monocytes (Bowdish, 2004).

Microbial lipopolysaccharides and lipotechoic acid induce immune response in airway epithelial lining via interaction with Toll-like receptors. Rhinovirus double stranded ribonucleic acid has been reported to be a ligand for Toll-like receptor 3. Rhinovirus infection induces increased expression of human β-defensins 2 and 3, and chemokines. Moreover, Toll-like receptor 9 facilitates the expression of interleukin-8 from colony epithelial cells in response to bacterial infection. Distinct types of Toll-like receptors from a range of cells induce unique signalling pathways that are specific to both cell type and microbe. For instance, Toll-like receptors and interleukin-1 receptors bind to the MyD88 adaptor molecule through the Toll-interleukin-1 domain. MyD88 recruits and results in the phosphorylation of serine/threonine kinase interleukin-1 receptor associated kinase and the latter consequently

interacts with TRAF6. TRAF6 mediates downstream signalling to mitogen activated protein kinases and transcription factors (Bals and Hiemstra, 2004).

1.9 Roles in innate immunity

As previously stated CAPs contribute to the first line of defence against bacterial, viral, protozoa and fungal infections and play a significant role in innate immunity (Hoskins and Ramamoorthy, 2008; Hsu et al., 2011; Torrent et al., 2007). Moreover, they provide the defence mechanism against infection in insects and plants which lack the T-cell and antibody based adaptive immunity (Scot et al., 2008). These peptides are toxic against a range of bacterial pathogens including drug resistant strains such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Stenotrophonomas maltophilia*, with minimum inhibitory concentrations in the range of 1-4µg\ml, but many are not toxic to mammalian cell membranes. Moreover, they have been reported to have antifungal activity via morphology integrity distortion and mitochondrion induced apoptosis (Hancock, 2001; Yeung et al., 2011). For instance, rabbit defensin NP-2 has been reported to cause permeabilisation in yeast *Candida albicans* (Hancok, 2001). Magainin has been reported to have a broad spectrum of toxicity and to be active against both Gram-positive and Gram-negative bacteria, fungi and protozoa (Matsuzaki, 1998). CAPs such as defensins, melittin and polyphemusin, have been reported to have antiviral activity (Hancok, 2001). Dermaseptin has been shown to be directly active against the human immunodeficiency virus (HIV) through interaction with and destabilisation of the viral envelope while the polyphemusin analogue T22 blocks the entry of chemokine receptor-dependant HIV strains into cells by interaction with the chemokine receptor CXCR4 on T cells (Yeung et al., 2011). Other peptides have been reported to be capable of blocking virus cell fusion (Hancok, 2001).

1.10 Immunomodulatory roles of CAPs

Cationic peptides are also involved in innate immunity through stimulating the chemo-attraction of monocytes and neutrophils, promotion of histamine release from mast cells, inhibition of tissue proteases and stimulation of wound healing (Hancock, 2001). Interestingly CAPs have been found to be capable of modulating the host immune response and inducing the adaptive immune response through stimulating the production of immune mediators and signalling molecules (Bechinger, 2010; Hoskins and Ramamoorthy, 2008;

Patrzykat and Douglas, 2003; Torrent et al., 2007). Human defence peptides, such as cathelicidin LL-37, have been established to selectively enhance and modulate the host immune system through a range of activities including direct stimulation of chemotaxis and/or through chemokine production, suppression of the synthesis of bacterial induced pro-inflammatory cytokines, regulation of neutrophil and epithelial cell apoptosis, modulation of cellular differentiation pathways, modulation of dendritic cell activation and differentiation, and promotion of angiogenesis and wound healing (Lau et al., 2004; Yeung et al., 2011). Moreover, peptides such as melittin and defensins participate in acute and chronic inflammation in humans (Patrzykat and Douglas, 2003). For instance, hBD-3 is expressed in response to inflammatory disorders such as Crohn's disease (Yeung et al., 2011).

1.11 Anti- cancer activity of CAPs

Currently, to our knowledge, there is no information on the signalling pathways for CAPs in response to genotoxic stress. However, a range of CAPs from different organisms have been shown to have anti-proliferative activity against cancer cells (Shcweizer, 2009).

CAPs have been reported to possess cancer selective-toxicity through a range of mechanisms. The peptides are capable of inducing cell death via mitochondrial membrane disruption, inhibition of blood vessel development thus preventing tumour progression and necrosis induction (Chen et al., 2010). The mechanisms of cell necrosis are localised on the cell membrane and involve either pore formation or disruption of membrane integrity (Maher and Mclean, 2004).

Changes in the membrane of cancer cells are significant for cancer progression without signals that normally control growth. These changes ultimately result in fundamental differences between cell membranes of malignant and normal cells and this has been proposed to result in the selective toxicity to cancer cells by CAPs (Hoskin and Ramamoorthy, 2007). The cancer-selective toxicity of CAPs has been linked to the electrostatic interactions between their positive charge and the highly negative charge on cancer cells (Hoskin and Ramamoorthy, 2007). The net negative charge on cancer cells is due to the increased expression of anionic molecules such as phosphatidyl serine and O-glycosylated mucins. Furthermore, the negative membrane potential contributes to the selective cytotoxicity. Conversely electrostatic interaction between CAPs and normal mammalian cell membrane is not favourable because the latter is composed largely of

zwitterionic phospholipids such as phosphatidylethanolamine, phoshatidylcholine and sphingomyelin resulting in a net neutral charge. Moreover, the cholesterol on the membrane interferes with the CAPs permeabilisation and disruption of the lipid bilayer (Hoskin and Ramamoorthy, 2007).

Chen et al., (2010) studied the *in vitro* anticancer activity of the cationic amphiphilic antibacterial peptide-CM4 (ABP-CM4) against human leukemia cells compared to normal mammalian cells; human embryonic kidney cell line (HEK-293) and mononuclear cells (PBMCs). The peptide was isolated from the haemolymph of the Chinese silkworm *Bombyx mori*. Conformational studies using circular dichroism showed that ABP-CM4 had a significant random coil conformation in water. Inversely, the peptide showed an increasingly characteristic α-helical secondary structure in 20% v/v 2,2,2-trifluoroethanol (TFE) in water and a well-defined α-helical conformation in 30 % and 50% v/v TFE in water. Furthermore, the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colometric assay results indicated dose-dependent cytotoxicity of ABP-CM4 against leukemia cells. The standard MTT assay measures cell viability after exposure to a growth inhibitor. The IC_{50} values of ABP-CM4 in inhibiting THP-1, K562 and U937 cells were about 14.2, 15.8 and 17.5 µM, respectively. Interestingly, the peptide showed no significant cytotoxicity against HEK-293 cell line and PBMCs even at the highest concentration evaluated, 80 µM. Trypan blue exclusion assays also confirmed that ABP-CM4 showed no significant cytotoxic activity against PMBCs at a concentration more than tenfold the IC_{50} obtained against leukemia cells. The results demonstrated selective cytotoxicity against leukemia cells (Chen et al., 2010).

A flow cytometry analysis showed that FITC-labelled ABP-CM4 had a high affinity for the cell membrane of leukemia cells as indicated by higher fluorescence compared to normal cell lines. Moreover, the increase in fluorescence was proportional to the peptide concentration (Chen et al., 2010). Confocal laser scanning microscopy examination further confirmed that ABP-CM4 interacts with and binds to the cytoplasmic membrane since the FITC-labelled ABP-CM4 was localized on cell surfaces (Chen et al., 2010). LDH release assay and fluorescent propidium iodide (PI) uptake assay indicated a cell membrane damage mechanism of ABP-CM4 against leukemia cells. The levels of LDH release and fluorescent PI uptake where proportional to the concentration of the peptide used to treat the cells (Chen et al., 2010).

Hsu et al., (2011) demonstrated that synthetic Pardaxin induced apoptosis enhances its antitumor activity in human fibrosarcoma cell line (HT-1080) and epithelial carcinoma cell lines (HeLa). Pardaxin is a 33-amino acid pore-forming toxin from the Red Sea Moses sole, *Pardachirus marmoratus*. The study showed that Pardaxin inhibited the proliferation of HT-1080 and HeLa cells in a dose-dependent manner after a 6-24 hour exposure at concentrations between 6-50µg/ml. The anti-proliferative effect of Paradaxin on HT-1080 cells was at the highest levels 6 hours after treatment. However, HeLa proliferation was inhibited after 24 hours. DNA fragmentation studies showed DNA fragmentation in HeLa cells and not in HT-1080 after exposure to 15µg/ml of pardaxin. LDH release assay showed that LDH release from HeLa and HT-1080 cells was dose dependent. This showed that pardaxin disrupted the cytoplasmic membrane integrity of HeLa and HT-1080 cells. Pardaxin had no effect on the cytoplasmic membranes of human red blood cells (HRBCs). Moreover, flow cytometry indicated an increased proportion of HeLa cells in the subG1 phase. The above results imply that pardaxin induces apoptosis in HeLa cells and not in HT-1080. Furthermore, Real-time reverse-transcription polymerase chain reaction analysis showed that pardaxin induces the synthesis of cytokines, down regulates the expression of invasion-related genes and probably has pleiotropic effects on different cells (Hsu et al., 2011).

Several other CAPs have been found to exhibit anti-proliferative activity against different cancer cell lines. Lehmann et al., (2006) established the antitumor activity of the antimicrobial peptide magainin II against bladder cancer cell lines (RT4, 647V and 486P) with a mean LC_{50} value of 198.1µM. Cecropins are a class of α-helical antimicrobial peptides derived from insects and have also been established to have cytotoxic effects on HL-60 human promyelocytic leukemia cell lines, in particular cecropin B1 (Hoskin and Ramamoorthy, 2008). Papo et al., (2003) designed a short cationic diastereomeric peptide composed of D- and L-leucines, lysines, and arginines that showed selective toxicity towards mouse melanoma, fibroblast and lung carcinoma cells and significantly inhibited lung metastasis in about 86% of mice, with no detectable side effects. The peptides displayed the ability to depolarize the membrane potential rapidly at a concentration of about 3 µM. Moreover**,** confocal microscopy studies verified that the cells died as a result of acute injury, swelling, and bursting thus, suggesting necrosis.

1.12 Development of CAPs as therapeutic agents

CAPs possess desirable attributes for the pharmaceutical development of cancer-selective drugs. They selectively and rapidly kill cancer cells without killing normal mammalian cells and have a broad spectrum of mechanisms of action (Ntwasa, 2012). However, most of the well-studied and documented CAPs for cancer-selective toxicity have been isolated from amphibians and marine organisms (Hoskin and Ramamoorthy, 2007). Moreover, they are secreted in response to microbial infection and injury (Leuschner and Hansel, 2004). Several challenges have been established to impede the therapeutic development of commercially viable peptide-based drugs (Ntwasa et al., 2012). Foreign CAPs may have great potential to elicit treatment-neutralizing antibodies and/or potentially dangerous allergic responses in cancer patients. They are also prone to proteolysis and inactivation in blood serum resulting in a short half-life *in vivo* thus hampering their systemic use. For instance, blood serum strongly inhibits HNP-mediated cytotoxicity and this is an obstacle to the systemic administration of these human α-defensins. Synthetic CAPs such as DP-1 and r7-kla that have been engineered for rapid cellular uptake and resistance to proteolysis require selective targeting to tumour sites to enhance cancer-selective toxicity following systemic administration. In addition, the exorbitant production cost of synthetic CAPs is a huge obstacle to their development as anticancer agents. It has been established that magainin II, isolated from a frog species, showed reduced cytotoxicity against a differentiated bladder cancer cell line in comparison to the undifferentiated cell line (Lehmann et al., 2006). The low toxicity to certain neoplastic cells observed in some studies may require frequent administration to keep doses high if those peptides are to be used as anticancer agents. Moreover, the low cytotoxicity observed against normal cells and the hemolysis of erythrocytes need to be taken into consideration if CAPs are to be further studied and developed for cancer-selective toxicity.

1.13 Review of the SAEIE08 cell line

SAEIE08 is a dung beetle *E. intermedius* embryonic cell line developed by Rodney Hull from the Flylab, University of the Witwatersrand. The karyotype of the beetle was established to be 2n = 23 + XY, resembling the majority of the Coleoptera species. The cell was reported to divide asymmetrically into two daughter cells of different sizes, with a doubling time of approximately 5 hours. The cell line rapid growth at 26^{o}C may be a result of the early

expression of cytokine, *Unpaired*-3, leading to the early induction of the JAK-STAT pathway in embryonic cell line (Alouna, 2012).

1.14 Camptothecin as a DNA damaging agent

Camptothecin (CPT), is an alkaloid that is often used as an anticancer agent as it is an inhibitor of DNA topoisomerase I and thus disrupts the process of DNA replication. Camptothecin is, therefore a genotoxic stressor (Seong et al., 2012). Topoisomerases are enzymes that regulate the level of DNA supercoiling, to facilitate interaction with proteins during replication and transcription. These enzymes bind to either single stranded or double stranded DNA and their actions result in temporary breaks to DNA during replication, transcription, recombination and chromatin remodelling (Champoux, 2001). Seong et al., (2012) reported that CPT binds to the topoisomeraseI-DNA complex introducing irreversible covalent linkage that prevents re-sealing of the topoisomerase 1 introduced DNA breaks. This causes replication-dependent double-strand breakages in the DNA of replicating cells. Thus the CPT-DNA-topoisomerase complex disrupts replication and causes unfavourable DNA topology leading to homologous and non-homologous recombination. DNA damage and/or the failure to repair the damage induce DNA repair pathways which are controlled by a range of genes including tumour response genes (Davis and Lin, 2011).

Drosophila melanogaster (Canton S) has been used as model system to study the effects of CPT on normal cells. In a previous study, CPT treatment was established to cause an increase in the transcription of the *Drosophila* homologue of p53 (Dmp53) revealing the activation of the p53 pathway. In this study, the transcription of a *Drosophila* member of the retinoblastoma binding protein 6 family, *Snama*, was reduced during CPT treatment, but up-regulated when the flies recovered from treatment. This up-regulation may be associated with glycolytic flux that was induced in recovering flies (Hull and Ntwasa, 2010). In another study, Dmp53 was reported to have roles in DNA damage-induced cell cycle arrest under specific circumstances and tissue regeneration in response to imaginal discs damage (Monk et al., 2012). Dmp53 is known to induce apoptosis by up-regulating the transcription of downstream effectors such as *reaper*, *head involution defective* and *sickle*. In this study CPT is therefore considered a suitable model for DNA damage since there is background information about its effect on insects. CPT does, however, have some disadvantages; it is insoluble in water and is solubilised here in DMSO which may also have adverse effects on cells.

1.15 Objectives of the study

The current study is exploratory and aims to screen for genotoxic induced CAPs of insect origin with increased anti-proliferative activity against cancer cells, no toxicity to normal mammalian cells and resistant to proteolysis.

The current study focused on the identification and characterisation of molecules induced in response to genotoxic stress from an embryonic cell line originating from the dung beetle *Euoniticellus intermedius*. Moreover, it is important to establish if the induction of CAPs expression is part of the response. *E intermedius* (Coleoptera: Scarabaeidae) is a well-known dung beetle found largely in the Afrotropical region. *E intermedius* has a wide ecological tolerance and survives under extreme environments and has been reported to be tolerant to a range of conventional pesticides (Kruger et al., 1999). The beetle is being studied in the Flylab as a model of innate immunity in coleopterans.

The objectives of the study are:

- To isolate camptothecin induced cationic amphiphilic peptides using a C18 column
- To assay for potential anti-proliferative activity of induced CAPs using radial diffusion assay as a model
- To evaluate differential gene expression following camptothecin treatment using the GeneFishing® technology
- To evaluate differential protein expression using 2-D gel electrophoresis
- To identify differential expressed proteins using mass spectroscopy

Chapter 2: Materials and methods

2.1 Materials

The materials used in this study are listed in the tables below. The materials were grouped into three categories: media, biological buffers and solutions, and primer.

Table 1: Media

Media	Composition/ supplier
Grace's Insect media	Highveld Biological (catalogue # L16)
Luria broth	1% Sodium chloride (Merck, Germany) 1% Tryptone (Biolab) 0.5% Yeast extract (Biolab)
Solid agar	1% Sodium chloride (Biolab) 1% Tryptone (Biolab) 0.5% Yeast extract (Biolab) 1.5% Agar (Biolab)
Tryptic soy broth	3% Tryptic soy (Biolab)
Underlay agar	0.03% Tryptic Soy Broth (Biolab) 1% Agarose (Biolab) 0.02% Tween (Merck Germany)
Overlay agar	6% Tryptic soy (Biolab) 1% Agarose (Biolab)

Table 2: Biological buffers and solutions

Buffer/solution	Composition
50X TAE electrophoresis buffer pH 8	24.2 w/v % Trisbase (Merck, Germany) 5.71 v/v % Glacial acetic acid (Merck) 3.72 w/v % $Na_2EDTA._2H_2O$ (Merck)
Buffer I	50mM glucose (Merck, Germany) 10mM EDTA (Merck, Germany) 0.25M TrisHCl pH 8 (Merck, Germany)

Buffer II	1% SDS (Prolabo catalogue #27926.238) 0.2N NaOH (Merck, Germany)
Buffer III	5M Potassium acetate (Merck, Germany)
Ethanol : Acetate	25 : 1 (Merck, Germany)
Camptothecin	50mM in DMSO (Merck, Germany)
Rehydration buffer	8mM Urea (Bio-rad, USA) 2% CHAPS (Bio-rad, Canada) 50mM DTT (Fermentas, Canada) 0.2% Biolyte (Bio-rad, Canada) 0.5% Bromophenol blue (Sigma, USA)

Table 3: Primer

Primer name	Sequence 5'- 3'
3' RACE forward specific primer	CGAGATCTTCTAGAAG

2.2 Tissue culture

The embryonic cell line (SAEIE08) from *E. intermedius* was cultured in 30ml Grace's insect media to give a concentration of 100cells\ml in the final volume. The cells were incubated at 25^{o}C for 12 hours to reach an exponential growth phase. The cells were treated with camptothecin dissolved in DMSO to give a final concentration of 50mM in the insect media. DMSO was used as a control treatment. The cells were incubated for a further 4 hours, and the cell concentration was estimated to be approximately 5 x 10^{6} cells\ml.

2.3 RNA extraction

Total RNA for GeneFishing® and RACE was extracted using the ZR RNA MiniPrep kit from Zymo Research (catalogue number: R1064) according to the manufacturer's protocol. A cell concentration of approximately 5 x 10^{6} cells\ml was used during the extraction. Extraction was performed for both camptothecin and DMSO treated cell cultures. The concentration and purity of total RNA was determined through standard spectroscopy and formaldehyde RNA gel (1% agarose) (Figure 3). The 260/280 ratio was used to determine the

purity of RNA. This ratio was measured using a Nanodrop and measures the level of protein contamination. A ratio of greater than 1.8 is acceptable.

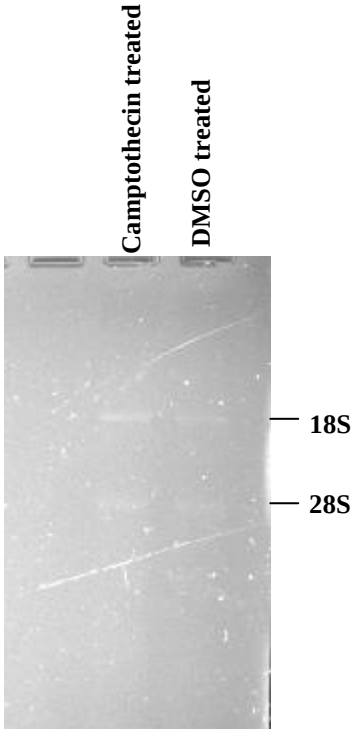


Figure 3: Total RNA extracted from the beetle cell culture. The integrity of RNA was determined using formaldehyde RNA gel (1% agarose). The gel shows both the 18S and 28S ribosomal RNA.

2.4 Protein extraction from lysed cells and concentration from the supernatant

Crude proteins from lysed cells were extracted from both the camptothecin treated and DMSO treated cell cultures using Tri-reagent from Sigma-Aldrich (catalogue number: T9424) following the manufacturer's protocol. The protein was however, washed in 100% ethanol by spinning for 5 minutes at 7500rpm after precipitation and dissolved in rehydration buffer for 2-D gel analysis and 1% SDS for C18 column purification. A cell concentration of approximately 5 x 10^6 cells\ml was used for the extraction. The protein concentration was determined using the Bradford reagent from Bio-rad (Cat # 10563) and a UV VIS spectrophotometer. The Bradford assay was performed according to the manufacturer's protocol. The standard curve (Figure 4) used was determined by Hull, (2012) and constructed in the spectrophotometer. The Bradford assay is a method used to determine protein concentration. The procedure involves binding of Coomassie Brilliant Blue G-250 dye to the proteins. The dye exists in three forms: cationic (red), neutral (green) and anionic (blue) and subsequently changes into a stable unprotonated blue form upon binding to proteins. The

unprotonated from has a wavelength of 592nm which is the wavelength used to determine protein concentration using a spectrophotometer.

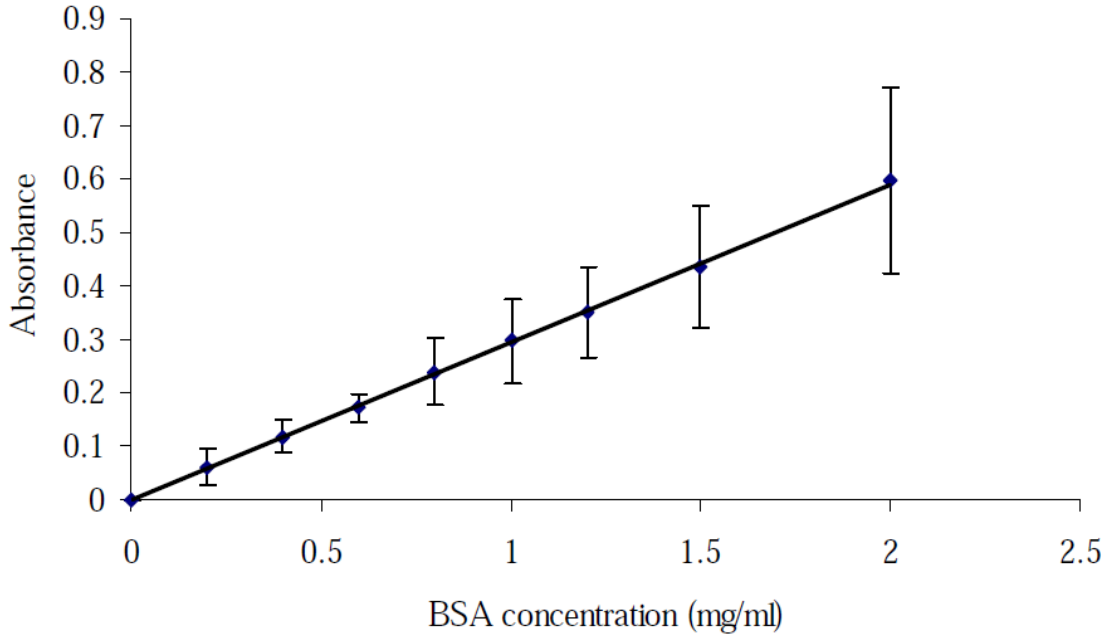


Figure 4: Standard curve for protein concentration using the Bradford assay. The curve was done in technical replicates and mean values used to construct the curve in the spectrophotometer. The equation of the line is y = 0.29564x -0.00004 and R^2 = 0.9994 (Hull, 2012).

Secreted proteins were concentrated from the supernatant**.** Camptothecin treated and untreated cell cultures were spun in 15ml centrifuge tubes for 5 minutes at 3.5rpm. The supernatant was used to concentrate the secreted proteins using Millipore's Amicon Ultra-4 centrifugal filter (Cat # UFC4 LCC 25). The filter enables retention of proteins from dilute samples, serum and cell culture samples through the nominal molecular weight limit membrane. The proteins were recovered from the bottom of the filter by sucking with a pipette. The lysate ~ 250µl was re-suspended in 250µl of 1% SDS. One volume of 20% trichloroacetic acid was added and the mixture incubated on ice for 30 minutes. The sample was spun at 12.5rpm for 10 minutes. The supernatant was discarded and pellet washed in 500µl cold acetone by spinning at 12.5rpm for 5 minutes. Acetone was discarded and pellet air dried. The pellet was re-suspended in rehydration buffer by pipetting and used in 2-D electrophoresis.

2.5 Isolation of the cationic amphiphilic proteins

The crude protein extract from the SAEIE08 cell line contains a complex mixture of proteins which needs further and specific purification to enable isolation of a specific group of proteins. In this study the Chromabond® C18 column from Macherey-Nagel (Cat # 0206/8) with a void volume of 0.8ml was used to isolate cationic amphiphilic peptides for radial diffusion assays. The column contains a hydrophobic, reverse phase, silica-based bonded phase that is capable of adsorbing cationic hydrophobic molecules from aqueous solutions. The column was activated with 2 volumes (0.8ml) of methanol. 0.8ml of the crude protein extract solubilised in 1% SDS was added to the column using a 10ml syringe and the flow through was collected. The column was washed with two volumes (0.8ml) of 5mM sodium phosphate buffer pH 8. The column was finally washed with 1ml of 80% acetonitrile in 5mM sodium phosphate buffer pH 8. Acetonitrile was removed from the samples through exposure to a stream of nitrogen.

2.6 Antimicrobial activity assays

The radial diffusion assays were based on the technique of Leher et al., (1991). Tryptic soy broth was inoculated with a single *Escherichia coli* colony and allowed to grow for 18 hours at 37^{o}C. 50µl of the culture was used to inoculate 50ml of fresh Tryptic soy broth and this was grown further for 3 hours at 37^{o}C. The culture was centrifuged and re-suspended in cold 10mM sodium phosphate buffer. The optical density of the culture was determined at 620nm and this was used to calculate the volume required to make a culture with 4 x 10^{6} colony forming units per ml in a final volume of 15 ml underlay agar using the formula: 0.2 $O.D_{620nm}$ = 5x 10^{7} CFU/ml. 15ml of the underlay agar with bacterial culture was prepared and plates were allowed to set. Holes were punched in the agar and the protein samples, tetracycline, phosphate buffer, acetonitrile and water controls were added to the holes. Tetracycline is a known antimicrobial and was used as the positive control while water was used as a negative control. Acetonitrile and phosphate buffer controls were used to verify that the solvents used in eluting proteins did not contribute to the antimicrobial activity of the proteins under investigation. Protein eluted with acetonitrile and sodium phosphate buffer were used as test samples. The plates were incubated for 3 hours at 37^{o}C. 15ml of overlay agar was then added

and plates incubated for 18 hours at 37^{o}C. The diameter of the clearings was measured. The assays were performed in triplicates for each test sample and control.

2.7 Chemical characterisation of the antimicrobial active agent using the Protein kinase assay

The inhibitory samples were treated with Proteinase K to determine the nature of the antimicrobial active molecules. Proteinase K was added to the samples to the final concentration of 100µg/ml. 100µg/ml Proteinase K and water were also included as controls. The reactions were incubated at 37^{o}C for 15 minutes and Proteinase K was inactivated by heating at 70^{o}C for 15 minutes.

The inhibition assay was repeated with the Proteinase K treated samples. Three technical replicates were used for each sample in the assays.

2.8 Differential expression of genes after camptothecin treatment

2.8.1 Genefishing Technology

The cDNA of differential expressed genes was synthesised using a GeneFishing® kit from SeeGene® (Catalogue number: K1026). The kit enables the identification of differentially expressed genes in 2 or more RNA samples. The kit works in three steps, which consist of the first strand cDNA synthesis step and a two-stage PCR. These steps use a set of three unique annealing controlled primers (Figure 5). The first step applies reverse transcription using dT-ACP 1 to synthesize cDNA from the mRNA. The 3′ end hybridizing sequence of dT-ACP 1 is complementary to the mRNA poly A tail and this results in a first strand cDNA transcript with the universal sequence of dT-ACP 1 at its 5′ end. The first strand cDNA is then diluted and placed in tube with an arbitrary ACP and dT-ACP 2 for the PCR step. The first PCR stage is a single PCR cycle under conditions that enable only the annealing of the arbitrary ACP with its 3′ end core to the first strand cDNA. This results in a second-strand cDNA with a complementary sequence of the universal sequence of dT-ACP 1 on its 3′ end and the universal sequence of the arbitrary ACP on its 5′ end. The second PCR stage enables the amplification of the targeted PCR product only. The conditions are set to exclusively allow both dT-ACP 2 and the arbitrary ACP to anneal to the 3′ and 5′ ends of the second strand

cDNA, respectively. The conditions also prevent the annealing of both primers to the first strand cDNA. The gene fishing experiment was performed following the manufacturer's protocol. The PCR products were electrophoresed in 1% agarose gel with ethidium bromide in 2X TAE electrophoresis buffer at 100Volts. A 1Kbp plus marker was used.The gel was kept for the recovery and purification of differentially expressed genes/bands.

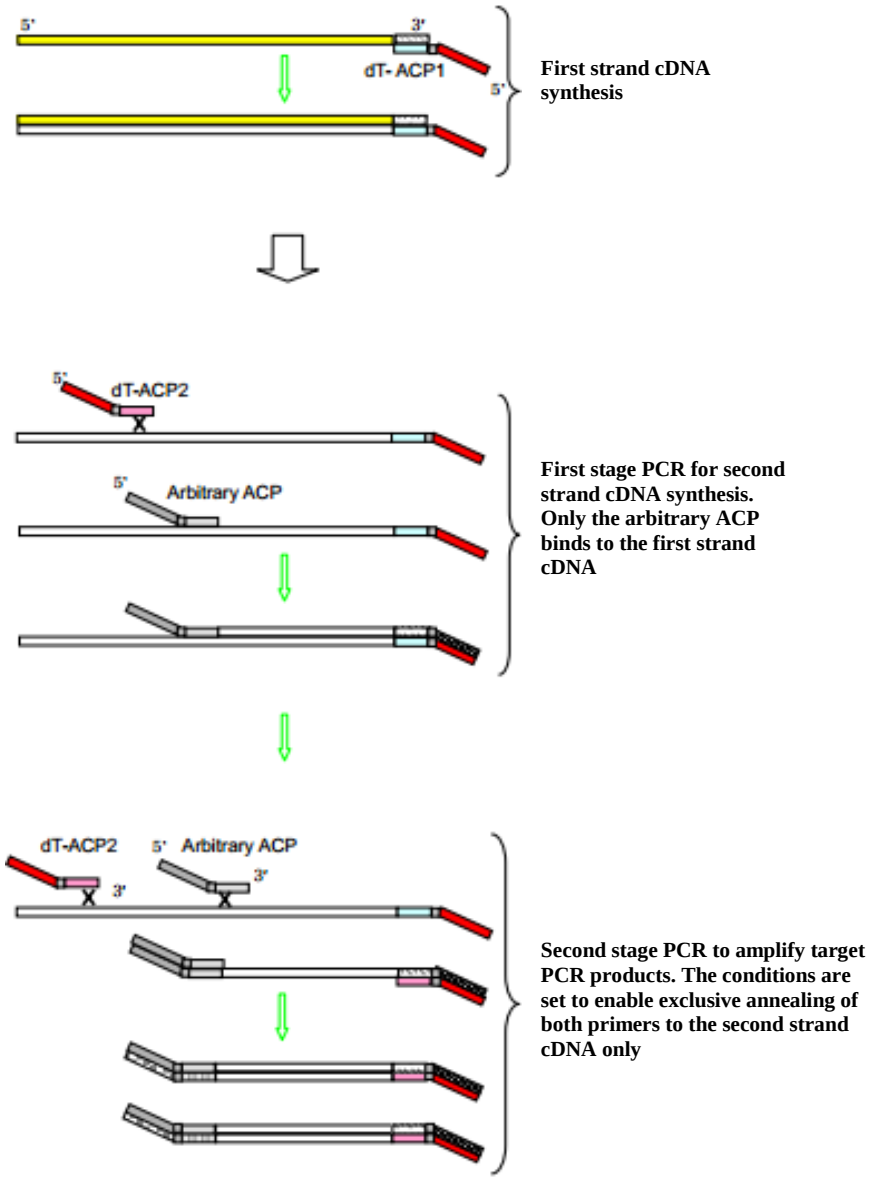


Figure 5: Flow chart of cDNA synthesis and GeneFishing® PCR (SeeGene® GeneFishing™ DEG premix kit user manual 2005, Catalogue number: K1026).

2.8.2 PCR products recovery and purification from electrophoretic gels

DNA purification from the gel was performed using with the Zymogen DNA recovery kit (Cat # D 4001). The kit provides an efficient method for high quality DNA purification and concentration from agarose gels without the use of organic denaturants. The kit purifies and concentrates DNA in a fast spin column. The bands of differentially expressed genes from gene fishing were excised from the gel under UV-light illumination. The purification was carried out following the manufacturer's protocol.

2.9 Cloning and sequencing of the GeneFishing® PCR product

2.9.1 Cloning

The recovered DNA was eluted with RNAase free water and cloned into a pGEM-T Easy Vector since gene fishing PCR products have poly-A tails. The ligation reaction was set-up according to the manufacturer's protocol. The total amount of insert DNA used was 25ng for each reaction. The tubes were sealed with parafilm and placed under water to prevent evaporation in the tube. The reaction was incubated for 12 hours at 4^{o}C and inactivated by incubating at -70^{o}C for 15 minutes

10µl of the ligation reaction was added to 40µl competent cells on ice, and was left to stand for 30 minutes. The cells were heat shocked for 45 seconds at 45^{o}C and cooled on ice for 2 minutes. 960µl of the recovery medium was added and the mixture was incubated at 37^{o}C with shaking at 250rpm for 90 minutes. 100µl of the transformation reaction was plated on pre-warmed agar with ampicilin/X-gal and IPTG. X-gal/IPTG was spread on the agar plate 30 minutes before plating and plates pre-warmed at 37^{o}C.

2.9.2 Sequencing

Gene fishing PCR products cloned in bacterial cells were sent to Inqaba Biotech® for sequencing.

2.10 3' Rapid amplification of cDNA Ends

Sequencing produced a partial sequence of a differentially expressed transcript which was used in 3' RACE to synthesise a full-length transcript of the gene (Figure 4). This technique

enables the generation of a full length sequence of RNA from cultured cells. The cDNA is generated using an Oligo-dT primer complementary to the natural polyA tail on 3′end of an mRNA transcript. The mRNA is then degraded before the PCR step. This technique was used because the PCR step requires only one specific primer (forward primer) and an anchor primer. A forward primer was designed from the partial sequence (Figure 10). The 3` RACE was performed using the 5´\3´ RACE kit, 2nd generation from Roche® (Cat # 03 353 621 001) according to manufacturer's protocol. The full-length sequence of the gene fishing transcript was amplified from total RNA from camptothecin treated cells. The PCR amplification program was performed as follows: Initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, 40 cycles of elongation at 72°C for 45 seconds, and a final elongation at 72°C for 5 minutes. PCR products were separated with electrophoresis. The whole PCR reaction was sent for sequencing by Inqaba biotech®.

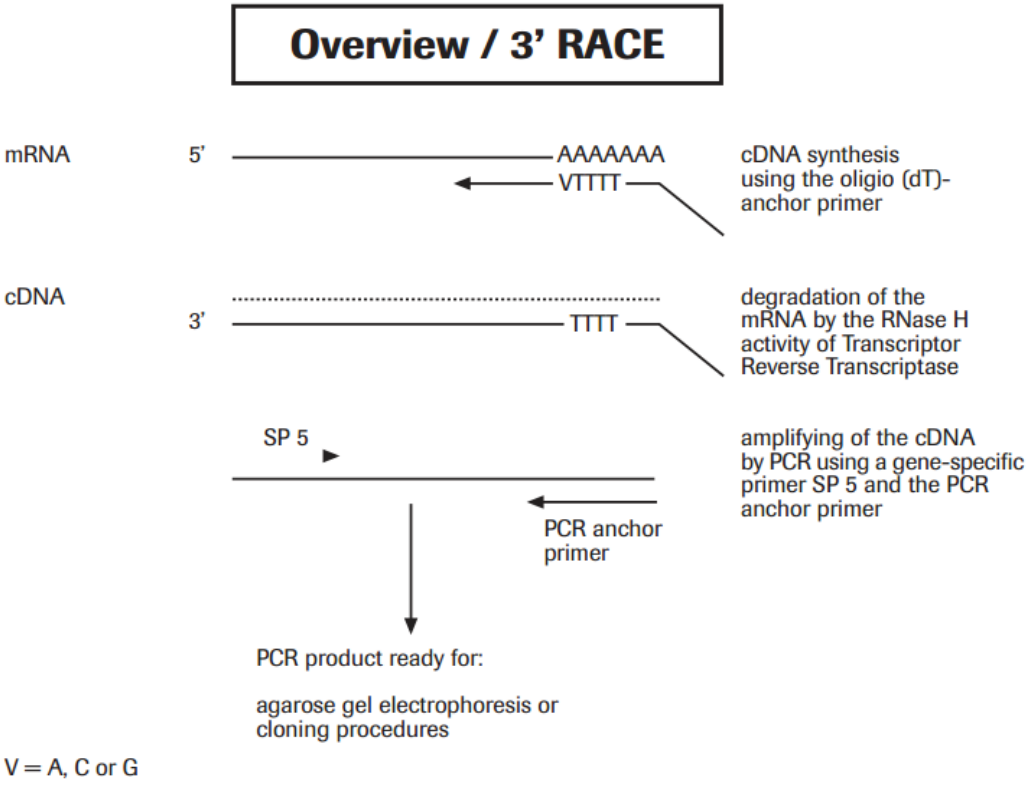


Figure 6: Overview of 3' RACE (Roche® Applied Science 5'\3' RACE kit, 2nd generation manual, Version October 2005, Cat # 03 353 621 001).

2.11 Proteomic analysis of molecules involved in genotoxic response of *E. intermedius* embryo cell line

2.11.1 2-D electrophoresis

A protein concentration of 100µg/ml was used for 2-D analysis. The protein sample was pipetted along the length of the rehydration tray and an 11cm IPG strip (pH 3-10) was laid carefully facing down avoiding the formation of bubbles. The strip was overlaid with mineral oil, sealed with parafilm and left to stand for 20 hours at room temperature. The wire electrodes of the isoelectric focusing tray were each covered with a paper wick and moistened with ~10µl of nanopure water. The IPG strip was removed from the rehydration tray and held for about 20 seconds to allow the mineral oil to drip off. The strip was placed facing down in the channel of the isoelectric focusing tray with its positive side on the anode left side of the tray. The strips were covered with mineral oil. The isoelectric focusing was performed in three steps. The first step was done at 250 Volts for 20 minutes with linear ramping, the second step at 8000 Volts for 2.5 hours with linear ramping and the last step was rapid ramping for 20000 Volts-hour. The IPG strip was removed from the isoelectric focusing tray and placed in a rehydration tray. 2 ml of equilibrating buffer I was added to the channel with the strip and shaken gently for 5 minutes. The buffer was discarded and replaced with equilibrating buffer II. The tray was gently shaken for 5 minutes and the buffer discarded. The strip was removed and dipped for about 30 seconds in 1X SDS running buffer in a measuring cylinder. The strip was over-laid on a precast gel. A paper wick loaded with 2µl pre-stained protein marker was placed next to the IPG strip. Overlay agarose was added and allowed to solidify. The PAGE was run at 100 Volts in 1X SDS running buffer. The gel was stained in Coomassie brilliant blue for 14 hours. The dye was de-stained for 4 hours and washed for 5 hours with several changes of distilled water.

2.11.2 Image analysis

A PDQuest 2-D analysis software version 6.2 (Bio Rad Cat #170-9630) was used to scan and upload the gel images. The camptothecin treated gel was used as the master gel. The spots from the camptothecin and DMSO treated gels were initially matched using the automated matching function. The spots were further matched manually with manual matching functions that enable deletion and addition of spots not detected or improperly detected during automated matching. Normalization was done automatically by the program based on the

given normalization formula: Normalized spot quantity = Raw spot quantity x scaling factor / Normalization factor (total quantity in all valid spots). The scaling factor was 10^6 parts per million

2.11.3 Mass spectroscopy

Spots representing differential expression were excised from the gels and sent for LC MS mass spectroscopy analysis at the Council for Scientific and Industrial Research, Pretoria, South Africa. The trypsin digested peptides were analysed using a Dionex Ultimate 3000 RSLC system coupled to a QSTAR ELITE mass spectrometer. Peaks v6.0 search engine (Bioinformatics Solutions Inc.) was used for comparison of the obtained MS/MS spectra with the MS protein sequence DataBase (MSDB) as well as the Flylab genome base. A False Discovery Rate (FDR) of 0.1% was used and proteins with more than one unique peptide reported.

2.11.4 Statistical and bioinformatics analysis

The mean OD values from the 2-D gels were calculated and changes in the expression of individual peptides were analysed using a one sided Wilcoxon Signed-Rank test at 10% significance level to compare differences in the mean since the data distribution is not normal. The Wilcoxon Signed-Rank t-test was also used to compare the differences in the mean inhibition in the activity assay. Peptide fingerprints from MS/MS were identified through manual search from the flylab genome base database (http://flylab.wits.ac.za/EI/est2uni/blast.php) (Khanyile et al., 2008) consisting of EST sequences of the adult transcriptome using TBLASTN. A BLAST was also performed from the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) using the UnitProtKB/Swiss-Prot(swissprot) database to identify proteins that had similar sequences to those identified by mass spectroscopy.

Chapter 3: Results

3.1 Results

The primary objective of the study is to investigate whether CAPs are activated by camptothecin and the molecular pathways involved in the process. GeneFishing® and 2-D analysis were used to determine the changes in gene expression at transcription and translation levels, respectively.

CAPs are an interesting group of molecules that are reported to play roles in apoptosis, stress response and to possess cancer selective toxicity as reviewed in this study. Moreover, it is in the view of this study that an understanding of the molecules and/or pathways involved in DNA damage paves a way for their exploitation to design novel cancer selective therapeutics. The objective of this study was to use GeneFishing® and 2-D gel analysis to analyse molecules involved in genotoxic stress response in an *E. intermedius* embryo cell line and to establish if the induction of CAPs is part of the response**.**

3.2 Antimicrobial activity assays

Genotoxic stress induces a series of response pathways in cells. The molecules induced in these response mechanisms are activated to either reverse the damage caused by the stress or cause cell apoptosis. This experiment aims to establish whether CAPs are specifically induced in response to genotoxic stress. In order to determine whether CAPs were expressed by the cell line, the C18 elutes of crude extracts from induced and un-induced cell cultures were assayed for anti-bacterial activity. This approach was used because CAPs are expected to have anti-bacterial activity.

The SAEIE08 cell line was used as a model in this study. The cell line was treated with camptothecin. The characteristic amphiphilic and cationic nature of CAPs was exploited to exclusively separate them from the crude protein extract from cell cultures using the C18 column. The column is designed to adsorb cationic hydrophobic molecules from an aqueous solution. Radial diffusion assays were subsequently used to determine whether camptothecin treatment induced molecules that possess anti-bacterial toxicity. The Gram negative, *E.coli* was used as a model for the toxicity assay because CAPs anti-bacterial activity employs the same mechanisms of action as during anti-proliferative activity against cancer cells. The

above is attributed to similarity in the overall characteristic of the membrane of cancer and bacterial cells.

Microbial inhibition was present in the samples eluted with acetonitrile (Figure 7a). This shows that the inhibitory molecules are cationic and amphiphilic which is consistent with the character of most CAPs. Interestingly acetonitrile purified samples from both camptothecin treated and untreated crude extracts showed microbial inhibition. This indicated constitutive expression of antimicrobial activity. However, inhibition by the former was significantly higher since the same concentration of the protein was used in the assays (T value = 0 < $T_{critical}$=2, for n = 3) (Figure 7a). This indicated that more activity was induced by camptothecin. Water was included as a standard negative control to enable comparison between microbial inhibition and non-inhibition (Figure 7b). No inhibition was shown in the water, sodium phosphate and acetonitrile control. Sodium phosphate and acetonitrile were included as controls since they were essential components of the elution buffers used to elute the proteins from the C18 column (Figure 7c). The positive control, tetracycline showed significant microbial inhibition (Figure 7c). Overall, the results show that the inhibitory molecules are constitutively active in the cells and that further expression is induced in response to camptothecin treatment. The constitutive activity may be attributed to the microbe-rich environment in which the beetles live.

To investigate the nature of the antimicrobial active molecules, the samples showing microbial inhibition were treated with Proteinase K. No antimicrobial inhibition was shown following the treatment of the inhibitory sample with Proteinase K (Figure 8a). This implies that the antimicrobial inhibition is a consequence of a protein since Proteinase K degrades proteins. Proteinase K alone also showed no inhibition (Figure 8c). Microbial inhibition was however, observed from untreated sample and from the tetracycline control.

Given these results, it may be proposed that probable cationic amphiphilic proteins are constitutively expressed by the cells and that camptothecin induces the production of more proteins.

The above observations pave the way to further study the pathways involved in the up-regulation of the anti-bacterial activity and to determine whether the induction of CAPs is part of the pathways and if they also play a role in genotoxic stress.

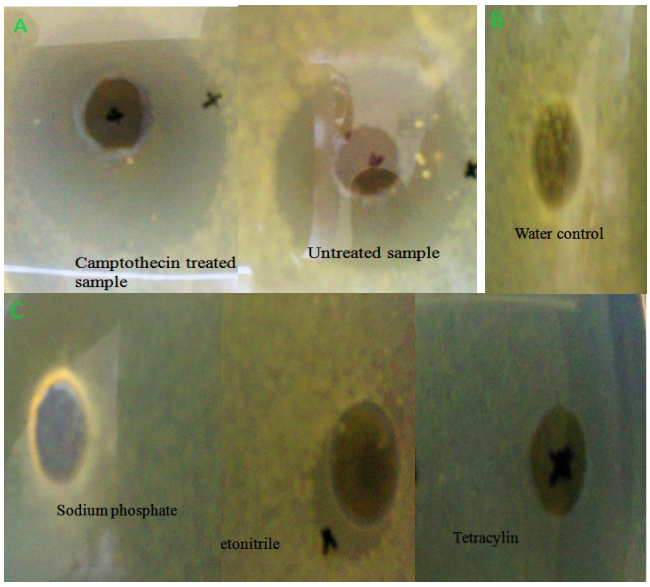


Figure 7: Radial diffusion assays. The crude protein extracts from *E intermedius* cells treated with camptothecin and untreated cells were purified with a C18 Sep pak vac column eluting with sodium phosphate buffer and acetonitrile. Figure 7 A shows inhibition by the acetonitrile purified samples. However, the sample from proteins extracted from camptothecin treated cells shows enhanced inhibition compared to the sample from the proteins extracted from untreated cells. T value $= 0 < T_{critical}=2$, for $n = 3$. The negative controls and positive control (tetracyclin) are represented in Figure 7 B and C.

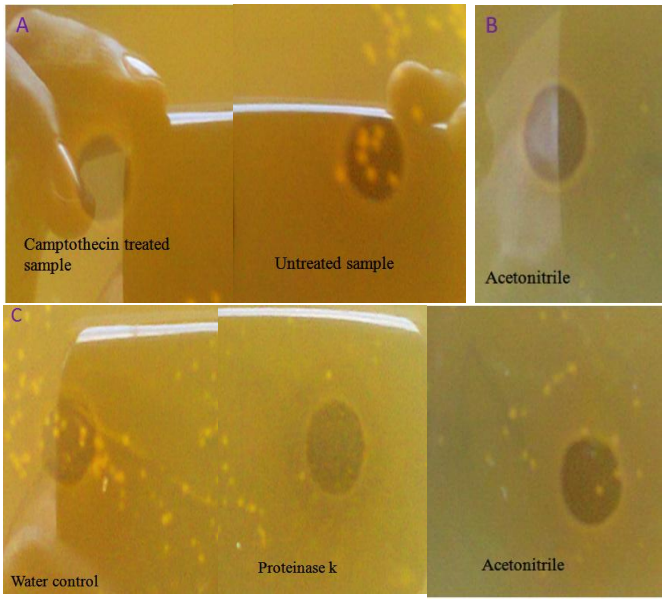


Figure 8: Characterisation of the inhibitory or toxic molecule. The sample that showed inhibition in the radial diffusion assay was treated with Proteinase K to verify that a peptide was responsible for the antimicrobial activity. Radial diffusion assays were used to test for microbial inhibition after treatment with Proteinase K. Figure 8A shows that no inhibition was seen after treatment with Proteinase K for both camptothecin treated and untreated samples. Similarly Figure 8 B and C show no inhibition by the controls.

3.3 Differential transcription of genes induced by camptothecin

Following the confirmation that the SAEIE08 cell line up-regulates the expression of probable antimicrobial proteins, the gene fishing technique in combination with 3' RACE was used to determine the identity of differentially transcribed genes following camptothecin exposure. The principle of gene fishing is based on the use of an annealing control primer (ACP) that anneals with high specificity to the intended template to allow amplification of the target product. The ACP is a tripartite structure with distinct 3′- and 5′-end portions separated by a regulator. The 3′-end core is designed to target the template via the hybridising sequence and is significantly complementary to a portion on the target nucleic acid template. The 5′-end core has a universal sequence and together with the regulator plays an important role in the annealing of the ACP to the template. RNA samples from camptothecin treated cells and from untreated cells were used in the gene fishing to identify differential expression between the two RNA samples (Figure 5). Electrophoresis was used to separate PCR products generated in the gene fishing experiment. The partial sequence of the up-regulated transcript generated by ACP 17 was used to design a forward primer to synthesise a full-length transcript of the gene using 3′ RACE (Figure 6). The full length sequence of the gene was generated from the RNA of camptothecin treated cells. The PCR product was separated by electrophoresis. The whole PCR reaction containing two bands was sent for sequencing by Inqaba Biotech®.

Camptothecin exposure also resulted in differential transcription of certain genes. Arbitrary complementary primers (ACP) 16- and 19-amplified fragments (300bp and 200bp, respectively) show down-regulation in the transcription of genes following campthotecin treatment (Figure 9b). Furthermore, ACP 17-amplified fragment (100bp) showing up-regulation in gene transcription (Figure 9b). ACP 18 also produced an up-regulated gene of ~ 250bp (Figure 9b).

The ACP 17-amplified transcript was cloned in pGem T-easy vector. Sequencing of the transformed clones produced a partial sequence of the gene. The sequence was subsequently used to design a forward primer (Figure 10) that was then used to perform 3' RACE to amplify the full sequence of the gene. Sequencing of the RACE PCR product produced two sequences and this is consistent with the two products shown in the electrophoretic gels (Figure 11). Only one of the sequences gave a likely match following a BLAST search in

NCBI nucleotide database. The sequence was highly similar to a yeast Yph1p protein homologue. Gene fishing coupled with 3'RACE results suggest that the gene for the Yph1p protein is constitutively expressed and also induced by camptothecin.

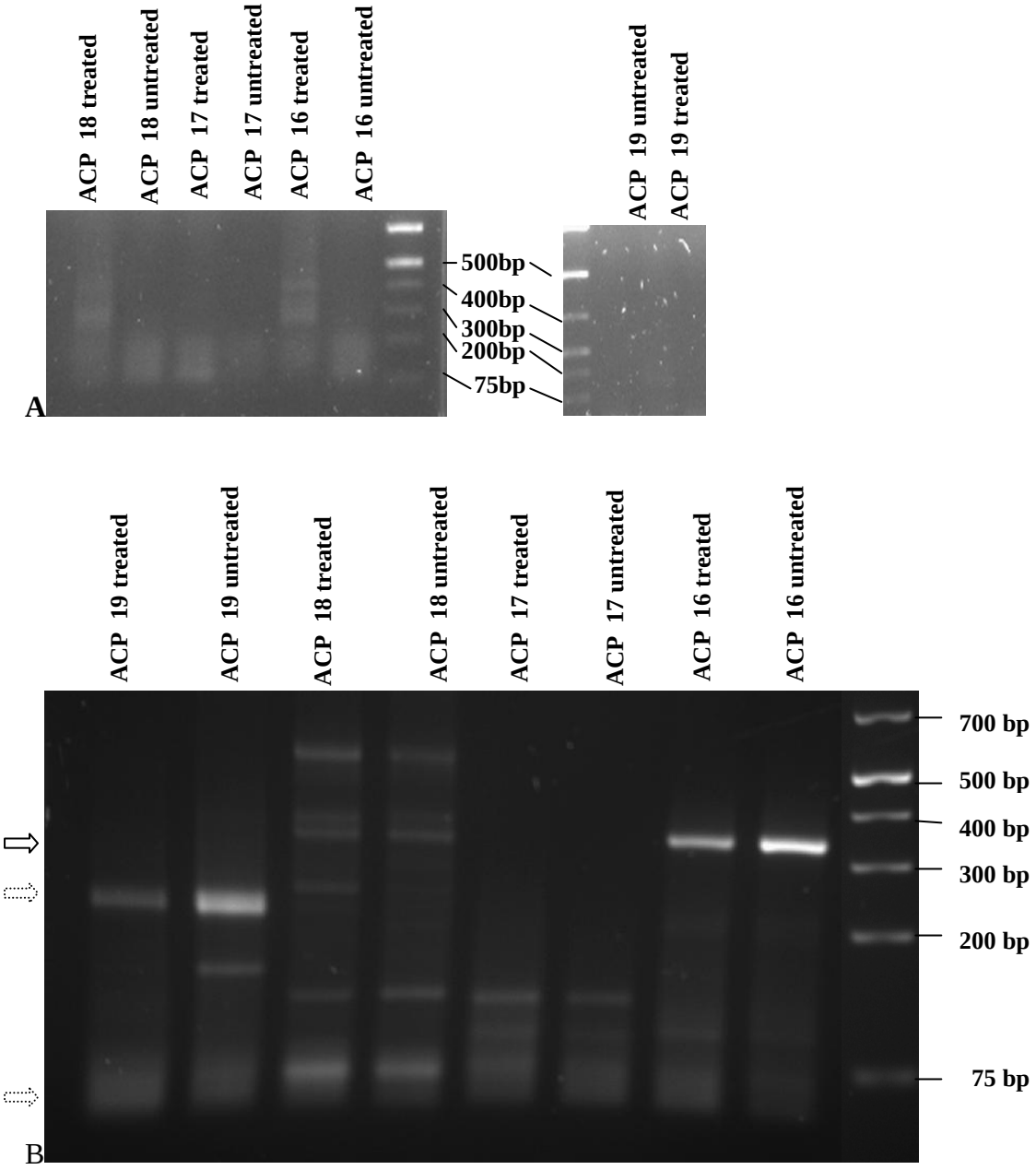
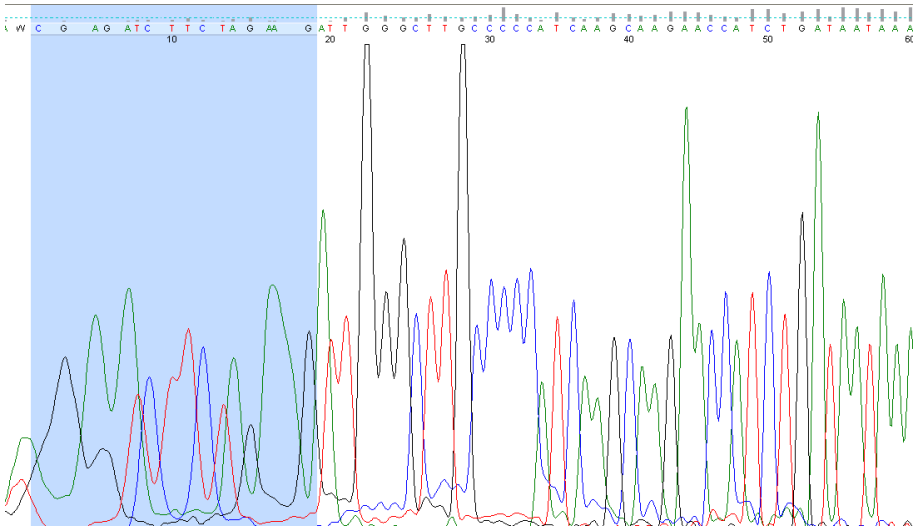


Figure 9: Gene fishing to identify differentially transcribed genes between camptothecin treated (T) and untreated/normal cells (U). Figure 9 A shows the arbitary complementary primers that amplified transcripts produced in response to camptothecin treatment in comparison to untreated\normal cells. B is a composite figure of figure A. The solid arrow shows the down-regulated transcript amplified with ACP 16. The dotted arrows show up-regulated transcripts amplified by ACP 18 and ACP 19.



5’ CGAGATCTTCTAGAAG 3’

Figure 10: Chromatogram showing the ACP 17 generated partial transcript that was used to design the forward primer for 3’ RACE.

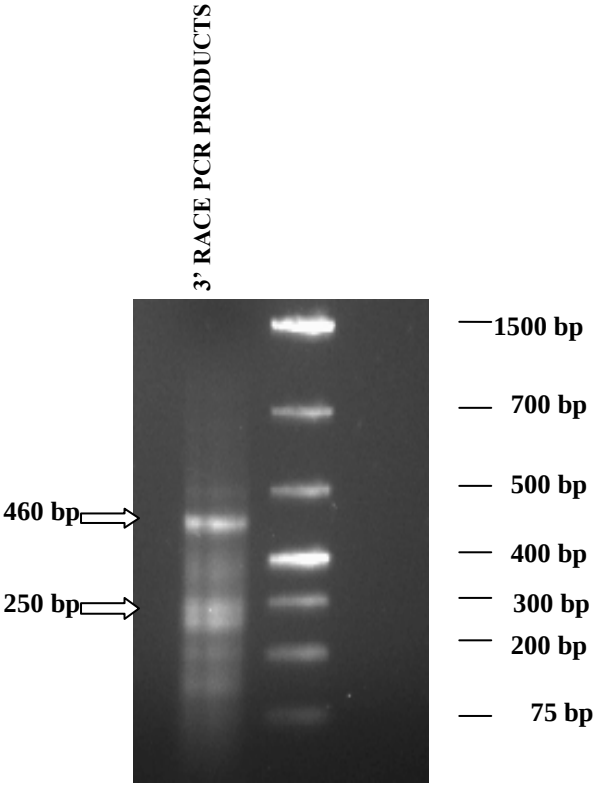


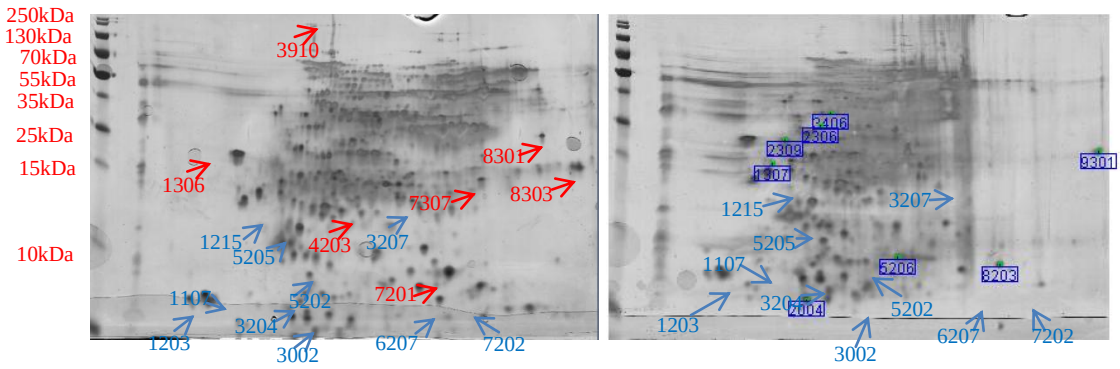
Figure 11: Rapid Amplification of cDNA Ends (3’ RACE) of the partial sequence obtained from differentially transcribed transcripts using the ACP17 GeneFishing® primer. The cDNA was amplified with a forward specific primer and PCR anchor primer. The gel shows two significant bands, one ~460bp and the other ~250bp.

3.4 2-D analysis of molecules involved in genotoxic response of E. intermedius embryo cell line

The activity assays showed that camptothecin treatment results in the up-regulation of the expression of proteins with CAP-like characteristics. As shown above, gene fishing results revealed that there were changes in gene transcription following camptothecin treatment to the cells (Figure 9). 2-D gel analysis and mass spectroscopy were subsequently used to identify the changes in protein expression following the exposure of the cells to camptothecin. The same concentration of the crude protein from camptothecin treated cells and non-treated cell cultures were separately exposed to 2-D electrophoresis on 11 cm pH 3-10 IPG strips. The 2-D gels for both camptothecin-treated and untreated samples were digitised using 2-D gel analysis software, PDQuest$^{(R)}$. The spots that were validated by PDQuest$^{(R)}$ software to be differentially expressed were excised from the gels and sent for sequencing using mass spectroscopy. The MSDB identified proteins were sorted according to their biological functions (Table 4). Furthermore, the peptide finger prints from mass spectroscopy were used to manually search the NCBI and Flylab genome base database (Table 5). The comparison in table 5 is important to enable genes to be cloned from adult beetles using sequences in the flylab genome base database since the database contains EST sequences of adult transcriptome of *E. intermedius*.

Gene fishing and 2-D gel analysis represented by Figure 7b and Figure 12, respectively show that camptothecin exposure leads to differential expression of certain genes. Gene expression analysis showed changes in the expression of a range of protein classes such as stress proteins, immunophilins, translation proteins, oxidative metabolism proteins, mitochondrial proteins and cytoskeletal proteins.

The expression of stress response proteins such as ubiquitin and heat-shock protein were up-regulated. There was a significant down-regulation of oxidative metabolism proteins such as ATP synthase and ADP/ATP synthase. The expression the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase was also down-regulated. Cytoskeletal proteins were both up-regulated and down-regulated. For instance myosin light chain was up-regulated while myosin heavy chain was down-regulated. Protein translation associated proteins such as upf 1 membrane protein, ribosomal protein S14e and elongation factor 1-alpha and mitochondrial maintenance protein, mmf 1 were up-regulated.



A (untreated) pH 3-10 B (treated) pH 3-10

Figure 12: 2-D electrophoresis analysis of differential expression of proteins between untreated (A) and Camptothecin-treated (B) in South African *Euoniticellus intermedius* embryonic cell line. Exponentially growing cells were treated with 50mM camptothecin and the crude protein extracted from cells after 4 hours using Trizol reagent. The isoelectric focusing was done with an 11 cm IPG strip and the 2nd dimension was run using a precast TGX gel from Biorad®. Spots numbered in Red are only present in the untreated (A) gel, spots marked in dark blue with a rectangle are found in treated only (B) and spots marked in blue are found in both gels.

Table 4. Proteins identified with MSDB and BLAST searches in NCBI database and Flylab base genomebase. Camptothecin exposure resulted in both up-regulation and down-regulation of protein expression. A one sided Wilcoxon Signed-Rank test at 10% significance level to verify if there was a significant change in the levels of protein expression between camptothecin treated cell line and untreated cell line. A T-value less than $T_{critical}$ indicates a significant difference in protein expression. $T_{critical} = 2$.

Spot	MSDB predicted protein	Spot MR (Kda)	Flylab genomebase matching peptide	Mean OD before	Mean OD after	T-value
Stress proteins						
2309	Yeast heat shock protein	20		4.8	75.2	0
5202	Ubiquitin	8		109.1	131.1	1
4203	Un-identified	11	Tribolium mrj (100%)	62.3	0.2	0
4203	Ubiquitin-60S ribosomal protein	11.5		62.3	0.2	0
Oxidative metabolism and mitochondrial						
7307	Mitochondrial peroxiredoxin	13		119.6	0.2	0
8303	Protein mmf1 mitochondrial	14.5		154.1	0.2	0
8303	Glyceraldehyde-3-phosphate deyhdrogenase	14.5		154.1	0.2	0
7202	ATP synthase	7		158.5	3.1	0
7202	ADP/ATP translocase	7		158.5	3.1	0
Protein translation						
1203	ribosomal rna small subunit methyltransferase	7.5	Glycosyl hydrolase family (100%)	44.2	121.8	0
5205	Un-identified	12.5	Elongation factor 1-alpha (100%)	13.8	57.9	1
5206	40S ribosomalprotein S14	8	Putative ribosomal protein S14e (100%)	0.3	88.1	0
9301	upf 1 membrane protein	17	Myosin heavy chain isoform 2 (100%)	0.3	124.3	0
3207	eukaryotic translation initiation factor 3 subunit	11		57.5	114.4	0
Cytoskeletal protein						
3207	Un-identified	11	Similar to Tropomyosin 1 (100%)	57.5	114.4	0
1215	myosin light chain	12	Myosin alkali light chain 1(100%)	10.4	37.6	1
7202	myosin heavy chain	7		158.5	3.1	0
6207	actin fragment	6.5		86.1	125.1	1
Immunophilins						
3204	FK506 Binding protein	7	FK506 Bingding protein (100%)	58.6	116.7	0
7201	Un-identified	7.5	Cyclophilin 1 (71%)	392.7	0.2	0

Table 5: Comparison of MS database identified proteins with the NCBI and Flylab base genomebase databases identified proteins.

Spot	NCBI predicted protein	Accession number	Flylab genomebase matching peptide	Flylab genome DB ID
2309	Heat shock 70 kDa protein	P12795.1		
5202	Ubiquitin	P19848.1		
4203			Tribolium mrj (100)	
4203	Ubiquitin -60S ribosomal protein L40	P0CH08.1		
7307	Mitochondrial peroxoredoxin PRX1	P34227.1		
8303	Protein mmf1 mitochondrial	P40185.1		
8303	Glyceraldehyde-3-phosphate dehydrogenase	P17819.1		
7202	ATP synthase F1 sector subunit beta	B0THN2.1		
7202	ADP/ATP translocase	P05141.7		
1203	ribosomal rna small subunit methyltransferase	Q4FPN5.1	Glycosyl hydrolase family (100%)	005275_1537_3307_c_s
5205	Elongation factor 1-alpha	Q07051.1	Elongation factor 1-alpha (100%)	CL17Contig1
5206	40S ribosomalprotein S14	P27069.1	Putative ribosomal protein S14e (100%)	CL171Contig1
9301	Buforin 1	P55897.1	Muscle myosin heavy chain isoform 2 (100%)	CL448Contig1
3207	Eurkaryotic translation initiation factor 3 subunit	P23301.3		
3207	Tropomyosin	P42637.1	Similar to Tropomyosin 1 (100%)	CL137Contig1
1215	Myosin-2 light chain	P53141.1	(Tribolium) Myosin alkali light chain 1(100%)	CL25Contig1
7202	myosin heavy chain D	P02567.3		
6207	actin 1	P85911.1		
3204	FK506 Binding protein	Q754K8.1	FK506 Bingding protein (100%)	009937_1646_3733_c_s
7201	Cyclophilin	P14832.3	Cyclophilin 1 (71%)	005522_1647_2340_c_s

Chapter 4: Discussion

4.1 Discussion

This study shows that the SAEIE 08 cell line constitutively expresses antimicrobial proteins. Moreover, more anti-bacterial proteins are induced upon challenge by camptothecin. Analysis of gene expression has not revealed any cationic peptides as expected but showed classes of biological molecules involved in a range of pathways. Gene fishing and 2-D gel analysis showed changes in the expression of proteins involved in stress response, oxidative metabolism, mitochondrial maintenance, cellular regulation and protein translation, following camptothecin exposure of the *E intermedius* embryonic cell line.

4.2 Cationic peptides detection

The C18 column was used to isolate cationic amphiphilic molecules expressed in the cell line before and after treatment with camptothecin. Radial diffusion assay and Proteinase K assay were then used to determine if the molecules have any antimicrobial activity and the nature of the molecules, respectively.

The microbial inhibition in the bioassays from samples eluted with acetonitrile demonstrated the presence of an antimicrobial molecule which is cationic and amphipathic. The C18 column contains a hydrophobic, silica-based bonded phase that is capable of adsorbing cationic hydrophobic molecules from aqueous solutions, and hence it requires a strong polar aprotic solvent to elute them. Furthermore, the Proteinase K assay verified that this inhibitory molecule is a protein. Bacterial cells were used as a model for the toxicity assays because cancer cells, like bacterial cells, have negatively charged phospholipid membranes which are targets of the cationic peptides (Hancock, 2001; Leuschner and Hansel, 2004). The electrostatic attraction between the negatively charged membranes and the cationic peptides, and the amphipathicity of the peptides play a major role in the membrane-localised mode of action of the cationic amphiphilic peptides (Jang et al., 2011; Lohner and Prossnigg, 2009). Although the study showed the induction of probable cationic amphiphilic proteins with antimicrobial activity, cationic amphiphilic peptides were not identified.

4.3 Changes in gene expression induced by genotoxic stress

Gene fishing and 2-D gel analysis were used to analyse the changes in gene expression following the exposure of the cell line to camptothecin. RNA samples from camptothecin-treated and untreated cell culture were used in GeneFishing® to identify changes in expression from a transcriptional level. Protein samples from camptothecin treated and from untreated cells were used in 2-D analysis to identity changes in expression at a translational level. The protein identities from mass spectroscopy were analysed and grouped according to their biological functions.

Overall gene expression analysis showed that there were significant changes in protein expression following camptothecin treatment. These changes in protein expression occurred particularly in the expression of proteins involved in stress response, oxidative metabolism, protein translation and cytoskeletal proteins.

Cell cycle regulator

Gene fishing and 3′ RACE resulted in the identification of a gene encoding a yeast Yph1p protein homologue. The expression of this gene was shown to be up-regulated. Yph1p is an 88 kDa protein and is highly conserved across species. Yph1p has been established to form complexes with proteins involved in cell cycle regulation, 60S ribosomal subunit biogenesis, DNA replication and regulation of chromatin structure, hence suggesting that the protein plays a critical role in these respective cellular processes. Interestingly, the mammalian ortholog of the protein has been shown to be up-regulated in metastatic cancer tissues giving further evidence to support its role in DNA replication, cell cycle regulation and checkpoint control (Du and Stillman, 2002).

Yph1p has been established to have two domains, a coiled-coil BRCT domain and a putative bipartite nuclear localisation signal domain (Du and Stillman, 2002). The former was first discovered at the C-terminus of the breast cancer protein BRCA1. A significant number of proteins have been found to have at least one BRCT motif and most of them have been suggested to have roles in cell cycle regulation, checkpoint control, and DNA damage repair. The BRCA1 BRCT domain interacts directly with a phosphorylated BRCA1 associated carboxyl terminal helicase, and this interaction is specifically cell cycle regulated and critical for DNA damage induced checkpoint control (Yu et al., 2003).

Since Yph1p contains a BCRT domain which is also found in most proteins with roles in cell cycle regulation, and DNA repair and regulation (Yu et al., 2003), the up-regulation of Yph1p in this study may be induced by the reported DNA damage caused by camptothecin treatment to normal cells (Hull and Ntwasa, 2010).

Stress response proteins

Camptothecin exposure resulted in significant changes in the expression of stress proteins.

The yeast heat-shock protein up-regulated in cells following exposure to camptothecin is a general stress protein induced when cells are exposed to temperatures slightly higher than their optimum growth temperature, or by a range of other environmental stresses such as genotoxic stress, inflammation and oxidative stress. For this reason they are also known as stress proteins (Verghese, 2012; Lindquist, 1984). The expression of ubiquitin was up-regulated following exposure to camptothecin. Ubiquitylation regulates many cellular processes including DNA repair pathways, damage avoidance mechanisms, cell cycle checkpoints, apoptosis (Bergink and Jentsch, 2009) and may be associated with genotoxic stress since it is up-regulated in this study.

An interesting observation was the down-regulation of MRJ protein. This protein is a member of the DNAJ/heat shock protein 40 family of chaperones (Hunter et al., 1999). Watson et al., (2007) showed that MRJ deficiency inhibits normal keratin turnover promoting the formation of large toxic keratin aggregates thus inhibiting the normal function of the trophoblast. This suggests a role of MRJ in preventing the formation of toxic inclusion bodies. Moreover, Andrews et al., (2012) showed that an MRJ small variant that is localised in the cytoplasm is capable of translocating to the nucleus in response to heat shock and hypoxia. Thus, MRJ is likely to be a heat shock protein that specifically responds to cellular stress and plays a limited role in genotoxic stress since it was down-regulated in this study. Furthermore, it is likely down-regulated to promote cell death in cells that have been damaged by camptothecin.

Immunophilins

Camptothecin exposure resulted in changes in the expression of FK506 binding protein and Cyclophilin A belonging to the immunophilin family. These proteins have similar roles such as receptor signalling, protein trafficking, transcription and regulation of other protein activity (Bang et al., 2008; Obchoei et al., 2011).

The expression of an FK506 binding protein was up-regulated in response to camptohecin treatment. FK506 binding proteins are a family of proteins that bind to FK506, an immunosuppressant drug. They act as immunomodulators when in complex with specific ligands. Recently they have been established to have roles in apoptosis through molecular interactions with receptors (Bang et al., 2008). Moreover, Aghdasi et al., (2001) established that the FK506 binding protein 12 is a physiological regulator of the cell cycle. They showed that the protein was a subunit of transforming growth factor beta and that the deletion of this protein resulted in cell cycle arrest in the G1 phase in mice fibroblasts.

In contrast to FK506 binding protein up-regulation, cylophilin A, another major immunophilin was down-regulated following camptothecin exposure. Cyclophilin A is multifunctional protein with similar roles to FK506 binding protein (Obchoei et al., 2011). The protein is also up-regulated in response to a range of inflammatory conditions. Furthermore, cyclophilin A secretion was established to be induced by oxidative stress via the activation of extracellular responsive kinase1/2 (Satoh et al., 2010). Cyclophilin A has also been reported to protect cells from hypoxia and infection (Obchoei et al., 2011).

In this study, the up-regulation of an FK506 binding protein may be associated with the increase in gene expression since some FK506 binding proteins have been reported to have roles as chaperones (Kang et al., 2008).

Oxidative metabolism and mitochondrial proteins

Treatment of the cell line with camptothecin resulted in the down-regulation proteins involved in oxidative metabolism and mitochondrial maintenance. ATP synthase, ADP/ATP translocase, glyceraldehyde-3-phosphaste dehydrogenase, mitochondrial peroxiredoxin and mitochondrial matrix factor-1 protein were shown to be down-regulated. The apparent down-regulation of certain proteins involved in oxidative metabolism observed in this study is different from the increase in oxidative metabolism observed in the *Drosophila* system after camptothecin treatment.

The down-regulation of mitochondrial peroxiredoxin, an H_2O_2 scavenger (Castro et al., 2011) may be a genotoxic stress response mechanism. High levels of H_2O_2 have been reported to induce cell death (Nonn et al., 2003). Moreover, Sung et al., (2011) reported that mitochondrial peroxiredoxin-3 is a potential target for novel cancer therapeutics since deletion (RNA silencing or expression knockdown) of peroxiredoxin-3 in HeLa cells

increased H_2O_2 concentration and ultimately increased sensitivity to cell death. Since camptothecin is known to increase the production of H_2O_2 (Hull and Ntwasa, 2010), the down-regulation of peroxiredoxin observed in this study may be related to H_2O_2 sensitive cell death. In this study, however, cell death was not investigated but inferred from studies in other organisms.

Protein translation

Camptothecin exposure resulted in up-regulation of ribosomal RNA small subunit methyltransferase, Upf 1 membrane protein, the eukaryotic translation initiation factor 3 subunit, translation elongation factor-1-alpha and ribosomal protein s14. The up-regulation of proteins involved in protein translation is consistent with the new expression of genes.

Cytoskeletal proteins

Camptohecin exposure induced higher expression of cytoskeletal protein such as tropomyosin, myosin light chain and actin in the cell line. Camptothecin treatment may be leading to increased tropomyosin expression in order for the cell to remain viable (Polevoda et al., 2003). Furthermore, these cells are not cancer cells even though they are a rapidly dividing immortal cell line, hence the increased expression of cytoskeletal proteins observed in this study may be important to maintain membrane integrity and cell viability following camptothecin treatment.

4.4 Camptothecin treatment and differential protein expression summary

Gene fishing and 2-D gel analysis showed changes in gene expression at transcription and translation levels, respectively following camptothecin treatment. Gene expression analysis has shown that *E. intermedius* cell line alters the expression of stress proteins, oxidative phosphorylation proteins, mitochondrial maintenance proteins, translation proteins, cytoskeletal proteins and immunophilins to deal with the effects of camptothecin treatment. However, the expression of probable cationic amphiphilic peptides was not revealed by this analysis. Moreover, the induction of stress response proteins can be related to changes in the cell metabolism and morphology following camptothecin exposure.

4.5 Conclusion

Although no novel CAPs were directly identified, the results showed that the cell line constitutively expressed probable cationic amphiphilic peptides which are further induced by camptothecin. Furthermore, camptothecin induced the differential expression of a well-known antimicrobial peptide, Buforin 1.

4.6 Future prospect

Since CAPs were not identified in the lysed cells protein extract, attempts were made to isolate and determine the activity of secreted CAPs. Secreted proteins were concentrated from the supernatant as describes in the materials and methods chapter. However, activity assays could not be performed since the protein yield from the cell culture media was significantly low. Furthermore, 2-D gel analysis resulted in low sensitivity to staining and poor resolution of the gels as result of low protein yields and high salt concentration in the protein samples. The use of an ultra-filtration protein isolation technique that combines desalting and concentration in a single step may reduce the salt concentration and increase the protein yield. Thus a high protein yield of secreted proteins may enable the detection and isolation of CAPs.

The use of fluorescence staining techniques instead of Coomassie and silver staining may increase sensitivity in detecting CAPs from 2-D gels.

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