ISOLATION, DESIGN, AND BIOLOGICAL PROPERTIES OF FISH-DERIVED
CATIONIC ANTIMICROBIAL PEPTIDES

by

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ABSTRACT

Cationic antimicrobial peptides are components of the host innate immune response against infections in almost all species. Although innate defenses in fish are particularly important due to a frequently late onset and short duration of the secondary immune response in these animals, few fish peptides have been studied to date.

This research identifies a novel H1-histone derived cationic peptide (HSDF-1) from coho salmon (*Oncorhynchus kisutch*), and describes the design, as well as the antibacterial, membrane, and intracellular activities of several other fish-based synthetic peptides of potential importance in aquaculture.

HSDF-1, a 26 residue species identical to the N-terminal segment of trout H1 histone, was isolated from the serum and mucus of disease-challenged coho salmon. The purified fractions inhibited the growth of antibiotic-supersusceptible *S.typhimurium*, as well as *A. salmonicida* and *V. anguillarum*. Synthetic HSDF-1 and its derivative HSDF-2 were inactive in antimicrobial assays, but they potentiated the antimicrobial activities of the flounder peptide pleurocidin, lysozyme, and crude lysozyme-containing extracts from coho salmon. Although the peptides inserted specifically into anionic lipid monolayers, synergy with pleurocidin did not appear to occur at the cytoplasmic membrane level.

The effects of pleurocidin and several of its derivatives on bacterial membranes and intracellular functions were further studied to gain insight into the mode of action of these fish-derived peptides. All peptides tested at their minimal inhibitory concentrations (MICs) inhibited macromolecular synthesis in bacteria, causing a decrease in the
incorporation of [3H]thymidine, [3H]uridine, or [3H]histidine into DNA, RNA, and proteins, respectively. However, many of the peptides applied at their MICs showed limited or negligible ability to damage bacterial cytoplasmic membranes, as observed using a fluorescent dye (diSC35) assay. The inhibition of macromolecular synthesis may thus be a result of the peptides entering bacterial cells and acting intracellularly in a direct fashion, as suggested by the fact that when two peptides were tested, they were able to translocate into liposomes without lysing them.

A combined approach of isolating antimicrobial peptides from nature and designing them from existing templates, should eventually provide the medical and veterinary clinicians with a useful arsenal of weapons in the face of developing resistance to conventional antibiotics.
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LIST OF ABBREVIATIONS

AU Acid-Urea

C6-NBD-PC 1-palmitoyl-2-[6-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-caproyl]-L-\(\alpha\)-phosphatidylcholine

CCCP carbonyl cyanide m-chlorophenyl hydrazone

CFU colony forming unit

CL cardiolipin

diSC\(_3\)5 dipropylthiacarbocyanine

DNS-PE dansyl phosphatidylethanolamine

EDTA ethylenediaminetetraacetate

ePC L-\(\alpha\)-phosphatidy-DL-choline from egg-yolk

ePG phosphatidylglycerol from egg yolk

FAO UN Food and Agriculture Organization

FIC fractional inhibitory concentration

Fmoc N-(9-fluorenyl) methoxycarbonyl

FPLC fast protein liquid chromatography

HEPES N-2-hydroxyethylpiperazine-N’-2-ethanesulfonate

HSDF histone-derived fragment

LPS lipopolysaccharide

LBP lipopolysaccharide binding protein

MIC minimum inhibitory concentration
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NACA</td>
<td>Network of Aquaculture Centers in Asia-Pacific</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPN</td>
<td>1-N-phenylnaphthylamine</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PE</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-ethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol</td>
</tr>
<tr>
<td>PMB</td>
<td>polymyxin B</td>
</tr>
<tr>
<td>RP</td>
<td>reverse phase</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxyethyl) amino methane</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
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STATEMENT OF AUTHORSHIP

Large sections of the Introduction have been published or submitted for publication as Patrzykat and Hancock (2001), Hancock and Patrzykat (2001), and Hancock and Patrzykat (1999). Also, several portions of the Materials and Methods, as well as portions of the Results chapters and Discussion, and several figures, have been published or submitted for publication as Jia et al. (2000), and Patrzykat at al. (2001, both references). In all instances where previously published materials, or materials submitted for publication, are reproduced in this thesis, they represent the original research and writing of the author. Prof. R.E.W. Hancock has co-authored all of the publications listed here and his reading of the thesis will serve to verify this statement of authorship.
1 INTRODUCTION

A recent FAO/NACA/WHO report identified aquaculture as one of the fastest growing food production sectors in the world, and focused in particular on food safety issues associated with products from aquaculture (Food Safety, 1999). One of the biggest challenges of aquaculture is its cost-effectiveness, and fish loss from infectious disease is a significant problem. While vaccination is commonly employed in the aquaculture setting, its effectiveness is limited by seasonal, environmental and age/size constraints (Smith et al., 2000). Antibiotics are therefore extensively used. The FAO/NACA/WHO report identifies several hazards associated with the use of antibiotics in aquaculture, including the transmission of bacterial antibiotic resistance genes from marine environments to humans, and antimicrobial drug residues in edible tissues (Food Safety, 1999). In addition, the authors of the report point out that intensive aquaculture is a relatively new field, and pharmaceutical companies have tended to borrow antimicrobials from other areas of veterinary medicine instead of developing chemotherapeutants specifically for use in the aquatic milieu.

As we have advanced into the era of bacterial resistance to conventional antibiotics, we are looking to natural immune defenses to provide us with novel solutions. It is in this search that we now have an opportunity to develop new agents for infectious disease control, derived from fish immune defenses, and intended for aquaculture use. Among the most promising contenders are cationic antimicrobial peptides. These peptides have been isolated from virtually all groups of living organisms, and shown to play a role in their innate defenses. Given that fish at times must rely heavily on their innate immunity (Bly and Clem, 1991; Bly and Clem, 1992), cationic peptides are of particular importance.
in this field. The increasing pace at which cationic antimicrobial peptides are being
commercialized, as well as the potential for developing transgenic species overproducing
these peptides, should ensure that peptide antimicrobials will eventually find their way to
the realm of aquaculture medicine. However, in order to make this happen, significant
research effort is still required: both to identify new peptide candidates and to understand
their activities.

1.1 Cationic Antimicrobial Peptides

An exhaustive survey of cationic peptide antimicrobials would have been
appropriate here a few years ago but, with more than 600 cationic peptides known today,
this is not possible anymore. Hence, the most common biological and biochemical
features, the most obvious trends, and several aspects specific to this thesis are surveyed
below.

1.1.1 Overview

Endogenous cationic antimicrobial peptides have been isolated from a multitude of
animal and plant species (reviewed by Hancock and Lehrer, 1998). They are recognized
as the major response to infection in more primitive species, and their induction appears
to be the primitive equivalent of the immune response. In higher animals, these peptides
tend to be induced as a local response to infection (Ganz and Lehrer, 1997). Indeed, it
has become clear in the past decade that cationic antimicrobial peptides represent a
ubiquitous response in nature for overcoming microbial infections. They are produced by
bacteria, fungi, plants, insects, amphibians, crustaceans, fish, birds, and mammals,
including man, either constitutively or in response to the presence of a microbe (Hancock and Diamond, 2000; Hancock and Scott, 2000; Boman, 1995; Ganz and Lehrer, 1997). Most natural peptides have a moderate spectrum of activity, and are usually present in modest amounts. Hosts generally compensate for this by producing an assortment of peptides with overlapping activities, and by up-regulating them in the response to the presence of microbes. Thus each animal can contain several antimicrobial cationic peptides, that are either redundant or possibly complementary in their activities, and that occur in single locations, such as the intestinal mucosa or the neutrophils.

In some instances, antimicrobial proteins and peptides have been shown to be processed products of larger proteins, including lactoferrin (Tomita et al., 1994), cathepsin G (Bangalore et al., 1990), and histone H2A (Park et al., 1996). More often however, cationic peptides are synthesized in nature by multienzyme complexes (gramicidins, polymyxins), or on the ribosome, with or without post-translational modifications (Bayer et al., 1995; Metz-Boutigue et al., 1998; Yorgey et al., 1994). While the former have furnished antibiotics that are used in current medical practice (polymyxin B, gramicidin S), the latter provide an extraordinary opportunity for peptide variation by mutation. In 1966, several small arginine-rich microbicidal peptides were found in the granules of polymorphonuclear leukocytes (Zeya and Spitznagel, 1966), and later on identified as β-sheet-structured defensins (Ganz et al., 1985). Among other early peptides were purothionins isolated from wheat plants (Fernandez de Caleya et al., 1972), cecropins isolated from silk moths (Steiner et al., 1981) and magainins isolated from frogs (Zasloff et al., 1987).
Only three structural characteristics are common among most cationic peptide antimicrobials: they are cationic, with excess lysines or arginines; they are small, being generally 6-45 amino acids in length; and their hydrophobic and hydrophilic residues are separated in the folded structure. The separation between hydrophobic and hydrophilic residues is of great consequence to the mode of peptide interactions with the amphipathic membranes of microbes, as will be described later.

While peptides generally share little sequence homology, they fall into four broad structural classes: (A) β-sheets stabilized by two to three disulphide bridges, (B) amphipathic α-helices formed upon membrane contact, (C) extended structures (again formed upon membrane contact) which are rich in tryptophan, proline and/or histidine, and (D) peptides with loops formed by a disulphide bridge (examples shown in Fig. 1A). A number of peptides from those classes have amidated C-termini, which enhances their activity. However, there are many variants amongst these basic classes, with at least eight sub-classes of β-sheet peptides having been described in plants alone (Garcia-Olmedo et al., 1998). Two main peptide folds have been recognized: amphipathic structures comprising a hydrophilic, positively charged face and a hydrophobic face, and a cationic double wing structure with two pockets of positive charge bracketing a hydrophobic core. The amphipathic α-helical peptides have received the most attention in the literature as prototypes for building improved peptides, since the design of α-helices is relatively straightforward.

Various peptides have been shown to possess antibacterial, antifungal, anti-viral and anti-parasitic properties, as well as a range of immunomodulatory activities. The best
Figure 1. Structures and mode of action of cationic antimicrobial peptides. (A) NMR-based molecular models of four structurally-diverse peptides are shown. Charged regions of the backbone are shown in teal and the hydrophobic residues in green. This figure has been adapted with Prof. R.E.W. Hancock’s permission from his recent review (Hancock 2001). (B) While there are several controversial theories about how cationic antimicrobial peptides (red) interact with the cytoplasmic membrane bilayer, it is generally agreed that unstructured peptides enter at least the outer leaflet of the bilayer, where they are folded, as depicted here. The various hypotheses as to the further character of membrane/peptide interactions are elaborated in the text. The figure has been adapted from a presentation slide originally presented by Dr. R.E.W. Hancock.
antimicrobial peptides kill susceptible bacteria \textit{in vitro} at concentrations ranging from 0.25 to 4 \( \mu \text{g/ml} \). While peptides are not the most potent antibiotics, they rapidly kill target cells, possess unusually broad spectra of activity, and show activity against some of the more serious antibiotic-resistant pathogens in the clinic. In addition, peptides can exhibit synergy with conventional antibiotics against resistant mutants, and they do not easily select resistant mutants \textit{in vitro}. Therefore they are being developed as a novel class of antimicrobial agents, with several peptides currently in clinical trials (Hancock, 2000), as well as being used as the basis for making transgenic, disease-resistant plants and animals (Broglie \textit{et al.}, 1991, Osusky \textit{et al.}, 2000).

1.1.2 Role in Innate Immunity

There are at least three lines of evidence emphasizing the role of cationic antimicrobial peptides in innate immunity. Firstly, the range of activities which the peptides exhibit within the host is indicative of their importance in innate immunity. Secondly, the pattern of expression of cationic antimicrobial peptides, as well as the fact that some peptides are produced in response to injury, suggest a role in early host defenses. And lastly, it has been shown that cationic peptides, when added exogenously, can be effective in protecting the host from bacterial infections in animal models.

In addition to killing pathogens directly, as described earlier, cationic peptides can interact directly with host cells to modulate inflammatory process and innate defenses (Hancock and Diamond, 1998; Hancock and Scott, 2000). Several activities have been reported in mammals, including stimulation of mast cell degranulation leading to histamine release and consequent increase in blood vessel permeability (vasodilation),
promotion of chemotaxis of neutrophils and T helper cells resulting in leukocyte recruitment to the infection site, promotion of non-opsonic phagocytosis, inhibition of fibrinolysis by tissue plasminogen activator, thus reducing the spreading of bacteria, tissue/wound repair through promotion of fibroblast chemotaxis and growth, and inhibition of tissue injury by inhibiting certain proteases such as furin and cathepsin (reviewed in Hancock and Diamond, 1988). In addition they neutralize the stimulatory effects of bacterial molecules such as Gram negative bacterial LPS, Gram positive bacterial lipoteichoic acid (LTA) and bacterial CpG-motif DNA, both directly and indirectly by interaction with the host cells they stimulate. In the case of LPS, this results in neutralization of the ability of this molecule to cause harmful sepsis and endotoxic shock (Gough et al., 1996). Collectively, these activities can all be considered part of the early response against infection and imply that peptides can provide protection before the specialized immune response takes over. This is because no clonal expansion is required for the activity of the peptides and no sophisticated pathogen recognition mechanism is involved. Indeed, many factors, including various bacteria, bacterial products such as LPS, and pro-inflammatory cytokines, can induce cationic peptides. The secretion and induction patterns of cationic peptides are described below.

Cationic antimicrobial peptides are found almost invariably at the interface between the host and environmental insults. Mammalian mechanisms of nonspecific host resistance include physiological barriers at the portal of entry (skin, mucosa), and relatively unspecific innate defenses (e.g. phagocytosis by polymorphonuclear leukocytes and macrophages, reticuloendothelial system, biochemical tissue constituents, and
inflammatory response including fever). The mucus layer, rich in glycoproteins, and proteinase-inhibitors, also contains cationic peptides which can be either constitutively expressed or induced upon infection. Among the most prominent mammalian mucosal peptides are the defensins (reviewed in Kaiser and Diamond, 2000).

When originally reported, these peptides were described as having a conserved cysteine motif and found to be constitutively expressed in human neutrophils (HNP-1,4) and gastrointestinal mucosa (HD-5,6). Those defensins are now called α-defensins, in contrast to a second group of defensins, lacking the strict cysteine motif and called β-defensins. The properties of β-defensins and their pattern of expression among mammals are relatively conserved: human β-defensin-1 (HBD-1), sheep β-defensin-1 (SBD-1), mouse β-defensin-1 (mBD-1) and pig β-defensin-1 are mainly constitutively expressed, while HBD-2, mBD-2 and mBD-3 are induced in response to infection. HBD-1 and several other type 1 β-defensins are expressed in the urogenital tract and kidney, while HBD-2 and other type 2 β-defensins are mainly expressed in the lungs and trachea. In addition, an assortment of non-defensin peptides have been isolated from mucosal surfaces of various animals, including the winter flounder peptide pleurocidin, which will be described in detail later on. Finally, one must realize that any antimicrobial peptides present on mucosal surfaces are expressed in the context of other mucus components, as well as in the context of other antimicrobial peptides, and a cooperative action of the various mammalian antimicrobial defenses has been reported (Yan and Hancock, 2001).

Aside from mucosal surfaces, many cationic peptides have been isolated from phagocytic cells such as neutrophils and macrophages, and implicated in non-oxidative
killing of bacteria. Collectively, this evidence, coupled to the fact that many cationic peptide genes are under the control of NFκB-like transcription factors, known to modulate various immune responses, strongly implicates cationic antimicrobial peptides in host defenses.

The ultimate confirmation of the assertion that cationic peptides can protect hosts from infections comes from animal model experiments where peptides are added exogenously to disease-challenged animals. A number of reports describing such experiments in diverse peptide/animal or peptide/plant models have been published, with the mode of peptide delivery ranging from topical to "genetic" (i.e. production of organisms transgenic for the peptides). Exogenously delivered peptide nisin has been shown to protect mice against *Streptococcus pneumoniae* infections (Goldstein *et al.*, 1998), while peptides D4B and LL-37 have been shown to protect mice from *P.aeruginosa* infections (Gamelli *et al.*, 1998; Kirikae *et al.*, 1998). Indeed, the LL-37 gene, when delivered in an adenovirus vector, was successfully expressed in mice and afforded a moderate level of protection against endotoxin and *E.coli* infections (Bals *et al.*, 1999). Other peptides have also been used for making transgenic, disease-resistant plants and animals, with the most recent success being a potato plant resistant to fungal and bacterial pathogens (Osusky *et al.*, 2000).

In the context of fish, a cecropin-derived peptide, LSB-37, was shown to enhance the resistance of the channel catfish *Ictalurus punctatus* to enteric septicemia caused by *E.ictaluri* (Kelly *et al.*, 1993). This, as well as corporate reports of peptide efficacy, will be described later on.
Taking into consideration all three lines of evidence: the immunomodulatory activity of cationic peptides, their expression pattern, and the protection offered to host by exogenously added peptides, the evidence is overwhelming that peptide antimicrobials play a significant role in early host defences. However, the specific nature of peptide involvement, and in particular the interaction between peptide antimicrobials and other components of the host defense, including their possible cooperative action in killing bacteria, still remain to be described.

1.1.3 Mode of Action of Antimicrobial Peptides

Cationic peptides are taken up across the outer membrane of Gram-negative bacteria by a process termed self-promoted uptake (Hancock and Lehrer, 1998). In this process, the cationic peptides interact initially with divalent cation binding sites on surface lipopolysaccharide (LPS), displace these divalent cations (because they have $10^3$-$10^4$ fold higher affinity for these sites), and being bulkier than the divalent cations they displace, cause distortion of the outer membrane structure. It is through these distortions that the cationic peptides pass across the membrane (i.e. self promote their uptake).

The above mechanism has several consequences. First, antimicrobial cationic peptides can promote the uptake of other agents, e.g. antibiotics and thus show synergy with conventional antibiotics, especially against antibiotic resistant mutants. Synergy of magainin and a β-lactam, cefepime, has also been shown in an animal model. Antimicrobial peptides can also show synergy with conventional antibiotics against Gram positive bacteria (e.g. they can reverse vancomycin resistance in vancomycin resistant
enterococci, VRE), and show synergy with antifungals against fungi. However, the mechanisms of synergy in these latter cases is not known. Secondly, as a consequence of their ability to bind LPS, cationic peptides (and cationic proteins like bactericidal permeability increasing protein, BPI) neutralize LPS and can protect galactosamine-sensitized mice against lethal endotoxaemia (Gough et al., 1996). Antimicrobial peptides were also able to bind to lipoteichoic acid which is the major molecule from Gram positive bacteria that has been implicated in sepsis by this group of bacteria. Thus peptides seem to have considerable potential against sepsis syndromes which affect up to 500,000 patients per year in North America, provided that structural aspects responsible for LPS and lipoteichoic acid binding are explored further. In any case, once in the periplasmic space, peptides have the cytoplasmic membrane accessible to them and it is at this level that controversy arises.

While most researchers agree that cationic peptides insert at least into the outer leaflet of the cytoplasmic membrane (Fig. 1B), a number of complex and controversial structure-function theories attempt to describe and explain what happens at this point. Generally, the theories differ as to the nature and impact of peptide-membrane interactions at the cytoplasmic membrane level.

One of the earliest and still popular propositions is the barrel-stave model, in which clusters of amphipathic peptides (or toroidal mixtures of peptides and lipids) are proposed to form hydrophilic pores embedded in the hydrophobic core of the cell membrane (Perez-Paya et al., 1995). The channels thus formed are said to lead to cell leakage of cytoplasmic constituents and hence cell death. While there is little doubt that
selected peptides at specific concentrations conform to this model, pore formation does not always accompany antimicrobial activity of cationic peptides (Wu and Hancock, 1998).

To account for the lack of pore formation Shai developed the so-called carpet model (Shai, 1995). In this model it was proposed that loss of cell membrane integrity occurs upon the membrane being covered by a “carpet” of peptides resulting in collapse of structural integrity. This model is favoured by many, but inconsistent with reports of intracellular effects of cationic peptides.

It has also been proposed (Hancock and Chappie, 1999) that cationic peptides reversibly and randomly cluster prior to, or upon, entering cell membranes, and form transient variable-sized, water-containing aggregates, comprising peptide and lipids (aggregate channel model). This leads to either depolarization of the cytoplasmic membrane, or, with kinetics ranging from microseconds to seconds, the dissociation of peptide aggregates leads to translocation through the bacterial cytoplasmic membrane, to access internal targets of peptide action. The “leakiness” of peptide-treated bacterial cells would thus be a consequence of the membrane disturbance and of ions being carried by the interstitial water. An advantage of the aggregate model is its ability to account both for membrane “leakiness” and for intracellular peptide effects. Multimodal models, where both membrane and intracellular targets are involved have also been proposed (Xiong et al., 1999; Friedrich et al., 2000).

The above theories notwithstanding, the argument is still of the “chicken or the egg” nature in that we do not know whether intracellular effects, or membrane effects
occur first. The meaning of “first” is also dual: it can mean “at an earlier time” or “at a lower peptide concentration.” A convincing argument can be built that once the cytoplasmic membrane is affected, intracellular effects will follow. On the other hand, several antibiotics are known to affect intracellular targets specifically, and peptides such as PR-39 have been shown to exhibit measurable intracellular effects, but not channel formation (Boman et al., 1993). A systematic approach to studying both membrane and intracellular effects in a well-controlled system should provide answers to these questions.

1.2 Antimicrobial Peptides of Marine Origin

While cationic antimicrobial peptides have been isolated from most species, they are a particularly important defense mechanism in invertebrates and lower vertebrates, whose immune systems lack several of the features present in mammals. Indeed some of the first peptides ever isolated came from insects. Since this early work, the focus has tended to move to peptides of mammalian origin, as medical applications appear to drive much of today’s research.

From this perspective, progress in researching and reporting cationic antimicrobial peptides of fish origin has been slow. This is unfortunate given that fish possess a limited range of antibody-mediated and cell-mediated secondary immune responses, and appear to depend greatly on non-specific defenses, thus being potentially good sources of cationic peptides. To date, only a few notable peptides have been isolated, and several synthetic derivatives of potential importance in controlling infectious diseases in fish have been developed (natural peptides are summarized in Table 1).
Table I. Overview of antimicrobial cationic peptides of marine origin

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Natural sources</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peptides originating from fish</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pardaxins</td>
<td>Toxic secretions of sole</td>
<td>Shai, 1994</td>
</tr>
<tr>
<td>Pleurocidin</td>
<td>Skin mucus secretions of winter flounder</td>
<td>Cole et al., 1997</td>
</tr>
<tr>
<td>Misgurin</td>
<td>Loach</td>
<td>Park et al., 1997</td>
</tr>
<tr>
<td>Histone peptides</td>
<td>Injured catfish</td>
<td>Park et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Channel catfish</td>
<td>Robinette et al., 1998</td>
</tr>
<tr>
<td>Other antimicrobial peptides and proteins</td>
<td>Skin mucosa of carp</td>
<td>Lemaitre et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Mucus cocoon of queen parrotfish</td>
<td>Viedler et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Mucus of rainbow trout</td>
<td>Smith et al., 2000</td>
</tr>
<tr>
<td><strong>Sample peptides originating from other marine organisms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tachyplesins</td>
<td>Japanese horseshoe crab</td>
<td>Nakamura et al., 1988</td>
</tr>
<tr>
<td>Polyphemusins</td>
<td>American horseshoe crab</td>
<td>Miyata et al., 1989</td>
</tr>
<tr>
<td>Panaeidins</td>
<td>Tropical shrimp</td>
<td>Destomieux et al., 1997</td>
</tr>
<tr>
<td>MGDs, mytilins, and myticins</td>
<td>Mussels</td>
<td>Mitta et al., 2000</td>
</tr>
</tbody>
</table>
1.2.1 Antimicrobial Peptides Isolated From Fish

The earliest and best-studied group of fish antimicrobial peptides were the natural pardaxins and scores of their synthetic derivatives. Pardaxins were isolated from the toxic secretions of at least two separate species of sole (Lazarovici et al., 1986; Shai, 1994), and described as predator-repellant peptides (Shai, 1994). The structures and activities of natural pardaxins have been studied extensively. Their potential for forming amphiphilic α-helices, and their ability to permeate phospholipid membranes in a manner comparable to that of dermaseptins and magainins, resulted in the inclusion of pardaxins among the cationic antimicrobial peptides. This classification is however inaccurate as natural pardaxins have a net charge of only +1, compared to much higher charges carried by conventional cationic peptides. In addition, natural pardaxins are hemolytic and highly neurotoxic, and hence are not host-friendly antimicrobial peptides. However, several pardaxin-derived, highly cationic synthetic peptides have been shown to exhibit improved antimicrobial activities and reduced toxicities (Oren and Shai, 1996), and will be described later on.

Perhaps the most conventional cationic antimicrobial peptide from fish described to date, is the 25-amino acid pleurocidin (Fig.2), isolated from skin mucous secretions of winter flounder Pleuronectes americanus (Cole et al., 1997). Pleurocidin is highly cationic and shares homology with frog dermaseptin and medfly ceratotoxin (Fig. 2B). It is active against a wide spectrum of bacteria, including the fish pathogens Aeromonas salmonicida and Vibrio anguillarum, and it has recently been localized to the mucin granules of skin and intestinal goblet cells (Cole et al., 2000). The pleurocidin gene has
Figure 2. Sequence homology and predicted structure of pleurocidin. A computer-generated probable α-helical structure of pleurocidin (prepared using SGI Insight II), with hydrophilic residues depicted in blue and hydrophobic residues depicted in red is shown in panel A. The sequence of pleurocidin (P) is homologous to that of insect ceratotoxin B (C) and frog dermaseptin (D), as shown in panel B.
been described (Cole et al., 2000) and its upstream region shows similarity to that found in mammalian host defense genes. Specifically, it includes consensus binding sequences identical to the NF-IL6 and alpha and gamma interferon response elements. In addition, the genes for several pleurocidin-like peptides have recently been cloned from the winter flounder (Douglas et al., 2001). Pleurocidin and its derivatives are therefore of great interest due to their potential to control bacterial disease in fish.

Another cationic antimicrobial peptide isolated from fish is the mudfish Misgurnus anguillicaudatus peptide misgurin (Park et al., 1997). This highly charged (+7) peptide was originally reported to be active against a range of gram-negative and gram-positive bacteria, as well as fungi. There have not been any further reports regarding the activity or expression of this peptide.

The discoverers of misgurin have also reported isolating a potent antimicrobial peptide from the mucosal secretions of injured catfish Parasilurus asotus (Park et al., 1998). This 19-amino acid peptide, parasin I, is highly basic, and 18 of its residues are identical to the N-terminal region of buforin I, an histone-H2A-derived peptide from toads isolated by the same group (Kim et al., 1996). While this discovery may seem unusual, histone proteins are well conserved across species and have since been implicated in antimicrobial activity in channel catfish Ictalurus punctatus (Robinette et al., 1998).

Several other reports related to cationic antimicrobial peptides merit mentioning in this section. Two novel antimicrobial proteins (27 kDa and 31 kDa) were isolated from the skin mucosa of carp Cyprinus carpio (Lemaitre et al., 1996) and a 21 kDa
antibacterial protein was isolated from the mucus cocoon of the queen parrotfish (Viedeler et al., 1999). While these are not cationic antimicrobial peptides, they reinforce the concept that a variety of innate antimicrobials are present at fish mucosal surfaces. This idea is further supported by a recent report of an unidentified cationic antimicrobial peptide being isolated alongside lysozyme and lysozyme-like proteins from the mucus of unstimulated rainbow trout *Oncorhynchus mykiss* (Smith et al., 2000) and other reports (Ebran et al., 1999).

### 1.2.2 Antimicrobial Peptides From Other Marine Organisms

The cationic antimicrobial peptides of other co-habitants of the aquatic environment, should also be considered when searching for sources of cationic peptides with potential for controlling disease in fish. However, such a large number of antimicrobial peptides are known that a comprehensive review of all falls beyond the scope of this introduction. The following provides a few prominent examples of cationic peptides from the aquatic milieu.

Both the Japanese (*Tachypleus tridentatus*) and the American (*Limulus polyphemus*) species of horseshoe crab, the oldest marine arthropod, have provided researchers with potent cationic antimicrobial peptides: tachypleins (Nakamura et al., 1988) and polyphemusins (Miyata et al., 1989), respectively. In addition, a large 6.5 kDa antibacterial peptide has been isolated from the hemocytes of the shore crab *Carcinus maenas* (Schnapp et al., 1996), and shown to have similarity to bactenecin-7. Tachypleins and polyphemusins have been extensively studied since they were discovered.
Motivated by infectious disease problems associated with panaeid shrimp aquaculture, researchers isolated three new antimicrobial peptides from the tropical shrimp *Panaeus vannamei* (Destoumieux et al., 1997, 1999, 2000). These large peptides (5.5 to 6.6 kDa) are proline-rich in their N-termini and have three disulphide bonds near their C-termini. The genes for penaeidins have been cloned and their C-termini were shown to be amidated. These peptides were further described by their discoverers as active against Gram-positive bacteria and fungi, but not against Gram-negative bacteria, and localized to shrimp granulocytes, where they are released upon microbial challenge (Destoumieux et al., 2000). While penaeidins are still rather obscure, they are a good example of the many individual efforts, currently under way, aimed at identifying new marine antimicrobials.

Another example of such efforts is the isolation and subsequent characterization of three types of cysteine-rich, cationic antimicrobial peptides from mussel hemocytes: *Mytilus galloprovincialis* defensins (MGDs), mytilins and myticins (Hubert et al., 1996, 1997; Mitta et al., 1999, 2000; Yang et al., 2000). Also, histidine-rich, amidated, α-helical antimicrobial peptides, clavanins, have been isolated from the solitary tunicate *Stryela clava* (Lee et al., 1997), as tunicate hemocytes are thought to be good models for studying the evolution of leukocyte-mediated host defenses. In conclusion, it appears that virtually all groups of marine organisms are potential sources of antimicrobial peptides for use in aquaculture, and for other applications.
1.2.3 Role of Cationic Peptides in Fish Immunity

Secondary, pathogen-specific immune responses in teleosts are confined to immunologically permissible temperatures and limited by fish size or age restrictions (Bly and Clem, 1991, 1992; Tatner, 1996). In addition, the fish secondary immune response is relatively brief. This drives fish to rely more heavily on their non-specific innate defenses than mammals.

As reviewed earlier, cationic antimicrobial peptides have already been isolated from fish mucosal secretions (Cole et al., 1997), which constitute the interface between environmental insults and the organism. Also, the context of the gene encoding flounder pleurocidin further indicates that the expression of fish antimicrobial peptides can be induced in response to immunogenic stimuli. Based on experience with other species, fish antimicrobial peptides are likely to be expressed by mucosal lining cells, macrophages and granulocytes. In conjunction with known antimicrobials, antimicrobial peptides offer protection against a wide spectrum of pathogens.

In addition, it is well known that fish can be exposed to relatively high doses of bacterial endotoxin (as much as 200 mg/kg of body weight), without exhibiting any clinical symptoms (Wedemeyer and Ross, 1960; Dalmo et al., 1997). While it is possible that this lack of response may be due to the fact that fish cellular responses are less manifest than those observed in mammals, another explanation is that fish possess highly efficient LPS detoxification and neutralization mechanisms. Since cationic antimicrobial peptides can bind lipopolysaccharide and thus inhibit LPS-mediated effects, they may account for the low sensitivity of fish to endotoxin.
As host-directed and pathogen-directed activities of cationic peptides are better described, we can expect that the extent of peptide involvement in fish immunity will also be better understood.

1.3 Applications for Peptides in Aquaculture

Cationic antimicrobial peptides are almost invariably active against wide spectra of bacteria. After accounting for factors such as media salinity and assay incubation temperatures, it can be therefore assumed that a peptide active against Gram-negative non-fish pathogens will also be active against fish bacteria. The main thrust in research to date has however been to test and engineer peptides of various origins for use against mammalian pathogens. It is only thanks to the rapid development of transgenic technologies, that peptides are now being tested for use in agriculture and aquaculture, as potential candidates for transgenically expressed disease control agents (Broglie et al., 1991, Osusky et al., 2000).

Several past and present attempts to optimize cationic peptides of marine and non-marine origin for use in aquaculture will be reviewed, and the logistic, environmental and financial issues involved in developing cationic peptide technologies will be identified in this section.
1.3.1 Therapeutic Potential and Mode of Delivery

In 1990, Kelly et al. (1990) described the activities of two synthetic cecropin-derived peptides against eight fish bacterial pathogens, including *V.anguillarum*, *A.salmonicida*, *A.hydrophila*, *Edwardsiella ictaluri*, and *Yersinia ruckeri*. The peptides exhibited activity against all fish bacteria tested. One of these cecropin-derived peptides, LSB-37, was later shown to enhance the resistance of the channel catfish *Ictalurus punctatus* to enteric septicemia caused by *E.ictaluri* (Kelly et al., 1993). The peptide in this study was delivered to live fish, together with a bacterial challenge, through injection and through an implanted osmotic pump. Only the latter administration method, which is considered to approximate the constant production of peptide from an active gene, was successful.

Significant sections of recent conferences on applied biotechnology (Pacific Rim Biotechnology Conference, Vancouver, 2000) and on Molecular Aspects of Fish Genomes and Development (Singapore, 2001) were devoted to transgenic technology in fish. Among the reports presented were the attempts, currently under way, to use cationic antimicrobial peptide genes to protect cultured hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) from *Streptococcus iniae* infections (Burns et al., 2001), as well as attempts to use cecropin B transgenes to protect fish from various bacterial infections (Chen, 2001).

While transgenic technology provides perhaps the best potential for developing cationic antimicrobial peptides into tools for aquaculture, it is not the only avenue. Provision of cationic peptide antibiotics in feed, or through baths or injections, should
also be considered, although at this time the cost of producing the required quantities of cationic peptides is prohibitive. As recombinant methods of producing cationic peptides develop, however, the cost may be comparable or lower than the cost of using conventional antibiotics. At this time however the problems associated with developing cationic peptide antibiotics are perhaps best illustrated by reviewing the development of peptide antimicrobial drugs for human use.

Only a few such peptides have entered clinical trials, with mixed success (mbiotech.com, intrabiotics.com, xoma.com, genaera.com). The most prominent failure to date was pexigan acetate developed by Magainin Inc. (now Genaera Corp.). Based on inadequate phase III trial design the Food and Drug Administration rejected the company’s New Drug Application (corporate press release of March 4, 1999) despite the fact that pexigan acetate was comparable to oral ofloxacin in treating diabetic foot ulcer infections (corporate press release of November 13, 1998).

On the brighter side, Micrologix Biotech Inc. has introduced 3 separate antimicrobial peptides related to the bovine neutrophil peptide indolicidin into clinical trials. The most advanced of these is MBI-226 which is in phase III clinical trials for prevention of catheter-related bloodstream infections. Preclinical studies demonstrated that MBI-226 was effective in animal models in reducing skin colonization by a variety of bacteria known to cause catheter-related infections, and also demonstrated good antifungal activity against *Candida albicans* in guinea pig skin. A randomized, double-blind phase I trial in 18 healthy volunteers demonstrated that MBI-226 eliminated 99.9% of common skin bacteria for prolonged periods (corporate press release of April 27,
Furthermore, it completely prevented short-term central venous catheter (CVC) colonization, while 5 out of 6 catheters in control individuals became colonized. Because of the seriousness of CVC infections in hospitalized patients, Micrologix has received fast track status from the FDA (corporate press release of September 7, 1999).

As illustrated above, despite the potential for success, the complexity involved in bringing cationic peptide antibiotics to the clinic is great, and transgenic technologies are, for obvious reasons, out of the question. This, however, is not the case in veterinary medicine and in agriculture, where a greater freedom to use unorthodox treatment approaches exists. And indeed, a reduction in the use of conventional antibiotics in those areas could lead to a general reduction in the levels of multiply resistant pathogens present in the environment.

While the ultimate applications and modes of delivery must certainly be considered, the bulk of fish cationic peptide research to date, as for all cationic peptides, is centered around understanding the rationale behind peptide activity on bacteria and the host, and developing rational approaches to designing new and better peptides. Most of the existing concerns regarding cationic peptide antibiotics can be addressed by manipulating the extensive pool of peptide structural motifs and activities.

1.3.2 Rational Engineering of Fish Peptides

While the mode of action of cationic peptides is not well understood, it is generally agreed that peptides need to interact with cell membranes as part of their action against microbes. This has been the assumption behind many of the rational
modifications to the existing peptide structures. Also, while microbicidal peptides have been reported to successfully kill Gram-negative and Gram-positive bacteria, fungi, enveloped viruses and even cancer cells in vitro, these activities can come at the cost of toxicity to healthy host cells. In such cases, rational modifications of existing peptides and customized delivery methods can reduce peptide toxicity (Moestrup et al., 1995; Ahmad et al., 1995) as well as enhance the desired activities (Dykes et al., 1998), and increase peptide stability (Rollema et al., 1995).

In addition, a wide variety of studies have been performed looking at structure/activity relationships (Hancock et al., 1995). These studies have generally indicated that the following properties can be important: overall charge, amphipathicity and formation of a hydrophobic face when folded into the final membrane-inserted conformation. Although peptide sequences vary greatly in nature, for any given peptide the permitted substitutions for any given amino acid in the peptide can be quite moderate.

Primary sequence modifications of natural peptides are commonly employed to increase the overall charge or amphipathicity of the peptides, improve their predicted folding patterns, or facilitate production. Some of the most successful alterations include amidation of the C-terminus, and amino acid replacements, insertions or deletions (Zhang et al., 1999; Shafer et al., 1996). While the rational approach has shown some success, one very effective peptide, CEME, was produced by empirically combining the N-terminus of cecropin and the C-terminus of melittin (Wade et al., 1992). In addition to rationally exploiting the structural patterns among existing cationic peptides, great potential thus exists for the employment of techniques such as random combinatorial
peptide libraries (Houghten et al., 1991, 1993; Goodson et al., 1999; Oh et al., 1999) or mutagenesis of DNA sequences encoding such peptides.

As previously described, the sole peptide pardaxin was the first pore-forming peptide isolated from fish. Although structurally close to melittin, with a proline hinge separating two helices, pardaxin carries a net charge of +1 compared to that of +6 for melittin. In an attempt to derive cationic antimicrobial peptides with antimicrobial activities, but without toxicities, from pardaxin, several variant peptides were constructed (Thennarasu and Nagaraj, 1995, 1996, 1997; Oren and Shai, 1996). In one case, three 18-amino acid constructs based on the N-terminal sequence of pardaxin were made, exhibiting different antimicrobial and hemolytic activities based on whether the proline hinge or lysine were present (Thennarasu and Nagaraj, 1996). In another case (Oren and Shai, 1996) eight separate pardaxin analogues were constructed and tested. An N-terminal fragment with additional positive charges exhibited good antimicrobial activities and was not hemolytic. In addition, studies aimed at elucidating the mode of action of pardaxin analogues were conducted (Oren and Shai, 1996) and the authors of these studies concluded that pardaxin inhibited bacterial growth by totally lysing the bacterial wall. Several other studies describing the activities of pardaxin have also been conducted, with particular focus on its interaction with lipid membranes (Rapaport et al., 1991, 1992, 1994, 1996). While these studies did not concentrate on testing of the peptides against fish pathogens, the results can be used as an indication that the engineering of natural fish peptides can produce better antimicrobials.
Significantly more work has been done on engineering other marine antimicrobial peptides, such as polyphemusins and tachyplesins, and understanding their modes of action, as they are good candidates for disease control agents in mammals (Iwanaga et al., 1994; Zhang et al., 2000). Better understanding of the structure/function relationships and mode of action of cationic antimicrobial peptides in general will eventually allow us to construct peptides with better antimicrobial activities against fish pathogens.

1.3.3 Environmental Impact of Developing Peptide Antimicrobials

One of the environmental impacts of aquaculture is that significant quantities of wild stocks are used in feeding cultured fish (Naylor et al., 2000). This is why reducing mortalities from infectious diseases in cultured species would improve not only the financial balance sheet of a fish farmer, but would have a downstream positive impact on the wild stocks, in that less of them would be required to produce a given amount of marketable cultured fish. The need to control infectious diseases in aquaculture should therefore be evident also from an environmentalist's point of view. The question remains whether cationic peptide technologies would be more or less environmentally sustainable than currently used antibiotics.

It is now known that pathogenic bacteria are becoming resistant to antibiotics. To delay this phenomenon, several measures have been put in place including the establishment of maximum residue levels in fish destined for consumption, and the requirement for elaborate approval procedures as well as guidelines for use of new antibiotics. This reflects the concern among the scientific and health communities that if we continue to overuse antibiotics we are facing a pre-antibiotic era all over again. This
is why we need to develop new agents, with lower potential for producing resistant bacteria. Given that cationic antimicrobial peptides are derived from nature, have wide spectra of activity, and do not induce resistance as readily as classical antibiotics, they should be even less objectionable to the environment. There is also a substantial concern that the intensive use of antibiotics in aquaculture leads to antibiotic residues in fish that can potentially induce resistance in people that consume such fish. The use of natural peptides that can be processed in the body by proteases might be considered a substantial advantage over conventional antibiotics in this regard.

The last comment concerns the use of peptide transgenes. Global scrutiny is evident, but will likely not stop the progress in this field. It will remain the responsibility of the scientist to ensure that transgenic species, with better survival characteristics, do not escape to natural habitats. This concern can and should be addressed at several levels: starting from confining transgenic animals to in-land systems, to producing triploid species and preventing transgenic animals from reproducing. Once the appropriate precautions are taken however, the transgenic technology carries an immense potential and lesser environmental impact than traditional antibiotics.

1.4 Rationale and Aims of this Study

Virtually every branch of medicine and veterinary medicine has contributed to the development of “superbugs” through indiscriminate use of antibiotics over the past decades. In the face of the impending crisis, intense research efforts are directed at finding alternative ways of controlling infections in human medicine and cationic antimicrobial peptides are among prime candidates for new drugs. Wide-ranging
introduction of cationic peptide drugs into the clinic is however still a relatively distant future due to prolonged drug approval procedures and because we lack cost-effective production technologies.

One way to extend the “useful lifespan” of existing antibiotics in human medicine is to slow down the development and transfer of resistant bacteria by discontinuing the use of antibiotics in veterinary medicine and intensifying research efforts to find replacements in this milieu. This approach holds particular promise in the context of cationic peptides and aquaculture since fish, even when vaccinated, rely heavily on their innate defenses and cationic peptides have been shown to be a part of natural defenses in many species of plants and animals. In addition, transgenic technologies have already been designed for use in aquaculture, and peptide genes could be directly introduced, or amplified, in fish, thus circumventing the need for peptide production. There is therefore a great potential advantage, from the commercial, veterinary and medical point of view, in developing cationic antimicrobial peptides for use in aquaculture.

The key question at this stage, and a major objective of this research, is to isolate or construct custom peptides for use in aquaculture. As discussed earlier the “right” peptide would be one that is effective against fish bacteria and one that cooperates well with host innate defenses. Two approaches to develop antimicrobials for use in salmon aquaculture are used in this research. One is to isolate natural antimicrobials from the tissues of diseased coho salmon, and the other to use existing fish antimicrobial peptides, especially pleurocidin, as templates to develop peptides effective against the salmon pathogens *A. salmonicida* and *V. anguillarum*.
As discussed earlier, rational development of effective antimicrobials requires an insight into their mode of action. The particular problem for cationic peptides is in reconciling their membrane-directed effects with their intracellular activities. The remainder of this research is thus directed at understanding these mechanisms in the context of peptide action on bacteria. Specifically, a large portion of this research is devoted to studying the effects of synthetic pleurocidin constructs at low concentrations, less toxic to the host.
2 MATERIALS AND METHODS

2.1 Bacterial Strains and Growth Conditions

All strains used in this study are listed in Table II. Most non-fish strains were grown at 37°C in Mueller-Hinton Broth (Difco Laboratories, Detroit), while the fish bacteria were maintained at 16°C in Tryptic Soy Broth (Difco, 5g/l NaCl). All strains were stored at -70°C until they were thawed for use and sub-cultured daily. The following strains, \textit{P.aeruginosa} K799 (parent of Z61), \textit{P.aeruginosa} Z61 (antibiotic supersusceptible), \textit{Salmonella typhimurium} 14028s (parent of MS7953s), \textit{Salmonella typhimurium} MS7953s (defensin supersusceptible), and \textit{Staphylococcus epidermidis} (human clinical isolate obtained from Dr. A. Chow, University of British Columbia) are from our laboratory stock collection.

\textit{Escherichia coli} strain CGSC 4908 (his-67, thyA43, pyr-37), auxotrophic for thymidine, uridine, and L-histidine (Cohen et al., 1963) was kindly supplied, free of charge, by the \textit{E.coli} Genetic Stock Centre (Yale University, New Haven, CT). Defined M9 minimum medium, supplemented with 5 mg/L thymidine, 10 mg/L uridine and 20 mg/L L-histidine (Sigma Chemical Co., St. Louis, MO), was used to grow \textit{E.coli} CGSC 4908 unless otherwise specified.

Field isolates of the salmonid pathogens \textit{Aeromonas salmonicida} and \textit{Vibrio anguillarum} were identified by typing and kindly provided by Dr. Julian Thornton, Microtek International Inc., Victoria, British Columbia, Canada.
Table II. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference/Source</th>
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<td>DC2</td>
<td>antibiotic supersusceptible mutant</td>
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<td>CGSC 4908</td>
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<td>Cohen <em>et al.</em>, 1963</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
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<td>K799 (H187)</td>
<td>wildtype isolate; parent for Z61</td>
<td>Angus <em>et al.</em>, 1982</td>
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<tr>
<td>Z61 (H188)</td>
<td>antibiotic supersusceptible mutant</td>
<td>Angus <em>et al.</em>, 1982</td>
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<td><strong>S. typhimurium</strong></td>
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<tr>
<td>C587 (14028s)</td>
<td>parent of C610</td>
<td>Fields <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>C610 (MS7953s)</td>
<td>phoP / phoQ mutant; defensin sensitive</td>
<td>Fields <em>et al.</em>, 1989</td>
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<tr>
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<tr>
<td>C621</td>
<td>clinical isolate</td>
<td>from Dr. A. Chow</td>
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<td><strong>A. salmonicida</strong></td>
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<tr>
<td>ASAL</td>
<td>field isolate</td>
<td>from Dr. J. Thornton</td>
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<td><strong>V. anguillarum</strong></td>
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<tr>
<td>VANG</td>
<td>field isolate</td>
<td>from Dr. J. Thornton</td>
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</table>
2.2 Design and Production of Peptides

All antimicrobial peptides used in this study were synthesized by N-(9-fluorenyl) methoxy carbonyl (Fmoc) chemistry at the Nucleic Acid Protein Service (NAPS) unit at the University of British Columbia. Peptide sequences are shown in Table VI. Peptide purity was confirmed by HPLC and mass spectrometry analysis in each case.

A series of peptides were designed based on the sequence of winter flounder pleurocidin and its two closest homologues: frog dermaseptin and insect ceratotoxin, as well as based on the sequence of the loach misgurin. A C-terminally amidated version of pleurocidin (P-CN) as well as a version with an additional lysine at the N-terminus were designed to determine the effects of adding additional charges at either terminus. Also, versions of pleurocidin with its N-terminus substituted with the N-terminus of dermaseptin (P-DER) or ceratotoxin (P-CER), or with its C-terminus substituted with that of misgurin (P-M) were constructed. In addition, versions of pleurocidin with both termini substituted were designed (CER-M, DER-M). The previously described P-DER was also constructed with a six histidine tag on its C-terminus, along with two peptides based on the sequence of the antimicrobial factor isolated from coho salmon (HSDF-1 and HSDF-2).

Further characteristics of the peptides are described later on and shown in Table VI.
2.3 Animals

Coho salmon (*Oncorhynchus kisutch*) were obtained from Chehalis River of British Columbia and maintained at 10°C in city water dechlorinated with sodium thiosulfate (free-flowing at 1 L/min in the winter and 2 L/min in the summer), unless otherwise stated. The fish were kept in 70 L tank and fed *ad libitum* by hand twice a day with commercial diet.

2.4 Determination of Inhibitory Concentrations

2.4.1 Minimum Inhibitory Concentrations

The antimicrobial activities of selected antimicrobial peptides were determined as minimal inhibitory concentrations (MICs) using the microtitre broth dilution method of Amsterdam (Amsterdam, 1996), as modified by Wu and Hancock (1999). Serial dilutions of the peptide were made in 0.2% BSA/0.01% acetic acid solution in 96-well polystyrene (Costar, Corning Incorporated, Corning, New York) microtiter plates. Bovine serum albumin (BSA) fraction V lyophilizate was purchased from Boehringer Mannheim (Germany). Bacteria were grown overnight to mid-logarithmic phase as described above, and diluted to give a final inoculum size of 10^6 cfu/ml. A suspension of bacteria was added to each well of a 96 well plate and incubated overnight at the appropriate temperature. In the case of *E.coli* CGSC 4908, supplemented M9 minimal medium was used. Inhibition was defined as growth lesser or equal to one-half of the growth observed in control wells, where no peptide was added.
2.4.2 Fractional Inhibitory Concentrations

The checkerboard microtitre assay was used to determine peptide-peptide synergy (Amsterdam, 1996). Serial dilutions of each peptide were made in TSB medium in 96-well polypropylene microtitre plates (Costar, Cambridge, MA). Each well was inoculated with 10 µl of approximately $10^6$ CFU/ml of the test organism. Samples of the bacterial inoculum were plated to ensure they were within the proper range. The FIC was determined after 48 hr incubation of the plates at $16^\circ$C. Synergy was defined as an FIC index of less than 0.5, calculated according to the following formula: $FIC\ index = (A) / (MIC\ A) + (B) / (MIC\ B)$, where (A) was the concentration of drug A in a well that represented the lowest inhibitory concentration in its row; (MIC A) was the MIC of drug A alone; (B) was the concentration of drug B in a well that represented the lowest inhibitory concentration in its row; and (MIC B) was the MIC of drug B alone. Since many MICs in the assay were higher than the maximal level of compounds tested, they were assumed to equal the highest concentration tested.

2.5 Purification and Identification of Peptides from Fish

2.5.1 Lysozyme Activity Assessment

One group of 6 coho salmon weighing 120-150g was challenged with LPS isolated from \emph{P.aeruginosa}, \emph{S.minnesota}, and \emph{E.coli}. Mixed LPS in saline was injected intra-peritoneally to a level of 7.5 mg/kg of fish. Two other groups of 6 coho salmon were injected with saline alone, and left untreated, respectively. At different time points post injection, mucus and blood were obtained from each fish, and the blood was
separated into cells and serum. The level of lysozyme activity in the serum was evaluated by the modified lysoplate assay of Osserman and Lawlor (1966), which utilizes *Micrococcus lysodeikticus* as the test agent. Mucus and serum were also diluted serially and the dilutions were tested for inhibitory activity against defensin susceptible *S. typhimurium* as described earlier.

2.5.2 Peptide Extraction and Purification.

Three groups of 20 coho salmon weighing 120-150g were challenged. The challenges consisted of: LPS isolated from *P.aeruginosa, S.minnesota, and E.coli*; clinical isolates of *V.anguillarum*; and handling stress. Mixed LPS in saline at the concentration of 7.5 mg/kg of fish, was injected intra-peritoneally, while *V.anguillarum* was introduced by immersing the fish for 15 min in a bath of $10^9$ bacteria per litre of Cortland saline. Handling stress consisted of holding the fish out of water for less than 5 min in a dip net. Following the challenge, fish were released into tanks and allowed to mount an immune response. When an adequate response was developed (1 day post challenge), as determined by measuring the levels of lysozyme in optimization studies, fish were sacrificed. The blood was obtained from each fish and separated into cells and serum. The head kidney, spleen, mucus, and gills were also harvested. All samples were pooled based on the sample-type and challenge-type. For example all spleens from the 20 LPS-challenged fish were pooled as one sample, while all spleens from the 20 *V.anguillarum* – challenged fish were pooled as a separate sample. The resulting 18 cross-pooled samples were then extracted by boiling in 10% acetic acid. Solid phase extraction with a Sep-Pak C18 cartridge (Waters) was performed using 60% acetonitrile...
in 0.1% trifluoroacetic acid (TFA) to elute the cartridge. The samples were lyophilized, resuspended in water, and tested for antimicrobial activity against a defensin-supersusceptible _S.typhimurium_ as described earlier. Samples exhibiting antimicrobial activities were applied to a Biogel P-30 size exclusion chromatography column (Bio-Rad), eluted with 50mM ammonium formate, lyophilized, resuspended in water, and re-tested for antimicrobial activity. Active samples were applied to a reverse-phase fast performance liquid chromatography (RP-FPLC) column (PepRPC, Pharmacia Biotech) in 0.1% TFA, eluted with a 0-60% gradient of acetonitrile in 0.1% TFA, and fractions were collected, monitored at 220nm, and tested for antimicrobial activity. The total protein content of the samples was assessed using the modified Lowry method (Markwell _et al._, 1978). The purity of active samples was assessed using acid-urea polyacrylamide gel electrophoresis (AU-PAGE) (Smith, 1994).

2.5.3 Peptide Identification

Following the purity assessment, active samples were subjected to amino acid analysis and Edman microsequencing at the Protein Microsequencing Laboratory at the University of Victoria, Victoria, BC. In addition MALDI mass spectroscopy was performed by the Mass Spectroscopy Facility, Department of Chemistry, UBC, Vancouver, BC.

2.5.4 Histone Protein Expression

Seven coho salmon were challenged with _V.anguillarum_ as described above, along with six control fish. Blood samples obtained 72 hr post incubation were separated
into cells and serum, and the latter was tested for antimicrobial activity. Acid extracts of the serum from each fish were also subjected to AU-PAGE (Smith, 1994) along with the previously purified histone peptide.

2.6 Assays to Assess Peptide Effects on Membranes

2.6.1 LPS Binding Assay

The relative binding affinity of each peptide for LPS was determined using the dansyl polymyxin B displacement assay of Moore et al. (1986), using LPS isolated from *E. coli* UB1005. In brief, bacterial LPS was allowed to bind to dansylated polymyxin B and the resulting level of fluorescence was measured. As the peptides were added, the drop in fluorescence, reflecting the displacement of dansyl polymyxin B by the peptides, was monitored. Maximal displacement of LPS was expressed as a percentage where 100% displacement of dansyl polymyxin B was taken as that observed with non-dansylated polymyxin B.

2.6.2 Outer Membrane Permeabilization Assay

The outer membrane permeabilization activity of the peptide variants was determined by the 1-N-phenyl napthylamine (NPN) uptake assay of Loh et al. (1984), using intact cells of *E. coli* UB1005. Briefly, the probe fluoresces only in the hydrophobic environment of the membrane. Hence, an increase in fluorescence upon addition of peptide is reflective of probe entering the membrane and thus of the ability of the peptide to facilitate this by permeabilizing the membrane.
2.6.3 Cytoplasmic Membrane Permeabilization Assays

The depolarization of the cytoplasmic membrane by the peptides was determined using the membrane potential sensitive cyanine dye diSC₃₅ by a modification of the method of Wu and Hancock (1999), as described in Friedrich et al. (2000). Dipropylthiacarbocyanine (diSC₃₅) was purchased from Molecular Probes (Eugene, Oregon).

Briefly, in HSDF experiments, the cytoplasmic membrane depolarization activity of the peptides was determined using *V.anguillarum* as the test organism. Bacterial cells in the mid-logarithmic phase of growth (OD₆₀₀ of 0.5) were centrifuged, washed in 5 mM HEPES, pH7.8, and resuspended in the same buffer to an OD₆₀₀ of 0.05. A stock solution of diSC₃₅ was added to a final concentration of 0.4 uM and quenching was allowed to occur at room temperature for 45 min. Then KCl was added to the cell suspension to a final concentration of 100 mM and allowed to equilibrate for 15 min. A 2-ml cell suspension was placed in a 1 cm cuvette and the desired concentration of tested peptide was added. Changes in fluorescence due to the disruption of the membrane potential in the cytoplasmic membrane were continuously recorded using a Perkin-Elmer model 650-10S spectrofluorimeter at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

In the remaining experiments *E.coli* CGSC 4908 was used as the test organism. Briefly, an equilibrated 1.5-ml suspension of bacterial cells in HEPES (5mM), diSC₃₅ (0.4uM), and KCl (100mM) was placed in a 1 cm cuvette and the desired concentration of tested peptide was added. Changes in fluorescence due to the disruption of the
cytoplasmic membrane potential were again continuously recorded using a spectrofluorimeter at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

Cell viability was assessed by plating aliquots withdrawn from a mixture identical to that used in the permeabilization experiment and prepared with the same bacterial culture.

2.6.4 Langmuir Monolayer Assays

Lipid monolayers were formed by applying the appropriate lipids dissolved in hexane or chloroform onto water contained in a circular Teflon trough (d=4.5 cm, total volume of 11.5 ml). The lipid composition of the monolayers varied and is shown with every result. Monolayers were allowed to equilibrate until a stable surface pressure was obtained (<0.2 mN/m drift in surface pressure Δπ). A small port in the side of the trough enabled injection of reagents into the subphase without disruption of the monolayer. The subphase was gently mixed with a magnetic stir bar at 45 rpm. Surface pressure measurements were obtained by using the Whilhelmy plate method (Mayer et al., 1983). The plate was cleaned with methanol three times and thoroughly rinsed with double-distilled water prior to each surface pressure measurement. The experiments were performed at 23°C.

2.7 Peptide Effects on Macromolecular Synthesis

Overnight cultures of *E.coli* CGSC 4908 were diluted 10⁻³ in synthetic media and allowed to grow to exponential phase (optical density at 600 nm of 0.3). The cultures
were spun down, resuspended in warm synthetic M9 media, and 500 mL aliquots were incubated with 15 ml of either [methyl-\(^3\)H]-thymidine (25 Ci/mmol), [5-\(^3\)H]-uridine (26 Ci/mmol), or L-[2,5-\(^3\)H]-histidine (46 Ci/mmol, all purchased from Amesham Pharmacia Biotech UK Ltd.), and an excess of the remaining two supplements. After 5 min of incubation at 37\(^\circ\)C, the peptides were added at the specified concentrations. Samples of 50 ml were removed immediately before peptide addition (0 min) and 5, 10, 20 and 40 min after peptide addition, and added to 5 ml ice-cold 10\% trichloroacetic acid (TCA) (Fischer Scientific, Fair Lawn, NJ, USA) with excess unlabelled precursors in order to precipitate the macromolecules. After 40 minutes on ice and 15 minutes at 37\(^\circ\)C the samples were collected over vacuum on Whatman 47 mm GF/C glass microfibre filters (VWR Canlab, Mississauga, ON, Canada) and washed twice with 10 ml 10\% ice-cold TCA. The filters were dried, placed in 7 ml scintillation vials with ReadySafe liquid scintillation cocktail (Beckman, Fullerton, CA) and counted in a Beckman LS600IC scintillation counter.

At the same time points, 5 ml samples were removed from non-radioactive parallel cultures otherwise identical to those containing radioactive precursor, diluted in 1 ml of buffer and plated on to LB plates with added supplements in order to obtain a viable count.
2.8 Assays to Determine Peptide Translocation Across Membranes

2.8.1 Liposome Preparation

The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (PG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE), phosphatidylglycerol from egg yolk (ePG), cardiolipin (CL), and 1-palmitoyl-2-[6-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-caproyl]-L-α-phosphatidylcholine (C6-NBD-PC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). L-α-phosphatidy-DL-choline from egg-yolk (ePC) and calcein were purchased from Sigma (St. Louis, Missouri).

Symmetrically labeled unilamellar liposomes were made from a 1:1 mixture of PC and PG. The mixture contained 0.5 mol% C6-NBD-PC. The lipids were dissolved in chloroform and dried under a stream of nitrogen, followed by 2 hr of vacuum drying. The lipid film was rehydrated with TSE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5), and extruded 10 times through two stacked filters with a pore size of 100 nm. For asymmetrically labeled liposomes (inner leflet only), the symmetrically labeled unilamellar liposomes were mixed with 1 M sodium dithionite in 1 M Tris-HCl (pH 7.5) and incubated for 15 min at 23 °C, which resulted in chemical quenching of the NBD groups in the outer leaflet of the bilayers. The liposomes were immediately separated from dithionite by gel filtration using Bio-Gel A 1.5m (Bio-Rad, Hercules, CA, 1.5 x 10 cm) at 23°C.

For calcein-encapsulated unilamellar liposomes, a PC:PG (1:1) lipid film was rehydrated with 5 mM sodium HEPES, pH 7.5, containing 100 mM calcein. The
liposome suspension was extruded ten times through two stacked polycarbonate filters (100 nm pore size) and free calcein was removed by passing the liposome suspension through a Sephadex G-50 column (Pharmacia Biotech AB, Uppsala Sweden, 1.5 x 10 cm) at 23°C and eluting with a buffer containing 20 mM sodium HEPES, 150 mM NaCl, 1 mM sodium EDTA, pH 7.5. Calcein-free PC:PG (1:1) unilamellar liposomes made in the same elution buffer were added to adjust final liposome concentrations.

2.8.2 Peptide Translocation Experiments

Unilamellar liposomes (ePC/ ePG/ DNS-PE, 50/45/5) were made containing 200 mM of chymotrypsin solution in buffer containing 150 mM NaCl, 20 mM HEPES, pH 7.4 according to Kobayashi et al. (2000). Trypsin-chymotrypsin inhibitor (Sigma), at a final concentration of 200 mM, was added to the liposomes to inactivate any enzyme present outside the unilamellar liposomes. Excitation of tryptophan residues in P-DER and PDH at 280nm lead to fluorescence transfer to the dansyl group in DNS-PE leading to an emission recorded at 510nm. A decrease in fluorescence after peptide addition indicated digestion of the internalized peptide by the enzyme within the liposomes.
3 RESULTS

3.1 CHAPTER ONE: Purification and Activity of Antimicrobial Fractions from Coho Salmon.

3.1.1 Introduction

While macrophages and granulocytes remain central cellular components of innate immunity, fish lack bone marrow and lymph nodes. The thymus, kidney and spleen are the most important lymphomyeloid tissues (Dalmo et al., 1997). It has long been known that while mammalian species are relatively sensitive to endotoxic lipopolysaccharide (LPS), some fish, including coho salmon and rainbow trout, fail to produce any clinical signs even when given 200 mg kg\(^{-1}\) body weight of *E.coli* LPS (Berczi et al., 1966; Wedemeyer and Ross, 1960). This, taken together with reports of salmonids developing resistance to bacterial infections prior to producing detectable levels of antibodies, which cannot be explained solely by macrophage activation or CRP-mediated opsonization (Kodama et al., 1987, 1989), draws attention to the existence of other important elements of fish defenses, such as cationic peptides.

The experiments in this chapter are directed at assessing the antimicrobial activity in various tissues of disease-challenged coho salmon and at isolating and identifying any specific protein/peptide components which contribute to this activity. Preliminary characterization of the isolated peptides will also be described.
3.1.2 Disease Challenge Optimization

The naturally occurring variability in the size of the salmon used for disease challenges is shown in Figure 3. The fish, weighed at the time of challenge, varied in size from 15 g to over 40 g, with a mean of 30.2 g and standard deviation of 8.2 g. This large variability indicates that caution should be used when interpreting differences in antimicrobial activity between the various treatment groups. Firstly, the quantity of sample available for each group may have been different due to fish size differences. Secondly, it is possible that fish of different sizes were in different developmental stages and thus responding to the infection in distinct ways.

In order to find the best time post challenge for harvesting the various tissues, serum lysozyme activity was assessed using a standard *Micrococcus lysodeikticus* assay. A specific increase in lysozyme activity due to an LPS disease challenge was observed 48 hours post injection (Fig. 4). At that time the control saline injection caused only a moderate increase in lysozyme activity, presumably due to the stress of injection itself. At day 6 and day 9 there was no longer a significant distinction between the level of lysozyme activity induced due to the LPS challenge and the stress of saline injection alone. Since specific induction of lysozyme activity was used to indicate the best time for harvesting the various tissues, a time point of 48 hours was selected.

3.1.3 Co-induction of Antimicrobial and Lysozyme Activity

Antimicrobial activities of the serum and mucus of LPS-challenged coho salmon were at least 4-fold higher than those of the controls 48 hours post injection (Fig. 5A). A
Figure 3. Variability in the size of salmon used for antimicrobial isolation experiments. In all cases where disease challenge protocol required fish anaesthesia, the fish were weighed. Forty fish representative of the population used in the isolation and lysozyme activity induction experiments were thus weighed and the results are shown.
Figure 4. Induction of lysozyme activity following an intraperitoneal injection of mixed bacterial LPS. The lysozyme activity in 1 ml of serum is shown on the y-axis (lysozyme activity units), as estimated by a standard *M. lysodeikticus* lysozyme activity assay. Six fish were tested in each group, and group averages, as well as their standard deviations are shown. The group without injection was sampled only at day 0, while the remaining two groups were sampled only post injection in order to reduce stress to the animals. Specific induction of lysozyme activity was used as a marker for harvesting fish tissues.
Figure 5. Induction of lysozyme activity, antimicrobial activity, and H1 histone-derived peptide after a disease challenge of coho salmon. (A) Lysozyme activity in coho salmon serum, as well as antimicrobial activity of coho serum and mucus are shown. The numbers on the y-axis represent two separate units. For the lysozyme activity bar, the numbers represent activity units \( \times 10^6 \) in 1 ml of serum. For the mucus and serum antimicrobial activity bars, y-axis units are the highest dilutions of the sample capable of inhibiting the growth of the supersusceptible *S. typhimurium* C610. An average of six fish and the standard deviation bar are shown for each lysozyme bar. Samples for antimicrobial activity were pooled from all fish, hence no error bars are shown. (B) Acid urea PAGE gel (15\%) and Coomassie blue staining were used to analyze acid extracts of serum samples from individual disease-challenged and unchallenged fish. Four of the challenged samples had antimicrobial activity against *S. typhimurium* C610 and have been designated as “active”. A band which appeared only in active samples from challenged fish, and which corresponded to an independently purified and identified histone fragment peptide HSDF-1 (H), is shown.
concurrent increase in lysozyme activity was observed in the serum of challenged fish. While the increase in lysozyme activity may have contributed to the increase in antimicrobial activity, lysozyme alone is not normally active against Gram-negative bacteria. Acid-urea PAGE analysis of acid extracts from the serum of unchallenged and challenged salmon revealed a band which was detectable in the microbicidal serum of challenged salmon, but not in unchallenged or challenged but non-microbicidal samples (Fig. 5B). The position of the band on the gel corresponded to that of an antimicrobial factor HSDF-1 independently purified from the mucus and serum of coho salmon (purification described in sections 3.1.4 and 3.1.5). The identity of two of the strongest bands was further confirmed through N-terminal Edman microsequencing.

3.1.4 Extraction and Purification of Active Fractions

Following an initial screen of 18 separate acetic acid extracts (Table III), two extracts were fractionated and purified to homogeneity as active single peak samples eluted from an RP-FPLC column. Other samples lost their activity at various points of the purification protocol, as indicated in Table III. It is important to note that in any of the cases where the activity was lost, the loss may be an artifact of insufficient sample size as previously mentioned in section 3.1.2, or it may have resulted from peptide damage in the purification procedure.

In the case of the active serum and mucus samples, every protein-containing peak was collected and tested (P1-P9 in Fig. 6, and P1-P5 in Fig.7), as well as the samples immediately before and after each peak. When a single peak extended over two or more eluate samples, these eluates were pooled. After every use, the column was washed in
Table III. Screening of coho tissues for antimicrobial activity. Samples are identified as described in the text. LPS, VIB, and HAN (handling) represent the three disease challenges, while SPL, KID, GIL, MUC, SER, and BCL represent the various tissues. Relative antimicrobial activities, assessed as maximum inhibitory dilutions against antibiotic supersusceptible *S.typhimurium*, of the solid-phase eluates (SP), size exclusion column eluates (SEC); and reversed-phase column eluates (RP) are indicated, with the highest activity indicated by the three “+” signs. Aborted experiments (“-”) are also indicated.

<table>
<thead>
<tr>
<th>Samples</th>
<th>SP</th>
<th>SEC</th>
<th>RP</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS-SPL</td>
<td>+++</td>
<td>+</td>
<td>no activity</td>
<td>-</td>
</tr>
<tr>
<td>VIB-SPL</td>
<td>++</td>
<td>no activity</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HAN-SPL</td>
<td>+</td>
<td>no activity</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LPS-KID</td>
<td>no activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VIB-KID</td>
<td>no activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HAN-KID</td>
<td>+++</td>
<td>+</td>
<td>no activity</td>
<td>-</td>
</tr>
<tr>
<td>LPS-GIL</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>impure sample</td>
</tr>
<tr>
<td>VIB-GIL</td>
<td>++</td>
<td>+</td>
<td>no activity</td>
<td>-</td>
</tr>
<tr>
<td>HAN-GIL</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LPS-MUC</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>identified</td>
</tr>
<tr>
<td>VIB-MUC</td>
<td>+++</td>
<td>++</td>
<td>no activity</td>
<td>-</td>
</tr>
<tr>
<td>HAN-MUC</td>
<td>++</td>
<td>+</td>
<td>no activity</td>
<td>-</td>
</tr>
<tr>
<td>LPS-SER</td>
<td>+++</td>
<td>+</td>
<td>no activity</td>
<td>-</td>
</tr>
<tr>
<td>VIB-SER</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>identified</td>
</tr>
<tr>
<td>HAN-SER</td>
<td>no activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LPS-BCL</td>
<td>no activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VIB-BCL</td>
<td>no activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HAN-BCL</td>
<td>no activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 6. Purification of the active component from the serum of *Vibrio*-challenged *coho salmon*. Following the acid extraction step, C18 extraction and concentration step, and gel filtration, the active sample was applied to a RP-FPLC column in 0.1% TFA. The column was eluted with acetonitrile in 0.1% TFA. Protein content of the eluates, monitored at 220nm, is shown. Acetonitrile concentration gradient is represented by the straight line. Each peak (P1-P9) was collected and tested for antimicrobial activity. Peak P7 was the only peak with activity and it was re-applied to the column to ensure its homogeneity. Peak P7 was active against *S.typhimurium*, *V.anguillarum*, and *A.salmonicida*. 
Figure 7. Purification of the active component from the mucus of LPS-challenged coho salmon. Following the acid extraction step, C18 extraction and concentration step, and gel filtration, the active sample was applied to a RP-FPLC column in 0.1% TFA. The column was eluted with acetonitrile in 0.1% TFA. Protein content of the eluates, monitored at 220nm, is shown. Acetonitrile concentration gradient is represented by the straight line. Each peak (P1-P5) was collected and tested for antimicrobial activity. Peak P4 was the only peak with activity and it was re-applied to the column to ensue its homogeneity. Peak P4 was active against *S.typhimurium*, *V.anguillarum*, and *A.salmonicida*. 
100% acetonitrile in 0.1% TFA to ensure that no peptides were left on the column. Of all peaks present in the sample derived from the serum of *Vibrio*-stimulated fish (VIB-SER), only peak P7 showed antimicrobial activity after being lyophilized and re-suspended in water (Fig. 6). Similarly, of all peaks present in the sample derived from the mucus of LPS-stimulated fish (LPS-MUC), only peak P4 showed antimicrobial activity after being lyophilized and re-suspended in water (Fig. 7). Samples immediately before and after the active peaks had no activity. Peaks VIB-SER P7 and LPS-MUC P4 were subsequently re-applied to the C18 column to ensure their purity.

The respective MICs of the LPS-MUC and VIB-SER samples, expressed in μg of total protein per ml, are shown in Table IV.

3.1.5 Identification of Active Fractions

The amino acid composition of the LPS-MUC sample revealed a high proportion of lysine, alanine, and proline. Screening of the VIB-SER sample against the SWISSPROT database using a web-based Expert Protein Analysis System AACompIdent tool (ca.expasy.org/tools/aacomp/) resulted in several matches, all of which were histone proteins from various species, including trout (Table IV). The amino acid sequencing data further confirmed that the sequence of the antimicrobially active component was identical to that of trout H1 histone (Table IV, Fig. 8 and Fig. 9).

The lengths of the sequences obtained for the LPS-MUC and VIB-SER samples were respectively 26 amino acids and 8 amino acids. MALDI mass spectroscopic analysis of each sample revealed a number of histone fragments, as indicated in Figure 9,
Table IV. Properties of RP-FPLC eluted samples. The proteins obtained through amino acid composition matching are unequivocally identified by their SWISSPROT access codes. They represent H1 histone proteins from various species.

<table>
<thead>
<tr>
<th></th>
<th>LPS-MUC peak P4</th>
<th>VIB-SER peak P7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antimicrobial activity against</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. typhimurim C610</em></td>
<td>100-150 µg total protein / mL</td>
<td>25-50 µg total protein / mL</td>
</tr>
<tr>
<td><em>V. anguillarum</em></td>
<td>50-100 µg total protein / mL</td>
<td>50-100 µg total protein / mL</td>
</tr>
<tr>
<td><em>A. salmonicida</em></td>
<td>50-100 µg total protein / mL</td>
<td>25-50 µg total protein / mL</td>
</tr>
</tbody>
</table>

**Amino acid analysis results:**
- High content of K, A, and P.
- No significant AAA matches to proteins in database

**SWISSPROT entry codes for top ten matches to amino acid composition**
1. H12_MOUSE
2. H1_SALTR
3. H103_CHICK
4. H13_MOUSE
5. H1_ANAPL
6. H1D_HUMAN
7. H11L_CHICK
8. H1A_HUMAN
9. H1_ONCMY
10. H13_RABIT

**Amino acid sequence obtained:**
- XEVAPAPAAAAPA
- KAPKKAAAKPKK

**MALDI MS analysis:**
- A series of species in the 2,200 - 2,500 Da range, including a match to acetylated version of the sequence above (2524 Da), and a 20,715 Da species
- Three clusters of peaks: 2423-2553 Da, 10350-10379 Da, and 20720-20893 Da
Figure 8. Identification of active component. The 26 amino acid sequence obtained from amino acid sequencing of LPS-MUC peak P4 was screened against a SWISSPROT data base using a VISUAL FASTA program available through the internet (www2.ebi.ac.uk/fasta3/). This sequence is shown in red. Sequence matches are unequivocally identified by their FASTA access codes, with the best matches showing as yellow lines. Both H1_SALTR and H1_ONCMY represent trout H1 hitones sequences.
Figure 9. Identification of the active component. The 26-amino acid sequence of the FPLC peak derived from the LPS-MUC sample is underlined and shown to be identical to the N-terminal sequence from trout H1 histone. The 8-amino acid sequence obtained from the VIB-SER-derived peak was also identical to the N-terminus of the histone. Mass spectroscopic data for both samples revealed a number of histone fragments. The most probable fragmentation pattern is shown by separating the fragments with period marks. Underlined section likely resulted in fragmentation into many short (~15 amino acid) sections. The sequences of the synthetic peptides HSDF-1 and HSDF-2 used in further experiments are also shown. Acetylation of the N-terminus is denoted by (Ac) and the C-terminal amidation is denoted by (NH$_2$)
suggesting that the samples were not homogenous despite appearing as a single RP-FPLC peak. Species in the 20 kDa range were identified, indicating that the entire histone might be present. Also, species in the 10 kDa range, species corresponding to the N-terminally acetylated 26 amino acid peptide identical to the N-terminus of the trout H1 histone, and several smaller species were identified. We named the 26 amino acid species HSDF-1 (histone-derived fragment 1). Amino acid analysis of the FPLC–purified samples from mucus and serum showed HSDF-1 concentrations to be 12 μg/mL and 207 μg/mL, respectively. Assuming 100% yield from the purification protocol, HSDF-1 concentrations in the original samples were estimated as 13 μg/mL in salmon mucus and 23 μg/mL in salmon blood. Given the 100% yield assumption, these concentrations almost certainly underestimate the actual values.

3.1.6 Antimicrobial and Synergistic Activity of Synthetic HSDFs

The results presented above identify a new candidate for an antimicrobial peptide. However, isolating working quantities of the peptide from fish is impractical, as it would require the sacrifice of a large number of coho salmon. One alternative was to produce the peptide synthetically, via F-moc chemistry, and perform the characterization on the synthetic peptide.

The experiments here were designed to describe the antimicrobial activities of synthetic HSDF-1 and its amidated, but not acetylated, homologue HSDF-2. Given that HSDF-1 was co-induced with lysozyme, synergy experiments aimed at evaluating the ability of HSDFs to potentiate lysozyme, as well as other substances, were also performed.
Synthetically produced 26 amino acid peptides equivalent to the N-terminally acetylated (native histones are acetylated) and C-terminally amidated variants of the N-terminus of histone H1 (Fig. 9), had no detectable inhibitory activities against *A. salmonicida* and *V.anguillarum*, even when used at concentrations in excess of 1 mg/ml, as shown in Table V. However, at concentrations as low as 16-32 µg/ml, they potentiated the activities of the flounder peptide pleurocidin, hen lysozyme, and extracts from the mucus and serum of coho salmon, against *V.anguillarum*. Both histone peptides potentiated the activity of pleurocidin against *A. salmonicida*, but neither potentiated the activity of hen lysozyme or extracts from the mucus and serum of coho salmon against this bacterium. As the sources of serum and mucus were pre-challenged with *V.anguillarum*, these samples would contain lysozyme and other pathogen-specific defenses, which could apparently be potentiated by histone peptides.

The synergistic activities of histone peptides and natural fish antibiotics are shown in terms of actual reduction in inhibitory concentrations, as well as the FIC indices (Table V). While the latter is the common way of expressing synergy, the former provides a more intuitive visualization of the experimental data, given that the histone peptides had no detectable minimal inhibitory concentrations by themselves. Given that synergy between HSDFs and pleurocidin was seen, the ability of these peptides to interact with lipid monolayers was further investigated.

3.1.7 Interaction of HSDF and Pleurocidin with Lipid Monolayers

The ability of pleurocidin and histone peptides to insert into lipid monolayers was investigated in three separate sets of experiments.
TABLE V. Fractional inhibitory concentrations for natural fish antibiotics. Inhibitory concentrations of natural fish antibiotics, as well as hen egg white lysozyme (HEWL), in the presence of HSDF-1 or HSDF-2, are shown. Two concentrations of each histone peptide are presented for two separate bacteria: *V.anguillarum* (VANG) and *A.salmonicida* (ASAL). FIC indices for natural fish antibiotics and histone peptides have been calculated according to the previously described formula and are shown. See text for details of calculations.

<table>
<thead>
<tr>
<th></th>
<th>HSDF-1</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>32</td>
<td>128</td>
<td><em>FIC Index</em></td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>vs ASAL</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>vs VANG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleurocidin</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>vs ASAL</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>vs VANG</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>HEWL</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>vs VANG</td>
<td>&gt;256</td>
<td>128</td>
<td>32</td>
<td>&lt;0.25</td>
<td>128</td>
<td>64</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>vs VANG</td>
<td>&gt;256 a</td>
<td>256 a</td>
<td>32 a</td>
<td>&lt;0.25</td>
<td>256 a</td>
<td>64 a</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>vs VANG</td>
<td>&gt;256 a</td>
<td>128 a</td>
<td>32 a</td>
<td>&lt;0.19</td>
<td>128 a</td>
<td>32 a</td>
</tr>
</tbody>
</table>

* Numbers show lysozyme concentrations, in µg/mL, in mucus and serum. Lysozyme concentration estimates in the extracts were performed using a standard *M.lysodeikticus* assay.
In the first set of experiments, the ability of HSDF peptides and pleurocidin to insert into monolipid monolayers was studied (Fig. 10). All peptides appeared to insert into the anionic lipid egg-PG, and pleurocidin and HSDF-2 additionally inserted into the anionic cardiolipin (Fig. 10) monolayer. None of the three peptides inserted into the neutral PC monolayer. This indicates that the peptides are specific for anionic lipids.

In the second set of experiments, the ability of the peptides to insert into *S. minnesota* LPS monolayers was investigated (Fig. 11) and in the last set the ability of HSDFs and pleurocidin to insert into a monolayer with a composition reflecting the makeup of a bacterial cytoplasmic membrane (PE/PC/CL ratio of 78/4.7/14.4) was studied (Fig. 12). The results of the last two sets of experiments were almost identical, with pleurocidin inserting both into the LPS monolayer and the membrane-mimicking monolayer, HSDF-2 showing some (significantly lower) extent of insertion, and HSDF-1 not inserting at all. In addition neither HSDF-1 nor HSDF-2 were able to synergistically affect the extent of pleurocidin insertion into the monolayers, although HSDF-2 appeared to have an additive effect with pleurocidin at the LPS monolayer.

### 3.1.8 Interaction of HSDF-1 and Pleurocidin with *V.anguillarum* Membrane

As the synthetic version of the naturally occurring peptide, HSDF-1 exhibited poor lipid-monolayer insertion characteristics, the question arose as to whether it would nonetheless be able to permeabilize its potential natural target, the *V.anguillarum* cytoplasmic membrane. In order to test this, membrane potential-dependent quenching of the fluorescent dye diSC₅5 was monitored. In this assay, if the membrane is depolarized
Figure 10. Peptide specificity for monolipid monolayers. The ability of pleurocidin and histone-derived peptides to insert into lipid monolayers is shown. Peptides were added to the monolayers to a final concentration of 6.4 μg/ml. Single lipid monolayers composed of egg-phosphatidyl-DL-glycerol (PG), phosphatidyl choline (PC), and cardiolipin (CL) were tested. Maximal surface pressure increase Δτ (mN/m) caused by each peptide addition is shown on the y-axis. Averages of two experiments are shown.
Figure 11. Effects of HSDF peptides and pleurocidin on an LPS monolayer. The ability of the peptides and peptide combinations to insert into an LPS monolayer was measured. The monolayer was formed by spreading *S.minnesota* LPS (0.5 mg/ml in chlorophorm/methanol/water 17:7:1) onto the buffer. A change in surface pressure $\Delta \pi$ (mN/m), representative of peptide insertion into the lipid monolayer is shown on the y-axis. Peptides were added to a final concentration of 6.4 $\mu$g/ml each. Averages of two experiments are shown.
Figure 12. Effects of HSDF peptides and pleurocidin on a lipid monolayer mimicking the *E.coli* cytoplasmic membrane. The ability of the peptides and peptide combinations to insert into a monolayer composed of POPE/eggPC CL (78:4.7:14.4) was measured. A change in surface pressure $\Delta \pi$ (mN/m), representative of peptide insertion into the lipid monolayer is shown on the $y$-axis. Peptides were added to a final concentration of 6.4 $\mu$g/ml each. The monolayer composition is similar to that of an *E.coli* cytoplasmic membrane. Averages of two experiments are shown.
(indicating permeabilization by the peptide) the probe will be released into the medium causing a measurable increase in fluorescence.

As shown in Figure 13, the HSDF-1 peptide did not permeabilize the cytoplasmic membrane of *V.anguillarum*, or potentiate permeabilization caused by the flounder pleurocidin in this assay.

### 3.1.9 Conclusions

In this chapter the H1 histone and its fragments were identified in the antimicrobial fractions at two separate sites in coho salmon challenged with distinct agents, indicating that histone proteins may be a relatively ubiquitous component of the host defenses. This finding is consistent with other histone research published to date. Human wound fluid contains histone H2B fragments (Frohm et al., 1996), and histone-like cationic proteins have been isolated from the cytoplasm of murine macrophages (Hiemstra et al., 1993). Recently, the presence of histone H1 in the cytoplasm and supernatants of villus epithelial cells has been reported (Rose et al., 1998), and antimicrobial activity in the skin of channel catfish has been attributed to histone-like cationic proteins (Robinette et al., 1998). The antimicrobial activity of calf thymus histone has been known since 1958 (Hirsch, 1958), but the recent findings described above identify several extranuclear sites where histone proteins may play an important protective role *in vivo*.

The presence of defined histone fragments in antimicrobial extracts has been shown previously in toads and catfish *Ictalurus punctatus* (Park et al., 1996, 1998),
Figure 13. Ability of naturally occurring peptides to permeabilize *V.anguillarum* cell membrane. The ability of HSDF-1 at 50 μg/ml (filled square), pleurocidin at 6.4 μg/ml (filled diamonds), pleurocidin at 12.8 μg/ml (triangles), and pleurocidin at 6.4 μg/ml with HSDF-1 at 50 μg/ml (crosses) to permeabilize *V.anguillarum* cell membranes is shown. Increase in fluorescence of a membrane sensitive dye diSC₃5 is indicative of membrane permeabilization. An additional 50 μg/ml of HSDF-1 was added at 600 s.
consistent with our finding of the 26-amino acid N-terminal fragment of H1 histone in coho salmon. Although mass spectroscopy identified multiple species of various sizes, the total amino acid content of the serum sample matched unambiguously that of other H1 histones, suggesting that all MS species may be of histone origin. In fact, specific histone processing, rather than increased histone production, could account for the increased presence of the 26-amino acid histone fragment in active samples. It remains unclear whether the histone proteins were secreted, or if they represent active debris from host cells damaged in the disease process, but precedents for the former exist (Hiemstra et al., 1993; Rose et al., 1998).

The lack of antimicrobial activity of the artificially synthesized peptides HSDF-1 and HSDF-2 against V. angillarum and A. salmonicida, as well as the increase in lysozyme activity in the original samples, led to the hypothesis that the antimicrobial activity associated with the presence of the 26-amino acid peptide may have arisen from its ability to synergize with other histone fragments and, in early extracts, other natural fish antibiotics. The ability of the two synthetic peptides to potentiate the flounder peptide pleurocidin, as well as lysozyme and lysozyme-containing serum and mucus extracts, lent strong support to the synergy hypothesis. Synergy with pleurocidin is of particular significance as a version of this peptide has been shown to protect coho salmon from V. anguillarum infections in vivo (Jia et al., 2000). The diSC35 system did not indicate that synergy took place at the cell membrane level. However, the preference of the cationic histone peptides for the monolayers of anionic lipids suggests that membrane composition may be a factor in determining bacterial susceptibility to histone peptides.
The findings of this chapter expand our understanding of the mode in which histone proteins can be involved in salmon immunity. In the light of recent concerns about residual antibiotics in aquaculture, enhancing natural immune responses presents a unique opportunity for infection control among farmed fish.
3.2 CHAPTER TWO: Antimicrobial and Membrane Activities of Pleurocidin-Derived Peptides

3.2.1 Introduction

While isolating antibiotics from natural sources (as reported in previous chapters) allows the researcher to tap into Nature’s toolkit of antibacterial remedies, progress using this avenue can be slow and somewhat dependent on luck. This is why alternative approaches ranging from combinatorial chemistry and large scale mutagenesis/screening technologies to single amino acid substitutions in existing peptides have been employed.

If a completely random 20 residue peptide were to be constructed based only on the natural amino acids, more than $10^{26}$ possible combinations would have to be screened. Properly introduced constraints would, of course, decrease this number, but even considering the advances in constructing and screening chemical and phage-display libraries, the technical and financial constraints of using combinatorial chemistry to find an optimal 20 amino acid peptide through a solely random approach are still prohibitive. A viable alternative is to modify existing cationic peptides and to study the effects of such modifications in an attempt to gain insights into the peptide mode of action. With that information one can return to the drawing board and construct even better peptides, or enhancers of existing peptides.

Knowing that the winter flounder pleurocidin is active against the salmon pathogens *A. salmonicida* and *V. anguillarum*, a series of pleurocidin-based peptides were constructed (sequences are provided in Table VI). The experiments in this chapter are
TABLE VI. Peptide Sequences (one letter amino acid code). Pleurocidin will also be later identified as P-0, pleurocidin amide as P-CN, P1 amide as P1-CN, and misgurin as M-0. Acetylation is depicted as (Ac) and amidation is depicted as (−NH$_2$).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleurocidin</td>
<td>GWGSFFKKAAHVGKHVGKAALTHYL</td>
</tr>
<tr>
<td>Pleurocidin amide</td>
<td>GWGSFFKKAAHVGKHVGKAALTHYL-NH$_2$</td>
</tr>
<tr>
<td>P1</td>
<td>KGWGSFFKKAAHVGKHVGKAALTHYL</td>
</tr>
<tr>
<td>P1 amide</td>
<td>KGWGSFFKKAAHVGKHVGKAALTHYL-NH$_2$</td>
</tr>
<tr>
<td>P-DER</td>
<td>ALWKTMLKKAAHVGKHVGKAALTHYL-NH$_2$</td>
</tr>
<tr>
<td>PDH</td>
<td>ALWKTMLKKAAHVGKHVGKAALTHYLHHHHHH</td>
</tr>
<tr>
<td>P-CER</td>
<td>SIGSAFKKAAHVGKHVGKAALTHYL-NH$_2$</td>
</tr>
<tr>
<td>Misgurin</td>
<td>RQRVEELSKFSKKGAAARRRK</td>
</tr>
<tr>
<td>P-M</td>
<td>GWGSFFKKAAHVGKHVGKAALGAAARRRK</td>
</tr>
<tr>
<td>DER-M</td>
<td>ALWKTMLKKAAHVGKHVGKAALGAAARRRK</td>
</tr>
<tr>
<td>CER-M</td>
<td>SIGSAFKKAAHVGKHVGKAALGAAARRRK</td>
</tr>
<tr>
<td>HSDF-1</td>
<td>Ac-AEVAPAPAAAAAPAKAPKKAACKPKK</td>
</tr>
<tr>
<td>HSDF-2</td>
<td>AEVAPAPAAAAAPAKAPKKAACKPKK-NH$_2$</td>
</tr>
</tbody>
</table>
designed to test the antimicrobial activities of these peptides against a range of fish and non-fish bacteria and to determine to what extent bacterial membranes are a target for these peptides.

3.2.2 Antimicrobial Activity of Pleurocidin Peptides

Two fish pathogens, A. salmonicida and V. anguillarum, were selected for the testing panel since they are common causes of infections in coho salmon and rainbow trout reared on fish farms. In addition, a standard MIC testing panel of bacteria from our laboratory culture collection were used.

Nine derivatives of two fish peptides, pleurocidin and misgurin, and sequence homologues of pleurocidin, dermaseptin, and ceratotoxin, were constructed. The primary sequences of these variants and their parent molecules are depicted in Table VI. Some of their other characteristics are shown in Table VII.

Antimicrobial activities of all the peptides against the bacteria described above, were tested and are shown in Table VIII. Addition of lysine to the N-terminus of pleurocidin (peptide P-1) did not significantly change the antimicrobial activity. However, amidation of both pleurocidin and P-1 substantially enhanced their antimicrobial activities. A hybrid of pleurocidin and dermaseptin (P-DER) had improved antibacterial activity compared to unamidated pleurocidin, whereas a hybrid of pleurocidin and ceratotoxin (P-CER) had reduced activity. Misgurin alone was inactive against the bacteria tested. When combined as a hybrid with ceratotoxin, the resulting peptide (CER-M) remained quite inactive. However, hybrids of pleurocidin (P-M) or
Table VII. Selected properties of the peptide constructs. The number of residues, net charge, percentage of hydrophobic residues (L, V, I, F, M) and predicted percentage content of α-helix, random strand, and extended coil in the secondary structures based on GOR secondary structure prediction method version IV (Methods in Enzymology, vol 266, p 540-553, 1996) available via the internet from Pole Bio-Informatique Lyonnais, Lyon, France (http://pbil.univ-lyon1.fr), are shown. Amidated peptides are shown to have an additional positive charge.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residues</th>
<th>Charge</th>
<th>Hydroph.</th>
<th>Secondary Structure Prediction</th>
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<td></td>
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<td></td>
<td></td>
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</tr>
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<td>20%</td>
</tr>
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<td>25</td>
<td>+5</td>
<td>24.0%</td>
<td>20%</td>
</tr>
<tr>
<td>P1</td>
<td>26</td>
<td>+5</td>
<td>23.1%</td>
<td>19%</td>
</tr>
<tr>
<td>P1-CN</td>
<td>26</td>
<td>+6</td>
<td>23.1%</td>
<td>19%</td>
</tr>
<tr>
<td>P-DER</td>
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<td>26.9%</td>
<td>38%</td>
</tr>
<tr>
<td>P-CER</td>
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<td>M-0</td>
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<td>63%</td>
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<tr>
<td>CER-M</td>
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<td>+8</td>
<td>17.2%</td>
<td>59%</td>
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Table VIII. Antimicrobial activity of peptide constructs.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Vibrio anguillarum</th>
<th>Aeromonas salmonicida</th>
<th>Salmonella Typhimurium</th>
<th>Pseudomonas aeruginosa</th>
<th>Staphylococcus epidermidis</th>
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<tr>
<td></td>
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<td>16</td>
<td>&lt;0.5</td>
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<td>1</td>
<td>2</td>
<td>&lt;0.5</td>
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<tr>
<td>M-0</td>
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<tr>
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<td>P-CER</td>
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</tr>
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<td>P-M</td>
<td>32</td>
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<td>16</td>
<td>0.5</td>
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</tr>
<tr>
<td>DER-M</td>
<td>&gt;64</td>
<td>2</td>
<td>32</td>
<td>0.5</td>
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<tr>
<td>CER-M</td>
<td>&gt;64</td>
<td>32</td>
<td>&gt;64</td>
<td>8</td>
<td>&gt;64</td>
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<tr>
<td>Polymyxin B</td>
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<td>&lt;0.5</td>
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<tr>
<td>Gentamicin</td>
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<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>16</td>
</tr>
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</table>
dermaseptin (DER-M) with misgurin showed antibacterial activities approaching those of pleurocidin. The PhoP/Q *S. typhimurium* mutant M579535 (defensin supersusceptible) demonstrated 8-64 fold lower MICs than its parent strain, whereas the outer-membrane-altered *P. aeruginosa* strain Z61 demonstrated lower MICs for only a few peptides (Table VIII).

3.2.3 Ability of Pleurocidin Peptides to Bind LPS

All peptides were able to displace dansylated polymyxin B which had been pre-bound to LPS (Fig. 14). The affinity and avidity of the interaction varied however, with P-M having a relatively high $I_{max}$ (95 per cent) and the relatively low IC$_{50}$ (4 µg/ml), thus making it the best LPS binder next to polymyxin B. Interestingly P-M was one of the less active peptides in antimicrobial assays. Collectively, the results obtained for the peptides argue against any pattern which could link peptide ability to bind LPS with antimicrobial activity.

3.2.4 Ability of Pleurocidin Peptides to Permeabilize Outer Membranes

In a similar manner, all antimicrobially active peptides except DER-M, when applied at a fixed concentration of 2.56 µg/mL, fully permeabilized the *E.coli* outer membrane reaching maximum permeabilization within 4 to 6 minutes (Fig. 15). While this does not explain differences in potency among active peptides, it confirms that all active peptides studied here were in fact able to breach the bacterial outer membrane.

Several peptides were selected for further studies based on data presented up to this point. They were: two pleurocidin variants with improved antimicrobial activity (P-
Figure 14. The ability of peptide constructs to bind LPS. The relative binding affinity of each peptide for LPS was determined by the dansyl polymyxin B displacement assay of Moore et al (1986), using LPS isolated from *E. coli* UB1005. The bars and the left hand side axis represent maximum percentage inhibition (also known as $I_{\text{max}}$) while the dots and the right hand side axis represent the concentration of peptide required to reach one-half of maximum inhibition (also known as $IC_{50}$).
Figure 15. The ability of peptide constructs to permeabilize the outer membrane. The outer membrane permeabilization activity of the peptide variants was determined by the 1-N-phenylnaphthylamine (NPN) uptake assay of Loh et al (1984), using intact cells of E. coli UB1005. Increase in fluorescence is indicative of outer membrane permeabilization.
DER and P-CN), the parent pleurocidin, and P-M, which exhibited unusually high affinity for LPS.

The LPS binding and outer membrane permeabilization data for the four peptides selected for further study are summarized in Table IX.

3.2.5 Effects of Pleurocidin Peptides on *E. coli* Cytoplasmic Membrane.

In order to evaluate the effects of the selected peptides on *E. coli* cytoplasmic membranes, the fluorescent probe diSC₃₋₅ was used. DiSC₃₋₅ self-quenches when it is concentrated inside the bacterial cells, and it distributes between cells and medium depending on the cytoplasmic membrane potential. If the membrane is depolarized, the probe will be released into the medium causing a measurable increase in fluorescence.

While pleurocidin caused maximal cytoplasmic membrane depolarization at ten times its MIC, accompanied by a two log order decrease in bacterial viable counts, it only caused about fifty per cent depolarization at its MIC and at five times its MIC (Fig. 16). This indicates that, compared with 10 x MIC, at the lower concentrations the cytoplasmic membrane was not fully permeabilized.

In contrast, P-CN caused maximal membrane depolarization at all concentrations tested, regardless of whether a decrease in cell viability was observed (Fig. 17).

The extent of cytoplasmic membrane depolarization observed for P-M appeared to be proportional to the peptide concentration added (Fig. 18), with ten times the MIC causing maximal membrane depolarization, and the MIC causing a moderate (about 30
Table IX. The ability of Pleurocidin, P-CN, P-M and P-Der to bind to *E.coli* LPS and permeabilize *E.coli* outer membrane.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MIC against <em>E.coli</em> (µg/ml)</th>
<th>LPS Binding</th>
<th>Time Required Until Maximum (100%) Outer Membrane Permeabilization is Reached</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleurocidin</td>
<td>2</td>
<td>78%</td>
<td>5 min</td>
</tr>
<tr>
<td>P-CN</td>
<td>1</td>
<td>85%</td>
<td>4 min</td>
</tr>
<tr>
<td>P-M</td>
<td>4</td>
<td>95%</td>
<td>6 min</td>
</tr>
<tr>
<td>P-DER</td>
<td>2</td>
<td>67%</td>
<td>5 min</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>2</td>
<td>100%</td>
<td>2 min</td>
</tr>
<tr>
<td>Mg++</td>
<td></td>
<td>60%</td>
<td>N/D</td>
</tr>
</tbody>
</table>

$^{a}$ $I_{max}$ represents maximal displacement of dansylated polymyxin B by the various peptides compared to the displacement caused by non-dansylated polymyxin B, which is taken as 100%. $IC_{50}$ is equivalent to one-half of the peptide concentration required for maximal LPS binding.
Figure 16. The ability of pleurocidin to permeabilize the cytoplasmic membrane of E.coli CGSC 4908. Bacterial survival (A) and permeabilization of the cytoplasmic membrane (B) are shown. Fluorescence of a membrane potential-sensitive dye, diSC₃, was measured. The peptide was added to its minimum inhibitory concentration (Δ), five times its MIC (×), and ten times its MIC (●). A control sample with no peptide added is also shown (●). Data representative of three separate experiments are shown.
Figure 17. The ability of P-CN to permeabilize the cytoplasmic membrane of E.coli CGSC 4908. Bacterial survival (A) and permeabilization of the cytoplasmic membrane (B) are shown. Fluorescence of a membrane potential-sensitive dye, diSC₃-5, was measured. The peptide was added to its minimum inhibitory concentration (△), five times its MIC (×), and ten times its MIC (●). A control sample with no peptide added is also shown ( ○ ). Data representative of three separate experiments are shown.
Figure 18. The ability of P-M to permeabilize the cytoplasmic membrane of E.coli CGSC 4908. Bacterial survival (A) and permeabilization of the cytoplasmic membrane (B) are shown. Fluorescence of a membrane potential-sensitive dye, diSC₃5, was measured. The peptide was added to its minimum inhibitory concentration (△), five times its MIC (×), and ten times its MIC (●). A control sample with no peptide added is also shown (○). Data representative of three separate experiments are shown.
per cent) permeabilization of the cytoplasmic membrane. Also, P-M at five and ten times
the MIC caused a four log order decrease in cell viability, P-M at its MIC caused minimal
decrease in viable cell counts.

P-Der, applied at five times its MIC, did not cause *E.coli* cytoplasmic membrane
permeabilization, estimated by the diSC₃5 assay, within 30 min (Fig. 19B) of adding the
peptide, despite causing a 10-fold decrease in viable counts (Fig. 19A). Conversely, ten­
fold MIC multiples of P-Der caused immediate maximum membrane depolarization (Fig.
19B) accompanied by a 500-fold decrease in cell viable counts within 15 minutes (Fig.
19A).

Overall, high multiples of the minimal inhibitory concentrations of all tested
peptides permeabilized the *E.coli* cytoplasmic membrane within the first three min after
peptide addition. Rapid cell membrane permeabilization was accompanied by cell death
either immediately, as observed in the case of P-CN (Fig. 17), or later within the 30 min
time course of the experiment. Delayed cell death, as observed in the case of P-M at five
times its MIC (Fig. 18), is indicative that factors other than membrane depolarization
itself might have modulated the lethal event.

This was reinforced further by examining membrane permeabilization and
lethality data for peptides added at their MICs. Addition of minimal inhibitory
concentrations of pleurocidin, P-CN, and P-M resulted in only 40%, 75%, and 30% of
maximal membrane permeabilization, respectively. And, as mentioned earlier, in the case
of P-DER, no membrane permeabilization was observed up to five-fold its MIC (Fig.
19AB).
Figure 19. The ability of P-DER to permeabilize the cytoplasmic membrane of E.coli CGSC 4908. Bacterial survival (A) and permeabilization of the cytoplasmic membrane (B) are shown. Fluorescence of a membrane potential-sensitive dye, diSC$_3$5, was measured. The peptide was added to its minimum inhibitory concentration (△), five times its MIC (☆), and ten times its MIC (●). A control sample with no peptide added is also shown (●). Data representative of three separate experiments are shown.
The data indicate that, although pleurocidin and its derivatives, especially when they are added at high multiples of their MICs, exert effects on the *E. coli* cytoplasmic membrane, not all antimicrobial activity can be attributed to the failure of the cytoplasmic membrane barrier.

### 3.2.6 Conclusions

In an attempt to improve the antimicrobial activity of pleurocidin, a series of different peptide constructs, including the hybrids of pleurocidin with its homologues, were synthesized. All three cationic peptide constructs with improved antimicrobial activities (P-DER, P-CN, and P-1-CN) were C-terminally amidated derivatives of pleurocidin. C-terminal amidation has been previously shown to improve the antimicrobial activity of many peptides (Falla *et al.*, 1996; Oren and Shai, 1996). Perhaps most surprising was the finding that in our studies the chemically synthesized misgurin exhibited no significant antimicrobial activity (contrary to results reported by Park *et al.*, 1997), and C-terminal misgurin hybrids showed reduced activities compared to their pleurocidin equivalents. Also, it appeared that the substitution of the N-terminus of pleurocidin with the N-terminus of dermaseptin had no effect on antimicrobial activity, while substitution with the N-terminus of ceratotoxin decreased this activity. There appeared to be no correlation between the LPS binding ability of the peptides, or their ability to permeabilize bacterial outer membranes, and their antimicrobial abilities. Four peptides were subsequently selected for further studies according to criteria described above.
At 10-fold their MICs, all four peptides caused rapid depolarization of the cytoplasmic membrane, cessation of macromolecular synthesis and cell death. Also, the extent to which the peptides bound LPS appeared to be unrelated to their antimicrobial activity, and all active peptides permeabilized the E.coli outer membrane at 6.4 μg/ml, regardless of their relative activity. These results are thus consistent with data published for many other peptides and they could be interpreted in terms of the cell membrane being the lethal target for cationic peptides applied at high concentrations. Although the destruction of the cytoplasmic membrane potential gradient is not per se a lethal event, it might be argued that the damage caused to the cell membrane could cause leakage of essential cell molecules and thus be responsible for the rapid cell death and cessation of macromolecular synthesis.

This above explanation could also hold true for P-CN tested at a lower concentration, closer to its MIC, since marked permeabilization of the cytoplasmic membrane was observed at that concentration.

However, a significantly different pattern was observed for pleurocidin, P-M, and especially P-DER. When used at its MIC, P-DER did not cause notable depolarization of the cytoplasmic membrane, or rapid killing, thus implying that other targets may exist for these peptides acting at concentrations close to their MICs. This concept is further reinforced by the fact that while there was still no detectable cytoplasmic membrane permeabilization when P-DER was added at five times its MIC, a one-log order decrease in cell viability was observed at that concentration. Hence the difference between the effects of P-DER on cell viability at one time its MIC and five times its MIC cannot be attributed to differential cytoplasmic membrane depolarization.
3.3 CHAPTER THREE: Intracellular Activities of Pleurocidin-Derived Peptides

3.3.1 Introduction

The results in Chapter Two suggest that neither LPS-binding properties, nor outer-membrane permeabilizing properties of the peptide constructs can adequately explain their relative potency. In addition, while there was a correlation between bacterial killing and the destruction of the bacterial cytoplasmic membrane when peptides were applied at high multiples of their MICs, damage to the cytoplasmic membrane was not always observed when peptides were added at their MICs. The question thus follows as to whether there may be other targets for cationic peptides acting at lower concentrations.

The experiments in this chapter are designed to investigate whether the previously selected four pleurocidin constructs, when applied at concentrations close to their MICs, would affect the ability of E.coli to synthesize DNA, RNA, and proteins.

3.3.2 Effects of Pleurocidin Peptides on E.coli Macromolecular Synthesis

In order to assess the effects of pleurocidin peptides on the synthesis of macromolecules in bacteria, the incorporation of the radioactive precursors [methyl-\(^3\)H]thymidine, [5-\(^3\)H]uridine, or L-[2,5-\(^3\)H]histidine into DNA, RNA and proteins, respectively, was monitored in an E.coli strain auxotrophic for these three precursors. E.coli cultures were incubated with one of the radiolabelled precursors at a time, as well as the two remaining unlabelled precursors, and the peptide was added. At the indicated time points after peptide addition the cultures were sampled, and the samples were precipitated on ice with 10 % TCA and filtered on Whatman 47 mm GF/C glass
microfibre filters. The filters were dried and assessed for the amount of radioactivity present. The amount of radioactivity thus represented only that quantity of each precursor which had been incorporated into its respective macromolecule.

Pleurocidin, added at ten times its MIC, caused inhibition of protein synthesis (Fig. 20D), DNA synthesis (Fig. 20B) and RNA synthesis (Fig. 20C) within 10 minutes of adding the peptide. In contrast, only DNA synthesis (Fig. 20B) and protein synthesis (Fig. 20D) were affected by pleurocidin at its MIC. In addition, cell viability was not affected at that concentration (Fig. 20A).

Pleurocidin amide, P-CN, caused immediate inhibition of DNA, RNA, and protein synthesis (Fig. 21BCD) when added at ten times its MIC, and immediate inhibition of protein and RNA synthesis (Fig. 21 CD) when added at its MIC. However, at its MIC, P-CN did not cause DNA synthesis inhibition (Fig. 21B) until 10 min after peptide addition.

The pleurocidin/misgurin hybrid P-M, added at ten times its MIC, caused an immediate cessation of protein and DNA, and RNA synthesis (Fig. 22BD). However, at its MIC P-M did not substantially inhibit protein synthesis (Fig. 22D) or cause cell death (Fig. 20A). Nonetheless, RNA synthesis (Fig. 22C) and later DNA synthesis (Fig. 22B) were inhibited at this concentration, suggesting that these events precede lethal or inhibitory effects, rather than result from them.

P-DER, added at ten times its MIC, caused immediate inhibition of RNA synthesis (Fig. 23C), followed by inhibition of protein synthesis (Fig. 23D), and DNA
Figure 20. The effects of pleurocidin on macromolecular synthesis in *E.coli* CGSC 4908. Bacterial survival (A), $[^3]$H-thymidine incorporation into DNA (B), $[^3]$H-uridine incorporation into RNA (C), and $[^3]$H-L-histidine incorporation into protein (D) were measured. The peptide was added to its minimum inhibitory concentration ($\triangle$), and ten times its MIC ($\times$). A control sample with no peptide added is also shown (□). Data representative of three separate experiments are shown.
Figure 21. The effects of P-CN on macromolecular synthesis in *E. coli* CGSC 4908. Bacterial survival (A), [\(^3\)H]-thymidine incorporation into DNA (B), [\(^3\)H]-uridine incorporation into RNA (C), and [\(^3\)H]-L-histidine incorporation into protein (D) were measured. The peptide was added to its minimum inhibitory concentration (△), and ten times its MIC (×). A control sample with no peptide added is also shown (○). Data representative of three separate experiments are shown.
Figure 22. The effects of P-M on macromolecular synthesis in E.coli CGSC 4908. Bacterial survival (A), $[^{3}\text{H}]$-thymidine incorporation into DNA (B), $[^{3}\text{H}]$-uridine incorporation into RNA (C), and $[^{3}\text{H}]$-L-histidine incorporation into protein (D) were measured. The peptide was added to its minimum inhibitory concentration (△), and ten times its MIC (×). A control sample with no peptide added is also shown (●). Data representative of three separate experiments are shown.
Figure 23. The effects of P-DER on macromolecular synthesis in *E.coli* CGSC 4908. Bacterial survival (A), \[^3\text{H}\]-thymidine incorporation into DNA (B), \[^3\text{H}\]-uridine incorporation into RNA (C), and \[^3\text{H}\]-L-histidine incorporation into protein (D) were measured. The peptide was added to its minimum inhibitory concentration (X), five times its MIC (Δ), and ten times its MIC (●). A control sample with no peptide added is also shown (○). Data representative of three separate experiments are shown.
synthesis (Fig. 23B). When added at its MIC, P-DER inhibited RNA synthesis within 5 min of peptide addition (Fig. 23C), followed by the inhibition of DNA synthesis (Fig. 23B). Protein synthesis did not appear to be substantially inhibited (Fig. 23D) and cell viability was not affected (Fig. 23A) at that concentration of P-Der within the time course of the experiment. Also, as mentioned earlier, P-DER at its MIC, an even five times its MIC, did not cause membrane permeabilization.

3.3.3 Comparison to RNA Polymerase Inhibitor Rifampin

Since several peptides appeared to inhibit RNA synthesis prior to inhibiting DNA or protein synthesis, the effects of rifampin, a specific inhibitor of RNA polymerase, on the macromolecular synthesis of *E. coli* CGSC4908 were tested for comparative purposes. At 64 μg/ml rifampin specifically inhibited RNA synthesis (Fig. 24C) without causing any cell death (Fig. 24A) or inhibiting DNA synthesis (Fig. 24B). Protein synthesis was inhibited 15 min after RNA synthesis inhibition was observed. In the context of macromolecular synthesis inhibition pattern rifampin was thus similar to P-DER at its MIC.

3.3.4 Effects of Protein and DNA Synthesis Inhibitors

In addition to Rifampin, the protein synthesis inhibitor chloramphenicol, and DNA gyrase inhibitor norfloxacin were tested as controls for the *E.coli* CGSC4908 system. As expected, the former inhibited protein synthesis (Fig. 25B) when applied at 16 μg/ml, while the latter inhibited DNA synthesis (Fig. 25A) when applied at 1 μg/ml.
Figure 24. The effects of Rifampin on macromolecular synthesis in *E. coli* CGSC 4908. Bacterial survival (A), $[^3]$H-thymidine incorporation into DNA (B), $[^3]$H-uridine incorporation into RNA (C), and $[^3]$H-L-histidine incorporation into protein (D) were measured. Rifampin was added to 8 μg/ml (△) and 64 μg/ml (×). A control sample with no antibiotic added is also shown (●). Data representative of three separate experiments are shown.
Figure 25. The effects of Chloramphenicol and Norfloxacin on macromolecular synthesis in *E.coli* CGSC 4908. [³H]-thymidine incorporation into DNA (A), and [³H]-L-histidine incorporation into protein (B) were measured. The antibiotics were added at 5 min. (△) to the concentration of 1 μg/ml for norfloxacin (A) and 16 μg/ml for chloramphenicol (B). A control sample with no peptide added is also shown for each experiment (●). Data representative of two separate experiments are shown.
3.3.5 Effects of the Uncoupler CCCP

To ensure that low level de-energization of the cell, such as could occur even with incomplete membrane permeabilization, could not in itself result in the macromolecular inhibition pattern observed, P-DER was tested in the *E.coli* CGSC4908 system in the presence of CCCP. When added at 50μM, CCCP did not have any effect on the system as tested (Fig. 26).

In addition, another control was randomly performed (due to the logistics of the experiments it was not possible to do this routinely). In this control, alongside the radioactivity in bacterial TCA extracts, radioactivity in *whole* bacterial cells was determined, to ensure that the radiolabelled precursors did in fact enter the bacterial cells. Invariably, high radioactive counts, indicative of high levels of precursors, were detected in whole bacterial cells, even when TCA extracts revealed that little precursor was incorporated into the macromolecules.

3.3.6 Conclusions

A summary of macromolecular synthesis inhibition data from this chapter, correlated with the previously described cytoplasmic membrane depolarization data, is shown in Table X. Concentrations of all peptides at 10-fold the MIC inhibited the incorporation of radioactive precursors into RNA, DNA, and protein in *E.coli* CGSC 4908. In the case of P-CN, the synthesis of all three types of macromolecules was inhibited within five min after peptide addition, while the other three peptides exhibited various time patterns of inhibition. Protein synthesis appeared to be inhibited first in the
Figure 26. The effects of CCCP and P-Der on macromolecular synthesis in *E. coli* CGSC 4908. [*³H*-thymidine incorporation into DNA (A), [*³H*-uridine incorporation into RNA (B), and [*³H*-L-histidine incorporation into protein (C) were measured. The peptide and/or CCCP were added at 0 min. Data representative of two separate experiments are shown.
Table X. Correlation between inhibitory concentration, cytoplasmic membrane depolarization, and inhibition of macromolecular synthesis\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MIC Concentration</th>
<th>Maximal Cytoplasmic Membrane Permeabilization at 3 minutes</th>
<th>Time of Initiation of Inhibition of Synthesis (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg/ml)</td>
<td>DNA</td>
<td>RNA</td>
</tr>
<tr>
<td>Pleurocidin</td>
<td>2 MIC</td>
<td>40%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MIC × 10</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>P-CN</td>
<td>1 MIC</td>
<td>75%</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>MIC × 10</td>
<td>100%</td>
<td>0</td>
</tr>
<tr>
<td>P-M</td>
<td>4 MIC</td>
<td>30%</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>MIC × 10</td>
<td>100%</td>
<td>5</td>
</tr>
<tr>
<td>P-Der</td>
<td>2 MIC</td>
<td>0%</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>MIC × 10</td>
<td>100%</td>
<td>10</td>
</tr>
<tr>
<td>CCCP</td>
<td>50 μM</td>
<td>N/D</td>
<td>-</td>
</tr>
<tr>
<td>Rifampin</td>
<td>64 µg/ml</td>
<td>N/D</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Maximal cytoplasmic membrane depolarization caused by each peptide at its MIC and MIC × 10, 3 min after the addition of peptide, is shown. The times, in minutes, between addition of peptides and the initiation of macromolecular synthesis inhibition, are shown.

\textsuperscript{b}Data correspond to the averages of three separate repeats and thus differ from the representative experiments presented in Figures 16-24.
case of pleurocidin, while RNA synthesis appeared to be inhibited first upon addition of high concentrations of P-M and P-DER.

Addition of MIC levels of all peptides still resulted in specific inhibition of macromolecular synthesis, despite the fact that only partial membrane depolarization, as described earlier, and virtually no killing was observed. The pattern of inhibition was similar, but not identical to that observed at high multiples of the MIC. Once again, RNA synthesis was inhibited first upon addition of P-Der and P-M, protein synthesis was inhibited first upon addition of pleurocidin, and the synthesis of all three macromolecules was inhibited upon addition of P-CN. Protein synthesis and RNA synthesis did not appear to be inhibited by MIC levels of P-DER, and pleurocidin, respectively, during the 40 minute course of the experiment. This finding is particularly important in case of P-Der, which appears to selectively inhibit RNA synthesis (Fig. 23C) at concentrations which have no effect on the inner membrane permeabilization (Fig. 23B). The effect of P-Der is similar to that observed with the RNA-polymerase-specific bacteriostatic inhibitor rifampin, but not for with general uncoupler CCCP, which also failed to alter P-DER effects on E.coli macromolecular synthesis.

Even without rapid killing and cell membrane permeabilization, discussed in the previous chapter, macromolecular synthesis, and in particular RNA synthesis, was inhibited by MIC concentrations of P-DER and PDH. The pleurocidin-dermaseptin hybrid, when used at its MIC, inhibited RNA synthesis about ten minutes before other effects were observed. In fact, the pattern of macromolecular synthesis in the presence of P-DER at its MIC appeared to be similar to that observed in the presence of the RNA-
polymerase inhibitor, rifampin. While it is too early to speculate on whether RNA inhibition is the primary target for sublethal concentrations of P-DER, our results clearly suggest that, for this peptide, membrane depolarization does not represent the bacteriostatic event.

This is consistent with recently published data for lysozyme-derived fragments (Pellegrini et al., 2000), where Pellegrini et al showed that short, positively charged peptides inhibited DNA and RNA synthesis prior to causing inner membrane permeabilization in *E.coli*. While in our experiments P-DER was the only peptide which did not cause inner membrane permeabilization while exerting intracellular effects, the results for all four peptides support the contention that intracellular targets may be a part of the antimicrobial mechanism of action at MIC. In this model, the incomplete or minimal cytoplasmic membrane depolarization which was observed, would be a consequence of the membrane disturbance and of ions being carried by the interstitial water while transient peptide aggregates span the membrane.

Overall, data in this chapter suggest that peptides can enter cells without permeabilizing the cytoplasmic membrane and access internal targets, albeit bacteriostatic ones. Recent reports establish a strong precedent for cationic peptides entering bacterial cells (Park et al., 1998) and targeting specific molecules, such as heat shock proteins (Otvos et al., 2000).
3.4 CHAPTER FOUR: Evidence for P-DER/PDH Translocation Through Membranes

3.4.1 Introduction

An analysis of results presented in the previous two chapters reveals that, when used at low concentrations, P-DER is capable of inhibiting the synthesis of macromolecules by bacteria without causing damage to their cytoplasmic membranes. This makes P-DER the best candidate for further investigation. A concise summary of P-DER data obtained so far is shown in Fig. 27.

A His-tagged version of P-DER, PDH, was constructed at this point due to its potential for expression and localization studies. The antimicrobial activity of PDH was determined (Table XI) and its properties in cytoplasmic membrane and macromolecular synthesis assays are summarized in Fig. 28. Essentially, PDH data mirrored those obtained for P-DER. In both cases, when used at concentrations close to their MICs, the peptides caused negligible cytoplasmic membrane depolarization while causing the inhibition of the synthesis of various macromolecules. This leads to the obvious question of how such intracellular outcomes are effected. Many different explanations could be given and will be discussed later in this thesis, but they must fall into one of two categories: those that require the peptide to cross the cytoplasmic membrane and act intracellularly, and those that do not require peptide entry into the cell. Indeed, there is very recent evidence for the entry of peptides such as buforin (Park et al., 1998, 2000) into bacterial cells.
Figure 27. Correlation between killing membrane effects and intracellular effects caused by P-DER in *E.coli* CGSC4908. Panel A shows the effects of P-DER added at its MIC while panel B shows the effects of P-DER added at ten times its MIC. The diamond shows percentage viability, the square shows percentage permeabilization of the cytoplasmic membrane, and the triangle, circle and cross show the rate of DNA, RNA, and protein synthesis, respectively, expressed as percentage of the rate in *E.coli* not treated with the peptide.
Table XI. Antimicrobial properties of PDH

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Minimum inhibitory concentration (μg/ml)</th>
<th>P-DER</th>
<th>PDH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salmonella typhimurium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14028s</td>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>MS7953s</td>
<td></td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K799</td>
<td></td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Z61</td>
<td></td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><strong>Staphylococcus epidermidis</strong></td>
<td></td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><strong>Vibrio anguillarum</strong></td>
<td></td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><strong>Aeromonas salmonicida</strong></td>
<td></td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 28. Correlation between killing membrane effects and intracellular effects caused by PDH in *E.coli* CGSC4908. Panel A shows the effects of PDH added at its MIC while panel B shows the effects of PDH added at ten times its MIC. The diamond shows percentage viability, the square shows percentage permeabilization of the cytoplasmic membrane, and the triangle, circle and cross show the rate of DNA, RNA, and protein synthesis, respectively, expressed as percentage of the rate in *E.coli* not treated with the peptide.
The findings presented so far, as well as structural data from our own laboratory (Rozek et al., 2000), have led to the development of a hypothetical working model. In this model the peptide inserts into the outer leaflet of the cytoplasmic membrane, and destabilizes the bilayer leading to an increase in the inherent rate of lipid exchange between the outer and the inner leaflet of the membrane, or lipid flip-flop. Eventually the peptide itself is “flipped” into the inner leaflet and thus enters the cell. In order to test whether this model could explain the activities of P-DER and PDH, the ability of these peptides to increase the rate of lipid flip-flop and to translocate through a lipid bilayer was tested in a liposome-based system. The system used was a well-established one (Matsuzaki et al., 1997) and has been utilized for other peptides in our laboratory (Zhang et al., 2001).

3.4.2 Ability of P-DER and PDH to Stimulate Lipid Flip-Flop and Lyse Liposomes

The induction of lipid flip-flop by P-DER and PDH was assessed using unilamellar liposomes with their inner leaflet labeled with C₆-NBD-PC. The rate of NBD group transfer from the inner leaflet to the outer leaflet was measured upon addition of peptide followed by a sodium dithionite quencher. The measurement reflected the fact that NBD group transfer into the outer leaflet resulted in their being quenched by sodium dithionite, and a decrease in fluorescence was observed.

The spontaneous rate of lipid flip-flop was also monitored in the course of the experiment as shown in Figure 29, and was found to be negligible.
Figure 29. The ability of P-DER to induce lipid flip flop across a liposome bilayer. Unilamellar liposomes composed of PC/PG (1:1) were asymmetrically labeled with the inner leaflet containing fluorescent PC (C6-NBD-PC) and a quencher present outside. The ability of peptides to induce the translocation of the fluorescently labelled lipids from the inner leaflet to the outer leaflet, leading to quenching of the fluorescence, was measured. Decrease in fluorescence is thus representative of lipid flip flop. A control sample with no peptide added until 500 s, and Triton X added at that point, is also shown. One of three repeats is shown. Statistically analysed data for percentage flip flop as a function of peptide concentration are shown in Fig. 31.
As shown in Figure 29, representing a sample P-DER experiment, the peptide caused various extent of lipid flip-flop at all concentrations tested within the 10 min course of the experiment. The percent flip-flop caused by each peptide concentration was calculated as follows: percent flip-flop = 100 x (F₀-Fₚ)/(F₀-Fₜ), where F₀, Fₚ and Fₜ represent the fluorescence intensity in the labeled liposomes without the peptide, with peptide, and with Triton X100, respectively. A plot of percent flip-flop as a function of peptide concentration is shown both for P-DER (Fig. 31) and PDH (Fig. 32). Averages of three independent experiments are plotted.

The respective abilities of P-DER and PDH to cause lipid flip-flop were similar. Both peptides stimulated lipid flip-flop at all concentrations tested, and both peptides caused 100% flip-flop at concentrations equal to or greater than 3 μg/ml within the course of the experiment (Fig. 31 and Fig. 32). In addition, as shown for P-DER in Figure 29, in both cases the rate of lipid flip-flop was higher at higher peptide concentrations, with P-DER at 3.84 μg/ml causing 50% flip-flop at 150 s, while P-DER at 1.28 μg/ml caused equivalent flip-flop only at 250 s.

In order to ensure that the observed flip-flop was not caused by a disruption of the bilayer, the leakage of calcein from unilamellar liposomes was measured. As calcein is self-quenching at high concentrations inside the liposomes, an increase indetectable fluorescence is indicative of calcein leakage. As shown in Figure 30, representing a sample P-DER experiment, the peptide caused various extents of calcein leakage at concentrations equal to or greater than 3 μg/ml. The percent leakage at each peptide concentration was calculated as follows: percent leakage = 100 x (Fₚ-F₀)/(Fₜ-F₀), where
Figure 30. The ability of P-DER to induce calcein release from liposomes. Calcein-filled liposomes composed of PC/PG (1:1) were used. An increase in fluorescence corresponds to the release of calcein and is indicative of liposome lysis. Triton X was used as a control to induce lysis. One of three repeats is shown. Statistically analysed data for percentage calcein release as a function of peptide concentration are shown in Fig. 31.
Figure 31. Correlation between lipid flip flop and calcein release caused by P-DER. Percentages of maximal lipid flip flop and calcein release are shown as a function of peptide concentration. Averages of three experiments and standard deviations are shown.
Figure 32. Correlation between lipid flip flop and calcein release caused by PDH. Percentages of maximal lipid flip flop and calcein release are shown as a function of peptide concentration. Averages of three experiments and standard deviations are shown.
F₀ and Fₚ denote the fluorescence intensity before and after peptide addition, and Fₜ represents the fluorescence intensity after addition of Triton X100. A plot of percent leakage as a function of peptide concentration is shown both for P-DER (Fig. 31) and PDH (Fig. 32). Averages of three independent experiments are plotted. The results show that while both P-DER and PDH were able to induce liposome lysis and calcein release at 4 µg/ml, neither did so at concentrations lower than 1 µg/ml.

It is evident from the plots of percentage flip flop and percentage calcein release as a function of peptide concentration (Fig. 31 and Fig. 32), there is a range of concentrations for each peptide (0.5-2 µg/ml for P-DER and 0.25-1.5 µg/ml for PDH) where measurable lipid flip-flop occurs without measurable calcein release. This indicates that the peptides can stimulate lipid translocation between the leaflets of the bilayer, without causing liposome lysis or gross permeabilization.

3.4.3 Ability of P-DER and PDH to Translocate into Liposomes

While the previous data show that P-DER and PDH can stimulate the translocation of lipids between bilayers, they do not indicate whether peptides themselves can translocate. In order to assess that, the ability of P-DER and PDH to access the interior of liposomes, made of an equimolar mixture ePC and ePG and containing the fluorescent probe DNS-PE, was measured. The liposomes contained active α-chymotrypsin, while any external enzyme was deactivated with a trypsin-chymotrypsin inhibitor. Resonance energy transfer from the P-DER or PDH tryptophan residue to DNS-PE resulted in an initial increase in fluorescence upon binding of the peptide to the
membrane. Upon translocation, the peptide was digested by the $\alpha$-chymotrypsin encapsulated in the liposomes, leading to a decrease in fluorescence intensity.

Evidence for peptide translocation is shown in Fig. 33. Both P-DER and PDH, when added at 1.28 $\mu$g/ml caused a decrease in fluorescence, which is characteristic of translocation. The extent of translocation was not as high as in the case of a positive control, polyphemusin, but nonetheless the ability to translocate was clear in the light of the negative control, misgurin.

3.4.4 Conclusions

Much of today's research on the mode of action of cationic antimicrobial peptides has been conducted at high multiples of the MICs, or high peptide-to-lipid ratios. As a consequence, most research identifies cell membranes as the major target for cationic peptide action. In Chapters Two and Three, the effects of near-MIC concentrations of pleurocidin and three of its derivatives on the $E. coli$ cytoplasmic membrane and on macromolecular synthesis were examined. P-DER, a hybrid of pleurocidin and dermaseptin, demonstrated the ability to inhibit intracellular functions without damaging the $E. coli$ cytoplasmic membrane at concentrations up to 5-fold its MIC. The results in the present chapter indicate that, when applied at 1.28 $\mu$g/ml, P-DER was also able to enter large unilamellar liposomes, but did not cause calcein leakage from the liposomes. The data are consistent with the peptide translocating into bacterial cells, without causing damage to the cytoplasmic membrane. Similar results were obtained for a His-tagged version of P-DER, PDH.

Yoshida et al., (2001) recently reported that pleurocidin can translocate into PC:PG (3:1) liposomes, but causes calcein leakage in that system. One hundred per cent
Figure 33. Ability of P-DER and PDH to translocate into liposomes. A decrease in fluorescence after addition of 1.28 μg/ml of peptide is indicative of digestion of the internalized peptide by liposome-entrapped chymotrypsin. Peptide internalization is thus shown. Polyphemusin and misgurin were used as a positive and a negative control, respectively. A result representative of three separate experiments is shown.
calcein release and peptide translocation were indeed reported when 10 μM pleurocidin was applied. However, the data of Yoshida et al. also indicate that at pleurocidin concentrations lower than 2.5 μM, the peptide translocated across membranes with reasonable efficiency (up to 60 per cent), while causing less than 30 per cent of calcein release.

A similar experimental design has been employed by Kobayashi et al. (2000) to describe the activities of the α-helical peptides magainin II and buforin II. Magainin II was an efficient promoter of lipid flip-flop and leakage, but did not translocate well across the bilayer, and buforin II was not very effective at promoting leakage and flip-flop, but translocated relatively well across lipid bilayers.

A significantly greater complexity of membrane interaction patterns were described by Zhang et al. (2001) who characterized a number of α-helical, β-sheet, extended and cyclic peptides. In their study the peptides fell into 3 broad groups with respect to their interactions with model membranes: (a) weak inducers of all tested membrane activities, (b) gramicidin S, with its limited ability to induce of flip-flop and calcein release, but good ability to insert into lipid monolayers, depolarize cytoplasmic membranes and form channels in planar lipid bilayers, and (c) strong inducers of most, but not all (depending on the peptide) membrane activities. Indeed, Zhang et al. reported significant variation within this last group, and in most cases the relationship between model membrane activities and bactericidal action was complicated (Zhang et al., 2001).

The results of the present chapter add to the complexity reported above. Three separate activities of P-DER and PDH were measured in the liposome assays. Firstly, the movement of lipids from one monolayer of the liposome bilayer to the other, also referred as lipid flip-flop, was assessed. Secondly, the ability of the peptide to cause the release of a fluorescent dye, calcein, trapped in the intravesicular space of the liposome, was
assessed. And lastly, the ability of the peptide to translocate across the liposome membrane was assessed. The results of these experiments revealed that lipid flip-flop occurred at peptide concentrations that were two-to-three fold lower than those causing calcein release. Also, P-DER and PDH at 1.3 μg/ml were able to enter large unilamellar liposomes, but did not cause calcein leakage from the liposomes. These data were consistent with the ability of the peptides to permeabilize the cytoplasmic membrane of *E.coli* and inhibit its intracellular functions.

Collectively, our data indicate that, at their minimal inhibitory concentrations, cationic peptides can translocate into bacterial cells to exert their antimicrobial effects intracellularly. In addition, data presented in this chapter are consistent with the previously proposed hypothetical model in which the peptide inserts into the outer leaflet of the cytoplasmic membrane, destabilizes the bilayer leading to an increase in lipid flip-flop, and eventually is itself “flipped” into the inner leaflet thus entering the cell. Such translocation is not unprecedented and has in fact been shown by Park *et al.* (25) for buforin II, although the specific mechanism of translocation in that particular case still remains unknown. Better understanding of the various aspects of peptide action on bacteria, especially those involving peptides at low multiples of their MICs, will provide the tools required to design more potent and less toxic antimicrobials.
4 GENERAL DISCUSSION

One advantage of using multiple approaches to finding cationic antimicrobial peptides for fish disease control was that the research had an impact on several different fields of science. At least three major contributions can be identified in the area of fish immunity and cationic peptides. Firstly, the experiments presented in this thesis for the first time demonstrated the involvement of H1 histone and its cationic peptide fragments in salmon immunity (Patrzykat et al., 2001). This finding was later corroborated by other researchers, working independently on Atlantic salmon Salmo salar (Richards et al., 2001). Secondly, several peptides for potential use in aquaculture were constructed and a patent, as well as licensing agreements to use them, are currently pending (Antimicrobial Antiendotoxic Peptides, 1998; Helix Biomedix Inc. corporate press release of August 3, 2001). One of these peptides, P-CN, has already been shown to protect coho salmon from V.anguillarum infections in an animal model (Jia et al., 2000). And lastly, the experiments linking membrane and intracellular effects of cationic antimicrobial peptides have contributed to a major shift in the paradigm for peptide mode of action over the past five years. When this research was initially undertaken, there were few reports describing peptide activities at low concentrations, close to their MICs, and most peptides were believed to exert their effects solely by damaging the cytoplasmic membranes of bacteria.

While the specific results were already discussed in the Conclusions section of each chapter, a general context for the contributions outlined above will be presented here, along with an assessment of the future potential of this research.
4.1 Histone Peptides

Histone proteins are well conserved across species. In our hands, a histone-H1-derived peptide, HSDF-1, was co-induced with lysozyme in coho salmon mucus and serum in response to infection (Patrzykat et al., 2001) and, possibly in conjunction with other histone fragments and the entire histone molecule, had antimicrobial activity against \textit{V.anguillarum}, and \textit{A.salmonicida}. A synthetically produced homologue of the fragment by itself it did not appear to have any antibacterial activity, but it was able to strongly potentiate the activities of pleurocidin, mucus and serum extracts, as well as egg lysozyme, against \textit{Vibrio anguillarum} and \textit{Aeromonas salmonicida}.

Three questions of general interest arise from these results. Firstly, histones are generally described as chromosomal proteins usually involved in DNA folding. In this context, is there enough evidence to conclude that histones, or their fragments, indeed have multiple functions, including one as antimicrobials? Secondly, are there specific enzymes which are likely to cleave histones into antimicrobial fragments? And thirdly, as histones are normally thought to be confined to eukaryotic nuclei, how can they exert their protective antimicrobial effects in the cytoplasm or outside of the mammalian cell?

With regards to the first question, the earliest report of antimicrobial activity attributable to a histone protein appeared in 1958 (Hirsh, 1958). Since then, several antimicrobial peptides derived from H2A histones of Asian toad and catfish have been identified by Park (Park et al., 1996, 1998), histones H1 and H2B have been identified as antimicrobial proteins in murine macrophages (Hiemstra et al., 1993), and antimicrobial H2B-histone-like proteins have been identified in the skin of another catfish (Robinette et
al., 1998). Several of these studies were conducted concurrently with experiments described in this thesis. Following the publication of our report (Patrzykat et al., 2001) implicating H1 histone-derived peptides in the immunity of coho salmon, researchers at the NRC Institute for Marine Biosciences in Halifax identified H1 histone as a player in the innate immunity of Atlantic salmon (Richards et al., 2001). The sharp increase in the number of reports describing antimicrobial activity of histone proteins and their fragments over the past few years may be a reflection of the fact that more and more researchers are searching for cationic peptide/protein antibiotics. Histones, with their high lysine and alanine content and their antimicrobial properties, would inevitably fall within the specifications of cationic peptide purification protocols. Thus, it appears that the drive to discover new cationic antibacterial peptides, has also led the researchers to discover new and exciting properties of histones – molecules which we have known for years.

The question of whether there exists a specific mechanism for cleaving histone proteins is a difficult one. On the one hand Kim (Kim et al., 2000) reported that pepsin was able to specifically cleave H2A histones to buforin I. These researchers concluded that this may be a specific regulatory mechanism for controlling the quantities of buforin I available for killing bacteria. On the other hand, one could imagine that the damage caused to host cells by bacterial infections should cause a release of histone proteins as well as non-specific proteases from cells and thus a fortuitous histone cleavage would produce the useful antimicrobial by-products, cationic antimicrobial peptides. The relative value of the two explanations can be perhaps evaluated when one considers
whether cell damage needs to occur at all in order for histone secretion to occur, which brings us to the third question.

Soluble histones H2A, H2B and H4 have been found in bovine milk and serum (Waga et al., 1987). Also, N-terminal fragments of histone H2B, as well as defensins, lysozyme and FALL-39, were found in human wound and blister fluid (Frohm et al., 1996), and H1 histone fragments were found in human ileum (Rose et al., 1998). How did they get there? In the last case, Rose et al. showed that H1 histone was in fact present in the cytoplasm of villus epithelial cells, and was released upon their detachment from the basement membrane, an event which in itself led to apoptosis. In fact, the presence of histone proteins outside of the nucleus is not unprecedented. It has been reported that mouse liver cells and Friend erythroleukemia cells contain large cytoplasmic pools of H1 histone (Zlatanova et al., 1990), and, upon activation of human peripheral blood lymphocytes, histone H2B shifts from its intranuclear location to the plasma membrane (Watson et al., 1995). Also consistent with the theory that histone proteins may be secreted and play a role outside of the nucleus are reports of histone H2A inhibiting the binding of gonadotropin-releasing hormone (Aten and Behrman, 1989), and the finding that histones H2A and H2B are the major constituents of the homeostatic thymus hormone (Reichhart et al., 1985). Collectively there is a significant body of evidence suggesting that histone proteins may in fact be secreted.

Overall, while it may be appropriate to consider histone-derived antimicrobial peptides separately from those produced naturally as peptides, their antimicrobial properties, and their role in host immunity should not be overlooked. The best example
of this to date is the H2A-histone derived buforin, with its spectrum of antimicrobial activity resembling that of "classical" cationic peptides. Buforin is of particular interest: since histones act in the nucleus and modulate DNA metabolism, could not buforin, or other antimicrobial peptides, be directed at intracellular targets within bacterial cells? As mentioned above, buforin has in fact been investigated in this context, and has recently been shown to translocate across lipid membranes (Kobayashi et al., 2000) and into bacterial cells (Park et al., 1998), which is consistent with our results for P-Der and PDH.

4.2 Peptide Mode of Action

Most reports of antimicrobial action of cationic peptides describe their effects on bacterial functions, ranging from general functions such as bacterial ability to form colonies or grow in broth assays, to more specific functions related to bacterial membranes or metabolism. In addition, peptide activity in artificial assay systems, such as monolayer or bilayer systems, or liposome systems, has been used to supplement data obtained from bacteria. Taking these approaches together, researchers have tried to model the mode of action of antimicrobial peptides, as was described in the Introduction. In Figure 34 I describe a model that attempts to explain the results of my liposome, cytoplasmic membrane depolarization, and macromolecular synthesis experiments. Briefly, the liposome experiments suggested that P-DER and PDH promote lipid flip-flop across the bilayer and themselves translocate across the bilayer without causing liposome lysis. This is consistent with the fact the P-DER and PDH did not cause the destruction of E.coli cytoplasmic membrane, but they were able to inhibit macromolecular synthesis in E.coli cells even at concentrations equal to or lower than five times the MIC.
Inhibition of Macromolecular Synthesis

Figure 34. Model for P-DER/PDH mode of action at low concentrations. Following interaction with the outer leaflet of the bilayer, peptides stimulate the inherent rate of lipid flip-flop and are eventually translocated into the bacterial cell alongside the lipids. Henceforth, either by targeting specific targets or by indiscriminately binding to many negatively charged targets, the peptides inhibit macromolecular synthesis.
Several questions arise from my findings. Firstly, is it reasonable to assume that the ability to translocate across the bilayer of the liposome reflects peptide ability to translocate into bacterial cells? Secondly, how, exactly, do peptides exert their effects intracellularly? Thirdly, which peptides, and at what concentrations, would be expected to have intracellular effects? And finally, what is the relative importance of intracellular and membrane effects in the mode of action of cationic peptides?

With respect to the question of whether the liposome experiment results are a sufficient indication of P-DER and PDH being able to translocate into live bacterial cells, one must remember that the translocation experiments were performed with peptides which had already been shown to inhibit macromolecular synthesis without damaging the cytoplasmic membrane of *E.coli*. This is strongly indicative of the peptides being able to reach intracellular targets. Hence, although generally liposome experiments are not fully equivalent to experiments performed in bacterial cells, as the complexity of a live respiring cell membrane is far greater than that of a liposome bilayer, in the case of P-DER and PDH corroborating evidence exists to suggest translocation. In addition, there is a precedent for a fluorescently labeled peptide, buforin II, entering bacterial cells and inhibiting intracellular functions (Park *et al.*, 1998)

Although it is evident from the existing data, proving conclusively that the pleurocidin/dermaseptin hybrid peptides can translocate into live bacteria is a logical follow-up to this project and will be discussed in *Future Directions*. In fact, in order to show that the results obtained for liposome studies applied to bacterial systems, an attempt was made to immunolocalize PDH in *E.coli* CGSC4908 cells, by exploiting the
His tag present on the C-terminus of the peptide. Due to technical reasons this attempt was unsuccessful, and its description is superfluous. It was concluded that immunolocalizing the His tag attached to P-DER was impractical, due to its poor epitope properties. Nonetheless, the combined evidence for inhibition of macromolecular synthesis without membrane damage in live *E.coli*, and for P-DER and PDH translocation across liposome bilayer membranes, leaves little doubt that these peptides can indeed enter bacterial cells.

With respect to the question of how peptides can exert their intracellular effects, various intracellular activities of cationic peptides have been reported, ranging from general inhibition of macromolecular synthesis (Boman *et al.*, 1995; Subbalakshmi and Sitaram, 1998) to a very recent report of pyrrhocoricin inhibiting the ATPase actions of the heat shock protein DnaK and preventing chaperone-assisted protein folding (Kragol *et al.*, 2001). In addition, non-specific interactions of peptides with nucleic acids have been reported (Park *et al.*, 1998). At this stage of our knowledge, the argument for or against a specific intracellular target is somewhat speculative. Otvos *et al.* (2000) reported that pyrrhocoricin and other insect peptides bind specifically to the *E.coli* heat shock proteins DnaK and GroEL (Otvos *et al.*, 2000), thus indicating that specific targets may exist within the bacterial cell. At the same time it has not been shown conclusively that this interaction is responsible for the killing of bacterial cells by these peptides. Indeed, the fact that cationic antimicrobial peptides are small, positively charged molecules indicates that they should bind non-specifically to nucleic acids within the bacterial cell. Indeed Zhang *et al.* (2000) demonstrated polyphemusin binding to plasmids in a gel shift assay.
This could lead to interference with bacterial replication, transcription, or translation. In addition peptides may bind to negatively charged domains of enzymes, transport proteins, or, for that matter, heat shock proteins. In fact, any of the possibilities described above may contribute to the ability of P-DER and PDH to inhibit macromolecular synthesis in *E.coli*.

The last two questions, regarding concentration-dependence and peptide-specificity of intracellular effects, as well as the relative impact of the peptides on membranes versus intracellular targets, will be discussed together. The reason for this is that, based on our own research, as well as on published reports, most cationic antimicrobial peptides are likely to disrupt bacterial membranes when used at sufficiently high concentrations. These concentrations are generally several fold higher than the minimal inhibitory concentrations, can be toxic to the host, and are therefore of little relevance in designing therapeutics. Indeed, a membrane permeabilizing activity may be seen as a factor in inducing toxicity. When studied at concentrations close to their MICs, some peptides, such as magainin II, are largely membrane-active, while others, such as P-DER, PDH, buforin (Park *et al.*, 2000), apidaecin (Castle *et al.*, 1999), or PR-39 (Boman *et al.*, 1995), exhibit largely intracellular effects at their MICs. Rather than rigidly separating cationic peptides into those which act on membranes and those which act intracellularly, research results suggest that the mode of action of these peptides depends on both the particular peptide and its concentration, with the resulting activities ranging from mainly membrane-directed, through multiple targets, to specific intracellular targets. Researchers have in fact recently begun introducing terms such as "secondary targets,"
and "minor targets" (Sharma and Khuller, 2001). Although not discussed elsewhere in this thesis, it is worth mentioning that the targeted bacterium itself, or its growth stage, may influence the details of the peptide/pathogen interactions. For example it is known that bacteria in their log phase are more susceptible to some antibiotics than bacteria in their stationary phase. While this "multimodality" poses a challenge for the researcher who wishes to improve peptides through rational modification, or for one who wishes to have a definite answer as to how antimicrobial peptides work, it is perhaps the best countermeasure against the constantly evolving bacterium with its arsenal of resistance mechanisms.

4.3 Future Directions

Several obvious future research questions arise from the data presented in this thesis. These include: (1) conclusive confirmation that P-DER and PDH enter live bacterial cells, (2) identification of specific intracellular targets for pleurocidin peptides, and (3) investigation of the mechanism of synergy of histone peptides with lysozyme and pleurocidin.

In order to demonstrate peptide translocation into live bacteria, fluorescent versions of the peptides can be constructed, or antibodies can be raised to existing peptides. Cell free in vitro assays can be used to isolate specific targets for peptide activity, and those results can be used to design experiments, which would demonstrate specific peptide effects in bacterial systems. The ability of histone peptides to potentiate pleurocidin effects in many of the proposed assays could be used to gain insight into the mechanism of synergy.
Perhaps the most exciting area for future investigation does not directly arise from this thesis, but rather complements it. Over the past several years cationic peptides have been shown to modulate various host defenses in mammals. There is little research on this subject in fish. The effects of exogenously added pleurocidin peptides on the host fish and its immunity could be assessed, especially given that pleurocidin amide can protect coho salmon from \textit{V.anguillarum} infections. This could be done by identifying the host response genes regulated by cationic peptides in salmon gene array studies, and further studying the target genes and their products in more classical experiments. Given how little is known about fish immune defenses compared to mammalian defenses, research into the effects of cationic antimicrobial peptides on the various components of fish immunity would provide many scientifically novel and exciting insights.

4.4 Relevance

The final comment of this discussion is about the relevance of this research, and its future potential. Hopefully by this point in the discussion the scientific relevance has been made clear. However, given that this project was undertaken and funded with the ultimate aim of developing new antimicrobials, the question remains as to whether the outcome is of any practical value. Research conducted by Dr. Xiaoyan Jia in Prof. Hancock’s laboratory suggests that it in fact is.

The amidated version of pleurocidin (P-CN), which was constructed as part of this thesis and shown to have improved \textit{in vitro} activity against \textit{V.anguillarum} and \textit{A.salmonicida}, was delivered gradually to \textit{V.anguillarum}-infected coho salmon via an implanted osmotic pump, and significantly reduced fish mortality from the infection (Jia
Figure 35. Protection of coho salmon from *Vibrio anguillarum* infections by the cationic peptide pleurocidin (amide). Coho salmon challenged with lethal doses of *Vibrio anguillarum* was protected by pleurocidin amide administered at 250 µg/day via an implanted osmotic pump (Jia *et al.*, 2000). Thirty-day cumulative mortalities are shown for groups treated with pleurocidin amide and a saline control (without peptide).
et al., 2000 - Fig.35). It therefore seems likely that a flounder-derived gene, when placed under the control of an appropriate promoter, could be expressed in coho salmon or other fish, and protect them from infection. The promising results, and the therapeutic potential of the pleurocidin constructs, warranted patent protection (Antimicrobial Antiendotoxic Peptides, 1998) and it is perhaps the best measure of the perceived future prospects that these peptides, along with several other peptides from our laboratory, have already been licensed to an outside biotechnology company (Helix Biomedix Inc. corporate press release of August 3, 2001). There is no doubt in the author’s mind that the remaining concerns about peptide efficacy or toxicity can be addressed by the combined approach of isolating new antimicrobials from nature, studying their properties, and modifying them, and their delivery methods, to the required specifications.

While several hypotheses regarding the mode of action of pleurocidin peptides have been advanced here, the definitive mode of action of P-DER has not been proven in this thesis. Rather, a number of previously accepted assumptions have been shown to be erroneous. The author contends that this in itself is significant and finds it appropriate to conclude with the following John Maynard Keynes quotation:

"The difficulty lies, not in the new ideas, but in escaping the old ones, which ramify, for those brought up as most of us have been, into every corner of our minds."

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APPENDIX A

AUTHOR'S CONTRIBUTIONS TO RESEARCH AND DEVELOPMENT

Articles published or accepted in refereed journals


Articles submitted to refereed journals

Other refereed contributions


Non-refereed contributions


Technology transfer


Patents
