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Anti-inflammatory, fibrinolytic and antimicrobial effects of lactoferrin-derived peptides

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Title (English)

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Abstract

The properties of 59 lactoferrin-derived peptides were investigated regarding important characteristics for a future product in preventing adhesion formation. The four assays performed were anti-inflammatory, fibrinolytic, cytotoxic and antimicrobial assays. The result was a number of peptides showed higher efficiency than existing PharmaSurgics' peptides in all the assays tested and they will now be further investigated.

Keywords

Lactoferrin, lactoferricin, post-surgical adhesion formation, wound healing, anti-inflammatory, fibrinolytic, cytotoxic, antimicrobial.

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Anti-inflammatory, fibrinolytic and antimicrobial effects of lactoferrin-derived peptides

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Populärvetenskaplig Sammanfattning

Laktoferrin är ett järnbindande protein med egenskaper som påverkar immunsystemet på ett positivt sätt, både genom att döda bakterier och verka anti-inflammatoriskt. Laktoferrin har lokaliserats i många kroppsvätskor såsom blod, tårar, svett och framförallt i bröstmjölk. En aktiv del av laktoferrin har lokaliserats till N-terminalen och man vill nu hitta en peptid som uppvisar samma egenskaper som laktoferrin men med högre effektivitet.
Målet är att hitta peptider som förhindrar sammanväxningar efter operationer, s.k. adhesioner, vilka kan ge upphov till kronisk smärta, nedsatt rörlighet av tarmar, infertilitet och komplikationer vid re-operationer. 59 peptider, baserade på den N-terminala delen av laktoferrin, med olika modifieringar designas och analyseras med avseende på egenskaper som anses relevanta för slutprodukten i fyra olika analyser; anti-inflammatorisk, fibrinolytisk, cytotoxisk och antimikrobiell.
Resultatet blev ett antal peptider som visade högre effektivitet än befintliga PharmaSurgics peptider för de testade egenskaperna. Vissa av dessa peptider ska undersökas vidare för att fastställa deras egenskaper ytterligare och se hur de uppför sig i djurstudier.

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Abbreviations

BSA	Bovine Serum Albumin
CD-14	Cluster of Differentiation 14
Chx	Cycloheximide
DMSO	Dimethyl-sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extra-Cellular Matrix
ELISA	Enzyme-Linked ImmunoSorbent Assay
FBS	Fetal Bovine Serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL-1β	Interleukin-1β
LPS	Lipopolysaccharide
LBP	LPS Binding Protein
PAI-1	Plasminogen Activator Inhibitor type 1
PBS	Phosphate Buffered Saline
PMA	Phorbol Myristate Acetate
SAR	Structural Activity Relationship
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor α
tPA	Tissue Plasminogen Activator

1. Introduction

When human body tissue is injured, due to surgery, trauma or infection, adhesions are very often formed (Ellis *et al* 1962). Adhesions are strands of scar tissue built-up by fibrin bands connecting tissue parts that normally are not attached (Holmdahl 1999). This phenomenon causes post-surgical complications such as chronic pain, decreased movement, bowel obstruction, infertility and re-operative complications, which all implicate big costs for society (Menzies *et al* 1990). The existing therapies on the market today are not good enough to substantially reduce these adhesions (Simmons *et al* 2005). But, PharmaSurgics is now developing a novel treatment for scar and adhesion prevention. PharmaSurgics has developed a number of first generation peptides that are good candidates for this mission, i.e. they fulfill a number of functions, such as being anti-inflammatory, antimicrobial, non-toxic in therapeutically relevant doses and fibrinolytic. The peptides are derived from the human protein lactoferrin, which is a glycoprotein naturally found in breast milk, blood and on mucosal surfaces of the body (Masson *et al* 1966) and plays an important role in human immune response system (Metz-Boutique *et al* 1984).

The multi-functional properties of the peptides derived from lactoferrin may open-up a wide field of applications, but the main-targets for PharmaSurgics' product are to prevent adhesions after flexor tendon (Figure 1a) and abdominal surgery (Figure 1b). Another field of interest in the future is topical application in wound healing. Due to frequent use of antibiotics there are many resistant and multi-resistant bacteria around us, this led to a search for new antimicrobial compounds with different mechanisms of action. The use of a product containing peptide might reduce these problems (Greathouse *et al* 2008; Bryers 2008).

a. b.

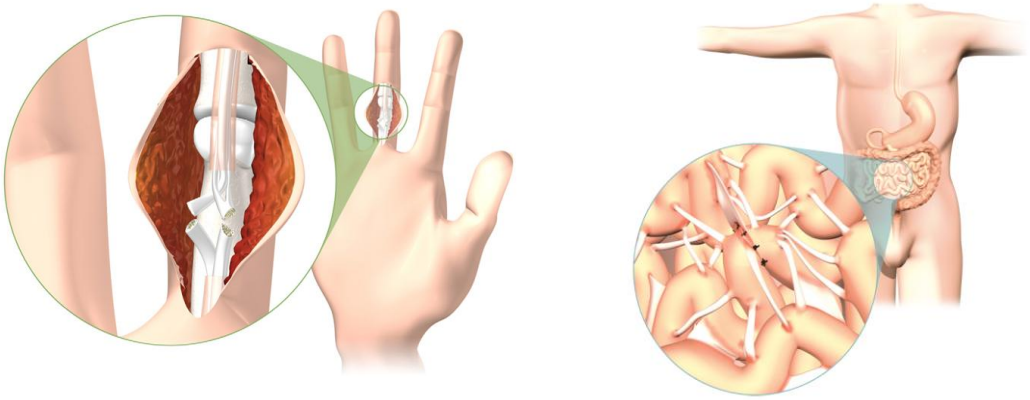

Figure 1: Main targets for PharmaSurgics products are to prevent adhesion formation after; a. flexor tendon injury and b. abdominal surgery. Adapted from PharmaSurgics with permission.

Through prior anti-inflammatory and fibrinolytic data on PharmaSurgics' first generation peptides followed by *in silico* analyses of their structure activity relationships, a group of 59 peptides with different modifications were designed and synthesized. The project assignment was primarily to screen these 59 peptides for their anti-inflammatory, fibrinolytic, cytotoxic and antimicrobial effects and to decide if there were any good candidates for a second generation of peptides with improved efficiency and safety variables. A screening project of this size has never been performed by PharmaSurgics before so the other part of the project was to design the study protocol, in a 96-well format, which could be used as a template for future screening studies.

2. Background

2.1. Lactoferrin

Lactoferrin is a cationic, multi-functional, iron-binding glycoprotein with antimicrobial and immunomodulatory properties (Puddu *et al* 2009; Britigan *et al* 2001).

Sörensen and Sörensen were the first to isolate lactoferrin from bovine milk in 1939 (Reviewed in Adlerova *et al* 2008) and lactoferrin was identified in human milk in 1951 (Goldman 2007). In 1960 it was determined that lactoferrin was the main iron-binding protein in human milk (Reviewed in Adlerova *et al* 2008) and later lactoferrin was also identified in a series of other external secretions. Lactoferrin was sequenced in 1984 (Metz-Boutigue *et al* 1984) and the 3D structure was solved in 1987 (Anderson *et al* 1987).

2.1.1. Occurrence

Lactoferrin is found in various exocrine secretions such as tears, blood, saliva, on mucosal surfaces and particularly in milk (Miller *et al* 2008). It is the second most abundant protein in human milk (Sun *et al* 1999), and the concentration varies between 7 g/l in colostrum and 1 g/l in mature milk (Sánchez *et al* 1992; Naidu and Naidu 2000).

The concentration of lactoferrin increases in all biological fluids during infection and inflammation, with the highest levels in the centre of infection (Britigan *et al* 2001). The protein is released from neutrophils into the serum through degranulation and hence the increase of lactoferrin seen during inflammation is due to this degranulation and not an up-regulation of lactoferrin biosynthesis (Ward *et al* 2002).

2.1.2. Structure and iron-binding

Lactoferrin, formerly known as lactotransferrin, is a single-chain glycoprotein with 703 residues (Hutchen and Lönnerdal 1997; Metz-Boutique *et al* 1984; Reviewed in Adlerova *et al* 2008). It is a bilobal protein with homologue N-and C-terminal halves. The two lobes have the same folding and contain two domains (Sun *et al* 1999; Metz-Boutique 1984; Anderson *et al* 1987); (Figure 2). The structure of lactoferrin is related to the serum iron-transport protein transferrin (Sanchez *et al* 1992; Anderson *et al* 1987; Miller *et al* 2008). Like other members of the transferrin family, lactoferrin weighs about 80 kDa and binds two Fe^{3+} ions reversibly with high affinity ($K_d \approx 10^{-20}$, approximately 300 times higher than transferrin) together with two CO_3^{2-}ions (Baker *et al* 2005; Hutchens and Lönnerdal 1997Miller *et al* 2008).

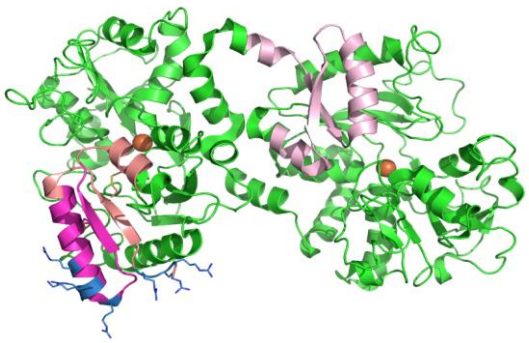

Figure 2: The ribbon-structure of lactoferrin. Orange beads represent the bound irons. Adapted from Protein Data Bank, pdb.org, PDB-code 1fck.

Lactoferrin is able to bind many other compounds beside iron, such as various surface molecules in cells (lipopolysaccharides, glycosaminoglycans) and other metal ions (Al^{3+}, Ga^{3+}, Mn^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+}); (Reviewed by Adlerova *et al* 2008; Legrand *et al* 2005).

2.1.3. Biological effects of lactoferrin

Lactoferrin displays pleiotropic immunomodulatory activities and many other biological functions related to the host immune defense system. The antimicrobial effect is the main benefit of lactoferrin and involves different mechanisms (Valenti and Antonini 2005; Masson *et al* 1966), e.g. blockade of microbial carbohydrate metabolism (Arnold *et al* 1982), destabilization of the bacterial cell wall (Valenti and Antonini 2005) and regulating monocyte/macrophage cytotoxic activity (Nishiya and Horwitz 1982; Duncan *et al* 1981; Mazurier *et al* 1989; Sánchez *et al* 1992).

The two main structural features contributing the biological effects of lactoferrin are the binding of iron (Fe^{3+}) and the properties of the basic N-terminal end.

The binding of iron reduces the formation of free-radicals, suppress tumor growth and prevents infections (Naidu and Naidu 2000). Since almost all bacteria are using iron as an essential growth nutrient the iron-binding property is important at sites of infection (Sánchez *et al* 1992; Reviewed Ward *et al*, 2002) to prevent the growth of microbes (Bullen *et al* 1972).

The basic N-terminal end is suggested to be responsible for the bactericidal function of lactoferrin, further commented in **2.1.4. Lactoferricin**

2.1.4 Lactoferricin

Lactoferricin is a cationic domain released from the basic N-terminus of lactoferrin during pepsin digestion (Figure 3). It consists of residue 1-47 of lactoferrin (Elass-Rochard *et al* 1995) with a surface exposed α-helix and a total weight of 5558 Da (Bellamy *et al* 1992).

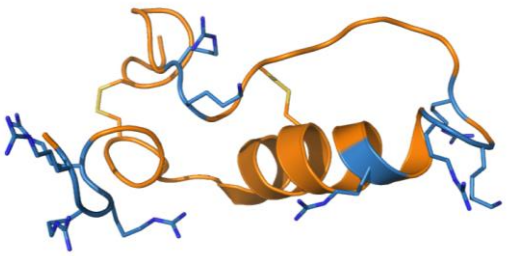

Figure 3: Structure of lactoferricin. Adapted from Protein Data Bank, pdb.org.PBD-code 1z6v.

Studies are suggesting that lactoferricin is the functional bactericidal part of lactoferrin (Tomita *et al* 2002). Lactoferricin has a more potent bactericidal activity than the intact native protein (Reviewed in Ward *et al* 2002; Tomita *et al* 2002) with a 2-fold better effectiveness against *E.coli* than undigested lactoferrin (Bellamy *et al* 1992). It is suggested that the microbicidal properties belong to the helix part of lactoferricin by its ability to interact with bacteria membrane and thereby destroying and killing the bacteria (Elass-Rochard *et al* 1995; Britigan *et al* 2001; Ellison and Giehl 1993).

Even though lactoferrin consists of two lobes with strong homology, no lactoferricin counterpart exists in the C-terminal (Bellamy *et al* 1992).

2.2. Wound healing

When the body is hurt due to surgery, trauma or infection the healing process starts instantly and continues for months up to years (Lorenz *et al* 2003). In most cases, at the end of the process an acellular and avascular scar is formed rather than a complete restoration of tissue and organ functions (Greenhalgh *et al* 1998).

The wound healing process is divided into three phases; Inflammatory phase, Proliferative phase and Maturation and Remodeling phase (Lorenz and Longaker 2003). The phases are not clearly separated but characterized by elaboration of cytokines and different growth factors secreted by specific cells (Lindholm 2003).

2.2.1. Haemostasis and Inflammatory Phase

The first phase of wound healing is the inflammatory phase that lasts for 3-4 days after trauma (Lindholm 2000). The first response in the wounded area is an intense contraction of the vessels to facilitate haemostasis as well as a coagulation process where trombocytes aggregate to form a clot and a fibrin plug arise (Martin 1997; Adlerova *et al* 2008). The trombocytes degranulate and release a multitude of growth factors that will attract cells to the wound area (Greenhalgh *et al* 1998). A parallel process to haemostasis is the fibrinolysis, which is a break-down process of the fibrin plugs, to facilitate cell migration and formation of new blood vessels in the area (Raftery 1979).

Neutrophils are the first cells to enter the wound area with the task to phagocyte debris, such as bacteria and damaged/dead tissue (Greenhalgh *et al* 1998). Neutophils will eventually be replaced by macrophages who become the predominant cell type in the wound area, the macrophages phagocytose the remaining debris as well as neutrophils. An important task for macrophages is also to release several growth factors and cytokines to prepare for new tissue (Greenhalgh *et al* 1998; Kiritsy *et al* 1993; Lindholm 2003).

2.2.2. Proliferative (Reconstitution) phase

The second phase is the proliferative phase also called the reconstitution phase, and it lasts for about 3-4 weeks (Greenhalgh *et al* 1998). The phase is initiated by macrophages releasing cytokines and growth factors which attract fibroblasts, myofibroblasts, new macrophages and endothelial cells, resulting in granulation tissue formation (Stadelmann *et al* 1998). Endothelial cells form new blood vessels and fibroblasts start to produce collagen and create a new extra-cellular matrix (ECM); (Holmdahl 1999).

Re-epithelialization takes place concurrently with inflammation and granulation tissue formation (Kiritsy *et al* 1993). The epithelial cells, including keratinocytes, migrate across the wound and will eventually create a protective layer.

The last step of the proliferative phase is to contract the wound and reduce its size. Contraction may last for several weeks and continues even after the wound is completely re-epithelialized (Stadelmann *et al* 1998).

2.2.3. Maturation and Remodeling phase

The last phase is the maturation and remodeling phase and it might last up to 2 years after wounding (Greenhalgh *et al* 1998). Collagen is degraded and the fibers are rearranged, cross-linked and aligned along tension lines (Lorenz and Longaker 2003), resulting in a stronger and remodeled type of collagen. The tensile strength increases and the wounded tissue might regain 80% of its original strength (Levenson *et al* 1965).

The result of a perfect wound healing process is a wound area which regains all strength and organ function with no sign of connective tissue parts (Martin 1997). This only occurs in fetus and sometimes in infants (Armstrong

and Ferguson 1995) and in all other cases an acellular and avascular scar appears with tensile and function loss (Greenhalgh *et al* 1998), i.e. an imperfect wound healing.

2.2.4 Excessive and prolonged Inflammation, Fibrinolysis and Proliferation

The wound healing process is based on a delicate balance between the cytokines and growth factors secreted during the different phases. If the inflammatory- or the proliferation phase is too pronounced or if the fibrinolysis is halted, new connective tissue is formed which increase the probability to form adhesions and scar tissue. Scar tissue is formed after surgery, not only topical but also inside the body. The formed fibrin and collagen bands connect tissue parts that normally are not attached (Kosaka *et al* 2008). These fibrin bands are called adhesions.

2.3. Post-operative Adhesions

Topical and internal wound healing processes are identical with the difference that inside the body tissues and organs lies close to each other and may be attached during the process. The attachments are scar tissue consisting of fibrin bands. These bands are called adhesions and usually arise due to wound healing process (Holmdahl 1999; Menzies *et al* 1990).

After surgery the fibrinolysis is disrupted and a fibrin structure is very often formed. If the fibrin is not degraded and dispersed, new blood vessels will arise in the fibrin web and when angiogenesis starts, the fibrin web is becoming a solid adherent (Ivarsson *et al* 1998).

Post-surgical adhesions often cause complications such as chronic pain, bowel obstruction, infertility, need of re-surgery and re-operative complications, which implicate big costs for society (Luijendijk *et al* 1996). Post-operative adhesions occur in 93% of all abdominal surgery (Menzies *et al* 1990; Menzies *et al* 1993; Ellis *et al* 1999; Holmdahl 1999) and adhesions are considered the main cause of small bowel obstruction (Menzies *et al* 2001).

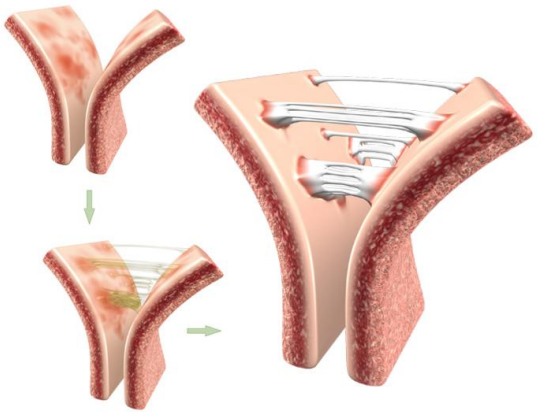

Figure 4: Adhesion formation. Adhesion formation between two tissue parts that normally are not attached. First an inflammation arises and the inflammatory process commence and fibrin is created. If the fibrin is not dispersed, firm adhesions will arise.
Adapted from PharmaSurgics with permission.

Prevention of adhesions is complex and involves many parameters, such as reduction of inflammation, break down of fibrin clots and prevention of bacteria to enter the site (Trew 2006; Olutoye 1996) and one action that is proved to decrease the formation of adhesions is the use of powder-free gloves, which is practiced every day in

clinical work (Dwivedi *et al* 2004). This action is not enough and today there are no pharmacologically active products in late clinical trials or on the market that are dealing with this. PharmaSurgics is developing a novel treatment which is expected to result in effective adhesion prevention. Hitherto several approaches have been evaluated, such as using anti-inflammatory agents (Yeo *et al* 2007), antibiotics (Hong *et al* 2008; Oncel *et al* 2001), and use of both chemical and physical barriers (Yaacobi *et al* 1996) but none of these has satisfactorily prevented the adhesion formation.

2.4. Mediators of inflammation and fibrinolysis and the role of lactoferrin

The inflammatory response is essential in wound healing and elimination of infections (Martin 1997; Roberts 1993). The inflammatory response may be triggered by pathogenic agents, such as lipopolysaccharide (LPS), or by pro-inflammatory signals, such as Interleukins (De Nardo *et al* 2009), which activate macrophages and stimulate the cytokine secretion and thereby initiate the inflammation (Stadelmann *et al* 1998). The initiated inflammation may trigger the adhesion formation.

2.4.1. Lipopolysaccharide, LPS

Lipopolysaccharide is the major component of the outer membrane of Gram negative bacteria (Stewart *et al* 2006) and is essential for growth and stability for the overall membrane structure (Naumann *et al* 1989). LPS is a bacterial endotoxin protein (Japelj *et al* 2005) and acts, together with LPS binding protein (LBP), by binding to the CD14/TLR-4/MD2-complex on macrophages (Andreakos *et al* 2004) and thereby initiating a cascade of host-mediated responses. The result is expression and secretion of cytokines, such as TNF-α and Interleukins, which leads to the inflammatory response (Andreakos *et al* 2004).

2.4.2. Tumor Necrosis Factor α, TNF-α

Tumor Necrosis Factor α (TNF-α) is a trans-membrane protein and a naturally occurring inflammatory cytokine in the body (Locksley *et al* 2001). It is produced by various cell types, such as macrophages, lymphocytes, endothelial cells and fibroblasts (Locksley *et al* 2001; Carswell *et al* 1975). The major inducer of TNF-α production is LPS, both *in vitro* and *in vivo* (Fiers *et al* 1991).

TNF-α is an important protector against parasitic, bacterial and viral infections (Fiers 1991) and its primarily role is to induce inflammation by regulation of immune cells to stimulate the recruitment of neutrophils and monocytes to the site of infection and activate them to abolish microbes (Idriss and Naismith 2000; Fiers 1991). A too high production of TNF-α causes a more pathogenic and harmful inflammation, it might even be fatal to the host (Fiers 1991).

2.4.3. Interleukin-1β, Il-1β

IL-1β is a cytokine involved in the inflammatory response against infection (De Nardo *et al* 2009). It is an endocrine substance produced by macrophages, neutrophils, epithelial and endothelial cells (Elass-Rochard *et al* 1998).

During infection IL-1β production rises and enters the blood stream. It will increase the expression of factors which promote an attachment between blood neutrophils and monocytes to the endothelium at the site of inflammation (Abbas *et al* 2007). One step in this process is the IL-1β activation of trombocytes whereby the

trombocytes undergo a conformational change from round smooth balls to sticky balls that attach to each other and form a fibrin plug (Oluyinka *et al* 1997). The fibrin formation is important in wound healing but also the first step in adhesion formation (Kucuk *et al* 2007).

2.4.4. Plasminogen Activator Inhibitor type 1, PAI-1

Plasminogen Activator Inhibitor type 1 (PAI-1) is produced by endothelial cells, liver and adipose tissue and the expression of PAI-1 is regulated and modulated by cytokines and growth factors (McMahon and Kwaan 2007/2008).

The fibrinolysis is based on the balance between the tissue Plasminogen Activators (tPA) and PAI-1, where PAI-1 is the main inhibitor of tPA (Elokdah *et al* 2004). tPA is an activator of fibrinolysis by transforming plasminogen into plasmin (Sulaiman *et al* 2002). An increase of PAI-1 will decrease the amount of active tPA and thereby decrease fibrinolysis and facilitate the formation of adhesion (Kosaka *et al* 2008).

2.4.5. The role of lactoferrin and the peptides

Lactoferrin has been reported to play a pivotal role in the antimicrobial activity, partly by binding iron (essential nutrient for bacteria); (Valenti *et al* 2005) and partly by binding other bacterial structures in the N-terminal part, such as LPS (Samuelsen *et al* 2004). This will modify the permeability barriers in the membrane and cause microbial cell injury and bacterial cell death (Britigan *et al* 2001; Ellison and Giehl 1993).

Neither the anti-inflammatory effects of lactoferrin nor the peptides are clearly surveyed but probably show anti-inflammatory properties by binding various structures, such as LPS and membranes (Legrand *et al* 2005). One suggestion is that lactoferrin inhibits the cytokine production, such as TNF-α and Interleukins, by competing with LPS binding protein (LBP) for the binding to LPS and thereby inhibits the binding to CD-14 on the surface of macrophages; the result is an inhibition of the inflammatory response (Håversen *et al* 2002). It is also suggested that lactoferrin enters the nucleus where it binds to DNA at three specific consensus sequences and thereby suppresses cytokine transcription (Håversen *et al* 2002). The PharmaSurgics' peptides are based on the N-terminal part of lactoferrin (Lactoferricin) and lack the iron-binding residues and will probably show effect by binding various structures as mentioned above.

The role of lactoferrin and the peptides in fibrinolysis is not examined but desirable.

2.5. Aim, Purpose and Strategy of the project

PharmaSurgics has previously identified peptides which have pronounced anti-adhesion activities. The candidate peptides fulfill the following properties;

- Anti-inflammatory
- Fibrinolytic
- Antibacterial against a wide spectrum of bacteria, including bacteria resistant to conventional antibiotics
- Non-toxic in therapeutically relevant doses
- Short sequences which result in easy, inexpensive manufacturing
- Protected by approved patent in both EU and the US

Based on earlier data on a limited number of PharmaSurgics' peptides, a peptide library was designed by SARomics Biostructures, a Lund based company specialized on structural activity relationships (SAR). The library consisted of 59 peptides in the range of 11 to 25 residues and six groups of modifications were defined;

- ***N-cap (Increase helix stability)***
  Helix content and stability of the peptide could be increased by insertion of preferred amino acids to the N-terminal boundary of helix/peptide.
- ***Leucine Spacing (Increase helix stability)***
  Spacing *i*, *i* + 3 or *i*, *i* + 4 between leucines is known to stabilize helices, *i* is the position of leucine.
- ***Perfect and imperfect amphipathicity***
  A perfect amphipathic helix has hydrophobic residues on one side and polar/hydrophilic residues on the other side, while in case of imperfect amphipathicity single amino acids are interrupting this organization. Amphipathicity is known to affect the ability of the peptide to interact with a biological membrane (Pérez-Payá *et al* 1995).
- ***Increase of positive and hydrophobic regions***
  Hydrophobicity affects membrane interactions.
  A relatively high net charge has been identified as important factor for antimicrobial activity (Pasupuleti *et al* 2008).
- ***Turn-like structure which interrupts helix structure***
  An antimicrobial and LPS-binding peptide with similar sequence as PharmaSurgics' peptides.
  Adopts a turn-like structure when bound to LPS and do not posses an α-helix (Japelj *et al* 2005).
- ***Variation of PharmaSurgics' peptides***
  Small modifications were introduced into previously known PharmaSurgics' peptides to investigate if this would improve their characteristics.

The purpose of this screening project is to understand the structure activity relationship (SAR) for lactoferrin-derived peptides and the aim was to find new candidates with improved characteristics compared to the first generation of peptides. Four different assays are performed to evaluate the efficacy and safety of the peptides;

- Anti-inflammatory Assay
- Fibrinolytic Assay

- Cytotoxic Assay
- Antimicrobial Assay.

3. Material and methods

3.1. The Peptides

The 59 peptide sequences were designed by SARomics (SARomics Biostructures, Lund, Sweden) and synthesized, without purification steps, from Sigma-Aldrich (St. Louis, MO, USA).

The peptide content of the 59 screened peptides was unknown and their purity depended on the length where average purity of 10mers ~86%, 15mers ~73%, 20mers ~61%. Among the 59 peptides were three peptide controls; Peptide 16, Peptide 50 and Peptide 52, with identical sequences to an existing peptide at PharmaSurgics, PeptideA. The peptide controls and thereby all the 59 peptides were compared to two internal controls of PeptideA purchased from Bachem (Bachem AG, Bubendorf, Switzerland) and Biopeptide (Biopeptide Co., San Diego, CA, USA). The two PeptideA from Bachem and Biopeptide had a higher and known purity and peptide content compared to the 59 peptides and were end-capped. Comparing the peptide controls with the internal controls will (1) facilitate comparison between the modified peptides and existing peptides at PharmaSurgics (2) control if the intra variation of the peptides from Sigma-Aldrich and (3) control the reliability of the methods used and if the peptides behave as PharmaSurgics are used to.

Peptides were dissolved in H_2O to a concentration of 3.2 mM and were centrifuged to avoid uneven results due to dissolved grains of peptide. The peptide solutions were aliquoted in Eppendorf-tubes and kept frozen in -20ºC until used. Although purity and peptide contents varied all the concentration calculations were based on the assumption of 100% peptide content.

3.2. Screening for anti-inflammatory effect in THP-1 cells

A monocytic cell line, THP-1, was used and differentiated with Phorbol Myristate Acetate (PMA) into macrophage-like cells. The cells were stimulated with lipopolysaccharide (LPS) in a concentration selected based on earlier *in vitro* experiments to give a maximum release of cytokines after six hours.

Cycloheximide (Chx) was used as a positive control and will decrease the secretion of TNF-α. Chx is an inhibitor of translation of all protein synthesis in eukaryote cells (Beyaert and Fiers 1994).

All the peptides were screened in this assay and in two concentrations; 40 µM or 320 µM.

3.2.1. Cultivation and seeding of the THP-1 cells

The THP-1 cell line (TIB-202; ATCC, Manassas, VA**,** USA) was maintained in Culture Medium [RPMI 1640 medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria), 1 mM Sodium Pyruvate (Sigma-Aldrich, St. Louis, MO, USA) and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; PAA Laboratories GmbH, Pasching, Austria)]. The cells were kept in tissue culture flasks (Sarstedt, Nümbrecht, Germany) in a humidified incubator at 37 ºC and 5% CO_2. The cells were sub-cultured twice a week to an initial cell density of 200.000 cells/ml.

THP-1 cells were centrifuged and cell density was adjusted to 10^6 cells/ml by adding new RPMI 1640 medium to. Cells were treated with Phorbol 12-myrisate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) and 100 µl cell suspension with 10 ng/ml PMA was added to each well in a 96-well cell culture plate (Sarstedt, Nümbrecht, Germany).

Plate was incubated for 48 hours at 37 ºC.

3.2.2. Stimulation of THP-1 cells

The cells were washed with AssayMedium (Culture Medium with 10% fetal bovine serum (FBS) exchanged to 5% heat inactivated FBS (PAA Laboratories GmbH, Pasching, Austria)). Cells were stimulated with 90 µl AssayMedium with a final concentration of 0.1 ng/ml LPS (*E. coli* serotype O55:B5; Sigma-Aldrich, St. Louis, MO, USA).

The plate was incubated for 30 minutes at 37 ºC.

The final peptide concentrations used were 40 µM or 320 µM and added in triplicates, i.e. 10 µl of 400 µM or 3.2 mM per well.

Three controls were used in quadruplicate; (1) unstimulated cells treated with just Assay Medium, giving a basal-level (2) cells stimulated with LPS (*E. coli* serotype O55:B5; Sigma-Aldrich, St. Louis, MO, USA), final concentration of 0.1 ng/ml but not treated with peptide and (3) LPS-stimulated cells treated with Cycloheximide (Chx, Sigma-Aldrich, St. Louis, MO, USA), final concentration of 50 ng/ml.

The plate was incubated for 6 hours at 37 ºC after stimulation with LPS.

The cell supernatants were collected and transferred to a 96-cone bottom plate (Nunc, Roskilde, Denmark) and centrifuged at 1500 rpm for 6 minutes, and transferred to a new 96-cone bottom plate.

Supernatants were kept frozen in -20 ºC until analyzed for TNF-α secretion by ELISA (R&D Systems, Minneapolis, MN, USA).

3.2.3. TNF-α Enzyme Linked ImmunoSorbent Assay, ELISA

An Enzyme-Linked ImmunoSorbent Assay (ELISA; R&D Systems, Minneapolis, MN, USA) was performed according to manufacturing protocol to measure the amount TNF-α secreted from the cells.

In short;

- A PolySorb Micro 96-well (Nunc, Roskilde, Denmark) plate was coated with Monoclonal Antibody against TNF-α (MAb; R&D Systems, Minneapolis, MN, USA) and left overnight in darkness and RT.
- The plate was washed and blocked with 1% Bovine Serum Albumin (BSA; PAA Laboratories GmbH, Pasching, Austria) and incubated for one hour.
- The plate was washed, standard and samples were added, incubated for 2 hours in RT.
- The plate was again washed and Biotinylated Antibody against TNF-α (BAb; R&D Systems, Minneapolis, MN, USA) was added. The plate was incubated for 2 hours.
- The plate was washed and ExtrAvidin-alkaline phosphatase (Sigma-Aldrich St. Louis, MO, USA) solution was added and plate incubated for 20 minutes in RT.
- The plate was washed and phosphatase-substrate solution (Sigma-Aldrich St. Louis, MO, USA) was added. Then the absorbance, OD_{405}, was read every 30 minutes until the absorbance reached $OD_{405} \approx 1.2$.

3.3. Screening for fibrinolytic effect in MeT-5A cells

A human mesothelial cell line, MeT-5A, was used. The cells were stimulated with IL-1β, at a concentration selected based on earlier *in vitro* experiments to give a maximum release of cytokines after six hours. Cycloheximide (Chx) was used as positive control.

All the peptides were screened in this assay but just the higher concentration of peptide was used, 320 µM.

3.3.1. Cultivation and Seeding of MeT-5A cells

The MeT-5A cell line (CRL-9444; ATCC, Manassas, VA**,** USA) was maintained in Culture Medium [M199 medium (Invitrogen, Paisley, UK) supplemented with 10% FBS (PAA Laboratories GmbH, Pasching, Austria), 1 M HEPES (PAA, Laboratories GmbH, Pasching, Austria), 10 mg/ml Hydrocortisone (MP Biomedicals, Irvine, CA, USA), 10 mg/ml Insulin (Sigma-Aldrich, St. Louis, MO, USA) and 1 mg/ml Epidermal Growth Factor (EGF; ImmunoKontact; AMS Biotechnology, Oxon, UK)]. The cells were kept in tissue culture flasks (Sarstedt, Nümbrecht, Germany) in a humidified incubator at 37 ºC and 5 % CO_2 and sub-cultured twice a week to an initial cell density of 30.000-60.000 cells/ml.

MeT-5A cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich, St. Louis, MO, USA) and treated with Trypsin (Invitrogen, Paisley, UK) to release adherent cells. The cell suspension was centrifuged and cell density was adjusted to 160.000 cells/ml by adding new Culture Medium. 100 µl of cell-suspension was added to each well in a 96 tissue culture flat well plate (Sarstedt, Nümbrecht, Germany).

The plate was incubated for 48 hours at 37 ºC.

3.3.2. Stimulation of MeT-5A cells

The cells were washed with Assay Medium (Culture Medium with 10% fetal bovine serum (FBS) exchanged to 5% heat inactivated FBS (PAA Laboratories GmbH, Pasching, Austria)). Cells were stimulated with 90 µl Assay Medium with a final concentration of 0.10 ng/ml IL-1β (R&D Systems, Minneapolis, MN, USA).

The final peptide concentration used was 320 µM and added in duplicates, i.e. 10 µl of 3.2 mM per well. Three controls were used; (1) unstimulated cells treated with just Assay Medium, giving a basal-level (2) cells stimulated with Interleukin-1β (IL-1β; R&D Systems, Minneapolis, MN, USA), final concentration of 0.1 ng/ml but not treated with peptide and (3) IL-1β-stimulated cells treated with Cycloheximide (Chx, Sigma-Aldrich, St. Louis, MO, USA), final concentration of 50 ng/ml.

The plate was incubated for 6 hours at 37 ºC after stimulation with IL-1β.

The cell supernatants were collected and transferred to a 96-cone bottom plate (Nunc, Roskilde, Denmark) and centrifuged at 1500 rpm for 6 minutes, transferred to a new 96-cone bottom plate.

Supernatants were kept frozen in -20 ºC until analyzed for PAI-1 production by ELISA (Trinity Biotech plc, Bray, Ireland).

3.3.3. PAI-1 Enzyme-Linked ImmunoSorbent Assay, ELISA

An Enzyme-Linked ImmunoSorbent Assay (ELISA; Trinity Biotech plc, Bray, Ireland) was performed according to TintElize PAI-1 protocol to measure the amount PAI produced.

In short;

- 100 µl PET-buffer (Trinity Biotech plc, Bray, Ireland) was added to each well of the ELISA plate and agitated for 1 minute.
- Standards and samples were added to both columns in one strip.
- Conjugate solution (Trinity Biotech plc, Bray, Ireland) was added to each well and the plate was incubated for 2 hours; agitated at 500-600 rpm.
- The strips were washed four times with PET-buffer and substrate was added; the plate was incubated for 15 minutes and agitated in RT.
- STOP solution (H_2SO_4) was added to each well and stored for 10 minutes, in darkness and RT.

- Absorbance was read at 492 nm.

3.4. Screening for cytotoxic effect in THP-1 cells

A monocytic cell line, THP-1, was used. The cells were stimulated with lipopolysaccharide (LPS). Triton-X 10% was used as positive control since it induces cell lysis (Pasupuleti *et al* 2008) and thereby reduces the amount of viable cells.

A selection of 29 peptides, based on the results from anti-inflammatory and fibrinolytic assay, was screened in the two peptide concentrations, 40 µM or 320 µM.

3.4.1. Cultivation and seeding of cells

Performed in the same way as described above under **"3.2.1. Cultivation and seeding of THP-1 cells***".*

3.4.2. Stimulation of THP-1 cells

Performed in the same way as described above under **"3.2.2. Stimulation of THP-1 cells"** with the exception that Triton-X 10% (ICN Biomedicals Inc, OH, USA) was used instead of Cycloheximide (Chx, Sigma-Aldrich, St. Louis, MO, USA) as a positive control and both peptides and controls were added in triplicates.

3.4.3. TACS MTT Assay

After the 6 hours of incubation, a MTT assay was performed according to the manufacture's instructions to measure the viability of the cells;

- 10 µl 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; R&D Systems, Minneapolis, MN, USA) was added to each well.
  Plate was incubated for 2 hours at 37 ºC.
- 100 µl Detergent Reagent (R&D Systems, Minneapolis, MN, USA) was added to each well.
  The plate was incubated over night at RT and embedded in foil.
- The plate was agitated for 10 minutes at 150 rpm before reading.
- The absorbance at 570 nm with a reference wavelength of 650 nm was monitored.

3.5. Screening for antimicrobial effect in S.aureus bacteria

The antimicrobial effect of the peptides against *Staphylococcus aureus* (*S. aureus*, #1800; CCUG, Göteborg, Sweden) was determined by MMC_{99} (Minimal Microbicidal Concentration) which is the concentration where 99% of bacteria are killed. All the peptides were screened in this assay.

3.5.1. Antimicrobial Assay

In short;

- Bacteria were grown on blood agar plates [Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated horse blood (National Veterinary Institute (SVA), Uppsala, Sweden)], a few colonies were transferred and cultured in 10 ml Brain Heart Infusion medium (BHI; Difco, BD Diagnostics, Franklin Lakes, NJ, USA) and incubated over night at 250 rpm at 37 ºC.
- 1 ml from the over-night culture was transferred to 10 ml fresh BHI medium and incubated for two hours at 250 rpm at 37 ºC to reach log-phase growth.

- The bacteria were centrifuged and the pellet was re-suspended in 1 ml fresh BHI-100 (BHI diluted 1:100 in H_2O). This bacteria suspension was diluted 1:40 and adjusted to give an OD_{600} = 0.125, which corresponds to $2*10^8$ bacteria/ml, and thereafter diluted 1:20 to get a concentration of 10^7 bacteria/ml.
- The peptides were serially diluted from 160 to 1.25 µM in BHI-100 in a 96-well culture plate (Nunc, Roskilde, Denmark). 100 µl of the dilutions were mixed with 5 µl bacteria suspension in a new 96-well culture plate (Nunc, Roskilde, Denmark). The plate was incubated for two hours at 37 ºC.
- 5 µl of the suspension was aspirated from each well and added as a drop onto blood agar plates [Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated horse blood (National Veterinary Institute (SVA), Uppsala, Sweden)], and incubated over-night at 37 ºC.

4. Results

4.1. Screening for anti-inflammatory effect in THP-1 cells

The purpose of this assay was to examine the anti-inflammatory properties of the peptides, i.e. to see how effectively the peptides decreased the secretion of TNF-α from LPS stimulated macrophage-like cells, THP-1. The screening included all the 59 peptides at the two concentrations, 40 µM and 320 µM.

Screening was performed in 96-well format and the stimulation-level (cells treated with LPS only) was set to 100% and all the values were compared to this level. The basal-level (cells treated with medium without LPS) was close to 0%.

To determine the quality of the peptide library the peptide controls of PeptideA (peptide 16, 50 and 52 from SIGMA) were compared to the internal controls of PeptideA (from Bachem and Biopeptide). The peptide controls and internal controls showed similar but varying values for both concentrations of peptide, still they were in the range of assay acceptance (Figure 5a and b).

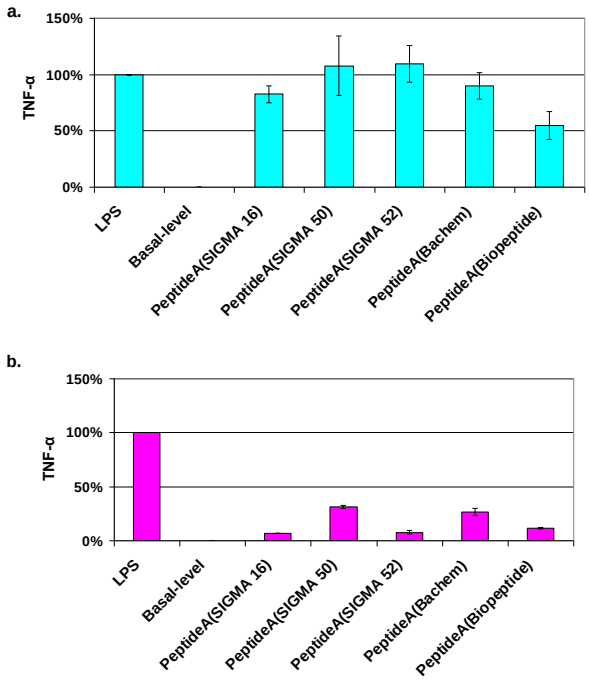

Figure 5: Determination of quality of peptide library in anti-inflammatory assay. THP-1 cells were differentiated into macrophage-like cells with PMA, stimulated with LPS and treated with peptide. The peptides ability to decrease TNF-α secretion was examined in an ELISA assay. Stimulation-level (cells stimulated with 0.1 ng/ml LPS, no peptide) was set to 100% corresponding to an approximate conc. of 403 pg/ml secreted TNF-α. Basal-level (unstimulated, no peptide) was 0%. The different *PeptideA* (stimulated with 0.1 ng/ml LPS, treated with peptide in a total concentration of 40 µM (a) and 320 µM (b), were added in triplicate). Values are mean values ± SEM.

The relative down-regulation for all the peptides, except PeptideA, of produced TNF-α showed in both high and low concentration, in Figure 6 (in tabularized form in Appendix 5). The peptides were divided into the six groups of modifications and the results were as follows;

- *N-cap* (1). All the peptides resulted in an increase of TNF-α, i.e. peptides were pro-inflammatory, with an obvious dose-response.
- *Leucine spacing* (2). All the peptides (except two) showed an anti-inflammatory effect in a dose-response fashion. A number of peptides had values close to zero, i.e. total quenching of TNF-α secretion.
- *Increase positive charge and hydrophobic groups* (3). The peptides showed an anti-inflammatory effect with over-all low values in both concentrations, without a clear dose-response.

- *Perfect/imperfect amphipathicity* (4). All the peptides at the higher concentration, except two, showed anti-inflammatory properties and a clear dose-response. A set of peptides showed very effective anti-inflammatory properties and decreased the secretion down to the basal-level.
- *Turn-like structure which interrupts helix structure* (5). The peptides showed pro-inflammatory properties with a clear dose-response.
- *Variation of PharmaSurgics' peptides* (6). Almost all peptides showed an anti-inflammatory dose-response to varying extent.

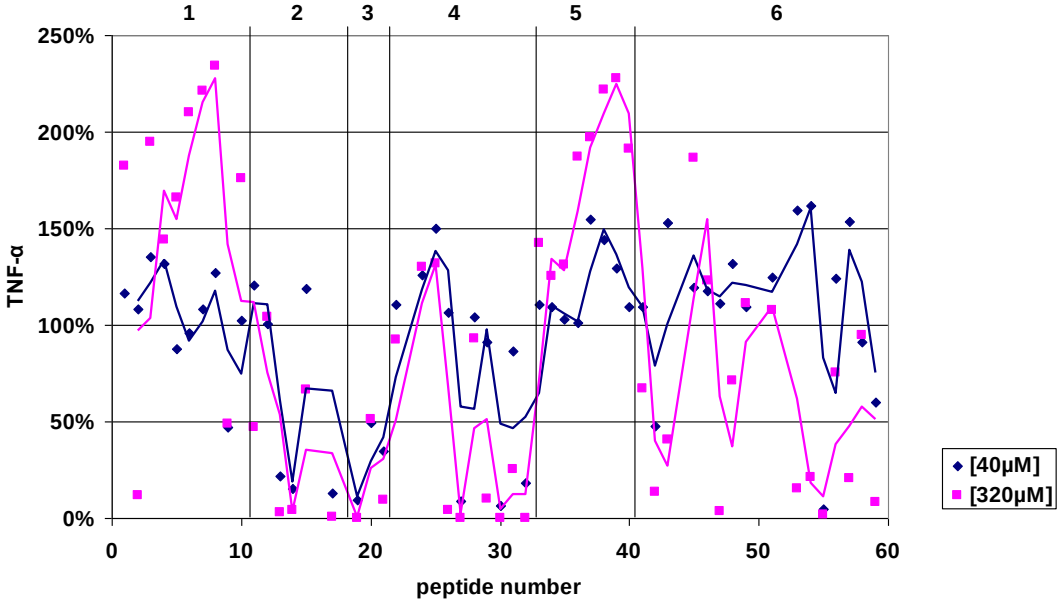

Figure 6: Relative TNF-α secretion from stimulated THP-1 in Anti-inflammatory assay, THP-1 cells were differentiated into macrophage-like cells with PMA, stimulated with LPS and treated with peptide (40 µM ◆ or 320 µM ■). The peptides ability to decrease TNF-α production was examined in an ELISA assay. Stimulation-level (cells stimulated with 0.1 ng/ml LPS, no peptide) was set to 100% corresponding to an approximate conc. of 403 pg/ml secreted TNF-α. Basal-level (unstimulated, no peptide) was 0%.
The peptides were divided into the six groups of modifications; (1) N-cap, (2) Leucine Spacing, (3) Increase positive charge and hydrophobic groups, (4) Perfect and imperfect amphipathicity, (5) Turn-like structure which interrupts helix structure and (6) Variation of PharmaSurgics' peptides. All the 59 peptides were screened, excluding PeptideA, and added in triplicate. Values are mean-values.
All the points were connected with the algorithm gliding mean-value with a period of 2.

4.2. Screening for fibrinolytic effect in MeT-5A cells

The purpose of this assay was to examine the fibrinolytic effect of the peptides, i.e. see how effectively the peptides decrease the production of PAI-1 in IL-1β stimulated MeT-5A cells. The screening included all the 59 peptides at the higher concentration of 320 µM.

The screening was performed in 96-well format and the stimulation-level (cells treated with IL-1β only) was set to 100% and all the peptide values were compared to this level. The basal-production of PAI-1 (cells treated with medium without IL-1β) was approximately 61%.

To determine the quality of the peptide library the three PeptideA from SIGMA were compared to the internal controls of PeptideA. They all showed similar values and the controls were within assay variations (Figure 7).

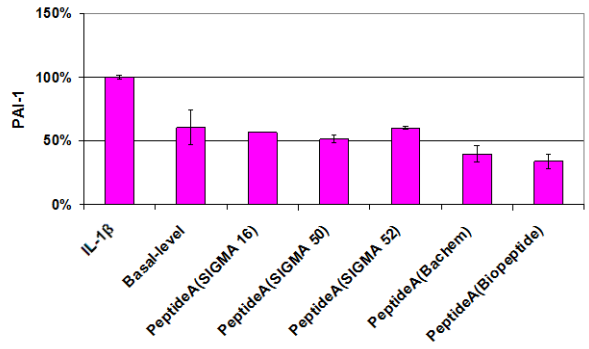

Figure 7: Determination of quality of peptide library in fibrinolytic assay. MeT-5A cells were stimulated with IL-1β and treated with peptide. The peptides ability to decrease PAI-1 production was examined in an ELISA assay. Stimulation-level (cells stimulated with 0.1 ng/ml IL-1β, no peptide) was set to 100%, corresponded to an approximate conc. of 103 pg/ml produced PAI-1. Basal-level (unstimulated cells, no peptide) was 60.73% of produced PAI-1.
Different *PeptideA* (cells stimulated with 0.1 ng/ml IL-1β, treated with peptide 320 µM, added in triplicate). Values were mean values ± SEM.

The relative down-regulation for all peptides, except PeptideA, of produced PAI-1 compared to the stimulation-level, i.e. IL-1β only, (Figure 8 and in tabularized form in Appendix 6) was over-all effective. The peptides were divided into the six groups of modifications and the broken line represents the basal-level of PAI-1 production.
All peptides resulted in a decrease of PAI-1 secretion compared to LPS-level with a number of peptides in each group, except one group, decreased the PAI-1 secretion below basal-level.

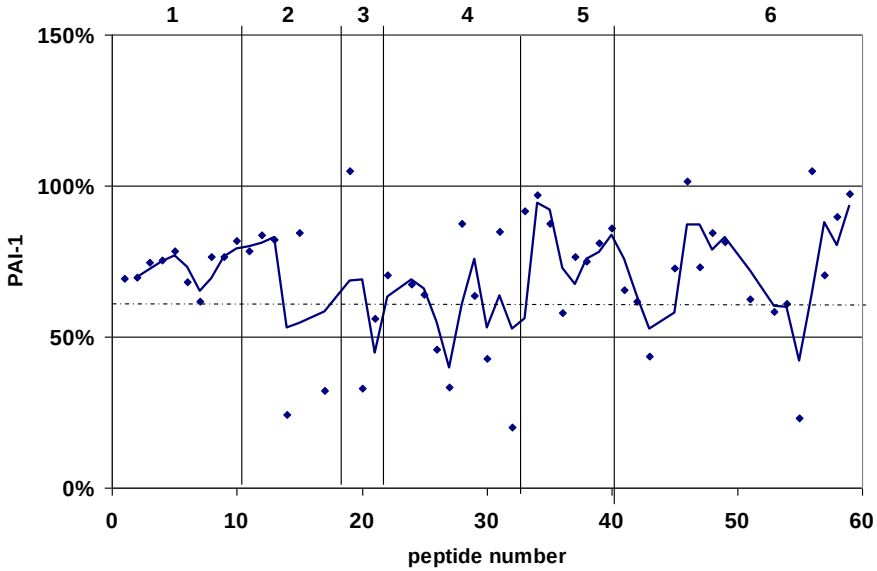

Figure 8: The relative PAI-1 production of stimulated MeT-5A cells in fibrinolytic assay. MeT-5A cells were stimulated with 0.1 ng/ml IL-1β and treated with peptide (320 µM◆). The peptides ability to decrease PAI-1 production was examined in an ELISA assay. Stimulation-level (cells stimulated with 0.1 ng/ml IL-1β, no peptide) was set to 100% corresponding to a mean-value of 103 pg/ml produced PAI-1. Basal-level (unstimulated cells, no peptide) was 61%.
The peptides were divided into the six groups of modifications; (1) N-cap, (2) Leucine Spacing, (3) Increase positive charge and hydrophobic groups, (4) Perfect and imperfect amphipathicity, (5) Turn-like structure which interrupts helix structure and (6) Variation of PharmaSurgics' peptides. Values are mean-values.
All the points were connected with the algorithm gliding mean-value with a period of 2.

4.3. Screening for cytotoxic effect in THP-1 cells

The purpose of this assay was to examine the cytotoxic effect of the peptides, i.e. to examine the viability of THP-1 cells when treated with peptide. The screening included a selection of 29 peptides, the selection was based on results from anti-inflammatory and fibrinolytic assay where the most effective peptides were selected.
The screening was performed in 96-well format and the stimulation-level (cells treated with LPS only) was set to 100% corresponding to total viability and the Triton-X level was set to 0%, i.e. close to total killing of cells.
To determine the quality of the peptide library the PeptideA from SIGMA were compared to the internal controls of PeptideA. The lower concentration of the peptide controls show similar values as the internal controls, high viability, and the controls were within assay variations (Figure 9a.). At the higher concentration of peptide the

peptide controls have considerably lower viability compared to internal controls and the variations have no current explanation (Figure 9b.).

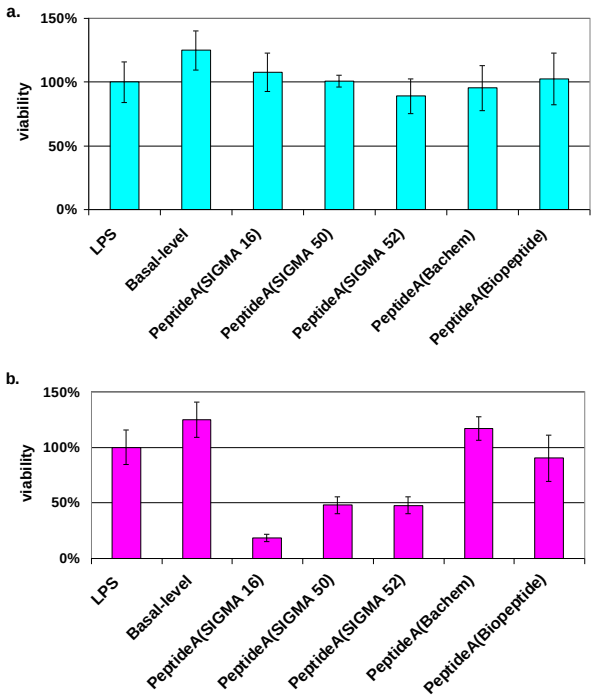

Figure 9: Determination of quality of peptide library in cytotoxic assay. THP-1 cells were differentiated into macrophage-like cells with PMA, stimulated with LPS and treated with peptide. The viability of cells was examined in an MTT assay. The stimulation-level (cells stimulated with 0.1 ng/ml LPS, no peptide) was set to 100% = total viability. Basal-level (unstimulated, no peptide) was 0% = no viability.
The different *PeptideA* (cells stimulated with 0.1 ng/ml LPS, treated with peptide, in total concentrations of 40 µM (a.) and 320 µM (b.), were added in triplicate). Values were mean values ± SEM.

The relative viability of all peptides, except PeptideA, in THP-1 cells when treated with peptide, both high and low concentration is shown in Figure 10 (in tabularized form in Appendix 7). The selection made resulted in a small number of peptides in each group which made conclusions about the modifications contribution to the results hard to draw. But still the peptides were divided into the groups of modifications and the results were as follows;

- *N-cap* (1). High viability in both concentrations of peptide.
- *Leucine spacing* (2). A divided group; two of peptides were highly cytotoxic and the other peptides resulted in high viability. A dose-response may be discerned in this group.
- *Increase positive charge and hydrophobic groups* (3). The peptides were cytotoxic to the cells at high concentration, but the lower concentration of peptide did not show any cytotoxicity.
- *Perfect/imperfect amphipathicity* (4). The peptides in the lower concentration resulted in high viability. The peptides show obvious cytotoxic effects in the higher concentration of peptide.
- *Turn-like structure which interrupts helix structure* (5). None of the screened peptides showed cytotoxicity in any concentration.
- *Variation of PharmaSurgics' peptides* (6). All the peptides resulted in high viability in the lower concentration. The viability of the cells treated with the higher concentration of peptide was lower and only two peptides showed high viability.

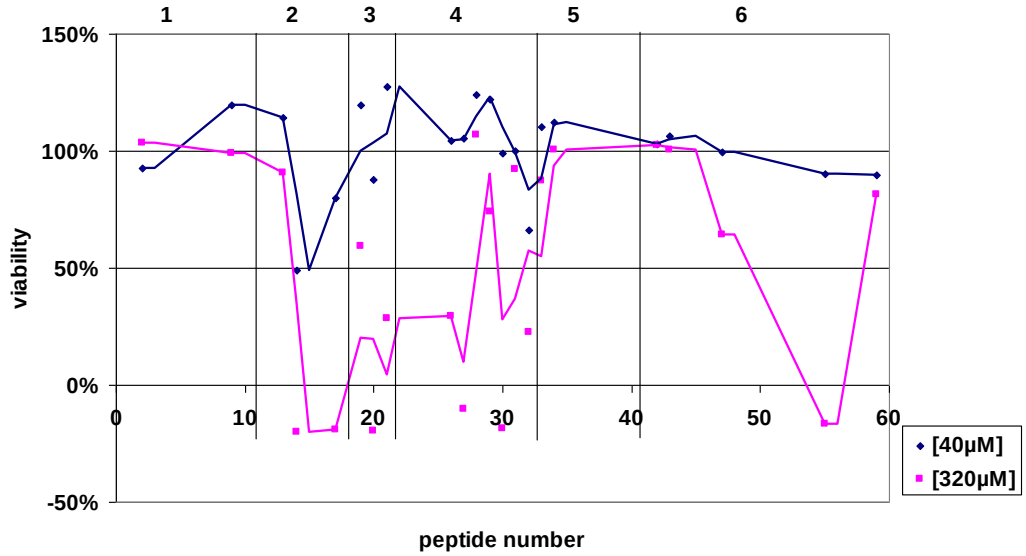

Figure 10: The relative viability of THP-1 cells when treated with peptide. THP-1 cells were differentiated into macrophage-like cells with PMA, stimulated with LPS and treated with peptide (40 µM ◆ or 320 µM ■). The viability of cells was examined in an MTT assay. Total viability (cells stimulated with 0.1 ng/ml LPS, no peptide) was set to 100%. Basal-level (unstimulated, no peptide) was 0%.
Peptides were divided into the six groups of modifications; (1) N-cap, (2) Leucine Spacing, (3) Increase positive charge and hydrophobic groups, (4) Perfect and imperfect amphipathicity, (5) Turn-like structure which interrupts helix structure and (6) Variation of PharmaSurgics' peptides. All 59 peptides are shown, excluding PeptideA and added in triplicate. Values are mean-values
All the points were connected with the algorithm gliding mean-value with a period of 2.

4.4. Screening for antimicrobial effect on S.aureus

The purpose was to examine the antimicrobial effect of the peptides, i.e. find the concentration where peptide kills 99% of added *Staphylococcus aureus*, in an antimicrobial assay. All the 59 peptides were serial diluted from 160 µM down to 1.25 µM and screened in this assay together with the internal controls (PeptideA).

To determine the quality of the peptide library the PeptideA from SIGMA were compared to the internal controls of PeptideA. All the variants of PeptideA showed similar values, separated by one dilution step which was within the assay variation (Figure 11).

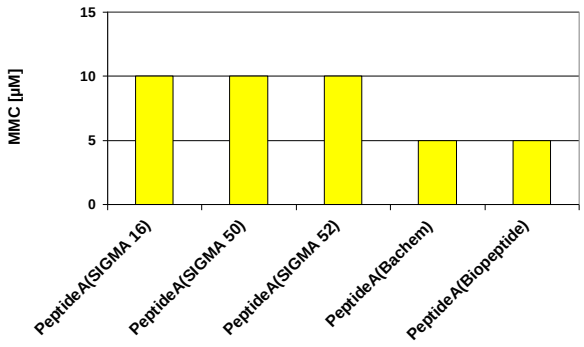

Figure 11: Determination of quality of peptide library in antimicrobial assay. Peptides serial diluted; 160 µM to 1.25 µM, 5µl peptide incubated with 100 µl 10^7 bacteria/ml *Staphylococcus aureus*, applied on blood agar plates and incubated over night. The MMC_{99} was found at concentration where peptide killed 99% of bacteria.

The MMC_{99} values for all the peptides, except PeptideA, on *S.aureus* from the antimicrobial assay are present in Figure 12 (in tabularized form in Appendix 8).

The over-all result was that the peptides were either very effective and killed bacteria at low peptide concentrations or they did not kill any bacteria at any tested peptide concentration. The peptides were divided into the six groups of modifications and the results from the antimicrobial assay were as follows;

- *N-cap* (1). Just two of the peptides showed microbicidal effect at the highest peptide concentration and the others did not kill any bacteria at any level (all the peptides set to ≥160µM).

- *Leucine spacing* (2). A divided group where three peptides were very effective and the other three did not show any bacteria-killing effect in the concentrations tested.
- *Increase positive charge and hydrophobic groups* (3). A group with effective peptides, all peptides showed low MMC_{99} values.
- *Perfect/Imperfect amphipathicity* (4). A group with consistently low MMC_{99}-values.
- *Turn-like structure which interrupts helix structure* (5). A divided group with one part of the peptides was microbicidal effective and the other part was not.
- *Variation of PharmaSurgics' peptides* (6). Consistently low values with a couple of exceptions.

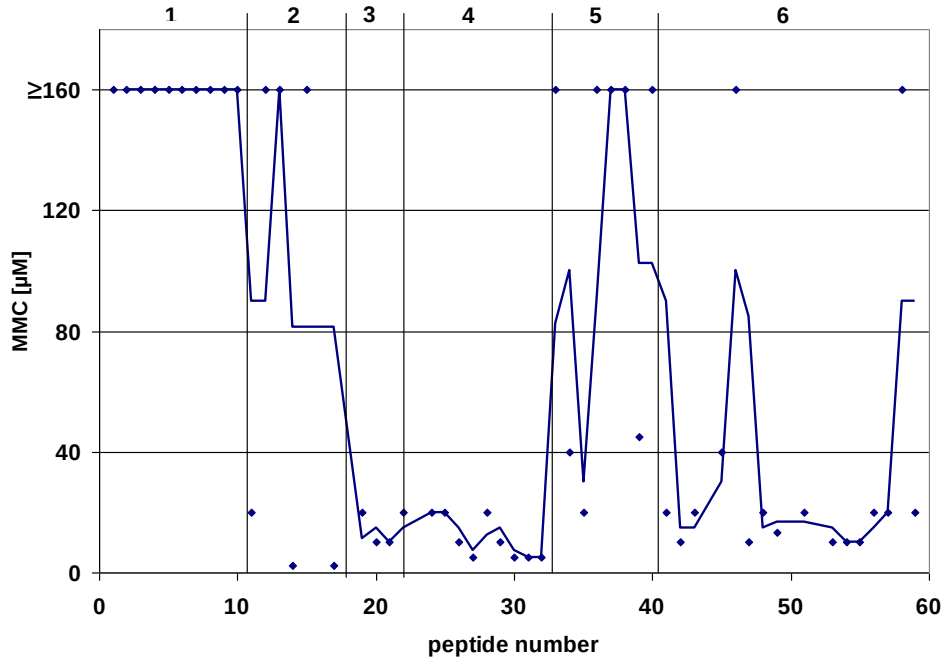

Figure 12: The antimicrobial assay, concentration where peptide kills 99% of bacteria. Peptides serial diluted; 160 µM to 1.25 µM, 5µl peptide incubated with 100µl 10^7bacteria/ml *Staphylococcus aureus*, applied on blood agar plates and incubated over night. The MMC_{99} was found at concentration where peptide killed 99% of all bacteria. All 59 peptides screened, excluding PeptideA.
All the points were connected with the algorithm gliding mean-value with a period of 2.

4.5. Correlations between assays

Correlation plots were made to see if there were any peptides effective in more than one of the performed assays, and thereby even more attractive as a candidate for further usage in wound healing and adhesion prevention.

The correlation between the results from the anti-inflammatory assay and the antimicrobial assay (Figure 13) showed that the peptides were effective in decreasing TNF-α production, i.e. anti-inflammatory, also had low MMC99 values, i.e. only a low concentration of peptide to kill bacteria. The opposite was also present; peptides that showed increased TNF-α production, i.e. pro-inflammatory properties, often needed high concentrations of peptide to show effect in the antimicrobial assay, i.e. low efficiency bacteria killers.
A peptide with high efficiency in both assays might be interesting candidate in a topical application of wound healing.

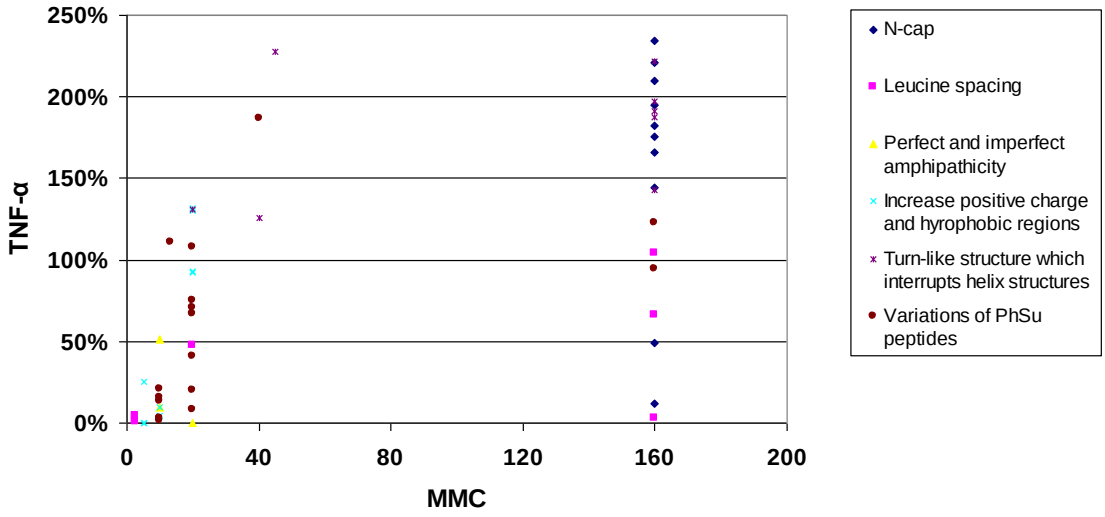

Figure 13: Correlation plot between results from anti-inflammatory assay and antimicrobial assay. Each point represents one peptide with a certain value from anti-inflammatory assay and antimicrobial assay, the symbols represent which group of modification the peptide belongs to. All peptides, excluding PeptideA, are shown.
Y-axis: THP-1 cells were differentiated into macrophage-like cells with PMA, stimulated with 0.1 ng/ml LPS and treated with peptide (320 µM). The peptides ability to decrease TNF-α production was examined in an ELISA assay.
X-axis: Peptides serial diluted; 160 µM to 1.25 µM, 5µl peptide incubated with 100µl 10^7bacteria/ml *Staphylococcus aureus*, applied on blood agar plates and incubated over night. The MMC_{99} was found at concentration where peptide killed 99% of bacteria.

A correlation plot between fibrinolytic assay and antimicrobial assay (Figure 14) shows that peptides decreased the PAI-1 production below the base-line always correlated with low MMC_{99} values. But, peptides that did not decrease the PAI-1 production below basal-level did not show a clear trend about the MMC_{99} value. In post-surgical abdominal applications are both these characteristics important to disperse fibrin bands and decrease the bacteria concentration in the wound.

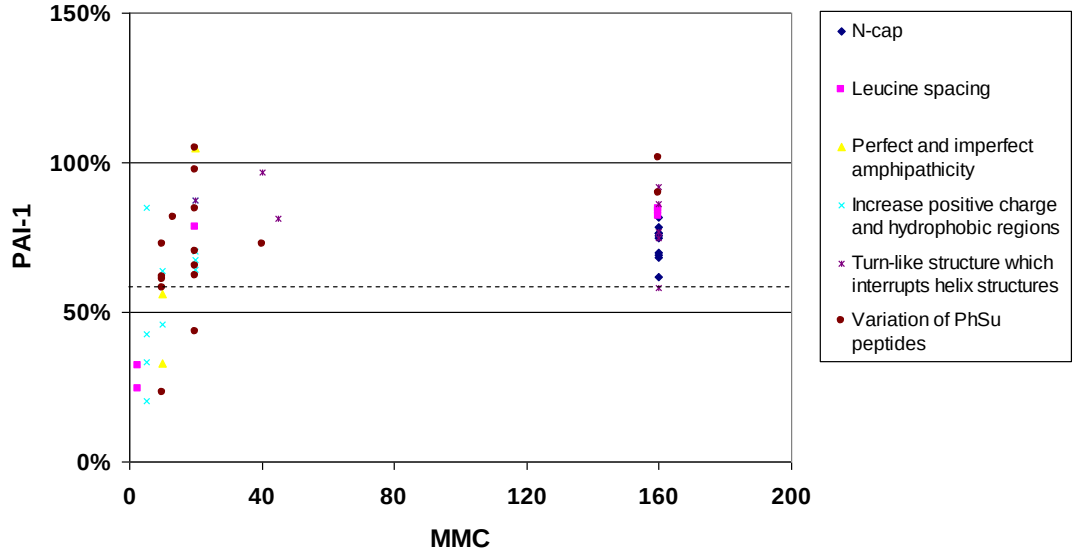

Figure 14: Correlation plot between fibrinolytic assay and antimicrobial assay. Each point represent one peptide with a certain value from fibrinolytic assay and antimicrobial assay, the symbol represent which group of modification the peptide belongs to. All 59 peptides present in both assays, excluding PeptideA.
Y-axis: MeT-5A cells stimulated with 0.1 ng/ml IL-1β and treated with peptide (320 µM). The peptides ability to decrease PAI-1 production was examined in an ELISA assay.
X-axis: Peptides serial diluted; 160 µM to 1.25 µM, 5µl peptide incubated with 100µl 10^7bacteria/ml *Staphylococcus aureus*, applied on blood agar plates and incubated over night. The MMC_{99} was found at concentration where peptide killed 99% of bacteria.

A correlation plot between fibrinolytic assay and anti-inflammatory assay (Figure15) showed that a pronounced reduction of PAI-1 production (below the base-line) fully correlates with a low TNF-α secretion (2-fold reduction). The opposite was not the case. Peptides that were both effective in fibrinolytic and anti-inflammatory assay might be interesting candidates for adhesion prevention in abdominal surgery.

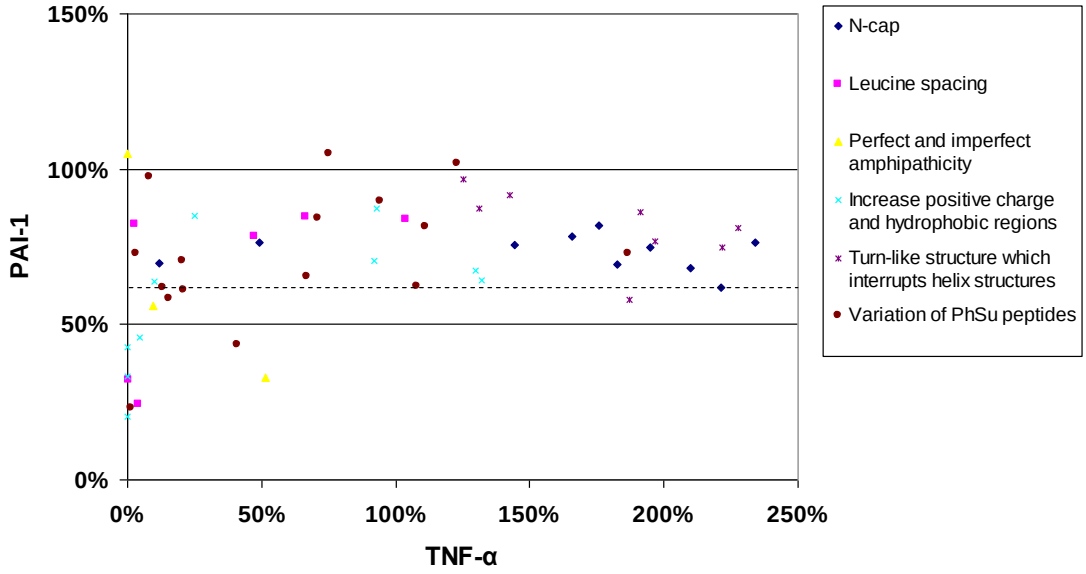

Figure 15: Correlation plot between Fibrinolytic Assay and Anti-inflammatory Assay. Each point represent one peptide with a certain value from fibrinolytic assay and anti-inflammatory assay, the symbol represent which group of modification the peptide belongs to. All 59 peptides present in both assays, excluding PeptideA.
Y-axis: MeT-5A cells treated with trypsin, stimulated with 0.1 ng/ml IL-1β and treated with peptide (320 µM). Their ability to decrease PAI-1 production was examined in an ELISA assay. Peptide concentrations compared to stimulation-level, set to 100% corresponding to 103.19 pg/ml produced PAI-1, basal-level = 60.73%.
X-axis: THP-1 cells were differentiated into macrophage-like cells with PMA, stimulated with 0.1 ng/ml LPS and treated with peptide (40 µM or 320 µM). Their ability to decrease TNF-α production was examined in an ELISA assay. Peptide concentrations compared to stimulation-level, set to 100% corresponding to 403.05 pg/ml secreted TNF-α and basal-level = 0%.

5. Discussion

5.1. Screening for anti-inflammatory effect in THP-1 cells

The anti-inflammatory assay examined the peptides ability to diminish the secretion of TNF-α from LPS stimulated THP-1 cells and thereby diminish the inflammatory response. A monocytic cell line, THP-1, was used and differentiated into macrophage-like cells with PMA. Macrophages are essential in the inflammatory response by producing cytokines and phagocytosis of cell debris and bacteria. In the search for products that decrease the adhesion formation it has been found that a decrease in inflammatory response, i.e. an anti-inflammatory agent, may decrease the adhesion formation (Dwivedi *et al* 2004).

The result showed several peptides which were effectively decreasing TNF-α secretion and fifteen of them lowered the TNF-α production more than 90% in the higher concentration of the peptide, 320μM. When looking at the ten most effective peptides in this assay in relation to the number of peptides in each group, the most effective modifications in creating an anti-inflammatory peptide were; *Leucine spacing, Increase positive charge and hydrophobic regions* and *Perfect and imperfect amphipathicity.* Interestingly two of the groups of modifications showed pro-inflammatory properties; *N-cap* and *Turn-like structure which interrupts helix structure*. The *N-cap* peptides are capped with amino acids or small groups on the N-terminal, which appears to lead to pro- rather than anti-inflammatory properties. The peptides in *Turn-like structure which interrupts helix structure* do not posses the helix-structure but are arranged into a turn-like structure when binding to LPS. The results indicate that the helix structure was important for the anti-inflammatory activities at the same time as leucines at the right positions, increasing the positive charge and amphipathicity are important.

The peptides, both anti- and pro-inflammatory, show a legible dose-response relationship.

5.2. Screening for fibrinolytic effect in MeT-5A cells

The fibrinolytic assay examined the peptides' ability to inhibit the secretion of PAI-1 from IL-1β stimulated MeT-5A cells and thereby their ability to break down fibrin clots. A mesothelial cell line, MeT-5A, was used, which are adherent cells that cover and protect most of the internal organs. These cells are important in the coagulation process, which is an essential step in formation of adhesions and scar tissue. Excessive fibrin deposition leads to adhesion formation and thus, reduction of PAI-1 expression is expected to lead to reduced scarring. (Bergström *et al* 2006).

All the peptides were effective in decreasing the production of PAI-1, it was hard to distinguish certain groups or single peptides that were very effective. To get an indication which modifications that improved the fibrinolytic properties, the ten most effective peptides in this assay were put in relation to the number of peptides in each group and the most effective groups in creating a fibrinolytic peptide were found; *Leucine spacing, Increase positive charge and hydrophobic regions* and *Perfect and imperfect amphipathicity.* Interestingly, these groups were the same ones improving the anti-inflammatory properties (5.1.).

5.3. Screening for cytotoxic effect in THP-1 cells

The cytotoxic assay examined the peptides cytotoxic effect on THP-1 cells when treated with peptide. The assay was based on the ability of viable cells to reduce 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to purple formazan, i.e. the larger amount of survived cells, the larger production of formazan and purple color. Only peptides with pronounced anti-inflammatory and/or fibrinolytic activities were screened in cytotoxic

assay. The relationship between the efficacy and cytotoxicity is described as the therapeutic window = TC_{50}/IC_{50}. TC_{50} (Toxic Concentration) is defined as the concentration of peptide where 50% of the cells died and IC_{50} (Inhibition Concentration) is the concentration of the peptide where TNF-α secretion was decreased to 50%. Since only two concentrations of the peptides were evaluated neither the IC_{50} nor the TC_{50} value could be determined and the results were therefore difficult to interpret. Groups of peptides with pronounced cytotxicity were also the groups with high efficacy in anti-inflammatory assay. The peptides showing cytotoxic effect at concentrations tested also exhibited a low IC_{50} and thereby might not be eliminated from the list of candidates.

5.4. Screening for antimicrobial effect in S.aureus bacteria

The antimicrobial assay examined the peptides microbial killing effect, i.e. the concentration where the peptide killed 99% of bacteria, in an antimicrobial assay. A bacteria strain usually found on skin, *Staphylococcus aureus*, was used. The ability to kill bacteria is important in any application of adhesion prevention (Oncel *et al* 2001).

A large number of peptides did not kill any bacteria in concentrations tested. In the *N-cap* group just two of the peptides killed bacteria at the highest level of concentrations tested.

The results varied in the groups *Leucine spacing, Turn-like structure which interrupts helix formation* and *Variations of PharmaSurgics peptides*. A number of the peptides did not kill bacteria at the concentration range tested while others were highly effective; some of them killed bacteria even better than PeptideA.

When looking at the ten most effective peptides in this assay in relation to the number of peptides in each group, the most pronounced microbicidal effect was seen in groups *Increase positive charge and hydrophobic regions* and *Perfect and imperfect amphipathicity,* which are the same groups as in anti-inflammatory and fibrinolytic assays.

5.5. Top-ten peptides

When evaluating the most effective peptides in anti-inflammatory, fibrinolytic and antimicrobial assays, six peptides appeared in the top-ten in all the assays;

- **Peptide 14** (Leucine Spacing)
- **Peptide 17** (Leucine Spacing)
- **Peptide 27** (Increase of positive charge and hydrophobic regions)
- **Peptide 30** (Increase of positive charge and hydrophobic regions)
- **Peptide 32** (Increase of positive charge and hydrophobic regions)
- **Peptide 55** (Variation of PharmaSurgics' peptides)

This arrangement shows that the modifications *Leucine spacing* and *Increase positive charge and hydrophobic regions* were most relevant to create peptides that are effective in all assays.

5.5. Correlations between assays

It was interesting to look at the correlations between two properties of one peptide in the search for new candidates in different medical applications, such as adhesion prevention in abdominal surgery and topical wound healing.

The correlations showed that the effective peptides in one assay were often effective in the other assays.

In a topical application the most important issues to consider are the prevention of inflammation and bacteria growth, i.e. anti-inflammatory assay and antimicrobial assay. Peptides highly effective in decreasing TNF-α

production were also microbicidal at low concentrations of peptide. A number of peptides showed pro-inflammatory properties, i.e. increasing the TNF-α production, together with efficiency in the antimicrobial assay. This combination could be desired in a therapy where pro-inflammatory propertiesare wanted to speed-up the inflammatory response and thereby prevent adhesion formation. All the peptides with low MMC_{99} values might be useful in these two different applications.

When looking at post-surgical abdominal application the correlation between the fibrinolytic assay and antimicrobial assay is interesting. This will distinguish a peptide effective in decreasing the fibrin formation and eliminating bacteria and thereby hinder adhesion formation. Peptides that have a low PAI-1 value (<basal-level) also showed a low MMC_{99} value and might therefore be candidates for this mission.

The third correlation plot, between anti-inflammatory assay and fibrinolytic assay, pointed out peptides both effective in diminishing the TNF-α and PAI-1 production, both events contributing to adhesion prevention. A low PAI-1 value implied a low TNF-α value. No peptides possessed a low PAI-1 value together with pro-inflammatory property, thereby the application with a pro-inflammatory and fibrinolytic peptide is not possible.

6. Conclusions and Future Perspectives

The objective of this project was to screen the 59 peptides for anti-inflammatory, fibrinolytic and antimicrobial activities which are important in wound healing and adhesion prevention, and to find peptides with improved properties compared to existing PharmaSurgics' peptides. The screening project also aimed to understand the structural activity relationship (SAR) for the lactoferrin peptides.

The screening resulted in peptides with improved efficacy compared to previously described lactoferrin-derived peptides. The data suggested that helix improvement with leucine spacing, increasing the positive charge plus hydrophobic regions and the amphipathicity are important structural features for the anti-inflammatory and fibrinolytic properties of the peptide. Some of these peptides will be further analyzed in *in vivo* experiments, which is beyond the scope of this project.

All data collected has been sent to SARomics for deeper Structural Activity Relationship (SAR) analysis and designing of a second peptide library to further improve and fine-tune the hypothesis on structural features defining the functional properties.

In upcoming screenings, all peptides should be included in all assays and a more exact value of IC_{50} and TC_{50} should be determined and thereby an exact number of the therapeutic window.

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Appendix 1

Subculturing THP-1 cells

General information

Cell concentration is maintained between 200.000 and 800.000 cells/ml.

The cells are subcultured two times a week, at an initial density of 200.000 cells/ml.

The working volume is 10 ml and 30 ml in respectively a T25 and a T75 flask.

Protocol

1. Pre-heat a tube with complete RPMI-medium (RPMI 1640 supplemented with 10% FBS, 1 mM Sodium Pyruvate and 20 mM HEPES) to RT or 37 °C.
2. Dissociate aggregated cells using a pipette (T25) or a 10 ml pipette (T75).
3. Transfer 10 µl cell suspension to a Bürker chamber.
   Count the cells in ~ 4 A-squares and calculate the cell density.
   Cell density, cells/ml = # cells per A-square * 10^4.
4. Transfer the right amount of cell suspension to a new flask to obtain a density of 200.000 cells/ml.
   E.g. for a T25 flask: V = 10 ml * 200.000 / Cell density
5. Add new pre-heated RPMI-medium (10 ml – V ml) to a new cell culture flask and transfer the calculated volume of cell suspension (V ml) to the new flask.
6. Label flask with content, date and passage. Incubate at 37 °C.

Appendix 2

Subculturing MeT-5A cells

General information

Subculture the cells when they are ~100% confluent, i.e. two times a week to initial concentration of 30.000-60.000 cells/ml.

The working volume is 5 ml and 10 ml in respectively a T25 and a T75 flask.

Protocol

1. Pre-heat Trypsin and complete M199-medium (M199 with 10% FBS, 3.3 nM EGF, 400 nM Hydrocortisone, 870 nM Insulin and 20 mM HEPES) to RT and let a bottle of D-PBS adjust to room temperature.
2. Determine the confluence by looking at the cells under the microscope.
3. Decant the spent medium from the culture flask with a pipette.
4. Add room-tempered D-PBS to the flask (2 ml to a T25 and 4 ml to a T75), rock the flask a couple of times before removing of the D-PBS.
5. Add Trypsin to the flask (2 ml to a T25 and 4 ml to a T75), rock the flask 20 times before pipetting of the Trypsin– put the cap on.
6. Place the flask in the incubator for approximately 3 minutes.
7. During incubation, add the pre-heated complete M199-medium to new flask(s), 5 ml to a T25 flasks or 10 ml to a T75.
8. After 3 minutes, check under the microscope that the cells are round and non-adherent, if not incubate the flask a little bit longer.
9. Add new complete M199 to the flask (3 ml to a T25 flask and 6 ml to a T75) and re-suspend the cells.
10. Transfer the cells to the pre-heated medium in the new flasks: Between 250 µl and 600 µl for T25 flasks and the double for T75 flasks.
11. Label flask with content, date and passage. Incubate at 37 °C.

Appendix 3

Protocol for Seeding and Stimulation of THP-1 cells

Seeding

1. Cell suspension is centrifuged and new RPMI-medium is added to reach a cell density of [10^6 cells/ml].
   100 µl cell suspension is required for each well. →96 wells * 100 µl = 9.6 ml → Prepare 11 ml.
2. Add PMA to cell suspension to a final concentration of 10ng/ml, i.e. if the volume of the cell suspension is 11 ml add 110 µl [1 µg/ml] PMA→ PMA [10 ng/ml].
   10 µl PMA [100 µl/ml] + 990 µl MQ → 1000 µl [1000 ng/ml].
3. Add 100 µl of the cell suspension with PMA to each well on a 96 flat-well plate with a multi-channel pipette.

Incubate the plate for 48 hours; 37 ºC.

Stimulation

All the following steps are performed with a multi-channel pipette.

4. Empty the wells of old medium.
5. The wells are washed by adding 90 µl AssayMedium (5% h.i. FBS in RPMI 1640 medium supplemented with 10% FBS, 1 mM Sodium Pyruvate and 20 mM HEPES).
   For a full 96-well plate: 96 wells * 180 µl = 19.2 ml → Prepare 22 ml
   1.1 ml h.i. serum + 20.9 ml RPMI- medium.
   11 ml AssayMedium + 12.21 µl LPS [100 ng/ml] → A mix with LPS [0.111 ng/ml] to get LPS [0.100 ng/ml] in the wells.
6. Empty the wells.
7. Cells are stimulated with 90 µl AssayMedium containing LPS [0.111 µg/ml].

Incubate plate for 30 minutes; 37 ºC.

8. Cycloheximide (Chx) is used as a positive control and a stock solution is prepared;
   10 µl Chx [100 mg/ml] + 990 µl H_2O→ 1000 µl [1 mg/ml]
   10 µl [1 mg/ml] + 190 µl H_2O→ 200 µl [500 µg/ml].
9. Add 10 µl of sample (peptide, Chx or H_2O) to each well.

Incubate plate for 6 hours, counting from stimulation with LPS at 37 ºC.

10. Collect the supernatants and transfer them to a V96-well plate.
11. Centrifuge the plate 1500 rpm; 6 minutes.
12. Collect ~85 µl from each well is transfer to a new V96-well plate.
13. Plate is frozen and kept in -20 ºC.

Appendix 4

Protocol for Seeding and Stimulation of MeT-5A cells

Seeding Cells

1. Wash the cells with D-PBS (~2 ml T25 and ~4 ml T75).
2. Add trypsin (~2 ml T25 and ~4 ml T75) to the cells and incubated for 3 minutes at 37 ºC.
3. Resuspend cell suspension in new M199 medium (3 ml T25 and 6 ml T75).
4. Centrifuge the cell suspension (300 rpm for 6 minutes) and re-suspende the pellet in new medium to reach a cell density of [160.000 cells/ml].
   100 µl cell suspension is required for each well → 96 wells * 100 µl = 9.6 ml → Prepare 12 ml.
5. Add 100 µl of the cell suspension to each well on a 96 flat-well plate → [16.000 cells/well].

Stimulation

All the following steps are performed with a multi-channel pipette.

6. Empty the wells of old medium.
7. The wells are washed by adding 90 µl (AssayMedium M199 medium supplemented with 1M HEPES, 10mg/ml Hydrocortisone, 10mg/ml Insulin and 1mg/ml Epidermal Growth Factor (EGF)).
   For a full 96-well plate: 96 wells * 180 µl = 19.2 ml → Prepare 22 ml
   1.1 ml h.i. serum + 20.9 ml M199- medium.
   11 ml AssayMedium + 12.21 µl IL-1β [100 ng/ml] → A mix with IL-1β [0.111 ng/ml] to get IL-1β [0.100 ng/ml] in the wells.
8. Empty the wells.
9. Cells are stimulated with 90 µl AssayMedium containing IL-1β [0.111 ng/ml].
10. Cycloheximide (Chx) is used as a positive control and a stock solution is prepared;
    10 µl Chx [100 mg/ml] + 990 µl H_2O→ 1000 µl [1 mg/ml]
    10 µl [1 mg/ml] + 190 µl H_2O→ 200 µl [500 µg/ml].
11. Add 10 µl of sample (peptide, Chx or H_2O) to each well directly after IL-1β stimulation.
    Incubate the plate for 6 hours at 37ºC.
12. Collect the supernatants six hours after IL-1β stimulation and transfer them to a V96-well plate.
13. Centrifuge the plate 1500 rpm for 6 minutes.
14. Collect ~85 µl from each well is transferred to a new V96-well plate.
15. Plate is frozen and kept in -20 ºC.

Appendix 5

Relative TNF-α production

The peptides are arranged in the order of the highest decrease of TNF-α production, down to the lowest.

	320µM		
	Average (%)	***Stdav***	***SEM***
LPS	***100,00%***		
Basal-level	***0,00%***		
Chx	***40,67%***		***0,0513***
peptide 19	0,00%	0	0
peptide 27	0,00%	0	0
peptide 30	0,00%	0	0
peptide 32	0,00%	0	0
peptide 17	0,61%	0,0028	0,0016
peptide 55	1,54%	0,0036	0,0021
peptide 13	2,75%	0,0045	0,0026
peptide 47	3,26%	0,0046	0,0026
peptide 14	4,17%	0,0115	0,0066
peptide 26	4,36%	0,0211	0,0122
peptide 16	6,92%	0,0038	0,0022
peptide 52	7,72%	0,0298	0,0172
peptide 59	8,11%	0,0292	0,0168
peptide 21	9,52%	0,0077	0,0044
peptide 29	9,94%	0,0263	0,0152
peptide 23	10,59%	0,0331	0,0191
peptide 2	11,83%	0,0234	0,0135
peptide 42	13,42%	0,0854	0,0493
peptide 53	15,45%	0,0582	0,0336
peptide 57	20,32%	0,0635	0,0367
peptide 54	21,03%	0,0104	0,0060
peptide 31	25,25%	0,0421	0,0243
peptide 44	25,60%	0,1122	0,0648
peptide 50	32,48%	0,1260	0,0728
peptide 43	40,76%	0,1047	0,0604
peptide 11	47,27%	0,2054	0,1186
peptide 9	48,96%	0,2820	0,1628
peptide 20	51,27%	0,0862	0,0498
peptide 15	66,32%	0,0870	0,0502
peptide 41	66,98%	0,0578	0,0333
peptide 48	71,00%	0,2643	0,1526
peptide 56	75,09%	0,0732	0,0423
peptide 22	92,11%	0,0208	0,0120
peptide 28	92,93%	0,2796	0,1614
peptide 58	94,48%	0,0145	0,0084
peptide 12	103,89%	0,1960	0,1132
peptide 18	105,61%	0,1544	0,0891
peptide 51	107,83%	0,2096	0,1210
peptide 49	110,96%	0,1568	0,0906
peptide 34	125,39%	0,2876	0,1660
peptide 35	131,02%	0,1810	0,1045
peptide 25	132,04%	0,2430	0,1403
peptide 24	134,34%	0,3515	0,2029
peptide 33	134,85%	0,2372	0,1369
peptide 4	144,18%	0,4947	0,2856
peptide 46	151,84%	0,5544	0,3201
peptide 5	165,64%	0,4202	0,2426
peptide 10	175,89%	0,1972	0,1139
peptide 1	182,45%	0,2677	0,1545
peptide 45	191,36%	0,3062	0,1768
peptide 3	194,84%	0,2195	0,1267
peptide 40	209,39%	0,4421	0,2553
peptide 6	209,95%	0,5306	0,3063
peptide 36	215,48%	0,2807	0,1621
peptide 7	221,18%	0,2274	0,1313
peptide 37	221,22%	0,4778	0,2758
peptide 8	234,09%	0,4467	0,2579
peptide 39	257,78%	0,1737	0,1003
peptide 38	258,02%	0,2190	0,1264

Peptide	40µM		
	Average (%)	***Stdav***	***SEM***
LPS	***100,00%***		
Basal-level	***0,00%***		
Chx	***40,67%***		***0,0513***
peptide 55	4,58%	0,0055	0,0032
peptide 30	6,28%	0,0133	0,0077
peptide 27	8,72%	0,0355	0,0205
peptide 19	9,62%	0,0890	0,0514
peptide 17	12,73%	0,0305	0,0176
peptide 14	15,26%	0,0623	0,0359
peptide 32	18,33%	0,0484	0,0279
peptide 13	21,89%	0,0528	0,0305
peptide 21	34,71%	0,0270	0,0156
peptide 9	47,06%	0,1550	0,0895
peptide 42	47,93%	0,0647	0,0374
peptide 20	49,39%	0,2147	0,1239
peptide 59	60,04%	0,2739	0,1581
peptide 16	82,58%	0,1301	0,0751
peptide 31	86,66%	0,0915	0,0529
peptide 5	87,47%	0,1093	0,0631
peptide 29	90,88%	0,0970	0,0560
peptide 58	91,10%	0,1281	0,0739
peptide 6	95,97%	0,0517	0,0299
peptide 36	96,32%	0,1390	0,0802
peptide 12	100,51%	0,2522	0,1456
peptide 10	102,14%	0,0185	0,0107
peptide 35	102,69%	0,2904	0,1677
peptide 28	103,88%	0,2377	0,1372
peptide 26	106,60%	0,1156	0,0667
peptide 7	108,14%	0,2727	0,1574
peptide 2	108,48%	0,2502	0,1445
peptide 34	109,18%	0,1963	0,1133
peptide 49	109,33%	0,0771	0,0445
peptide 41	109,50%	0,0975	0,0563
peptide 52	109,78%	0,2824	0,1631
peptide 22	110,76%	0,0287	0,0166
peptide 47	111,45%	0,0445	0,0257
peptide 46	111,95%	0,0997	0,0575
peptide 18	113,11%	0,5129	0,2961
peptide 1	116,21%	0,2908	0,1679
peptide 40	116,69%	0,0619	0,0357
peptide 15	118,80%	0,3855	0,2226
peptide 11	120,55%	0,1606	0,0927
peptide 56	124,33%	0,1150	0,0664
peptide 51	124,85%	0,2944	0,1700
peptide 8	127,00%	0,4724	0,2727
peptide 24	128,08%	0,2834	0,1636
peptide 4	131,78%	0,1687	0,0974
peptide 48	131,83%	0,0814	0,0470
peptide 50	134,19%	0,1423	0,0822
peptide 33	134,76%	0,1109	0,0640
peptide 3	135,23%	0,1482	0,0856
peptide 45	135,93%	0,3233	0,1866
peptide 37	136,81%	0,4837	0,2793
peptide 44	140,43%	0,0751	0,0433
peptide 39	148,50%	0,4859	0,2805
peptide 25	150,17%	0,2566	0,1481
peptide 43	152,77%	0,4889	0,2823
peptide 57	153,38%	0,3607	0,2082
peptide 23	155,78%	0,0722	0,0417
peptide 53	159,14%	0,2320	0,1339
peptide 54	161,83%	0,1570	0,0907
peptide 38	172,08%	0,3709	0,2141

Appendix 6

Relative PAI-1 production

The peptides are arranged in the order of the highest decrease of IL-1β production, down to the lowest.

	320µM		
	Average (%)	***Stdav***	***SEM***
IL-1	***100,00%***		***0,0164***
Basal-level	***60,73%***		***0,1365***
Chx	***28,66%***		***0,0000***
peptide 32	20,25%	0,0634	0,0449
peptide 55	23,21%	0,0057	0,0040
peptide 14	24,22%	0,0000	0,0000
peptide 17	32,27%	0,0419	0,0296
peptide 20	33,06%	0,0295	0,0208
peptide 27	33,42%	0,1614	0,1141
peptide 30	42,62%	0,0197	0,0139
peptide 43	43,47%	0,0471	0,0333
peptide 26	45,90%	0,0416	0,0294
peptide 44	47,28%	0,0057	0,0040
peptide 50	51,25%	0,0391	0,0276
peptide 21	56,03%	0,0359	0,0254
peptide 16	56,61%	0,0013	0,0009
peptide 36	58,12%	0,0240	0,0169
peptide 53	58,17%	0,0776	0,0548
peptide 52	60,26%	0,0088	0,0062
peptide 54	61,15%	0,0492	0,0348
peptide 42	61,75%	0,2033	0,1437
peptide 7	61,84%	0,1708	0,1208
peptide 51	62,31%	0,0643	0,0455
peptide 23	62,54%	0,0095	0,0067
peptide 29	63,65%	0,0126	0,0089
peptide 25	64,10%	0,1551	0,1097
peptide 41	65,57%	0,1242	0,0878
peptide 24	67,53%	0,0120	0,0085
peptide 6	68,09%	0,1466	0,1037
peptide 1	69,30%	0,1178	0,0833
peptide 2	69,85%	0,0563	0,0398
peptide 22	70,43%	0,0429	0,0303
peptide 57	70,50%	0,0642	0,0454
peptide 45	72,78%	0,0533	0,0377
peptide 47	72,93%	0,0731	0,0517
peptide 3	74,71%	0,0609	0,0430
peptide 38	74,93%	0,0069	0,0049
peptide 4	75,50%	0,0065	0,0046
peptide 8	76,42%	0,1283	0,0907
peptide 9	76,47%	0,0399	0,0282
peptide 37	76,63%	0,1734	0,1226
peptide 11	78,32%	0,1342	0,0949
peptide 5	78,46%	0,1257	0,0889
peptide 39	81,22%	0,1160	0,0820
peptide 49	81,58%	0,0038	0,0027
peptide 10	81,88%	0,0039	0,0028
peptide 13	82,11%	0,0111	0,0079
peptide 12	83,73%	0,0877	0,0620
peptide 48	84,38%	0,0574	0,0406
peptide 15	84,61%	0,0556	0,0393
peptide 31	84,89%	0,0314	0,0222
peptide 40	86,17%	0,1822	0,1289
peptide 35	87,51%	0,0460	0,0326
peptide 28	87,52%	0,0058	0,0041
peptide 58	89,87%	0,0214	0,0152
peptide 33	91,70%	0,1086	0,0768
peptide 34	96,80%	0,0467	0,0330
peptide 59	97,36%	0,0252	0,0178
peptide 46	101,69%	0,0599	0,0424
peptide 18	101,83%	0,1158	0,0819
peptide 56	104,92%	0,0238	0,0168
peptide 19	105,07%	0,0648	0,0458

Appendix 7

Relative viability

The peptides are arranged in the order of viability; from the highest viability, 100%, down to total killing of cells, 0%.

	320µM		
	Average (%)	***Stdav***	***SEM***
LPS	***100,00%***		***0,1588***
Basal-level	***124,87%***		***0,1546***
Triton X	***0,00%***		***0,0218***
peptide 28	106,68%	0,0817	0,0472
peptide 23	105,87%	0,2331	0,1346
peptide 2	103,32%	0,1887	0,1090
peptide 42	102,31%	0,1725	0,0996
peptide 34	100,64%	0,1871	0,1080
peptide 43	100,51%	0,1181	0,0682
peptide 9	99,11%	0,3903	0,2253
peptide 31	92,30%	0,1314	0,0759
peptide 13	90,82%	0,0595	0,0344
peptide 33	87,03%	0,0614	0,0355
peptide 59	81,13%	0,1354	0,0782
peptide 29	74,20%	0,1059	0,0611
peptide 47	64,44%	0,0787	0,0454
peptide 19	59,18%	0,1301	0,0751
peptide 50	48,14%	0,1317	0,0760
peptide 52	47,88%	0,1277	0,0738
peptide 26	29,59%	0,1555	0,0898
peptide 21	28,19%	0,0950	0,0548
peptide 32	22,72%	0,7727	0,4461
peptide 16	18,24%	0,0576	0,0332
peptide 27	-10,40%	0,0314	0,0181
peptide 55	-16,69%	0,0102	0,0059
peptide 30	-18,49%	0,0080	0,0046
peptide 17	-19,13%	0,0193	0,0111
peptide 20	-19,52%	0,0123	0,0071
peptide 14	-20,28%	0,0231	0,0133

	40µM		
	Average (%)	***Stdav***	***SEM***
LPS	***100,00%***		***0,1588***
Basal-level	***124,87%***		***0,1546***
Triton X	***0,00%***		***0,0218***
peptide 14	127,30%	0,1409	0,0814
peptide 32	125,26%	0,1851	0,1069
peptide 17	124,01%	0,1619	0,0934
peptide 20	121,82%	0,0715	0,0413
peptide 52	119,64%	0,1826	0,1054
peptide 59	119,64%	0,1725	0,0996
peptide 55	114,41%	0,0927	0,0535
peptide 2	112,07%	0,1049	0,0605
peptide 30	110,14%	0,1987	0,1147
peptide 47	107,53%	0,2611	0,1508
peptide 31	106,55%	0,0836	0,0483
peptide 50	105,23%	0,2476	0,1429
peptide 42	104,21%	0,3018	0,1742
peptide 26	103,08%	0,0743	0,0429
peptide 27	100,77%	0,0769	0,0444
peptide 43	100,13%	0,1579	0,0911
peptide 16	99,49%	0,1736	0,1002
peptide 33	99,23%	0,0555	0,0321
peptide 34	92,73%	0,2946	0,1701
peptide 13	90,37%	0,0367	0,0212
peptide 9	89,86%	0,3727	0,2152
peptide 19	88,96%	0,2350	0,1357
peptide 29	87,63%	0,1238	0,0715
peptide 28	80,10%	0,1454	0,0840
peptide 23	66,11%	0,1301	0,0751
peptide 21	49,23%	0,0958	0,0553

Appendix 8

Relative antimicrobial effect

The peptides are arranged in the order of the lowest concentration needed to kill 99% of bacteria up to the highest concentration.

	[µM]
peptide 14	2.5
peptide 17	2.5
peptide 27	5
peptide 30	5
peptide 31	5
peptide 32	5
peptide 16	10
peptide 20	10
peptide 21	10
peptide 26	10
peptide 29	10
peptide 42	10
peptide 44	10
peptide 47	10
peptide 50	10
peptide 52	10
peptide 53	10
peptide 54	10
peptide 55	10
peptide 49	13.3
peptide 11	20
peptide 19	20
peptide 22	20
peptide 23	20
peptide 24	20
peptide 25	20
peptide 28	20
peptide 35	20
peptide 41	20
peptide 43	20
peptide 48	20
peptide 51	20
peptide 56	20
peptide 57	20
peptide 59	20
peptide 34	40
peptide 45	40
peptide 39	45
peptide 6	160
peptide 9	160
peptide 58	160
peptide 1	>160
peptide 2	>160
peptide 3	>160
peptide 4	>160
peptide 5	>160
peptide 7	>160
peptide 8	>160
peptide 10	>160
peptide 12	>160
peptide 13	>160
peptide 15	>160
peptide 18	>160
peptide 33	>160
peptide 36	>160
peptide 37	>160
peptide 38	>160
peptide 40	>160
peptide 46	>160