

## 4.1 Final publishable summary report

## Executive summary

Biomaterial-associated infection (BAI) is a major cause of failure of indwelling medical devices. *Staphylococcus aureus* and coagulase-negative staphylococci are the most common causative agents of BAI. Since established BAI are very difficult to treat using antibiotics, alternative approaches to combat such infections are urgently needed. In the EU FP7 consortium BALI, “Biofilm Alliance”, we have utilized a unique combination of two highly innovative technologies; Synthetic Antimicrobial Anti-biofilm Peptides (SAAPs), and a Polymer-Lipid Encapsulation matrix (PLEX) coating, to prevent BAI.

The project had three overall **aims**:

1. Develop novel Synthetic Antimicrobial and Antibiofilm Peptides (SAAPs) with antimicrobial, anti-inflammatory and anti-biofilm activity and understand the mechanism of action of these SAAPs
2. Incorporate these SAAPs in controlled-release coatings
3. Assess efficacy of SAAP-containing release coatings to prevent BAI by reducing biofilm formation and peri-implant tissue colonization by testing in animal models

The **BALI consortium** consisted of three SME’s and five prominent research groups (two of which are university medical centers) located in five different countries. The depth and breadth of this consortium enabled a substantial impact on developing the essential knowledge and innovative technologies aimed at anti-biofilm and antimicrobial agents and advanced drug delivery systems.

## Main results of BALI

- Novel SAAPs derived from the human antimicrobial proteins LL-37 and Thrombocidin-1, with potent activity even against bacteria which are resistant to all conventional antibiotics
- Proof of concept of the PLEX coating to prevent infection in mouse and rabbit implant infection models with the antibiotic doxycycline and with selected SAAPs
- Detailed information on mode of action of the top-hit SAAPs
- Development of a toxicity data package for further clinical development of hit-SAAP P148

## Output of BALI / Dissemination

Until now BALI generated 7 peer-reviewed scientific publications, 2 patent applications, 57 abstracts and oral or poster presentations and 17 articles in popular press and 4 radio interviews. Ten other manuscripts for peer reviewed scientific publication are presently in preparation. BALI co-organized the International Meeting on Antimicrobial Peptides (IMAP) in Graz (Austria) in 2014, and organized the public BALI meeting as a satellite meeting of the IMAP in Leipzig (Germany) in 2016.

## Potential impacts and use

Due to the rapidly ageing population, infections caused by biofilm-forming micro-organisms are becoming a growing problem. Therefore, the SAAP eluting delivery system that allows the disruption of biofilms, prevent biofilm formation and decrease the infection rate has the potential to have a huge impact on the health of patients being treated. Besides decreasing biofilm associated infections, it also reduces the development of resistance for antibacterial and antifungal drugs. The concept of the SAAP delivery coating can potentially be applied to all types of implants. Therefore, this potentially has a huge impact on the reduction of costs associated with biofilm infection and the number of biofilm infections as a result of implants.

A major asset of the BALI project is that it provides new tools to prevent biofilm formation and increasing development of antimicrobial resistance. Finding alternatives to traditional antibiotic chemotherapy is urging and as highlighted by the European Centre for Disease Prevention and Control (ECDC)/ European Medicines Agency (EMA) in their joint technical report and interviews: “A future without effective antibiotics will exacerbate a situation where already at least 25,000 patients in the EU each year die from infections due to multidrug-resistant bacteria. Patients suffering from healthcare-associated infections will be particularly hard hit.” In addition, infections due to any of the selected antibiotic-resistant bacteria, among which strong biofilm forming species, resulted in approximately 2.5 million extra hospital days and extra in-hospital costs of more than EUR 900 million. In the United States the Centers for Disease Control and Prevention (CDC) estimates that approximately 1.7 million patients contract a hospital infection of which approximately 100,000 die annually. Thus, the control of biofilm formation by pathogens with a propensity to be multi-resistant will have a major benefit for patients in European hospitals and elsewhere in the world.

## Summary description of project context and objectives

### *Problem definition*

Biofilms have been found to be involved in a wide variety of infections in the body, by one estimate 80% of all infections. Biofilms have specific characteristics that enable them to withstand the conventional antibiotic therapies. Biofilms are notoriously resistant to antibiotics and even long-term use of high concentrations of antibiotics does not ensure the eradication of the bacteria within biofilms. As a consequence, the current treatment strategies to prevent or treat biofilms are inadequate. Approximately 3% of all patients receiving implants develop biofilm associated infections, and antibiotic treatment of implant-associated infections is unsuccessful in up to 32 to 82% of the cases and results in chronic infection and inflammation.

The main problem is that there is no effective strategy to control biofilm formation, and as a result biofilm associated infections cannot be prevented or treated adequately.

For implant-associated biofilm infections the current treatment strategies are based on the administration of antibiotics. These antibiotics are administered systemically or locally through polymeric drug delivery systems. These treatment strategies are inadequate mainly because of:

1. Reduced or incomplete penetration of antimicrobials into biofilms;
2. Inactivation or degradation of the antibiotics before they can exert their desired effect;
3. Reduced metabolic activity of bacteria within the biofilm, rendering antibiotics ineffective;
4. Antibiotics cause release of pro-inflammatory microbial compounds facilitating microbial adhesion at the site of inflammation and impairing the host defense system;
5. At the site of the infection the concentration of the effective agent is too low, and/or its presence is too short to be effective.

Thus, even when an antimicrobial agent is available it is difficult to target the infection because the local delivery of the agent is insufficient. Currently available polymeric drug delivery systems do not overcome these problems because the release rate of the agent cannot be controlled. Moreover, since the number of required long term systemic therapies with conventional antibiotics in BAI remains high, resistance towards these antibiotics will increasingly develop. This contributes to the worldwide problem of antimicrobial resistance.

### *Concept*

Formation of biofilms and their associated infections can be prevented, and existing biofilm-associated infections in patients with orthopedic implants can be treated, if novel potent antimicrobial agents can be administered locally in effective concentrations for the required period.

### *Novel microbicidal agents*

To achieve this goal, novel potent microbicidal agents will be developed that overcome the shortcomings of conventional antibiotics. The unique effect of second generation SAAPs on biofilm infections will be threefold: they will 1) prevent biofilm formation and disperse existing biofilms, 2) kill the bacteria or fungi at and around the site of release, and 3) orchestrate immune responses by neutralizing pro-inflammatory microbial endotoxins such as lipoteichoic acid (LTA), peptidoglycan (PG) and lipopolysaccharides (LPS) and activating macrophages to enhance their phagocytic and microbicidal activity. This immune control is necessary to prevent the tissue surrounding implants to become a novel niche for the pathogens.

The basis is the recently developed peptide, OP-145, that shows significant antimicrobial and anti-biofilm properties *in vitro*. OP-145 has shown to disperse biofilms, kill the bacteria and neutralize their inflammatory endotoxins. Moreover, safety and efficacy of OP-145 against BAI has already been demonstrated in chronic suppurative otitis media Phase I and II clinical trials.

The molecular mechanisms of OP-145 and other peptides interacting with cell membranes will be studied to unveil the critical structures that account for the desired properties. This knowledge will be used to develop second generation SAAPs that both disperse and kill biofilm bacteria and fungi and also restrict associated inflammation that otherwise may result in tissue injury and survival of pathogens.

### *Novel delivery platform*

The efficacy of the novel microbicidal agent will be dramatically enhanced when administered in close proximity to its target, over prolonged periods of time and at high local concentrations. The PolyPid unique polymer and lipid based drug delivery platform is a recently developed and well characterized proven

technology that can be tailored to allow prolonged and pre-determined release rates of SAAPs, to ensure the required local concentrations over a desired period. In the present project, this platform will be applied to develop coatings for implants resulting in a tool that can control biofilms and consequently prevent implant associated biofilm infections and treat existing BAI.

The SAAP loaded PolyPid coating will be tested *in vivo* in animal models and in a first in human Phase 1 clinical study. Proof of concept for prevention of biofilm formation will be obtained in a mouse subcutaneous implant infection model. This is a model for soft tissue infection of indwelling implants such as catheters, shunts, pacemaker leads, etc. Within this model the prevention of BAI will be assessed as well as the treatment by re-implantation after infection of the primary implant. Proof of concept for treatment efficacy in bone infection will be obtained in a rabbit model for revision surgery for infected orthopedic implants. Following this, a Phase 1 clinical study in humans with infected intramedullary nails will be performed.

OP-145 is a good candidate to be incorporated in PolyPid coatings of implants. The consortium however, anticipated that even better performing SAAPs may be developed. So the prime candidate for the *in vivo* studies is OP-145, until SAAPs with better characteristics have been developed.

### Objectives

To address the problem of biofilm infection, the objectives of project BALI are based on two pillars:

1. The development of novel potent microbicidal agents (second generation SAAPs) that overcome the shortcomings of conventional antibiotics. These agents will be biofilm dispersing and immune-orchestrating Synthetic Antimicrobial and Anti-biofilm Peptides (SAAPs) that will be active against a wide spectrum of bacterial and fungal species.
2. The development of innovative delivery formulations for the second generation SAAPs by tailoring a unique and well characterized drug-delivery platform (PolyPid). This platform allows prolonged and pre-determined release rates of SAAPs, ensures local delivery of high concentrations over a sufficient duration and can be developed into coatings for different types of implants.

Thus, the overall objective of project BALI is:

***To develop a unique combination of immune orchestrating Synthetic Antimicrobial and Anti-biofilm Peptides (SAAPs) incorporated in novel controlled release drug delivery formulations that can control biofilm infections.***

A recently developed SAAP that has successfully been tested up to clinical phase II trials, OP-145, will be the first candidate to be incorporated in the novel delivery system ("PolyPid formulation"). Newly developed SAAPs will be referred to as second generation SAAPs.

To reach the overall objective, several sub-objectives are defined:

- Next to OP-145, to develop even more effective and safe novel Synthetic Antimicrobial and Anti-biofilm Peptides (SAAPs) and show their activity profile *in vitro*;
- To elucidate the working mechanism of OP-145 and other second generation SAAPs to further improve their efficacy;
- To incorporate OP-145 and other second generation, potent SAAPs in unique tailored polymer-lipid based structures (PolyPid formulations) that will serve drug delivery systems or as coatings for implants;
- To obtain *in vivo* proof of concept in mouse and rabbit models, of the efficacy against BAI for the OP-145 and other second generation SAAPs that are incorporated into the novel formulations;
- To develop GMP production and perform GLP preclinical safety studies of the novel OP-145 and other second generation SAAP formulations;
- To show safety and efficacy in a first human Phase I clinical study with intramedullary nails coated with the SAAP-loaded PolyPid formulations.

## Description of the main S&T results/foregrounds

### 1. Development and synthesis of SAAPs

#### 1.1. OP-145

Large amounts of OP-145 were synthesized, checked by UPLC-MS and Maldi-Tof mass spectrometry (purity = 100%), and distributed to all collaborators within the BALI project.

#### 1.2. Bead diffusion assay

To find peptides with improved antimicrobial activities, we developed a bead diffusion assay, in which peptides are coupled to beads. These beads are spread on agar plates inoculated with bacteria. Clear zones are formed around beads carrying antimicrobial active peptides. The assay was adapted in such a way that OP-145 beads did not result in a clear zone. Two libraries of peptides based on the sequence of OP-145 were synthesized on beads: (1) 800,000 peptides, in which the neutral and negatively charged amino acids were substituted by other neutral and negatively charged amino acids; (2) 800,000 peptides, in which the positively charged amino acids were substituted by other positively charged amino acids. As a pilot, 0.3% of each library was tested. Based on the results, we selected library 1 to test approximately 80,000 peptides.

#### 1.3. Second generation SAAPs

We have synthesized 3 sets of second generation OP-145 derived peptides: set 1 (P2-P19) and set 3 (P139-P163) are peptides in which several amino acids have been replaced compared to OP-145; set 2 (P83-P112) are peptides in which only 1-5 amino acids have been replaced compared to peptide P3. Moreover, different variants of OP-145 that have chemical modifications, including retro-inverso forms, have been synthesized (set 4). Furthermore, we synthesised a set of second generation thrombocidin-1 (TC-1) derived peptides (set 5), and an alanine-scan of the most potent peptide (set 6). To select peptides with improved activity, we first screened for antimicrobial activity in PBS and subsequently in the presence of 50% pooled human plasma.

Set 1: In a first attempt to identify peptides with enhanced antimicrobial activity, we generated a set of peptides in which several amino acids have been replaced compared to OP-145 (P2-P19). The substitutions were performed in such a way that the variant peptide, like OP-145, was predicted to adopt an amphipathic helical structure. At various positions amino acids were replaced randomly, taking into account that only conservative substitutions were made. None of the peptides showed significantly improved antimicrobial activity in PBS against a methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* as compared to OP-145. Some peptides showed reduced antimicrobial activity, but this could not be linked to specific amino acid substitutions.

Set 2: Conserved substitutions were made randomly throughout the peptide sequence of P3 (P83-P112). Substitutions were also performed in such a way that the predicted amphipathic structure was maintained. In contrast to the previous set, here we replaced only 1-5 amino acids per peptide in order to get information on how marginal changes would affect the activity. None of the peptides showed increased antimicrobial activity against MRSA and *P. aeruginosa* in PBS as compared to OP-145.

Set 3: Again conserved substitutions were performed in such a way that the predicted amphipathic structure was maintained. Substitutions were made randomly (P139-P163). Peptides P139-P163 had similar antimicrobial activity as OP-145 against *S. aureus* JAR060131 in PBS. In the presence of 50% plasma, however, most peptides had an increased antimicrobial activity as compared to OP-145. P148 and P159 have the highest activity in plasma, with a LC99.9 of 12.8  $\mu$ M, which is 16-fold lower than the LC99.9 of OP-145 in plasma. On the other peptides with improved activity, P145 was randomly selected for further studies. Differences in activity could not be linked to specific amino acid substitutions.

Set 4: It is known that the activity of peptides can be maintained (or even improved) by using their retro-inverso (RI) form. One RI peptide consists of D-amino acids and is thus resistant to enzymatic degradation. For generating RI peptides D-amino acids were used and the amino acid sequence was synthesized in reversed order. The RI-variant of OP-145 and P3 were synthesized. These variants showed similar antimicrobial activity as OP-145 and P3 against *S. aureus* JAR060131 in PBS and in the presence of 50% plasma.

Set 5: The activity of the TC-1 derived peptide L3 was previously improved by replacing residues by lysines at several positions in the peptide, with L3-I14K being most potent. The initial approaches to design peptides with enhanced antimicrobial activity were to increase cationicity of the C-terminus by adding one or several lysine (K) residues, to increase hydrophobicity by replacing central tyrosine (T) for tryptophan (W) and to increase hydrophobicity of the N-terminus by adding phenylalanine (F) or W residues or some more subtle modifications (TC2 – TC25). The reference TC peptide L3-I14K (i.e. TC12) has potent bactericidal activity in low-salt buffer, but lacks activity in PBS (LC99.9 > 60  $\mu$ M). TC19 (the two central T substituted by W) is the only variant from the first series of modified peptides with improved activity in PBS and in 50% plasma when compared to L3-I14K.

Cyclisation is a strategy used for improvement of plasma stability of antimicrobial peptides. The presence of cysteine residues however complicates cyclisation of TC-derived peptides. Substitution of cysteines by other residues can facilitate the cyclisation of TC-derived peptides. Several peptide variants were designed where internal cysteines were substituted by 2-aminobutanoic acid (Abu), a cysteine analogue that lacks the ability of disulfide bond formation (TC26-TC30). In peptides TC28 and TC29 a dibromohexane spacer has been added to span the distance between the C- and N-terminus based on the previously described NMR structure of the L3 peptide. Unfortunately, all Abu-containing peptides lacked antibacterial activity, even in the low salt buffer. Thus, Abu is not suited to replace cysteine residues. Also cysteine to isoleucine substitution (TC37) or cysteine to tryptophan (TC44) resulted in loss of activity compared to TC19.

Since we aimed to design peptides with enhanced activity in physiologically relevant media (PBS, and preferentially also in 50% plasma), we designed novel variants based on peptide TC19, the only TC-derived peptide with activity in PBS and plasma from the first series. We designed various peptides (TC41 - TC80) based on the sequence of peptide TC19, with replacement of tryptophan (W) by other hydrophobic aromatic (phenylalanine, F) or hydrophobic bulky residues (tyrosine, Y), addition of extra hydrophobic residues at the N-terminus, replacement of cysteines by tryptophan, addition of an extra hydrophobic residue in the central region, N-terminal acetylation as a means to increase the hydrophobicity, or replace lysine (K) to arginine (R) and vice versa, and histidine to lysine substitutions. All peptides (TC41-TC80) were bactericidal at a fixed concentration of 60  $\mu$ M in PBS, but only peptide TC43, TC57, TC63, TC69, TC70 to TC75 were active in the presence of 50% plasma. Bactericidal activity compared to TC19 is maintained, but not substantially improved, by acetylation (TC69) or histidine to lysine substitution (TC75). Addition of an extra tryptophan to the N-terminus or in central region resulted in loss of activity. Replacement of tryptophan by phenylalanine or tyrosine in the central region resulted in reduced activity, and addition of these residues at the N-terminus or in the central region again resulted in loss of activity. Lysine (K) to arginine (R) and arginine to lysine substitutions only resulted in minor changes in bactericidal activity.

Set 6: An alanine-scan of TC19 was produced in which single amino acids were substituted by alanine. Thus, residues essential for microbicidal activity of TC19 could be identified, and also possibilities for further improved activity can be identified. Several substitutions of amino acids in the N-terminal region by alanine resulted in improved activity against *S. aureus* JAR060131, especially when tested in the presence of 50% human plasma. Subsequently, several combinations of these alanine substitutions were tested, but this did not further enhance the activity of these TC19 derivatives. Also substitution with amino acids other than alanine did not result in improved microbicidal activity compared to TC19.

In conclusion, P145, P148, P159 and TC19 were the second generation SAAPs with the most promising bactericidal activity in the presence of 50% plasma. We therefore proceeded to characterize the *in vitro* properties of these second generation SAAPs in further detail.

#### 1.4. Large scale synthesis

Large amounts of highly purified P145, P148, P159, P276 and TC19 were generated, and distributed to all collaborators within the BALI project for experimentation.

## 2. Antimicrobial characterization of SAAPs

### 2.1. Peptide library

Testing of 10% of bead library 1 resulted in at least 10 peptides with improved antimicrobial activity in plasma as compared to OP-145 and similar activity as our selected second generation SAAPs. Further optimization of the assay is necessary to find peptides with improved antimicrobial activity in plasma as compared to the second generation SAAPs.

## 2.2. Second generation SAAPs

Compared to OP-145, the second generation SAAPs P145, P148, P159 and TC19 all show increased antimicrobial activity in plasma against several bacterial species – including multi-drug resistant *Acinetobacter baumannii* and *Enterococcus faecium* – as well as one fungal species. These SAAPs had a slightly lower anti-biofilm activity than OP-145, but could all inhibit biofilm formation by 61-82%. The immunomodulatory activities of the SAAPs differed: compared to OP-145, the second generation SAAPs P145, P148 and P159 showed increased LPS-neutralizing activities; P145, P148 and P159, but not TC19, showed increased LTA-neutralizing activities; P145 and P148 showed a reduced ability compared to OP-145 to inhibit *S. aureus*-induced cytokine production; P159 and TC19 showed reduced change of monocytes differentiation towards pro-inflammatory macrophages. P148 induced higher levels of cytotoxicity towards erythrocytes and monocytes than OP-145, whereas TC19 did not lyse isolated erythrocytes or monocytes at the tested concentrations. Importantly, no resistance development in *S. aureus*, *S. epidermidis* and *Escherichia coli* was observed towards peptides P145, P148, P159 and TC19 after serial culturing in the presence of subinhibitory concentrations of the peptides up to passage 22.

To summarize, the second generation SAAPs P145, P148, P159 and TC19 displayed improved antimicrobial activities compared to OP-145. The combination of antimicrobial and anti-biofilm activities made us select P145, P159 and TC19 for characterization of the *in vivo* efficacy of second generation SAAPs, either injected or released from controlled release PolyPid coatings on implants in animal studies.

## 3. Biophysical characterizations of SAAPs

### 3.1. OP-145

OP-145, termed previously P60.4 is a synthetic 24-amino acid derivative of the human cathelicidin LL-37 and has antimicrobial and anti-biofilm activity, but low chemotactic activity as compared to its parent peptide LL-37. In order to gain insight into the mode of action biophysical studies on liposomes composed of phosphatidylglycerol (PG) and phosphatidylcholine (PC) mimicking bacterial and mammalian cell membranes were performed including leakage, structural (X-ray) and thermodynamic studies. Similarly, to earlier findings on LL-37 the peptide interacted with both lipid systems inducing however different extent of perturbation. Leakage experiments revealed that OP-145 induced complete release of entrapped fluorescence marker molecules from PG liposomes, while in case of PC liposomes only about one third was set free under the same experimental condition. Microcalorimetry showed that increasing peptide concentration led to a decrease of the pre- and main transition enthalpy of PC with a concomitant appearance of a low enthalpic, broad transition underlying the main transition of pure PC. These characteristics are indicative for a detergent-like action of OP-145 leading to disintegration of the multilamellar PC liposomes into disk-like aggregates as confirmed by small-angle X-ray scattering experiments. The perturbation of PG liposomes was more clearly detected in cooling scans showing a phase separation into peptide-enriched and -poor lipid domains that is in accordance with the degree of membrane thinning determined by X-ray scattering upon cooling. This suggests that OP-145 induces a quasi-interdigitated structure like LL-37.

### 3.2. Second generation SAAPs

The following strategies for modifications of OP-145 were performed, whereby (i) and (ii) should increase cell specificity improving the therapeutic index and (iii) should increase the stability *in vivo*: (i) Insertion of a cationic amino acid (lysine) replacing isoleucine at position 12 to break the hydrophobic face of the peptide (termed P1236-04), (ii) amino acid substitutions affecting the fraction of the hydrophobic face along the helix axis and increasing the net positive charge (P145, P159) and (iii) adding a polar polymer (PEG) at the N- and C-terminus of OP-145, respectively, (P1236-12, P1236-02).

All novel SAAPs were more effective in permeabilizing PG membranes (bacterial mimics) as compared to the parent peptide OP-145. While P159 and pegylated OP-145 did not improve the selectivity between the bacterial and mammalian membrane mimetic systems, this was the case to some extent for P145 and in particular for P1236-04 as deduced from DSC and leakage experiments. Both the rapid leakage and total permeabilization of PG liposomes at very low concentrations of P1236-04 suggest the formation of stable peptide pores, while negligible leakage was observed for PC even at very high peptide concentrations.

Furthermore, two peptides having a promising biological activity profile were included in the biophysical characterization. The synthetic Bactericidal Peptide 2 (BP2), based on LPS binding domains, and TC19, derived from thrombocidin-1, exhibited an unselective behavior affecting both membrane mimetic



systems. TC19 like P1236-04 clearly discriminated between the bacterial and mammalian model membranes not affecting PC bilayers in its thermodynamic behavior and structural properties and inducing leakage at very low concentrations only in PG liposomes.

The two peptides, TC19 and P1236-04, which show membrane specific activity, are characterized by structural flexibility and negligible  $\alpha$ -helix formation in the presence of bacterial mimics. While TC19 per se exhibits low amphipathicity, the amphipathic character of P1236-04 is broken by the cationic amino acid residue in the hydrophobic face, which reduces their ability to interact with mammalian mimics. DSC and leakage experiments however indicate that both peptides act differently on PG bilayers. Stable pore formation of P1236-04 and lipid segregation by TC19, which induces large defects within the bilayer, are those models, which describe best their molecular mode of action. Both result in high membrane permeability.

### 3.3. lead compound (i.e. P148)

As a lead compound P148 was selected, which in contrast to the parent peptide OP-145 and other designed peptides is characterized by higher total hydrophobicity, an increased net cationic charge and improved amphipathicity. All these features are considered to facilitate membrane partitioning and result in stronger permeabilization of membranes. Indeed, P148 is more effective in permeabilizing bacterial mimetic membranes as compared to the parent peptide OP-145. On the other hand, P148 did not improve the selectivity between the bacterial and mammalian membrane mimetic systems. Both leakage and total permeabilization of PG liposomes mimicking bacterial membranes at very low peptide concentration demonstrate strong perturbations of PG membranes owing to membrane thinning and creation of defects induced by peptide insertion. Leakage of PC/cholesterol liposomes representative for mammalian membranes was also observed at lower peptide concentrations as compared to the parent peptide. P148 clearly adopted an  $\alpha$ -helical conformation in the presence of mammalian membrane mimics, whereas in the presence of bacterial membrane mimics the detection of a defined secondary structure was limited due to formation of peptide aggregates. Finally, P148 formed hexameric peptide aggregates in aqueous solution as well as in the presence of lipid membranes.

## 4. Development of SAAP-releasing PLEX coatings

### 4.1. Coating procedure parameters

Adherence studies showed that TAN disks supported the adhesion of the PLEX coating to the surface much better than to stainless steel disks, probably due to the micro-structure nature of its surface. Therefore, TAN was selected for the *in vivo* studies.

Dipping and spraying of the coating to the TAN materials were both evaluated as coating methods, examined by uniformity, thickness, loading capability, release rate and profile. A previously developed doxycycline-PLEX formulation showed that the spray coating method resulted in a more uniform layer, the coating thickness can be adjusted, and thereby controlling the loading. The doxycycline release profile showed an initial release followed by a zero order kinetics release over more than 3 weeks.

### 4.2. Characterization of the OP-145-PLEX formulation

OP-145 was incorporated in the Polymer-Lipid Encapsulation Matrix (PLEX) formulation on titanium aluminium niobium (TAN) discs. The coating consists of poly lactic-co-glycolic acid (PLGA) as the biodegradable polyester, dipalmitoyl phosphatidyl choline (DPPC) for the saturated fatty acid role and the antimicrobial agent. Cholesterol was also added to the final formulation.

Using DSC (Differential scanning calorimetric) technique it was demonstrated that OP-145 peptide did not interact with the polymer (PLGA). In contrast, strong interaction between the peptide and the lipid (dipalmitoyl-PC, DPPC) was demonstrated. These interactions were characterized demonstrating that each OP-145 molecule can interact with up to five DPPC molecules within the formulation. Therefore, DPPC was selected as main ingredient for the formulation. Using FTIR (Fourier transform infrared spectroscopy) analysis, no interactions between OP-145 and any of the formulation substances was defined.

### 4.3. Characterization of the SAAP-PLEX formulation

PLEX formulations with P145 and P276 were prepared, resulting in a zero order kinetic release profile with different initial short term release: P145 release profiles demonstrated an initial short term release of 30-60% during the first 3 days followed by zero order kinetics release for 50 days, and P276 release profiles demonstrated an initial short term release of 48-60% during the first 1 day followed by zero order kinetics



release for 50 days. TC19 was integrated in a modified formulation, resulting in a release profiles with an initial short term release of about 70% during the first 3 days followed by zero order kinetics for 3 weeks.

## 5. Murine infection models

### 5.1. Development of BAI model

The established biomaterial-associated infection (BAI) mouse model<sup>1</sup>, originally developed to study *S. epidermidis* injected along silicon elastomer biomaterials, was adapted in three ways: i) to apply bacteria on a titanium biomaterial surface, ii) to study *S. aureus* infections and iii) to study immune regulation.

#### *Adaption of mouse BAI model to apply bacteria on a titanium surface*

The novel model where *S. epidermidis* bacteria are inoculated on the titanium biomaterial prior to implantation showed a larger reservoir of bacteria present on the implant surface compared to the model with injection of bacteria along the implant, but did not result in different levels of tissue colonization. This indicates that this novel model is suited to study biomaterial-associated infection where bacteria are introduced on the biomaterial. Survival of bacteria in tissue did not depend on the numbers of bacteria applied on the implanted biomaterial, but a higher bacterial load resulted in substantially higher levels of colonization of the biomaterial, and is therefore highly suited to assess the efficacy of antimicrobial coatings for anti-biofilm activity.

#### *Adaptation of the mouse BAI model for infection with S. aureus.*

To be able to study *S. aureus* infections in the BAI model, we performed dose-response studies with injecting *S. aureus* bacteria along the implants. We determined the inoculum size of *S. aureus* that is cleared by the host in the absence of a titanium biomaterial, but which caused colonization of the implant as well as the surrounding tissue in the majority of mice with a subcutaneous titanium implant. This illustrated that the presence of a titanium implant hampers the immune responses of mice to eradicate subcutaneously injected *S. aureus*.

#### *Development of mouse model for the assessment immune regulation*

We set up a model to investigate peptide efficacy to prevent dysregulation of immune responses, e.g. to neutralize inflammatory compounds from bacteria *in vivo*. Therefore, we have developed suitable methods for immunohistochemistry, transcriptome analysis and cytokine determination to determine the efficacy of SAAPs to prevent immune dysregulation related to implant infections in mouse experimental biomaterial-associated infection.

### 5.2. Efficacy of OP-145 to prevent BAI in mice

First, the *in vivo* efficacy of OP-145 against *S. aureus* JAR060131 was assessed when injected as free peptide along a titanium biomaterial, compared to injection of the conventional antibiotic doxycycline. Injection of OP-145 did not significantly reduce the colonization of the implant surfaces or the tissues surrounding the implants compared to the PBS injection, whereas doxycycline both reduced the numbers of CFU and the culture positivity of the implant surfaces and in the surrounding tissue compared to the PBS injection.

Next, we assessed the *in vivo* efficacy of OP-145 when incorporated in the PLEX coating on titanium against a doxycycline-resistant MRSA, compared to incorporation of the conventional antibiotic doxycycline in the PLEX coating. The OP-145-PLEX coatings significantly reduced the numbers of CFU on the implant surfaces compared to the uncoated control. Even with the use of a doxycycline-resistant MRSA strain, the doxycycline in the coating resulted in a reduction in numbers of CFU on the implant surface compared to uncoated controls. The numbers of bacteria in tissues surrounding the OP-145- or doxycycline-PLEX coated implants were not significantly reduced compared to uncoated controls.

In conclusion, when applied in a coating OP-145 is more effective in reducing the numbers of CFU on the implant surfaces compared to an injected solution containing the peptide, even with the use of the doxycycline-resistant MRSA strain. Doxycycline is still effective in reducing the bacterial colonization of implants of the doxycycline-resistant MRSA strain, but not in reducing the tissue colonization.

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<sup>1</sup> Broekhuizen et al. (2008), *Staphylococcus epidermidis* is cleared from biomaterial implants but persists in peri-implant tissue in mice despite rifampicin/vancomycin treatment, Journal of Biomedical Materials Research Part A.

## *6. Clinical investigation*

A study protocol was developed for a prospective case study to assess the safety and efficacy of a OP-145-PLEX-coated intramedullary nail system to be used in open diaphyseal tibia shaft fractures. The promising pre-clinical results with the novel second generation SAAPs (e.g. SAAP-148) within the BALI project lead to the decision not to execute the clinical study with the first generation OP-145 and to reallocate the budget to further pre-clinical investigations of the second generation lead SAAPs.

## *7. Pre-clinical trials*

With regards to pre-clinical toxicology testing of the most promising second generation SAAP P148, the following five studies have been done: 1) a 5-day intravenous repeated dose toxicity study of P148 (as well 6 other peptides) in rats (male and female), 2) a 14-day intravenous repeated dose toxicity study including toxicokinetics, local tolerance and safety pharmacology testing of P148 in rats, followed by a 7-day recovery period, 3) an acute dermal toxicity study of P148 in rabbits, 4) a single dose dermal application of P148 vs standard of care, and 5) a 14-day dermal repeated dose toxicity study including toxicokinetics and safety pharmacology testing of P148 in rabbits.

## Potential impact and the main dissemination activities and exploitation of results

### Potential impact

In the BALI project we have taken very significant steps towards development of new strategies in the treatment of infections. This is highly warranted, as antibiotic resistance (antimicrobial resistance, AMR) is emerging. This resistance will increase over the coming years and decades because of the excessive use of traditional antibiotics in humans and animals.

AMR results into resistance of a microorganism to antimicrobial drugs that were originally very effective to treat infections caused by the microorganism. Resistant bacteria are able to withstand treatment by antibiotics and frequently these bacteria are resistant to multiple drugs, or classes of drugs. As a result, standard treatments have become ineffective, resulting in prolonged illness, higher health care expenditures, and a greater risk of death, which, if the infections persist, poses a significant risk of spreading the resistant strains in the community.

*Antimicrobial resistance is becoming a global public health treat.*

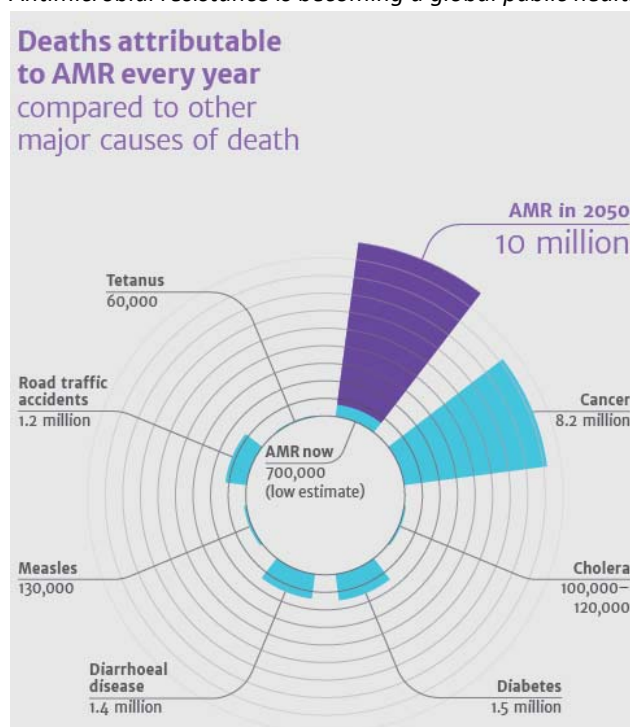


Figure 1 Source <http://amr-review.org>

Unfortunately, the excessive use of traditional antibiotics in humans and animals has resulted in the emergence of multidrug resistance of various microorganisms. This causes bacteria to become increasingly resistant to existing antibiotics, with the **rising mortality** rate and **extended hospitalization** for patients translating into **soaring treatment** and **societal costs**. AMR has gradually become a major global public health threat, which is recognized by governments and authorities like WHO, CDC, ECDC, etc. Antibacterial resistance kills nearly fifty thousand people per year across the EU and USA, whilst inducing healthcare costs of nearly 18 billion Euro.<sup>2</sup>

Since 2014 a remarkable number of alarming reports have come out on the impact of AMR. Just one example is a report on a deadly epidemic that could have global implications, which is quietly sweeping India. Among its many victims are tens of thousands of new-borns dying because antibiotics no longer work. These infants are born with bacterial infections that are resistant to most known antibiotics, and more

than 58,000 died last year as a result. While that is still a fraction of the nearly 800,000 new-borns that die annually in India, Indian paediatricians say that the rising toll of resistant infections could soon swamp efforts to improve India's abysmal infant death rate.<sup>3</sup>

As another example, the death rate for patients with serious infections caused by common bacteria treated in hospitals can be about twice that of patients with infections caused by the same non-resistant bacteria. For instance, people with MRSA (methicillin-resistant *Staphylococcus aureus*, another common source of severe infections in the community and in hospitals) are estimated to be 64% more likely to die than people with a drug-sensitive form of the infection. Recently, pan-drug resistant (PDR) and even so-called extensively drug resistant (XDR) bacteria have started to appear, taking the treatment situation to **a critical point**. Examples of XDR and PDR bacteria that place a huge burden on the global healthcare systems include carbapenem-resistant bacteria, such as KPC *Klebsiella* and *Acinetobacter*. Both of these organisms are belonging increasingly to XDR, and are increasingly found in the EU, the US and the rest of the world. They will continue to kill a high percentage of infected patients until new prevention and treatment methods become available.

<sup>2</sup> WHO Antimicrobial Resistance Global Report on Surveillance 2014

<sup>3</sup> [http://www.nytimes.com/2014/12/04/world/asia/superbugs-kill-indias-babies-and-pose-an-overseas-threat.html?\\_r=0](http://www.nytimes.com/2014/12/04/world/asia/superbugs-kill-indias-babies-and-pose-an-overseas-threat.html?_r=0)

The AMR problem is gradually gaining ‘momentum’ and we are reaching a critical stage in which we have to act. If no new drugs will be developed, we will encounter serious problems as a society where infections cannot be treated properly, causing illnesses and deaths. The UK’s chief medical officer, Dame Sally Davies, warned that the rise of antibiotic-resistant superbugs could lead to an “apocalyptic scenario” in which people would die of minor infections and basic operations would become deadly. She equated the threat of antibiotic resistance to terrorism and natural disasters, and called on parliament to place it on the government’s official register of national emergencies. UK’s Prime Minister David Cameron warned that it could take medicine back to the Dark Age.<sup>4</sup>

#### *Lack of development of novel antibiotics*

The lack of novel antibiotics is significantly compromising the survival and recovery of patients suffering from these infections, as well as our ability to fight simple bacterial infections in the future. Between 1940 and 1962, more than 20 new classes of antibiotics were approved and marketed. Since then, some new molecules – belonging only to two new classes – have reached the market. Now, not enough analogues are reaching the market to keep up with antibiotic resistance. The recently discovered teixobactin<sup>5</sup> is the first molecule that belongs to a new class of antibiotics, which is now in early stage preclinical development. Furthermore, every conventional (new) type of antibiotic will inevitably result in some form of resistance in the targeted bacteria. Therefore, there is a constant need to develop new agents to keep up with the acquisition of resistance among pathogenic bacteria. Antimicrobials are vital for reducing the risk of complications in relation to complex medical interventions as well as to reduce spread of (multi-drug) resistant bacterial strains.

There are various reasons why the development of new antibiotics has failed in the last decades. While some argue that the pharmaceutical industry is only interested in developing drugs for chronic diseases, we see that the major hurdle is a simple intrinsic problem: the ‘low-hanging fruits’ have simply been plucked. Drug screens for new antibiotics tend to re-discover the same lead compounds or with minor modifications towards approved drugs, to which bacteria can be resistant already. In general, discovery and development of antibiotics has become scientifically more complex, more expensive, and more time consuming over time, whereas the new compounds do not reach the market once they are approved because of drug-sparing regimens.

If one could propose a completely new class of antimicrobial compounds, this problem would be circumvented, thus creating very significant added value, both economic as well as societal, that others cannot easily copy.

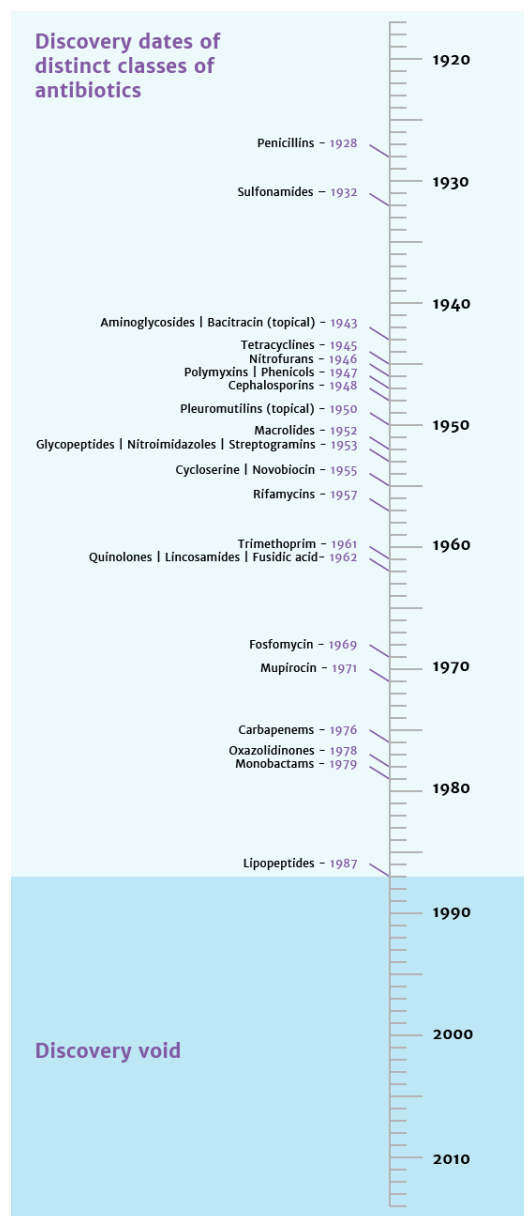


Figure 2. Source <http://amr-review.org/home>.

#### *SAAPs as alternative to classic antibiotics*

Synthetic Antimicrobial and Anti-biofilm Peptides (SAAPs) have a distinct mechanism of action from conventional antibiotics. The key advantage is that the development of resistance against these peptides is highly unlikely, due to their multiple modes of action. Thereby, The SAAP platform that has been developed in the BALI program addresses the fast growing and unmet medical need for new treatment options for patients suffering from difficult to treat infections due to antimicrobial resistance.

<sup>4</sup> <http://www.newsweek.com/revolutionary-new-antibiotic-alternative-could-save-world-superbug-apocalypse-282771>

<sup>5</sup> <http://www.nature.com/nature/journal/v517/n7535/full/nature14098.html>

### Synthetic Antimicrobial and Anti-biofilms Peptides (SAAPs)

Antimicrobial peptides based on naturally occurring antimicrobial peptides (AMPs) have been around for some time. Unfortunately, the first generation AMPs had limitations that prevented further development, especially with regard to their (limited) potency and high cost of goods, resulting in poor commercial perspectives. Several peptides have been tested in clinical studies against a range of topical infections, but their development was halted for these reasons.

*In vitro* comparison studies have shown that our initial peptide P60.4Ac (i.e. OP-145) is significantly more potent than first generation AMPs, active at a sub-micromolar range. Therefore, we anticipate lower dosages, higher efficacy and fewer side effects and consequently lower cost of goods.

Earlier we identified the LL-37-derived peptide P60.4Ac as highly effective peptides for the elimination of MRSA from medical devices and 3D human skin equivalents (resulting in the EC-funded FP7 project Biofilm Alliance (BALI) program). Furthermore, these peptides were found to rapidly and effectively kill both Gram-positive and Gram-negative pathogens including MRSA, antibiotic resistant *P. aeruginosa* (including extended-spectrum beta-lactamase (ESBL) producing strains), PDR *A. baumannii*, antibiotic-resistant *E. faecium* and *E. coli*, and NDM-1 drug-resistant *Klebsiella pneumoniae*. New peptides were subsequently identified in the BALI program and the Top Institute Pharma-funded project “Innovative peptides for atopic dermatitis (IPAD)”. From these families, we have selected the cathelicidin-based peptide SAAP-148 as the lead molecule based on best activity and toxicity profile. In addition to SAAP-148, we currently have about six other peptides as backup molecules, which are cathelicidin-based. These six have very similar but slightly different characteristics as SAAP-148, warranting their suitability as backup molecules in case SAAP-148 will fail during development.

#### Advantages of AMPs over conventional antibiotics:

- Microbicidal activity
- Gram-positive and -negative
- Fast activity
- Anti-inflammatory activity
- Antibiofilm activity
- High barrier to resistance

The BALI consortium has selected SAAP-148 as its lead candidate. This molecule has unique characteristics with regards to potency against bacteria, immuno-modulatory characteristics, endotoxin-binding activities, activity in the presence of plasma, and anti-biofilm aspects. This makes the peptide suitable for application in different therapeutic indications, as will be outlined in more detail in this business plan.

### Clinical positioning of the BALI SAAPs

The most promising peptide that was developed within BALI is SAAP-148. We have generated and are continuing to generate a large body of *in vitro* and *in vivo* efficacy and safety data for SAAP-148. Extensive toxicology reports for SAAP-148 are currently being generated (intravenous (IV) dose-escalating study in rats, and IV repeat-dose study in rats [GLP]) to demonstrate that SAAP-148 can be safely used in the anticipated dose-range we are targeting.

The antibacterial activities of the peptides in our project have been confirmed in *in vitro* killing and anti-biofilm assays also involving a full range of bacteria, including antibiotic-resistant bacteria (*P. aeruginosa* and *S. aureus*), forming the ESKAPE panel<sup>6</sup>. In addition to the direct antibacterial killing activity of these peptides on various laboratory as well as clinical bacterial strains, we have studied the anti-biofilm activity.

### Main dissemination activities and exploitation of results

#### Peer-reviewed scientific publications

Within the BALI project, 7 abstracts for (international) conferences were published in peer-reviewed scientific journals, and 7 scientific papers were published:

- T.F. Moriarty *et al.*, EFORT Open Reviews (2016), Orthopaedic device-related infection: current and future interventions for improved prevention and treatment.
- W-J. Metsemakers *et al.*, Journal of Controlled Release (2015), A doxycycline-loaded polymer-lipid encapsulation matrix coating for the prevention of implant-related osteomyelitis due to doxycycline-resistant methicillin-resistant *Staphylococcus aureus*.

<sup>6</sup> clinical relevant Gram-positive species *E. faecium* and *S. aureus* and Gram-negative species *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and Enterobacteriaceae, such as *E. coli* and *Enterobacter cloacae*.

- A. de Breij *et al.*, Journal of Controlled Release (2016), Prevention of *Staphylococcus aureus* biomaterial-associated infections using a polymer-lipid coating containing the antimicrobial peptide OP-145.
- N. Malanovic *et al.*, Biochim. Biophys. Acta – Biomembr. (2015), Phospholipid-driven differences determine the action of the synthetic antimicrobial peptide OP-145 on Gram-positive bacterial and mammalian membrane model systems.
- N. Malanovic and K. Lohner, Biochim. Biophys. Acta – Biomembr. (2016), Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides.
- N. Malanovic and K. Lohner, Pharmaceuticals (2016), Antimicrobial Peptides Targeting Gram-Positive Bacteria.
- K. Lohner, Curr. Top. Med. Chem. (2016), Membrane-active Antimicrobial Peptides as Template Structures for Novel Antibiotic Agents.

Moreover, 10 manuscripts are in preparation or already submitted.

#### *Outreach*

Results obtained in the BALI project have been presented in 35 oral and 14 poster presentations in international conferences, mainly within Europe, but also at conferences in Malaysia and the USA, and in 5 oral and 3 poster presentations in national conferences.

A known number of 18 (online) articles appeared in the (national) press in context of the BALI project. After a press release, 4 interviews were given on local and national radio stations. BALI was also partner in the organization of the International Meeting on Antimicrobial Peptides (IMAP) in 2014 in Graz (Austria).

#### *Public BALI meeting*

The final meeting for the BALI consortium was organized as a satellite meeting at the IMAP conference, which took place August 31<sup>st</sup> 2016 in Leipzig, Germany. During the public BALI meeting, representatives of the BALI consortium, Dr. A. de Breij (LUMC), Dr. R. van Leeuwen (Madam Therapeutic) and Dr. S.A.J. Zaat (AMC; PI of BALI) presented recent work from BALI. Moreover, different key note speakers were invited, namely Prof. Giovanna Batoni (Pisa, IT), Dr. Karin Thevissen (Leuven, BE), Dr. Annette Moter (Berlin, DE), Prof. Henk Haagsman (Utrecht, NL) and Prof. Ralf Hoffmann (Leipzig, DE). The joint key note lecture was shared between BALI and IMAP, and given by Prof. Andrej Trampuz (Berlin, DE).



**The address of the project public website, if applicable as well as relevant contact details.**

*Public consortium website*

[www.bali-consortium.eu](http://www.bali-consortium.eu)

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## Selection of group pictures of yearly BALI assemblies

### *Israel*



### *Austria*

