Send Orders for Reprints to reprints@benthamscience.ae

Current Topics in Medicinal Chemistry, 2018, *18*, 2068-2079

REVIEW ARTICLE

RNAIII Inhibiting Peptide (RIP) and Derivatives as Potential Tools for the Treatment of *S. aureus* Biofilm Infections

Michele Ciulla, Antonio Di Stefano*, Lisa Marinelli, Ivana Cacciatore and Giuseppe Di Biase

Department of Pharmacy, University "G. d'Annunzio" of Chieti - Pescara, Chieti, Italy

ARTICLE HISTORY

Received: April 17, 2018
Revised: September 11, 2018
Accepted: September 24, 2018

DOI:
10.2174/1568026618666181022120711

Abstract: *S. aureus* under the biofilm mode of growth is often related to several nosocomial infections, more frequently associated with indwelling medical devices (catheters, prostheses, portacaths or heart valves). As a biofilm, the biopolymer matrix provides an excellent growth medium, increasing the tolerance to antibiotics and host immune system. To date, the antimicrobial therapy alone is not effective. A novel strategy to prevent biofilm formation is based on the interference with the bacterial cell–cell communication, a process known as quorum sensing (QS) and mediated by the RNA-III-activating peptide (RAP) and its target protein TRAP (Target of RAP). The RNAIII inhibiting peptide (RIP) is able to inhibit *S. aureus* pathogenesis by disrupting QS mechanism competing with RAP, thus inhibiting the phosphorylation of TRAP. This alteration leads to a reduced adhesion and to the inhibition of RNAIII synthesis, with the subsequent suppression of toxins synthesis. The present paper will provide an overview on the activity and potential applications of RIP as biofilm inhibiting compound, useful in the management of *S. aureus* biofilm infections. Moreover, medicinal chemistry strategies have been examined to better understand which modifications and/or structure alterations were able to produce new derivatives of this QS inhibitor with an improved antibiofilm activity.

Keywords: *S. aureus* Biofilm, Nosocomial infections, Quorum sensing mechanism, Cell-cell communication inhibition, RNAIII inhibiting peptide, Structure-activity relationship.

1. INTRODUCTION

Bacterial growth is characterized by two life forms, one being as single cells and the other being in sessile aggregates, commonly referred to as biofilm. In recent years, the biofilm modality of growth was found in several bacterial cell settings, often found in close association with surfaces and interfaces, in the form of multicellular aggregates [1]. These aggregates are normally present in nature, but they can also develop human microbial biofilm infections - especially related to the utilization of implants or medical devices [2] - redefining the description of biofilm in medical field as "*a coherent cluster of bacterial cells imbedded in a matrix, which are more tolerant to most antimicrobials and the host defense, than planktonic bacterial cells*" [3].

Indeed, the biofilm-embedded microorganisms have several advantages, such as a broad concentration of environmental nutrients, the ability to evade the clearance mechanisms produced by the host, and also a strong tolerance to antimicrobial factors [4, 5].

In particular, this characteristic is explained through both the inability of the antimicrobial molecules to diffuse into the polymeric matrix and the alteration of the cellular growth state, with the production of many slow-growing cells that are tolerant to high levels of antibiotics [6].

It is estimated that over 60% of bacterial infections in humans involve biofilm formation [1]. In particular, biofilm plays a central role in healthcare-associated infections (HAIs), especially those related to the implant of medical devices, intravascular and urinary catheters, orthopedic implants or cardiac valves [7].

Several studies have shown that, since the end of the '90s, the number of HAIs has risen dramatically, estimating that there are about 4 million individuals annually which contract nosocomial infections in the European hospitals, whereas in the corresponding US clinics they are estimated to be between 2 - 4 million [8, 9]. These results not only have a consequence in the hospitalization cost, estimated around 5 billion dollars per year, but primarily in the increase in mortality rate which, in some cases, can reach 35% for the most difficult-to-treat infections, such as those originating from the heart valves [10, 11]. In particular, among the HAIs bacteria, *S. aureus* is one of the most clinically relevant pathogens due to its resistance to antibiotics and the increased use of indwelling medical devices [12]. *S. aureus* biofilms, once established, are resistant to the antimicrobial treatments and the host response, becoming the etiological agent of many recurrent infections. To date, the antimicrobial therapy alone is not effective [7]. In conjunction with surgical intervention - such as debridement, incision and drainage or the removal

*Address correspondence to this author at the Department of Pharmacy, University "G. d'Annunzio" of Chieti - Pescara, Chieti, Italy;
Tel/Fax: +39-0871-355-4708; E-mail: antonio.distefano@unich.it

Current Topics in Medicinal Chemistry

 © 2018 Bentham Science Publishers

indwelling medical device - antimicrobial therapy is often prolonged and takes place in the outpatient setting. The majority of patients with surgically managed biofilm infections can be treated orally with one or more antibiotics: β-lactam agents, fluoroquinolones, aminoglycosides, glycopeptides (vancomycin), linezolid or daptomycin [13, 14].

Beside these common approaches, a number of strategies have been developed in the last two decades to prevent or attenuate the biofilm growth. Preventing attachment of bacteria to surfaces can be achieved by the use of antibacterial coatings, anti-adhesion surfaces, vaccines [15-17]. On the contrary, the treatment or disruption of established biofilms can be achieved with matrix degrading enzymes, dispersal triggering agents, small-molecule inhibitors or targeting the regulatory networks [18-20]. Among the molecules able to inhibit the regulatory networks of bacteria, a new approach for the reduction of *S. aureus* biofilm virulence is represented by the RNAIII inhibiting peptide (RIP) [21]. RIP is able to effectively counteract bacterial cell-cell communication, commonly named as Quorum Sensing (QS), resulting in a reduced adhesion of biofilm to surfaces and inhibition of toxins and bacterial virulence.

The present paper provides an overview on the activity and potential applications of RIP as biofilm inhibiting compound, useful in the management of *S. aureus* biofilm infections. Moreover, medicinal chemistry strategies have been highlighted to investigate which modifications and/or structural alterations were able to improve the therapeutic profile of this QS inhibitor, and at the same time, produce new with an improved antibiofilm activity.

2. *S. AUREUS* BIOFILM FORMATION

S. aureus is a Gram-positive, ubiquitous bacterial species, able to produce a multilayered biofilm embedded in a matrix of extracellular polymeric substance, inside a glycocalyx layer, and characterized by an altered phenotype with regard to growth, gene expression and protein production [22].

Four types of subpopulations have been identified into the polymeric matrix, different in terms of metabolic characteristics: cells with aerobic metabolism, with fermentative metabolism, quiescent (slow growth) or deaths. The metabolic state is largely dependent on the area of the matrix in which the cells are present: in the most superficial layer, with greater availability of oxygen and nutrients, we will find highly metabolically active cells; in the deeper layers, where mainly anoxic environment is present, there will be fermentative or quiescent cells [5]. As a microbial biofilm, three main phases characterize its formation (Fig. **1**). At the beginning, free-floating microorganisms adhere to the surface through weak, reversible adhesion via van der Waals forces and hydrophobic effects. Subsequently, if the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures or proteins, with the subsequent excretion of a slimy, polymeric substance that builds the matrix which encloses and holds bacteria together, allowing the biofilm growth. The final stage of biofilm formation is known as detachment or dispersion, and is the stage in which the biofilm is established and free-floating bacteria (which turn immediately into the planktonic growth phase), or biofilm fragments can be released in the surroundings, allowing the spread of the infection in different host's sites [23].

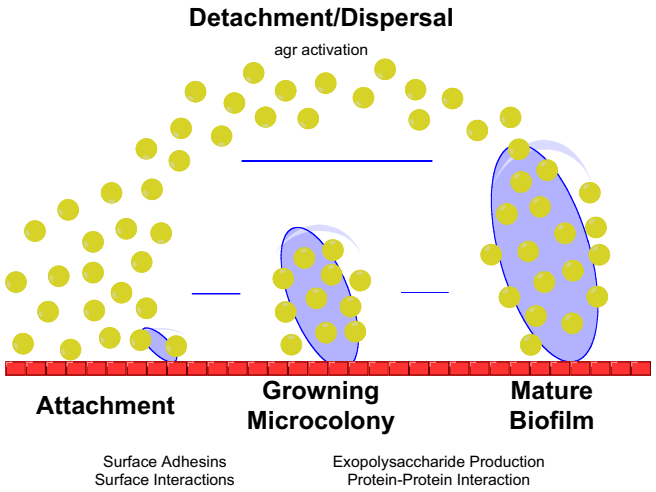


Fig. (1). Biofilm modality of growth on solid surface or animal tissue.

In the case of *S. aureus*, the virulence and the ability to develop pathologies in the host are given by the production of exotoxins such as proteases, enterotoxins, hemolysins and the toxic shock syndrome toxin (TSST1). These molecules play also a fundamental role in the survival of the bacterial cells. The aforementioned exotoxins are not produced during the entire life cycle of the microorganism, but only in the post-exponential growth phase. For cells associated in biofilm, the production of exotoxins occurs during the last stages of biofilm development, when cells are found in large numbers and at a high density [12].

3. MECHANISM OF *S. AUREUS* QUORUM SENSING COMMUNICATION

During the conversion from the free floating mode of growth to the biofilm modality, there is a process of signaling between bacterial cells thanks to the continuous exchange of numerous chemical messengers.

In *S. aureus* two main mechanisms of QS have been recognized, QS1 and QS2, both essential for the production of toxins and the transition from a fluctuating to a sessile form and *vice versa* [24]. In particular, the RNAIII-activating peptide (RAP) is the autoinducer of QS mechanisms. RAP is a 277 amino acids protein that induces the phosphorylation of TRAP, its target protein and master regulator of *S. aureus* pathogenesis (Fig. **2**). TRAP is a 21 kDa protein that is histidine-phosphorylated in the presence of RAP [24]. TRAP expression is constitutive, but its phosphorylation is regulated by RAP and reaches the peak of phosphorylation in the mid-exponential phase of growth. In particular, QS1 consists of the activation of the TRAP receptor by the auto-inducer protein RAP, produced by bacterial cells. When the RAP reaches a threshold concentration, it activates its own TRAP receptor, inducing phosphorylation on a histidine residue. The activation of TRAP occurs in the initial exponential growth phase, when the bacteria are at a low concentration

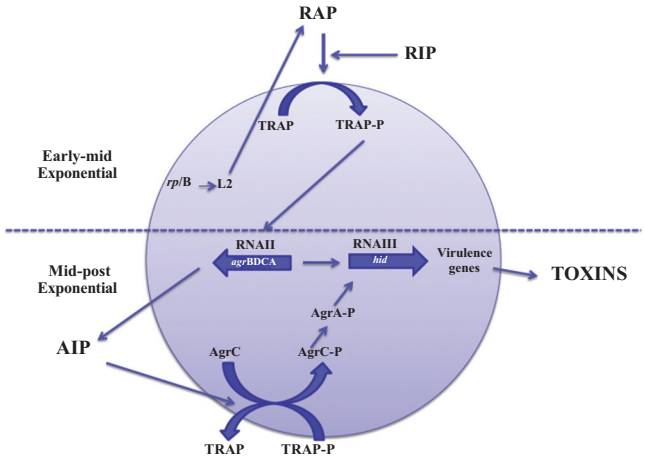


Fig. (2). Proposed *S. aureus* QS mechanism and toxin production *via* TRAP-RAP interaction and agr regulation.

within the biofilm. The phosphorylation of TRAP receptor is followed by the induction to the production of adhesion proteins, leading to a growth of the bacterial population and the passage of new cells from the fluctuating form to the sessile form [25]. The phosphorylation of TRAP also leads to trigger the QS2 mechanism, based on the activation of the gene locus *agr*. The *agr* system encodes two divergently transcribed transcripts, RNAII and RNAIII, promoted by the P2 and P3 promoters, respectively, which are mainly active during the mid-exponential growth phase of the bacteria [26]. In particular, the activation at the level of the P2 promoter induces the transcription of RNAII, a polycistronic transcript composed of 4 Open Reading Frames (ORFs): *agr*A, *agr*B, *agr*C and *agr*D. The *agr*A and *agr*C genes encode a classical two-component signal transduction pathway composed of the AgrC signal receptor and the AgrA response regulator. The *agr*D gene product is a pro-peptide that is processed and secreted through AgrB, which is an integral membrane protein. The protein produced is the Autoinducing Peptide (AIP), which is able to bind and activate the phosphorylation of AgrC [27]. The phosphorylation cascade involves the phosphorylation of AgrA, leading to the up-regulation of RNAIII synthesis. The latter is a *trans*-acting RNA, which regulate several mRNAs at the post-transcriptional level. This transcript acts as an mRNA that encodes several peptides, and more important, it controls the switch between the early expression of surface proteins and the late expression of several exotoxins, acting as a regulator in the production of bacterial toxins and surface adhesive proteins [28, 29]. In particular, RNAIII represses the synthesis of membrane proteins that favor adhesion to the surface during the mid-exponential phase, such as protein A and fibronectin-binding proteins, generating an initial detachment of cells from the site and the return to the fluctuating form. On the contrary, during the post-exponential phase, it stimulates the production of toxins such as enterotoxins B, C, D and E (Staphylococcal Enterotoxins type B, C, D, E, or SEB, SEC, SED and SEE), hemolysins α and β, Toxic shock syndrome toxin 1 (TSST-1), thus leading to the onset of virulence [30, 31]. The two QS phases are connected to each other and are phase-dependent: once AIP is made in the mid-exponential phase of growth, it indirectly down-regulates the phosphorylation of TRAP. This is reflected in the production of adhesion proteins induced by TRAP, leading to their reduction, and at the same time, encouraging the return from the sessile to the fluctuating form. The QS mechanisms, therefore, act to avoid a biofilm overcrowding, which could lead to nutrient deficiency for the entire population, as well as to promote the dissemination in different districts of the host organism [32]. In this wide panel, QS mechanisms can be inhibited using the selective TRAP receptor inhibitor RIP. Competing with RAP for the receptor binding, RIP leads to the blockade of the QS mechanisms, thus interfering with the various phases of biofilm formation and the development of the virulence [33].

4. RIP STRUCTURE AND MECHANISM OF ACTION

RAP is a 277 amino acids protein, containing the NH_2-terminal sequence IKKYKPITN, ortholog of the ribosomal protein L2 and one of the peptides involved in the QS process [34]. The RIP peptide is an heptapeptide isolated initially from the supernatant of cultures of *S. xylosus*, with the amino acid sequence identified as YSPXTNF, where X may represent a residue of W or C [33]. Subsequently the isolation, in 1998 Balaban *et al.* determined that the optimal amino acid sequence was YSPWTNF [17]. The authors synthesized and tested the new RIP peptide for its ability to interfere with the RNAIII production *in vitro*, demonstrating that the inhibiting capacity was comparably to the native RIP.

Moreover, Gov *et al.* in 2001 developed the definitive synthetic RIP structure, with the amino acids sequence YSPWTNF and the terminal –COOH portion in the amide form (Fig. **3**) [33]. The resulting synthetized RIP was equally active if compared with the native peptide. Furthermore, it was soluble in water, could inhibit the RNAIII synthesis at 5 µg per 106 *S. aureus* cells and it was stable at high temperatures. Notably, the structural similarities with the terminal -NH_2 portion of RAP are probably responsible for the ability of RIP to bind the TRAP receptor and antagonize the action of RAP [21]. The antagonist effect on the TRAP receptor leads to the inhibition of phosphorylation on the histidine residue, interrupting the QS mechanisms upstream. In fact, the failure to activate TRAP does not lead to the induction of the agr gene through the P2 promoter, causing the absence of the four agr A, B, C and D factors and thus blocking the QS1. The lack of production of the *agr* factors leads to a blockage of the QS2, with the consequent failure to induce RNAIII. By interfering with these mechanisms, RIP causes in the cell an opposite response compared to the physiological bacterial behavior. In fact, during the initial-exponential phase, RIP stops the production of adhesion proteins, avoiding in this way that the cells adhere to the surfaces and form populations able to develop biofilms. Moreover, several studies showed that RIP globally decreases the adhesion of bacterial cells to surfaces also during the mid-exponential phase [35]. RIP also has an effect on the events occurring during the post-exponential phase, such as the production of exotoxins, avoiding the onset of diseases related to the microorganism [32]. The molecular mechanisms through which RIP disrupts the QS transmission sequences activated by the TRAP-RAP mechanism have been confirmed in several studies.

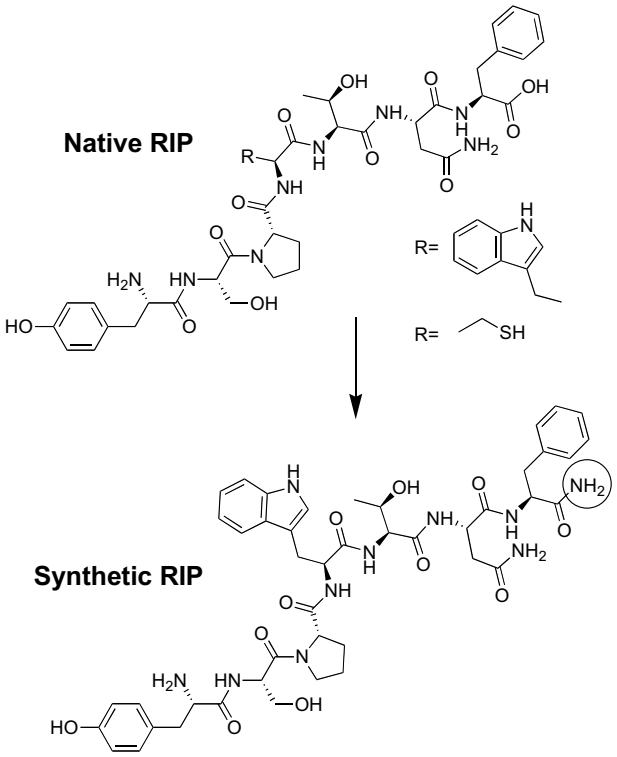


Fig. (3). Native RIP and its derivative synthetic structure, with R = W and the terminal -COOH portion in the amide form (circle).

In particular, Vieira-da-Motta *et al.* assessed this effect by observing the RIP influence on both enterotoxin A, B, C, D, and E, and on hemolysin α and β [36]. For the measurement of enterotoxins, the supernatant deriving from the centrifugation of bacterial cultures treated with RIP or with DMSO was analyzed, and the quantification was performed with a visual immunoassay. A reduction of SEB production of 89.8% was observed, 25.4% for SEC, 12.8% for SED, 44.8% for the SEE. On the other hand, there was no reduction of the SEA toxin, confirming the fact that it is not an agr-dependent toxin produced. To evaluate the hemolytic activity reduction of *S. aureus* in the presence of RIP, the same authors produced cultures enriched with and without the QS inhibitor. After centrifugation, the supernatant with the hemolysins produced by the bacterium was taken from these cultures. Different supernatants were placed into 96-well plates, then rabbit erythrocytes were added and incubated. The healthy cells were left to sediment and the supernatant was analyzed through UV spectroscopy (λ = 405 nm) to measure the optical density (OD) of the residual hemoglobin. It was observed that treatment with RIP led to a decrease of 86% in hemolytic activity compared to control cells.

Balaban *et al.* incubated three different *S. aureus* cultures in a buffer containing 32P labeled phosphate groups, which was used by bacteria to phosphorylate the TRAP receptor in the event of its activation [24]. The presence of the radioactive atom allowed the observation of the receptor activation during the analysis. The three cultures differ in the presence of RAP, RIP or blank buffer. After incubation, the cells were analyzed in SDS-PAGE and the presence of the TRAP-P receptor observed by autoradiography. The observation showed that the lane derived from bacterial cultures incubated in buffer without RAP or RIP and the lane derived from cells incubated with RAP expressed a high density band, corresponding to a protein of 21.5 kDa, the phosphorylated TRAP receptor. The most intense band was detected on the RAP lane, with respect to the control, confirming that RAP stimulates the phosphorylation, while the absence of any bands on the RIP lane confirmed that the heptapeptide inhibits the phosphorylation of TRAP. In the same study, the authors investigated the competitive inhibition mechanism of RIP to the RAP-TRAP system. Also, in this case, the presence of radioactively labeled TRAP-P was evaluated by SDS-PAGE and observed by autoradiography. Six cultures of the same strain of *S. aureus* were prepared with different buffers. In the first culture, RAP (50 μL) was added to the buffer, in the second saline without RAP or RIP, in the third only RIP (50 μL), in the fourth, fifth and sixth 50 μL of RAP were added, with the addition of decreasing doses of RIP (50, 25, 12.5 μL, respectively). It was possible to notice that in the culture which presented 50 μL of RIP and 50 μL of RAP, there was no phosphorylation of TRAP, due to the inhibition by RIP which cancels the effect of RAP present into the buffer. Competitive antagonism was established by the minimal presence of TRAP-P in those cultures with a lower concentration of RIP, confirming that the inhibition of the receptor is dose-dependent.

Gov *et al.* assessed the block of the production of RNAII and RNAIII mediated by RIP [33]. Wild-type *S. aureus* cells were incubated with RAP or Phosphate Buffer Saline (PBS) for 40 min or with RIP or control buffer for 2.5 h. Subsequently, bacterial cells were collected, RNA purified and northern blotted. RNAII was detected by hybridizing the membrane with RNAII-specific radiolabeled DNA followed by hybridization with RNAIII-specific radiolabeled DNA, and the membrane autoradiographed. Compared to the control, the absence in the synthesis of RNAII and RNAIII was observed in bacteria treated with RIP.

4.1 RIP *In Vitro* and *In Vivo* Studies

Several studies were performed both *in vitro* and *in vivo* to demonstrate the efficacy of RIP in the bacterial cells reduction related to difficult-to-treat infections caused by *S. aureus* biofilm.

Giacometti *et al.* tried to evaluate the preventive effect of RIP against MSSA (Methicillin-susceptible *S. aureus*) and MRSA (Methicillin-resistant *S. aureus*) infections due to the biofilm formation on implantable devices [37]. For the experiments, a subcutaneous pocket was made on adult male Wistar rats. A 1 cm^2 sterile collagen-sealed Dacron graft was aseptically implanted into the pocket. Immediately prior to implantation, the Dacron grafts were soaked for 20 min in a sterile solution of 10 μg/mL of RIP. Part of the animal groups received solutions containing also antibiotics including levofloxacin, rifampin, quinopristine-dalfopristin, and mupirocin (Table **1**). This group of implants were used to evaluate the effect of local antibiotic prophylaxis on the formation of bacterial biofilms in the presence of antibiotic alone or with RIP, in order to estimate a possible synergistic effect. In addition to local prophylaxis, systemic prophylaxis and its effect on bacterial colonization were also evaluated. Antibiotics used for this type of prophylaxis were cefazolin,

imipenem, teicoplanin and levofloxacin which were administered intraperitoneally 30 minutes before the implantation. In this way, it was also possible to observe the synergistic effects between RIP and the parenteral antibiotic prophylaxis. Once the rat skin pockets were closed by skin clips, a saline solution containing MSSA or MRSA was inoculated. The measurement of the bacterial concentration present on the implant took place after 7 days from the graft implantation.

As it is possible to see in Table **1**, RIP treatment alone showed a reduction of the bacterial inoculum in both the experiments if compared to the untreated grafts, but the inhibition was not sufficient to completely eradicate the infection. It was possible to notice how the association of RIP with the antibiotics expressed better effect if compared to the use of single antibiotic or RIP alone. Best results were achieved by the use of the RIP associated with systemic teicoplanin, local mupirocin and local quinupristine-dalfopristine used as prophylaxis. In combination with these antibiotics, RIP was able to achieve 100% inhibition. Carbapenems (imipenem), cephalosporins (cefazolin), and quinolones (levofloxacin) were less effective, but the addition of RIP strongly increased their inhibition potentials. The most relevant fact was that the antibiotic-RIP association was also active on MRSA strains, which are more difficult to eradicate with conventional therapies than the more sensitive MSSA. Moreover, the incapacity of RIP alone to remove *S. aureus* biofilm infection was also an indication that the interaction is only at the QS level, and the intervention of the host immune system or an antibiotic therapy may be necessary to complete the protocol for the eradication.

To assess the action of RIP in single therapy, Balaban *et al.* in 2007 evaluated the peptide as an anti-biofilm agent against *S. aureus* in a multiple dose therapy [38]. Moreover, in the same studies, the action was also observed in association with teicoplanin. For the experiments, the bacterial contamination was evaluated applying the protocol mentioned before, using the skin pocket implants covered with collagen and inserted subcutaneously on rat models. The initial contamination of the implant was caused by the inoculation of a bacterial culture, while RIP therapy involved an intraperitoneal administration, in order to evaluate the effect of the systemic circulation. Several RIP protocols were applied: 0 (untreated), 10, 20 and 30 mg/kg of body weight, administrated once a day, for 1, 4 or 7 days. Furthermore, the administration of the above-mentioned doses occurred immediately after the inoculation of the bacterial cells or after 2 days; the evaluation of the bacterial number took place at day 10, after removal of the implant and the CFU (Colony Forming Units) counts. The intraperitoneal route was also chosen for the combined RIP/teicoplanin treatment. In particular, the study of a possible synergism between RIP and teicoplanin was carried out treating individuals for 7 days, starting from the 3rd day after the inoculation of the microorganisms, with a daily dose of 10 mg/kg for RIP and 3 mg/kg for teicoplanin. For some animals, the implant was removed on day 3, in order to confirm the presence of biofilm before starting the therapy. Results showed that treatment of rats with RIP was most effective when RIP was administered in multiple doses, with suggestions of dose- and duration-dependent effects on biofilm reduction. It was confirmed also that RIP treatment, when it is combined with antibiotics like teicoplanin, results in augmented activity compared to the activity of both agents alone. These results provide encouraging support for the further evaluation of RIP as a therapeutic agent for device-associated infections, including those caused by drug-resistant strains.

Bacterial biofilms create problems regarding the chronicity of wounds or skin ulcerations, since many of them do not respond to the common treatments available on the market. The resistance that biofilm gives to bacterial cells is the cause of their persistence on the infected wounds and their difficult eradication with consequent healing.

Simonetti *et al.* tested the effect of RIP on these types of wounds infected with *S. aureus* cells, in order to verify the possible effect on the bacterial reduction [39]. Groups of animals were treated with RIP, teicoplanin or with the medical device Allevyn®, a particular type of dressing applied to the wound. To evaluate any synergistic effects, the three therapies were also combined with each others. A mouse model of surgical wound infection was used to investigate the efficacy of drug-free and RIP-soaked Allevyn® compared to that of parenteral administration of teicoplanin. After 24 hours from the inoculum of *S. aureus* colonies animals received the following therapies: Allevyn® without RIP replaced every 2 days; Allevyn® containing 20 μg of RIP replaced every 2 days; teicoplanin intraperitoneally, given once a day for 7 days (7 mg/kg of body weight); combination of Allevyn® and teicoplanin therapy; combination of therapy with Allevyn® enriched with RIP and teicoplanin. At the end of the therapy cycles, animals were sacrificed and the skin portion containing the incision was homogenized in buffer and subjected to CFU counts by the method of subsequent dilutions. RIP did not demonstrate any *in vitro* activity as expected by its mechanism of action (minimum inhibitory concentration or MIC > 256 mg/L). The highest inhibition of bacterial load was obtained in the group that received RIP-soaked Allevyn® and intraperitoneal teicoplanin (13 CFU/mL), underlying the contribution that RIP has as a strong QS reducing agent which can be administered as common antibiotic therapies.

In another study, Schierle *et al.* presented a novel murine cutaneous wound system that directly demonstrates delayed re-epithelialization caused by the presence of *S. aureus* biofilm [40]. In particular, full-thickness excisional punch wounds were created through the skin down to the *panniculus carnosus*, and the wound contraction was prevented by the use of a flexible external splint. This technique allowed to evaluate the wounds re-epithelialization grade and observe the course of the infection in the presence of *S. aureus* biofilm, avoiding wounds contraction. Animals were divided into three groups: the first received topically on the wound infected 0.1 mL of a 1 mg/mL of RIP solution; the second group received topical oxacillin at a concentration of 10 mg/L; the last group did not receive any treatment. The therapies were provided into a single administration the third day from the bacteria inoculation. Results showed that wound re-epithelialization was significantly delayed by bacterial biofilm. Compared to the uninfected mice, in which re-epithelialization is completed at day 9, animals infected showed a more slow healing kinetics. Treatment with oxacil-

Table 1. Summary of experimental results derived from the RIP treatments in combination with local and systemic antibiotic profilaxis.

Antibiotic	Bacterial Concentration (log CFU/mL) + SD		Route of Administration
	MRSA	MSSA	
Control	7.73 ± 0.11	7.49 ± 0.07	/
RIP	4.58 ± 0.09	4.64 ± 0.06	Local
Cefazolin	7.49 ± 0.04	5.64 ± 0.25	IP
Cefazolin + RIP	4.40 ± 0.09	3.40 ± 0.13	IP
Teicoplanin	2.90 ± 0.05	1.93 ± 0.03	IP
Teicoplanin + RIP	<1	<1	IP
Imipenem	4.91 ± 0.19	3.89 ± 0.12	IP
Imipenem + RIP	3.84 ± 0.09	2.86 ± 0.09	IP
Levofloxacin	5.84 ± 0.13	4.59 ± 0.16	IP
Levofloxacin + RIP	4.68 ± 0.06	3.69 ± 0.08	IP
Mupirocin	2.91 ± 0.16	2.84 ± 0.17	Local
Mupirocin + RIP	<1	<1	Local
Rifampin	4.85 ± 0.15	4.92 ± 0.14	Local
Rifampin + RIP	2.96 ± 0.09	3.57 ± 0.04	Local
Quinupristin-Dalfopristin	1.68 ± 0.08	1.64 ± 0.19	Local
Quinupristin-Dalfopristin + RIP	<1	<1	Local
Levofloxacin	5.9 ± 0.15	4.89 ± 0.14	Local
Levofloxacin + RIP	4.18 ± 0.19	3.93 ± 0.09	Local

Local: Local antibiotic prophylaxis.
IP: Intraperitoneal antibiotic prophylaxis.

lin did not lead benefits to the skin regeneration, keeping the kinetics of re-epithelialization substantially similar to that of individuals not treated. The treatment of biofilm infected wounds with RIP was able to eradicate biofilm colonization and restore the normal wound healing kinetics. Significantly, wounds re-epithelialized more rapidly when treated with RIP if compared with untreated controls (day 7 epithelial gap: 1.1 mm *vs.* 2.4 mm, respectively).

Balaban *et al.* examined whether RIP reduces adherence of *S. aureus* to host cells and dialysis catheter polymers *in vitro* [41]. For the adherence test, HaCat human skin keratinocytes or human epithelial HEp-2 cells were incubated with labeled *S. aureus* with or without RIP, whereas for the polymer test, *S. aureus* strains were grown on polystyrene wells in the presence or absence of RIP. Results showed that the heptapeptide decreased bacterial adherence to human cells as well as the attachment to polymeric material, demonstrating the potential role of RIP in the reduction of bacterial adhesion and the number of adherent *S. aureus* cells.

Domenico *et al.* assessed the combination efficacy of RIP and bismuth ethanedithiol (BisEDT), a new generation of bismuth agents with a greater antibacterial activity [42]. Experimental studies were conducted by the use of the rat graft model applied in the study mentioned before [36]. The authors included a control group, a contaminated group, and different groups that received RIP, BisEDT, rifampin, BisEDT and rifampin, RIP and rifampin, BisEDT and RIP-soaked grafts, respectively. Before implantation, the graft segments were soaked for 30 min in 0.1 mg/L of BisEDT, 10 mg/L of RIP, or 10 mg/L of rifampin, alone or in combination therapy. Results showed a modest adherent bacterial reduction among RIP-treated groups and BisEDT treated animals, while RIP–rifampin or BisEDT–rifampin treatment showed a higher reduction of *S. aureus* cells on the graft if compared with the control group. Finally, the groups treated with the BisEDT–RIP combination showed the minimal quantitative cultures of *S. aureus*. Moreover, among the resistant strains, the only combined therapy that was significantly better than single-agent therapy was BisEDT–RIP. Another positive indication was that none of the agents exhibited any toxicity and no animals died or had clinical evidence of drug-related adverse effects.

Cirioni *et al.* tested the efficacy of RIP, ciprofloxacin, imipenem, and vancomycin into an *in vitro* susceptibility test on planktonic *S. aureus* and its biofilms mode of growth [43]. Moreover, the same authors assessed the treatment of central venous catheters infections (CVC), using a silastic catheter inserted into the jugular vein and advanced into the superior *vena cava* of adult rats. The rat CVC–associated infection model included a control group (no CVC infection) and a contaminated group that did not receive any antibiotic prophylaxis. There werealso a contaminated group that received CVCs filled with 0.1 mL of RIP at 1 mg/mL and contaminated groups that received RIP-filled CVCs or no RIP-filled CVCs plus ciprofloxacin, imipenem, or vancomycin. Regarding the *in vitro* susceptibility test, the activity of the three antibiotics against the biofilm was at least 2-fold lower than that against the freely growing planktonic cells, but when bacterial cells were first treated with RIP and then with antibiotics, no difference in MICs and MBCs (Minimum Bactericidal Concentration) against planktonic cells was observed, but MICs and MBCs against biofilms were drastically reduced. RIP alone did not show any microbicidal or microbiostatic activity, as it was expected on the basis of its mechanism of action. The rat CVC–associated infection model exhibited similar results, in which vancomycin, ciprofloxacin, and imipenem exerted only a weak antimicrobial activity, with results not significantly different when compared with the untreated controls. In contrast, when the catheters were impregnated with RIP, catheter/venous tissues showed a significant reduction of the bacterial biofilm. This is an ulterior confirmation that RIP could be a potent and useful tool for the eradication of *S. aureus* biofilm into a synergistic action with different antibiotics: the peptide is able to disrupt the QS mechanism and disturb the biofilm formation, in this way, antibiotics may have better access to the bacterial cells, exerting their bactericidal activity.

4.2. Use of RIP to Treat Human Chronic Wound Infections

In literature, there are two cases of application of RIP in order to treat difficult chronic wound infections in patients which were unresponsive to the ordinary antibiotic treatments, in order to demonstrate the potential power of RIP in clinical application for controlling biofilm-based infectious diseases [44].

In particular, the first case reported a man with severe peripheral vascular disease and diabetes mellitus sustained minor trauma to his right little toe. MRSA and several other bacterial strains were found in his minor diabetic foot ulcer. Over the next 6 months, the patient had aggressive conservative wound care, but despite the therapies, there was a continual dying back into the forefoot. Perfusion and oxygenation studies showed that there were adequate blood flow and oxygenation of the tissues for healing, but the wound was getting worse constantly. An extensive osteomyelitis across the forefoot was compared within 4 months, and after 7 months with different therapies, the foot was clearly worsening. Since the patient refused the major limb amputation, after the 8th month, a gel preparation of RIP at 1.7 μg/mL was applied topically to the wound on a daily basis, followed by the continued use of cubicin 4 mg/kg for 3 weeks. By that time, the wound had shown significant improvement. After 3 weeks, the antibiotic was stopped, but the RIP gel was continued for 3 months with steady improvement. After that, Apligraf® wound therapy was instituted every 2–4 weeks for the next 8 months to completed healing. This case demonstrated that RIP as a topical therapy, used in combination with intravenous antibiotics, was able to stop the biofilm that was slowly eroding through the foot.

A second case regarded a man with severe insulin-dependent diabetes mellitus presented with a hot, swollen left foot. He was febrile and hypotensive and was immediately admitted to the hospital for intravenous antibiotics and surgical management of his left foot. The patient refused the surgical amputation of the left lower extremity, even if severe osteomyelitis was compromising the tarsal and metatarsals. The organisms cultured were MRSA and group D enterococci. The patient was given cubicin 6 mg/kg without any appreciable improvements. He was administered with outpatient therapy with daily cubicin 6 mg/kg and was injected with RIP in the tarsal area (25 mg/5 mL). This injection was done on a daily basis for three weeks. The local wound care consisted of the daily removal of any debridement and necrotic material, followed by the topical application of lactoferrin, xylitol, and RIP 1.7 μg/mL. After 1 week, the wound was much improved and the foot edema and erythema completely resolved within 10 days.

The injection of RIP in the area of *S. aureus* biofilm osteomyelitis markedly changed the patient's clinical course and led to the resolution of the osteomyelitis as well as healing of the diabetic foot ulcer. These results clearly demonstrated that RIP application produced a better resolution of chronic biofilm infections, highlighting that the approach of direct biofilm suppression may be a valuable medical tool.

5. RIP Structure-activity Relationship

The study of molecular structure is highly important in medicinal chemistry. It allows investigating which part of the structure acts as a pharmacophoric region, and which part is possible to modify in order to improve pharmacokinetic features or physic-chemical properties, in order to obtain better molecules compared to the lead compound [45].

Gov *et al.* in 2001 performed *in vitro* and *in vivo* studies on several derivatives in order to better understand the structure and function of RIP and investigate whether amino acids modification can improve the QS inactivation [33]. Based on the similarity between the NH_2-terminal sequence of RAP (YKPITN) and the structure of RIP (YSPWTNF-NH_2, **1**) - which is the base of the competitive inhibition of the TRAP receptor - the authors synthetized the NH_2-terminal derivative peptides **2-7** (Fig. **4**). Moreover, COOH-terminal derivatives **8-10** (Fig. **4**) were synthetized to assess the importance of the amide terminal group. All peptides were tested for their ability to inhibit RNAIII synthesis *in vitro* and to inhibit *S. aureus* biofilm *in vivo*.

Although RIP was able to inhibit *S. aureus* biofilm in both *in vitro* and *in vivo* experiments, all its derivatives were able inhibit RNAIII *in vitro*, but not all inhibited infections *in vivo*. In particular, several RIP derivatives inhibited the synthesis of RNAIII *in vitro*, including **6** by 68%, **3** by 70%, **8** by 80%, **9** by 88%, and **5, 10, 4, 7** and **2** by 100%. For the

in vivo experiments, mice received a subcutaneous dose of bacteria to induce local infection, and at the same time received a subcutaneous dose of RIP derivatives. Animal treated with derivatives **3, 4, 5, 6, 8**, and **9** died or presented lesions on the injection site after the incubation period, while mice treated with **7** and **2** showed no lesion at all and they were completely protected. The results of the *in vivo* and *in vitro* experiments indicate that peptides that were most effective in suppressing *S. aureus* also *in vivo* are peptides **2** and **7**, which contain K and I at positions 2 and 4, respectively. Derivative **2** exhibited the highest inhibitory activity *in vivo*, under the experimental conditions tested, but conformational studies did not show any particular secondary conformation, suggesting that the enhanced inhibitory activity of peptide **2** may be allowed to the local interactions with the TRAP receptor, probably due to the high similarity with the RAP terminal sequence YKPITN, in which derivative **2** differs only for a more amide-terminal F residue.

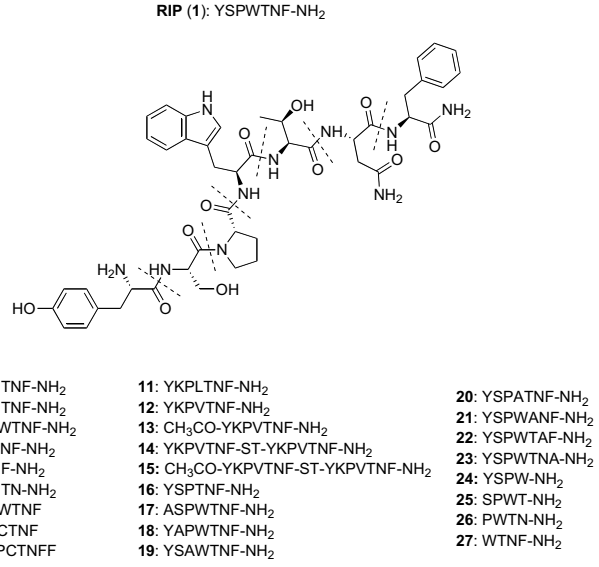


Fig. (4). RIP derivative **1 - 27**.

The derivative **2** was tested by Vieira-da-Motta *et al.* for the possible influence on both enterotoxin A, B, C, D, and E, and on hemolysin α and β, in the same experiment exposed in the RIP structure and mechanism of action section [36]. All the results were similar to the originator compound RIP, demonstrating the high level of activity of this derivative despite the amino acid modifications.

Starting from derivative **2**, Zhou *et al.* replaced the I residue at position 4 with two others amino acids with similar hydrophobicity and structure, V and L, to enhance the *in vivo* activity (derivatives **11** and **12**) [46]. They also operated terminal modifications or oligomerization of the synthesized structures to improve their stability *in vitro* and *in vivo*. In particular, derivative **13** was prepared by the acetylation of the $-NH_2$ terminal group of peptide **12**, derivative **14** was synthetized by the dimerization of peptide **12** using an S-T bridge, and finally derivative **15** resulted in the acetylation of the $-NH_2$ terminal of peptide **14**. *In vitro* inhibition of *S. aureus* was assessed by the measurement of the biofilm OD, and results showed that derivative **11** expressed a similar reduction in the absorption if compared with derivative **2**, which was better of the originator RIP, indicating that the biofilm formation of *S. aureus* was reduced. Peptide **12** significantly decreased the OD compared with RIP, derivative **2** and **11**, whereas among the derivatives with terminal and oligomerization modifications, **13** and **14** showed a better reduction in biofilm formation than peptide **12**. Derivative 15 was the peptide with the best antibiofilm activity among all the derivative tested, highlighting the most significant inhibition effect to *S. aureus* adhesion on polystyrene plates.

Since peptide **15** demonstrated the best inhibition activity *in vitro*, the authors performed an *in vivo* evaluation of its antibiofilm function in a rat urinary tract infection model. The colonization of mice urinary tract was performed through an MRSA inoculum, followed by an intraperitoneal administration (10 mg/kg) of derivative **12**, **15** or vancomycin. The number of bacterial colonies was verified in the bladder, kidney, urine, and stent. The bacterial counts decreased in the bladder, urine, and kidney in the derivative **12**-treated group, but derivative **15** showed a better biofilm inhibitory activity than other derivatives tested, with similar results if compared with vancomycin. Bacterial titers in the urine, kidney, and bladder of MRSA infected rats were suppressed effectively by peptide 15, and the number of bacteria that adhered to the implanted stents was also remarkably reduced. These results indicated that the modifications produced to obtain derivative **15** effectively increased the capacity to reduce intercellular adhesion and accumulation into the polymeric matrix, interfering with the MRSA biofilm formation.

Balaban *et al.* in another study performed an interesting experiment regarding RIP and the synthetized derivative **16** (Fig. **4**), which presented the loss of W in position 4 [32]. They tested the peptides in several models of *S. aureus* infections, including: keratitis and osteomyelitis (tested in rabbits), mastitis (tested in cows) and septic arthritis (tested in mice). Bacteria were pre-incubated with the RIP or with carrier buffer as a control, to mimic the application of RIP to devices (such as catheters or sutures) or during procedures (such as surgical procedures) normally associated with biofilm infections. For the osteomyelitis model, results showed a strong reduction of bone disruption in animals treated with RIP compared to the control. The septic arthritis model demonstrated that RIP treatment of bacteria reduces the frequency and delays the onset of staphylococcal sepsis, with a reduction in animals death. RIP and derivative **16** were used for the keratitis experiment. Rabbits injected with bacteria and RIP showed a distinct reduction in pathology 18 h postinfection, while animals treated with **16** showed no reduction in pathology at that time. Finally, with the cow mastitis model demonstrated that RIP can effectively prevent *S. aureus* infection protecting animals from the infection after the bacteria inoculation. In addition, RIP effectively inhibited the occurrence of clinical mastitis which demonstrated the potential therapeutic values in already infected animals.

An interesting analysis of the RIP structure and the contributions of each single amino acids was performed by Baldassare *et al.* in 2013, which synthetized a series of peptides which differ from the originator due to the presence of a residue of A at different positions (Fig. **4**, **17-23**) [47]. This amino acid usually is used to replace other residues since its

insertion does not alter the secondary structure of the peptide, which is in any case active. Furthermore, truncated analogues **24-27** were synthetized to evaluate the minimum amino acid sequence which may have anti-biofilm activity. All the RIP derivatives were synthesized on the solid phase using Fmoc chemistry on a rink amide resin. The *in vitro* antimicrobial activity of RIP derivatives **17-27** was tested against *S. aureus* strain Smith adherent cells in comparison with daptomycin, used as a reference. Based on the RIP mechanism of action, all the derivatives expressed high MIC values compared to daptomycin (>128 μg/mL and 4 μg/mL, respectively), showing no *in vitro* killing activities against the selected strain. The *in vivo* studies were assayed in a rat model of vascular graft infection, creating a subcutaneous pocket on each side of the median line by a 1.5 cm incision. Aseptically, the grafts were soaked in a solution of the RIP derivatives **17-27** and then implanted into the pockets. Then, the pockets were closed by the use of skin clips and bacterial colonies were inoculated on the graft surface. After 7 days the grafts were explanted, and the results by CFU count showed that derivative **21** had a lower activity than RIP, confirming that the T residue is essential for the activity. On the contrary, substitution of the residues of S in position 2 and F in position 7, respectively, resulted in the greater power of derivatives **18** and **23**, indicating that these amino acids are not essential for the activity of RIP. Interestingly the compound **25**, a truncated derivative of RIP showed the greatest activity compared to all the other compounds synthesized.

In particular, Cirioni *et al.* tested derivative **18** to evaluate MIC and MBC against *in vitro* and *in vivo S. aureus* biofilms models [48]. Using the same technique described above, the study included a control group, a contaminated group that did not receive any antibiotic prophylaxis and three contaminated groups that received intraperitoneal daptomycin, derivative **18**-soacked graft, or daptomycin plus **18**-soaked graft, respectively. The *in vitro* studies showed that MIC and MBC values for daptomycin were lower in the presence of derivative **18**. *In vivo* results highlighted that daptomycin alone showed a strong reduction in bacterial CFU, while group treated with derivative **18** showed also significant differences in the contaminated group with a reduction in bacterial cells number. The drug combination showed the lowest bacterial CFU among the treated groups, indicating the positive interaction which intervenes between derivative **18** and daptomycin. These data could be explained considering the capacity of RIP analogs to inhibit the production of polymeric matrix and virulence factors in *S. aureus*, thus facilitating the biofilm eradication by the antimicrobial agents and the host immune system.

Simonetti *et al.* assessed the efficacy of the derivative **23** and intraperitoneal tigecycline in preventing prosthesis biofilm in an animal model of *S. aureus* infection [49]. *In vitro* and *in vivo* studies were performed with the same models described for the derivative **18**, with the use of tigecycline instead of daptomycin. In particular, tigecycline showed *in vitro* MIC and MBC values of 8.00 and 16.00 mg/L, while in the presence of **23**, the antibiotic showed MIC and MBC four-fold lower (2 mg/L and 4 mg/L, respectively). *In vivo* model included the rat groups treated with tigecycline, which showed a positive bacterial reduction. Derivative **23**-treated group showed also a good activity compared to the contaminated group. A stronger inhibition of bacterial growth was observed in rats treated with the combination of derivative **23** and tigecycline, with the higher CFU reduction among the treated groups. The combination proved to be effective in reducing bacterial counts to levels comparable with those observed in uninfected animals.

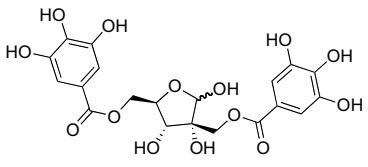
Finally, the same authors in another study tested the derivative **25** using the same *in vivo* model described before, establishing a wound through the panniculus carnosus on the back subcutaneous tissue of each animal, using MRSA and MSSA *S. aureus* strains [50]. After the inoculum of the bacterial colonies, treatment was initiated in infected animals and intraperitoneal tigecycline (7 mg/Kg) was administered daily for 7 days, while topical treatment of derivative **25** was applied every 2 days. The administration was realized with the Allevyn® foam wound dressing, previously soaked with the tetrapeptide. Data analysis showed that, for the MSSA strain, treatment with **25** alone had a slight effect, reducing the mean bacterial numbers from 3.8 x 109 of the contaminated group to 4.9 x 107 CFU/g. Tigecycline had a strong effect compared with infected animals. Nevertheless, the highest inhibition of infection was achieved in the group that received **25**-soaked Allevyn® and parenteral tigecycline, reducing the bacterial number to 2.8 x 103 CFU/g. Comparable results were observed for the MRSA strain. In particular, **25**-soaked Allevyn® showed a reduction from 4.5 x 109 of the infected control to 3.7 x 108 CFU/g. Tigecycline had a strong effect when compared with untreated control. Similarly for the MRSA strain, the highest inhibition of contamination was achieved in the group that received **25**-soaked Allevyn® and systemic tigecycline, reducing bacterial number to 4.6 x 103 CFU/g. Further studies also demonstrate that derivative **25** acts by favoring wound re-epithelialization, re-establishing proper healing kinetics.

A particular derivative developed by Yang and collaborators was created from a random 12-mer phage-displayed peptide library based on RAP, in order to select new RAP-binding peptides able to reduce the QS mechanism [51]. Early exponential wild-type *S. aureus* cells were incubated for 90 min together with different positive phage clones, the total RNA was extracted, Northern blotted, and detected using radiolabeled RNAIII specific DNA. Results showed that among the developed derivatives **28-36** (Fig. **5**), only peptide **29** and **30** demonstrated significant effects on the inhibition of RNAIII. In particular, peptide 30 expressed the higher capacity of binding RAP, thus interfering with the TRAP-RAP mechanism and reducing the QS activation. For this promising properties, it was assayed into an *in vivo* murine cutaneous *S. aureus* infection model. Bacterial cells were injected with or without peptide **30** subcutaneously with cytodex beads into immunocompetent hairless mice. After 27 h post-injection, the size of the lesion was measured. All animals developed a lesion, but the mean size of lesions in the mice treated with peptide **30** was smaller than that in control animals, indicating that the derivative was able to actively bind RAP also *in vivo*, thus reducing the biofilm infection.

The synthesis of codrug derivatives allows to obtain new chimeric derivatives with a potentiated efficacy, a synergistic action between the two linked molecules which at the same time are able to exert their action with different mechanisms

[52]. Balaban *et al.* created a chimeric peptide composed of the Dermaseptin Derivative DD13 and RIP (derivative **37**, Fig. **5**) to evaluate the inhibition of bacterial proliferation and suppression of QS mechanism *in vitro* [53]. Moreover, the efficacy of derivative **37** in preventing staphylococcal infection was assessed in a rat graft infection model with MRSA, as previously reported by Cirioni *et al.* [39]. The basis of this construct was the potential synergistic activity of the two-component: inhibition of RNA III synthesis by RIP, with the ability of DD13 to reduce bacterial proliferation. *In vitro* results showed that DD13 and derivative **37** displayed a quasipotent bactericidal activity, with an MIC of 2 mg/L, whereas RIP was basically inactive. Moreover, peptide **37** efficiently inhibited RNA III synthesis, while DD13 was totally inactive as expected and RIP appeared to be less efficient than the new derivative. *In vivo* experiments forecasted the soaking of the collagen-coated Dacron grafts with 10, 20, or 50 mg/L of either RIP, DD13 or derivative **37** before being implanted in rats. Bacteria were injected into the implants, and the implants were removed after a week, with the consequential determination of the bacterial load. Results demonstrated the high antibiofilm activity profile of the chimeric peptide compared to the single components, especially at the lower concentration of 10 mg/L in which only derivate **37** was able to provide a high efficiency in biofilm reduction.

28: WHWTNWGKTSPA
29: FHWWQTSPAHFS
30: WPFAHWPWQYPR
31: HPWYYSMYWRSP
32: IHPLLLYNYYFG
33: HRNHLMDLSGPH
34: PPYPDFLMQPPH
35: SFPQPLAPAPTI
36: TSVKLELGVTAS
37: ALWKTLLKKVLKAYSPWTNFC-NH_2



38

Fig. (5). RIP derivative **28** - **38**.

Finally, Kiran *et al.* performed an interesting screening for small molecule non-peptide analogs of RIP using a computer search with the Integrated Scientific Information System (ISIS) software from Elsevier MDL (Hayward, CA) against the Available Chemicals Database (ACD), a library of 300,000 commercially available small-molecule compounds [54]. Using the RIP as a molecular model, it was possible to identify molecules with specific features and regions, called pharmacophores, that are necessary for the recognition of the ligand by a biological macromolecule and are responsible for the pharmacological activity [55]. In this study, the pharmacophore was defined in terms of distance calculation, leading to the discovery of hamamelitannin (**38**, Fig. **5**) as a small molecule non-peptide analog of RIP. Hamamelitannin is a natural product found in the bark and the leaves of *Hamamelis virginiana*, and it was tested in both *in vitro* and *in vivo S. aureus* biofilm model. *In vitro* results showed that hamamelitannin did not affect the bacterial growth, whereas it demonstrated to be effective in the competition with RAP, blocking RNAIII and hemolysins production, inhibiting cells attachment. Surprisingly, hamamelitannin demonstrated to be an excellent inhibitor of device-associated infections *in vivo*. Grafts presoaked with 30 mg/L of the small drug exhibited no signs of infection, even though the animals were challenged with a high bacterial concentration. These data confirmed that hamamelitannin inhibits staphylococcal virulence by acting as a QS inhibitor.

CONCLUSION

S. aureus is a clinically relevant pathogen, etiological agent of many chronic infections and wound diseases, difficult to treat and to eradicate when present under the biofilm growth modality. A biofilm could be defined as a microbially-derived sessile community, embedded in a polymeric matrix, which allows to increase the nutrients concentration, and above all, it confers antimicrobial resistance and protection from the host immune system. To date, the antimicrobial therapy alone is not effective. A new approach for the eradication of *S. aureus* biofilm is represented by the disruption of the bacterial regulatory network. In particular, *S. aureus* biofilm growth modality is regulated by a bacterial cell-cell communication, a process known as QS mechanism and mediated by RAP and its target protein TRAP. The well-know RIP peptide competes with RAP, thus inhibiting the phosphorylation of TRAP and leading to a reduction of bacterial cells adhesion and to the inhibition of RNAIII synthesis, with the subsequent suppression of toxin synthesis. Medicinal chemistry approach was adopted in order to better characterize the structure-activity relationship of the heptapeptide, understanding which amino acids or structure modifications are essential for the blockage of the TRAP-RAP mechanism, setting a starting point for the development of derivatives with improved antibiofilm activity. Since the mechanism of action of RIP is based on inhibition of QS, RIP derivatives showed high MIC values compared to the antibiotic therapy, showing no *in vitro* killing. In the case of RIP and a large part of RIP derivatives, the best treatment is represented by antibiotic prophylaxis combined with the interruption of bacterial QS mechanism, demonstrating how the peptides can act as a promoter of the drug's antimicrobial action.

These studies confirm the importance of RIP and its derivatives in the treatment of chronic and nosocomial infections related to the development of biofilms by resistant strains of *S. aureus*. These peptides could overcome the main causes of therapies failure against the biofilm infections: the pharmacological resistance of the bacteria and the opposition to the host immune system. The possibility to act directly on the biofilm formation allows to restore and improve the activity of the antimicrobial agents as well as the immune system against the biofilm-embed cells, leading to the complete eradication of chronic and complicated *S. aureus* infections.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Costerton, J.W.; Stewart, P.S.; Greenberg, E.P. Bacterial biofilms: a common cause of persistent infections. *Science*, **1999**, *284*(5418), 1318-1322.

[2] Hall-Stoodley, L.; Costerton, J.W.; Stoodley, P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol*., **2004**, *2*(2), 95-108.

[3] Bjarnsholt, T.; Jensen, P.O.; Moser, C.; Høiby, N. *Biofilm Infections*; Springer: New York, **2011**.

[4] Stewart, P.S.; William Costerton, J. Antibiotic resistance of bacteria in biofilms. *Lancet,* **2001**, *358* (9276), 135-138.

[5] Gil, C.; Solano, C.; Burgui, S.; Latasa, C.; García, B.; Toledo-Arana, A.; Lasa, I.; Valle, J. Biofilm matrix exoproteins induce a protective immune response against staphylococcus aureus biofilm infection. *Infect. Immun*., **2014**, *82*(3), 1017-1029.

[6] Archer, N.K.; Mazaitis, M.J.; Costerton, J.W.; Leid, J.G.; Powers, M.E.; Shirtliff, M.E. Properties, regulation and roles in human disease staphylococcus aureus biofilms. *Virulence*, **2011**, *2*(5), 445-459.

[7] Donlan, R.M. Role of biofilms in antimicrobial resistance. *ASAIO J.*, **2000**, *46*(6), S47-S52.

[8] Francolini, I.; Donelli, G. Prevention and control of biofilm-based medical-device-related infections. *FEMS Immunol. Med. Microbiol*., **2010**, *59*(3), 227-238.

[9] Scott, R.D. The direct medical costs of healthcare-associated infections in U.S. hospitals and the benefits of prevention. CDC Report: Atlanta. **2009**.

[10] Wenzel, R.P. Health care–associated infections: major issues in the early years of the 21st century. *Clin. Infect. Dis*., **2007**, *45*(Suppl 1), S85-S88.

[11] Pittet, D.; Tarara, D.; Wenzel, R.P. Nosocomial bloodstream infection in critically ill patients. excess length of stay, extra costs, and attributable mortality. *JAMA*, **1994**, *271*(20), 1598-1601.

[12] Arciola, C.R.; Campoccia, D.; Speziale, P.; Montanaro, L.; Costerton, J.W. Biofilm formation in staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. *Biomaterials,* **2012**, *33*(26), 5967-5982.

[13] Zimmerli, W. Clinical presentation and treatment of orthopaedic implant-associated infection. *J. Intern. Med.*, **2014**, *276*(2), 111-119.

[14] Smith, K.; Perez, A.; Ramage, G.; Gemmell, C.G.; Lang, S. Comparison of biofilm-associated cell survival following *in vitro* exposure of meticillin-resistant staphylococcus aureus biofilms to the antibiotics clindamycin, daptomycin, linezolid, tigecycline and vancomycin. *Int. J. Antimicrob. Agents*, **2009**, *33*(4), 374-378.

[15] Fu, J.; Ji, J.; Yuan, W.; Shen, J. Construction of anti-adhesive and antibacterial multilayer films via layer-by-layer assembly of heparin and chitosan. *Biomaterials*, **2005**, *26*(33), 6684-6692.

[16] Arciola, C.R.; Montanaro, L.; Caramazza, R.; Sassoli, V.; Cavedagna, D. Inhibition of bacterial adherence to a high-water-content polymer by a water-soluble, nonsteroidal, anti-inflammatory drug. *J. Biomed. Mater. Res.*, **1998**, *42*(1), 1-5.

[17] Balaban, N.; Goldkorn, T.; Nhan, R.T.; Dang, L.B.; Scott, S.; Ridgley, R.M.; Rasooly, A.; Wright, S.C.; Larrick, J.W.; Rasooly, R.; Carlson J.R. Autoinducer of virulence as a target for vaccine and therapy against staphylococcus aureus. *Science*, **1998**, *280* (5362), 438-440.

[18] Park, J.; Jagasia, R.; Kaufmann, G.F.; Mathison, J.C.; Ruiz, D.I.; Moss, J.A.; Meijler, M.M.; Ulevitch, R.J.; Janda, K.D. Infection control by antibody disruption of bacterial quorum sensing signaling. *Chem. Biol*., **2007**, *14*(10), 1119-1127.

[19] Chung, P.Y.; Toh, Y.S. Anti-biofilm agents: recent breakthrough against multi-drug resistant staphylococcus aureus. *Pathog. Dis.*, **2014**, *70*(3), 231-239.

[20] Marinelli, L.; Di Stefano, A.; Cacciatore, I. Carvacrol and its derivatives as antibacterial agents. *Phytochem. Rev.,* **2018**, *17*(4), 903-907.

[21] Novick, R.P.; Ross, H.F.; Figueiredo, A.M.S.; Abramochkin G. activation and inhibition of the staphylococcal AGR System. *Science,* **2000**, *287*(5452), 391.

[22] Di Stefano, A.; D'Aurizio, E.; Trubiani, O.; Grande, R.; Di Campli, E.; Di Giulio, M.; Di Bartolomeo, S.; Sozio, P.; Iannitelli, A.; Nostro, A.; Cellini L. Viscoelastic properties of staphylococcus aureus and staphylococcus epidermidis mono-microbial biofilms. *Microb. Biotechnol*., **2009**, *2*, 634-641.

[23] Mack, D.; Becker, P.; Chatterjee, I.; Dobinsky, S.; Knobloch, J.K.M.; Peters, G.; Rohde, H.; Herrmann, M. Mechanisms of biofilm formation in staphylococcus epidermidis and *Staphylococcus Aureus*: Functional molecules, regulatory circuits, and adaptive responses. *Int. J. Med. Microbiol.*, **2004**, *294*(2-3), 203-212.

[24] Balaban, N.; Goldkorn, T.; Gov, Y.; Hirshberg, M.; Koyfman, N.; Matthews, H.R.; Nhan, R.T.; Singh, B.; Uziel, O. Regulation of *Staphylococcus Aureus* pathogenesis via target of RNAIII-Activating Protein (TRAP). *J. Biol. Chem.*, **2001**, *276*(4), 2658-2667.

[25] Lowy, F.D. Staphylococcus Aureus Infections. *N. Engl. J. Med.*, **1998**, *339*(8), 520-532.

[26] Novick, R.P.; Ross, H.F.; Projan, S.J.; Kornblum, J.; Kreiswirth, B.; Moghazeh, S. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA Molecule. *EMBO J.*, **1993**, *12*(10), 3967-3975.

[27] Lina, G.; Jarraud, S.; Ji, G.; Greenland, T.; Pedraza, A.; Etienne, J.; Novick, R.P.; Vandenesch, F. Transmembrane topology and histidine protein kinase activity of AgrC, the Agr signal receptor in *Staphylococcus Aureus*. *Mol. Microbiol.*, **1998**, *28*(3), 655-662.

[28] Chevalier, C.; Boisset, S.; Romilly, C.; Masquidal, B.; Fechter, P.; Geissmann, T.; Vandenesch F.; Romby, P. *Staphylococcus aureus* RNAIII binds to two distant regions of *coa* mRNA to arrest translation and promote mRNA degradation. *PLoS Pathog*., **2010,** *6*(3), e1000809.

[29] Liu, Y.; Mu, C.; Ying, X.; Li, W.; Wu, N.; Dong, J.; Gao, Y.; Shao, N.; Fan, M.; Yang, G. RNAIII activates map expression by forming an RNA-RNA complex in *Staphylococcus aureus*. *FEBS Lett.*, **2011**, *585*, 1873-3468.

[30] Arvidson, S.; Tegmark, K. Regulation of virulence determinants in *Staphylococcus aureus*. *Int. J. Med. Microbiol.*, **2001**, *291*, 159-170.

[31] Morfeldt, E.; Taylor, D.; von Gabain, A.; Arvidson, S. Activation of alpha-toxin translation in *Staphylococcus Aureus* by the Trans-Encoded antisense RNA, RNAIII. *EMBO J.*, **1995**, *14*(18), 4569-4577.

[32] Balaban, N.; Collins, L.V.; Cullor, J. S.; Hume, E. B.; Medina-Acosta, E.; Vieira da Motta, O.; O'Callaghan, R.; Rossitto, P.V.; Shirtliff, M. E.; Serafim da Silveira, L.; Tarkowski A.; Torres J.V. Prevention of diseases caused by staphylococcus aureus using the peptide RIP. *Peptides*, **2000**, *21*(9), 1301-1311.

[33] Gov, Y.; Bitler, A.; Dell'Acqua, G.; Torres, J.V.; Balaban, N. RNAIII Inhibiting Peptide (RIP), a global inhibitor of staphylococcus aureus pathogenesis: structure and function analysis. *Peptides*, **2001**, *22*(10), 1609-1620.

[34] Korem, M.; Sheoran, A.S.; Gov, Y.; Tzipori, S.; Borovok, I.; Balaban, N. Characterization of RAP, a quorum sensing activator of *Staphylococcus Aureus*. *FEMS Microbiol. Lett.*, **2003**, *223*(2), 167-175.

[35] Yarwood, J.M.; Schlievert, P.M. Quorum sensing in *Staphylococcus* infections. *J. Clin. Invest*. **2003**, *112*(11), 1620-1625.

[36] Vieira-Da-Motta, O.; Ribeiro, P.D.; Dias Da Silva, W.; Medina-Acosta, E. RNAIII Inhibiting Peptide (RIP) inhibits agr-regulated toxin production. *Peptides*, **2001**, *22*(10), 1621-1627.

[37] Giacometti, A.; Cirioni, O.; Gov, Y.; Ghiselli, R.; Del Prete, M.S.; Mocchegiani, F.; Saba, V.; Orlando, F.; Scalise, G.; Balaban, N.; Dell'Acqua G. RNA III inhibiting peptide inhibits *in vivo* biofilm formation by drug-resistant *Staphylococcus Aureus*. *Antimicrob. Agents Chemother.*, **2003**, *47*(6), 1979-1983.

[38] Balaban, N.; Cirioni, O.; Giacometti, A.; Ghiselli, R.; Braunstein, J. B.; Silvestri, C.; Mocchegiani, F.; Saba, V.; Scalise, G. Treatment of staphylococcus aureus biofilm infection by the Quorum-Sensing Inhibitor RIP. *Antimicrob. Agents Chemother.,* **2007**, *51*(6), 2226-2229.

[39] Simonetti, O.; Cirioni, O.; Ghiselli, R.; Goteri, G.; Scalise, A.; Orlando, F.; Silvestri, C.; Riva, A.; Saba, V.; Madanahally, K.D.; Offidani A.; Balaban N.; Scalise G.; Giacometti A. RNAIII-inhibiting peptide enhances healing of wounds infected with methicillin-resistant *Staphylococcus Aureus*. *Antimicrob. Agents Chemother.*, **2008**, *52*(6), 2205-2211.

[40] Schierle, C.F.; De La Garza, M.; Mustoe, T.A.; Galiano, R.D. Staphylococcal biofilms impair wound healing by delaying reepithelialization in a murine cutaneous wound model. *Wound Repair Regen.*, **2009**, *17*(3), 354-359.

[41] Balaban, N.; Gov, Y.; Bitler, A.; Boelaert, J.R. Prevention of *Staphylococcus aureus* biofilm on dialysis catheters and adherence to human cells. *Kidney Int.*, **2003**, *63*(1), 340-345.

[42] Domenico, P.; Gurzenda, E.; Giacometti, A.; Cirioni, O.; Ghiselli, R.; Orlando, F.; Korem, M.; Saba, V.; Scalise, G.; Balaban, N. BisEDT and RIP act in synergy to prevent graft infections by resistant staphylococci. *Peptides*, **2004**, *25*(12), 2047-2053.

[43] Cirioni, O.; Giacometti, A.; Ghiselli, R.; Dell'Acqua, G.; Orlando, F.; Mocchegiani, F.; Silvestri, C.; Licci, A.; Saba, V.; Scalise, G.; Balaban, N. RNAIII-Inhibiting peptide significantly reduces bacterial load and enhances the effect of antibiotics in the treatment of central venous catheter–associated staphylococcus aureus infections. *J. Infect. Dis.*, **2006**, *193*(2), 180-186.

[44] Wolcott R.D. Clinical wound healing using signal inhibitors. In: *Control of Biofilm Infections by Signal Manipulation*; Naomi Balaban, Ed.; Springer-Verlag: Berlin Heidelberg, **2008**; Vol. *2*, pp. 157-170.

[45] Sozio, P.; Marinelli, L.; Cacciatore, I.; Fontana, A.; Türkez, H.; Giorgioni, G.; Ambrosini, D.; Barbato, F.; Grumetto, L.; Pacella, S.; Cataldi, A.; Di Stefano, A. New flurbiprofen derivatives: synthesis, membrane affinity and evaluation of *in vitro* effect on β-amyloid levels. *Molecules,* **2013**, *18*(9), 10747-10767.

[46] Zhou, Y.; Zhao, R.; Ma, B.; Gao, H.; Xue, X.; Qu, D.; Li, M.; Meng, J.; Luo, X.; Hou, Z. Oligomerization of RNAIII-Inhibiting peptide inhibits adherence and biofilm formation of methicillin-resistant *Staphylococcus aureus in vitro* and *in vivo*. *Microb. Drug Resist.*, **2016**, *22*(3), 193-201.

[47] Baldassarre, L.; Fornasari, E.; Cornacchia, C.; Cirioni, O.; Silvestri, C.; Castelli, P.; Giocometti, A.; Cacciatore, I. Discovery of novel rip derivatives by alanine scanning for the treatment of *S. aureus* Infections. *Med. Chem. Comm.*, **2013**, *4*, 1114.

[48] Cirioni, O.; Mocchegiani, F.; Cacciatore, I.; Vecchiet, J.; Silvestri, C.; Baldassarre, L.; Ucciferri, C.; Orsetti, E.; Castelli, P.; Provinciali, M.; Vivarelli, M.; Fornasari, E.; Giacometti, A. Quorum sensing inhibitor FS3-Coated vascular graft enhances daptomycin efficacy in a rat model of staphylococcal infection. *Peptides*, **2013**, *40*, 77-81.

[49] Simonetti, O.; Cirioni, O.; Mocchegiani, F.; Cacciatore, I.; Silvestri, C.; Baldassarre, L.; Orlando, F.; Castelli, P.; Provinciali, M.; Vivarelli, M.; Fornasari, E.; Giacometti, A.; Offidani, A. The efficacy of the quorum sensing inhibitor fs8 and tigecycline in preventing prosthesis biofilm in an animal model of staphylococcal infection. *Int. J. Mol. Sci.*, **2013**, *14*(8), 16321-16332.

[50] Simonetti, O.; Cirioni, O.; Cacciatore, I.; Baldassarre, L.; Orlando, F.; Pierpaoli, E.; Lucarini, G.; Orsetti, E.; Provinciali, M.; Fornasari, E.; Di Stefano, A.; Giacometti, A.; Offidani, A. Efficacy of the quorum sensing inhibitor fs10 alone and in combination with tigecycline in an animal model of staphylococcal infected wound. *PLoS One*, **2016**, *11*(6), 1-12.

[51] Yang, G.; Cheng, H.; Liu, C.; Xue, Y.; Gao, Y.; Liu, N.; Gao, B.; Wang, D.; Li, S.; Shen, B.; Shao, N. Inhibition of *Staphylococcus Aureus* pathogenesis *in vitro* and *in vivo* by RAP-Binding peptides. *Peptides*, **2003**, *24*(11), 1823-1828.

[52] Cacciatore, I.; Di Giulio, M.; Fornasari, E.; Di Stefano, A.; Cerasa, L.S.; Marinelli, L.; Turkez, H.; Di Campli, E.; Di Bartolomeo, S.; Robuffo, I.; Cellini, L. Carvacrol Codrugs: A new approach in the antimicrobial plan. *PLoS One*. **2015**, *10*(4), e0120937.

[53] Balaban, N.; Gov, Y.; Giacometti, A.; Cirioni, O.; Ghiselli, R.; Mocchegiani, F.; Orlando, F.; D'Amato, G.; Saba, V.; Scalise, G.; Bernes, S.; Mor, A. A chimeric peptide composed of a dermaseptin derivative and an rna iii-inhibiting peptide prevents graft-associated infections by antibiotic-resistant staphylococci. *Antimicrob. Agents Chemother.,* **2004**, *48*(7), 2544-2550.

[54] Kiran, M.D.; Adikesavan, N.V.; Cirioni, O.; Giacometti, A.; Silvestri, C.; Scalise, G.; Ghiselli, R.; Saba, V.; Orlando, F.; Shoham, M.; Balaban, N. Discovery of a quorum-sensing inhibitor of drug-resistant staphylococcal infections by structure-based virtual screening. *Mol. Pharmacol.,* **2008**, 73(5), 1578-1586.

[55] Yang, S.Y. Pharmacophore modeling and applications in drug discovery: challenges and recent advances. *Drug Discov. Today*, **2010**, *15*(11-12), 444-450.