Solonamide B Inhibits Quorum Sensing and Reduces Staphylococcus aureus Mediated Killing of Human Neutrophils

Nielsen, Anita; Månsson, Maria; Bojer, Martin S.; Gram, Lone; Larsen, Thomas Ostenfeld; Novick, Richard P.; Frees, Dorte; Frøklæ, Hanne; Ingmer, Hanne

Published in: P L o S One

Link to article, DOI: 10.1371/journal.pone.0084992

Publication date: 2014

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Nielsen, A., Månsson, M., Bojer, M. S., Gram, L., Larsen, T. O., Novick, R. P., ... Ingmer, H. (2014). Solonamide B Inhibits Quorum Sensing and Reduces Staphylococcus aureus Mediated Killing of Human Neutrophils. P L o S One, 9(1), [e84992]. DOI: 10.1371/journal.pone.0084992

DTU Library
Technical Information Center of Denmark

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Solonamide B Inhibits Quorum Sensing and Reduces *Staphylococcus aureus* Mediated Killing of Human Neutrophils

Anita Nielsen¹, Maria Månsson², Martin S. Bojer¹, Lone Gram², Thomas O. Larsen², Richard P. Novick³, Dorte Frees¹, Hanne Frøkiær¹, Hanne Ingmer¹*¹

¹Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, ²Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark, ³Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, New York, United States of America

**Abstract**

Methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be a serious human pathogen, and particularly the spread of community associated (CA)-MRSA strains such as USA300 is a concern, as these strains can cause severe infections in otherwise healthy adults. Recently, we reported that a cyclodesipeptide termed Solonamide B isolated from the marine bacterium, *Photobacterium halotolerans* strongly reduces expression of RNAIII, the effector molecule of the agr quorum sensing system. Here we show that Solonamide B interferes with the binding of *S. aureus* autoinducing peptides (AIPs) to sensor histidine kinase, AgrC, of the agr two-component system. The hypervirulence of USA300 has been linked to increased expression of central virulence factors like α-hemolysin and the phenol soluble modulins (PSMs). Importantly, in strain USA300 Solonamide B dramatically reduced the activity of α-hemolysin and the transcription of psma encoding PSMs with an 80% reduction in toxicity of supernatants towards human neutrophils and rabbit erythrocytes. To our knowledge this is the first report of a compound produced naturally by a Gram-negative marine bacterium that interferes with agr and affects both RNAIII and AgrA controlled virulence gene expression in *S. aureus*.

**Citation:** Nielsen A, Månsson M, Bojer MS, Gram L, Larsen TO, et al. (2014) Solonamide B Inhibits Quorum Sensing and Reduces *Staphylococcus aureus* Mediated Killing of Human Neutrophils. PLoS ONE 9(1): e84992. doi:10.1371/journal.pone.0084992

**Editor:** Gunnar F. Kaufmann, The Scripps Research Institute and Sorrento Therapeutics, Inc., United States of America

**Received:** March 28, 2013; **Accepted:** November 22, 2013; **Published:** January 8, 2014

**Copyright:** © 2014 Nielsen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by funding from the Programme Committee for Food, Health and Welfare under the Danish Strategic Research Council. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: hi@sund.ku.dk

**Introduction**

*Staphylococcus aureus* is a serious human pathogen that causes a variety of diseases, such as skin and soft tissue infections, bacteremia, and toxic shock syndrome [1,2]. The organism is well known for its ability to develop resistance to a wide range of antibiotics and in consequence only few treatment options are now available for the most resistant strains [3]. Resistance to methicillin is particularly widespread, and nosocomial infections with methicillin resistant *S. aureus* (MRSA) strains are one of the most serious risk factors associated with hospitalization [4]. While the hospital associated *S. aureus* strains are generally opportunistic pathogens incapable of infecting healthy individuals [5] a more aggressive group of strains have emerged since the early 1990s that is both highly virulent and transmissible giving rise to infections in the community, thus termed community associated, methicillin resistant strains (CA-MRSA). The CA-MRSA strains belong to several sequence types with USA300 (ST8) being the most common in the US [6,7]. Importantly, these strains are able to infect healthy individuals often giving rise to skin and soft tissue infections that in some instances may turn out to be lethal [8,9].

Two of the most important virulence factors of CA-MRSA are α-hemolysin [10] and the phenol soluble modulins, the PSMs [11]. α-hemolysin is a pore forming α-toxin that lyses immune cells such as phagocytes, erythrocytes, and lymphocytes [12]. Also, α-hemolysin is required for *Staphylococcus aureus* phagosomal escape after internalization in a cystic fibrosis epithelial cell line [13]. PSMs are a class of secreted surfactant-like, amphipathic, alpha-helical staphylococcal peptides and they are remarkable at recruiting, activating and subsequently lysing human neutrophils. There are four alpha-types and two beta-type PSMs. The alpha-type PSMs are about 20–25 amino acids in length, and especially PSM*β3* is responsible for the lysis of human neutrophils. The beta-type PSMs are longer, about 40–45 amino acids and lack cytolytic activity [11,14]. Neutrophils constitute an essential part of the innate immune system, as they hold strong phagocytic activity and are recruited to the site of infection in high numbers [15]. Thus, the production of PSMs is critical for the ability of *S. aureus* to evade the host immune system and as such is determining for the outcome of the infection [11].

The exceptionally high expression of toxins and exoenzymes by CA-MRSA strains such as USA300 relies on the agr two-component quorum sensing system encoded by *agrACDB* [16,17]. With increasing cell density, autoinducing peptides (AIPs) produced by *agrB* and *agrD* [18] accumulates in the extracellular space and binds to the sensor histidine kinase, AgrC. Upon AIP binding, AgrC activates the response regulator, AgrA that stimulates transcription of a small regulatory RNA, RNAIII,
Figure 1. Solonamide B does not influence RNAIII expression in the presence of a constitutively active AgrC. Cultures of RN10829 (P2- agrA; P3-blaZ) containing either pagrC-I-WT (A, denoted WT) or pEG11 (B, denoted Const.) were grown to OD_{600} 0.4–0.5 where 1/10 a volume of AIP-containing supernatant and 5 μg/mL or 10 μg/mL Solonamide B (SolB) or DMSO was added. Controls not containing AIP were included to confirm the inducible and constitutive nature of reporters in A and B, respectively. Samples were obtained at various time points after addition and were analyzed for β-lactamase activity (P3 expression). A single representative experiment is depicted with bars representing the mean +/− standard error of the mean (SEM) from triplicate determinations of β-lactamase activity. Growth monitored for the WT strain confirms that the reduction in P3 expression is not due to growth impairment (C).

doi:10.1371/journal.pone.0084992.g001
responsible for the expression of α-hemolysin and other toxins in the late exponential growth phase [19,20]. AIPs consist of seven to nine amino acids in which the central cysteine residue is covalently linked to the terminal carboxylate, forming a thiolactone ring [21,22]. Between strains, AIP structure varies giving rise to at least four AIP classes where an AIP activates agr of strains only belonging to the same class but represses agr of the other classes [20]. In contrast to most toxins, expression of the PSMs is controlled directly by AgrA that binds to the promoter region of the pmaA and pmaB operons respectively and activates transcription [23].

In common to both community and hospital associated S. aureus infections, resistance to antibiotics is an increasing problem and we urgently need new approaches to prevent and treat S. aureus infections caused by resistant strains [24–27]. Anti-virulence compounds may offer an alternative to antibiotics, as they target the expression or activity of virulence factors, rather than growth or viability [28,29]. Examples of anti-virulence therapies include neutralization of toxins using antibodies [30], prevention of adhesion [31] or interference with virulence gene regulation [32]. Advantages to such approaches may be that the host microbiota is left unharmed and that there is likely to be less selection of drug-resistance [33]. In a search for compounds that reduce virulence gene expression in S. aureus we discovered that Solonamide B which is produced by a marine Photobacterium halotolerans, reduces expression of α-hemolysin and rnaIII and increases expression of spa encoding protein A in both strain 8325-4 and USA300 [34]. The purpose of the present study was to determine the mode of action and the extent to which RNAIII and AgrA controlled virulence factors were affected. We show here that Solonamide B is likely to interact directly with the agr quorum sensing system and that the activity of the compound is influencing expression of both RNAIII and AgrA controlled toxins.

Materials and Methods

Bacterial Strains and Growth Conditions

S. aureus strains used in this study included: Strain NCTC 8325 (RN1), and 8325-4 [35] and FPR3757 USA300 a multidrug resistant CA-MRSA isolate implicated in outbreaks of skin and soft tissue infection [36]. For competition assays PC203 (S. aureus 8325-4, spa::lacZ) [38] and RN6911 (S. aureus 8325-4 Δagr) [19] were also employed. During the study we constructed a series of lux-reporter strains. Plasmid from S. aureus strain RN9723 containing the P3::luxABCDE construct (R. Novick) was phage transduced into S. aureus strains with different agr group backgrounds (RN1, RN6607, MOZ53, RN4850, R. Novick) obtaining strain AN1: (RN1 8325 agr gr.I, P3::luxABCDE, CMR), AN2: (RN6607 agr gr.II, P3::luxABCDE, CMR), AN3: (MOZ53 agr gr.III, P3::luxABCDE, CMR), AN4: (RN4850 agr gr.IV, P3::luxABCDE, CMR). Strain RN10829/pEG11 [40], a β-lactamase reporter strain substituting the native

Figure 2. Solonamide B competitively interferes with AIP binding to AgrC. Agar plates containing the spa-lacZ reporter strain PC203 were supplied with 10 μl DMSO containing 0, 0.8, 4, 20, 100 and 500 μg Solonamide in DMSO and 20 μl of supernatant of overnight culture of 8325-4 (indicated as “WT”) or RN6911, Δagr mutant cells (indicated as “Δagr”) or TSB medium resulting in no (B) or the indicated concentrations of solonamide B (A, C, D).

doi:10.1371/journal.pone.0084992.g002
Agr locus with a chromosomal integration of P2-agrA and P3-blaZ and a plasmid from which a constitutive active variant of AgrC (agrC-I-R238H) is expressed, was used to assess AgrC-dependent effects of Solonamide B. Strain RN6911 [19] was used to generate bacterial supernatant not containing auto-inducing peptide (AIP) (strain 8325-4 was used to obtain supernatant containing AIP gr.I.). Unless otherwise stated, bacteria were grown in Tryptone Soya Broth (TSB), Oxoid, (1:10 volume/flask ratio), at 37°C with shaking at 200 rpm.

Activity of Solonamide B in WT and Constitutive Active AgrC reporter Strains

Plasmid pagrC-I-WT was generated by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Agilent Technologies) using primers 5'-aacaacgaaatgcgcaagttccgtcatgattatgtcaatatcttaa-3' (AgrCHtoR_Fwd) and 5'-ttaagatattgacataatcatgacggaacttgcgcatttcgttgtt-3' (AgrCHtoR_Rev) containing the desired nucleotide substitution (underlined) and plasmid pEG11 [40] as template following the manufacturer’s instructions. Correct reversion to AgrC-I-WT was confirmed by sequencing and by displaying wild-type characteristics, i.e., an AIP-I-dependent induction of β-lactamase activity in strain RN10829. Cultures of reporter strains, containing either pagrC-I-WT or pEG11, were diluted to OD600 0.05 in fresh TSB (without antibiotics) and grown to OD600 0.4–0.5 (37°C, 225 rpm) followed by addition of 5 or 10 μg/mL Solonamide B (final concentration) or DMSO (solvent) and 1/10 the volume of spent medium containing or free from AIP-I. Bacterial samples were taken at indicated time points, cooled instantly in an ice-water bath and frozen at −80°C. The respective culture OD600 was recorded. β-lactamase activity of samples was subsequently determined by the nitrocefin hydrolysis method [21]. Briefly, 50 μL cell culture was added an equal volume of 0.2 mM nitrocefin (Oxoid) in 0.1 M phosphate buffer (pH 7.0) and measuring changes in OD486 over time in a PowerWave XS microplate reader (BioTek) at 37°C. Activities

Figure 3. Assessment of competitive action of Solonamide B in P3-blaZ reporter strain. The inhibitory activity of 5 μg/mL Solonamide B (SolB) on RNAIII expression controlled by wild type AgrC was assessed when challenged by increasing concentrations of AIP. β-lactamase activity was determined with and without Solonamide B for three independent cultures (#1–#3) 30 minutes after addition of Solonamide B and induction by AIP. Bars represent means +/- SEM from triplicate activity determinations (A). Average inhibition obtained by Solonamide B at increasing AIP concentrations. Bars represent the mean +/- SEM reduction in β-lactamase activity observed from the three biological replicates (B). P-values between AIP treatments were calculated by Student’s t-test (two-sided).
doi:10.1371/journal.pone.0084992.g003

Figure 4. Structures of AIP II and Solonamide B. AIP II (left), Solonamide B (right).
doi:10.1371/journal.pone.0084992.g004
were calculated as arbitrary units based on respective conversion velocities ($V_{\text{max}}, \Delta \text{OD}_{486/\text{time}}$) normalized to the sample cell densities.

**Competition Assay**

The reporter assay were made according to Nielsen et al. (2010) [37] using the *S. aureus* 8325-4 derived *spa::lacZ* reporter strain [38] or strain RN10829 carrying pagC-I-WT. Sterile filtered supernatants from ON-cultures were made from *S. aureus* 8325-4 (wt) [35] and *S. aureus* 8325-4 Δagr [19] grown in TSB. For AIP competition experiments using the P3-β-lacZ reporter, 5 μg/mL Solonamide B was challenged by addition of a fixed 1/5 volume of culture supernatant, adjusted to contain 5%, 13%, or 20% AIP-I-containing supernatant (final concentration), and the effect was monitored in the AgrC-I-WT reporter strain. Solonamide B were purified from cultures of the marine bacterium *Photobacterium halotolerans* as described by Mannson et al. (2011) [34].

---

**Figure 5. Solonamide B inhibits agr-group I, II and IV.** The P3:β-lux reporter strains AN1 agr I, AN2 agr II, AN3 agrIII and agr IV AN4 were grown with DMSO (control), Solonamide B 5 μg/mL, and Solonamide B 10 μg/mL. Luminescence was recorded after 2 ½ hours of growth in the presence of the compound. The luminescence is scored in % luminescence relative to the control (DMSO) set to 100% for each agr group individually and standard deviations are calculated based on three replicates.

doi:10.1371/journal.pone.0084992.g005

**Figure 6. Solonamide B specifically reduces α-hemolysin production.** 1–256 fold dilutions of sterile filtered 24 hour culture supernatants from USA300 cultures grown with DMSO, 5 μg/mL or 10 μg/mL of Solonamide B, were tested for its ability to lyse rabbit erythrocytes. 0.1% triton X was used as a positive control. Hemolysis was scored in percentage relative to the DMSO control set to 100%. Data are a representative of at least two independent, biological replicates.

doi:10.1371/journal.pone.0084992.g006

**Figure 7. Solonamide B reduces psmA and agrA expression.** Strains USA300 or 8325-4 were grown exponentially to OD_{600} = 0.4 with either 5 μg/mL Solonamide B, DMSO or nothing added. RNA was purified from samples collected at OD_{600} = 0.7 and 1.7, and analyzed by Northern blotting. The membrane was probed with radioactive labeled probes targeting agrA and psmA. doi:10.1371/journal.pone.0084992.g007
Effect of Solonamide B on agr-group I, II, III and IV

Strain AN1, AN2, AN3 and AN4 were used for testing the effect of Solonamide B on *S. aureus* with different agr backgrounds (agr gr. I, II, III and IV). Cells were grown until OD_{600} = 0.4 and diluted 10-fold before distribution into a 96 well microtiter plate (100 µL per well). Solonamide B dissolved in DMSO was added to the microtiter well to a final concentration of 3 and 10 µg/mL. The plate was incubated at 37°C in a microplate reader with shaking every 10 minutes. Luminescence was recorded after 2½ hour incubation at OD_{600} = 0.5 in microtiter plates. For *agr* group III luminescence was recorded every 20 minutes in the absence of shaking.

Rabbit Erythrocyte Hemolysis Assay

1% suspension of rabbit erythrocytes were prepared by spinning 2 ml of rabbit blood at 3000 g for 10 min at 10°C, where after supernatant was removed. Pellet was dissolved in 2 mL of PBS containing 0.1% BSA (bovine serum albumin). Centrifugation and washing was repeated until the supernatant was clear (approx 3 times). To prepare a 1% erythrocyte solution 100 µL erythrocytes were dissolved in 10 ml PBS containing 0.1% BSA. To prepare culture supernatants *S. aureus* USA300 (FPR3757) cultures were spun at 5000 g for 10 minutes to pellet the bacteria. The supernatant was transferred to new eppendorf tubes, and dilution rows (2-fold dilutions) were made in TSB. 100 µL of 1% erythrocytes suspension was added to 100 µl culture supernatant in a 96 well microtiter plate and incubated for 30 minutes at 37°C and 30 minutes at 4°C. 0.2% Triton X in TSB (final concentration of 0.1%) was used as a positive control, and TSB was used as a negative control. The microtiter plate was spun at 2000 rpm for 2 minutes, supernatant was transferred carefully to a new plate and read at OD_{540}.

Transcriptional Analysis of agrA and psmα by Northern Blot

Northern blot analysis was performed as described previously [39]. The strains used were *S. aureus* 8325-4 and FPR 3757 USA300, and were grown in Erlenmeyer flask, shaking at 185 rpm. Growth was monitored by measuring optical density at OD_{600}. Start inoculum was OD_{600} = 0.03. Solonamide B was added at OD_{600} = 0.4. Samples for RNA purification were taken at OD_{600} = 0.7 and 1.7. Probes targeting AgrA and PSMα transcripts were amplified by PCR using the primers AgrA fwd: 5’ cgataaccttaggagc 3’, AgrA rev: 5’ cgatgcatagctgc 3’, PSMα rev: 5’ tatccaaagactgaactct 3’ and PSMα fwd:5’ cctctcaaataatggctcata 3’ (from TAG Copenhagen A/S, Denmark) resulting in probe lengths of 584 bp (AgrA) and 176 bp (PSMα) respectively.

*S. aureus* lysis of Human Neutrophils

Sterile filtered supernatants from 7 h and 22,5 h *S. aureus* LAC/ FPR3757 (USA300) cultures grown in 10 mL TSB media/ 100 mL Erlenmeyer flask, with a start inoculum OD_{600} = 0.02 and either DMSO (control) or Solonamide B (5 and 10 µg/mL) added to the cultures were tested for their capacity to lyse human neutrophils. Bacterial supernatants were collected from three independent experiments on different days, and stored at −20°C until used. Lysis of human neutrophils was determined using Cytotoxicity Detection Kit plus (LDH) from Roche (measuring lactate dehydrogenase release), manufacturer’s protocol was followed. Supernatants diluted 3- and 9-fold and undiluted supernatants were tested. Neutrophil lysis was determined spectrophotometrically at A490 nm-A630 nm after 1 h incubation at 37°C of 100 µL neutrophils (50,000 cells per well)+50 µL supernatant/TSB in microtiter wells. Data is a representative of 3 biological replicates, and each supernatant was tested at least in replicate wells. Neutrophils were isolated by Ficoll-hypaque purification from fresh heparinized human blood from healthy volunteers according to the regulations of National Committee on Health Research Ethics in Denmark. Using blood for assessment of effects of microorganisms or microbial components does not require approval from the committee [law no 593 of June 14, 2011]. Blood was mixed with equal volume of dextran (3%)/saline (0.9%), and incubated at room temp. for 20 min. The upper leukocyte rich layer was saved and centrifuged 10 min at 5°C at 250 g. Pellet was resuspended in 0.9% NaCl (equal to start volume), Ficoll-hypaque was gently layered under the cell suspension and centrifuged for 40 min. The red blood cells present in the pellet were lysed and cleared by washing with ice cold 0.2% NaCl for 30 sec. followed by adding 1.6% NaCl and centrifuging for 6 min at 250 g until colorless pellet was obtained and neutrophils were resuspended in ice-cold PBS/glucose (10 mM).

Results

Solonamide B Interferes with agr Activation

From the marine bacterium *Photobacterium halotolerans* we recently identified a cyclopeptide antibiotic, Solonamide B that interferes with virulence gene expression in *S. aureus* without inhibiting growth. The reduction in hla and RNAIII expression and increase inspa transcription provided circumstantial evidence that Solonamide B may interfere with *agr* activation [34]. To address this issue we examined the effect of the compound on RNAIII expression in a strain carrying an AgrC variant with a substitution at R238H in the histidine kinase domain that allows AgrC to be active independently of AIPs [40]. If, indeed, solonamide B acts through AgrC we predicted that RNAIII expression in cells carrying the constitutive variant would be unaffected by the compound in comparison to cells expressing the wild type AgrC. At solonamide B concentrations not affecting growth RNAIII (fig. 1C) promoter (P3) activity was monitored in strain RN10829 carrying a constitutive AgrC reporter fusion in addition to plasmids pmgC-I-WT or pEG11 expressing wild type or constitutive AgrC, respectively. Since this strain does not produce AIP, agr was induced by addition of 1/10 a volume of spent medium obtained from wild type 8325-4 cells in stationary growth phase. We found that in the presence of the constitutive AgrC (fig. 1B), Solonamide B did not affect the P3 promoter activity at concentrations that reduced RNAIII expression significantly in cells expressing wild type AgrC (fig. 1A). Thus, our results show that the effect of Solonamide B on virulence gene expression is mediated via interactions with AgrC. Furthermore, we noted a tendency towards a dose-dependency on Solonamide B (fig. 1A) suggesting competitive activity.

To address if Solonamide B interferes competitively with AIP binding to the AgrC receptor we added varying amounts of Solonamide B to wells in our reporter assay also containing fixed amounts of culture supernatant from either stationary phase 8325-4 cells or from *agr* mutant cells (fig. 2). Here, we observed that a fixed concentration of solonamide B induced *spa* expression of the incorporated reporter strains both in the presence of spent medium of wild type and *agr* mutant cells thus indicating inhibition of *agr* activity (fig. 2A). When reducing the amount of solonamide B only the lowest concentration (corresponding to 0.05 mM in the well) was unable to induce *spa* expression in competition with spent medium of wild type cells (fig. 2C). However, at the same
Solonamide B Inhibits Agr of *S. aureus*

A concentration we did see expression of *spa* when competed with supernatant obtained from *agr* mutant cells lacking AIP production thus confirming that solonamide B competes with AIP for AgrC binding (fig. 2D). A more quantitative measure of the competition was obtained with the P3-*bla*Z reporter strain exposed to a fixed concentration of Solonamide B (5 μg/mL) and dilutions of the spent supernatant from stationary phase, wild type cells. The results (fig. 3) show that 50% inhibition was reached.

**Figure 8. Solonamide B protects against *S. aureus* mediated neutrophil lysis.** Sterile filtered supernatants of *S. aureus* USA300 (S.a.) cultures grown for 7 hours or overnight (ON) with either Solonamide B at 5 μg/mL or 10 μg/mL or DMSO (control) were added to isolated human neutrophils. The control supernatant where only DMSO was added was tested in 1-fold, 3-fold and 9-fold dilutions (A). Undiluted (1:1) supernatants from Solonamide B (SolB) treated cultures are shown together with the control in (B) and 3-fold dilutions (1:3) are shown in (C). Lysis was monitored by lactate dehydrogenase (LDH) release. Data represents 3 independent experiments, using the average of triplicate wells from each experiment. Asterisks indicate SolB treated cultures resulting in lysis statistically significant from the corresponding control. *, p<0.05, **, p<0.01; ***, p<0.001. doi:10.1371/journal.pone.0084992.g008
Solonamide B Inhibits more than One agr Specificity Group

Interestingly, the cyclic structure of Solonamide B resembles the backbone of the native AIPs [fig. 4] that are thiolactones containing seven to nine amino acid residues in which the thiol of the central cysteine is linked to the alpha-carboxyl of the C-terminal amino acid residue [34,41]. As the thiolactone ring of AIPs is involved in agr repression while the peptide tails are linked to activation [42] we examined if Solonamide B inhibits more than one AIP specificity group. To this end we grew S. aureus P3::lux reporter strains belonging to the different agr specificity groups in the presence or absence of Solonamide B and measured luminescence after 2½ hr incubation at OD600 = 0.3 in the microtiter plate. As observed in fig. 5 luminescence expressed by the P3 promoter for agr group I and II, was reduced 5 fold when 5 and 10 µg/mL Solonamide B was added compared to the control wells containing DMSO. The strain carrying the agr specificity group IV showed a 2–3 fold decrease in P3::lux expression when treated with Solonamide B, and the luminescence declined with the higher concentration of Solonamide B. The expression pattern remained unchanged after 3½, 4½, 5½ h and ON incubation (data not shown). For agr group III an effect was seen already after 20 minutes resulting in 25% and 35% reduction with 5 and 10 µg/mL Solonamide B, respectively compared to the DMSO control with the effect declining over time (data not shown).

Altogether these results show that Solonamide B reduces agr expression in all the specificity groups, as measured by P3 activity, with the least effect towards agr group III.

Solonamide B Interferes with Hemolysin Production

Hemolysis is one of the major virulence factors in S. aureus pathogenesis [43]. As we had previously seen that hla expression was strongly reduced by Solonamide B [34] we sought to confirm that hemolytic activity was also affected. We chose to use rabbit erythrocytes for the hemolysis assay as they are known to be particularly susceptible to α-hemolysin [44,45]. When testing supernatants from S. aureus cultures grown with and without Solonamide B on rabbit erythrocytes we observed that Solonamide B reduced production of hemolysin(s) in S. aureus strain USA300 (fig. 6). In contrast, the solvent DMSO did not inhibit hemolysin production (fig. 6). When comparing the lytic activities, 50% hemolysis of rabbit erythrocytes was observed with a 64 fold dilution of the USA300 culture supernatants whereas it was seen for an eight fold dilution of supernatant from USA300 cells treated with 5 µg/mL solonamide B and at a three fold dilution for those treated with 10 µg/mL solonamide B. Thus, hemolysin activity is reduced in the presence of Solonamide B.

Solonamide B Reduces psmx Transcription

The PSM phenol soluble modulins are implicated as key virulence factors of CA-MRSA such as USA300 and in contrast to most S. aureus virulence genes, their expression is controlled directly by the AgrA response regulator [23]. To determine the potential of Solonamide B as an anti-virulence compound targeting CA-MRSA strains, we examined the effect of Solonamide B on the transcription of the psmx operon encoding the alpha-type PSMs. Importantly, when bacterial cells were cultured in the presence of Solonamide B there was a dramatic effect on psmx expression as the presence of the compound essentially abolished expression in both strain 8325-4 and USA300 (fig. 7).

To assess if this effect is correlated with agrA expression we probed the agr P2 transcript with a probe covering agrA and found it to be significantly decreased in both strains but most so in USA300. Thus, the reduced expression of psmx is likely caused by reduced expression of agrA.

Solonamide B Reduces PSM Expression and Neutrophil Killing

Neutrophils are a part of the innate immune system, and as the first leukocytes that infiltrate affected tissues they are one of the primary defenses against Staphylococcal infection [46,47]. S. aureus produces PSMs of which the alpha type PSM is the most important for neutrophil lysis, with PSMα3 having the most pronounced effect [11,23]. Since our transcriptional analysis revealed that Solonamide B dramatically reduces expression of psmx we examined if it may be protective of S. aureus mediated neutrophil killing. To this end we monitored toxicity of sterile filtered CA-MRSA USA300 supernatant grown with and without Solonamide B on human neutrophils (fig. 8). As above, growth rate and maximum cell density of S. aureus was identical with and without Solonamide B (data not shown). Importantly, we found that Solonamide B offered a significant reduction in the toxicity of 7 h and ON culture supernatants of USA300. Thus, the transcriptional effects on both hla and psmx are translated into reduced toxin production; though other factors than PSM may contribute to neutrophil lysis as well.

During these studies we did not observe lysis of human neutrophils or of bovine erythrocytes at any of the tested concentrations (up to 20 µg/mL) of Solonamide B thus indicating that Solonamide B displays low toxicity (data not shown).

Discussion

In times where antibiotic resistance is evolving towards most known antibiotics and the development of new antibiotics is lacking far behind we are searching for alternatives to treat serious infectious diseases. One approach that has received considerable interest is anti-virulence therapy where the virulence of the pathogen is targeted rather than viability. In this context we recently isolated a compound from the marine bacterium Photobacterium halotolerans that interfered with virulence gene expression in S. aureus. The compound termed Solonamide B inversely affected transcription of hla and spa indicating that it interferes with activation of the agr quorum sensing system [34]. We have addressed this hypothesis further by investigating the effect of Solonamide B in mutant cells expressing a variant of the AgrC sensor histidine kinase that is active irrespectively of the presence of the AIP auto-inducing peptides [14]. In this strain background the expression of rnaIII was unaffected by the compound strongly indicating that Solonamide B interferes with virulence gene expression by compromising AgrC activation and thus, quorum sensing.

A characteristic property of the S. aureus agr system is the presence of at least four agr subclasses, in which an AIP from one class induces agr in strains of its own class but represses agr of the other subclasses [18,48]. Structural studies of the AIPs have revealed that the macrocyclic ring of the AIPs is responsible for inhibition of agr, while the peptide tail is required for agr-activation [18,49]. It has been shown that truncated versions of group II AIPs inhibit all four agr groups, and that AIP analogs with oxygen or...
Solonamide B Inhibits Agr of S. aureus

In contrast to many other virulence factors in S. aureus, AgrA [23] and also the transcription of agrA was significantly reduced by the Solonamide B. Thus our data show that Solonamide B not only interferes with production of virulence factors belonging to the RNAIII regulon but also expression of agrA and the AgrA controlled PSMs.

When comparing the ability of Solonamide B to compete with AIP for binding we found that 5 µg/mL solonamide corresponding to 0.3 mΜ provide 50% inhibition in spent medium of wild type cells containing approximately 0.25 mΜ AIP (estimated as 5% of 5 mΜ, Alexander Horwill, personal communication). Thus, although we with the examined compound see a significant reduction in S. aureus virulence, structural optimizations may increase the inhibitory potential even further. In conclusion we show that Solonamide B interferes with agr activation by binding to the AgrC sensor histidine kinase and thereby preventing interactions between AgrC and the AIPs. As an anti-virulence compound Solonamide B demonstrates potential due to its low toxicity, its inhibitory effect towards the known classes of agr and its ability to reduce expression of the PSMs involved in the severe CA-MRSA infections.

Acknowledgments

Morten Alhede is thanked for kindly providing access to instrumentation. This is Galathea 3 contribution no. P102.

Author Contributions

Performed the experiments: AN MM MB. Analyzed the data: AN MM LG TOL RPN DF HFI MB. Contributed reagents/materials/analysis tools: TOL LG RPN. Wrote the paper: AN MM LG HI.

References

1. Archer GL (1998). Staphylococcus aureus: A well-armed pathogen. Clinical Infectious Diseases 26: 1179–1181.
2. Lowy FD (1998). Staphylococcus aureus infections. N Engl J Med 339: 520–532.
3. Sakoulas G, Moelling RC Jr (2008) Increasing antibiotic resistance among methicillin-resistant Staphylococcus aureus strains. Clin Infect Dis 46 Suppl 5: S360–S372.
4. Hidron AI, Edwards JR, Patel J, Sievert DM, Horan TC, et al. (2008) NHSN annual update: Antimicrobial-resistant pathogens associated with healthcare-associated infections: Annual summary of data reported to the national healthcare safety network at the centers for disease control and prevention, 2006-2007. Infect Control Hosp Epidemiol 29: 996–1011.
5. Thompson RL, Cabezudo I, Wenzel RP (1982) Epidemiology of nosocomial infections in California hospitals. Infect Control 3: 390–397.
6. Loughman JA, Fritz SA, Storch GA, Hunstad DA (2009) Virulence gene expression in human community-acquired Staphylococcus aureus infection. J Infect Dis 199: 294–301.
7. Kazakova SV, Hageman JC, Mataza M, Sinivasan A, Phelan L, et al. (2005) A clone of methicillin-resistant Staphylococcus aureus among professional football players. N Engl J Med 352: 486–475.
8. Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005) Methicillin-resistant Staphylococcus aureus in community-acquired skin infections. Emerg Infect Dis 11: 920–920.
9. Herold BC, Immergluck LC, Maranan MC, Lauderdale DS, Gaskin RL, et al. (1998) Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. JAMA: the Journal of the American Medical Association 279: 593.
10. Bubek Wardenburg J, Bae T, Otto M, Delo FR, Schneewind O (2007) Poring over pores: Alpha-hemolysin and panton-valentine leukocidin in Staphylococcus aureus pneumonia. Nat Med 13: 1405–1406.
11. Wang R, Braugher KK, Kretschmer D, Bach TH, Queck SY, et al. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 13: 1510–1514.
12. Bhakdi S, Tranum-Jensen J (1991) Alpha-toxin of Staphylococcus aureus. Microbiol Rev 55: 733–751.
13. Jarry TM, Menni G, Cheung AL (2008) The expression of alpha-haemolysin is required for Staphylococcus aureus phagosomal escape after internalization in CFT-1 cells. Cell Microbiol 10: 1801–1814.
14. Otto M (2010) Basis of virulence in community-associated methicillin-resistant Staphylococcus aureus. Annu Rev Microbiol 64: 143–162.
15. Janeway JC, Travers P, Walport M, Shlomchik MJ (2005) Immuno biology, the immune system in health and disease. USA and UK: Garland Science publishing. 823 p.
16. Cheung GY, Wang R, Khan BA, Sturdevant DE, Otto M (2011) Role of the accessory gene regulator agr in community-associated methicillin-resistant Staphylococcus aureus pathogenesis. Infect Immun 79: 1927–1935.
17. Reseci P, Kreiswirth B, O'Reilly M, Schlievert P, Gruss A, et al. (1986) Regulation of exoprotein gene expression in Staphylococcus aureus by agr. Mol Gen Genet 202: 58–61.
18. Novick RP (2003) Antioxidation and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol 48: 1429–1449.
19. Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, et al. (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J 12: 3967–3972.
20. George EA, Mair TW (2007) Molecular mechanisms of agr quorum sensing in virulent staphylococci. ChemBiochem 8: 847–855.
21. Ji G, Beavis RC, Novick RP (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci U S A 92: 12055–12059.
22. Chan WC, Coyle BJ, Williams P (2004) Virulence regulation and quorum sensing in staphylococcal infections: Competitive AgrC antagonists as quorum sensing inhibitors. J Med Chem 47: 4633–4641.
23. Queck SY, Jameson-Lee M, Villanuz AE, Bach TH, Khan BA, et al. (2008) RNAIII-independent target gene control by the agr quorum-sensing system: Insight into the evolution of virulence regulation in Staphylococcus aureus. Mol Cell 32: 150–158.
24. Deresinski S (2006) Antistaphylococcal vaccines and immunoglobulins: Current status and future prospects. Drugs 66: 1797–1806.
25. Werner G, Stommenger B, Witte W. (2008) Acquired vancomycin resistance in clinically relevant pathogens. Future Microbiol 3: 547–562.
26. Shaham M. (2011) Antivirulence agents against MRSA. Future Med Chem 3: 773–777.
27. Krucke GW, Grimes DE, Grimes RM, Dang TD (2009) Antiadhesive resistance in staphylococcus aureus-containing cutaneous abscesses of patients with HIV. Am J Emerg Med 27; 344–349.
28. Chatterjery AE, Person E, Hung DT (2007) Targeting virulence: A new paradigm for antimicrobial therapy. Nat Chem Biol 3: 541–548.
29. Rasko DA, Sperandio V (2010) Anti-virulence strategies to combat bacteria-mediated disease. Nat Rev Drug Discov 9: 117–128.
30. Ragle BE, Bubeck Wardenburg J (2009) Anti-alpha-hemolysin monoclonal antibodies mediate protection against Staphylococcus aureus pneumonia. Infect Immun 77: 2712–2718.
31. Firon N, Ashkenazi S, Mirelman D, Olek I, Sharon N (1987) Aromatic alpha-glycosides of mannose are powerful inhibitors of the adherence of type 1 fimbriated Escherichia coli to yeast and intestinal epithelial cells. Infect Immun 55: 472–476.
32. Henzer M, Wie H, Andersen JB, Riedel K, Rasmussen TB, et al. (2003) Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors. EMBO J 22: 3803–3815.
33. Cegelski L, Marshall GR, Eldridge GR, Hultgren SJ (2008) The biology and future prospects of antivirulence therapies. Nat Rev Microbiol 6: 17–27.
34. Mansson M, Nielsen A, Kjærulff L, Gotfredsen CH, Wietz M, et al. (2011) Inhibition of virulence gene expression in Staphylococcus aureus by novel depsipeptides from a marine photobacterium. Marine Drugs 9: 2537–2552.
35. Novick RP, Morse SI (1967) In vivo transmission of drug resistance factors between strains of Staphylococcus aureus. J Exp Med 125: 46–59.
36. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, et al. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. Lancet 367: 731–739.
37. Nielsen A, Nielsen KF, Frees D, Larsen TO, Ingmer H (2010) Method for screening compounds that influence virulence gene expression in Staphylococcus aureus. Antimicrob Agents Chemother 54: 509–512.
38. Chan PF, Foster SJ (1998) The role of environmental factors in the regulation of virulence-determinant expression in Staphylococcus aureus 0323–4. Microbiology 144 (Pt 9): 2469–2479.
39. Jelsbak L, Ingmer H, Valihrach L, Cohn MT, Christiansen MH, et al. (2010) The chaperone ClpX stimulates expression of Staphylococcus aureus Protein A by rot dependent and independent pathways. PLoS One 5: e12752.
40. Geisinger E, Muir TW, Novick RP (2009) Agr receptor mutants reveal distinct modes of inhibition by staphylococcal autoinducing peptides. Proc Natl Acad Sci U S A 106: 1216–1221.
41. Jensen RO, Winzer K, Clarke SR, Chan WC, Williams P (2008) Differential recognition of staphylococcus aureus quorum-sensing signals depends on both extracellular loops 1 and 2 of the transmembrane sensor AgrC. J Mol Biol 381: 300–309.
42. Mayville P, Ji G, Beavis R, Yang H, Goger M, et al. (1999) Structure-activity analysis of synthetic autoinducing thiolactone peptides from Staphylococcus aureus responsible for virulence. Proc Natl Acad Sci U S A 96: 1218–1223.
43. Graves SF, Kobayashi SD, DeLeo FR (2010) Community-associated meticillin-resistant Staphylococcus aureus immune evasion and virulence. J Mol Med [Berl] 88: 109–114.
44. Bernheimer AW, Schwartz LL (1963) Isolation and composition of staphylococcal alpha toxin. J Gen Microbiol 30: 135–168.
45. Bhakdi S, Muhly M, Fussle R (1984) Correlation between toxin binding and hemolytic activity in membrane damage by staphylococcal alpha-toxin. Infect Immun 46: 318–323.
46. Fischetti VA, Novick RP, Ferretti JJ, Pormento DA, Reed JL (2006) Gram-positive pathogens. United States: ASM Press, Washington, DC 20036–2904. 849 p.
47. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A (2012) Neutrophil function: From mechanisms to disease. Annu Rev Immunol. 30: 459–89.
48. Ji G, Beavis R, Novick RP (1997) Bacterial interference caused by autoinducing peptide variants. Science 276: 2027–2030.
49. Lyon GJ, Wright JS, Muir TW, Novick RP (2002) Key determinants of receptor activation in the agr autoinducing peptides of Staphylococcus aureus. Biochemistry 41: 10095–10104.
50. Lyon GJ, Mayville P, Muir TW, Novick RP (2000) Rational design of a global inhibitor of the virulence response in Staphylococcus aureus, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. Proc Natl Acad Sci U S A 97: 13330–13335.
51. Jarraud S, Lyon GJ, Figureido AM, Lima G, Vandenbosch F, Etienne J, Muir TW, Novick RP (2000) Exfoliatin-producing strains define a fourth agr specificity group in Staphylococcus aureus. J Bacteriol. 182: 6517–22.
52. Lovey PD (2007) Secrets of a superbug. Nat Med 13: 1418–1420.