**Staphylococcus aureus** biofilm susceptibility to small and potent β\(^{2,2}\)-amino acid derivatives

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Small antimicrobial β\(^{2,2}\)-amino acid derivatives (Mw < 500 Da) are reported to display high antibacterial activity against suspended Gram-positive strains combined with low hemolytic activity. In the present study, the anti-biofilm activity of six β\(^{2,2}\)-amino acid derivatives (A1–A6) against *Staphylococcus aureus* (ATCC 25923) was investigated. The derivatives displayed IC\(_{50}\) values between 5.4 and 42.8 \(\mu\)M for inhibition of biofilm formation, and concentrations between 22.4 and 38.4 \(\mu\)M had substantial effects on preformed biofilms. The lead derivative A2 showed high killing capacity (log \(R\)), and it caused distinct ultrastructural changes in the biofilms as shown by electron and atomic force microscopy. The anti-biofilm properties of A2 was preserved under high salinity conditions. Extended screening showed also high activity of A2 against *Escherichia coli* (XL1 Blue) biofilms. These advantageous features together with high activity against preformed biofilms make β\(^{2,2}\)-amino acid derivatives a promising class of compounds for further development of anti-biofilm agents.

**Keywords:** biofilm; antimicrobial peptide; peptidomimetic; β-amino acid; electron microscopy; *Escherichia coli*

**Introduction**

Biofilm-associated chronic infections pose a growing healthcare problem and are frequently caused by the spherically-shaped, Gram-positive bacterium *Staphylococcus aureus* (Kiedrowski & Horswill 2011). *S. aureus* is a pathogen of high virulence and biofilm infections of *S. aureus* origin require not only long term antibiotic treatment but often necessitate surgical intervention (Darouiche 2004). Biofilms are surface-attached, matrix-enclosed, three dimensionally-structured bacterial agglomerates with a life-cycle characterized by different stages, ie adherence, colonization, maturation and dispersion (Otto 2013). Pre-conditioning of the surfaces of implanted or inserted medical devices by host proteins and poor vascularization, facilitate the adherence of bacterial cells and aggravates counter measures of the immune system (Kiedrowski & Horswill 2011). Furthermore, biofilms display extraordinary antibiotic resistance and an up to 1,000-fold increase of minimal inhibitory concentrations (MICs) of antimicrobial drugs is often a consequence (Davies 2003; Schillaci 2011).

The biofilm matrix acts as a physical barrier that substantially impairs the activity of antibiotics against biofilms (Davies 2003; Tote et al. 2009). The matrix is formed by an extracellular polymeric substance (EPS) and consists of a conglomerate of extracellular DNA (eDNA), proteins, and polysaccharides (Davies 2003; Flemming & Wingender 2010). Also differentiation of bacteria within the biofilm to metabolically inactive persister cells hampers biofilm eradication by eluding antibiotics targeting active cell processes. Additionally, the typical bacterial resistance mechanisms known from planktonic cells contribute to biofilm defense (Batoni et al. 2011; Schillaci 2011).

During recent years, cationic antimicrobial peptides (AMPs) have increasingly gained importance as a new promising class of antibiotic agents (Baltzer & Brown 2011). The second version of the antimicrobial peptide database (APD2) already counts nearly 1,800 antibacterial peptides, that are manifold regarding structure and mechanism of action (Brogeden 2005; Wang et al. 2009). Furthermore, many AMPs are active against antibiotic-resistant strains and, of importance to the current work, are able to unfold their antimicrobial properties against biofilms of varied origin (Baltzer & Brown 2011; Batoni et al. 2011).

Hansen et al. (2011) have reported that small (Mw < 500 Da) amphipathic β\(^{2,2}\)-amino acid derivatives show antimicrobial activity against both Gram-positive and negative strains, including the multi-resistant bacteria methicillin resistant *S. aureus* (MRSA) and methicillin resistant *Staphylococcus epidermidis* (MRSE), as well as low hemolytic activity against human red blood cells.

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(RBC). Of note, the permeability of the derivatives through a phospholipid vesicle based barrier, resembling colon uptake by passive diffusion, has also been successfully demonstrated (Flaten et al. 2006). This property together with high stability against proteases (Hansen et al. 2010; Tørfoss et al. 2012) emphasize the potential of developing such β2,2-amino acid derivatives into future antimicrobial peroral drugs.

In the present study, the anti-biofilm properties of six (A1–A6) antimicrobial β2,2-amino acid derivatives were investigated (structures are presented in Figure 1) which have been recently developed (Hansen et al. 2011, 2012). *S. aureus* (ATCC 25923) was used as model biofilm-forming strain for investigating the anti-biofilm activity of the derivatives. Changes in the biofilm morphology upon treatment with the lead derivative A2 were furthermore investigated by atomic force microscopy (AFM) and by scanning and transmission electron microscopy (SEM/TEM). The salt sensitivity of A2 was also assessed since high salinity has been demonstrated to impair the efficiency of many AMPs (Yu et al. 2011). Moreover, the effect of the lead derivative A2 on an extended set of representative bacterial strains was measured, including *S. aureus* (Newman), *Staphylococcus epidermidis* (S. epidermidis) (ATCC 35984 and ATCC 12228), and *Escherichia coli* (E. coli) (XL1 Blue).

**Material and methods**

**Bacterial growth conditions and biofilm formation**

All strains used were stored at −70 °C in tryptic soy broth (TSB) containing 20% glycerol. Fresh cultures were started prior to every experiment from the glycerol stocks by incubating overnight in TSB (1:300 dilution) at 37 °C, 200 rpm. The liquid cultures were prepared by diluting the pre-cultures 1,000 times (*S. aureus* ATCC 25923 and Newman strains, as well as *E. coli* XL1 Blue) or 100 times (*S. epidermidis* ATCC 35984 and ATCC 12228 strains) in fresh TSB, under aerobic conditions at 37 °C, 200 rpm until exponential growth was reached (4–5 h), corresponding to a bacterial concentration of 10⁸ CFU ml⁻¹. Biofilms were formed by transferring 200 µl of exponentially grown suspensions (10⁶ CFU ml⁻¹) to 96-well plates (Nunclon™ Δ surface). Subsequently, the cultures were incubated for 18 h under equivalent conditions as stated above and described earlier (Sandberg et al. 2008).

**Derivatives A1–A6**

The β2,2-amino acid derivatives (A1–A6) were synthesized as described earlier (Hansen et al. 2011, 2012) The derivatives were isolated as di-trifluoroacetate salts and analyzed with an analytical RP-HPLC C18-column and

![Figure 1. The β2,2-amino acid derivatives A1–A6 screened for anti-S. aureus biofilm activity. Derivatives A1–A3 and A4–A6 display the same lipophilic bulk moieties but have different C-terminal ends, ie a 1,2-diaminoethane C-terminal group in A1–A3 (amide derivatives) and a N,N-dimethylaminoethanol C-terminal group in A4–A6 (ester derivatives).](image-url)
UV detection at 214 and 254 nm showing purity above 95%. Stock solutions in dry DMSO were prepared prior to the experiments. Final DMSO concentrations did not exceed 2%, which has been shown to be well tolerated by *S. aureus* biofilms (Sandberg et al. 2008).

**Potency determination of A1–A6 using planktonic S. aureus**

To assess the antimicrobial activity of A1–A6, planktonic cultures of *S. aureus* (~2 × 10⁶ CFU ml⁻¹) were incubated with increasing concentrations of the β²,²-amino acid derivatives. Turbidity changes (at 595 nm) were followed with a Varioskan Multimode reader (Thermo Scientific, Vantaa, Finland) using a kinetic loop programmed to make measurements every 15 min at 37 °C for 18 h. During that period, the plate was constantly shaken at 240 shakes min⁻¹ on background mode. The percentage inhibition of bacterial growth in relation to untreated suspensions was calculated using the absorbance readouts from the last measurement point (after 18 h). With those values, half inhibitory concentrations (IC₅₀) were calculated. The plates were also visually inspected after each incubation. The lowest concentrations of the derivatives in wells showing no turbidity were reported as the MIC values.

**Impact on S. aureus biofilm formation**

The ability of the β²,²-amino acid derivatives to hamper biofilm formation and to act on formed *S. aureus* biofilms was investigated by two different staining methods adapted from Sandberg et al. (2008, 2009). Resazurin (Sigma-Aldrich, St Louis, MO, USA) staining was applied for evaluating biofilm viability while crystal violet (2.3% w/v, Sigma-Aldrich, St Louis, MO, USA) staining was applied for evaluating biofilm biomass. To assess the impact on biofilm formation (prior-to exposure), *S. aureus* cultures were incubated with different concentrations of the β²,²-amino acid derivatives at 37 °C and 200 rpm for 18 h (Table 1). When the post-exposure assay was carried out, biofilms were first formed for 18 h, as described above. After exchanging the planktonic phase with fresh TSB, the β²,²-amino acid derivatives were added and plates were incubated for an additional 24 h. At the end of the exposure periods, the planktonic phase was carefully removed and 200 μl of resazurin 20 μM in PBS (Lonza Walkersville Inc, Walkersville, USA) were added and the well plate was incubated (200 rpm, RT, 30 min, darkness). Fluorescence was measured with a Varioskan Multimode reader (λₑₓ 570 nm, λₑₘₐₜ 590 nm); viability was calculated as a percentage of the untreated control and IC₅₀ values determined. Subsequently, the supernatant was gently removed and 170 μl of the crystal violet solution were added with a multidrop dispenser (Thermo Scientific, Vantaa, Finland) and incubated for 5 min. The dye was removed and the wells were washed twice with deionized water followed by the addition of 200 μl of 96% ethanol. After 1 h the photometric absorbance (λ 590 nm) was measured using a Varioskan Multimode reader. The suitability of measuring biofilm biomass right after performing the viability assay has been shown earlier (Skogman et al. 2012). For the experiments performed with other strains (*Staphylococcus* spp. and *E. coli*), the biofilm viability quantification was conducted with the resazurin assay in the same way as described here, except that for *E. coli* the incubation time with the fluorescent probe was extended to 40 min.

**Biofilm log reduction (log R) assay**

Derivative A2 was selected as the lead derivative for a more detailed investigation of the effects on *S. aureus* biofilms. Biofilms were preformed in 96-well plates as described above and after 18 h they were exposed to A2 (10, 25, 50, 100, 200 and 400 μM) or penicillin G (400 μM). After incubation for 24 h, the planktonic phase was carefully removed and remaining biofilms were recovered by thoroughly scraping the well surfaces and suspending the recovered biofilms in TSB. Serial dilutions in TSB were prepared and aliquots plated on tryptic soy agar (TSA) plates (Sigma-Aldrich, St Louis, MO, USA). The agar plates were incubated at 37 °C and colonies counted the following day. The log reduction (log R) was determined by subtracting the log [CFU ml⁻¹] values of the treated biofilms from the log [CFU ml⁻¹] value of the untreated controls. Efficient recovery of biofilm was checked by resazurin staining of the scraped wells.

**Atomic force microscopy**

For morphology studies by AFM, biofilms were formed on custom-made polystyrene discs (Nunclon™ Δ surface)

| Cmp. | Test concentration ranges [μM] |
|------|-------------------------------|
|      | *S. aureus* biofilm formation |
|      | Prior-to | Post-to |
| A1   | 1–30       | 3–100      |
| A2   | 1–30       | 3–100      |
| A3   | 0.25–10    | 3–100      |
| A4   | 3–100      | 3–150      |
| A4   | 3–50       | 1–100      |
| A6   | 1–10       | 1–100      |

The μM concentrations of A1–A6 were calculated based on their di-trifluoroacetate salts.
as previously described (Sandberg et al. 2008). Biofilms were prepared for 18 h according to the prior performed assays and treated with 10, 50, 200 and 400 μM of compound A2. Penicillin G (400 μM)-treated and untreated biofilms were used as controls. The samples were analyzed using a NTEGRA Prima atomic force microscope (NT-MDT, Moscow, Russia), positioned on an active vibration isolation table (TS-150, Table Stable Ltd, Zwillikon, Switzerland) that was further placed on a stone table to eliminate external vibrational noise. Intermittent-contact mode under ambient conditions (RH = 35 ± 7%, T = 25 ± 1 °C) and rectangular silicon cantilevers (NSG30, NT-MDT) were used for topography imaging. 1024 × 1024 pixels images were recorded in the repulsive regime using a damping ratio of 0.5–0.6 and a scan speed of 0.2–0.25 Hz.

**Scanning and transmission electron microscopy**

SEM and TEM were applied using a setup similar to the log R assay to further investigate the impact of the lead derivative A2 on biofilm morphology over larger areas and to measure intracellular alterations. *S. aureus* biofilms were formed for 18 h on 2.0 mil Aclar® films mounted on live cell carriers (Leica Microsystems GmbH, Wetzlar, Germany) that were subsequently incubated for 24 h with 100 and 200 μM of A2, or 400 μM of penicillin G used as the control antibiotic. Excess liquid was gently removed from the discs and small drops of 1-hexadecene were carefully placed on the disc edges to cover the Aclar® film. Treated biofilms and untreated controls were cryofixed using a Leica high-pressure freezer (Leica EM PACT2, Leica Microsystems GmbH, Wetzlar, Germany).

For SEM studies, the frozen specimens were freeze substituted with a mixture of acetone, 2% osmium tetroxide, and 0.1% uranyl acetate for 17 h with a temperature gradient rising from −90 to −30 °C. The temperature was further increased to 0 °C over a period of 9 h and the fixative was replaced by pure acetone. After critical point drying the discs were directly mounted on aluminum stubs using silver paint. Prior to examination the specimens were sputter coated for 90 s. Samples were analyzed on a JEOL JSM-6300 scanning electron microscope (JEOL, Akaishima, Japan) and image acquisitions carried out via an EDAX Phoenix EDAM III data acquisition module (EDAX Inc., Mahwah, NJ, USA).

In the TEM studies, the frozen specimens were freeze substituted as described above and infiltrated with Epon resin. The biofilm surface was orientated to the thicker part of the resin bloc, and the biofilm top and base layer could thereby be identified during microscopy. The ultrathin 70 nm sections were prepared and placed on formvar carbon-stabilized copper grids. Uranyl acetate (5%) and Reynold’s lead citrate were used for staining and contrasting. Samples were analyzed on a JEOL-1010 transmission electron microscope (JEOL, Akaishima, Japan) and images were taken with an Olympus Morada side-mounted TEM CCD camera (Olympus soft imaging solutions GmbH, Münster, Germany).

**Impact of high salinity on anti-biofilm properties**

To investigate the salt susceptibility of the lead derivative A2, 18 h preformed *S. aureus* biofilms were prepared as described above and TSB was supplemented with sodium chloride to obtain final concentrations of 150 and 200 mM. The amounts of sodium chloride added to TSB were adjusted, taking into account that TSB contains 86 mM sodium chloride as part of the basic formulation. The biofilms were incubated with the lowest concentration of A2 that caused > 95% destruction of *S. aureus* biofilm (35 μM). Gentamicin was used as control as in earlier studies (Hansen et al. 2011). Biofilm viability was assessed 24 h after adding A2 and gentamicin by resazurin staining.

**Statistical analysis and data processing**

For each experiment a minimum of at least three replicates per treatment were included on every plate and at least two independently performed experiments (biological replicates) were carried out. The IC_{50} values for antimicrobial and anti-biofilm effects on *S. aureus* were calculated from at least eight concentration points by non-linear regression analysis (sigmoidal dose-response fitting with variable slope) using GraphPad Prism software version 5.01 (GraphPad Software, San Diego, CA, USA). Sigma Plot software version 12 (Systat Software Inc, San Jose, CA, USA) was used to plot graphs. AFM image processing and analyzing was carried out with Scanning Probe Image Processor software (SPIP™, Image Metrology, Denmark). TEM images were acquired with help of the program iTEM version 5.0 (Olympus soft imaging solutions GmbH, Münster, Germany), and the program Genesis version 4.61 (EDAX Inc., Mahwah, NJ, USA) was used to acquire SEM images.

**Results**

**IC_{50} determination of A1–A6 using planktonic S. aureus**

It has recently been reported that the β^{2,2}-amino acid derivatives A1–A3 display high antimicrobial activity against *S. aureus* and MRSA, but no information existed on the antimicrobial activity of derivatives A4–A6 (Hansen et al. 2011). The antibacterial effects of these new compounds against planktonic growth of *S. aureus*
were therefore examined first, and to ensure comparable outputs, the effects of A1–A3 were also measured (Table 2). The structurally related pairs A2 and A5, as well as A3 and A6 displayed similar IC_{50} values when bacterial growth was recorded for 18 h. Even though A1 showed an IC_{50} value of the same magnitude as A2 and A5, its counterpart A4 was almost six times less effective. The most potent pair of β^2-amino acid derivatives were A3 and A6 with IC_{50} values below 10 μM. However, when the derivatives were compared based on previously published cytotoxicity data (Hansen et al. 2011, 2012), derivatives A1, A2 and A5 possessed the best selectivity, i.e., a more than 37- to 49-fold preference for S. aureus than for human RBC. The 95% confidence intervals (Table S1), growth curves (Figures S1–S3) and the determined MIC values (Table S2) are presented in the Supplementary material (Supplementary material is available via a multimedia link on the online article webpage).

**Anti-biofilm potencies of A1–A6 prior to or post-S. aureus biofilm formation**

The impact of the β^2-amino acid derivatives was assessed prior to biofilm formation (biofilm prevention) or on existing 18 h biofilms (post-biofilm exposure) (Table 2). Resazurin staining showed that A2–A3 and A5–A6 were more potent in preventing S. aureus bacterial biofilms than derivatives A1 and A4. Also, similar to the trend observed for the planktonic results, A1 was more than twofold more potent than the structurally related counterpart A4. When biofilm biomass was assessed by crystal violet staining the IC_{50} values were of the same magnitude as for the resazurin staining, thus indicating that effective reduction of S. aureus biofilm biomass was most likely associated with the reduction in the core of viable cells (Table 2). More than 90% metabolic inhibition (as measured with the resazurin reduction) was achieved with concentrations just above the IC_{50} values (Table 3). Overall, A2 and A3 were the most potent derivatives whereas A4 was the least potent derivative (Table 2).

During the investigations of the effects on formed biofilms, it was observed that the determined IC_{50} values for all derivatives were approximately one and a half- to sixfold higher compared to the results obtained for preventing biofilm formation (Table 2). Derivative A4 was the only compound for which an IC_{50} value could not be estimated when assayed at concentrations up to 150 μM. The concentrations resulting in more than 90% inhibition of metabolic activity of S. aureus biofilms displayed a similar ranking as the prior determined IC_{50} values (Table 3). Derivative A3 was the most efficient compound acting on S. aureus biofilms and closely followed by A2. The derivatives A5 and A6 displayed lower potency that was approximately twice as low as for their structurally related counterparts A2 and A3. Even though A3 seemed to be the most potent compound among the six derivatives, the better potency/
toxicity profile of A2 led to a more detailed investigation of this compound.

**Determination of log reduction of biofilms by A2**

Formed (18 h) biofilms treated with the lead derivative A2 or penicillin G were investigated by subsequently performed plate counting, which remains the gold standard for quantification of viable bacteria. Only 25 μM A2 was needed to reduce the number of viable *S. aureus* biofilm bacteria by 1 log unit, which was as efficient as 400 μM penicillin G (Figure 2). By stepwise increasing the concentration of A2 a gradual decrease of viable bacteria could be achieved. At 200 μM, A2 caused nearly sixfold log reduction of biofilms, which is regarded as a high killing efficacy. Calculation of the biocompatibility index (BI), as the ratio of the concentration of A2 causing 50% of hemolysis, and the concentration causing at least a 3 log reduction (in this case a 4 log reduction) of biofilm bacteria gives an excellent value of 4.57, suggesting that A2 would be a safe antimicrobial agent to use. Of note, it is desirable that antimicrobial agents should have BI > 1, as originally proposed by Müller and Kramer (2008).

**Atomic force microscopy**

AFM is a high-resolution, non-destructive and fixation/staining free technique which can be utilized for surface characterization of biological samples (Li et al. 2010). Biofilms were treated with increasing concentrations of the lead derivative A2, similar to the log R assay, and the biofilm surface was scanned using AFM (Figure 3). It appeared that A2 at a concentration of only 10 μM was able to decrease the biofilm, and that the remaining bacteria displayed the same morphology as observed in the control. By raising the A2 concentration to 50 μM the bacteria appeared collapsed after treatment as indicated by flattened circular structures compared to the untreated control and penicillin G-treated biofilms. Of note, the two highest A2 concentrations were able to nearly completely remove the biofilm since both density and morphology were severely affected compared to the controls (Figures 3 and S4). Treatment of biofilms with the control antibiotic penicillin G (400 μM) resulted in lower bacterial density. However, the morphology of *S. aureus* cells seemed not to differ from untreated bacteria as indicated by their sharp defined shapes. Blurry structures appeared to surround the bacterial agglomerates treated with penicillin G. This was not visible in the images of the A2-treated or the untreated biofilms.

**Scanning and transmission electron microscopy**

Both SEM and TEM were applied upon high-pressure cryofixation to gain a deeper morphological insight in outer and inner structures of *S. aureus* biofilms. SEM images of untreated control cells showed that the bacteria were very well interconnected and were coated with the EPS, ie a tight network of bacteria was formed (Figure 4, Ia and b). The EPS produced was tightly packed around the Staphylococci and varied in thickness on top and within the biofilm. Dividing bacteria were prominent in all biofilm layers. SEM and TEM images of penicillin G – treated biofilms showed that *S. aureus* cells were also well interconnected but were massively covered with EPS when compared with the control cells (Figure 4, Iia and b). In contrast, biofilms treated with 200 μM A2 seemed to be dispersed (Figure 4, IIIa). Flattened, disc shaped, bacterial structures were visible as observed in the AFM images (Figures 3 and 4, IIIa and c), but interconnecting biofilm structures could not be observed in the same way as for untreated or penicillin G-treated biofilms. TEM images also showed that derivative A2 substantially reduced the thickness of the biofilms, and had a pronounced impact on the morphology of the remaining bacterial cells (Figures 4, IIIb and c and S4).

In addition, *S. aureus* biofilms treated with 100 μM A2 were investigated in order to better follow the impact of A2 with help of TEM. A defined EPS structure was not visible (Figure 4, IIId) and consecutively the biofilm appeared as a loose aggregation of bacteria as seen in the SEM images (Figure 4, IIIa). The bacteria seemed heavily affected, as shown by lysed or partly lysed cells. This *S. aureus* morphology was not visible when
Figure 3. AFM images of treated and untreated preformed *S. aureus* biofilms. A2 treatment at increasing concentrations shows its impact on biofilm morphology and density. Control biofilms grown in plain TSB and biofilms treated with 400 μM penicillin G were used as controls. Sample topography appears according to the color scale bar and z-ranges for the acquired images lie between 0.57 and 1.61 μM.
compared with the penicillin G-treated bacteria (Figure 4, IIId), and it was surprising that this effect was spread over the entire biofilm. Also bacteria in the protected deeper layers of the biofilm appeared to be exposed to the influence of derivative A2.

Salt sensitivity of derivative A2

High salinity can have a negative impact on AMP potencies (Yu et al. 2011) but this has so far not been assessed for these β2,2-amino acid derivatives. The present results showed that treatment of *S. aureus* biofilms formed in TSB supplemented with increasing concentrations of sodium chloride, still resulted in an efficient potency of A2. Even the highest salt concentration of sodium chloride (200 mM) did not compromise the anti-biofilm activity of A2 (Figure 5). The control antibiotic gentamicin was only able to reduce the biofilm burden by 50% in TSB, even though it was applied at a much higher concentration than A2. As expected, a further decrease in the potency of gentamicin was observed at the highest sodium chloride concentration of 200 mM, which only led to a 37% inhibition of viability of the preformed biofilms.

Anti-biofilm effects of A2 in other strains

To further assess the potential clinical application of the anti-biofilm activity of the lead derivative A2, experiments were performed with other representative strains (Table 4). The concentration at which A2 caused 50% inhibition of biofilm viability when tested prior-to and post-to *S. aureus* (ATCC 25923) biofilm formation was chosen, corresponding to 10 and 25 μM, respectively.
using preformed biofilm systems. The results showed that the lead derivative A2 caused around 50% inhibition of biofilm formation by S. aureus (Newman), showing a similar potency as observed against S. aureus (ATCC 25923). A lower susceptibility was registered in the post-exposure experiment, which may be due to a general higher chemoresistance in S. aureus (Newman) biofilms. No inhibitory effects were detected against the two strains of S. epidermidis (ATCC 35984 and ATCC 12228, Table 4). In contrast, A2 was highly potent against the E. coli (XL1 Blue) strain, causing more than 60% inhibition of biofilm formation, and more than 70% inhibition of viability of pre-formed E. coli biofilms. Thus, the lead derivative A2 displayed a wider spectrum of anti-biofilm actions against both Gram-positive and Gram-negative strains.

Discussion

Rising numbers of resistant pathogenic bacterial strains, the seriousness of biofilm infections, and the lack of favorable treatment options drives the interest in discovering new antibacterial compounds with potent anti-biofilm effects (Lynch & Abbanat 2010). Even though agents with anti-biofilm properties are discovered and new therapy strategies have evolved over recent years, diagnosis and treatment of biofilm-associated infections still remain serious medical challenges (Batoni et al. 2011; Kiedrowski & Horswill 2011; Hall-Stoodley et al. 2012).

Motivated by the high antimicrobial activity and optimized drug properties of small β2,2-amino acid derivatives, the current study was conducted to clarify whether these derivatives could have potential as anti-biofilm agents. The β2,2-amino acid derivatives A1–A3 were selected based on previous studies where two para-trifluoromethyl benzyl (A1), 2-naphthyl-methylene (A2) and para-tertbutyl benzyl (A3) side-chains were demonstrated to be the most favorable bulky groups for assuring high antimicrobial activity (Hansen et al. 2011). As a successful structural motif for the C-terminal cationic group, the 1,2-diaminoethane group of A4 contributed to increased potency and selectivity. In the present study ester bond containing derivatives A4–A6 with a C-terminal N,N-dimethylaminoethanol group were also included. Their high potency against malignant cells and especially the ease of synthesis has made them attractive templates for further optimization of β2,2-amino acid derivatives (Hansen et al. 2012).

The IC50 values against planktonic S. aureus for five of the six β2,2-amino acid derivatives were shown to be between 5.5 and 12.3 µM (Table 2). The derivatives displayed also a 3- to nearly 50-fold preference for S. aureus as opposed to human RBC, and A1, A2 and A5 were shown to be the most selective derivatives. Overall, the potencies were in the same range as reported for short antimicrobial peptidomimetics or longer cathelicidin-derived peptides (Haug et al. 2004; Pompilio et al. 2011). The amide derivatives A1–A3 and the ester derivatives A4–A6 were comparatively active with the exception of A4. Even though A4 showed similar structural features as the other derivatives, it was surprisingly inactive. This indicated that C-terminal modifications had a considerable impact when using para-trifluoromethyl benzyl side-chains compared to the other two side-chain structures as lipophilic groups in β2,2-amino acid derivatives.
Biofilms can, as consequence of their organization, be regarded as multicellular organisms and are not comparable with their planktonic counterpart due to their structural features and functional differences (Shapiro 1998; Davey & O’Toole 2000). The susceptibility of biofilm bacteria to antimicrobial agents compared to planktonic bacteria therefore varies tremendously (Batoni et al. 2011). Currently, an important factor in tackling biofilms is the prevention of cell attachment and their formation in first line (Glinel et al. 2012). To intervene in biofilm formation, the prevention of wound infections or the use of implants with engineered surfaces that limit biofilm formation, are so far clearly preferred to any treatment strategy since the eradication of biofilms needs high concentrations of antibiotics which may promote multiple drug resistance (Bruellhoff et al. 2010). Anti-biofilm molecules that are effective at low concentrations, even in pre-formed biofilms, would therefore be a suitable alternative.

Derivative A4 demonstrated the lowest potency of all six derivatives and A1 showed higher IC$_{50}$ values for S. aureus biofilm prevention compared to planktonic S. aureus cells (Table 2). The other four compounds (A2, A3, A5 and A6) displayed high activity regarding the prevention of biofilm development since the IC$_{50}$ values were in the same range as for the planktonic assay. These findings suggest that these compounds rapidly reduce the viability of planktonic bacteria before the actual biofilm formation process can start.

However, in clinical settings biofilm prevention may not always be feasible and compounds able to eradicate existing biofilm infections are highly desirable counter measures. Currently only a few AMPs and mimetics with activity against preformed biofilms have been reported, such as the lantibiotic gallidermin, the β-peptoid-peptide hybrid oligomers, and the cationic steroid antibiotics (CSA)-13 (Saising et al. 2012; Liu et al. 2013; Nagant et al. 2013). In this report, the activity of β$_{2,2}$-amino acid derivatives against 18 h preformed S. aureus biofilms was therefore also investigated (Table 2).

It has been shown that biofilm resistance to AMPs is partly attributed to EPS constituents such as poly-N-acetylg glucosamine (PNGAG) and eDNA, which are both abundant in biofilms of various S. aureus strains (Izano et al. 2008; Wu & Xi 2009; Skogman et al. 2012). Biofilms are able to prevent AMPs from reaching their target due to the cationic character of PNGAG that repels the positively charged AMPs (Peschel et al. 2001; Otto 2006). Furthermore, the negatively charged phosphate groups of the DNA backbone has been hypothesized to electrostatically attract cationic AMPs and thus eDNA may compromise antimicrobial activity by complexation (Lewenza 2013). In spite of this, no substantial decrease in the antibiotic activity of the β$_{2,2}$-amino acid derivatives was observed here. In fact, IC$_{50}$ values below 27 μM for A1–A3 and below 39 μM for A5–A6 were obtained when tested against preformed biofilms (Table 2). Of note, recent findings suggest that interaction of small antimicrobial peptidomimetics with eDNA possibly cause a converse effect leading to dispersion of Pseudomonas aeruginosa biofilms (Kapoor et al. 2011). Whether this mechanism also occurs for the anti-biofilm activity of β$_{2,2}$-amino acid derivatives, is a matter for future investigations.

A previously demonstrated positive impact on the membrane interaction of β$_{2,2}$-amino acid derivatives due to higher rotational freedom of the C-terminal ester cationic groups (Hansen et al. 2012) was not supported by the results obtained here. The more rigid amide derivatives seemed better suited to interact with S. aureus biofilms, as shown by lower IC$_{50}$ values, and this might be a good approach for developing novel anti-biofilm compounds. Of note, A1 could reconstitute its activity with respect to destruction of preformed biofilms and was among the most potent derivatives with IC$_{50}$ values of the same magnitude as the most active derivative A3. This also illustrated that the lipophilicity of the derivatives was not the sole parameter regarding their antimicrobial potency, which was in agreement with observations made in earlier studies (Hansen et al. 2011).

Considering the small size of the compounds it is surprising that they display similar anti-biofilm activities in comparison to much larger peptides (2000–3000 Da) (Dean et al. 2011; Pompilio et al. 2011, 2012; Saising et al. 2012) or the 13 amino acid peptide PTP-7 (Kharidia & Liang 2011). Already small antibiofilm peptidomimetics have been reported (Flemming et al. 2009), but here it has been shown that anti-staphylococcal biofilm activity can be achieved by even smaller AMP derived compounds (Table 3).

Derivative A2 was selected for more detailed studies of anti-biofilm activity, since it displayed the best activity-to-cytotoxicity ratio. Different classes of commonly used antibiotics are known to be ineffective against biofilms even at very high concentrations, and can therefore rather promote bacterial/biofilm resistance than bring an enduring removal of microbial burden (Proser et al. 1987; Rachid et al. 2000; Tote et al. 2009). The log $R$ results showed that A2 (25 μM) was 16-fold more potent than penicillin G, which was 50- to 100-fold more potent against planktonic S. aureus (Figure S3). Furthermore, increased concentrations of A2 caused almost complete removal of bacterial cells. In fact, the achieved log $R$ of around 6 implied that only a few hundred bacteria remained in the plate wells from originally over a hundred million S. aureus cells. With respect to otherwise immunotolerant biofilm infections a substantial reduction in the biofilm burden is expected to assist the immune system in effectively tackling and clearing the remaining pathogens in vivo (Jensen et al. 2010). In addition, the
calculated biocompatibility index (BI) for A2 (using the concentration of the peptide that caused a 4 log reduction in pre-existing biofilms) was higher than 1 (BI = 4.57), and the value would be even higher if only a 3 log reduction was taken into account. Thus, in agreement with Müller and Kramer (2008), this suggests that the lead derivative A2 may be considered as a seemingly safe antimicrobial agent and a candidate for future in vivo animal studies.

AFM was applied in accordance with the log R assay setup to investigate topographical alterations in both A2 treated and untreated S. aureus biofilms. The acquired images were in good agreement with the log R assay and A2 was able to efficiently reduce S. aureus biofilms from polystyrene surfaces, especially at concentrations higher than 50 μM (Figure 3). Also morphological changes were registered in biofilms treated with 50 μM A2 which differed structurally from the untreated and the penicillin G-treated controls. The observed flattened structures indicated a mechanism of action leading to decreased membrane stability and lower mechanical stiffness of the bacteria. Earlier TEM studies conducted on planktonic S. aureus treated with A2 showed cell-disintegrating effects, which strengthens this hypothesis (Hansen et al. 2011).

To further investigate A2 biofilm interactions, SEM and TEM studies using high-pressure cryofixation of treated and untreated S. aureus biofilms were carried out. Webster et al. (2004) have shown that rapid freezing and freeze substitution are superior to conventional fixation methods and leave biofilms in very well preserved states. The treatment of biofilm specimens with organic solvents during conventional sample processing can result in undesired extraction of matrix components or even lead to destruction of ultrastructures. Here, by utilizing improved fixation techniques, biofilms were preserved most adequately in order to follow the impact of A2 on an ultrastructural level. Concentrations of 100 and 200 μM were chosen to obtain images with a frequent occurrence of effects on S. aureus biofilm. The results revealed that A2 was able to reduce the biofilm burden noticeably as indicated by the absence of bacteria and matrix (Figure 4, IIIa–c). The antibiotic penicillin G did reduce the biofilm to some extent, however, the drug seemed to stimulate EPS production by the S. aureus biofilm (Figure 4, Ila and b) that most likely displayed a counter measure as has been previously reported (Skogman et al. 2012).

The question arose if A2 caused the substantial decrease in EPS due to an effect on the biofilm matrix, whether it was a bactericidal effect, or a combination of both. Lysed bacteria in the top and the basal layers of the biofilm (Figure 4, IIIId) indicated that A2 must have had access to individual bacteria within the biofilm structure. Biofilms are known to be an assembly of specialized cells, displaying highly organized structures and exhibiting water channel assemblies to guarantee nutrient supply by variations in bacterial density throughout the matrix (Davey & O’Toole 2000). Pores or channels within the biofilm could have functioned as entrance ports for the lead derivative A2 facilitated by its small size. Derivative A2 seemed therefore capable of diffusing deep into the biofilm and to exert its antimicrobial properties on otherwise covered biofilm bacteria. Protective mechanisms, due to the presence of PNAG and eDNA that have been postulated to inhibit other AMPs, were apparently insufficient to hamper the anti-biofilm activity of A2.

During the SEM and TEM investigations flat and circular shaped bacteria were observed, which were also recognized during the AFM studies (Figure 3). As already mentioned above, a membrane destabilizing effect could be the cause of the collapse of the spherical shaped S. aureus cells. If the cell membrane in fact was the main target could not be revealed, yet. Studies are currently being conducted to clarify the antimicrobial mechanism of action of A2 and other β2,2-amino acid derivatives.

A peculiar feature of some AMPs is the loss of efficacy in environments with elevated levels of salt. Formation of amphipathic structures and structural stability are important for antibiotic effects of AMPs but can be altered in body fluids such as for instance in lungs of cystic fibrosis patients (Bals et al. 1998; Park et al. 2004). The impact of increased levels of sodium chloride, the most abundant salt in physiological conditions, was studied with respect to the anti-biofilm activity of A2. The aminoglycoside antibiotic gentamicin, which is known for its sensitivity to environments with higher concentrations of sodium chloride was used as control (Rubenis et al. 1963). Even in non-supplemented TSB, gentamicin at a concentration of 400 μM was not able to reduce the biofilm burden as effectively as A2, which was used at a more than 11-fold lower concentration. The biofilms grown in TSB with different sodium chloride concentrations were all susceptible to A2 treatment, demonstrating no salt sensitivity of this derivative. Of note, it has been shown earlier that naphthyl substituents can positively influence the salt sensitivity of AMPs and can be utilized to improve and maintain AMP activity in salt rich environments (Yu et al. 2011). Additionally, creating smaller peptidomimetics, as displayed by β2,2-amino acid derivatives, may be a promising strategy to aid overcoming the AMP salt sensitivity.

The lead derivative A2 was additionally tested in an extended panel of representative bacterial strains. Previously, evidence has been provided of antibacterial activity of A2 against suspended MRSA (ATCC 33591), MRSE (ATCC 27626), E. coli (ATCC 25922) and P. aeruginosa (ATCC 27853) (Hansen et al. 2011). In the present study, it was additionally shown that A2 prevents biofilm formation by S. aureus strains (ATCC
25923 and Newman) with similar potencies, and that A2 displays an even higher anti-biofilm effect against E. coli (XL1 Blue). With respect to the lack of anti-biofilm effects against S. epidermidis, differences between the strains used in the two studies offer a plausible explanation. Nevertheless, the results obtained in the present study demonstrate that this antimicrobial β2,2- amino acid derivative displays a wider spectrum of anti-biofilm actions, and that the effects of A2 are not only detectable in the model bacterium S. aureus (ATCC 25923).

In view of physiological conditions and biofilm defense mechanisms new anti-biofilm compounds must possess an improved activity profile compared to conventional antimicrobial drugs. Additionally, these compounds should preferably not lead to the development of resistance. AMPs are in this context promising molecules to meet the demands of anti-biofilm therapeutics. So far, no antibiotic and only one disinfectant (commercially sold by Sterilex Corporation, USA) has been approved by a regulatory agency to be specifically used against bacterial biofilms. Despite the increased efforts in AMP research during the last two decades, reports of AMPs with anti-biofilm properties are relatively few (Jorge et al. 2012). Furthermore, just a handful of antimicrobial peptides are currently used in clinical settings because of challenges regarding synthesis, production costs, stability, and/or toxicity (Gordon et al. 2005; Straus & Hancock 2006; Baltzer & Brown 2011). It has been demonstrated that it is possible to overcome these hurdles by developing potent and small antimicrobial β2,2-amino acid derivatives (Hansen et al. 2011). In the present study it was shown for the first time that this novel AMP derived compound class is also active against preformed S. aureus biofilms. The β2,2-amino acid derivatives represent a new promising class of compounds to tackle the complexity of biofilm infections by combining drug-like properties with high activity against S. aureus biofilms.

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