Journal Pre-proof

α,α-disubstituted β-amino amides eliminate *Staphylococcus aureus* biofilms by membrane disruption and biomass removal

Dominik Ausbacher, Lindsey A. Miller, Darla M. Goeres, Philip S. Stewart, Morten B. Strøm, Adyary Fallarero

PII: S2590-2075(23)00048-5

DOI: https://doi.org/10.1016/j.bioflm.2023.100151

Reference: BIOFLM 100151

To appear in: *Biofilm*

Received Date: 23 April 2023

Revised Date: 12 August 2023

Accepted Date: 24 August 2023

Please cite this article as: Ausbacher D, Miller LA, Goeres DM, Stewart PS, Strøm MB, Fallarero A, α,α-disubstituted β-amino amides eliminate *Staphylococcus aureus* biofilms by membrane disruption and biomass removal, *Biofilm* (2023), doi: https://doi.org/10.1016/j.bioflm.2023.100151.

CRediT authorship contribution statement

Dominik Ausbacher: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Initial draft, review & editing, Funding acquisition, Project administration.

Lindsey A. Miller: Methodology, Investigation, Writing – review & editing.

Darla M. Goeres: Conceptualization, Methodology, Resources, Supervision, Validation, Writing review & editing.

Philip S. Stewart: Conceptualization, Methodology, Resources, Supervision, Writing – review & editing

Morten B. Strøm: Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition, Project administration

Adyary Fallarero: Conceptualization, Methodology, Resources, Supervision, Validation, Writing review & editing

α,α-disubstituted β-amino amides eliminate *Staphylococcus aureus* biofilms by membrane disruption and biomass removal

Running title: **α,α-disubstituted β-amino amides eliminate *S. aureus* biofilms**

Dominik Ausbacher [a1*], Lindsey A. Miller [b], Darla M. Goeres [b], Philip S. Stewart [b], Morten B. Strøm [a], Adyary Fallarero [c]

[a] Natural Products and Medicinal Chemistry Research Group, Department of Pharmacy, UiT - The Arcitc University of Norway, N-9037 Tromsø, Norway

[b] Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717, USA

[c] Drug Research Program, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, FI-00014 Helsinki, Finland

*Corresponding author. Dominik Ausbacher, Natural Products and Medicinal Chemistry Research Group, Department of Pharmacy, UiT - The Arcitc University of Norway, N-9037 Tromsø, Norway. *E-mail address:* Dominik.Ausbacher@uit.no

[1]Present address. Hospital Pharmacy of North Norway Trust, 9038 Tromsø, Norway. *E-mail address:* Dominik.Albert.Ausbacher@sykehusapotek-nord.no

Abstract

Bacterial biofilms account for up to 80% of all infections and complicate successful therapies due to their intrinsic tolerance to antibiotics. Biofilms also cause serious problems in the industrial sectors, for instance due to the deterioration of metals or microbial contamination of products. Efforts are put in finding novel strategies in both avoiding and fighting biofilms. Biofilm control is achieved by killing and/or removing biofilm or preventing transition to the biofilm lifestyle. Previous research reported on the anti-biofilm potency of α,α-disubstituted β-amino amides **A1**, **A2** and **A3**, which are small antimicrobial peptidomimetics with a molecular weight below 500 Dalton. In the current study it was investigated if these derivatives cause a fast disintegration of biofilm bacteria and removal of *Staphylococcus aureus* biofilms. One hour incubation of biofilms with all three derivatives resulted in reduced metabolic activity and membrane permeabilization in *S. aureus* (ATCC 25923) biofilms. Bactericidal properties of these derivatives were attributed to a direct effect on membranes of biofilm bacteria. The green fluorescence protein expressing *Staphylococcus aureus* strain AH2547 was cultivated in a CDC biofilm reactor and utilized for disinfectant efficacy testing of **A3**, following the single-tube method (American Society for Testing and Materials designation number E2871). **A3** at a concentration of 90 µM acted as fast as 100 µM chlorhexidine and was equally effective. Confocal laser scanning microscopy studies showed that chlorhexidine treatment lead to fluorescence fading indicating membrane permeabilization but did not cause biomass removal. In contrast, **A3** treatment caused a simultaneous biofilm fluorescence loss and biomass removal. These dual anti-biofilm properties make α,α-disubstituted β-amino amides promising scaffolds in finding new control strategies against recalcitrant biofilms.

Introduction

The lack of innovative antibiotics poses a serious threat to human health [1]. Antimicrobial resistance leads to more than 35 000 deaths in the EU each year, according to estimates presented in a recently released report [2]. Not just resistant bacteria, but also bacterial biofilms with an intrinsic tolerance to antibiotics complicate successful treatment of infections [3]. On the other hand, industrial installations in sectors such as oil and gas, water supply and food processing suffer of biocorrosion and contamination due to biofilms. Countermeasures are costly and require expenses of many hundred billions of dollars [4]. Biofilms develop when planktonic bacteria form agglomerates that can adhere to a surface [5]. In patients, catheters, artificial heart valves or prosthetic joints are prone to bacterial attachment and biofilm formation [6]. In addition, biofilms can also be the cause of faulty wound healing and wound chronicity [7]. Biofouling on shipping vessels or leaks in oil and gas pipelines due to microbially influenced corrosion cause not just undesired investments but have a heavy environmental impact, too [4]. Besides bacteria, these agglomerates consist of an extracellular biofilm matrix, which contributes to the formation of three-dimensional biofilm structures and microenvironments [8]. Specialized bacteria exist in a biofilm, such as slow metabolizing persisters, or bacteria with high mutation rates, that promote environmental adaption of biofilms and high tolerance against antimicrobial treatments [8,9]. These features make biofilms a challenging target for treatment, especially in health care settings where harsh physical and chemical treatments are not an option. New approaches, including effective anti-biofilm compounds, are needed in order to develop new strategies against these recalcitrant pathogen formations [10]. Biofilms can be controlled by killing and/or removing the biofilm or preventing transition to the biofilm lifestyle [11,12] High-throughput methods are commonly used for screening of libraries consisting of natural products or synthetic molecules in order to identify compounds with anti-biofilm properties [13,14]. Besides

identifying potential hits and development of lead compounds, substantial efforts have been invested in determining the mode-of-action of new compounds [15,16]. These studies are crucial for improvements regarding potency and toxicity, drug formulation, and administration, but also regarding utilizing synergism and avoiding antagonism in combination treatments [17,18]. In the medical community, anti-biofilm compounds approved by the regulatory agencies do not exist and the only chance to fight microbial biofilms is by using antibiotics or surgical removal of biofilm infected tissues or replacement of medical devices. One of most recent and innovative antibiotics introduced to the market is the antimicrobial depsipeptide daptomycin. This drug was approved by the Food and Drug Administration back in 2003 but innovative antimicrobial drugs or compound classes with a clearly novel mechanism of action are needed now and will be needed in the future [19]. A promising compound is the natural product teixobactin. This antimicrobial peptide is in late-stage preclinical development and potent against multi-resistant gram-positive bacteria [20,21]. Both, daptomycin and teixobactin have rather complex structures with potential attack points for degradation. Hydrolases in actinomycete WAC4713, for instance, have been reported to confer resistance by being able to hydrolyze the depsipeptide ester-bond in daptomycin [22]. Of note, the same structural element is present in teixobactin, however, no resistance development could be observed *in vitro*, yet [20,21]. Therefore, many groups investigate how to utilize the mode of action of natural products and/or potent antimicrobial peptides while equipping them with drug-like properties [23]. The group of Strøm has shown that it is possible to create novel and potent compounds by simplifying and condensing complex structures of natural products [24,25,26]. Hansen *et al.* for instance was able to transfer the antimicrobial motif of larger antimicrobial peptides to small molecules with enzymatic stability [27]. Furthermore, the group developed so called α,α-disubstituted β-amino amides which are peptidomimetics showing potency in the same range as larger

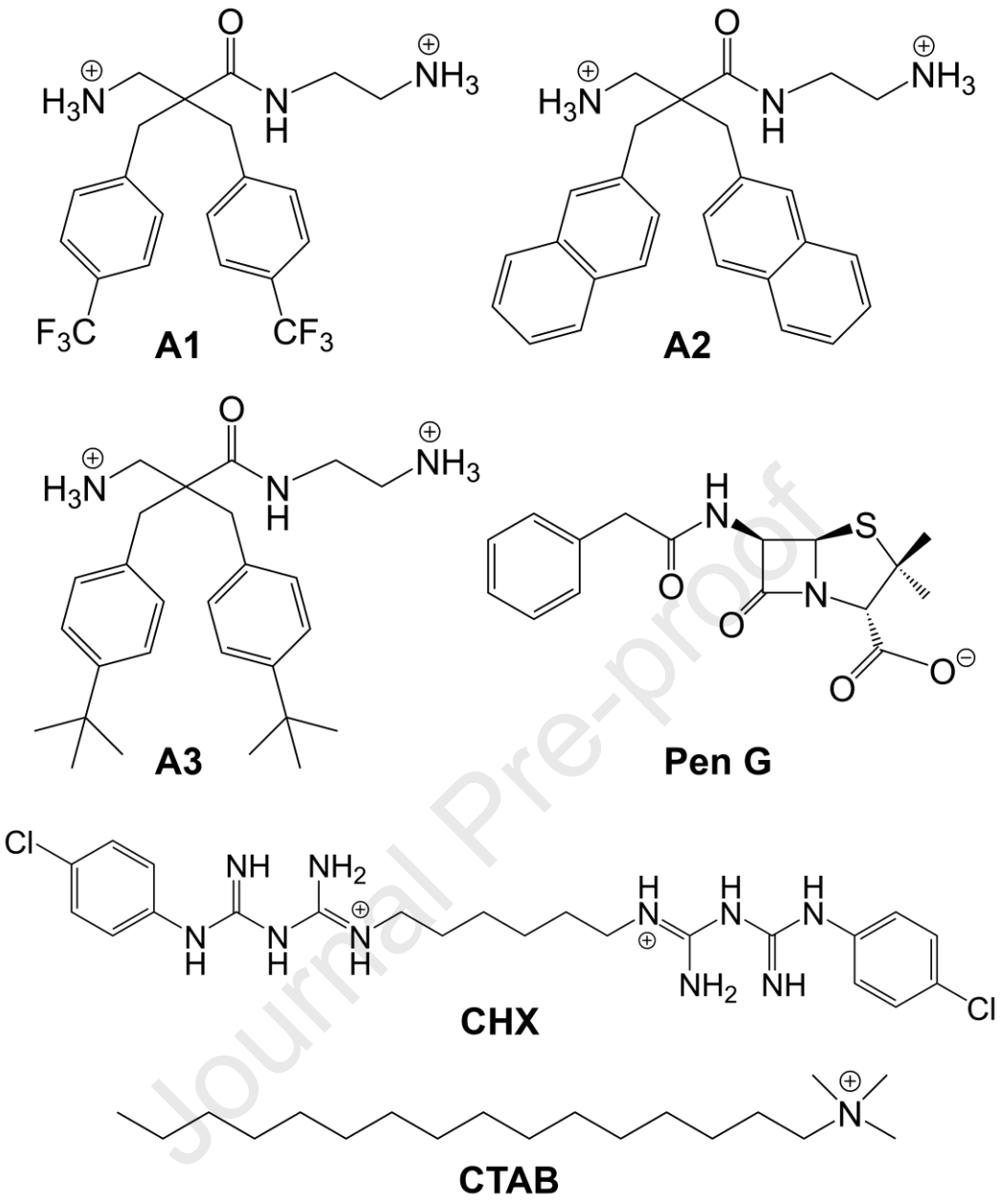
antimicrobial and anticancer peptides [28,29] . These derivatives are easily synthesized, have a preference for gram-positive bacteria, including antibiotic resistant strains like MRSA and MRSE and have drug-like properties [28,30]. The activity spectrum also includes *Staphylococcus aureus* (*S. aureus*) biofilms, and both *in vitro* and microscopy studies have suggested that these derivatives possess microbicidal and biofilm removal properties [31]. In the present study, we investigated if these derivatives act on the bacterial membrane in *S. aureus* biofilms. This would suggest membrane related effects, like for instance permeabilization, would occur relatively fast. Quantitative and qualitative assays on biofilms formed in 96-well plates were conducted similar to a previously used screening setup, however using much shorter incubation times [31]. The control compounds chlorhexidine (CHX) and cetylpyridnium bromide (CTAB) have been included for comparison since both compounds have similarities with the α,α-disubstituted β-amino amides like cationic charge and an amphipathic structure. In addition, CHX and CTAB have a membrane perturbing mechanism of action [32] . Both, CHX and **A3,** were further challenged with *S. aureus* biofilms that were cultivated in a CDC biofilm reactor. Disinfectant efficacy testing by using an adaption of American Society for Testing and Materials (ASTM) standard method E2871 [33] guided the confocal laser scanning microscopy studies using a treatment-flow-cell. A reporter system consisting of green fluorescent protein (GFP) expressing biofilm bacteria together with calcein red-orange fluorescence staining was used in order to determine if and to which extent bacterial membranes, and the biofilm as a whole, were affected during treatment with **A3**.

This knowledge will help to tune derivative potency in future and to further explore derivative properties in suitable formulations and applications for tackling biofilm challenges in health care and/or industrial settings.

99 # Material and methods

100 ## The α,α-disubstituted β-amino amides A1 - A3 and chemicals

101 The α,α-disubstituted β-amino amides (**A1** - **A3,** FIG. 1) were synthesized according to a
102 previously published procedure [28]. The derivatives were isolated as di-trifluoroacetate salts
103 and purity above 95% was determined with an analytical RP-HPLC C_{18}-column and UV
104 detection at 214 and 254 nm. Prior to the experiments stock solutions of all derivatives were
105 prepared in DMSO. DMSO concentrations did not exceed 2% and were well tolerated by *S.*
106 *aureus* biofilms as reported earlier [34]. Chlorhexidine dichloride (CHX),
107 cetyltrimethylammonium bromide (CTAB) and penicillin G sodium (Pen G) (all Sigma
108 Aldrich, Schnelldorf, Germany) were used as treatment controls (FIG 1). Autoclaved Milli-Q
109 water was used to prepare stock solutions of CTAB and Pen G whereas DMSO was used for
110 CHX.



111

112 **FIG 1. Compounds with antimicrobial properties used for mechanistic**
113 **investigations on *S. aureus* biofilms**. Representation of amphipathic α,α-disubstituted
114 β-amino amides lead structures **A1** - **A3** with anti-biofilm properties and the disinfectants
115 chlorhexidine (CHX) and cetylpyridnium bromide (CTAB) in cationic state. Penicillin G was
116 used as control antibiotic represented as anion.

117

Bacterial strains and growth condition

The reference strain *S. aureus* ATCC25923 and the green fluorescent protein (GFP) expressing *S. aureus* strain AH2547, kindly provided by Dr. Alex Horswill, were used for the experiments. AH2547 contains the plasmid pCM29 [35]. For AH2547 tryptic soy broth (TSB) and tryptic soy agar (TSA) were supplemented with 10 µg/ml chloramphenicol for plasmid retention.

All strains were stored as glycerol stocks at -70°C and TSA streak plates were prepared prior to the experiments. Three colonies were picked, inoculated in TSB and incubated at 37°C and 200 rpm, overnight. For well-plate based assays the liquid cultures were prepared by diluting the overnight cultures 1000 times in fresh TSB. The inoculum was incubated under aerobic conditions at 37°C, 250 rpm until late exponential growth was reached (4 h), corresponding to a bacterial concentration of 10^8 CFU/ml. Biofilms were formed by transferring 200 µl of exponentially grown suspensions (10^6 CFU/ml) to 96-well plates (Nunclon^{TM} Δ surface; Thermo Fisher Scientific, Vantaa, Finland). Subsequently, the cultures were incubated for 18 h under equivalent conditions as stated above and described earlier [34].

For experiments involving the CDC biofilm reactor, biofilms were formed on glass coupons according to ASTM Method E2562 [36] and Buckingham-Meyer *et al* [37]. A CDC reactor containing 500 ml full strength TSB and chloramphenicol (10 µg/ml) was inoculated with 1 ml of a 10^9 CFU/ml overnight GFP expressing *S. aureus*. The biofilms were grown in batch conditions at 37°C, 125 rpm for 24 h. Subsequently, continuous flow with one-tenth TSB was applied for another 24 h at 37°C and 125 rpm until start of sampling.

Assessment of metabolic activity and biomass after 1 h treatment of *S. aureus* ATCC 25329 biofilms.

The impact of α,α-disubstituted β-amino amides on biofilms of *S. aureus* ATCC 25923 formed in 96-well plates was investigated by two different staining methods as described earlier [31]. To investigate the immediate response of biofilms after exposure to the derivatives, biofilms were formed for 18 h, as described above. The planktonic phase was replaced with fresh TSB or compound containing TSB. Based on previous studies, derivatives at concentrations of $2xIC_{50}$ (50 µM for **A1** and **A2**; 45 µM for **A3**) and 50 µM of CHX and CTAB as well as 400 µM of Pen G were added. Untreated controls were treated with an equivalent of DMSO in TSB and the plates were incubated for 1 h. At the end of the exposure periods, the planktonic phase was carefully replaced with 20 µM resazurin in PBS (Lonza Walkersville Inc., Walkersville, USA) and the 96-well plate was incubated (200 rpm, RT, 40 min, darkness). Fluorescence was measured (λex 570 nm, λem 590 nm) with a Varioskan Multimode reader (Thermo Fisher Scientific, Vantaa, Finland). Metabolic activity was determined as percentage of untreated control. Subsequently, the supernatant was gently removed and 170 µl of the crystal violet solution was added and incubated for 5 min. The dye was removed and wells washed twice with deionized water. The dye was dissolved in 96% ethanol (200 µl/well). After 1 h the photometric absorbance (λ 590 nm) was measured using a Varioskan Multimode reader. Susceptibility of the *S. aureus* strain AH2547 to our derivatives was determined by using a similar 96-well setup. GFP production of the strain was exploited and loss of GFP fluorescence was recorded after exposure to biofilm treatments, further described in the supplementary information to this article.

Qualitative and quantitative detection of membrane integrity by SYTO9 and propidium iodide staining

A membrane integrity assay and fluorescence microscopy studies (LIVE/DEAD BacLight Bacterial Viability Kit; EVOS FL imaging system, Thermo Fisher Scientific, Vantaa, Finland) were performed according to the manufacturer's instructions. Formed biofilms were exposed to treatments as described above for 1 h and staining was subsequently applied. Fluorescence of green SYTO9 and red propidium iodide (PI) was determined using a Varioskan Multimode reader and the EVOS FL imaging system was used for fluorescence microscopy.

Detection of intracellular ATP leakage from *S. aureus* ATCC 25329 biofilms

The ATP leakage assay was adapted from Manner *et al.* [38]. In brief, *S. aureus* ATCC 25923 biofilms were formed and treated as described above. Untreated biofilms and negative control wells were incubated with TSB. After 1h, the planktonic suspensions were removed and collected for each treatment. The suspensions were filtered using 0.22 µm syringe filters. Subsequently, 4x100 µL of each filtrate were transferred to a clear-bottom 96-well plate (Isoplate-TC; Perkin Elmer, Waltham, MA, US). The CellTiter-Glo® reagent (Promega, Madison, WI, US) was prepared according to the manufacturer instructions. Luciferin luminescence was measured using Varioskan Flash Multimode Plate Reader.

Disinfectant efficacy testing on CDC reactor cultivated *S. aureus* biofilms

A modification of ASTM method E2871 (single-tube-method) was used to gain a better knowledge of derivative efficacy. Log reduction in viable biofilm cells exposed to 90 µM (4 x IC_{50}) of **A3**, 100 µM CHX and 400 µM of penicillin G was measured for 1 h, 2 h and 3 h [33]. Briefly, coupons containing biofilms of *S. aureus* AH2547 were removed from the CDC

reactor, rinsed and then transferred to 50 ml conical tubes with tweezers. Subsequently, 4 ml of TSB solution or compound containing TSB solutions were carefully added to the tubes, and the tubes were incubated at 37°C under static conditions. At each specific time point, 36 ml D/E broth for compound neutralization purposes were added and biofilms were disaggregated by sonication and vortexing according to ASTM E2871. The diluted samples were drop plated on TSA plates, incubated overnight at 37°C and enumerated. D/E broth was validated to neutralize **A3** to concentrations up to 200 µM according to the procedure in the standard test method for evaluation of inactivators of antimicrobial agents (ASTM E1054) (data not shown).

Treatment-flow-cell assay with detection of membrane integrity and biomass removal by confocal microscopy

A dual dye leakage indicator system was established by using the FilmTracer™ Calcein red-orange biofilm stain and bacterial expression of GFP. Fading due to diffusion of the small calcein dye (Mw 789.55 Da) from the biofilm was intended to indicate small pore formation which would to a lower extent affect the leakage of the intracellular, large GFP (238 amino acids, 27 kDa). Fading of both calcein-red-orange and GFP, would thus indicate formation of large pores or membrane collapse. Biofilms were stained with the FilmTracer™ Calcein red-orange dye according to the manufacturer's instructions. After rinsing the coupons for removing unbound stain, the coupons were transferred to the treatment-flow-cell (model FC310; Biosurface Technologies, Bozeman, Mt, USA) and video microscopy experiments were conducted as described previously [39]. In brief, untreated controls were treated with full strength TSB supplemented with DMSO as vehicle control. Images were acquired of the

bright field, GFP, and RFP channel using a Leica SP5 confocal laser-scanning microscope. The z-stack step size was set to 10 µm.

Software and statistical analysis

SigmaPlot 14.5, (Systat Inc., Chicago, IL, USA) was used for plotting of graphs and statistical analysis (Student's t-test). Included asterisks in figures indicate significant differences with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. A p-value of < 0.05 was considered statistically significant.

Overlay images of treatment-flow-cell results were created with Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA) whereas movie generation was carried out with IMARIS® (Bitplane AG, Zurich, Switzerland).

Results

A1, A2 and A3 affect *S. aureus* biofilm viability within 1 hour

The initial experiments investigated the immediate impact of different treatments on *S. aureus* (ATCC 25329) biofilms. The α,α-disubstituted β-amino amides **A1** - **A3** reduced biofilm viability to approximately 50% after 1 h of treatment at concentration of 45 to 50 µM (FIG. 2 A). We observed comparable effects after treatment with 50 µM of the disinfectant CHX. In contrast, treatment with 50 µM of the quaternary ammonium compound CTAB or 400 µM of Pen G did not reduce biofilm viability even though these compounds inhibited planktonic *S. aureus* ATCC25329 at 2.5 µM and 0.12 µM (TAB S1). Substantial biofilm removal was not observed for any of the treatments, however, derivative **A3** yielded approximately 30% removal. CHX seemed to act similarly to the α,α-disubstituted β-amino amides and appeared

as a suitable control for further studies. The presence of human serum albumin did not significantly lower the potency of the derivatives **A1 - A3** or CHX (FIG S1). However, CTAB and Pen G suffered loss of activity under the presence of the plasma protein (FIG S1).

A1, A2 and A3 cause membrane permeabilization in *S. aureus* biofilms

The SYTO9/propidium iodide assay is useful for assessment of bacterial cell membrane integrity. Intact bacteria are stained green with the membrane permeable dye SYTO9. Bacteria with compromised membranes are additionally susceptible to staining with the red fluorescent dye PI, which is otherwise excluded from viable cells due to its positive charge. Derivatives **A1 - A3** as well as CHX showed increased PI staining, i.e., absence of any barrier function of the bacterial membrane within 1 h of treatment with 45 – 50 µM, compared to 50 µM CTAB, 400 µM Pen G or untreated controls (FIG 2B).

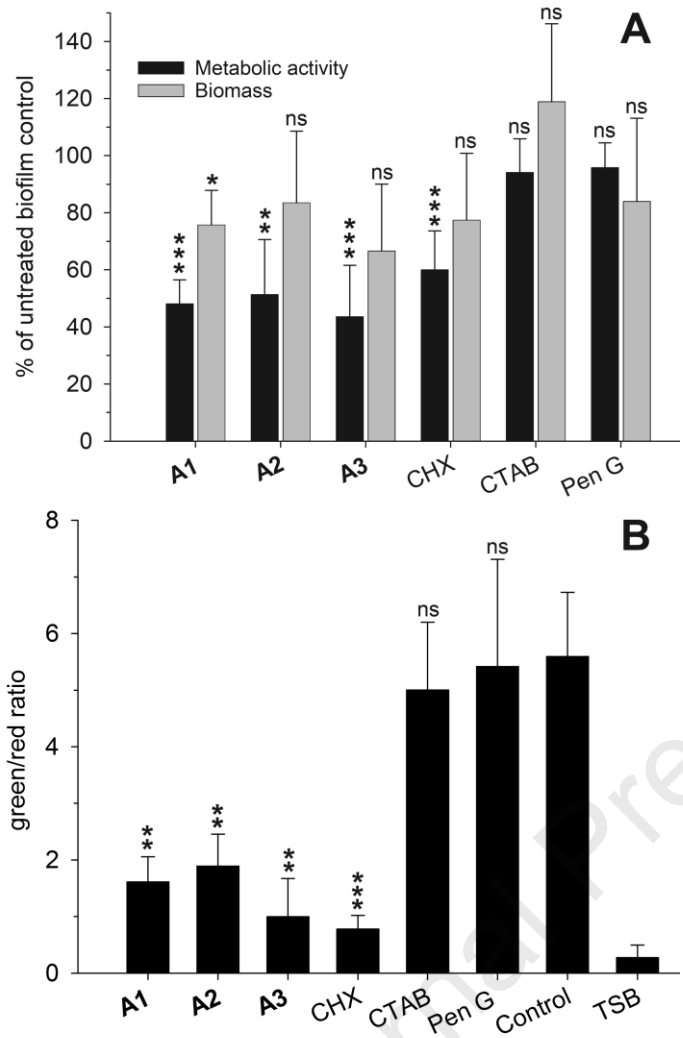


FIG 2. **Metabolic activity, biomass and membrane integrity assays with *S. aureus* ATCC 25329.** (A) Resazurin based metabolic activity assessment and crystal violet based biomass staining. (B) Detection of the green/red ratio in TSB containing wells, untreated control biofilms and treated biofilms bacteria for assessment of membrane integrity assessment based on the green SYTO9 and red PI dye. (Results shown under (A) and (B) display the mean with standard deviation of at least three independent experiments).

The SYTO9/propidium iodide staining is additionally suitable for fluorescence microscopy studies. Imaging of treated and subsequently stained biofilms (FIG 3) showed similar staining patterns as observed in the quantitative well-plate based fluorescence measurements (FIG 2B). The most pronounced PI staining was noted when the biofilm was treated with **A3** and

CHX, and to a slightly lower extent for the **A1** and **A2** treated biofilms. In contrast, only a few bacteria were PI positive in the CTAB and Pen G treated biofilms. Minimal PI staining was detected in the untreated control biofilms.

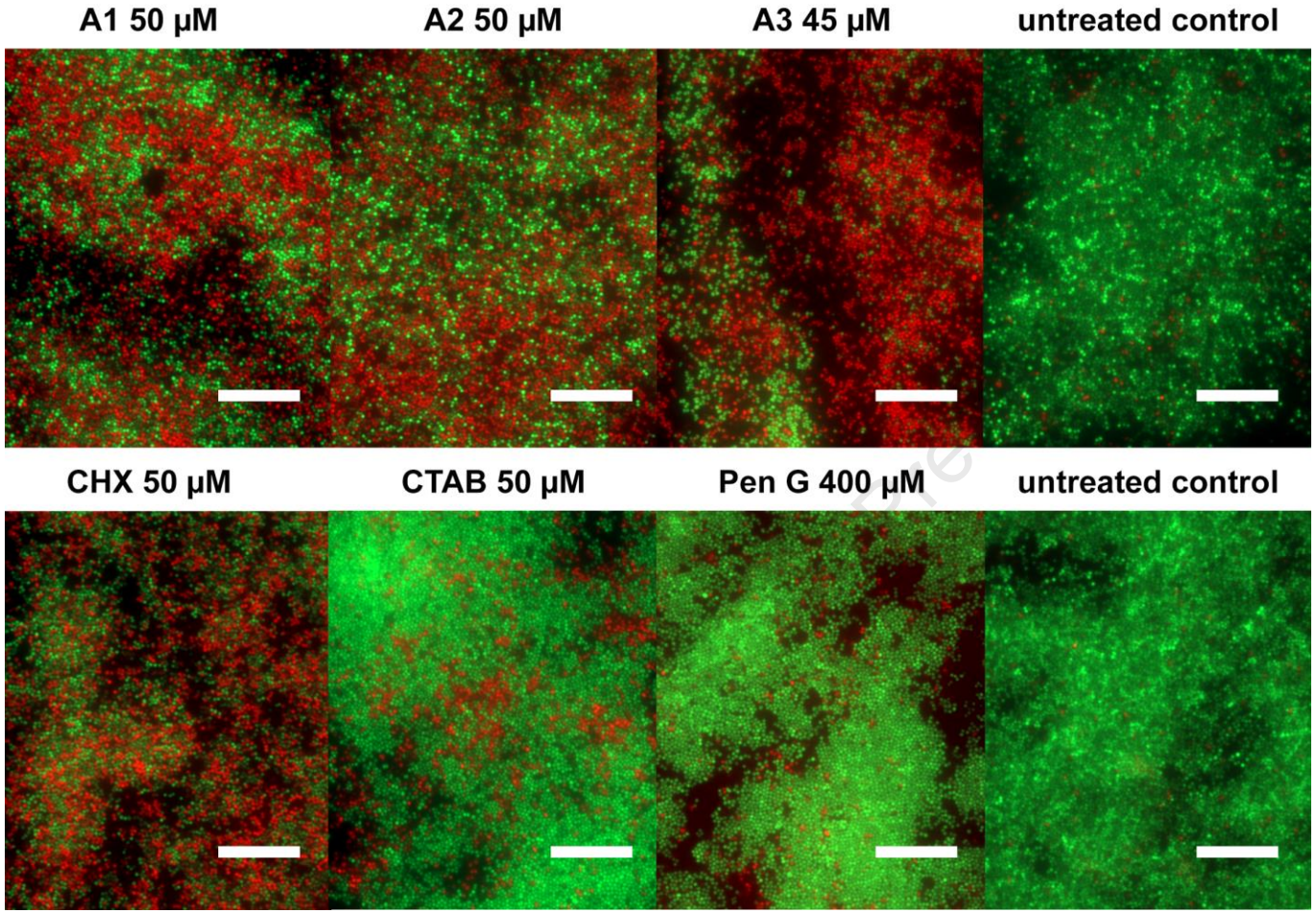


FIG 3. Fluorescence microscopy of *S. aureus* ATCC 25329. Biofilms were treated 1 h with derivatives and controls and stained with the green SYTO 9 and red PI dye staining kit. Green represents intact cells whereas red indicates bacteria with compromised membrane integrity. Scale bars represent 200 µm.

α,α-disubstituted β-amino amides cause ATP efflux from *S. aureus* biofilms

The ATP dependent luciferin-luciferase reaction was utilized to investigate membrane damage and ATP leakage upon incubation with the test compounds. The assay showed that α,α-disubstituted β-amino amides as well as CHX caused a pronounced release of ATP and supported our findings regarding disrupted cell integrity of derivative or CHX treated biofilm bacteria (FIG 4). The surface-active compound CTAB showed ATP release to a much lower extent compared to the derivatives and CHX. Released ATP after Pen G treatment was negligible and comparably low as observed for untreated controls.

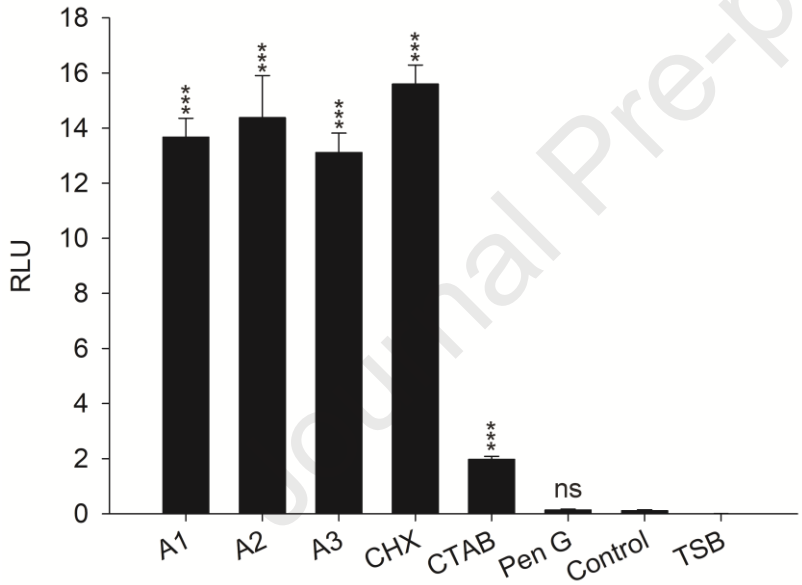


FIG 4. **ATP release assays with *S. aureus* ATCC 25329.** ATP release as measure of membrane integrity of biofilm bacteria by ATP - luciferin/luciferase-based luminescence assay. (Results display the mean with standard deviation of at least three independent experiments).

Log reduction on CDC reactor cultivated *S. aureus* AH2547 biofilms by A3, CHX and Pen G

Preliminary experiments in 96-well plates indicated that **A3** was the most potent of our derivatives against planktonic *S. aureus* AH2547 and able to prevent biofilm formation (FIG S2). Additionally, susceptibility of preformed *S. aureus* AH2547 biofilm was assessed in 96-well plates and showed it was necessary to increase the concentration of the derivatives to 4xIC_{50} to achieve a biofilm reduction of 40% or higher. A concentration of 90 µM of **A3** for 3 h reduced preformed biofilms to the same extent as was achieved after a 3 h of treatment with CHX. CHX with a concentration of 100 µM was used as control based on doubling the concentration of **A3**. These assay parameters were selected for the single-tube-method experiments. The single-tube-method is a validated standard test method well suited for determination of anti-biofilm efficacy testing [40]. Derivative **A3** at 90 µM and CHX at 100 µM killed biofilm cells at a rate of approximately 1 log unit per hour (FIG 5). After 3 h, both compounds showed a 3.5 log reduction (99.97% reduction of biofilm) of viable cell counts whereas Pen G showed a reduction of only 1 log unit.

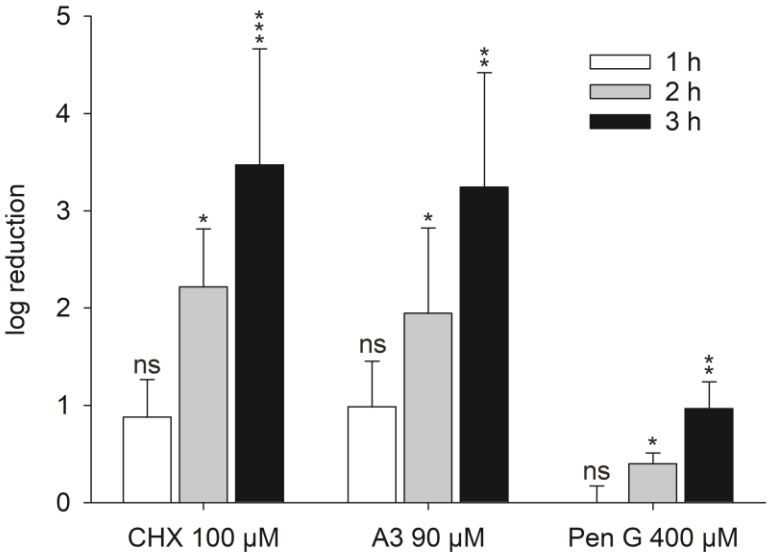


FIG 5. Determination of log-reduction in viable cells of *S. aureus* AH2547 biofilms by single tube method. Coupons were sampled from the CDC biofilm reactor and subsequently exposed to treatments for 1 h, 2 h and 3 h. After enumeration, the log (colony counts) of treated biofilms were subtracted from log (control counts) resulting in log reduction. At least two coupons for each treatment were used and a minimum of three independent experiments performed (results display the mean with standard deviation).

A3 causes membrane permeabilization and biomass removal of *S. aureus* biofilms under flow conditions

The effect of **A3** and CHX under flow conditions was assessed using the treatment-flow-cell. The treatment-flow-cell is a useful tool to investigate removal properties of biofilm treatments and is engineered to fit coupons sampled from a CDC biofilm reactor. The assessment of biofilm killing efficacy and removal events using coupons collected from a single CDC biofilm reactor experiment can be performed, as described previously [39]. No morphology changes were noted in the bright-filed or fluorescence images when the biofilm was treated

312 with full-strength TSB (FIG 6 and Fig S4). In contrast, treatment with 90 µM **A3** resulted in a
313 decrease of fluorescence intensity after 60 min and fluorescence loss was additionally
314 intensified by removal of biofilm over the remaining 120 min. The biofilm bacteria
315 membranes seemed to disintegrate after 60 min since both GFP and calcein red-orange faded
316 simultaneously before dispersal of the biofilm. CHX (100 µM) showed a similar effect on
317 AH2547 biofilms, however, fluorescence loss was less pronounced compared to the **A3**
318 treated biofilms. In addition, the biofilm appeared to develop a rougher topography in the
319 bright-filed images, and did not disperse as promoted by **A3**. Treatment with Pen G (400 µM)
320 started with erosion and resulted in complete dispersal of the biofilm indicated by removal of
321 visible structures in both the fluorescent and bright-field images.

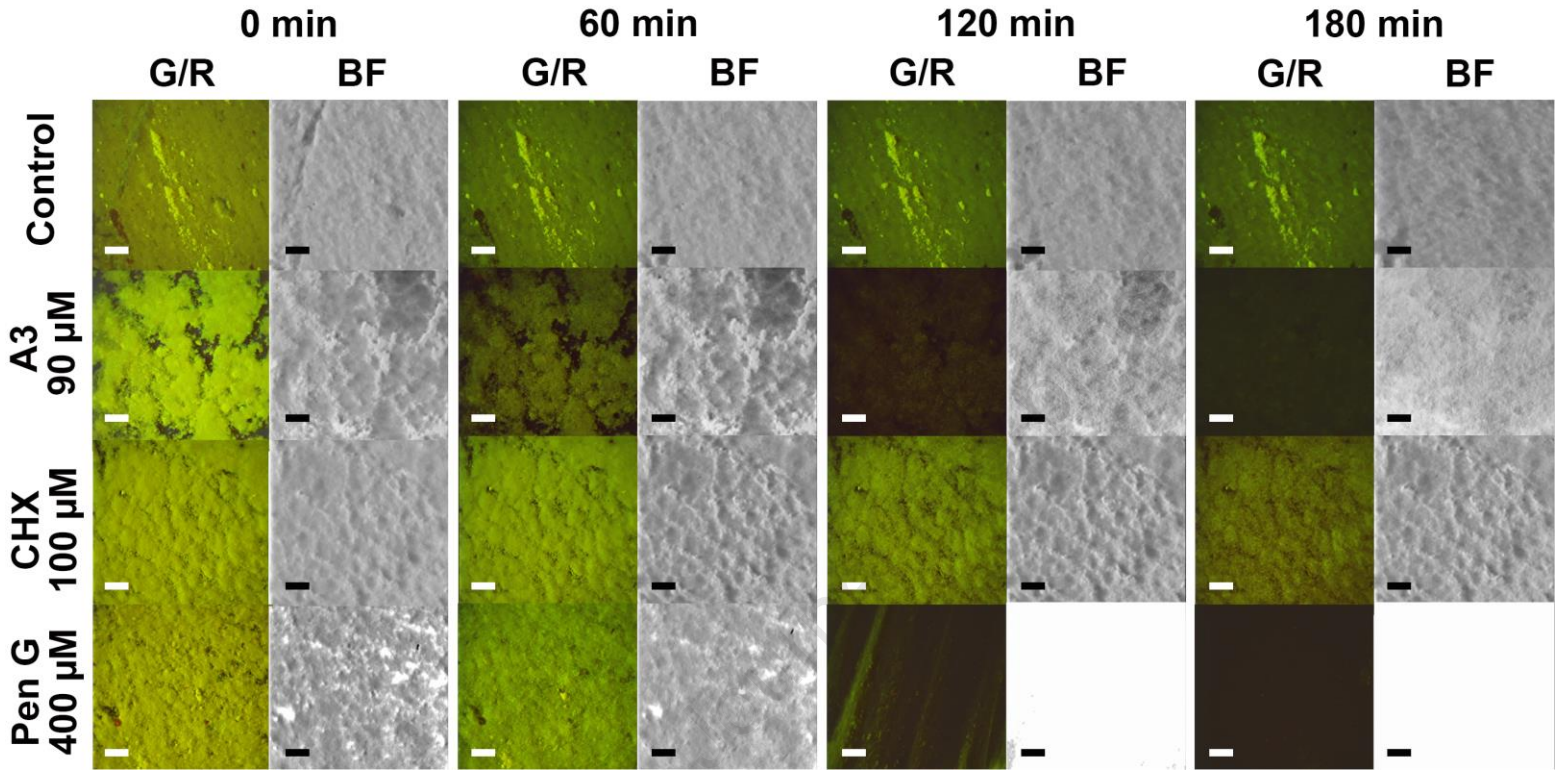


FIG 6. Treatment-flow-cell/CLSM video microscopy experiments of *S. aureus* AH2547 biofilms. GFP expressing and calcein red-orange stained biofilms as overlay images with green/red (G/R) fluorescence. Bright field images reveal the presence of biofilm (dark) independent of fluorescence and facilitate interpretation of the impact on membrane integrity and biofilm removal during a period of 3 h. Dark G/R images indicate loss of fluorescence and/or non-fluorescent biofilm structures whereas white bright field images indicate absence of biofilm. Scale bar represents 100 µm.

Discussion

The mode-of–action studies showed that α,α-disubstituted β-amino amides are capable of efficiently both killing and removing biofilms. Easily cultivable biofilms in 96-well plates and use of known assay parameters from our previous antibiofilm screening studies facilitated our approach [31]. The resazurin based metabolic activity assays showed that the most potent derivatives **A1** - **A3** were able to decrease biofilm viability at 45 – 50 µM within 1 h, similar to the cationic control disinfectant CHX (FIG 2A). Surprisingly, CTAB did not affect biofilm viability to the same extent as CHX even though we measured similar susceptibility of planktonic *S. aureus* (TAB S1). Similar to values reported by Manner *et al.*, Pen G at 400 µM was not active against the biofilms [38]. In microtiter plate assays, little biofilm removal was observed for any treatment, which suggested that biofilm removal was time dependent/flow dependent for these compounds or a downstream event after killing of biofilm bacteria. To our surprise, the presence of 300 µM human serum albumin (HSA), which is present in wound exudates [41], did not inhibit the potency of the derivatives because tetra- and hexapeptides which contained α,α-disubstituted β-amino amides as building blocks showed decreased activity against lymphoma cells, when co-incubated with HSA for 6 hours [42]. Binding of lipophilic groups to HSA was located on drug site II. Similar results were obtained for other small synthetic mimics of antimicrobial peptides when HSA (550 µM) was present during MIC assays with vagrious bacterial strains [43]. Of note, Sivertsen *et al*. suggested that lipophilic side chains larger than a benzyl group may reduce binding to HSA [44]. That α,α-disubstituted β-amino amides were not affected by HSA may be attributed to their bulky lipophilic groups. In addition, the derivatives were used in high concentrations over 24 h against adherent biofilms compared to shorter treatment of cells/bacteria in suspension [42,43]. Due to their fast mechanism of action, release of biofilm constituents may have interacted with HSA during the 24 h incubation time and thus reduced HSA binding

properties. In contrast, potency of CTAB and Pen G decreased which may be due to the reported high affinity of HSA to bind acidic drug molecules and molecules with long aliphatic chains [45,46].

The quantitative and qualitative green SYTO9 and red PI dye uptake analyses (FIG 2B and FIG 3) confirmed the viability data. During these experiments approximately 50% of *S. aureus* biofilms showed signs of membrane damage within 1 h of treatment with the derivatives. Similar results were obtained with CHX even though incorporation of the hydrophobic moiety in microbial membranes plays only a minor role in the proposed mode-of-action for CHX [32]. Treatment with the cationic derivatives **A1** – **A3** resulted in similar membrane damaging effects as seen with CHX, whereas CTAB or Pen G did not show any membrane damaging properties during the incubation period. Thus, CTAB appeared to be unable to perturb microbial membranes under the experimental conditions, which also explains its low impact on biofilm viability. It has been reported that staining with SYTO9 and PI can result in mixed states, which make differentiation between live and dead bacteria challenging [47]. Therefore, we additionally performed a membrane disintegration study based on the leakage of intracellular ATP. The ATP assay confirmed the SYTO9 and PI staining experiments. Treatment with derivatives **A1** - **A3** as well as CHX resulted in a considerable leakage of ATP. Similar observations of ATP leakage due to pore formation were reported for small, dehydroabietic acid derived compounds and the bacteriocin Nisin A [38,48]. We observed only a minor ATP leakage after CTAB treatment and no effect after incubation with Pen G.

Biofilms of GFP expressing *S. aureus* strain AH2547 grown in a CDC biofilm reactor allowed us to challenge the α,α-disubstituted β-amino amides in a different assay system. We adapted the standardized ASTM method 2562 [36] to grow CDC reactor biofilms under higher shear forces and for a longer time compared to the 96-well plate setup. Susceptibility

of planktonic *S. aureus* AH2547 against our derivatives was in the same range as previously described for *S. aureus* ATCC25923 [31]. However, *S. aureus* AH2547 biofilms seemed more tolerant to **A3** treatment, similar to our previous observations with the *S. aureus* Newman strain [31]. Treatment of harvested *S. aureus* AH2547 biofilms with 90 µM **A3** or 100 µM CHX resulted still in equally high log reductions and kill rates of over 99.99% (FIG. 5). These findings in combination with our results from our 96-well plate assays suggested a similar mode-of-action of **A3** and CHX. These two compounds have an equal number of covalent bonds between the two positively charged moieties. The two (p-chlorophenyl)guanide units in CHX are linked by a six carbon hexamethylene bridge, which are important for its activity by bridging two neighboring phospholipid head groups [32]. This results in inhibition of metabolic functions of the membrane and can ultimately lead to structural integrity loss, which may also be the case for our derivatives. However, the treatment-flow-cell experiments revealed a notable difference between CHX and **A3** regarding their biofilm removal properties (FIG 6). We found indications that **A3** was able to additionally remove biofilm. *In silico* simulations suggest that **A2** and **A3** only partly incorporate the hydrophobic moiety into bacterial membranes ("can-can" pose which also correlates with greater anti-biofilm activity) in contrast to the proposed mode-of-action of CHX [49,32]. Koivuniemi *et al.* suggested that the insertion of only one hydrophobic side chain may lead to local aggregation of the compounds driven by the hydrophobic effect which results in a collective behavior of these compounds that disrupts the bacterial membrane [49]. Additionally, an exposed hydrophobic arm may impact the protective peptidoglycan macronet outside of the bacterial membrane. The observed effects of membrane disintegration as well as decreased biofilm cohesion in the treatment flow cell experiments would fit with the proposed behavior for **A2** and **A3**. However, further studies are needed to pinpoint localization and interaction of α,α-disubstituted β-amino amides in biofilms. Simultaneous killing and removal of biofilm is a favorable outcome for anti-biofilm treatments because of maintenance of tissue/medical device functionality and limiting the chance of biofilm re-

404 establishment on a preconditioned surface. Therefore, biofilm removal is an equally important
405 biofilm controlling strategy in addition to killing the biofilm as highlighted by Gloag *et al*.
406 [50]. For instance, the non-biocidal peptide A has been shown to effectively disperse and
407 prevent biofilms also after grating to surfaces [11,51]. Various antimicrobial treatments such
408 as glutaraldehyde, the antimicrobial peptide Nisin, or quaternary ammonium compounds are
409 able to kill biofilm bacteria, however, they all lack the capability to remove the biofilm [52].
410 Even though the bactericidal impact of CHX can contribute to biofilm erosion, a considerable
411 amount of biomass remained in comparison to treatment with **A3** or Pen G. Crosslinking of
412 anionic matrix components has been suggested for CHX's lack of removal properties, and a
413 decrease of biofilm deformability after CHX treatment has been observed [53,54]. Even
414 though **A3** is di-cationic, it did not share the inability of reported cationic compounds of
415 removing biofilm biomass.

416 Our results show that α,α-disubstituted β-amino amides affect *S. aureus* biofilms as fast as the
417 antiseptic CHX. Biofilm treatment leads to membrane permeabilization and application of
418 these derivatives under flow conditions causes biofilm removal. These properties make α,α-
419 disubstituted β-amino amides attractive candidates for the development of new anti-biofilm
420 control strategies.

Acknowledgments & Funding

This work was supported by a personal overseas grant to D.A. and the ‘Fellesløftet’ grant 214493/F20 by the Research Council of Norway.

References

[1] Harbarth S, Theuretzbacher U, Hackett J**.** Antibiotic research and development: business as usual? J Antimicrob Chemother 2015;70:1604-7.

[2] European Centre for Disease Prevention and Control**.** Assessing the health burden of infections with antibiotic-resistant bacteria in the EU/EEA, 2016-2020. 17-11-2022;

[3] Stewart PS**.** Prospects for Anti-Biofilm Pharmaceuticals. Pharmaceuticals 2015;8:504-11.

[4] Miguel Cámara, Green W, MacPhee CE, Rakowska PD, Raval R, Richardson MC et al. Economic significance of biofilms: a multidisciplinary and cross-sectoral challenge. NPJ Biofilms and Microbiomes 2022;8:42.

[5] Sauer K, Stoodley P, Goeres DM, Hall-Stoodley L, Burmølle M, Stewart PS et al. The biofilm life cycle: expanding the conceptual model of biofilm formation. Nat Rev Microbiol 2022;20:608-20.

[6] Darouiche RO**.** Treatment of Infections Associated with Surgical Implants. N Engl J Med 1-4-2004;350:1422-9.

[7] Goswami AG, Basu S, Banerjee T, Shukla VK**.** Biofilm and wound healing: from bench to bedside. European Journal of Medical Research 2023;28:157.

[8] Coenye T, Bové M, Bjarnsholt T**.** Biofilm antimicrobial susceptibility through an experimental evolutionary lens. NPJ Biofilms Microbiomes 18-10-2022;8:82.

[9] Lambert G, Estévez-Salmeron L, Oh S, Liao D, Emerson BM, Tlsty TD et al. An analogy between the evolution of drug resistance in bacterial communities and malignant tissues. Nat Rev Cancer 2011;11:375-82.

[10] Abdelhamid AG and Yousef AE**.** Combating Bacterial Biofilms: Current and Emerging Antibiofilm Strategies for Treating Persistent Infections. 2023;12:

[11] Herzberg M, Berglin M, Eliahu S, Bodin L, Agrenius K, Zlotkin A et al. Efficient Prevention of Marine Biofilm Formation Employing a Surface-Grafted Repellent Marine Peptide. ACS Appl Bio Mater 19-4-2021;4:3360-73.

[12] Skogman ME, Kanerva S, Manner S, Vuorela PM, Fallarero A**.** Flavones as Quorum Sensing Inhibitors Identified by a Newly Optimized Screening Platform Using Chromobacterium violaceum as Reporter Bacteria. Molecules 10-9-2016;21:

[13] Melander RJ, Basak AK, Melander C**.** Natural products as inspiration for the development of bacterial antibiofilm agents. Nat Prod Rep 1-11-2020;37:1454-77.

[14] Harvey AL, Edrada-Ebel R, Quinn RJ**.** The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discovery 2015;14:111-29.

[15] Editorial**.** Mechanism matters. Nat Med 2010;16:347.

[16] Nonejuie P, Burkart M, Pogliano K, Pogliano J**.** Bacterial cytological profiling rapidly identifies the cellular pathways targeted by antibacterial molecules. Proc Natl Acad Sci U S A 2013;110:16169-74,S16169-1.

[17] Bollenbach T**.** Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution. Curr Opin Microbiol 2015;27:1-9.

[18] Crowley PJ and Martini LG**.** Formulation design: new drugs from old. Drug Discovery Today: Ther Strategies 2004;1:537-42.

[19] Terreni M, Taccani M, Pregnolato M**.** New Antibiotics for Multidrug-Resistant Bacterial Strains: Latest Research Developments and Future Perspectives. Molecules 2-5-2021;26:

[20] Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP et al. A new antibiotic kills pathogens without detectable resistance. Nature 22-1-2015;517:455-9.

[21] Shukla R, Lavore F, Maity S, Derks MGN, Jones CR, Vermeulen BJA et al. Teixobactin kills bacteria by a two-pronged attack on the cell envelope. Nature 2022;608:390-6.

[22] D'Costa VM, Mukhtar TA, Patel T, Koteva K, Waglechner N, Hughes DW et al. Inactivation of the lipopeptide antibiotic daptomycin by hydrolytic mechanisms. Antimicrob Agents Chemother 2012;56:757-64.

[23] Svenson J, Molchanova N, Schroeder CI**.** Antimicrobial Peptide Mimics for Clinical Use: Does Size Matter? Front Immunol 2022;13:915368.

[24] Igumnova EM, Mishchenko E, Haug T, Blencke HM, Sollid JUE, Fredheim EGA et al. Amphipathic sulfonamidobenzamides mimicking small antimicrobial marine natural products; investigation of antibacterial and anti-biofilm activity against antibiotic resistant clinical isolates. Bioorg Med Chem 15-9-2018;26:4930-41.

[25] Paulsen MH, Engqvist M, Ausbacher D, Anderssen T, Langer MK, Haug T et al. Amphipathic Barbiturates as Mimics of Antimicrobial Peptides and the Marine Natural Products Eusynstyelamides with Activity against Multi-resistant Clinical Isolates. J Med Chem 12-8-2021;64:11395-417.

[26] Dey H, Simonovic D, Norberg-Schulz H, I, Vasskog T, Fredheim EGA, Blencke HM et al. Synthesis and Antimicrobial Activity of Short Analogues of the Marine Antimicrobial Peptide Turgencin A: Effects of SAR Optimizations, Cys-Cys Cyclization and Lipopeptide Modifications. Int J Mol Sci 10-11-2022;23:

[27] Hansen T, Alst T, Havelkova M, Strøm MB**.** Antimicrobial activity of small β-peptidomimetics based on the pharmacophore model of short cationic antimicrobial peptides. J Med Chem 2010;53:595-606.

[28] Hansen T, Ausbacher D, Flaten GE, Havelkova M, Strøm MB**.** Synthesis of cationic antimicrobial $\beta^{2,2}$-amino acid derivatives with potential for oral administration. J Med Chem 2011;54:858-68.

[29] Hansen T, Ausbacher D, Zachariassen ZG, Anderssen T, Havelkova M, Strøm MB**.** Anticancer activity of small amphipathic $\beta^{2,2}$-amino acid derivatives. Eur J Med Chem 5-10-2012;58:22-9.

[30] Paulsen MH, Engqvist M, Ausbacher D, Strøm MB, Bayer A**.** Efficient and scalable synthesis of α,α-disubstituted $\beta^{2,2}$-amino amides. Org Biomol Chem 2016;14:7570-8.

[31] Ausbacher D, Fallarero A, Kujala J, Määttänen A, Peltonen J, Strøm MB et al. *Staphylococcus aureus* biofilm susceptibility to small and potent $\beta^{2,2}$-amino acid derivatives. Biofouling 2014;30:81-93.

[32] Gilbert P and Moore LE**.** Cationic antiseptics: diversity of action under a common epithet. J Appl Microbiol 2005;99:703-15.

[33] ASTM International. E2871-13: Standard Test Method for Evaluating Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm Grown in CDC Biofilm Reactor using Single Tube Method. In: West Conshohocken, PA.: ASTM International; 2013,

[34] Sandberg M, Määttänen A, Peltonen J, Vuorela PM, Fallarero A**.** Automating a 96-well microtitre plate model for Staphylococcus aureus biofilms: an approach to screening of natural antimicrobial compounds. Int J Antimicrob Agents 2008;32:233-40.

[35] Pang YY, Schwartz J, Thoendel M, Ackermann LW, Horswill AR, Nauseef WM**.** agr-Dependent Interactions of *Staphylococcus aureus* USA300 with Human Polymorphonuclear Neutrophils. J Innate Immun 2010;2:546-59.

[36] ASTM International. E2562-12: Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor. In: West Conshohocken, PA: ASTM International; 2012,

[37] Buckingham-Meyer K, Goeres DM, Hamilton MA**.** Comparative evaluation of biofilm disinfectant efficacy tests. J Microbiol Methods 2007;70:236-44.

[38] Manner S, Vahermo M, Skogman ME, Krogerus S, Vuorela PM, Yli-Kauhaluoma J et al. New derivatives of dehydroabietic acid target planktonic and biofilm bacteria of *Staphylococcus aureus* and effectively disrupt bacterial membrane integrity. Eur J Med Chem 2015;102:68-79.

[39] Ausbacher D, Lorenz L, Pitts B, Stewart PS, Goeres DM**.** Paired methods to measure biofilm killing and removal: a case study with Penicillin G treatment of Staphylococcus aureus biofilm. Lett Appl Microbiol 2018;66:231-7.

[40] Goeres DM, Walker DK, Buckingham-Meyer K, Lorenz L, Summers J, Fritz B et al. Development, standardization, and validation of a biofilm efficacy test: The single tube method. J Microbiol Methods 2019;165:105694.

[41] James TJ, Hughes MA, Cherry GW, Taylor RP**.** Simple biochemical markers to assess chronic wounds. Wound Repair Regen 2000;8:264-9.

[42] Sivertsen A, Tørfoss V, Isaksson J, Ausbacher D, Anderssen T, Brandsdal BO et al. Anticancer potency of small linear and cyclic tetrapeptides and pharmacokinetic investigations of peptide binding to human serum albumin. J Pept Sci 2014;20:279-91.

[43] Svenson J, Brandsdal BO, Stensen W, Svendsen JS**.** Albumin Binding of Short Cationic Antimicrobial Micropeptides and Its Influence on the in Vitro Bactericidal Effect. Journal of Medicinal Chemistry 1-7-2007;50:3334-9.

[44] Sivertsen A, Isaksson J, Leiros HK, Svenson J, Svendsen JS, Brandsdal BO**.** Synthetic cationic antimicrobial peptides bind with their hydrophobic parts to drug site II of human serum albumin. BMC Struct Biol 23-1-2014;14:4.

[45] Kratochwil NA, Huber W, Muller F, Kansy M, Gerber PR**.** Predicting plasma protein binding of drugs: a new approach. Biochem Pharmacol 1-11-2002;64:1355-74.

[46] Lee P and Wu X**.** Review: Modifications of Human Serum Albumin and Their Binding Effect. Curr Pharm Des 2015;21:1862-5.

[47] Berney M, Hammes F, Bosshard F, Weilenmann HU, Egli T**.** Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. Appl Environ Microbiol 2007;73:3283-90.

[48] Okuda K, Zendo T, Sugimoto S, Iwase T, Tajima A, Yamada S et al. Effects of bacteriocins on methicillin-resistant *Staphylococcus aureus* biofilm. Antimicrob Agents Chemother 2013;57:5572-9.

[49] Koivuniemi A, Fallarero A, Bunker A**.** Insight into the antimicrobial mechanism of action of $\beta^{2,2}$-amino acid derivatives from molecular dynamics simulation: Dancing the can-can at the membrane surface. Biochimica et Biophysica Acta (BBA) - Biomembranes 2019;1861:183028.

[50] Gloag ES, Fabbri S, Wozniak DJ, Stoodley P**.** Biofilm mechanics: Implications in infection and survival. Biofilm 2020;2:100017.

[51] Jia R, Yang D, Dou W, Liu J, Zlotkin A, Kumseranee S et al. A sea anemone-inspired small synthetic peptide at sub-ppm concentrations enhanced biofilm mitigation. International Biodeterioration & Biodegradation 2019;139:78-85.

[52] Davison WM, Pitts B, Stewart PS**.** Spatial and temporal patterns of biocide action against *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemother 2010;54:2920-7.

[53] Brindle ER, Miller DA, Stewart PS**.** Hydrodynamic deformation and removal of *Staphylococcus epidermidis* biofilms treated with urea, chlorhexidine, iron chloride, or DispersinB. Biotechnol Bioeng 2011;108:2968-77.

[54] Stewart PS**.** Biophysics of Biofilm Infection. Pathog Dis 2014;70:212-8.

Supplementary information

Table S1. MIC data of chlorhexidine (CHX), cetyltrimethylammonium bromide (CTAB) and penicillin G (Pen G) against planktonic *Staphylococcus aureus* ATCC 25329

MIC of control compounds [µM]		
CHX	**CTAB**	**Pen G**
2.25	**2.50**	**0.12**

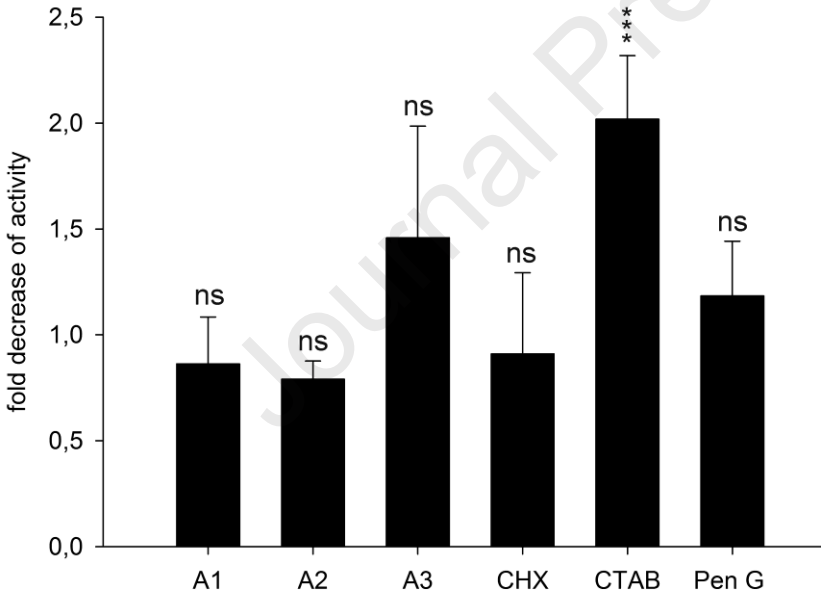


FIG S1. Impact of human serum albumin on anti-biofilm potency by resazurin based metabolic activity assessment. *S. aureus* ATCC 25923 biofilms were formed in 96-well plates for 18 h. The biofilms were concurrently treated with the α,α-disubstituted β-amino amides and control substances in the absence and presence of 300 µM HSA (>99%, Sigma-Aldrich, Schnelldorf, Germany), e.g. representing HSA levels in wound exudate [1],

for 24 h. After applied resazurin staining, treatment efficacies were compared. (Results display the mean with standard deviation of three independent experiments).

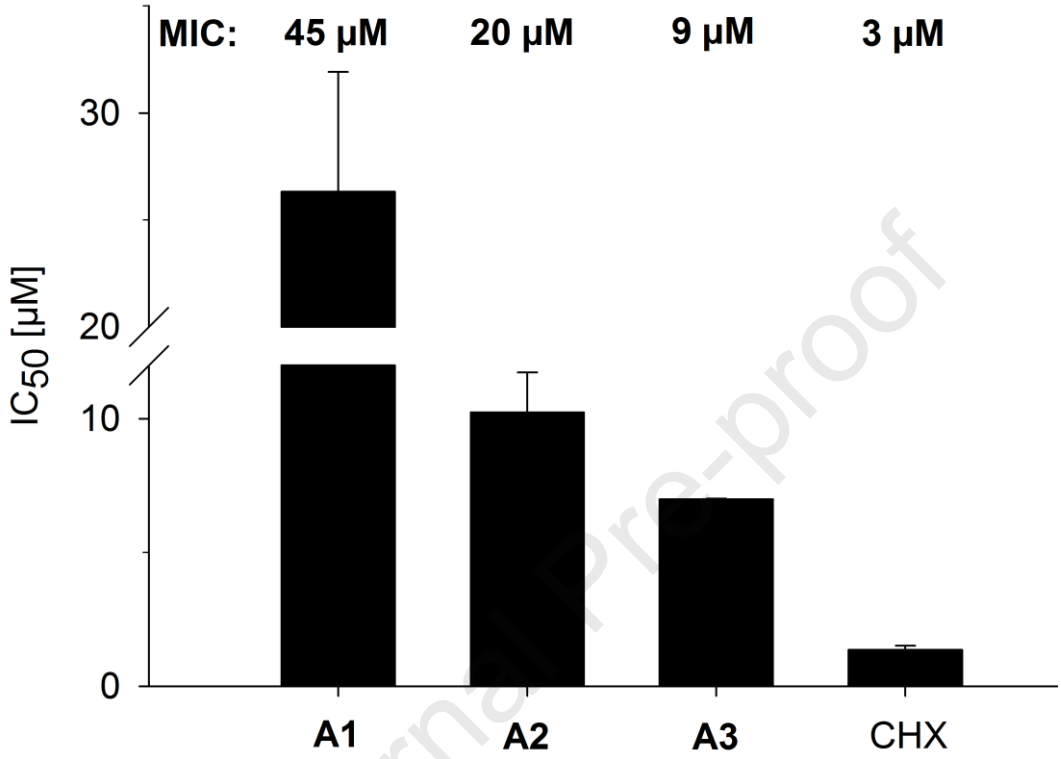
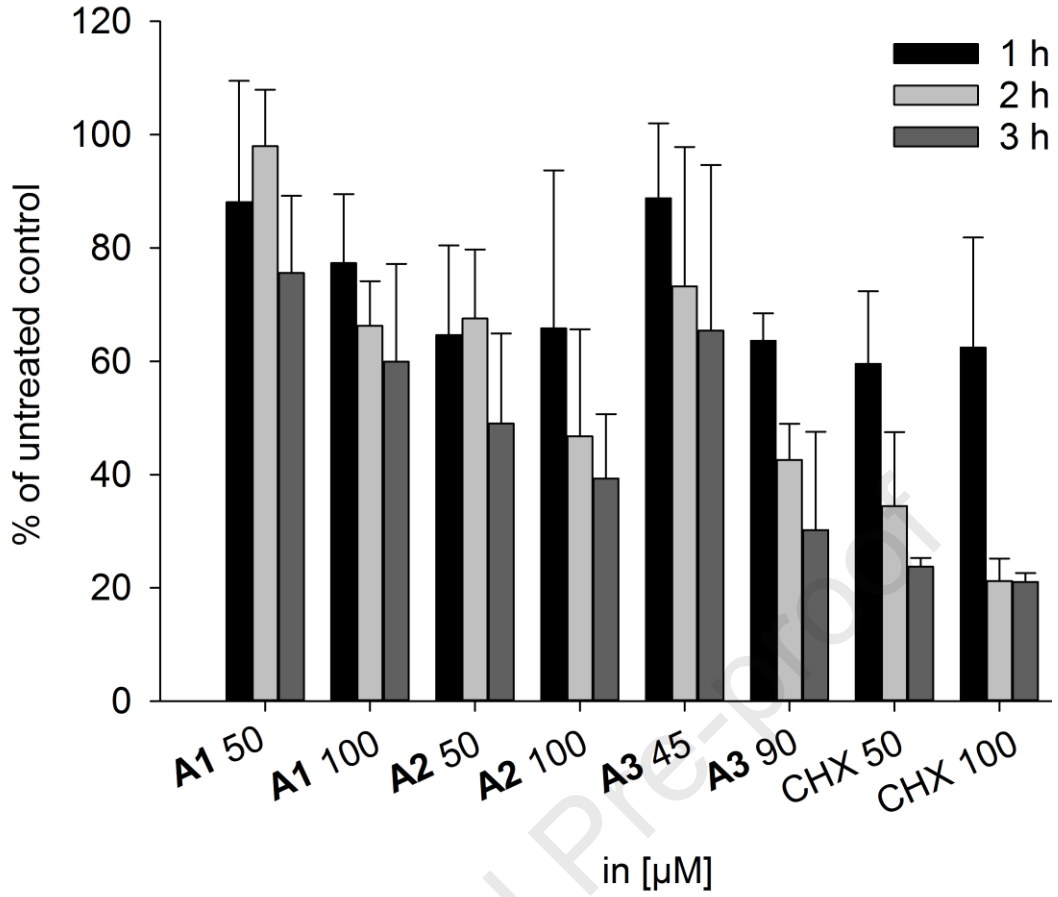


FIG S2. Susceptibility of planktonic *S. aureus* AH2547. MIC data of **A1** - **A3** and CHX and biofilm prevention after replacement of the planktonic phase with pure TSB. (Bars represent the mean of three independent experiments and error bars indicate the 95% confidence interval).



589

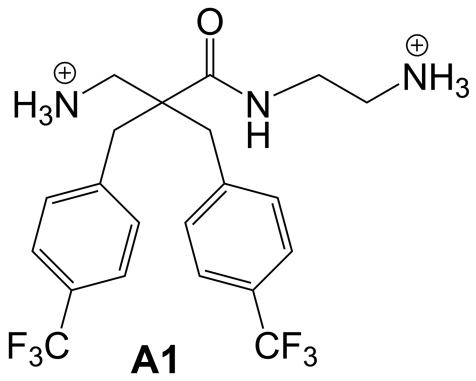
590 **FIG S3. Susceptibility assessment of *S. aureus* AH2547 biofilms cultivated in**
591 **96-well plates over 18 h.** The pre-formed biofilms were treated with chlorhexidine and
592 α,α-disubstituted β-amino amides for 1 - 3 h. After exchange of the planktonic phase, GFP
593 fluorescence of the remaining biofilm was detected, and compared with untreated controls
594 (results display the mean ± SD of three independent experiments.

595

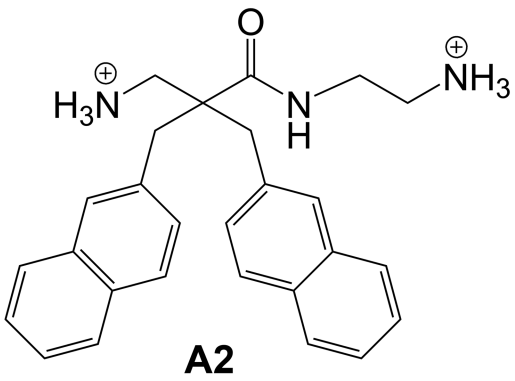
Figure S4 (online version of the article). Representative time-lapse video of treatment-flow-cell experiments displaying the GFP, RFP and bright field channels. (A) Exposure to full-strength TSB for 3h and 3 min. (B) Exposure to full strength TSB (3 min) and subsequently to **A3** (90 µM) for 3h. (C) Exposure to full strength TSB (3 min) and subsequently to CHX (100 µM) for 3h. (D) Exposure to full strength TSB (3 min) and subsequently to Pen G (400 µM) for 3h.

References

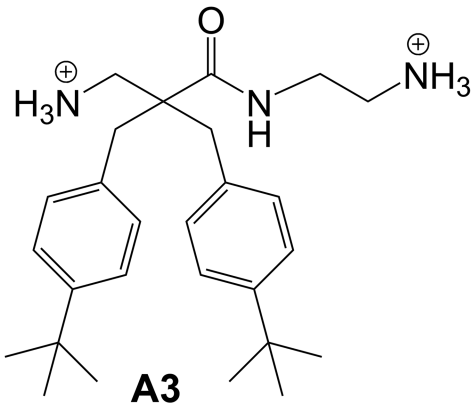
[1] James TJ, Hughes MA, Cherry GW, Taylor RP. Simple biochemical markers to assess chronic wounds. Wound Repair Regen 2000;8:264-9.



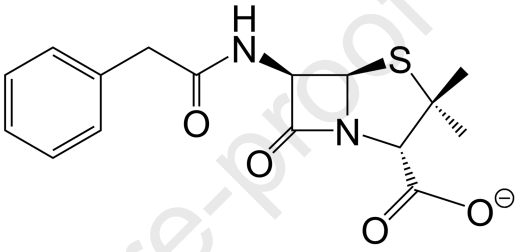
A1



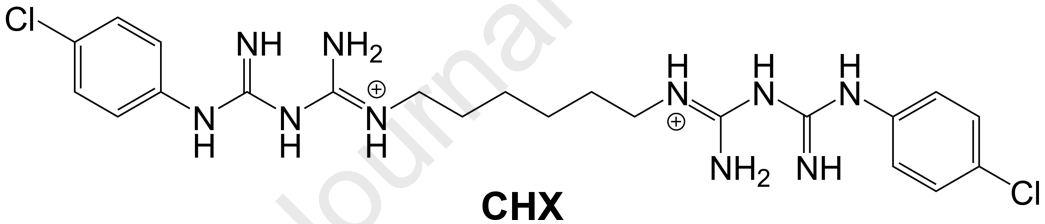
A2



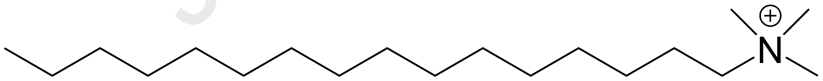
A3



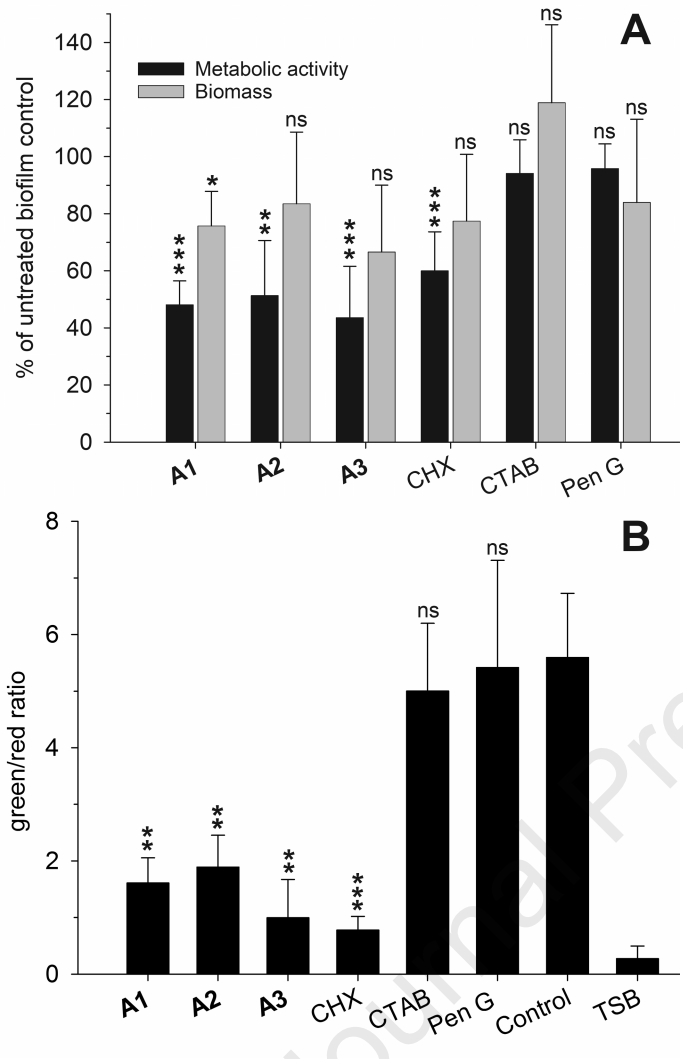
Pen G

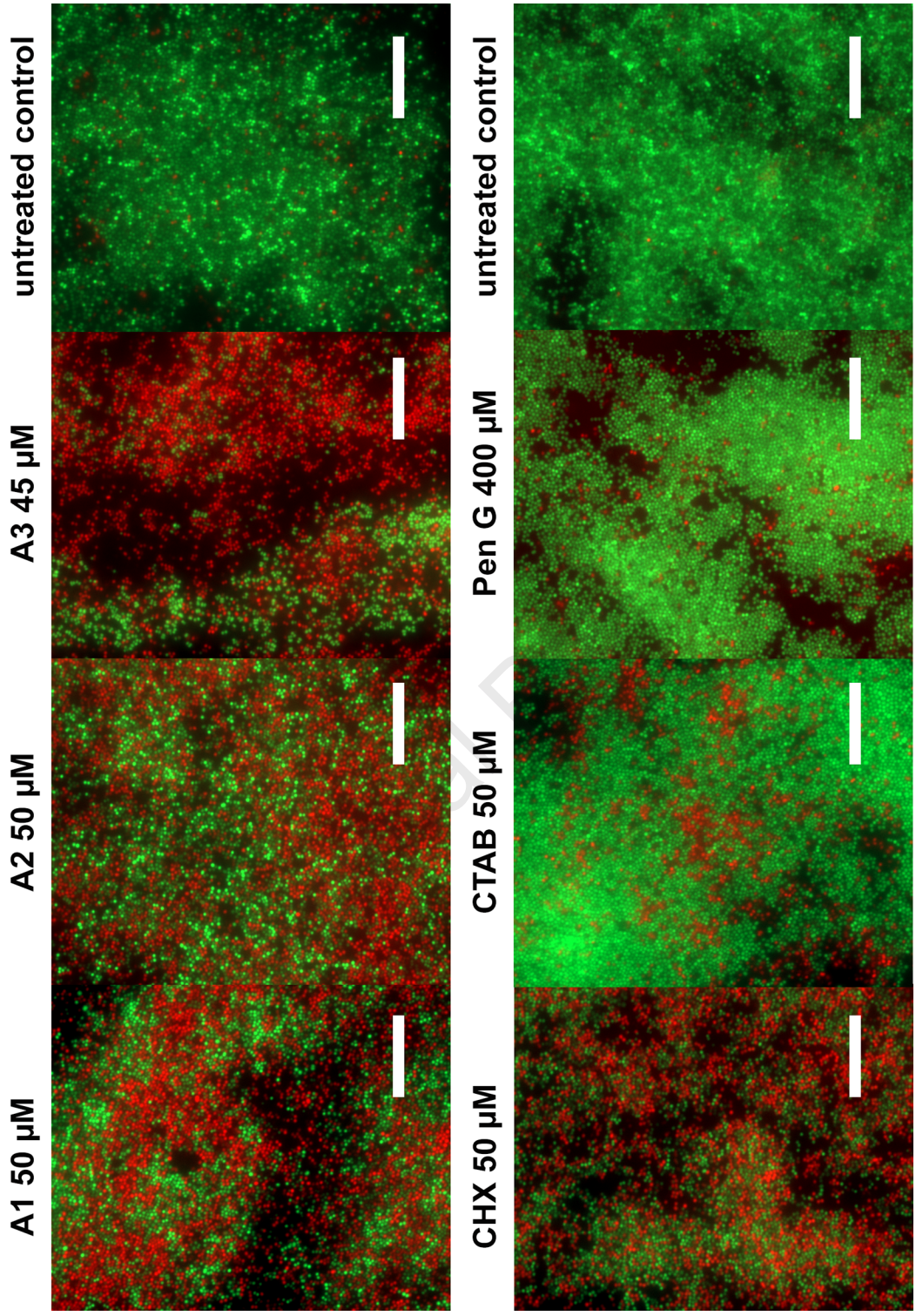


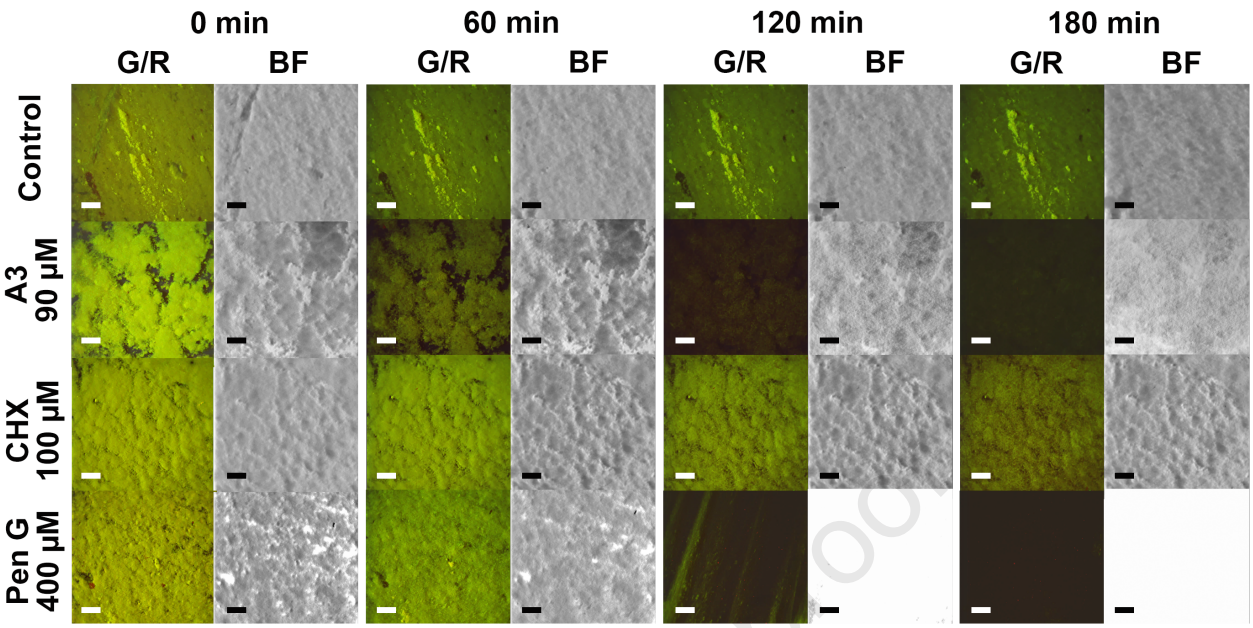
CHX

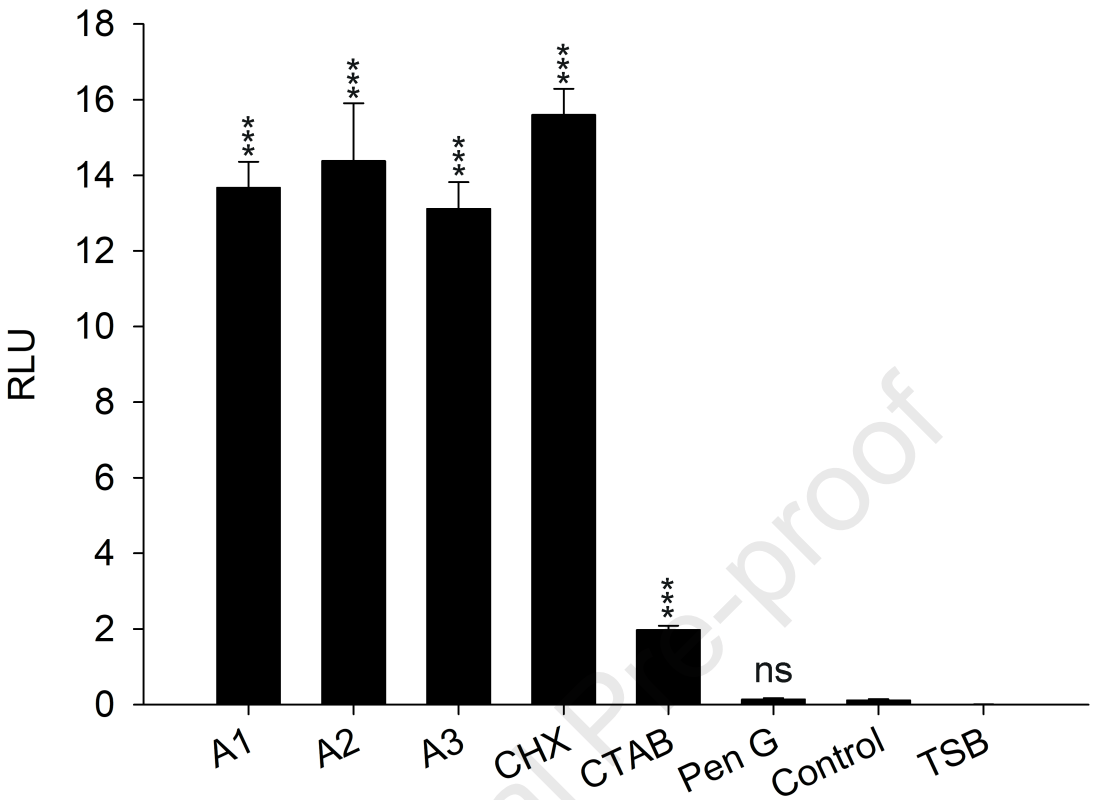


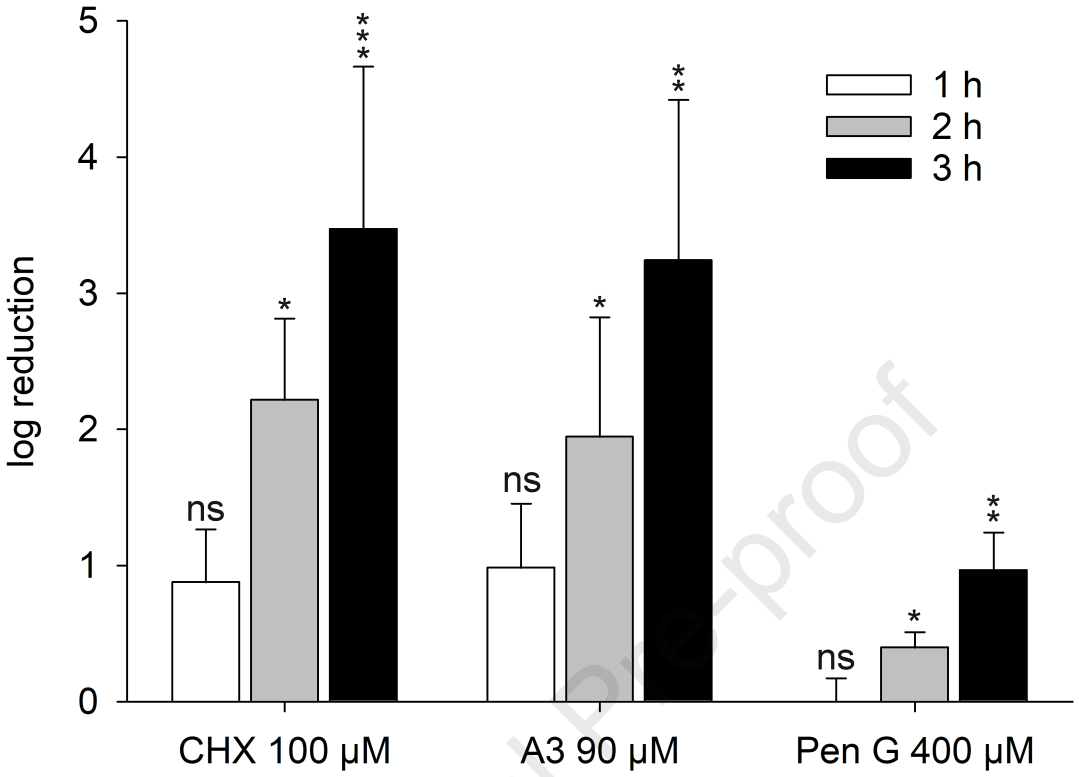
CTAB




untreated control
A3 45 µM
A2 50 µM
A1 50 µM
untreated control
Pen G 400 µM
CTAB 50 µM
CHX 50 µM







Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Given her roles as an Editor, D.M. Goeres had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to a different editor. A. Fallarero is currently employed by Thermo Fisher Scientific, but this work has no association with her current employment relationship. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.