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Anti-biofilm properties of the antimicrobial peptide temporin 1Tb and its ability, in combination with EDTA, to eradicate *Staphylococcus epidermidis* biofilms on silicone catheters

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ABSTRACT

In search of new antimicrobials with anti-biofilm potential, in the present study activity of the frog-skin derived antimicrobial peptide temporin 1Tb (TB) against *Staphylococcus epidermidis* biofilms was investigated. A striking ability of TB to kill both forming and mature *S. epidermidis* biofilms was observed, especially when the peptide was combined with cysteine or EDTA, respectively. Kinetics studies demonstrated that the combination TB/EDTA was active against mature biofilms already after 2–4-h exposure. A double 4-h exposure of biofilms to TB/EDTA further increased the therapeutic potential of the same combination. Of note, TB/EDTA was able to eradicate *S. epidermidis* biofilms formed *in vitro* on silicone catheters. At eradicating concentrations, TB/EDTA did not cause hemolysis of human erythrocytes. The results shed light on the anti-biofilm properties of TB and suggest a possible application of the peptide in the lock therapy of catheters infected with *S. epidermidis*.

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Temporin; biofilm; antimicrobial peptides; lock therapy; EDTA; *Staphylococcus epidermidis*

Introduction

The colonization of medical devices such as intravascular catheters, urinary catheters and orthopedic implants by microorganisms able to form biofilms is at present a serious health-care problem.

Staphylococcus epidermidis is one of the major nosocomial pathogens, often associated with biofilm-related medical device infections (Gomes et al. 2014). It is generally agreed that *S. epidermidis* biofilms develop in at least four sequential phases (Fey & Olson 2010): after an initial adhesion phase, which is typically measured *in vitro* after 1–2-h exposure to a surface, attached cells form small clusters or 'microcolonies' with biofilm-like properties (accumulation phase). In the presence of appropriate nutritional conditions microcolonies grow to form a mature biofilm (maturation phase) that usually acquires a characteristic 3-D architecture within 18–24 h of *in vitro* growth. A final dispersal phase limits biofilm expansion and involves the detachment of cells from the biofilm that, *in vivo*, may lead to dissemination of infection to surrounding tissues or to the blood. The best studied component of the extracellular polymeric substance (EPS) of staphylococcal biofilms is the polysaccharide intercellular adhesin (PIA), a polymer of β-1,6-linked *N*-acetyl-glucosamine with partially *N*-deacetylated amine groups, synthesized and secreted by the gene products of the *ica* operon, *icaA*, *icaD*, *icaB* and *icaC* (Fey & Olson 2010). *Ica*-negative clinical isolates of *S. epidermidis*, exhibiting a marked biofilm-forming ability, have also been described (Kogan et al. 2006; Brancatisano et al. 2014). Biofilm accumulation in these isolates seems to be protein-mediated as their biofilms are sensitive to protease treatment, but resistant to polysaccharide-degrading enzymes (Kogan et al. 2006; Brancatisano et al. 2014). It is well known that cells in biofilms are intrinsically refractory to antimicrobial drugs (Høiby et al. 2010). This is due to several factors that include: (1) the overall low growth rate of cells in biofilms, (2) the presence of the extracellular matrix that hampers the diffusion of antibacterial compounds through the biofilm layers, (3) the presence of ecological niches with low oxygen and nutrient concentrations that favor cells entering into a dormant state ('persisters'), and (4) the facility in exchanging mobile genetic elements carrying resistance genes, due to cell vicinity. The growing use of medical devices and

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the consequent increment in biofilm-associated infections indicate the urgent need to identify new drugs active against microbial biofilms.

Over the last few years, antimicrobial peptides (AMPs) have been proposed as a new source of anti-biofilm agents as they display several features suitable for targeting cells in the biofilm mode of growth (Batoni et al. 2016). An updated list of AMPs displaying activity against biofilms of clinically relevant microorganisms is freely available at www.baamps.it (Di Luca et al. 2015). Most AMPs have shown promise in preventing biofilm formation, while molecules able to treat mature biofilms are only rarely reported and intensive research to design AMPs with optimized anti-biofilm properties or to improve further the activity of existing AMPs is highly desirable. In this respect, AMP-based anti-biofilm strategies aimed at combining AMPs with synergistic compounds may represent a good, but still relatively poorly investigated approach. The combination of conventional and unconventional antimicrobial compounds with AMPs may improve both the prophylactic and the therapeutic efficacy of some AMPs by preventing the insurgence of resistance or by lowering the drug dosage and, thus, reducing the possible toxic side effects. Furthermore, the heterogeneity of microbial biofilms makes combinatorial strategies particularly attractive, as there might be the need to target cells in different metabolic states or in different environmental niches. Possible combinatorial strategies include the use of AMPs with: (1) conventional antibiotics (Maisetta et al. 2009; Mataraci & Dosler 2012), (2) compounds that dissolve and/or digest the biofilm matrix or inhibit its synthesis (Chaignon et al. 2007; Donelli et al. 2007; Wu et al. 2011), and (3) inhibitors of quorum sensing, the bacterial communication machinery often involved in biofilm formation (Brackman & Coenye 2015). Among these strategies, one of the most promising is the use of compounds that affect the biofilm extracellular matrix in synergy with bactericidal agents in order to facilitate the targeting of microorganisms entrapped in the matrix (Chaignon et al. 2007; Donelli et al. 2007; Kaplan et al. 2012). In this regard, it has been reported that sulfhydryl compounds, including L-cysteine (Cys), inhibit staphylococcal biofilm formation by inhibiting PIA biosynthesis (Wu et al. 2011). It is also known that metal cations (calcium, magnesium, iron) may be involved in maintaining the structure of the biofilm extracellular matrix and that chelating agents like ethylenediaminetetra-acetic acid (EDTA) may destabilize the biofilm structure (Banin et al. 2006). EDTA is also endowed with antimicrobial properties and has been used with success in combination with antibiotics against biofilms of a variety of clinical strains (Lebeaux et al. 2015).

The present study focuses on the anti-biofilm properties of temporin 1Tb (TB), a frog skin-derived AMP previously shown to exert potent antibacterial properties against clinically relevant bacterial species in planktonic form (Mangoni et al 2008). The possibility of improving the anti-biofilm potential of TB by combining the peptide with Cys or EDTA was also investigated.

A striking ability of TB to kill both forming and mature *S. epidermidis* biofilms was observed, especially when the peptide was used in combination with Cys or EDTA, respectively. Interestingly, TB in combination with EDTA was able to eradicate *S. epidermidis* biofilms formed *in vitro* on silicone catheters, suggesting the possible use of TB-combinatorial strategies in the lock therapy of central venous access devices colonized by *S. epidermidis* biofilms.

Materials and methods

S. epidermidis *strains*

Three clinical isolates of *S. epidermidis* (Se30, SeBER, SeVIC) and the reference strain ATCC 35,984 were used in the study. All the strains have been previously characterized in detail with respect to their drug susceptibility profile, biofilm forming ability, site of isolation and the nature of the biofilm EPS (Brancatisano et al. 2014). The main characteristics of the strains are summarized in Table 1. The strains were grown in tryptone soy broth (TSB) (Oxoid, Basingstoke, UK) supplemented with 0.25% glucose (TSB/Glc) overnight at 37°C or on Tryptone soy agar (TSA) (Oxoid for 24 h at 37°C for colony-forming unit (CFU) determination.

TB and other antimicrobial compounds

Synthetic TB (LLPIVGNLLKSLL-NH2) (purity > 98%) was purchased from the Peptide Specialty laboratories GmBH (Heidelberg, Germany). The peptide was diluted in milli-Q water to obtain a stock solution of 2 mg ml^{-1} and kept frozen at −80°C until use. Disodium EDTA and L-cysteine (Cys) were obtained from Sigma Aldrich

Table 1. Main features of the *S. epidermidis* strains used in the study.

S. epidermidis strain	Isolation site	Biofilm forming ability	PIA[a]-producing ability
ATCC 35,984	Catheter	Strong biofilm producer	PIA-positive
Se30	Catheter	Strong biofilm producer	PIA-positive
SeBER	Catheter	Moderate biofilm producer	PIA-negative[b]
SeVIC	Blood	Strong biofilm producer	PIA-negative

[a]Polysaccharide intercellular adhesion.
[b]PIA-negative strains had an extracellular matrix made mainly of proteins (Brancatisano et al. 2014).

(St Louis, MO, USA). A stock solution of 0.5-M EDTA was prepared by adjusting the pH to 8.0 with NaOH. This solution was then diluted to 50-mM (18.6 mg ml^{-1}) in milli-Q water to obtain the working solution. A stock solution of 400-mM Cys was prepared in milli-Q water. Both solutions were dispensed into aliquots and stored at −20°C.

Determination of the minimum inhibitory concentration (MIC) of TB, Cys and EDTA against planktonic bacteria in biofilm-like conditions

The MIC values for TB, Cys and EDTA against planktonic *S. epidermidis* cells were evaluated by the microdilution method in the same experimental conditions used thereafter for the biofilm assays. To this end, stationary phase *S. epidermidis* cells, obtained from cultures grown overnight, were diluted 1:100 in biofilm promoting medium (BPM, see next paragraph). Aliquots of 90 µl of bacterial suspensions were incubated for 24 h at 37°C in polypropylene tubes in the presence of TB at concentrations ranging from 6.25 to 100 µg ml^{-1}, Cys from 78 to 2,500 µM, or EDTA from 0.125 to 32 mg ml^{-1}, in a final volume of 100 µl. The MIC values were determined as the lowest concentrations of the different compounds at which no bacterial growth was visible.

Biofilm inhibition assay by TB

S. epidermidis strains were cultured overnight in TSB/Glc at 37°C with shaking. Bacterial suspensions were diluted 1:100 in BPM (TSB diluted 1:1 with 10-mM sodium phosphate buffer at pH 6.6 [SPB], with added glucose at 0.25%). A volume of 90 µl of each diluted bacterial suspension was dispensed into flat-bottom polystyrene 96-well microtiter plates (Corning Costar, Lowell, MA, USA) and 10 µl of TB solution were added to reach final peptide concentrations ranging from 12.5 to 50 µg ml^{-1}. Wells without peptide were set up as positive controls. Plates were incubated at 37°C without shaking for 24 h. At the end of the incubation period, biofilm biomass was assessed by crystal violet (CV, bioMérieux, Firenze, Italy) staining as previously described (Brancatisano et al. 2014). In parallel, to evaluate the viable biofilm-embedded bacteria, cells were mechanically detached from biofilms at the end of the incubation period by means of a pipette tip, vigorously vortexed, serially diluted 10-fold, and finally plated on TSA. The number of CFU per well was determined after incubation of the plates at 37°C for 24 h. The assays were performed in triplicates, and the results expressed as the percentage biofilm biomass reduction compared with wells without peptide or as Log_{10} CFU ml^{-1} ± the standard error of the mean (SEM).

Biofilm treatment assays by TB

Biofilms of the four different *S. epidermidis* strains were left to form in 96 well plates for 24 h in BPM in the absence of TB or other antimicrobials. After repetitive washing with SPB to remove planktonic cells, established biofilms were treated with 50 or 100 µg ml^{-1} TB in a final volume of 100 µl BPM. After 24-h incubation, biofilms were washed again with SPB and bacterial cells were mechanically removed by scraping with pipette tips and vigorous vortexing. To determine the number of viable bacteria surviving the treatment, 10-fold dilutions were plated onto TSA. The results were expressed as number of Log_{10} CFU ml^{-1} obtained in at least three independent experiments ± SEM.

Combination studies

The possibility of improving the anti-biofilm potential of TB was investigated by combining the peptide with Cys or EDTA against forming or mature biofilms of two arbitrarily chosen strains of *S. epidermidis*, representative of PIA-positive (ATCC 35,984) and PIA-negative (SeVIC) strains, respectively. Biofilm inhibition assays were carried out by co-incubating bacterial cells, prepared as described above, with sub-inhibitory concentrations of TB (range 6.25–25 µg ml^{-1}) and different concentrations of Cys (range 39–1,250-µM) or EDTA (range 1.5–12 µg ml^{-1}). After 24-h incubation, the total biofilm biomass was evaluated by CV staining and the results expressed as percentage biofilm biomass reduction compared to the control wells (bacteria incubated in medium only).

The ability of TB, in combination with EDTA or Cys to treat mature biofilms was assessed by exposing 24 h-old biofilms of the two representative *S. epidermidis* strains to sub-inhibitory concentrations of TB (range 25–100 µg ml^{-1}) and different concentrations of Cys (range 10–40-mM) or EDTA (range 2–8 mg ml^{-1}). Following incubation, the anti-biofilm effect was evaluated in terms of biofilm-associated viable counts as described previously.

Among all the combinations tested, in both biofilm inhibition and biofilm treatment assays, only the most powerful combinations, in terms of a reduction in biofilm biomass or viable count respectively, are shown in the 'Results' section.

Evaluation of the kinetics of the anti-biofilm effect of TB combined with EDTA against mature biofilms

The kinetics of the anti-biofilm effect of TB combined with EDTA were evaluated against established biofilms of the *S. epidermidis* ATCC 35,984. To this aim, 24 h-old biofilms were exposed for 2, 4 and 24 h to the TB-EDTA combination (100 µg ml^{-1} TB, 4 mg ml^{-1} EDTA) and the number of viable biofilm-associated cells determined as

reported above. In some experiments, the numbers of viable bacteria in the supernatants of biofilms treated with the combination or incubated in medium only were also evaluated. The effect of two consecutive exposures to the TB/EDTA combination on the number of residual cells was also assessed by exposing 24 h-old biofilms of *S. epidermidis* ATCC 35,984 to the combination for 4 h, washing with SPB, and again exposing the biofilms to the fresh combination for a further 2 or 4 h. Following washing and harvesting of the biofilm cells, the CFU number was assessed by plating serial dilutions of the bacterial suspensions onto TSA plates, as described previously.

Confocal laser scanning microscopy (CLSM) and image analyses

For biofilm CLSM study, *S. epidermidis* ATCC 35,984 was prepared as reported above. Briefly, 200 µl of 1:100 diluted overnight bacterial suspensions were dispersed into the inner wall of a glass bottom dish (WillCo GWSt-3512 Wells BV, Amsterdam, The Netherlands) and incubated at 37°C. After 24 h, biofilms were washed three times with 200 µl of 10-mM SPB (pH 6.6) and incubated with or without the TB/EDTA combination (100 µg ml^{-1} TB, 4 mg ml^{-1} EDTA) for 4 h. After three washes with SPB a second treatment was performed.

Biofilm cell viability after the TB/EDTA treatment was determined by staining cells with Syto9 and propidium iodide (PI) using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Life technologies) as recommended by the manufacturer. After staining, the wells were rinsed three times with 200 µl of SPB, before capturing images. Stained biofilms were examined under a Leica CLSM (TCS SP5, Leica, Heidelberg, Germany) using a 63 × /1.2 NA water immersion objective (Leica Microsystems). The pinhole aperture was set to 1.0 Airy. Each sample was sequentially excited at 488 (Syto9) and 561 (PI) nm and emissions were monitored at 500–540 and 600–650 nm, respectively. For each condition, two independent biofilms were analyzed. Images were acquired with 512 × 512 pixel resolutions in at least four different regions by collecting XY plans at different distances from the bottom of the biofilm (Petri glass). For each sample, different Z-plan images were also collected. All acquired data were processed and analyzed by ImageJ software version 1.37 (NIH Image; http://rsbweb.nih.gov/ij/).

Killing activity of TB against S. epidermidis *cells recovered from exponentially growing cultures or from 24 h-old biofilms*

S. epidermidis ATCC 35,984 was cultured to mid-log phase in TSB for 3 h at 37°C with shaking. Bacterial cells were then washed and resuspended in SPB pH 7.4 at a concentration of 10^7 CFU ml^{-1}. An aliquot of 10 µl of the bacterial suspension was mixed with 90 µl of SPB containing increasing peptide concentrations ranging from 6.25 to 50 µg ml^{-1} and 1% TSB. The bacteria-peptide suspensions were incubated for 4 h at 37°C with continuous rotation, serially diluted and plated on TSA for CFU counting. In parallel, *S. epidermidis* ATCC 35,984 cells were obtained from 24 h-old biofilms by mechanical detachment with a pipette tip and vigorous vortexing. Bacterial cells were re-suspended in SPB with 1% TSB, exposed to different concentrations of TB, and enumerated on TSA plates following the same procedure described above.

Eradication assay of S. epidermidis *biofilms obtained on segments of silicone catheters after sequential exposures to TB in combination with EDTA*

Overnight *S. epidermidis* ATCC 35,984 cultures were diluted 1:1,000 in fresh BPM. A volume of 200 µl was transferred to wells of 96-well plates containing sterile silicone catheter segments (length 3 mm, diameter 4 mm; Teflex Medical, Monza-Brianza, Italy). The plates were incubated at 37°C for 24 h in static conditions to allow biofilms to form. At the end of the incubation period, the catheter segments were transferred into new wells and washed three times with 200µ µl of SPB. The catheters were moved again into new wells containing TB (50 or 100 µg ml^{-1}), EDTA (4 mg ml^{-1}) or both in a final volume of 200µ µl of SPB. Wells containing catheter segments in SPB alone were set up as controls. Following a 4-h incubation at 37°C with agitation, the treated and un-treated catheters were washed three times with SPB and incubated for an additional 4 h as before. The catheter segments were then transferred into new wells, washed three times with 200 µl SPB and placed in propylene tubes containing 1 ml of SPB. One set of catheter segments was vigorously vortexed for 10 s, sonicated for 10 min in a water bath sonicator (45 kHz and 80 Watt, WWR, Radnor, PA, USA) and vortexed again for 10 s to dislodge biofilm forming bacteria. The bacterial suspensions so obtained were serially diluted and plated on TSA for CFU counts. Another set of catheter segments was not subjected to the dislodging procedure, but immediately placed into tubes containing 1 ml of fresh TSB. In this case, after incubation for 72 h at 37°C with agitation, bacterial growth was evaluated by visual examination of the turbidity of the cultures.

Hemolysis assay

The hemolytic activity of TB was evaluated by determining at OD_{450} the amount of hemoglobin released

from a 4% suspension of fresh human red blood cells (RBCs) exposed to different concentrations of the peptide. To this aim, human RBCs from healthy volunteers were harvested by centrifuging venous blood at 1,700 × *g* for 10 min at room temperature. After discarding the buffy coat layer, the RBC pellet was washed three times in PBS and diluted to 8% (v/v) in PBS. Aliquots of the 8% RBC solution (100 µl) were transferred in triplicates into wells of a 96-well microtiter plate and mixed with 100 µl of TB solutions at concentrations ranging from 6.25 to 320 µg ml^{-1}. RBCs incubated in PBS served as negative controls, while RBCs incubated in 0.2% Triton X-100 (Sigma) were used as positive controls (100% lysis). The microtiter plate was incubated at 37°C for 60 min and centrifuged at 1,000 × *g* for 20 min at 4°C. The supernatant (100 µl) was then transferred to a new plate and the OD_{450} was measured in a microtiter plate reader. The percentage hemolysis was calculated according to the following formula: [(OD_{450} in the peptide suspension – OD_{450} of the negative control in PBS)/(OD_{450} of the positive control – OD_{450} of the negative control in PBS)] × 100. The hemolytic activity of the combination TB (100 µg ml^{-1})/EDTA (4 mg ml^{-1}) as well as that of EDTA used alone up to16 mg ml^{-1}, was also assessed following the same protocol.

Statistical analysis

Differences between the mean values of groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test or by Student's t test. Data presented represent the mean ± the SEM of at least three independent experiments. A p value <0.05 was considered statistically significant.

Results

Activity of TB, alone and in combination with Cys and EDTA, in preventing biofilm formation of PIA-positive and PIA-negative S. epidermidis strains

The MIC values of TB, Cys and EDTA against planktonic *S. epidermidis* cells (two PIA-positive and two PIA-negative strains) in the same experimental conditions used thereafter for the biofilm inhibition assay (ie stationary phase cells diluted 1:100 in BPM) were first determined (Table 2). In the case of TB, MIC values of 50 µg ml^{-1} were recorded for the ATCC 35,984 and the SeVIC strains, while for the Se30 and SeBER strains the MIC values were 25 µg ml^{-1}. No growth inhibition was observed when the four *S. epidermidis* strains were incubated with Cys up to the concentration of 2,500-µM, while the MIC values of EDTA were 1,000 µg ml^{-1} for all the tested strains.

Table 2. MIC values of TB, Cys and EDTA in biofilm-like conditions.

S. epidermidis strain	TB[a]	Cys[b]	EDTA[a]
ATCC 35,984	50	> 2,500	1,000
Se30	25	> 2,500	1,000
SeBER	25	> 2,500	1,000
SeVIC	50	> 2,500	1,000

[a]values expressed in µg ml^{-1}.
[b]values expressed in µM.

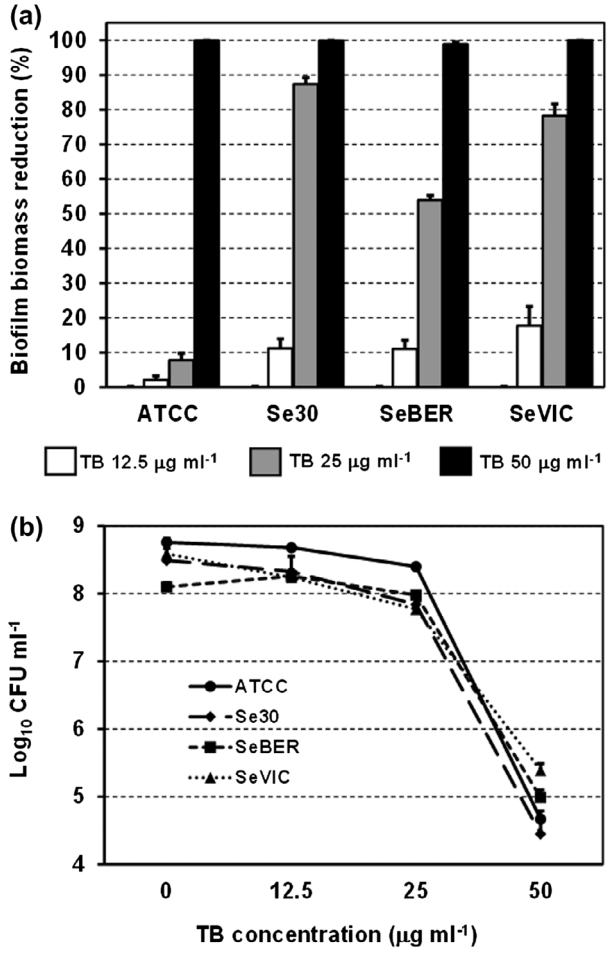

Figure 1. Inhibitory effect of TB on biofilm formation by different clinical isolates of *S. epidermidis*. Bacterial cells of PIA-positive (ATCC; Se30) and PIA-negative (SeBER; SeVIC) *S. epidermidis* strains were incubated in 96-well plates in the presence of different concentrations of TB. Cultures without peptide were used as positive controls. Following incubation at 37°C for 24 h, the anti-biofilm effect was evaluated as a percentage of biofilm biomass reduction after CV staining (a) or as biofilm-associated viable bacteria counts (b).

The ability of TB to inhibit biofilm formation was evaluated by CV staining after incubation of *S. epidermidis* strains for 24 h with different concentrations of the peptide. As shown in Figure 1a, a striking ability of TB to reduce the biomass of forming biofilms of both PIA-positive and PIA-negative strains was observed, compared to cells

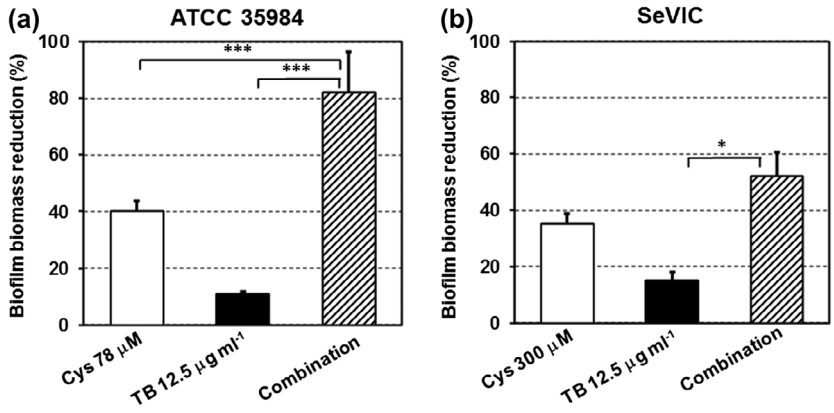

Figure 2. Inhibitory effect of TB combined with cysteine on *S. epidermidis* biofilm formation. *S. epidermidis* ATCC 35,984 (a) and SeVIC (b) cells were incubated with TB or cysteine (Cys) used alone and in combination. After incubation for 24 h at 37°C, the anti-biofilm effect was evaluated as a percentage of biofilm biomass reduction after CV staining. *$p<0.05$; ***$p<0.001$.

incubated in medium only. At a concentration of 50 μg ml^{-1} (~36-μM), the percentage inhibition was 100% for all four strains tested. These results correlated well with those obtained evaluating the number of biofilm-associated *S. epidermidis* viable cells (Figure 1b). In this case, at the concentration of 50 μg ml^{-1}, around 3 Log_{10} reduction in the CFU number ml^{-1} was observed, compared to the bacteria incubated without the peptide, for all the four strains tested.

The ability of Cys and EDTA to potentiate the activity of TB against forming biofilms was assessed, as a percentage of biofilm biomass reduction, against one PIA-positive (ATCC 35,984) and one PIA-negative (SeVIC) *S. epidermidis* strains chosen arbitrarily. When sub-inhibitory concentrations of TB were used in combination with Cys against the ATCC 35,984 strain, the biofilm biomass reduction was statistically higher than that caused by Cys or TB used alone (Figure 2a). In particular, TB at the sub-inhibitory concentration of 12.5 μg ml^{-1} (~9-μM) combined with 78-μM Cys caused more than an 80% reduction in the biomass of forming biofilms (Figure 2a). The effect of the combination was less evident against the PIA-negative strain SeVIC, even when the Cys concentration was increased to 300-μM (Figure 2b). Combining TB with EDTA did not cause any significant improvement in the ability of the peptide to inhibit biofilm formation of the two strains tested (data not shown).

Activity of TB alone and in combination with cysteine or EDTA in treating mature biofilms of PIA-positive and PIA-negative S. epidermidis *strains*

The activity of TB in the treatment of established biofilms of *S. epidermidis* was evaluated as the number of viable biofilm-associated cells after incubation for 24 h with the peptide. As shown in Figure 3, at concentrations of 50 and/

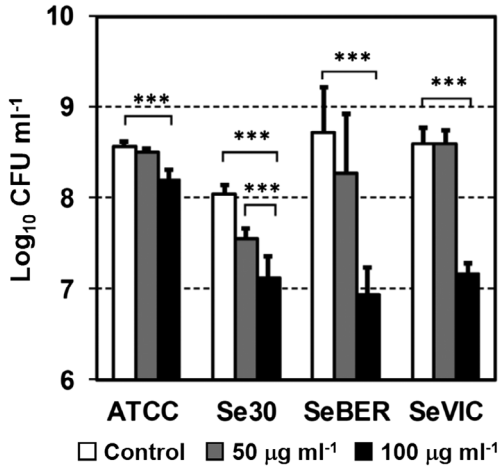

Figure 3. Effect of TB on preformed biofilms of different clinical isolates of *S. epidermidis*. Bacterial cells of PIA-positive (ATCC; Se30) and PIA-negative (SeBER; SeVIC) *S. epidermidis* strains were incubated in 96-well plates for 24 h to allow biofilm formation. After removing planktonic cells, established biofilms were treated with 50 and 100 μg ml^{-1} of TB for further 24 h and the anti-biofilm effect was evaluated as biofilm-embedded viable bacterial counts. Cultures without peptide were used as positive controls. ***$p<0.001$.

or 100 μg ml^{-1} TB was able to cause a statistically significant reduction in the Log_{10} number of CFU ml^{-1} of all tested strains, compared to the untreated control biofilms. At the higher peptide concentrations tested, the reduction in viable counts ranged from around 0.5 Log_{10} CFU ml^{-1} for the ATCC 35,984 strain (80% reduction) to almost 2 Log_{10} CFU ml^{-1} for the SeBER strain (99% reduction).

As bacteria within mature biofilms are less sensitive to antimicrobial agents than log-phase bacteria, the activity of TB on planktonic bacteria obtained by mechanical disruption of 24 h-old biofilms was evaluated. To this aim, bacteria dislodged from mature biofilms of *S. epidermidis*

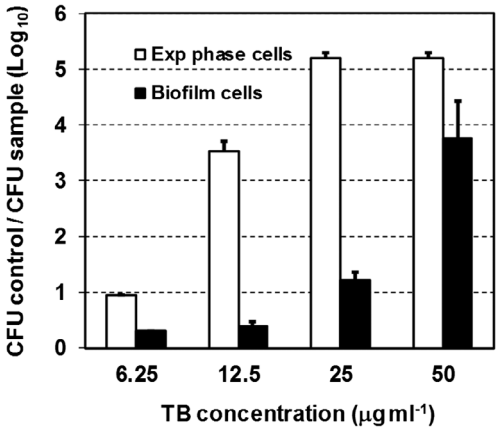

Figure 4. Bactericidal effect of TB against bacterial cells mechanically detached from mature biofilms of *S. epidermidis* ATCC 35,984 compared to mid-log phase cells. Planktonic bacteria harvested from exponentially growing cultures or from 24 h old biofilms were incubated for 4 h with different concentrations of TB in SPB with added 1% TSB at 37°C. The cells surviving the treatment were enumerated by CFU count. Data are expressed as ratio between the Log_{10} CFU ml^{-1} of untreated bacteria (control) and TB-treated bacteria (sample).

ATCC 35,984 were treated with different concentrations of TB in SPB with the addition of 1% TSB for 4 h. Thereafter, the number of bacteria resisting the treatment was determined and compared with that obtained from killing assays performed in parallel against log-phase bacteria. Figure 4 shows, for each experimental condition, the ratio between the Log_{10} CFU ml^{-1} of the control (bacteria incubated in medium only) and the sample (bacteria incubated with different concentrations of TB). TB treatment caused a reduction in the number of viable bacteria obtained from biofilms of about 1 Log10 (90%) at 25 μg ml^{-1} and >3 Log10 (99.9%) at 50 μg ml^{-1} (Figure 4). Interestingly, the bactericidal concentration of TB against bacteria harvested from biofilms was only 4-fold higher than that observed against log-phase bacteria (12.5 μg ml^{-1}) (Figure 4).

Unlike what was observed with forming biofilms, no evident enhancement of TB activity against mature biofilms was observed when the peptide was combined with Cys (data not shown). In contrast, the ability of TB to reduce the CFU number of established biofilms was greatly enhanced when the peptide was combined with EDTA (Figure 5). Indeed, at a concentration of 100 μg ml^{-1}, TB combined with EDTA (4 mg ml^{-1}) caused > 3 Log_{10} ml^{-1} reduction in the CFU number of the PIA-positive *S. epidermidis* ATCC 35,984 strain (Figure 5a), compared to the control. An almost 1 Log_{10} ml^{-1} reduction in CFU number was instead observed when sub-inhibitory concentrations of both EDTA (0.03 mg ml^{-1}) and TB (25 μg ml^{-1}) were used to treat mature biofilms of the PIA-negative *S. epidermidis* SeVIC strain (Figure 5b).

Kinetics of the anti-biofilm effect and possible mechanism of the anti-biofilm action of the TB/EDTA combination against mature biofilms of S. epidermidis ATCC 35984

The kinetics of the anti-biofilm effect of the TB/EDTA combination against established biofilms of the *S. epidermidis* ATCC 35,984 strain were then assessed. As shown in Figure 6, the enhancement of TB activity by EDTA was already evident after incubation for at both 2 and 4 h, two time points at which EDTA alone had no effect. The reduction in the CFU number caused by the combination at 4 h was almost 2.5 Log_{10} ml^{-1} compared to EDTA and 1.5 Log_{10} ml^{-1} compared to TB used alone.

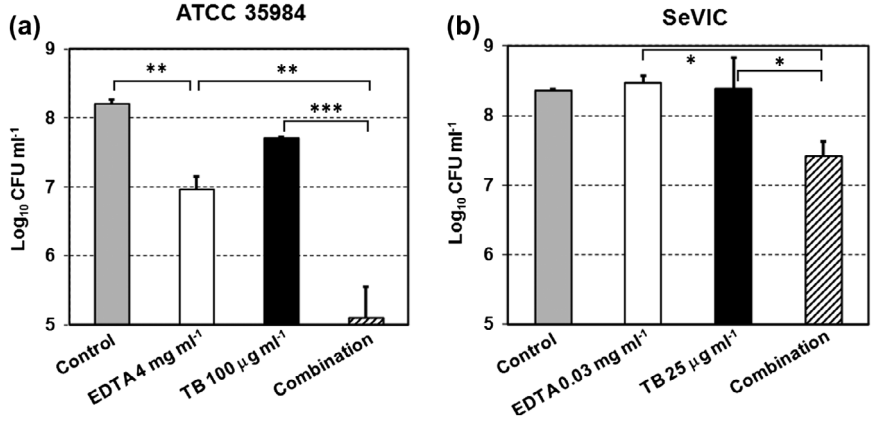

Figure 5. Effect of TB combined with EDTA against preformed biofilms of *S. epidermidis*. 24 h-old biofilms of *S. epidermidis* ATCC 35,984 (a) and SeVIC (b) were exposed to TB or EDTA used alone or in combination for 24 h at 37°C. After incubation, the biofilm associated viable count (CFU) was evaluated by plating the bacteria scraped from the wells. Control: bacteria incubated in medium only. *p<0.05; **p<0.01; ***p<0.001.

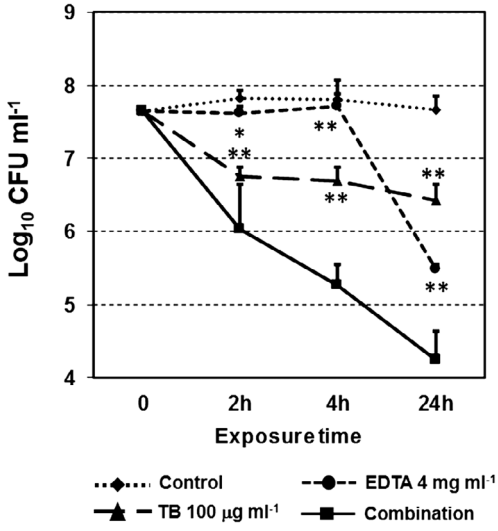

Figure 6. Kinetics of the anti-biofilm activity of TB in combination with EDTA on preformed biofilms of *S. epidermidis* ATCC 35,984. 24 h-old biofilms were exposed to TB and EDTA alone and in combination. At different exposure times, the anti-biofilm effect was evaluated as biofilm-associated viable counts (CFU). Control: bacteria incubated in medium without antimicrobial agents. *$p<0.05$; **$p<0.01$.

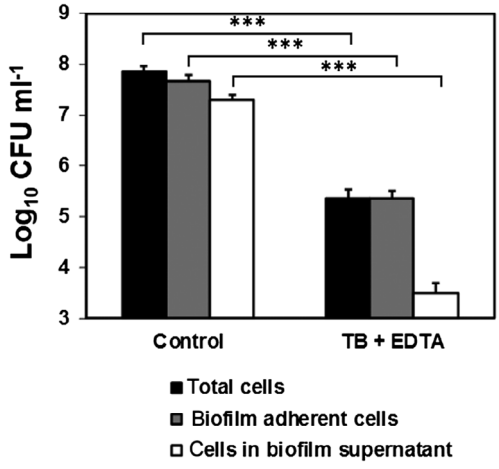

Figure 7. Evaluation of the anti-biofilm mechanism of action of the TB/EDTA combination against mature biofilms of *S. epidermidis* ATCC 35,984. 24 h-old biofilms were exposed for 4 h to TB (100 μg ml^{-1}) together with EDTA (4 mg ml^{-1}) or to medium only (control). The viable count (CFU) was then evaluated both in the biofilms and in the biofilm supernatants. ***$p < 0.001$.

It has been reported recently that cationic peptides may degrade established biofilms by killing embedded bacteria or by causing the detachment of viable cells (Segev-Zarko et al. 2015). In order to gain insights into the possible mechanisms of the anti-biofilm action of the TB/EDTA combination against mature biofilms of *S. epidermidis*, CFU were enumerated both in the biofilms and in the biofilm-supernatants after treatment for 4 h. As shown in Figure 7, following the treatment, the number of CFU was greatly reduced not only in the biofilm, but also in the biofilm supernatant, suggesting that the observed reduction in the viability of biofilm-embedded bacteria was due to a direct killing effect.

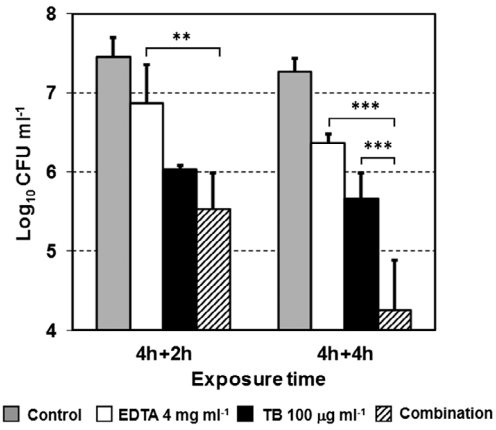

Figure 8. Anti-biofilm effect of two sequential exposures of established biofilms of *S. epidermidis* ATCC 35,984 to the combination TB/EDTA. 24 h-old biofilms were incubated for 4 h with TB (100 μg ml^{-1}) in combination with EDTA (4 mg ml^{-1}) and subsequently washed and exposed again for 2 h or 4 h to the same combination. **$p<0.01$; ***$p<0.001$.

To investigate whether the therapeutic potential of TB combined with EDTA could be further improved by multiple exposures of mature biofilms to the combination, 24 h-old biofilms of *S. epidermidis* ATCC 35,984 were exposed to TB/EDTA for 4 h, washed and exposed again to the same fresh combination for further 2 or 4 h. Compared to the 4-h exposure (Figure 6), no evident improvement in the therapeutic potential was observed by exposing mature *S. epidermidis* biofilms to the combination for 4 h followed by 2 h (Figure 8). In contrast, two subsequent exposures, of 4 h each caused a further reduction in the CFU numbers compared to a single 4 h treatment (2.4×10^4 CFU ml^{-1} *vs* 2.1×10^5, respectively; $P<0.05$, Student's t test), allowing the number of viable biofilm associated cells comparable to that obtained after a single exposure to TB+EDTA for 24 h (1.6×10^4 CFU ml^{-1}) to be determined.

Evaluation of the activity of the TB/EDTA combination on mature biofilms of S. epidermidis *by CLSM*

The effect on mature biofilms of *S. epidermidis* ATCC 35,984 of a double exposure to the TB/EDTA combination was evaluated by confocal fluorescence microscopy imaging (Figure 9). In agreement with the data obtained by the CFU count, 24 h-old biofilms treated twice with the TB/EDTA combination harbored high amounts of dead cells (stained red, Figure 9b), compared to the un-treated biofilms (Figure 9a). In addition, the biofilm architecture

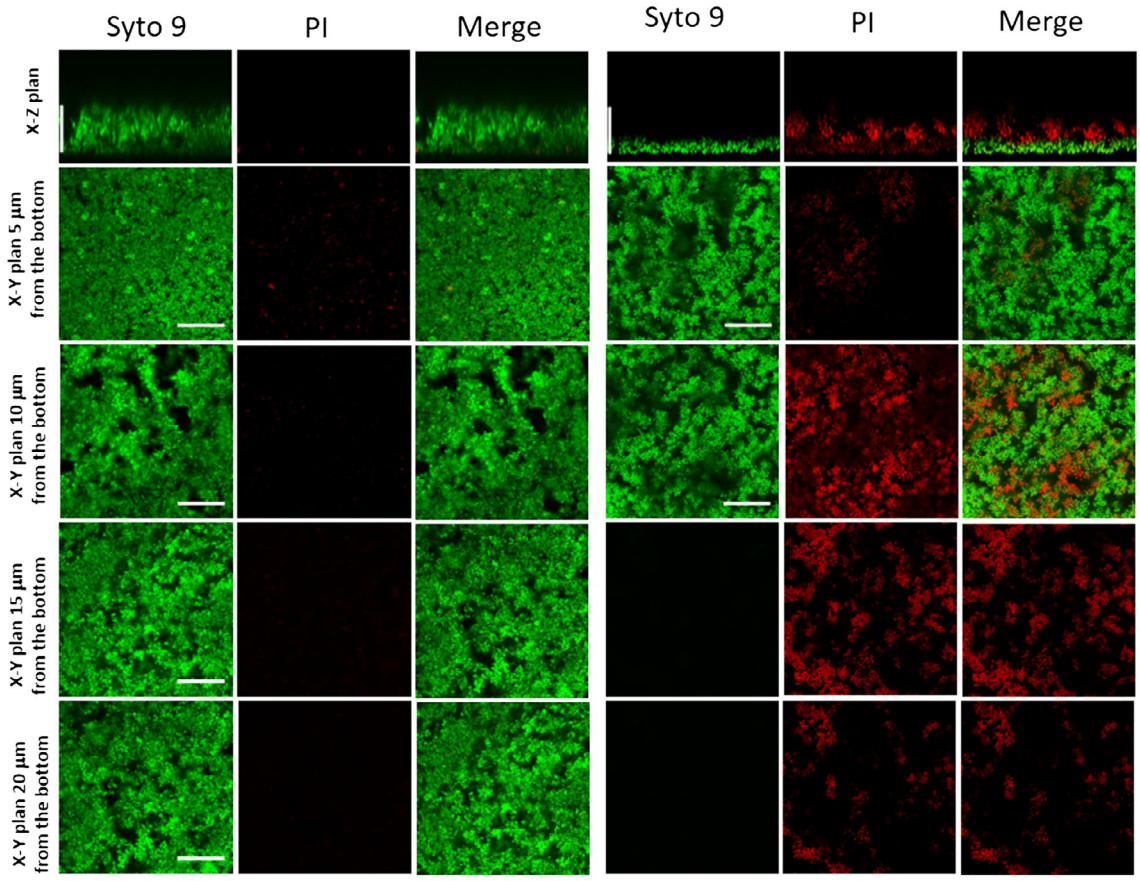

Figure 9. Effect of TB/EDTA combination on *S. epidermidis* biofilm morphology and viability. (a) Untreated 24 h-old biofilm of *S. epidermidis* ATCC 35,984; (b) 24 h-old biofilm of *S. epidermidis* ATCC 35,984, exposed to 100 µg ml^{-1} of TB and 4 mg ml^{-1} of EDTA for 4 h twice. Staining with green fluorescent labeled Syto 9 (488/500–540 nm) indicates live cells; staining with red fluorescent propidium iodide (PI) (561/600–650 nm) indicates dead cells; Scale bar: 30 µm.

was greatly altered by the treatment, displaying large areas of disaggregation and a thickness reduction, compared to the control biofilms.

Testing the TB/EDTA combination as 'lock therapy' in an in vitro *model of catheter-associated bacterial biofilms*

Lock therapy is a technique used in clinical settings for the treatment of long-term intravascular catheter-related bacteremia (Fortún et al. 2006). It consists in the instillation of high concentrations of an antimicrobial agent directly into the biofilm-containing catheter for exposure times sufficiently long to eradicate the biofilm. Due to the above reported ability of the TB/EDTA combination to treat established biofilms of *S. epidermidis*, the potential of such combination as lock solution for the salvage of infected catheters was evaluated. In order to mimic the lock therapy procedure *in vitro*, segments of silicone catheters were incubated with suspensions of *S. epidermidis* ATCC 35,984 for 24 h to allow the formation of mature

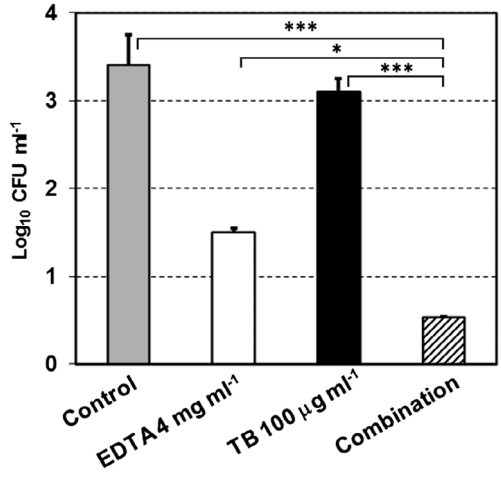

Figure 10. Anti-biofilm effect of TB in combination with EDTA against biofilms of *S. epidermidis* ATCC 35,984 preformed on segments of silicone catheters. 24 h-old biofilms were exposed to EDTA and TB used alone and in combination for 4 h + 4 h. Cells were detached from biofilms by brief sonication and plated to enumerate CFU. *p<0.05; ***p<0.001.

biofilms on the catheter surfaces. After washing, colonized catheter segments were exposed twice for 4 h each to TB (100 μg ml^{-1}) in combination with EDTA (4 mg ml^{-1}). As shown in Figure 10, biofilm-cell count levels compatible with those described for *in vivo* generated biofilms (Kite et al. 2004) were recovered from untreated catheters. In contrast, the number of viable cells harvested from catheters treated with the combination was around 3 Log_{10} ml^{-1} lower than that obtained from the control catheters. The number of CFU ml^{-1} was so low (from 0 to 6, mean value 2 CFU) that no visible bacterial growth was detected when replicates of treated catheters ($n = 6$) were placed in fresh bacterial growth medium for 72 h, suggesting that the antimicrobial lock treatment could significantly reduce or potentially eradicate catheter-associated biofilms of *S. epidermidis*.

Hemolytic activity of TB, the TB/EDTA combination, and EDTA

To evaluate the cytotoxic potential of TB, its ability to lyse human RBCs was tested in a standard hemolysis assay. No hemolytic activity was observed at TB concentrations ranging from 20 to 160 μg ml^{-1}. A hemolytic effect of 45% was observed at a TB concentration of 320 μg ml^{-1}. Therefore, concentrations of TB active against *S. epidermidis* biofilms did not produce hemolysis on human erythrocytes (data not shown). Of note, no detectable hemolytic effect was also exhibited by the combination TB (100 μg ml^{-1})/EDTA (4 mg ml^{-1}) or by EDTA alone, assayed up to 16 mg ml^{-1}.

Discussion

The involvement of *S. epidermidis* biofilms in infections associated with indwelling medical devices and the possible development of antibiotic resistance by this bacterial species is receiving significant attention (Raad et al. 1998; Fey & Olson 2010). The potential of AMPs as a therapeutic alternative to conventional antibiotics to combat both increasing antibiotic resistance and the biofilm lifestyle is increasingly emerging (Park et al. 2011; Strempel et al. 2015; Batoni et al. 2016). Indeed, AMP-activity against bacterial membranes is less specific than that of conventional antibiotics, thus lowering the possibility of inducing bacterial resistance. In addition, AMPs display many properties suitable for an 'ideal' anti-biofilm agent, not least the activity against metabolically inactive or poorly active cells, such as those in biofilms, circumventing the restriction of many traditional antibiotics that require actively growing bacterial cells to act (Batoni et al. 2016).

The frog skin-derived TB belongs to a large family of small mildly cationic AMPs produced by the skin of *Rana temporaria* (Mangoni et al. 2016). It adopts a α-helical conformation in membrane mimicking environments and acts by perturbing the membrane of microbial cells without significant hemolytic or cytotoxic activity against eukaryotic cells (Mangoni 2006). The peptide was previously shown to display rapid killing effects against multi-drug-resistant, clinically relevant, bacterial pathogens and particularly against Gram-positive species (Mangoni et al. 2008). Recently it has been reported that TB, encapsulated in chitosan nanoparticles, is able to exert a strong and prolonged bactericidal effect against numerous clinical isolates of *S. epidermidis* (Piras et al. 2015). Interestingly, it has been also demonstrated that the peptide is able to penetrate eukaryotic cells, kill intracellular bacteria, and promote wound-healing (Di Grazia et al. 2014). All these properties are important in view of a therapeutic development of TB whose anti-biofilm properties have not been previously reported. In the present study, TB showed a striking ability to inhibit biofilm formation of different clinical isolates of *S. epidermidis* irrespective of whether the biofilm EPS was composed mainly of proteins or PIA. Unlike conventional antibiotics, that usually act against biofilms at concentrations much higher than their MIC, the inhibitory effect of TB was obtained at peptide concentrations close to the MIC values (25–50 μg ml^{-1}) recorded for the different strains in biofilm-like conditions, suggesting that such an effect was due to a direct killing activity of the peptide against biofilm-forming cells. Most conventional antibiotics work best against metabolically active bacterial cells (ie in exponential growth phase) as they target active cell processes such as protein, cell wall, or nucleic acids synthesis. Therefore, their activity is greatly reduced against biofilm cells that, overall, exhibit a low growth rate. TB mainly acts by causing membrane permeabilization (Rinaldi et al. 2001) and therefore it is highly likely to act also against metabolically inactive or slow growing cells.

Mature biofilms are known to be much more refractory than forming biofilms to antimicrobials including conventional antibiotics and AMPs. In the case of AMPs, this is mainly attributed to the multiple interactions that they may establish with components of the biofilm EPS (Batoni et al. 2016). These interactions may reduce the bioavailability of the peptides and lower their anti-biofilm potential. When TB was assayed against both PIA-dependent and protein-dependent preformed biofilms of *S. epidermidis*, a statistically significant reduction in the CFU number was observed for all tested strains at a concentration of 100 μg ml^{-1}. In addition, TB was bactericidal against planktonic bacteria recovered by mechanical

disruption of preformed *S. epidermidis* biofilms, demonstrating its potential to act on metabolically inactive cells. Such an effect was observed at a concentration of 50 μg ml^{-1}, only 4-fold higher than the concentration needed to kill log-phase bacteria. This observation is relevant if it is considered that most conventional antibiotics exert a bactericidal effect towards biofilm-derived bacteria at concentrations much higher than their MBC values (up to 100-fold).

Due to the high antimicrobial resistance level of bacteria organized in biofilms and to the intrinsic heterogeneity of sessile bacterial communities, combination therapies are increasingly taken into consideration (Batoni et al. 2016). Therefore, the possibility of improving the anti-biofilm potential of TB by combining the peptide with Cys or EDTA against forming or mature biofilms of *S. epidermidis* was investigated.

TB in combination with Cys, but not with EDTA, was potentiated in inhibiting biofilm formation of *S. epidermidis*, although the inhibitory effect was more evident against the PIA-positive *S. epidermidis* ATCC 35,984 than the PIA-negative *S. epidermidis* SeVIC strain. The former strain was also more susceptible to the inhibitory effect of Cys alone than the latter strain (about a 40% reduction in biofilm biomass at a concentration of 78-μM for the ATCC 35,984 strain *vs* 300-μM for the SeVIC strain, Figure 2, $P<0.05$). These results are in agreement with the observation that sulfhydryl compounds are able to reduce the biosynthesis of EPS (eg PIA) by *S. epidermidis* (Wu et al. 2011). In turn, this could facilitate the direct interaction of the peptide with bacterial cells. Conversely, the lack of a synergistic effect with EDTA at the early stages of biofilm development may indicate that, in forming biofilms, the possible disaggregating effect of EDTA plays a minor role. A reverse effect was observed when TB was combined with Cys or EDTA against established biofilms of *S. epidermidis*. In this case, EDTA strongly potentiated the effect of TB against the PIA-positive *S. epidermidis* ATCC 35,984 strains. The ability of EDTA to destabilize the polysaccharides of the EPS may facilitate the diffusion of the peptide through the biofilm layers, improving its anti-biofilm potential. Although to a minor extent, EDTA potentiated the effect of TB also against the PIA-negative strain *S. epidermidis* SeVIC. A possible explanation is that polysaccharides other than PIA are possible components of biofilm EPS of PIA-negative strains of *S. epidermidis* (Spiliopoulou et al. 2012). In addition, possible disaggregating effects of EDTA could be due to sequestration of cations irrespective of the prevalent component constituting the EPS. Cys did not show any evident improving effect on TB activity against formed biofilms. It can be argued that once the biofilm is mature the inhibitory effect of Cys on extracellular matrix biosynthesis might be less relevant, at least in the *in vitro* model employed.

Among the combinations tested on preformed biofilms, the TB (100 μg ml^{-1})/EDTA (4 mg ml^{-1}) combination was the most promising. Hence, kinetics studies were performed in order to establish whether this combination could exert its anti-biofilm effect at earlier time points. Interestingly, a striking increase in the TB anti-biofilm effect was already evident after incubation for 4 h, an action time that can be considered rather short in comparison to conventional antibiotics. The mechanism of the anti-biofilm effect of the TB/EDTA combination on established biofilms was investigated by evaluating not only the viable count associated with the biofilm, but also that present in the biofilm supernatant. These experiments demonstrated that the reduction in the number of viable biofilm-embedded cells following TB/EDTA treatment was due to a real killing effect and not simply to the detachment of viable cells. This observation is relevant since, as previously outlined (Segev-Zarko et al. 2015), the detachment of live bacteria from a biofilm might be very detrimental as it promotes their spreading to the surrounding tissues or even to the bloodstream. The rapid effect exerted by the combination could be a consequence of the fast killing kinetics of TB (Mangoni et al. 2008) facilitated by the sequestration of cations by EDTA that, in turn, could destabilize the biofilm matrix and favor peptide penetration. The continuous drop in survival (Figure 6) could also be indicative of the ability of the combination to kill persister cells, metabolically inactive bacterial cells known to be highly represented in mature biofilms. This hypothesis is supported by preliminary data demonstrating that TB is able to kill experimentally generated persister cells of *S. epidermidis* (authors' unpublished observation).

The anti-biofilm potential of the TB/EDTA combination could be further improved by exposing mature biofilms of *S. epidermidis* ATCC 35,984 to the combination for two consecutive periods of 4 h, suggesting that the second exposure may target bacteria localized in the deeper layers of the biofilm that resisted the first treatment. The ability of the TB/EDTA combination to kill bacterial cells across the biofilm layers was demonstrated by CLSM analysis. Such analysis also revealed large areas of disaggregation in the biofilm architecture, supporting the hypothesis that EDTA may favor the penetration of TB into the biofilm, facilitating its interaction with bacterial cells and subsequent killing.

Among biomaterial-associated infections, those related to the use of central venous catheters (CVC) are particularly relevant. By several mechanisms, bacteria from the skin can migrate along the external and/or internal surface of the catheters, form biofilms, and represent the starting point of CVC-related

bloodstream infections (Yousif et al. 2015). Removal of large CVCs is not always feasible, and replacement is expensive and associated with a procedural risk for the patient. On the other hand, systemic antibiotic administration frequently fails to achieve sterilization. Among the numerous approaches used to prevent intraluminal contamination of catheters is the so-called lock therapy (Sousa et al. 2011; Justo & Bookstaver 2014). This consists in closing the catheter and exposing its internal surface to 1–2 ml of a solution containing a highly concentrated antibiotic (up to 1,000 times the MIC) in order to eradicate the biofilm and an anticoagulant to prevent thrombosis (Sousa et al. 2011; Justo & Bookstaver 2014). After 12–24 h, the lock solution is removed, but repetition of the treatment at 24 h intervals for up to 15-days might be needed for complete eradication. Although clinical guidelines recommend the lock therapy in non-complicated cases of long-term catheter stay, the actual lock therapies have several limits regarding both the antimicrobial and the anticoagulant agents (Mermel et al. 2009; Justo & Bookstaver 2014). Among the major limits, there is the need to use high concentrations of antibiotics that may cause systemic toxicity and/or promote the selection and diffusion of multidrug resistant strains. Regarding the anticoagulant agent, heparin is frequently used, but it has several disadvantages (McIntyre et al. 2004). It causes the development of thrombosis mediated by anti-heparin antibodies and, at clinical relevant concentrations, it may enhance biofilm formation (Shanks et al. 2006). Moreover, locking of long-term intravenous catheters reduces their availability for several days and to date no antibiotics singularly used are able to eradicate CVC-associated bacterial biofilms in an acceptable time-span. For all these reasons, there is a pressing need to identify more effective lock solutions to eradicate intraluminal biofilms and reduce the time during which the catheter is unavailable. In this regard, EDTA used alone or in combination with antibiotics has recently emerged as a promising adjuvant of antimicrobial catheter lock solutions due to its dual anticoagulant and anti-biofilm activity (Chauhan et al. 2012). In the present study, it was demonstrated that TB in combination with EDTA could greatly reduce the viable count of *S. epidermidis* biofilms grown on the surface of polystyrene plates and kill bacteria released from the biofilm. These results suggest a possible use of the TB/EDTA combination for the lock therapy of catheters infected with *S. epidermidis*, one of the most common bacterial species involved in the colonization of this type of medical devices. As the characteristics of the surface used for biofilm formation might greatly influence the phenotype of tolerance towards antimicrobial agents (Beloin et al. 2014; Lebeaux et al. 2015), the combination TB/EDTA was assayed against *S. epidermidis* biofilms established on segments of silicone catheters, simulating the antibiotic lock therapy.

The results obtained demonstrated that two consecutive exposures of 4 h each to 100 μg ml^{-1} of TB combined with 4 mg ml^{-1} of EDTA substantially reduce the CFU count and potentially eradicate *S. epidermidis* biofilms from the catheter surfaces. Of note, this effect was obtained at concentrations and exposure times lower than those reported in similar studies in the literature. For instance, minocycline tested at 3 mg ml^{-1} in combination with 30 mg ml^{-1} EDTA was able to eradicate *S. epidermidis* biofilms after incubation for 24 h, while a combination of vancomycin (3 mg ml^{-1}) and heparin (100 U ml^{-1}) had no significant impact in reducing colonization of catheter segments by *S. epidermidis* (Raad et al. 2003). In another study, EDTA was tested alone at a concentration of 40 mg ml^{-1} for 21 h in *in vitro* models of catheter-associated biofilms (Percival et al. 2005). Recently, the antibiofilm effect of gentamicin, used at 5 mg ml^{-1}, was found to be increased by EDTA at 30 mg ml^{-1} against clinical isolates of *S. epidermidis* (Lebeaux et al. 2015). It is noteworthy that the TB/EDTA combination at the eradicating concentration (100 μg ml^{-1} TB, 4 mg ml^{-1} EDTA) was not hemolytic towards human erythrocytes. Thus, the *in vitro* data provide encouraging evidence for a potential application of the TB/EDTA combination in the lock therapy of catheters colonized with *S. epidermidis* biofilms. Studies in animal models of CVC-related infections will be needed to demonstrate the efficacy of such combination *in vivo*.

In conclusion, the present study offers a detailed analysis of the anti-biofilm potential of TB against clinical isolates of *S. epidermidis*. The peptide was demonstrated to inhibit the formation of both polysaccharide-dependent and protein-dependent biofilms of *S. epidermidis* and reduce, to a certain extent, the viable count of mature biofilms of both types. The anti-biofilm properties of TB could be greatly enhanced by combining the peptide with Cys or EDTA against forming or mature biofilms of *S. epidermidis*, respectively. The anti-biofilm effect of the TB/EDTA combination against established biofilms was principally due to direct killing rather than to a mere disaggregating effect. Finally, the strong killing ability of the TB/EDTA combination was observed towards biofilms obtained on silicone catheters, suggesting the possible use of such a combination in the lock therapy of catheters colonized with *S. epidermidis*. The possible use of TB in combination with EDTA in the lock solution may reduce the risk of development of resistance compared to conventional antibiotics, while taking advantage of

the intrinsic anti-biofilm and anticoagulant properties of EDTA.

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G.M., S.E., and G.B. conceived and designed the experiments and wrote the paper. G.M., L.G., S.B., C.M., F.L.B. and M.D.L. performed the experiments and analyzed the data.

Disclosure statement

No potential conflict of interest was reported by the authors.

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