Article

Strategies to Prevent Biofilm Infections on Biomaterials: Effect of Novel Naturally-Derived Biofilm Inhibitors on a Competitive Colonization Model of Titanium by *Staphylococcus aureus* and Human Cells

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Abstract: Biofilm-mediated infection is a major cause of bone prosthesis failure. The lack of molecules able to act in biofilms has driven research aimed at identifying new anti-biofilm agents via chemical screens. However, to be able to accommodate a large number of compounds, the testing conditions of these screenings end up being typically far from the clinical scenario. In this study, we assess the potential applicability of three anti-biofilm compounds (based on natural compounds) as part of implanted medical devices by testing them on *in vitro* systems that more faithfully resemble the clinical scenario. To that end, we used a competition model based on the co-culture of SaOS-2 mammalian cells and *Staphylococcus aureus* (collection and clinical strains) on a titanium surface. Additionally, we studied whether these derivatives of natural compounds enhance the previously proven protective effect of pre-incubating the titanium surface with SaOS-2 cells. Out of the three tested leads, one showed the highest potential, and can be regarded as a promising agent for incorporation into bone implants. This study emphasizes and demonstrates the importance of using meaningful experimental models, where potential antimicrobials ought to be tested for protection of biomaterials in translational applications.

Keywords: Biofilm; Co-culture; *Staphylococcus aureus*; SaOS-2; biomaterials; implanted devices

1. Introduction

Antimicrobial resistance is one of the major healthcare challenges that is currently faced by mankind. By switching into the biofilm state, bacteria can withstand antibiotic chemotherapy, and this is increasingly regarded as the most important nonspecific mechanism of antimicrobial resistance [1,2]. Biofilms are defined as a community of cells encased within a self-produced matrix that adhere to biological or non-biological surfaces [3,4]. Because implanted medical devices can be ideal substrates for bacteria to attach, biofilm-mediated infections are one of the leading causes of prosthesis implantation failures. Biomaterial-associated infections (BAIs) represent a great clinical
concern, because they cause increased morbidity and distress in patients, along with high economic costs due to increased hospitalizations [5].

When implanting a biomaterial, the desired outcome is the correct integration of such material with the host tissue. However, this ideal outcome is often impacted by the presence of bacterial cells at the moment of implantation. According to the concept of “race for the surface” [6], if host cells are able to colonize the surface of the device first, the chances of bacterial cells to adhere to such surface are lower, therefore lowering the risk of implant infection [7]. A frequent route of infection for implants occurs during surgery [8], as microorganisms can be introduced on the implant surface, providing them with an advantage to colonize the unprotected surface and create a biofilm [9]. Taking this in consideration, a reasonable approach would be to design an antimicrobial material or coating, which promotes tissue integration. In that direction, it would be advantageous to precondition the material with host cells [10]. Staphylococcus aureus is found asymptomatically on the skin [11] and its presence there enhances the risk of infection in the surgical site, which is why it is regarded as a frequent causative agent of implant-related infections, especially in orthopaedics [12].

Thus far, there is a limited repertoire of compounds that are able to act on biofilms at sufficiently low concentrations, especially in the case of S. aureus [13]. In order to tackle this problem, several strategies have been proposed, which include, among others, i) the screening of compound libraries to identify new small molecules able to inhibit or disassemble biofilms [14], ii) medicinal chemistry-driven approaches directed towards synthetic modifications of known antibiotics to increase their effectiveness on biofilms [15] and iii) development of novel nano-formulations to improve antibiotic penetration on biofilms [16]. Our laboratory has embraced the exploration of natural compound sources and those studies [17-19] have resulted in the identification of three promising anti-biofilm leads, shown in Figure 1.

The first two compounds are dehydroabietic acid (DHA) derivatives, N-(abiet-8,11,13-trien-18-oyl) cyclohexyl-L-alanine and N-(abiet-8,11,13-trien-18-oyl) D-tryptophan, coded DHA1 (shown in Figure 1a) and DHA2 (shown in Figure 1b), respectively. These two compounds were synthetically developed in [23] (coded 11 and 9b, respectively in this publication), by combining the abietane moiety with amino acids, which had separately been shown to display anti-biofilm properties [17,20-22]. Compounds DHA1 and DHA2 were both demonstrated to prevent biofilm formation as well as to effectively disassemble pre-formed S. aureus biofilms [23] and they represent the most potent abietane-type anti-biofilm agents that have been reported thus far. The third antimicrobial candidate (show in Figure 1c) is a flavan derivative: 6-chloro-4-(6-chloro-7hydroxy-2,4,4-trimethylchroman-2-yl)benzene-1,3-diol, coded FLA1, that has also been earlier shown to prevent bacterial colonization and decrease the viability of existing S. aureus biofilms [18] (coded 291 in that publication).

![Figure 1](image-url). Chemical structure of the two DHA derivatives, (a) N-(abiet-8,11,13-trien-18-oyl) cyclohexyl-L-alanine and (b) N-(abiet-8,11,13-trien-18-oyl) D-tryptophan, coded DHA1 and DHA2, as well as the flavan-derivative (c), 6-chloro-4-(6-chloro-7hydroxy-2,4,4-trimethylchroman-2-yl)benzene-1,3-diol, coded FLA1.

These three lead compounds are good candidates to protect medical devices from biofilm infections. Among the strategies used to protect implants are the modification of the material surfaces to inhibit bacterial adhesion [24,25] and the application of passive coatings to enhance tissue integration or compatibility. Altogether, these strategies aim at diminishing the rate of implant infection [26], which is essential not only to prevent biofilm formation per se but also to avoid aseptic loosening, which is also an important cause of prosthesis failure [27]. It is conceivable that these three
lead compounds could be used to develop new materials loaded with them, or that they could be incorporated into coating solutions.

However, moving from in vitro to translational studies can be highly challenging. Often, antimicrobial screening is carried out on materials like polystyrene, which might give dramatically different results from those obtained in more clinically-relevant surfaces, such as titanium. Similarly, in antimicrobial screens, collection strains of bacteria are often used, in which virulence and adherence capability might differ from those found in wild type strains isolated from implants [28,29]. In addition, promotion of tissue integration is not often tested on antimicrobial screens and if it is included, the testing is done separately from the antibacterial properties [30-34]. This is less than ideal as anti-biofilm/antibacterial capability and tissue integration should be tested together. In vitro co-culture models have been applied to better mimic the clinical situation. They do not only assess the effects of the leads or materials on bacterial and mammalian cells at the same time but they also provide with information of the antimicrobial effect of the interaction between these two cell types [9,35].

Our goal in this investigation was to study the applicability of these naturally compound-derived anti-biofilm leads (DHA1, DHA2 and FLA1) for protection of biomaterials, by using an in vitro system that better resembles the clinical conditions. Biofilm inhibiting effects of these leads were studied using collection and clinical bacterial strains. In addition, the anti-biofilm effects were measured on a competitive colonization model of titanium surfaces, exposed simultaneously to Staphylococcus aureus strains and human osteosarcoma cells (SaOS-2). Additionally, as it has been previously shown that the incubation of biomaterials with human cells before implantation can be an effective strategy to prevent bacterial adhesion and biofilm formation [36], we tested if the naturally-derived anti-biofilm leads would enhance this preventive effects, by testing them in titanium surfaces that had been pre-incubated with SaOS-2 cells.

2. Materials and Methods

4.1 Compounds

The two Dehydroabietic acid derivatives (DHA2 and DHA1) were synthesized according to [23]. Their spectral data were identical to those reported in [23]. The flavan-derivative coded FLA1, 6-chloro-4-(6-chloro-7-hydroxy-2,4,4-trimethylchroman-2-yl)benzene-1,3-diol, was purchased from TimTec (product code: ST0756729, www.timtec.net). Control antibiotics were purchased from Sigma-Aldrich: rifampicin (13292-46-1) and penicillin G (69-57-8).

4.2 Bacterial strains

Bacterial studies were performed with the collection strain Staphylococcus aureus ATCC 25923 (American Type Culture Collection, Manassas, Virginia) and five clinical strains isolated from hip prostheses and osteosynthesis implants, at the Hospital Fundación Jiménez Díaz (Madrid, Spain) [37] (S. aureus P1, P2, P4, P18 and P61).

4.3 Effects on biofilm viability in 96-wells microplates

Bacteria were cultured in 30g/L Tryptic Soy Broth (TSB, Fluka Biochemika, Buchs Switzerland) under aerobic conditions at 37°C, 220 rpm for 4 h to reach exponential phase. For forming biofilms, these exponentially grown cultures (10^6 CFU/mL) were added into flat bottomed 96-wells microplates (Nunclon Δ surface, Nunc, Roskilde, Denmark). The anti-biofilm effects of the compounds were assessed prior to biofilm formation and post-biofilm formation. This was carried out as described earlier [17]. Briefly, compounds (at a concentration of 50 µM, 2% DMSO) were added simultaneously with the bacterial suspension and effect were examined after incubation at 37°C, 200 rpm for 18 h, to assess the prevention of biofilm formation. For the post-biofilm formation testing, biofilms were first formed during 18 h (37 °C, 200 rpm), compounds were added and plates were incubated for 24 h at 37°C, 200 rpm. Untreated biofilms (only exposed to culture media and 2% DMSO), cell-free wells containing only TSB and wells containing biofilms and 2% of DMSO were included as controls.
Rifampicin and penicillin were used as positive controls, at the same concentration of the studied compounds. The effects on the biofilm viability were assessed following the protocol of [38] by resazurin staining. Briefly, the biofilms were washed twice (200 µL per well) with Phosphate Buffered Saline (PBS) and stained with 20 µM resazurin for 20 min at room temperature (RT), 200 rpm. The top fluorescence of the reduced resazurin was measured at \( \lambda_{\text{excitation}} = 560 \text{ nm} \) and \( \lambda_{\text{emission}} = 590 \text{ nm} \) using a Thermo Scientific Varioskan LUX Multimode Microplate Reader.

4.4 Competition model on a titanium surface

4.4.1. Culture of human cells

Human osteosarcoma SaOS-2 cells (89050205, European Collection of Authenticated Cell cultures (ECACC)) were grown in Minimal Essential Medium (MEM) (Sigma Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma Aldrich) containing 500 UI/mL penicillin and 0.1 mg/mL streptomycin (Sigma Aldrich). Cells were maintained at 37°C in 5% CO2 in a humidified incubator.

4.4.2. Cytotoxicity of compounds in 96 well plates

Before proceeding with the co-culture studies, the possible cytotoxicity of the compounds to SaOS-2 cells was assessed in opaque-walled well plates, using Promega CellTiter-Glo® Luminescent Cell Viability Assay. This assay is based on the quantification of the ATP present, which signals the presence of metabolically active cells, and therefore allows calculating the number of viable cells. To do this, 50000 cells per well were seeded. They were exposed to a concentration of 50 µM of each compound (0.25% DMSO), cells without treatment were used as control and effects of DMSO (0.25%) were also measured. Media alone well (with MEM) were used as blanks, and in the case of rifampicin, a blank containing only this compound was also included, as its red color may cause interference during the luminescence measurement. After 24 h incubation Promega CellTiter-Glo® assay was carried out, and the luminescence was measured using a Thermo Scientific Varioskan LUX Multimode Microplate Reader.

4.4.3. Culture of staphylococci and human cells

The bacteria \textit{S. aureus} (25923 or the clinical strains) was pre-cultured overnight at 37°C in 5 mL of Trypticase Soy Broth (TSB) medium [39]. It was later centrifuged at 4500 \( \times g \) for 10 min, the supernatant was discarded and the pellet washed three times with PBS. The optical density of the bacterial suspension was measured at \( \lambda \) of 600 nm with a Thermo Scientific Multiskan Sky Microplate Spectrophotometer according to the McFarland standard. The final concentration of the \textit{S. aureus} suspension was 10^6 CFU/mL. This suspension was then added to titanium coupons (0.4 cm height, 1.27 cm diameter, BioSurface Technologies Corp., Bozeman, MT, USA), onto which the different compounds or the control antibiotics had been added at a concentration of 50 µM and inserted in the different wells of a 24 well plate (Nuncclon Delta surface, Nunc, Roskilde, Denmark).

On the other hand, for the SaOS-2 cells, the media was refreshed 24 hours before the experiment started, in order to clear possible traces of antibiotics on the maintenance media. On the day of the experiment the cells were detached with a Trypsin:EDTA solution and re-suspended in MEM 10% FBS, afterwards they were seeded at a concentration of 10^5 cells/mL on the titanium coupons, onto which the bacterial suspension had been added as well as the studied compounds and control antibiotics (as described in the previous paragraph). The well plates containing the coupons were maintained in co-culture with the 10^6 \textit{S. aureus} and 10 5 SaSO-2 cells solution in a total volume of 1 mL of MEM:PBS 5% FBS for 24 hours, as originally described in [40]. Titanium coated with added rifampicin or penicillin (50 µM) were used as positive controls. As negative controls, titanium coupons without addition of the tested compounds or the control antibiotics were exposed to both cellular systems (\textit{S. aureus} and SaOS-2 cells) at the concentrations previously described. In addition, bacterial controls (exposed or not to the compounds, without SaOS-2 cells), SaOS-2 cells controls (exposed or not to the compounds, without bacterial cells) and a DMSO control (0.25 % DMSO...
coating titanium coupon in 10⁶ cells/mL, were also included. Furthermore, an extra bacterial control
(10⁶ CFU/mL of S. aureus on 1 mL of MEM:PBS 5% FBS ) was set in order to assess the possible
background caused by S.aureus in the measurement of SaOS-2 cells viability by luminescence.

For these studies, one of the S. aureus clinical strains, P2, was selected and compared to the effect
on the reference collection strain (S. aureus ATCC 25923). The clinical S. aureus strain P2 was chosen
as it was the one with the highest biomass-containing biofilm when compared to the collection strain
S. aureus 25923 (Supplementary Figure 1).

4.5 Competition model on a titanium surface pre-incubated with SaOS-2 cells

Following the protocol of Perez-Tanoira et al [10] a concentration of 10⁵ SaOS-2 cells/mL was
seeded on titanium coupons and incubated for 24 h. After the end of the incubation period, the cell
medium was removed and samples were washed three times with sterile PBS to remove any non-
adherent human cells. The different compounds were added to the coupons at a concentration of 50
µM. A suspension containing 10⁴ CFU/mL of S. aureus and 10⁵ SaOS-2 cells/mL on 1 mL of MEM:PBS
5% FBS, was added to each coupon and incubated for 24 hours. As a negative control, a titanium
coupon that had been pre-incubated with the SaSO-2 cells and added bacterial cells as described
before but not exposed to compounds, was used. As a bacterial control a suspension of 10⁴ CFU/mL
of S. aureus on 1 mL of MEM:PBS 5% FBS was added to a titanium coupon without pre-incubation
with SaOS-2 cells. As a cellular control, titanium was incubated with 10⁵ SaOS-2 cells/mL on 1 mL
of MEM:PBS 5% FBS, without addition of bacterial cells. Additionally another bacterial control (10⁴
CFU/mL of S. aureus on 1 mL of MEM:PBS 5% FBS ) was set in order to assess the possible background
caused by S.aureus in the measurement of cell viability by luminescence.

4.6. Measurement of SaOS-2 cells viability

Coupons were washed one with PBS and cells detached with 500 µL of a 1:10 trypsin:EDTA
solution. Then, 500 µL of MEM (containing 10% FBS ) was added to neutralize the trypsin:EDTA
solution. The resulting (1 mL) suspension was centrifuged at 150×g. Supernatant was discarded and
100 µL of MEM containing the cellular pellet was transferred to an opaque-walled well plate and
Promega CellTiter-Glo® Luminescent Cell Viability Assay was carried out. The possible background
signal corresponding to S. aureus was subtracted from the co-culture wells

4.7. Bacterial adherence and biofilm formation

Titanium coupons were washed with TBS to remove remaining planktonic cells and then they
were transferred into Falcon tubes containing 1 mL of 0.5% (w/v) Tween® 20-TSB solution. After that,
the tubes were sonicated in an Ultrasonic Cleaner 3800 water sonicator (Branson Ultrasonics,
Danbury, CT, USA) at 25°C, for 5 min at 35 kHz. The tubes were mixed vigorously for 20 s prior to
and after the sonication step. Serial dilutions were performed from the resulting bacterial
suspending, and plated on Tryptic Soy Agar (TSA) plates.

4.8. Fluorescence imaging

Titanium coupons were incubated in the same exact conditions as described in 4.4 and 4.5. After
incubation with the different compounds, coupons were washed three times with PBS. The dried
coupons were stained for 2 min with a rapid fluorescence staining method using acridine orange (BD
Diagnostics, Sparks, MD, USA) at a concentration of 0.1 mg/mL. After the staining, coupons were
rinsed with sterile water, to remove of the excess of dye. Images were taken with an Invitrogen EVOS
M500 Imaging System (Thermo Scientific, USA). On each coupon, 8-10 fields were viewed and
photographed at magnifications of 10x and 20x. All experiments were performed in duplicates and
experiments repeated twice.

4.9. Statistical analysis
The data is reported as mean of at least 3 samples ± SEM. Data were analysed using GraphPad Prism 8 for Windows. Non-parametric tests were used. For statistical comparisons, Welch’s unpaired t test and One-way ANOVA with Bonferroni correction were applied.

3. Results

2.1. Effect on the prevention and killing of biofilms formed by S. aureus clinical strains in 96-wells microplates

The three anti-biofilm leads studied here had been earlier tested using only collection strains [18,23]. Thus, prior to starting their effects on biomaterials, we set-out to test their anti-biofilm efficacy using five different clinical S. aureus strains, isolated from hip prostheses and osteosynthesis implants [37]. These results were compared to the effect of the compounds on S. aureus ATCC 25923, the collection strain previously used as reference. The effects on the biofilm viability were measured using a redox staining assay (resazurin-based) on biofilms grown on the polystyrene surface of 96-wells microplates.

As it can be seen in Figure. 2a, all the three compounds caused over 90% prevention of biofilm formation by all clinical strains, as well as the previously tested reference strain, with an effect fairly similar to rifampicin, a control antibiotic. In contrast, penicillin G was only efficient against the collection strain and one of the clinical strains (P61). When the effect was measured on clinical strains, on pre-formed biofilms (Figure 2b), the inhibitory effects of the three compounds was reduced (compared to Figure 2a), but still a significant inhibitory effect was measured in all cases and it was higher than the one reported by penicillin G. Rifampicin remained an effective antibiotic able to cause over 60% inhibition of the viability of pre-formed biofilms (Figure 2b).

![Figure 2](image_url)

Figure 2. Effect of the two dehydroabetic acid (DHA) derivatives (DHA1 and DHA2), the flavonoid-derivative (FLA1) and two commercial control antibiotics (rifampicin and penicillin G), at a concentration of 50 µM, in the prevention (a) or bactericidal (b) of S. aureus biofilms. Biofilms formed by one collection strain (ATCC 25923) and 5 different clinical isolates were tested. ‘*’ indicates differences with the collection strain (p*<0.033; p**<0.002; p***<0.001). Results are expressed of mean of inhibition ± SEM of three technical replicates, experiment repeated two times.

The antimicrobial capability of the three compounds to prevent biofilm formation by clinical strains was further tested on titanium coupons, as it could defer from the effects on prevention on the polystyrene surface of the 96-well plates. Supplementary Figure 2 shows how the change on the material already has an effect on the effectiveness of the compounds. As it can be seen, compound DHA2 suffers a drop of activity against both the collection (Supplementary Figure 2 a) and the clinical strain (Supplementary Figure 2 b), and the compound FLA1 when tested against the clinical strain (Supplementary Figure 2 b). Penicillin G is as well not so efficient when preventing biofilm prevention in titanium, and in concordance with the results obtained in 96-well microplates, it is less efficient against the clinical strain.

2.2 Effect on the prevention of S. aureus biofilms in a competitive colonization model on titanium coupons
Prior to testing the capacity of the compounds on the competitive colonization model, their possible cytotoxic effects towards SaOS-2 cells was assessed on polystyrene 96-well microplates and on titanium coupons (Supplementary Figures 3 and 4, respectively). None of the compounds showed cytotoxicity at a concentration of 50 µM in either of the materials.

Figure 3 shows the effects of the compounds on SaOS-2 viability, as well as S. aureus 25923 and P2 viable attached cells when bacteria and human cells are simultaneously co-cultured. The presence of either collection or clinical S. aureus strains induce a slight proliferative effect on the SaOS-2 cells, but this effect is not statistically relevant. Only compound FLA1 diminished SaOS-2 viability in the presence of the clinical strain P2 (Figure 3b).

On the other hand, the presence of SaOS-2 cells caused a significant reduction of the attached viable S. aureus ATCC 25923 when compared with the material incubated only with bacteria (p=0.007). All the compounds reduced the viability of bacterial cells in co-cultured with SaOS-2 cells, compared to the S. aureus ATCC 25923 control (Figure 3a). Compound DHA1 managed to cause a 4-log reduction when compared to the bacteria grown alone (S. aureus ATCC 25923 bar; p<0.001) and more than a 2-log reduction when compared to the co-culture control (SaOS-2 + S. aureus ATCC 25923 bar; p<0.001). The reduction of bacterial adhesion obtained after treatment with compound DHA2 is slightly smaller but yet significant (p=0.0247), being the number of S. aureus ATCC 25923 attached in this group 1-log less than in the co-culture control. Compound FLA1 caused a reduction of the viable S. aureus ATCC 25923 comparable to the one obtained with DHA1 (p<0.001). The control antibiotic rifampicin completely prevented S. aureus ATCC 25923 biofilm formation while penicillin G caused a significantly reduction (p<0.001 in both cases).

Considering the clinical S. aureus strain P2 (Figure 3d), the presence of mammalian cells did not cause a reduction of the attached viable bacteria. Compound DHA1 significantly reduced the viable attached S. aureus P2 by almost 2-log when compared to both bacterial (S. aureus strain P2) controls (p<0.001). Compounds DHA2 and FLA1 did not cause any significant reduction of the viable clinical bacteria in this competitive colonization assay. Rifampicin and penicillin G significantly reduced the viable attached clinical bacterial strain when compared to both bacterial controls, but penicillin G lost some activity, as it was measured with compound DHA1.

![Figure 3](image)

**Figure 3.** Effect of the two DHA derivatives (DHA1 and DHA2), the flavonoid-derivative (FLA1) on the competitive colonization assay performed in titanium coupons. (a), (b) Results corresponding to the viability of SaOS-2 cells when ATCC 25923 (a) or P2 clinical S. aureus strain (b) were used. (c), d) Results corresponding to the effects of attached viable S. aureus measured when ATCC 25923 (c) or P2 clinical strain (d) were used. Percentage of viability of SaOS-2 cells was calculated with respect to
untreated controls after 24-h incubation on titanium coupons, using glow luminescence signal resulting from ATP production by viable SaOS-2 cells. The background luminescence signal caused by S. aureus in the co-culture groups was subtracted as indicated in materials and method section. Viable counts (log of CFU/mL) of S. aureus 25923 and the clinical strain P2, respectively were also measured after 24-h incubation on titanium coupons when co-cultured with SaOS-2 cells. “*” represents differences with the bacterial control (S. aureus) and “#” represents differences with the co-culture control (S. aureus + SaOS-2). (p<0.033;p**<0.002;p***<0.001) Results are expressed as mean ± SEM of three technical replicates, experiments repeated three times.

Figure 4 shows fluorescence microscope images of the titanium coupons in the competitive colonization model, using the reference collection strain (S. aureus ATCC 25923). Figure 4 confirms that the presence of S. aureus ATCC 25923 does not cause a reduction in the number of SaOS-2 cells. A slight change on the SaSO-2 cells morphology can be noticed, when comparing the SaSO-2 monoculture control (Figure 4a) with the co-culture control (Figure 4c). The presence of bacteria seems to cause a slight reduction on the size of the cells and a loosening on its adhesive shape. This change on morphology is not observed in the samples treated with the control antibiotics, given its high antibacterial activity, but it can be noticed in the samples treated with DHA1, DHA2 and FLA1, as they do not completely prevent the biofilm formation. The S. aureus ATCC 25923 remaining on those samples might be the responsible of these changes in SaOS-2 cells morphology.

**Figure 4.** Representative fluorescence microscope images of titanium coupons treated under different conditions in the competitive colonization model. Upper row of the images (Figures 4a-c) correspond to the controls: a) titanium covered by 10⁵ human cells/mL (cell control); b) titanium covered by 10⁴ CFU/mL of S. aureus ATCC 25923 (bacterial control), and c) 10⁴ CFU/mL of S. aureus ATCC 25923 and 10⁵ human SaOS-2 cells/mL (co-culture control). Middle row of images (Figures 4d-f) correspond to titanium coupons coated with the two DHA derivatives (d) DHA1 and (e) DHA2, and the flavonoid-derivative FLA1 (f) and co-cultured with S. aureus ATCC 25923 and 10⁵ human SaOS-2 cells/mL. The bottom row of images (Figures 4g-h) are representative images of titanium coupons coated with the two control antibiotics g) rifampicin and h) penicillin G, all at a concentration of 50 µM and co-
cultured with $10^4$ CFU/mL of *S. aureus* ATCC 25923 and $10^5$ human SaOS-2 cells/mL. The samples were stained with acridine orange (BD Diagnostics, Sparks, MD, USA)

2.3. Effect on the prevention of *S. aureus* biofilms in a competitive colonization on titanium coupons pre-incubated with SaSO-2 cells

Figure 5 shows the effects of the compounds in coupons pre-incubated with SaSO-2 cells, when using both the reference *S. aureus* collection (ATCC 25923) as well as the clinical strain (P2). Only when bacterial collection strain and SaOS-2 cells were simultaneously incubated with compounds DHA1 or 9 b, a proliferative effect was found ($p=0.004$ and $0.037$, respectively) compared to control (Figure 5a). Co-culture of the clinical isolate P2 caused a significantly decrease on the mammalian cells viability ($p<0.001$) in contrast to collection strain which did not produce a significant effect (Figure 5b). In the controls, mammalian cells did not survive when co-cultured in the presence of *S. aureus* P2 strain, neither the SaSO-2 cells treated with the compounds DHA2 and FLA1. However, a significant proliferative effect of SaSO-2 cells was found in the presence of compound DHA1. Exposure to control antibiotics (rifampicin and penicillin G) also resulted in the protection of SaSO-2 cells and prevented the bacteria-induced cytotoxicity (Figure 5b).

Figures 5c and d show the effects of the compounds on the viable attached bacteria when using coupons pre-incubated with SaSO-2 cells. The pre-incubation of titanium with SaOS-2 cells significantly reduced the number of attached bacteria, in the case of the collection (ATCC 25923) strain (Figure 5c). In this case, exposure to compound DHA1 as well as the control antibiotics caused a total reduction of *S. aureus* ATCC 25923 attachment ($p<0.001$ in all cases). Compounds DHA2 and FLA1 caused a reduction of the viable attached bacteria, when compared to the bacterial (ATCC 25923) control ($p<0.001$, in both cases), and the reduction was also significant when compared to the cellular pre-coated coupons (SaOS-2 + *S. aureus* 25923 bar, Figure 5c) ($p<0.001$ and $p=0.035$, respectively).

In contrast, results of figure 5d show how the positive impact of cellular pre-conditioning was not detected for the clinical *S. aureus* strain P2. Interestingly, exposure to compound DHA1 caused a significant reduction on the attached bacteria as it significantly reduced 1-log the viable attached P2 strain when compared to both the bacteria control and the control corresponding to pre-coating of titanium with SaOS-2 cells (SaOS-2 + *S. aureus* P2 strain) ($p<0.001$). The activity of rifampicin was preserved against the clinical strain, while penicillin G, despite losing some activity when compared to its effect against the collection strain, also significantly reduced the viable bacteria (clinical P2 strain) attached ($p<0.001$ in both cases).
Figure 5. Effect of the two DHA derivatives (DHA1 and DHA2), the flavonoid derivative (FLA1) on competitive colonization on titanium coupons with cellular pre-coating. (a), (b) Results corresponding to the viability of SaOS-2 cells when ATCC 25923 (a) or P2 clinical S. aureus strain (b) were used. c, d) Results corresponding to the effects of attached viable S. aureus measured when ATCC 25923 (c) or P2 clinical strain (d) were used. Percentage of viability of SaOS-2 cells was calculated with respect to untreated controls after 24-h incubation on titanium coupons, using glow luminescence signal resulting from ATP production by viable SaOS-2 cells. The background luminescence signal caused by S. aureus in the co-culture groups was subtracted as indicated in materials and method section. Viable counts (log of CFU/mL) of S. aureus 25923 and the clinical strain P2 were also measured after 24-h incubation on titanium coupons when co-cultured with SaOS-2 cells. “*” Represents differences with the bacterial control (S. aureus) and “#” represents differences with the co-culture control (S. aureus + SaOS-2). (p<0.033; p<0.002; p<0.001/ p<0.033;p<0.002;p<0.001). Results are expressed as mean ± SEM of three technical replicates, experiments repeated three times.

Finally, Figure 6 shows fluorescence microscope images of the titanium coupons in the competitive colonization model using titanium coupons that had been pre-coated with SaSO-2 cells. The protective effect of the pre-coating with SaOS-2 cells is visible, as the cells do not show such an acute change of morphology as the one observed when the SaOS-2 cells were directly co-cultured with S. aureus ATCC 25923 (Figure 4c versus Figure 6c). The different treatments (Figures 6d-h) do not appear to affect the morphology or the number of SaOS-2 cells when compared to the cellular monoculture control (Figure 6a).

| Controls |
|------------------|------------------|------------------|
| (a) Cell control: SaOS-2 | (b) Bacteria control: S. aureus 25923 | (c) Co-culture: SaOS-2 + S. aureus 25923 |
| DHA derivatives (Co-culture) | Flavonoid-derivative (Co-culture) |
| (d) DHA1 (50μM) | (e) DHA2 (50μM) | (f) FLA1 (50μM) |
| Control antibiotics (Co-culture) | |
| (g) RIF (50μM) | (h) PEN (50μM) |

Figure 6. Representative fluorescence microscope images of titanium coupons treated under different conditions in a competitive colonization model with cellular pre-coating. Upper row of the images (Figures 6a-c) correspond to the controls: a) titanium covered by 10⁵ human cells/mL (cell control); b) titanium covered by 10⁴ CFU/mL of S. aureus ATCC 25923 (bacterial control), and c) 10⁴ CFU/mL of S. aureus ATCC 25923 and 10⁵ human SaOS-2 cells/mL (co-culture control). Middle row of images
4. Discussion

In this study, the applicability of three previously discovered antimicrobial compounds for protection of biomaterials against *S. aureus* biofilms was studied. To examine such effects a competitive model was utilized, allowing to investigate, in a more realistic scenario, the implantation of a biomaterial, which provides a substratum to host either tissue-cell integration or bacterial colonization. These two phenomena are in conflict, because after the adherence of either one, the surface is less prone to colonization by the other. Tissue integration and bacterial contamination of medical devices have been extensively studied as independent phenomena i.e. [41]. However, only recently, experimental assays have been established in which a fair competition can be investigated during the development of new biomaterial-coating strategies [40,42]. Using such tools, it has been recently shown that the incubation of typical implant materials with human SaOS-2 cells before implantation represents an innovative and effective way to reduce the bacterial living space available and prevent *S. aureus* adhesion, thus protecting biomaterials against biofilm formation [10]. Such approach would offer an attractive concept that could be further enhanced by the presence of antimicrobial compounds.

In current study, we hypothesized that two DHA derivatives (DHA1 and DHA2) and a flavonoid derivative (FLA1), which our group had previously reported as promising anti-biofilm leads [18,23], could find applicability in the protection of medical devices against infections. Here, we started by evaluating their antimicrobial efficacy against clinical bacterial strains [37]. In the case of *S. aureus*, its origin is particularly relevant. Besides being a virulent agent of implant infections, it is part of the normal bacterial flora of human skin and mucosal surfaces. This makes it obvious that the inter strain virulence, and as a consequence the efficacy of the tested compounds, may drastically change [43]. In fact, our results show how, despite being very efficient at both preventing and killing biofilms formed by the reference *S. aureus* ATCC 25923 strain, penicillin G loses great part of its activity when tested against most of the clinical strains (all except P61, Figure 2a and b). On the contrary, compounds DHA1, DHA2, FLA1 as well as the control rifampicin kept their preventive activity against all the clinical strains (Figure 2a). All of the tested compounds were also able to inhibit at least 30% of the viability of already formed biofilms by both the reference collection and the clinical strains.

Moreover, efficacy preventing biofilm formation of the different compounds was tested on titanium, given its relevance as biomaterial used for orthopaedic implants. In Supplementary Figure 2, it can be seen that compound DHA1 preserved its antimicrobial activity when tested on titanium. However, compound DHA2 was shown to lose its prevention capability on this material. This change is even more evident with the clinical strain, where neither the compound DHA2 nor FLA1 manage to prevent biofilm formation.

Titanium treated with DHA1, DHA2 or FLA1 was studied in a competitive colonization model with both bacteria and human (SaSO-2) cells. We used a *S. aureus* concentration of 10^6 CFU/mL as more than 10^5 CFU of *S. aureus* is necessary to establish a prosthesis infection and 10^6 CFU/mL in surgery without an implant. On the other hand, it has been proposed that using concentrations higher than 10^6 CFU/mL might be questionable and not relevant from a clinical perspective [44,45]. According to present results, and in concordance with the ones reported by [40], the presence of *S. aureus* did not significantly affect the viability of the SaOS-2 cells (Figure 3a and b). Previously, Yue, C et al. [46] have shown that low bacterial concentrations increase cell adhesion, likely as a result of the stress response caused by bacteria on the mammalian cells, which are then forced to compete more effectively and withstand cellular detachment. This effect was observed in both the collection strain and the clinical strain. None of the compounds produced a negative effect on the SaOS-2

(Figures 6d-f) correspond to titanium coupons coated with the two DHA derivatives (d) DHA1 and (e) DHA2, and the flavonoid derivative FLA1 (f) and co-cultured with *S. aureus* ATCC 25923 and 10^5 human SaOS-2 cells/mL. The bottom row of images (Figures 6g-h) are representative images of titanium coupons coated with the two control antibiotics g) rifampicin and h) penicillin G, all at a concentration of 50 µM and co-cultured with 10^6 CFU/mL of *S. aureus* ATCC 25923 and 10^5 human SaOS-2 cells/mL. The samples were stained with acridine orange (BD Diagnostics, Sparks, MD, USA).
cellular viability, but surprisingly, the cells treated with the compound FLA1 died when exposed to the clinical S. aureus P2 strain (Figure 3 b). From fluorescence imaging (Figure 4), it can be seen how the presence of bacteria has an effect in the morphology of the SaOS-2, despite not affecting the number of attached cells. It can be observed that this change in morphology is reduced in presence of antimicrobial treatments such as rifampicin (Figure 4 g), likely due to the fact that this antibiotic reduces practically to zero the presence of bacteria.

As mentioned before, the success of an anti-infective prosthesis relies not only on the efficacy to eradicate bacteria, but also in its ability to promote bone-implant osseointegration. Titanium is an inert material that does not accelerate this process, which is why many approaches towards implant development aimed at bio-functionalizing titanium to improve its bioactivity. The difficulty remains in modifying the titanium in such a way that it promotes osseointegration while being antimicrobial, as in many occasions, the antimicrobial agents incorporated are cytotoxic to the osteoblasts. Nie B, et al. [47] developed a biofunctionalized titanium with bacitracin that proved to be antimicrobial and cytocompatible in vitro. The efficacy of such titanium on preventing infection and improving osteoinductivity was later proven in vivo [48]. This is a good example of how an antimicrobial that is intended to form part of a prosthesis, has not only to be proven as antimicrobial but also cytocompatible in vitro, before considering it suitable for further experimentation, as both qualities are essential for a correct integration of the prostheses. The in vitro system described in this study not only assessed both qualities in the same assay, but it also gives information on the effect that the antimicrobial has on the adherence on the material of each cell type, mammalian and bacteria, when they are present together.

The second part of this study aimed at assessing the utility of the compounds in enhancing the positive effect of pre-incubating of materials with human SaOS-2 cells [36]. As previously demonstrated, the development of an infection would highly depend on what type of cells colonize the surface of an implant first, the cells of the host or the invading bacteria cells. Unfortunately, during surgery, bacterial cells frequently are in advantage, as they can be introduced onto an implant before integration with host tissue even starts. Giving advantage to the host cells would facilitate tissue integration and diminish the risk of bacterial infection [9]. By combining the protective effect of pre-conditioning titanium with mammalian cells with the previously reported anti-biofilm capability of the studied compounds, it was expected to accomplish a drastic reduction of the biofilm formation.

The pre-exposure of SaOS-2 to S. aureus 25923 generates the same slight proliferation effect on the mammalian cells at 24 h (Figure 5 in comparison to Figure 3). This proliferation is less acute in the case of rifampicin and penicillin, probably due to their high efficacy of killing the bacterial cells. None of the compounds appeared to affect the morphology of the cells. Perez-Tanoira, R et. al 2017 ([36]) reported a drop on the proliferation of SaOS-2 cells at 48 h exposed to the same concentration of the clinical isolate S. aureus 15981. In our study, the presence of the clinical S. aureus strain P2 dramatically decreases SaOS-2 cells viability in co-culture. It could be explained by a higher virulence of this strain. This drop on the cell viability can also be observed on the groups treated with DHA2 and FLA1, probably due to the lack of antibacterial activity of those compounds (Figure 5 b).

In this study, we analysed three compounds that based on our earlier results were regarded as promising candidates to prevent S. aureus biofilms related infections. However, out of the three, we demonstrated here that only compound DHA1 remains effective in conditions that are more likely to be encountered during an in vivo bacterial infection in an orthopaedic implant. In the case of the compound DHA2, its lack of activity, might be given by the fact that the concentration used here is slightly below its MIC [23]. As with most antimicrobials, a common concern is the unspecific cytotoxicity, both DHA derivatives had already been tested on mammalian cells, specifically HL cells (originating from the human respiratory tract). Compound DHA1 did not cause any reduction on the viability of this cell line, but with a concentration of 100 µM of DHA2, only 23 % of the cells remained viable. This is why a concentration of 50 µM was chosen, as in a 96-well microplate system it did not show cytotoxic effect but kept a considerably strong anti-biofilm capability. An increase of the concentration on the co-culture system would have probably shown a higher antimicrobial effect,
but also a slight cytotoxic effect, so it would have anyway limit the efficacy of DHA2 as a possible implant coating as it does not look like a good promoter of tissue integration. On the contrary, the compound FLA1 was used in a dosage that was expected to be effective [18], and despite the effectiveness shown in 96-wells microplate, the lack of activity in the competitive colonization system indicates that it is not suitable for protection of implants.

In this study, the compound DHA1 was shown to be a promising candidate to form part of a bone implant. However, the competitive colonization model used here is not meant to replace in vivo experimentation, and it can still be further improved, for instance by utilizing primary osteoblast cells. Indeed, in this scenario utilizing autologous osteoblast of the patient would be indispensable to avoid possible rejection of the prosthesis. In addition, this co-culture model can be further enriched by the addition of immune cells, such as neutrophils, which are also present at the moment of implantation. In any case, as it stands, it does offer a deeper insight into the protective capacity of antimicrobial compounds, particularly those intended for protection of bone implants.

5. Conclusions

These results highlight the importance of developing new protocols in vitro that would more truthfully select the best antimicrobial candidates for specific applications and that would minimize the translational gap between the results of a preliminary screen and the clinical scenario. Additionally, we concluded that the DHA derivative, DHA1, would be a promising candidate for coating of biomaterials in order to prevent biofilm formation. This compound shows a preventive activity of S. aureus biofilm formation by both collection and clinical strains, and it also displays a positive effect on the adhesion of mammalian cells to titanium.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Biofilm biomass of S. aureus and five clinical strains. Figure S2: Effect of the two DHA derivatives (DHA1 and DHA2), the flavonoid derivative (FLA1) on the prevention of biofilm formation of (a) S. aureus 25923 or (b) S. aureus P2 on titanium surfaces. Figure S3: Effect of the two DHA derivatives (DHA1 and DHA2), the flavonoid derivative (FLA1) and two control antibiotics (rifampicin and penicillin) on SaOS-2 viability when cultured in 96 wells polystyrene plates. Figure S4: Effect of the two DHA derivatives (DHA1 and DHA2), the flavonoid-derivative (FLA1) and two control antibiotics (rifampicin and penicillin) on SaOS-2 viability when cultured on titanium coupons.


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