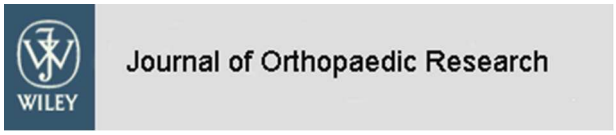

Antibacterial activity of a new broad-spectrum antibiotic covalently bound to titanium surfaces

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TITLE

Antibacterial activity of a new broad-spectrum antibiotic covalently bound to titanium surfaces

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Antibacterial SPI031-coated titanium surfaces

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AUTHOR CONTRIBUTION

In vitro experiments were designed by E.G., M.Fa., N.V. and J.M. and performed by E.G. Microscopic analysis was performed by A.K and M.L. *In vivo* experiments were carried out by S.K. and P.V.D. Cytotoxicity was assessed by M.Fr. and B.D. Ti discs were produced by F.I. and covalently coated by M.E. The surface roughness profile was determined by A.B. and J.Vl., S.C.A.R., H.P.S, J.Va., K.D.B., K.T. and B.P.A.C. coordinated the study. The manuscript was written by E.G. and edited by N.V. and J.M. All authors have read and approved the final manuscript.

ABSTRACT

Biofilm-associated infections, particularly those caused by *Staphylococcus aureus*, are a major cause of implant failure. Covalent coupling of broad-spectrum antimicrobials to implants is a promising approach to reduce the risk of infections. In this study, we developed titanium substrates on which the recently discovered antibacterial agent SPI031, a N-alkylated 3, 6-dihalogenocarbazol 1-(sec-butylamino)-3-(3,6-dichloro-9H-carbazol-9-yl)propan-2-ol, was covalently linked (SPI031-Ti). We found that SPI031-Ti substrates prevent biofilm formation of *S. aureus* and *Pseudomonas aeruginosa in vitro*, as quantified by plate counting and fluorescence microscopy. To test the effectiveness of SPI031-Ti substrates *in vivo*, we used an adapted *in vivo* biomaterial-associated infection model in mice in which SPI031-Ti substrates were implanted subcutaneously and subsequently inoculated with *S. aureus*. Using this model, we found a significant reduction in biofilm formation (up to 98 %) on SPI031-Ti substrates compared to control substrates. Finally, we demonstrated that the functionalization of the titanium surfaces with SPI031 did not influence the adhesion and proliferation of human cells important for osseointegration and bone repair. In conclusion, these data demonstrate the clinical potential of SPI031 to be used as an antibacterial coating for implants, thereby reducing the incidence of implant-associated infections.

KEY WORDS

Staphylococcus aureus, *Pseudomonas aeruginosa*, SPI031, biofilm, titanium

INTRODUCTION

The use of implant devices has risen exponentially in the last decades. However, the introduction of foreign material into the human body significantly increases the risk of infection.[1] As such, implant-associated infections have become a major cause of implant failure.[2,3] These infections are difficult to treat and in most cases, removal of the implant followed by a long-term antimicrobial treatment is the only remedy.[4] This leads to increased morbidity and mortality and puts a great financial burden on healthcare systems.[3]

Implant-related infections are frequently caused by Gram-positive staphylococci such as *Staphylococcus aureus* and by Gram-negative rod-shaped bacteria such as *Pseudomonas aeruginosa*.[5] It is widely accepted that biofilm formation plays a key role in the development of these infections.[6,7] These biofilms consist of a microbial community embedded in a matrix of extracellular polymeric substances. Generally, biofilms are 10 to 1000 times more resistant to antimicrobials, severely hampering the successful treatment of biofilm-associated infections.[8]

Various approaches have been reported to reduce the infection rates associated with implant devices, including the development of antimicrobial coatings on implant surfaces.[9,10] These coatings can be classified as active or passive. Active coatings are designed to release high amounts of antimicrobial agents, such as antibiotics and antiseptics, immediately after implantation.[11] However, this strategy has some limitations such as an elevated local toxicity and a reduced long-term activity.[9] Therefore, recent research has focused on the development of passive coatings that do not release antimicrobial agents but inhibit microbial adherence to the implant and/or kill the pathogen by contact.[12–19]

Recently, we identified a N-alkylated 3, 6-dihalogenocarbazol 1-(sec-butylamino)-3-(3,6-dichloro-9H-carbazol-9-yl)propan-2-ol as an antibacterial compound. This compound, SPI031, exhibits broad-spectrum antibacterial activity against Gram-positive and Gram-negative pathogens, including *S. aureus* and *P. aeruginosa.*[20] In the present study, we investigated the potential of SPI031 for use as a passive implant coating. We show that titanium (Ti) surfaces can be successfully functionalized with SPI031. In addition, we demonstrate that SPI031-functionalized Ti substrates significantly prevent colonization by *S. aureus* and *P. aeruginosa in vitro*. Moreover, we corroborated these observations *in vivo*, using a *S. aureus* biomaterial-associated murine infection model. Finally, we show that the functionalized Ti substrates support osseointegration potential *in vitro*.

MATERIAL AND METHODS

Bacterial strains and chemicals

S. aureus SH1000 cells[21] and *P. aeruginosa* cells PA14[22] were grown at 37 °C in Trypticase Soy Broth (TSB, Becton Dickinson Benelux) or on solid TSB medium containing 1.5 % agar. SPI031 was supplied by the Centre for Drug Design and Discovery (CD3, Leuven, Belgium).

Covalent binding of SPI031 to Ti discs

To enhance osseointegration, one side of round titanium discs (commercially pure titanium, grade 2; height: 2 mm; diameter: 5 mm) were first roughened by bead blasting with high purity Al_2O_3 particles, followed by etching using an acid mixture and finally by washing with isopropanol. A 3D surface roughness analysis was performed by white light interferometry (Wyko NT 3300 Optical Profiler, Veeco Instruments, Mannheim, Germany), measuring a

total of ten positions distributed over two samples. The roughness data were analyzed using the MountainsMapH Premium software (Digital Surf, Besançon, France), showing an average surface roughness, S_a, of 0.69 ± 0.10 µm, while the ten point height (average height of the 5 highest and 5 lowest points), S_z, and the developed interfacial area ratio, S_{dr}, amounted 8.90 ± 2.00 µm and 2.69 ± 1.11 %, respectively. Next, discs were functionalized by coupling with Fmoc-protected 3-aminopropyl-triethoxy silane[23] and were deprotected by piperidine in tetrahydrofuran (90:10). Discs were first placed in a hydrolysis vessel containing 45 mL n-heptane / hexamethylene diisocyanate (85:15) for 3 h at room temperature. Subsequently, discs were rinsed with n-heptane and were covalently linked to SPI031 by placing them in a solution containing 30 mL DMSO and 0.050 g SPI031 for 16 h with gentle agitation. Finally, the covalently linked SPI031-Ti discs were washed three times with DMSO, three times with demineralised, pyrogen-free water and finally with acetone, and were subsequently allowed to dry.

Quantification of SPI031 attached to Ti discs by high-performance liquid chromatography (HPLC)

The amount of immobilized SPI031 on the Ti discs was measured via HPLC upon hydrolysis, resulting in release of bound compound. To this end, SPI031-Ti discs were placed in hydrolysis vessels containing 1 mL demineralized water, 0.5 mL isopropanol and 1 mL triethylamine. The vessels were locked and heated to 60 °C for 1 h. Subsequently, the solvents were evaporated at 60 °C and the residues were dissolved in 0.5 mL demineralized water. The amount of SPI031 in the residues was quantified using HPLC equipped with a C18 column

(50 x 2.1 mm). The SPI031-Ti discs remained stable for at least 12 months when stored at 4 °C.

In vitro quantification of biofilm formation on SPI031-Ti discs

Before biofilm formation, control-Ti and SPI031-Ti discs were incubated in Fetal Bovine Serum (FBS, Life Technologies, Europe) overnight at 37 °C, to mimic the *in vivo* environment of the human tissue. Next, the discs were washed in PBS and transferred to the wells of a 24-well plate. Sterile silicon tubes (9 mm OD x 5 mm ID × 15 mm L) (VWR International) were placed over the discs to exclude the, non-rough, sides and bottoms. Subsequently, 0.2 mL of a 1 x 10^4 cells/mL suspension of *S. aureus* or *P. aeruginosa* in 1/20 TSB was added to the discs and biofilms were allowed to grow for 24 h at 37 °C under static conditions. Biofilms formed on the discs were quantified by colony forming unit (CFU) counting. To this end, discs were washed with PBS to remove non-adherent cells, transferred to centrifuge tubes containing 1 mL PBS, vortexed for 1 min, sonicated at 45,000 Hz in a water bath sonicator (VWR USC 300-T) for 10 min and vortexed again. This procedure effectively removed the biofilms attached to the discs. The resulting suspensions containing the detached cells were serially diluted in a 10 mM $MgSO_4$ solution and spread on TSB agar plates. After 24 h of incubation at 37 °C, colonies were counted and CFUs per mL were calculated.

Confocal laser scanning microscopy (CLSM) analysis

S. aureus and *P. aeruginosa* biofilms were grown as described above on control-Ti and SPI031-Ti discs, and were stained using the LIVE/DEAD BacLight stain (Molecular Probes,

USA) following the manufacturer's instructions. Biofilm images were acquired using a CLSM (Leica TCS SP5, Heidelberg, Germany) in an inverted microscope configuration. Biofilms were observed using a HCX PL APO CS 63x/1.2 water-immersion objective. During CLSM analysis, 25 digital images with 2048 x 2048 resolutions were taken with X-Y scan a few µm above the surface plane. The laser emissions used to excite the LIVE and DEAD stains were argon (488 nm) and HeNe (594 nm), respectively. Images obtained by CLSM were analyzed in Matlab using an in-house developed software macro to quantify the area fraction of live and dead cells in a thin optical section close to the surface of the disc. This macro is made to automatically run large series of images by subtracting the background, removing artifacts smaller than 20 pixels and calculating the area covered by objects over a set intensity.

In vivo quantification of biofilm formation on SPI031-Ti discs

All *in vivo* experiments were approved by the Animal Ethical Committee of the KU Leuven (project number P125/2011). Female pathogen-free BALB/c mice (20 g, 8 weeks of age) were purchased from Janvier (France). Mice were maintained in individually ventilated cages (4 mice/cage) and were allowed *ad libitum* access to sterile food and water. 24 h before surgery, the mice were immunosuppressed by the addition of 0.4 mg/L dexamethasone to the drinking water. The immunosuppression was maintained throughout the entire experiment. At the day of surgery, mice were anesthetized intraperitoneally with a mixture of ketamine (Ketamine1000®; Pfizer, Puurs, Belgium) and medetomidine (Domitor®; Pfizer) (45 mg/kg ketamine and 0.6 mg/kg medetomidine), followed by shaving and disinfecting of the lower back by iodine isopropanol (1 %) and administration of local anesthesia (xylocaine gel 2 %,

AstraZeneca, Zoetermeer, the Netherlands). A subcutaneous incision of approximately 2 cm long and 1 cm wide was made for the implant of 1 disc. After implant, the wound was closed with surgical staples, disinfected and locally anesthetized with xylocaine gel. An intraperitoneal injection of atipamezole was used to reverse anesthesia (Antisedan; Pfizer, 0.5 mg/kg for mice). 24 h after implant, the mice were anesthetized with a mixture of ketamine and medetomidine as described above. Next, the discs were inoculated with a bacterial culture of *S. aureus* which was washed and resuspended in sterile saline (0.9 %) to a concentration of 1 x 10^8 cells/mL. 100 µl of the bacterial inoculum was injected subcutaneously into the area around the disc. Anesthesia was reversed with an intraperitoneal injection of atipamezole as mentioned above. After 4 days of biofilm formation, mice were euthanized by cervical dislocation. The skin was disinfected (0.5 % chlorhexidine in 70 % alcohol) and the discs and the surrounding tissue were removed. Biofilm formation on the discs was quantified by CFU counts. Briefly, discs were washed two times with PBS, sonicated at 40,000 Hz in a water bath sonicator (Branson 2210) for 10 min and vortexed for 30 s in 1 mL PBS. The tissue surrounding the discs was weighed and homogenized. The resulting bacterial suspensions (discs and surrounding tissues) were diluted and plated on TSB agar plates in duplicate. After 24 h of incubation at 37 °C, CFUs were counted.

Effect of SPI031-coating on the osseointegration response *in vitro*

To test the osseointegration potential of SPI031-coated discs, human bone marrow derived stromal cells (MSC) and human microvascular endothelial cells (HMVEC) were used. MSC were cultured in advanced Dulbecco's Modified Eagle Medium (Life Technologies, USA) supplemented with 10 % FBS, 1x GlutaMAX and 0.05 mg/mL gentamicin (Gibco, Carlsbad,

CA). HMVEC were grown in medium 131 supplemented with Microvascular Growth Supplement (Life Technologies, USA). Cells were seeded at a cell density of 5000 cells/cm^2 and cultured in 5 % CO_2 at 37 °C for one passage. After reaching 95 % confluence, cells were trypsinized (Trypsin-EDTA, Sigma Aldrich) and counted with a hemocytometer. Cells of the 4^{th} passage were used for the experiments.

Subsequently, the control-Ti and SPI031-Ti discs were placed into the wells of a 24-well plate and were seeded with cells at a cell density of 9000 cells/disc. The discs were kept in the incubator to allow attachment and after 30 min, additional culture medium was added. Cells were cultured for 5 or 12 days, and were then fixed with formalin for 15 min and washed 3 times with PBS. Then, cells were incubated in a Phalloidin solution (stock solution 0.1 mg/mL in methanol diluted 1:20 in PBS) to stain actin filaments. After 30 min of incubation in the dark at room temperature, the discs were washed 3 times with PBS and were incubated in a Vectashield/DAPI (Vector Laboratories, USA) solution to stain nuclei. All samples were imaged with a 40x objective on a Nikon T300 fluorescent microscope.

Statistical analysis

All *in vitro* experiments were carried out in technical duplicates and were independently repeated at least 3 times. The *in vivo* experiment was performed twice using 11 mice implanted with control-Ti discs and 15 mice implanted with SPI031-Ti discs. Statistical significance of data was determined by applying a student's t-test (Mann-Whitney test) using GraphPad Prism version 5 (GraphPad Software, USA). Differences were considered significant if $*p < 0.05$.

RESULTS

SPI031 prevents biofilm formation by *S. aureus* and *P. aeruginosa* on Ti discs *in vitro*

We covalently linked SPI031 to titanium discs as described in Materials and Methods. The amount of SPI031 coated on the discs was 54.8 pmol/cm^2 as determined by HPLC. To examine the antibiofilm activity of the SPI031-Ti discs *in vitro*, control-Ti and SPI031-Ti discs were inoculated with *S. aureus* and *P. aeruginosa* cells and biofilms were grown for 24 h where after CFUs were determined. As shown in Figure 1, there was a significant reduction of bacterial colonization of the substrates (70 % for *S. aureus* and 87 % for *P. aeruginosa*, $p < 0.05$). To confirm these results, biofilm growth on the control-Ti and SPI031-Ti discs was visualized by CLSM using the LIVE/DEAD stain consisting of a mixture of the SYTO 9 (green) and propidium iodide (red) nucleic acid fluorescent stains. SYTO 9 is used to label all cells in a bacterial population while propidium iodide only penetrates cells with damaged membranes. CLSM imaging also revealed a significant reduction of biofilm formation on SPI031-Ti discs, compared to the control-Ti discs (Figure 2A). Next, the area fraction covered by the biofilms on the discs was calculated. As seen in Figure 2B, the viable area fraction of staphylococcal and pseudomonal biofilms was significantly reduced on SPI031-Ti discs.

SPI031 prevents biofilm formation by *S. aureus* in a murine infection model

The activity of the SPI031-Ti discs under *in vivo* conditions was assessed using an adapted model of biomaterial-associated infection, which was originally developed to study *S. epidermidis* biofilm development on titanium and silicone substrates.[24] Briefly, the control-Ti and SPI031-Ti discs were implanted subcutaneously and the tissue adjacent to the discs was challenged with ~10^7 *S. aureus* cells to allow *in vivo* biofilm formation. After 4 days of

biofilm development, control-Ti discs contained on average 5.39 $\log_{10}$ CFUs. In comparison, SPI031-Ti discs contained on average 4 $\log_{10}$ CFUs, which corresponds to a significant reduction of 98 % ($p < 0.05$) (Figure 3A). Individual data points of each mouse are shown in Supplementary Figure S1. In addition, analysis of the tissue surrounding the implanted discs revealed a similar amount of bacteria colonizing the tissue surrounding the control-Ti and SPI031-Ti discs (Figure 3B), indicating that SPI031 was not or only minimally released from the discs.

SPI031-Ti does not affect osseointegration potential

Osseointegration, the close interaction of living bone tissue with implants, is an important factor in implant success.[25] Therefore, we assessed if the coated discs affected adhesion and proliferation of osteogenic (MSC) and vasculogenic (HMVEC) cells, two cell types that are known to be involved in osseointegration and bone repair.[25] In this assay, cells were visualized by staining their actin filaments (Phalloidin) and DNA (DAPI) after 5 or 12 days of incubation on control-Ti and SPI031-Ti discs, and cell attachment and growth were evaluated visually. The surfaces of the control-Ti and SPI031-Ti discs supported attachment and growth of MSC and HMVEC cells equally, suggesting that a suitable level of osseointegration can occur upon implantation of SPI031-coated implants (Figure 4).

DISCUSSION

Biomaterial-related infections are a major problem in implant surgery and impose a huge economic burden on healthcare systems.[2,3] Covalent immobilization of antibacterial agents on implant surfaces has been explored to inhibit these infections.[12–19] Although these coatings

have proven their effectiveness *in vitro*, it remains important to develop coatings with new antibacterial agents, thereby lowering the risk of developing antibiotic resistance.

In this study, we evaluated the antibiofilm properties and biocompatibility of titanium surfaces covalently coated with the new antibacterial compound SPI031, a N-alkylated 3,6-dihalogenocarbazol 1-(sec-butylamino)-3-(3,6-dichloro-9H-carbazol-9-yl)propan-2-ol. Silanization was used to covalently link the new antibacterial compound to titanium surfaces. This technique has previously been demonstrated to be an effective method for covalent coating of bioactive molecules to metallic surfaces[26] and the procedure employed in this study is carried out at ambient conditions, implying that this coating strategy is suitable for large-scale production of coated implants.[19] Furthermore, to our knowledge, this is the first study describing the development of an implant coating using a new non-peptide antibiotic, thereby possibly overcoming some disadvantages associated with peptide-based coatings such as reduced *in vivo* activity and potential toxicity.[27]

We demonstrated previously that SPI031 exhibits broad-spectrum antibacterial activity. Moreover, this compound does not exert a cytotoxic effect on several human cell types at the tested concentrations.[20] In the present study we examined the antibacterial activity of covalently bound SPI031 *in vitro* by challenging SPI031-Ti substrates with *S. aureus* and *P. aeruginosa*, two bacterial strains frequently involved in implant-related infections.[5] Significantly less bacterial cells were found on the SPI031-Ti discs, compared to the control-Ti discs. Of note, the coated discs were more active against the Gram-positive bacteria *S. aureus* than against the Gram-negative bacteria *P. aeruginosa*. This is likely caused by the fact that, in contrast to Gram-positive bacteria, Gram-negative bacteria possess an outer

membrane, making them less susceptible to antibacterial agents.[28] These differences in cell wall structure can possibly explain the observed differences in sensitivity.

To investigate the antibacterial activity of the SPI031-Ti substrates *in vivo*, a mouse biomaterial-associated model of infection was used. Strikingly, under these conditions *S. aureus* biofilm development on SPI031-Ti substrates was reduced by 98 %, compared to control substrates. It is likely that this reduction in staphylococcal colonization is sufficient to allow further clearance of the infection by the host defense mechanism.[29] It is noteworthy to mention that in this study *S. aureus* biofilm development was followed for 4 days. However, no difference was found between the number of bacteria in the tissue surrounding the control-Ti and SPI031-Ti discs, indicating that SPI031 was not released from the implant. Covalent coatings are known for their long-lasting effects[30], suggesting that potential biofilm formation on the coated discs can be inhibited for longer periods of time.

We previously demonstrated the *in vitro* and *in vivo* efficacy of covalently bound vancomycin, a glycopeptide antibiotic active against Gram-positive bacteria.[19] Our SPI031-coated substrates appear to be as active as these vancomycin-coated substrates. Moreover, since the SPI031-coated substrates are effective against both Gram-positive and Gram-negative bacteria, we believe this coating has even a wider range of application potential.

Next to an anti-bacterial strategy, osseointegration is a prerequisite for successful implant therapy.[25] Therefore, the osseointegration potential of the discs was evaluated *in vitro*. No differences in the adhesion and proliferation of mammalian cells important for osseointegration and bone repair were found between the control-Ti and SPI031-Ti discs. These results further support the potential of our coating technique to decrease the incidence of implant-related infections, without compromising osseointegration.

In summary, we were able to covalently link the new antibacterial compound SPI031 to titanium surfaces. Furthermore, these functionalized surfaces showed significant antibacterial activity both *in vitro* and *in vivo* without affecting the *in vitro* osseointegration potential. As such, these results demonstrate the clinical potential of our antibacterial coating. Future studies will be directed at further investigating the toxicity, biocompatibility and osseointegration of this coating *in vivo* and at evaluating its effectiveness using a clinically relevant orthopedic infection model.

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FIGURE LEGENDS

Figure 1: *In vitro* characterization of biofilm formation of *S. aureus* (A) and *P. aeruginosa* (B) on SPI031-Ti discs. Control-Ti and SPI031-Ti discs were inoculated with *S. aureus* and *P. aeruginosa* cells and incubated for 24 h. The results are expressed as percentage of biofilm formation on SPI031-Ti discs relative to control-Ti discs. Data represent the mean ± standard errors of the means (SEM) of 3 independent experiments (*$p < 0.05$).

Figure 2: CLSM analysis of biofilm formation of *S. aureus* and *P. aeruginosa* on SPI031-Ti discs. Control-Ti and SPI031-Ti discs were inoculated with *S. aureus* and *P. aeruginosa*

cells and incubated for 24 h. (A) CLSM images of *S. aureus* and *P. aeruginosa* biofilms grown on control-Ti and SPI031-Ti discs. Biofilms were stained with SYTO 9 (green; viable cells) and propidium iodide (red; dead cells). (B) The area fraction of live and dead biofilm cells visualized in Figure 2A. Data represent the mean ± SEM of 3 independent experiments (*$p < 0.05$).

Figure 3: ***In vivo* characterization of biofilm formation of *S. aureus* on SPI031-Ti discs.** Sampling was performed after 4 days of biofilm development. (A) Growth of *S. aureus* biofilms on control-Ti and SPI031-Ti discs. (B) *S. aureus* cells found in tissue surrounding control-Ti and SPI031-Ti discs. Data represent the mean ± SEM of 2 independent experiments (*$p < 0.05$).

Figure 4: *In vitro* analysis of the osseointegrative potential of SPI031-Ti discs. (A) Visualization of human microvascular endothelial cells bone marrow derived stromal cells grown on control-Ti and SPI031-Ti discs. (B) Visualization of human bone marrow derived stromal cells microvascular endothelial cells grown on control-Ti and SPI031-Ti discs.

Figure S1: *In vivo* characterization of biofilm formation of *S. aureus* on control-Ti and SPI031-Ti discs. Sampling was performed after 4 days of biofilm development. Single data points per mouse and mean ± SEM per group are shown.

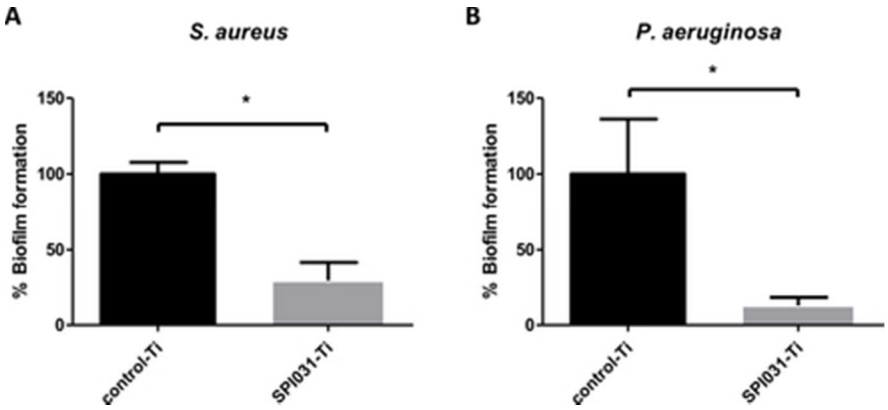

Figure 1: In vitro characterization of biofilm formation of S. aureus (A) and P. aeruginosa (B) on SPI031-Ti discs. Control-Ti and SPI031-Ti discs were inoculated with S. aureus and P. aeruginosa cells and incubated for 24 h. The results are expressed as percentage of biofilm formation on SPI031-Ti discs relative to control-Ti discs. Data represent the mean ± standard errors of the means (SEM) of 3 independent experiments (*$p < 0.05$).
37x16mm (300 x 300 DPI)

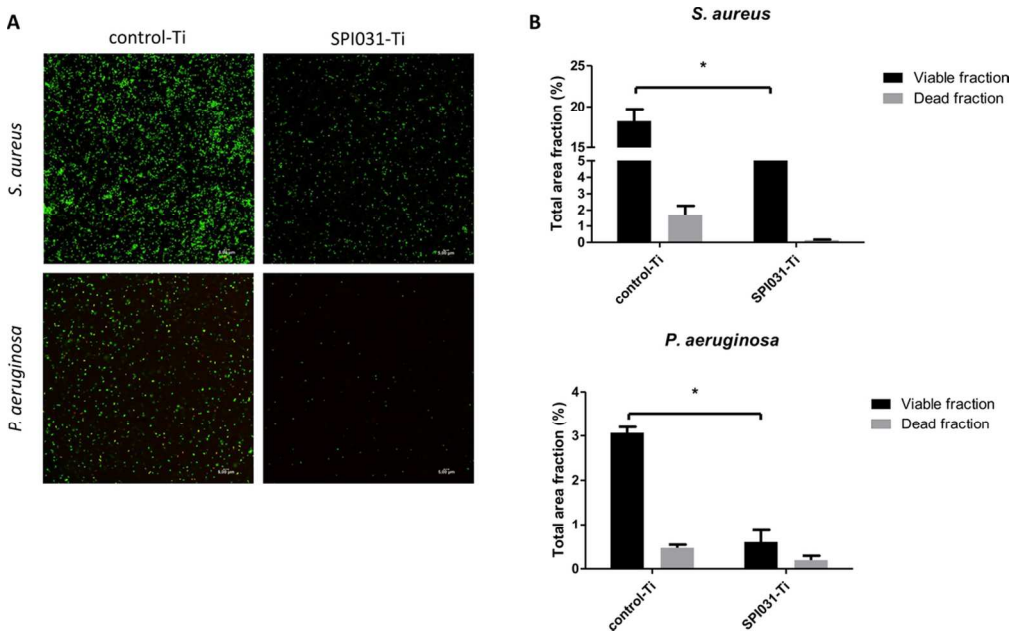

Figure 2: CLSM analysis of biofilm formation of S. aureus and P. aeruginosa on SPI031-Ti discs. Control-Ti and SPI031-Ti discs were inoculated with S. aureus and P. aeruginosa cells and incubated for 24 h. (A) CLSM images of S. aureus and P. aeruginosa biofilms grown on control-Ti and SPI031-Ti discs. Biofilms were stained with SYTO 9 (green; viable cells) and propidium iodide (red; dead cells). (B) The area fraction of live and dead biofilm cells visualized in Figure 2A. Data represent the mean ± SEM of 3 independent experiments (*p < 0.05).
105x65mm (300 x 300 DPI)

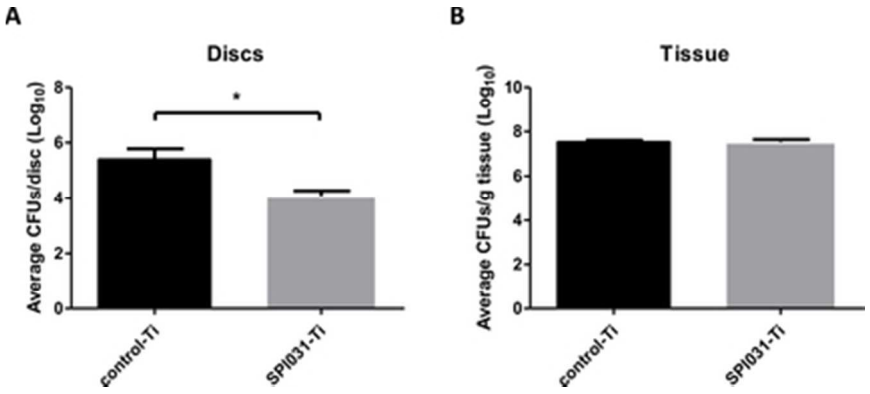

Figure 3: In vivo characterization of biofilm formation of S. aureus on SPI031-Ti discs. Sampling was performed after 4 days of biofilm development. (A) Growth of S. aureus biofilms on control-Ti and SPI031-Ti discs. (B) S. aureus cells found in tissue surrounding control-Ti and SPI031-Ti discs. Data represent the mean ± SEM of 2 independent experiments (*p < 0.05).

36x16mm (300 x 300 DPI)

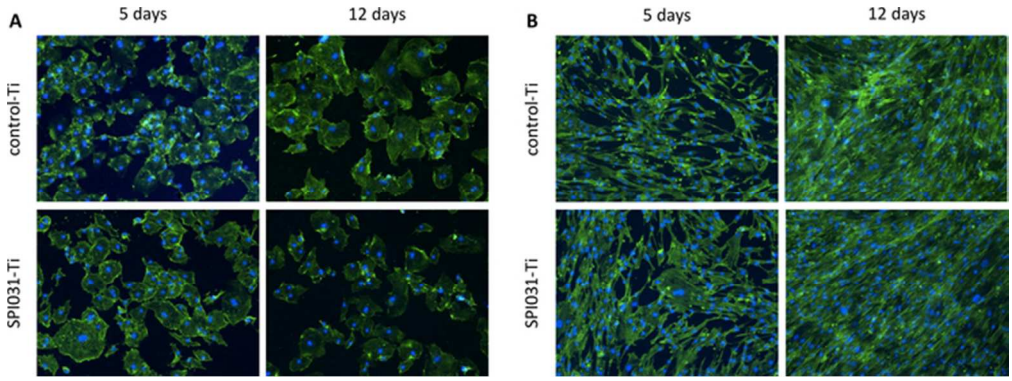

Figure 4: In vitro analysis of the osseointegrative potential of SPI031-Ti discs. (A) Visualization of human microvascular endothelial cells bone marrow derived stromal cells grown on control-Ti and SPI031-Ti discs. (B) Visualization of human bone marrow derived stromal cells microvascular endothelial cells grown on control-Ti and SPI031-Ti discs.
62x23mm (300 x 300 DPI)

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

[1]The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, [2]School of Veterinary Science, University of Bristol, Bristol, UK, [3]School of Biological Sciences, University of Bristol, Bristol, UK, [4]National Heart and Lung Institute, Imperial College London, UK, [5]Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Title page
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Abstract and Keywords
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	a. Introduction, Paragraphs 1-3 b. Introduction, Paragraph 4
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Introduction, Paragraph 4
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Material and Methods, Paragraph 6
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	a. Material and Methods, Paragraph 8 b. Material and Methods, Paragraph 6 c. Material and Methods, Paragraph 6

Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	a. Material and Methods, Paragraph 6 b. Material and Methods, Paragraph 6 c. Material and Methods, Paragraph 6 d. Material and Methods, Paragraph 6
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	a. Material and Methods, Paragraph 6 b. Material and Methods, Paragraph 6

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010[1]

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	a. Material and Methods, Paragraph 6 b. Material and Methods, Paragraph 6 c. Material and Methods, Paragraph 6
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	a. Material and Methods, Paragraph 8 b. The number of available control and SPI031-coated discs determined the amount of mice used in this study. c. Material and Methods, Paragraph 8
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	a. Mice were randomly divided into two groups b. Mice were treated randomly and one by one in the two groups
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Material and Methods, Paragraph 6
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	a. Material and Methods, Paragraph 8 b. For each test, the experimental unit was an individual animal. c. Material and Methods, Paragraph 8
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Material and Methods, Paragraph 6

Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	a. Material and Methods, Paragraph 8 b. Data of all animals was used
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Results, Paragraph 2
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	a. No adverse events were seen b. No adverse events were seen
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	a. Discussion, Paragraph 4 and 5 b. No specific limitations were observed c. This study was performed simultaneously with another study (Kucharíková *et al.*, 2015), thereby lowering the number mice implanted with control-Ti discs.
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Discussion, Paragraph 7
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Acknowledgments

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.