Halicin remains active against *Staphylococcus aureus* in biofilms grown on orthopaedically relevant substrates

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Aims

Biofilm infections are among the most challenging complications in orthopaedics, as bacteria within the biofilms are protected from the host immune system and many antibiotics. Halicin exhibits broad-spectrum activity against many planktonic bacteria, and previous studies have demonstrated that halicin is also effective against *Staphylococcus aureus* biofilms grown on polystyrene or polypropylene substrates. However, the effectiveness of many antibiotics can be substantially altered depending on which orthopaedically relevant substrates the biofilms grow. This study, therefore, evaluated the activity of halicin against less mature and more mature *S. aureus* biofilms grown on titanium alloy, cobalt-chrome, ultra-high molecular weight polyethylene (UHMWPE), devitalized muscle, or devitalized bone.

Methods

S. aureus-Xen36 biofilms were grown on the various substrates for 24 hours or seven days. Biofilms were incubated with various concentrations of halicin or vancomycin and then allowed to recover without antibiotics. Minimal biofilm eradication concentrations (MBECs) were defined by CFU counting and resazurin reduction assays, and were compared with the planktonic minimal inhibitory concentrations (MICs).

Results

Halicin continued to exert significantly (p < 0.01) more antibacterial activity against biofilms grown on all tested orthopaedically relevant substrates than vancomycin, an antibiotic known to be affected by biofilm maturity. For example, halicin MBECs against both less mature and more mature biofilms were ten-fold to 40-fold higher than its MIC. In contrast, vancomycin MBECs against the less mature biofilms were 50-fold to 200-fold higher than its MIC, and 100-fold to 400-fold higher against the more mature biofilms.

Conclusion

Halicin is a promising antibiotic that should be tested in animal models of orthopaedic infection.

Bone & Joint Research

Article focus

- Does halicin remain antibacterially active against *Staphylococcus aureus* biofilms grown on different orthopaedically relevant substrates?

- How does the activity of halicin against biofilms compare with the activity of vancomycin against biofilms on different orthopaedically relevant substrates?

Key messages
- Halicin remains active against *S. aureus*-Xen36 biofilms grown on different orthopaedically relevant substrates.
- Halicin exerts substantially more activity than vancomycin against both less and more mature biofilms.

Strengths and limitations
- A strength of this study is that the effects of halicin were measured on *S. aureus* biofilms grown on multiple orthopaedically relevant substrates, including titanium alloy, cobalt-chrome, ultra-high molecular weight polyethylene, devitalized muscle, and devitalized bone.
- A limitation of this study is that only *S. aureus* biofilms were compared. Future studies will focus on other bacterial biofilms common in orthopaedic infections.
- Future studies should test the effects of halicin on biofilms in orthopaedic infection animal models.

Introduction

Bacterial biofilms readily form on orthopaedic implants and musculoskeletal tissues. These biofilm infections are often due to *Staphylococcus aureus* and are among the most challenging complications in orthopaedics.[1-5] Biofilms protect bacteria from both the host immune system and antibiotics.[6,7] A primary mechanism responsible for antibiotic tolerance by biofilm bacteria is induction of quiescence, as most conventional antibiotics specifically target proliferating bacteria.[7-15] The minimum biofilm eradication concentrations (MBECs) for many antibiotics are substantially higher than the minimum inhibitory concentration (MIC) for planktonic bacteria, and are also substantially higher than the level that is clinically safe for therapeutic use.[16-19]

Halicin (SU3327), a nitrothiazole compound without structural similarity to any known antibiotic, was recently found to have broad-spectrum activity against planktonic bacteria.[20] Halicin was identified by a deep learning artificial intelligence approach and is effective against many bacteria strains including both methicillin-sensitive *S. aureus* and methicillin-resistant *S. aureus*.[20] Moreover, halicin did not induce resistance in vitro, and was also effective against persister cells in planktonic cultures.[20] Because planktonic persister cells are phenotypically similar to bacteria residing in biofilms, halicin might also kill quiescent bacteria within biofilms. Indeed, halicin was recently shown to remain active against *S. aureus* in both less mature and more mature biofilms grown on polystyrene substrates.[18] Similarly, other investigators found that halicin remains active against antimicrobial-resistant *S. aureus*, *Acinetobacter baumannii*, and *Escherichia coli* in immature 24-hour biofilms and mature seven-day biofilms grown on polypropylene substrates.[21] More recently, halicin was found to synergize with doxycline to reduce biomass of 24-hour *Enterococcus* spp. biofilms grown on polymeric substrates.[22]

Antibiotic susceptibility of bacterial biofilms varies depending on the substrates on which the biofilms are grown.[23-26] For example, susceptibility of *Staphylococcus* spp.

biofilms to antibiotics varied up to 250-fold depending on whether biofilms were grown on stainless steel, ultra-high molecular weight polyethylene (UHMWPE), or polymethyl-methacrylate (PMMA).[23] Moreover, the differences between substrates were substantially dependent on the tested antibiotic.[23] Antibiotic susceptibility of biofilms on surrounding tissues such as muscle and bone is perhaps more important to consider, as infected orthopaedic implants are commonly removed surgically, but it is difficult to remove all the necrotic tissue that harbours biofilms.[27,28] In that regard, *Staphylococcus* spp. biofilms grown on devitalized bone were up to 2,500-fold less susceptible to antibiotics than biofilms on PMMA, depending on the antibiotic that was tested.[24] Similarly, antibiotic susceptibility of biofilms grown on devitalized muscle or devitalized bone varied between 100-fold less and 100-fold more than biofilms on polystyrene depending on the strain of bacteria that was tested.[26] Taken together, the above studies show that it is not possible to predict a priori how biofilm growth on different substrates will affect susceptibility to a specific antibiotic.[23-26] This study therefore evaluated effects of halicin against both less mature and more mature *S. aureus* biofilms grown on orthopaedically relevant substrates, including titanium alloy (Ti6Al4V), Cobalt-Chrome (Co-Cr), UHMWPE, devitalized muscle, and devitalized cortical bone. Vancomycin was used for comparison as an antibiotic known to be affected by biofilm maturity.

Methods

Preparation of orthopaedically relevant substrates

Ti6Al4V discs (medical grade ELI 23, 12.7 mm diameter, Titanium Industries, USA) were autoclaved, sonicated in acetone for 30 minutes, and incubated in alkali ethanol (0.1 N NaOH and 95% ethanol at 32°C, overnight) and then in 25% nitric acid (room temperature, overnight).[29] Co-Cr and highly crosslinked UHMWPE discs were manufactured from a knee arthroplasty prosthesis. The Co-Cr discs (11.4 mm diameter, 5.0 mm thickness) were sonicated in alkali ethanol (0.1 N NaOH and 95% ethanol) for 30 minutes, and then incubated in alkali ethanol (0.1 N NaOH and 95% ethanol at 32°C, overnight). The UHMWPE discs (11.0 mm diameter, 5.1 mm thickness) were sonicated in 100% ethanol for 30 minutes, and then incubated in 100% ethanol overnight. Muscle and bone samples were harvested from New Zealand White rabbits. The rabbits were euthanized for other studies, and an ARRIVE checklist is therefore not appropriate for this study. Pieces of rabbit fore limb muscle (biceps brachii, triceps brachii, or brachialis) and cortical bone (humerus, radius, or ulna) without marrow were obtained immediately after euthanasia and stored at -20°C.[26] For each experiment, devitalized muscles were cut into approximately 4 mm³ pieces (median 60 g; interquartile range (IQR) 50 to 66), and devitalized cortical bones were cut into pieces approximately 5 mm in length (median 12 g; IQR 10 to 15).

Biofilm growth

A single colony of kanamycin-resistant, methicillin-sensitive *S. aureus*-Xen36 (Perkin Elmer) was incubated overnight with 200 RPM at 37°C in 3 ml of Luria-Bertani (LB) broth containing kanamycin (200 ug/ml) to avoid confounding growth of other bacteria.[30] Overnight cultures were diluted in LB broth without kanamycin to optical density (OD) 600 of 0.07 (approximately

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1.5 × 10⁸ CFUs/ml), and were further diluted 200-fold to approximately 6.7×10^5 CFUs/ml in LB without kanamycin. The diluted bacterial suspensions were added to discs (Ti6Al4V, Co-Cr, and UHMWPE) and devitalized tissue pieces (muscle and bone) in 24-well (1.5 ml) or 48-well (1.0 ml) plates, respectively. Plates were then sealed with a gas-permeable membrane (SealMate; Excel Scientific, USA) and incubated at 37°C in a humidified incubator with shaking at 110 RPM for one, three, five, or seven days. For biofilms grown for more than one day, substrates were gently rinsed three times with sterile PBS after the first 24 hours of incubation and then incubated in fresh LB broth without kanamycin (metallic and polymeric discs) or with kanamycin (biological tissues). The broth was changed every 24 hours.[18,30,31]

Antibiotics

Halicin (Catalogue #3607; Tocris Bioscience, USA) was dissolved to 50 mM in 100% dimethyl sulfoxide (DMSO) and stored at -20°C. For each experiment, the stock was diluted to 10 mM in 100% DMSO followed by serial dilution in 100% DMSO. The serial dilutions were added (1/10 volume) to each experimental well to obtain the indicated final concentrations of halicin in LB broth (Thermo Fisher Scientific, USA) with 10% DMSO. Control wells for halicin experiments therefore also contained LB broth with 10% DMSO. Selected experiments also included halicin groups with final concentrations of 20% DMSO as vehicle or with 10% or 20% Kolliphor HS 15 (Catalogue #42966; Sigma, USA) as vehicle. Kolliphor HS 15 is an amphiphilic solubilizer that is Food and Drug Administration-approved for parenteral administration and has been used as vehicle for halicin in mice.[32] Importantly, preliminary experiments found that 10% to 20% DMSO or 10% to 20% Kolliphor HS 15 by themselves had no detectable effect on bacterial viability within the biofilms (Supplementary Figure aa). However, 10% DMSO inhibited the anti-biofilm activity of vancomycin (Supplementary Figure ab). Vancomycin (Sigma) was therefore routinely dissolved to 200 or 800 μM in LB broth without DMSO. For each experiment, vancomycin stock solutions were serially diluted in LB broth without DMSO to obtain the indicated final concentrations. Control wells for vancomycin therefore also contained LB broth without DMSO.

MBEC assay

Biofilms were gently rinsed three times with sterile PBS to remove non-adherent bacteria, then incubated for 20 hours at 37°C in wells containing the indicated concentrations of halicin, vancomycin, or vehicle controls in LB broth without kanamycin. After the antibiotic challenge, biofilms were gently rinsed three times with sterile PBS, transferred to fresh LB broth without kanamycin, and allowed to recover from the antibiotic challenge for 24 hours at 37°C. After the recovery period, biofilms were disrupted by sonication (40 kHz for 30 minutes, BRANSON 3800; Branson Ultrasonics, USA) on a solid stainless steel insert tray in LB broth containing 0.3% Tween-80 (1.5 ml/well in 24-well plates for discs; 1.0 ml/well in 48-well plates for tissue pieces).[18] Bacterial viability was assessed by colony counting after serial tenfold dilutions in PBS and incubation on LB agar containing kanamycin (200 ug/ml) at 37°C for 24 hours. Bacterial viability was also assessed by resazurin reduction assays. For these assays, fluorescence was measured (excitation: 530 nm, emission:

595 nm, and gain: 60) with a GENios plus plate reader (Tecan, USA) after incubation for 60 minutes at 37°C with 15 μg/ml of resazurin (Alfa Aesar, USA). Background fluorescence in wells without bacteria was subtracted from experimental fluorescence values. MBECs were defined as the lowest concentration of halicin or vancomycin that induced a ≥ 3 log reduction in CFUs or a ≥ 75% decrease in resazurin reduction.[33,34] Medians of four to seven independent experiments for each drug concentration were used to calculate MBECs and are connected by dashed lines in the figures. Symbols in the figures denote the medians for each drug concentration from an independent experiment.

Statistical analysis

PRISM software version 9.4.1 was used for statistical analysis (GraphPad Software, USA). The Mann-Whitney U test was used to compare halicin and vancomycin MBECs. A p-value < 0.05 was considered statistically significant.

Results

To determine whether halicin and vancomycin remain antibacterially active against *S. aureus* within biofilms grown on orthopaedically relevant substrates, MBEC results are reported in comparison to the MIC values we previously measured with the same strain of *S. aureus* (25 μM for halicin and 0.5 μM for vancomycin).[18] For the less mature 24-hour and three-day biofilms on Ti6AlV4 discs, halicin's MBECs, assessed by either CFU assays or resazurin reduction assays, were ten-fold higher than the MIC (red symbols in Figure 1a, in Supplementary Figure ba, and in Supplementary Figure ca). In contrast, vancomycin's MBECs against those less mature biofilms on Ti6AlV4 discs were 100-fold higher than the MIC (blue symbols in Figure 1a, in Supplementary Figure ba, and in Supplementary Figure ca). For the more mature five-day and seven-day biofilms on Ti6AlV4 discs, halicin's MBECs were 20-fold higher than the MIC (red symbols in Figure 1b, in Supplementary Figure bb, and in Supplementary Figure cb), while vancomycin's MBECs were 200-fold higher than the MIC for five-day biofilms and 400-fold higher for seven-day biofilms (blue symbols in Figure 1b, in Supplementary Figure bb, and in Supplementary Figure cb). Since results on the Ti6Al4V discs were similar between the 24-hour and three-day biofilms (Figure 1a, Supplementary Figure ba, and Supplementary Figure ca) and between the five-day and seven-day biofilms (Figure 1b, Supplementary Figure bb, and Supplementary Figure cb), all further experiments focused exclusively on the less mature 24-hour biofilms and the more mature seven-day biofilms.

For Co-Cr discs, halicin's MBECs against less mature 24-hour and more mature seven-day biofilms, assessed by either CFU or resazurin reduction assays, were both ten-fold higher than the MIC (red symbols in Figures 2a to b and in Supplementary Figure da and db). In contrast, vancomycin's MBEC against the less mature and more mature biofilms were 100-fold to 200-fold higher than the MIC (blue symbols in Figures 2a to b and in Supplementary Figure da and db).

For UHMWPE discs, halicin's MBEC against less mature 24-hour biofilms measured by CFU assays was ten-fold higher than the MIC (red symbols in Figure 3a), and five-fold higher when measured by resazurin reduction assays (red symbols in Supplementary Figure ea). In contrast, vancomycin's MBEC
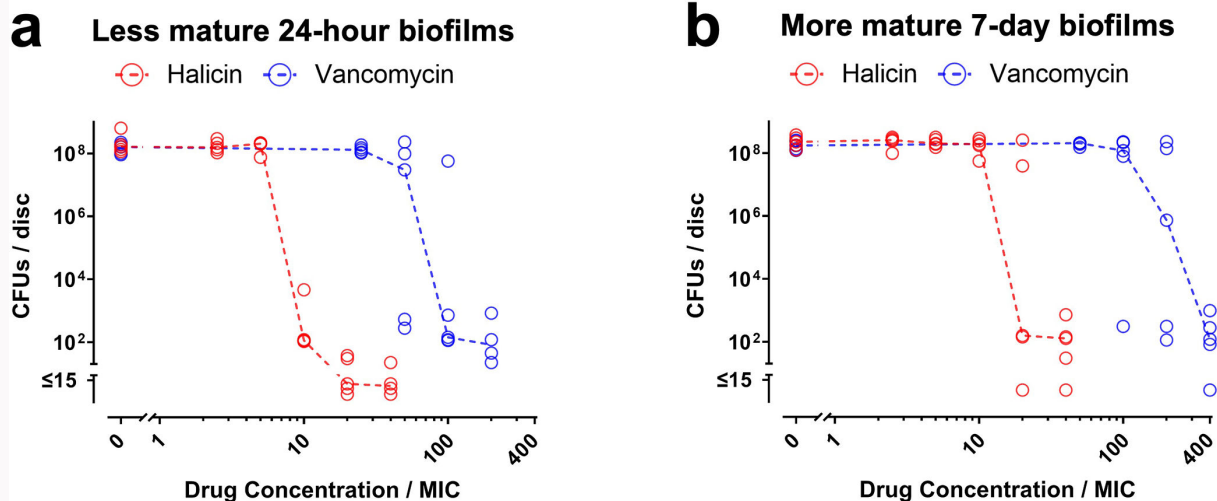
Fig. 1

Halicin remains active against *Staphylococcus aureus*-Xen36 biofilms grown on Ti6Al4V discs. a) Less mature 24-hour biofilms and b) more mature seven-day biofilms on Ti6Al4V discs were exposed to the indicated concentrations of halicin (red symbols) or vancomycin (blue symbols) for 20 hours. Effects on biofilm viability were determined by CFU assays. Dashed lines connect medians of five independent experiments for each drug concentration. Each symbol denotes the median for each drug concentration from an independent experiment, with four Ti6Al4V discs per symbol.
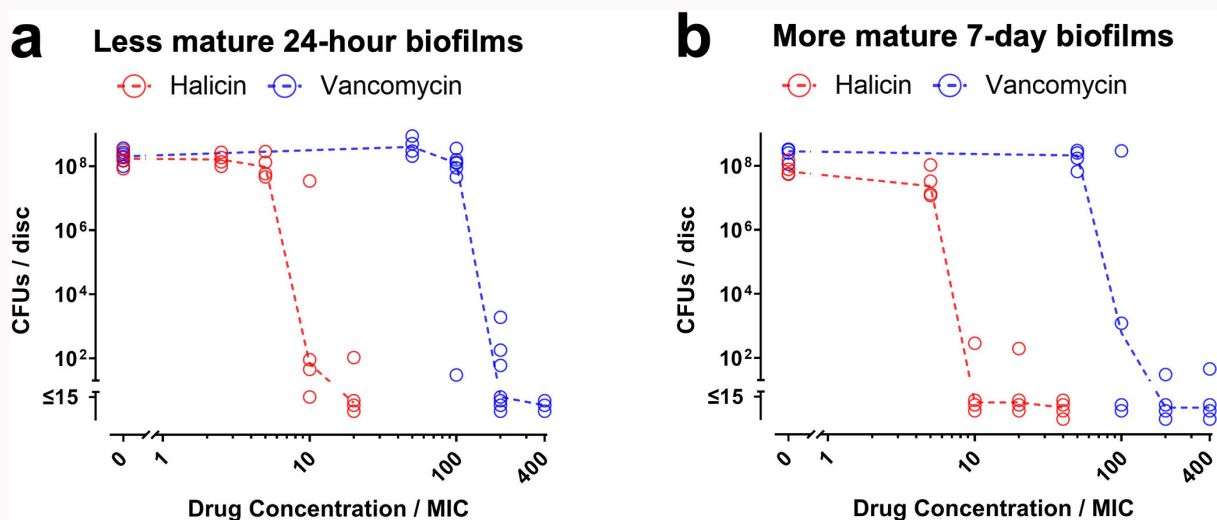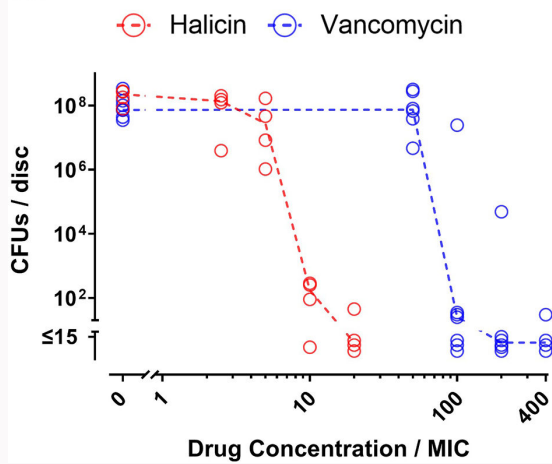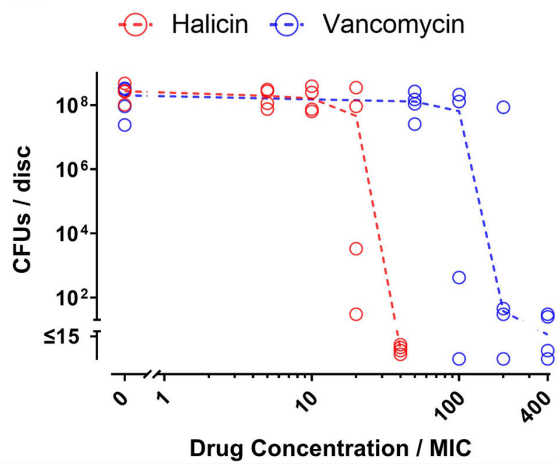

Fig. 2

Halicin remains active against *Staphylococcus aureus*-Xen36 biofilms grown on cobalt-chromium (Co-Cr) discs. a) Less mature 24-hour biofilms and b) more mature seven-day biofilms on Co-Cr discs were exposed to the indicated concentrations of halicin (red symbols) or vancomycin (blue symbols) for 20 hours. Effects on biofilm viability were determined by CFU assays. Dashed lines connect medians of four to seven independent experiments for each drug concentration. Each symbol denotes the median for each drug concentration from an independent experiment, with two to three Co-Cr discs per symbol. MIC, minimum inhibitory concentration.

against the less mature biofilms was 100-fold higher than the MIC (blue symbols in Figure 3a and in Supplementary Figure ea). For the more mature seven-day biofilms on UHMWPE discs, halicin's MBEC measured by CFU assays was 40-fold higher than the MIC (red symbols in Figure 3b), and 20-fold higher when measured by resazurin reduction assays (red symbols in Supplementary Figure eb), while vancomycin's MBEC was 200-fold higher than the MIC (blue symbols in Figure 3b and in Supplementary Figure eb).

For devitalized muscle, halicin's MBEC against less mature 24-hour biofilms, assessed by either CFU or resazurin reduction assays, was 40-fold higher than the MIC (blue symbols in Figure 4a and in Supplementary Figure

fa). Vancomycin's MBEC against the less mature biofilms was 50-fold higher than the MIC (blue symbols in Figure 4a and in Supplementary Figure fa). For the more mature seven-day biofilms on muscle samples, halicin's MBEC measured by CFU assays was 40-fold higher than the MIC (red symbols in Figure 4b), and 20-fold higher when measured by resazurin reduction assays (red symbols in Supplementary Figure fb), while vancomycin's MBEC was 100-fold higher than the MIC (blue symbols in Figure 4b and in Supplementary Figure fb).

For devitalized cortical bone, halicin's MBEC against less mature 24-hour biofilms measured by CFU assays was 40-fold higher than the MIC, and 20-fold higher when measured by resazurin reduction assays (red symbols in Figure 5a and in

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Fig. 3

Halicin remains active against *Staphylococcus aureus*-Xen36 biofilms grown on ultra-high molecular weight polyethylene (UHMWPE) discs. a) Less mature 24-hour biofilms and b) more mature seven-day biofilms on UHMWPE discs were exposed to the indicated concentrations of halicin (red symbols) or vancomycin (blue symbols) for 20 hours. Effects on biofilm viability were determined by CFU assays. Dashed lines connect medians of four to seven independent experiments for each drug concentration. Each symbol denotes the median for each drug concentration from an independent experiment, with two to three UHMWPE discs per symbol. MIC, minimum inhibitory concentration.
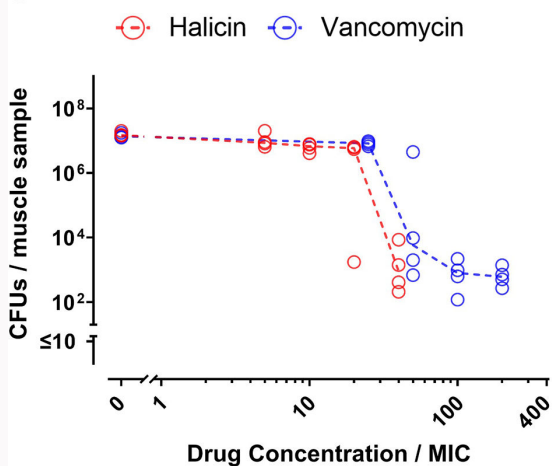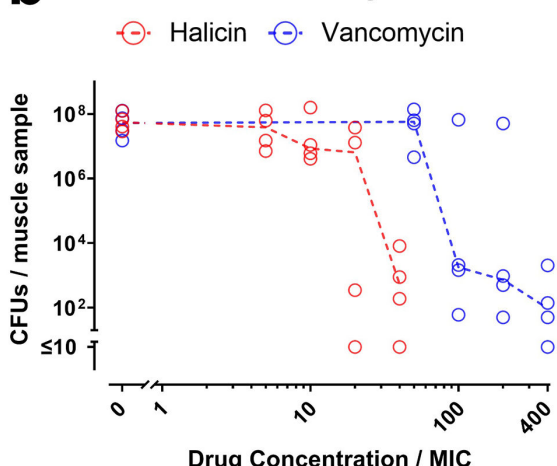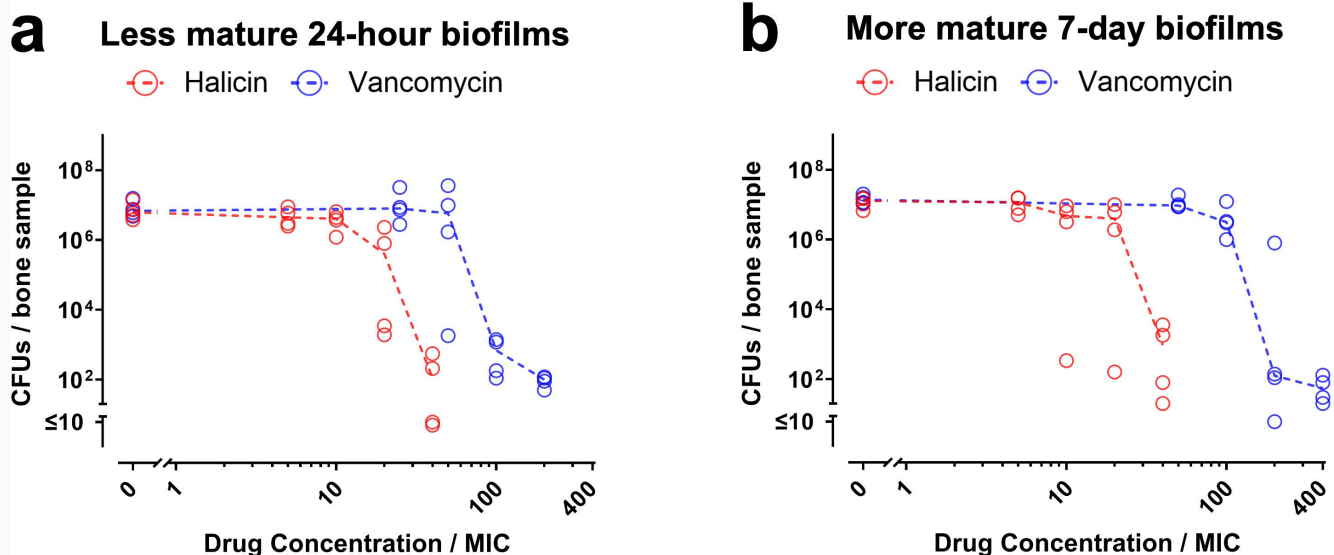

Fig. 4

Halicin remains active against *Staphylococcus aureus*-Xen36 biofilms grown on devitalized muscle. a) Less mature 24-hour biofilms and b) more mature seven-day biofilms on muscle samples were exposed to the indicated concentrations of halicin (red symbols) or vancomycin (blue symbols) for 20 hours. Effects on biofilm viability were determined by CFU assays. Dashed lines connect medians of four independent experiments for each drug concentration. Each symbol denotes the median for each drug concentration from an independent experiment, with three muscle samples per symbol.

Supplementary Figure ga). In contrast, vancomycin's MBEC against the less mature biofilms was 100-fold higher than the MIC (blue symbols in Figure 5a and in Supplementary Figure ga). For the more mature seven-day biofilms on cortical bone samples, halicin's MBEC was 40-fold higher than the MIC (red symbols in Figure 5b and in Supplementary Figure gb), while vancomycin's MBEC was 200-fold higher than the MIC (blue symbols in Figure 5b and in Supplementary Figure gb).

MBECs for halicin and vancomycin determined by CFU assays are compared in Table I. Mann-Whitney U test showed that halicin MBECs, expressed as fold change versus MIC, are lower ($p < 0.01$) than vancomycin MBECs against both the less mature 24-hour biofilms and the more mature seven-day biofilms. MBECs determined by resazurin reduction assays are identical to those in Table I, which were determined by CFU assays, with three exceptions where the halicin MBECs determined by resazurin reduction are two-fold less than those determined by CFU assays (compare red symbols in Figures 3 and 5a, with red symbols in Supplementary Figures ea, eb, and ga).

To further assess the translational relevance of our study, we determined whether the antibiofilm effects of halicin depended on the use of DMSO as a vehicle in the previous experiments of this study. For this purpose, halicin

Fig. 5

Halicin remains active against *Staphylococcus aureus*-Xen36 biofilms grown on devitalized cortical bone. a) Less mature 24-hour biofilms and b) more mature seven-day biofilms on bone samples were exposed to the indicated concentrations of halicin (red symbols) or vancomycin (blue symbols) for 20 hours. Effects on biofilm viability were determined by CFU assays. Dashed lines connect medians of four independent experiments for each drug concentration. Each symbol denotes the median for each drug concentration from an independent experiment, with three bone samples per symbol.

Table I. Halicin remains active against *Staphylococcus aureus*-Xen36 biofilms grown on orthopaedically relevant substrates. Minimal biofilm eradication concentrations from colony-forming unit assays are reported as fold change versus the planktonic minimal inhibitory concentrations, which we previously reported for this strain of *S. aureus*.[18]

Substrate	Less mature 24-hour biofilms MBECs (fold change vs MIC)		More mature 7-day biofilms MBECs (fold change vs MIC)	
	Halicin	**Vancomycin**	**Halicin**	**Vancomycin**
Ti6Al4V	10× MIC	100× MIC	20× MIC	400× MIC
Cobalt-chrome	10× MIC	200× MIC	10× MIC	100× MIC
UHMWPE	10× MIC	100× MIC	40× MIC	200× MIC
Muscle	40× MIC	50× MIC	40× MIC	100× MIC
Bone	40× MIC	100× MIC	40× MIC	200× MIC

p-values determined by Mann-Whitney U test were < 0.01, comparing MBECS for halicin to MBECs for vancomycin for both 24-hour and seven-day biofilms.

MBEC, minimum biofilm eradication concentration; MIC, minimal inhibitory concentration; UHMWPE, ultra-high molecular weight polyethylene.
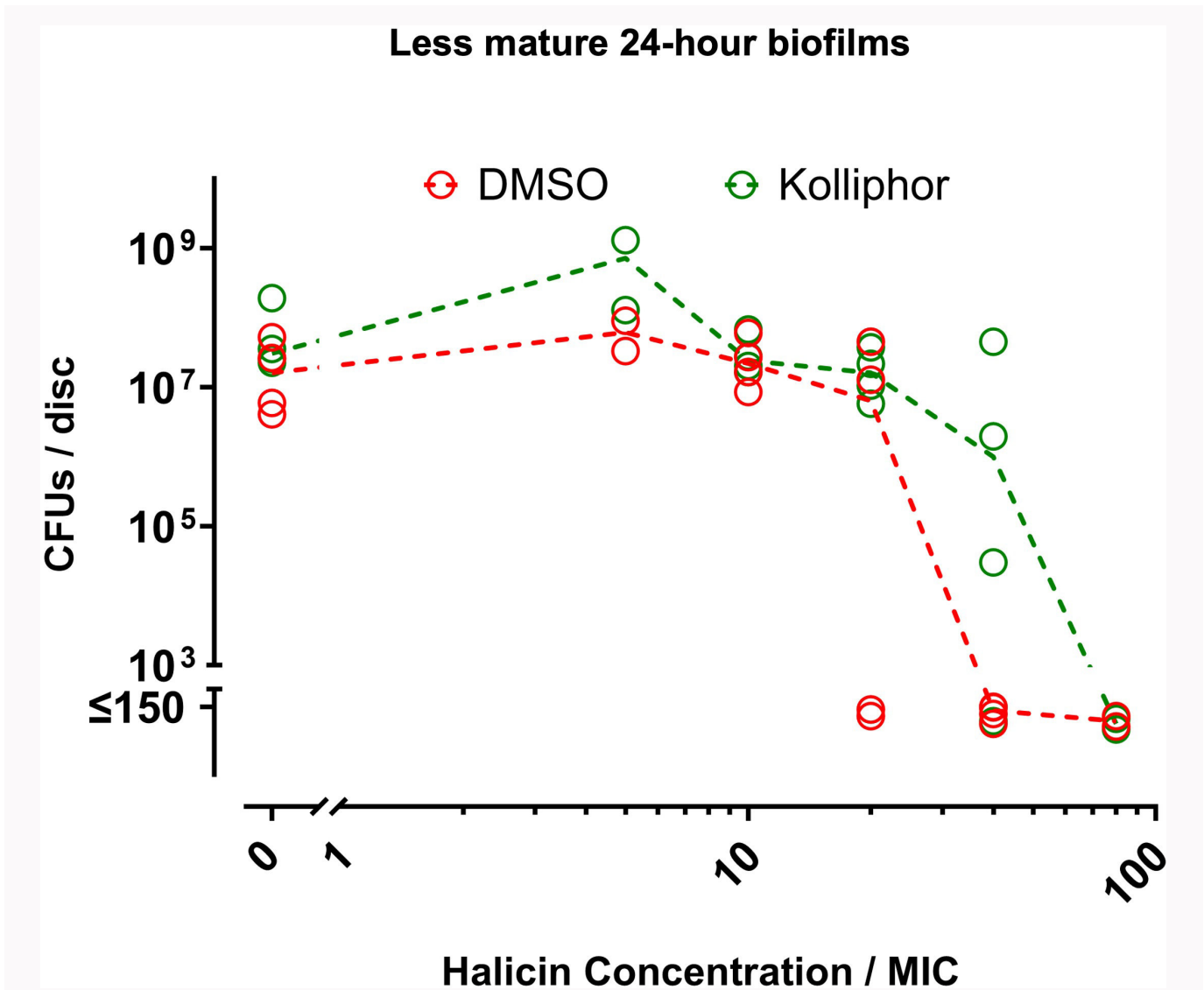
with DMSO as vehicle was compared to halicin with Kolliphor HS 15 as vehicle. Kolliphor HS 15 is an amphiphilic solubilizer that is FDA-approved for parenteral administration and has been used as vehicle for halicin in mice.[32] The halicin MBEC with DMSO as vehicle was two-fold less than the halicin MBEC with Kolliphor HS 15 vehicle (Figure 6). Thus, halicin continues to exert the majority of its antibiofilm activity regardless of vehicle.

Discussion

Previous studies found that halicin kills bacteria in bio-films grown on polystyrene or polypropylene substrates.[18,21,22] However, biofilm susceptibility to antibiotics can be altered by growth on different substrates.[23-26] This study therefore determined whether halicin remains active against *S. aureus* biofilms grown on orthopaedically relevant substrates. We found that halicin's MBECs against both less mature and more mature biofilms are ten-fold to 40-fold higher than its MIC. In contrast, vancomycin's MBECs are significantly larger: 50-fold to 200-fold higher than its MIC against the less mature biofilms, and 100-fold to 400-fold higher than MIC against the more mature biofilms. The decreased susceptibility to vancomycin confirms biofilm formation and maturation in our study.[35,36]

Induction of bacterial quiescence is a hallmark of biofilm maturation and a major cause of tolerance to most antibiotics.[7-15] Halicin's effectiveness against bacterial biofilms shows that it remains active against quiescent bacteria, and is consistent with previous findings that similar concentrations

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Less mature 24-hour biofilms

Fig. 6

Halicin activity against *Staphylococcus aureus*-Xen 36 biofilms does not depend on DMSO vehicle. Less mature 24-hour biofilms on Ti6Al4V discs were exposed for 20 hours to the indicated concentrations of halicin with either dimethyl sulfoxide (DMSO) (red symbols) or Kolliphor HS 15 (green symbols) as vehicle. Final concentrations of DMSO or Kolliphor HS 15 were 20% in LB broth without kanamycin for groups with halicin at 80 × minimal inhibitory concentration (MIC) and 10% for all other halicin groups. Controls without halicin therefore included groups with 10% or 20% of either DMSO or Kolliphor HS 15 as vehicle. The concentrations of either vehicle did not affect biofilm viability (Supplementary Figure aa). Effects on biofilm viability were determined by colony-forming unit (CFU) assays. Dashed lines connect medians of four independent experiments for each group. Each symbol denotes the median for each group from an independent experiment, with four Ti6Al4V discs per symbol.

of halicin remain active in planktonic cultures of metabolically repressed bacteria and quiescent persister bacteria.[20]

Our results also show distinct halicin MBECs for the biofilms grown on inorganic substrates compared with the biofilms grown on devitalized tissues. Thus, halicin MBECs against less mature and more mature biofilms grown on discs of Ti6Al4V, Co-Cr, or UHMWPE were ten-fold to 20-fold higher than its MIC. In contrast, halicin MBECs against biofilms on devitalized muscle or devitalized cortical bone were 40-fold higher than its MIC, irrespective of whether the biofilms were grown for 24 hours or seven days. However, the distribution of vancomycin MBECs did not differentiate between inorganic and biological substrates. The different patterns of MBECs for halicin and vancomycin are consistent with the conclusion that MBECs determined on different substrates can vary in an unpredictable manner.[23-26]

The major limitation of our study is that the clinically achievable serum level of halicin is not known; therefore, it cannot be compared to our measured MBECs. Future pharmacokinetic studies are needed to resolve this question. A second limitation is that we only tested a single strain of bacteria. However, halicin is also effective against biofilms of other *S. aureus* strains as well as biofilms of *A. baumannii*, *E. coli*, and *Enterococcus*.[21,22] Halicin also has broad-spectrum activity against planktonic cultures of many Gram-positive and Gram-negative bacteria, including multidrug-resistant clinical isolates.[20,37] Halicin is the first completely novel antibiotic identified by deep-learning artificial intelligence approaches.[20] Follow-up studies revealed that halicin has potent broad-spectrum activity, but the antibacterial mechanism of halicin remains uncertain.[20] Evidence from planktonic cultures suggests inhibition of bacterial proton motive force as a

potential mechanism.[20] That mechanism would be consistent with halicin's activity against planktonic cultures of quiescent persister bacteria and bacteria in biofilms, as both remain dependent on the proton motive force.[38] Future studies are needed to determine whether halicin inhibits the proton motive force of bacteria in biofilms. Another limitation of our study is that we did not use bioluminescent techniques to confirm biofilm formation in our cultures. Bioluminescent imaging, however, is primarily advantageous for longitudinal in vivo studies.[29,39,40] In contrast, CFU counting is the gold-standard technique for in vitro biofilm studies,[41,42] and our resazurin reduction assays provide confirmation in a high-throughput/low-cost setting.[18,42] The *S. aureus*-Xen36 strain is nonetheless advantageous for our in vitro studies, as it also contains a kanamycin-resistance gene cassette.[30] Kanamycin can therefore be used to limit confounding growth of other bacteria.[30] Importantly, we were careful to exclude kanamycin from the cultures during the antibiotic treatments to avoid potential confounding synergistic effects.

Halicin was previously found to be effective against non-orthopaedic infections in mice.[20,22] Those studies demonstrated the feasibility of in vivo halicin treatment for gastrointestinal infections, wound infections, and a subcutaneous abscess biofilm model. In the current study, we found that halicin remains active against *S. aureus* in biofilms formed on orthopaedically relevant substrates, including Ti6AL4V, Co-Cr, UHMWPE, devitalized muscle, and devitalized bone. We therefore conclude that halicin is a promising antibiotic that should be tested in animal models of orthopaedic infection.

Supplementary material
Graphs comparing the effects of DMSO and Kolliphor HS 15 on biofilms, and halicin and vancomycin on biofilms grown on Ti6AL4V, cobalt-chrome, and ultra-high molecular weight polyethylene discs, and devitalized muscle cortical bone.

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Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.