

Halicin-Rifampicin Combinations Are More Effective Than Monotherapy Against Biofilm-Residing *Staphylococcus Aureus* and Prevent Emergence of Rifampicin Resistance

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Abstract

Background: Biofilms shield bacteria from immune defenses and antibiotics, contributing to implant-associated infections, particularly those caused by *Staphylococcus aureus* (*S. aureus*). Halicin, a repurposed antimicrobial with broad-spectrum activity, has shown promise against planktonic and biofilm-residing bacteria. Building on our previous work, we investigated whether halicin enhances the efficacy of conventional antibiotics when used in combination against *S. aureus*. **Methods:** The treatment efficacies of combination therapies of Halicin and conventional antibiotics were tested against planktonic *S. aureus* and *S. aureus* biofilms grown on titanium substrates of varying maturities. The emergence of antibiotic resistance in treated biofilm bacteria was also characterized. **Results:** In checkerboard assays against planktonic bacteria, halicin synergized with gentamicin, tobramycin, and cefazolin but not with rifampicin or vancomycin. In biofilms grown on titanium substrates, halicin combined most effectively with rifampicin, reducing viable bacteria by >3 logs in less-mature and >5 logs in more-mature biofilms compared to rifampicin alone. Compared to untreated biofilms, combination therapy reduced viable bacteria by >5 logs in all cases. This combination also prevented rifampicin resistance seen with rifampicin monotherapy. Halicin only modestly enhanced aminoglycoside activity against less-mature biofilms and had no significant effect when combined with vancomycin or cefazolin. **Conclusions:** These findings highlight that antibiotic-halicin synergy differs between planktonic and biofilm states, underscoring the importance of biofilm-specific studies. Highlighting the translatability of our results, the doses of conventional antibiotics combined with halicin required to affect planktonic and biofilm-residing *S. aureus* are all achievable systemically and in bone.

Keywords: Biofilm eradication, implant-associated infections, antibiotic resistance, *S. aureus*.

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Introduction

Biofilms were first described in the biomedical literature in the 1970s as a structured microbial community surrounded by an extracellular matrix [1,2]. Biofilm formation is tightly regulated through cell-to-cell communication, environmental cues, and quorum-sensing gene regulation, leading to phenotypic growth changes [3]. Biofilms protect bacteria from most conventional antibiotics and the host immune system, leading to chronic infections that are difficult to treat clinically [4,5]. For example, biofilms on orthopedic implants can cause devastating complications, includ-

ing multiple surgeries for the patient, loss of function, impaired activities of daily living, amputation, and sepsis [6]. *Staphylococcus aureus* (*S. aureus*) is one of the most common causes of recalcitrant biofilm infections and can form biofilms on a wide range of materials, including catheters, metal implants, and necrotic tissue [7,8].

Interrogation of the Drug Repurposing Hub database with a deep learning model predicted the nitro-thiazole compound halicin as a possible antibacterial [9]. Follow-up studies demonstrated broad-spectrum activity against both gram-positive and gram-negative planktonic bacteria [9].

Importantly, halicin was also effective against planktonic persister cells [9]. Planktonic persister cells and bacteria in biofilms have many similar characteristics, including quiescence-induced tolerance to most conventional antibiotics [10,11]. Subsequent studies from our lab documented that halicin was also active against *S. aureus* biofilms at concentrations similar to those active against planktonic persisters [12]. We found that halicin was active against both less-mature and more-mature biofilms grown on metallic, polymeric, and biologic substrates relevant to clinical infections [12,13]. Halicin's activity against *S. aureus* biofilms on those substrates establishes its potential usefulness in bone and joint infections surrounding orthopaedic implants. Other investigators simultaneously demonstrated halicin's activity against gram-negative bacterial biofilms [14–16]. Furthermore, doses of halicin that are effective in murine infection models cause minimal toxicity, making it a promising candidate for further investigation as an antibiotic for clinical use [17,18].

There has been an alarming spread of antibiotic resistance worldwide [19,20]. The development of novel antibiotics for the clinical setting is associated with high costs and low returns for pharmaceutical companies, leading to a decrease in the generation of new antibiotic compounds [21]. Therefore, conventional antibiotics are often used in combination, as they can have synergistic therapeutic effects and reduce the emergence of resistance [22–28]. Further, antibiotics such as rifampicin are effective against bacterial biofilms, especially when used in combination with other antimicrobials [25,26,29]. Combinatorial use can also reduce the emergence of bacterial resistance to rifampicin, which cannot be used as monotherapy because it commonly causes resistance [28–31]. Halicin's antimicrobial and antibiofilm properties have not yet been studied in combination with conventional antibiotics. However, combination therapy can increase antibacterial efficacy and allow for lowering the doses of each drug to decrease both the emergence of resistance and adverse events [29]. This underscores the future potential of combination therapies that include halicin [32]. We therefore evaluated the combinatorial effects of halicin with conventional antibiotics on *S. aureus* in planktonic cultures and in biofilms of differing maturity.

Methods

Antibiotics

Halicin (Catalog #3607, Tocris Bioscience, Minneapolis, MN, USA) was dissolved in 100% dimethyl sulfoxide (DMSO) or PBS containing 25% Kolliphor-HS 15 (Catalog #42966; Sigma, Louis, MO, USA). Rifampicin (Catalog #R3501, Millipore Sigma, Burlington, MA, USA) was dissolved in 100% DMSO. Stocks of all other antibiotics (all from Millipore Sigma) were made in Milli-Q water. Stock solutions of halicin (25 or 50 mM), rifampicin (50 or 102.4 mM), gentamicin (100 mM), and kanamycin (85.5

mM) were all stored in aliquots at -20°C . Stock solutions of tobramycin (50 mM), cefazolin (10 mM), and vancomycin (10 mM) were stored in aliquots at 4°C . Vehicle concentrations were equal in all wells of each experiment and are listed in the figure captions.

Bacteria Strains and Growth Conditions

Methicillin-sensitive, kanamycin-resistant *S. aureus*-Xen36 (Catalog # 119243, Perkin Elmer) was used for all experiments as in our previous halicin studies [12,13] except that log-phase growth was obtained by sub-culture of overnight cultures derived from a single colony. Subcultures were incubated (2 hours, 37°C , 200 RPM) in Luria Bertani (LB) broth (Thermo Fisher Scientific) containing 200 $\mu\text{g/mL}$ kanamycin. The log-phase bacterial suspension was centrifuged (5 minutes at 1500 g), and the bacteria were resuspended in LB broth without kanamycin to an absorbance of 0.07 at 600 nm and were further diluted 200-fold to approximately 7×10^5 CFUs/mL in LB without kanamycin for all experiments [13]. The bacteria concentrations in those suspensions were confirmed by Colony Forming Units (CFU) counting.

Checkerboard Assays-Planktonic Bacteria

The combinatorial planktonic bacteria inhibitory effect of halicin with five distinct conventional antibiotics was tested using checkerboard assays on 96-well flat-bottom plates (Catalog # 12-565-210, Thermo Fisher, Waltham, MA, USA). Eighty microliters of the diluted *S. aureus*-Xen36 suspensions were added to duplicate wells containing serial dilutions of halicin and one of the other antibiotics (10 μL of each). All assays included both inoculated and sterile control wells with vehicles without antibiotics. The 96-well plates were sealed (Catalog # DY992, Bio-technie, Minneapolis, MN, USA) and incubated for 20 hours at 37°C . Absorbances at 600 nm were measured using a SpectraMax i3 plate reader (Molecular Devices), and background absorbance in wells without bacteria was subtracted from experimental values. A 95% decrease in absorbance compared to inoculated wells without antibiotics was defined as inhibition of bacterial growth for calculation of fractional inhibitory concentration indices (FICIs).

Biofilm Assays

S. aureus-Xen36 biofilms were grown on sterile titanium alloy discs (medical grade Ti6Al4V ELI 23, 12.7 mm diameter, Titanium Industries, Rockaway, NJ) in 24-well tissue culture plates (Catalog #07-200-84, Corning Incorporated, Corning, NY, USA). Less-mature and more-mature biofilms were obtained by incubation (37°C , 110 RPM) for 24 hours or 7 days, respectively, as we previously described [13]. LB broth was exchanged daily during formation of the more-mature biofilms. The biofilms were gently rinsed with sterile PBS one time to remove non-adherent bacteria and were then challenged for 20 hours at 37°C with 1.5

mL of LB broth containing various doses of one antibiotic in the presence and absence of a fixed dose of the other antibiotic. Each combination biofilm assay included control biofilms treated with vehicle alone, as well as titanium discs incubated without bacteria to confirm sterility. After the antibiotic challenges, biofilms were gently rinsed with sterile PBS and incubated in fresh LB broth (24 hours, 37 °C) to allow quiescent bacteria to recover [12,13,33]. Biofilms were then gently rinsed in sterile PBS and sonicated (40 kHz for 30 minutes, BRANSON 3800, Branson Ultrasonics, Brookfield, Connecticut) in PBS containing 0.3% Tween-80 on a solid stainless-steel insert (Catalog #100-410-174, Branson Ultrasonics, headquartered in Brookfield, CT, USA) [33]. To assess effects on the number of viable bacteria, biofilm sonicates were serially diluted in PBS, and CFU were counted after incubation (37 °C, 24 hours) on LB agar containing kanamycin (200 µg/mL).

Antibiotic Resistance Assays on Sonicates

Sonicates of mature biofilms were prepared as described in the previous paragraph. Resistance assays included sonicates from the following groups: biofilms incubated with vehicles only, halicin monotherapy, rifampicin monotherapy, combination therapy sufficient to partially reduce the CFUs, and combination therapy that had little or no effect on CFUs (see outlined groups in Fig. 3B). Sensitivity of sonicates to monotherapy with either halicin or rifampicin was measured as described previously for the planktonic checkerboard assays. Briefly, rifampicin or halicin minimum inhibitory concentration (MICs) against bacteria in the biofilm sonicates were compared to the MICs of naïve *S. aureus*. A > 4-fold increase in sonicate MIC was considered resistance.

Statistical Analysis

Data from planktonic combination experiments were analyzed by calculating the fractional inhibitory concentration index ($FICI = (MIC_{AB}/MIC_A) + (MIC_{BA}/MIC_B)$). $FICI \leq 0.5$ indicates synergism, $0.5 < FICI \leq 4$ indicates no interaction, and $FICI > 4$ indicates antagonism [34,35]. Data from the antibiotic combination biofilm assays were analyzed by mixed effects models. Data from halicin monotherapy biofilm experiments were analyzed using a 1-way ANOVA. Rifampicin and halicin resistance data were analyzed using a 2-way ANOVA with Dunnett's multiple comparisons test comparing antibiotic treatment groups to the vehicle-treated control. p values < 0.05 were considered significant. All statistical analyses and figure preparations were completed using Prism (version 10.3.1, GraphPad Software, San Diego, CA, USA) and verified by a biostatistician.

Results

Checkerboard Assays on Planktonic Bacteria

Representative heat maps of each distinct conventional antibiotic combination with halicin are depicted in Fig. 1. Gentamicin, tobramycin, and cefazolin had FICIs indicative of synergism (≤ 0.5) [34,35] with halicin (Fig. 1). In contrast, neither vancomycin nor rifampicin synergized with halicin in these planktonic checkerboard assays (FICIs = 0.75–1.1, Fig. 1). All concentrations of the conventional antibiotics used in these experiments are achievable systemically and in bone (Table 1).

Biofilm Assays

Initial experiments determined the effects on less-mature biofilms of each conventional antibiotic in the presence and absence of a fixed dose of halicin. 250 µM halicin was chosen for these experiments as it did not detectably inhibit viable bacteria counts in either the less- or more-mature biofilms grown on titanium alloy discs (Supplementary Fig. 1A,B). Less-mature biofilm experiments initially used DMSO as a vehicle for halicin. Conventional antibiotics that were substantially more potent when combined with halicin in those experiments were advanced to experiments with less-mature and more-mature biofilms using Kolliphor-HS as a vehicle for halicin. Kolliphor-HS 15 is more translationally relevant as it is FDA approved for use in humans [53–55]. In contrast, high doses of DMSO are toxic in animals and humans when administered orally or parenterally (>10% v/v solutions) [56–58]. To provide context for our results, achievable antibiotic concentrations systemically and in bone are listed in Table 1.

Rifampicin monotherapy had only modest effects on viable bacteria counts in less- or more-mature biofilms at all tested doses, including 1500 µM (Fig. 2A–D), which is > 16-fold higher than achievable systemically and > 6-fold higher than achievable in bone (Table 1). However, rifampicin was substantially more potent in the presence of 250 µM halicin against less-mature (Fig. 2A,B, $p < 0.001$) and more-mature (Fig. 2C, $p = 0.021$) biofilms. Thus, compared with monotherapy, the combination of halicin with all doses of rifampicin > 15 µM reduced viable bacteria number in the less-mature biofilms by > 3 logs (Fig. 2A,B) and in the more-mature biofilms by > 5 logs (Fig. 2C). When compared to untreated biofilms, combination therapy with halicin and rifampicin at doses > 15 µM reduced viable bacteria by > 5 logs in all cases. To further reduce the possibility of confounding results due to vehicles, additional experiments were performed with the minimal practical concentrations of the vehicles (0.05% DMSO and 0.25% Kolliphor-HS 15). At these vehicle concentrations, rifampicin was again substantially more potent in the presence of halicin against more-mature biofilms (Fig. 2D, $p = 0.016$). Rifampicin (>15 µM) combined with 250 µM halicin reduced viable bacteria to undetectable levels (CFUs

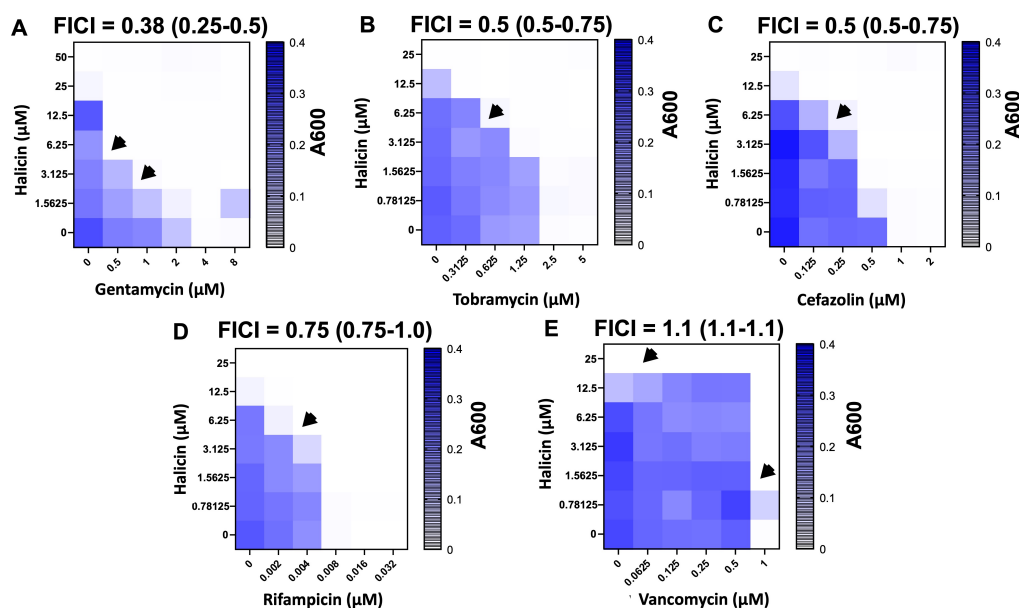


Fig. 1. Halicin combinations with gentamicin (A), tobramycin (B), and cefazolin (C) synergistically inhibits the growth of *S. aureus* in planktonic cultures. Median (and range) of FICI values from $n = 3$ independent experiments are indicated above the heatmaps. Each heatmap depicts results from a representative checkerboard assay that provided the median FICI value. Blue shading indicates mean absorbance values at a wavelength of 600nm (A600) from duplicate wells. Arrowheads signify experimental conditions used to calculate FICI values. FICI, fractional inhibitory concentration index. The figure was designed using GraphPad Prism, version 10.3.1, GraphPad Software, San Diego, CA, USA.

Table 1. Achievable concentrations in systemic circulation (C_{max}) and in bone after systemic ($C_{bone,sys}$) or local ($C_{bone,local}$) treatment.

Antibiotic	$C_{max}(\mu\text{M})$	$C_{bone,sys}(\mu\text{M})$	$C_{bone,local}(\mu\text{M})$	References
Cefazolin	400–800	441	500–650	[36–40]
Gentamicin	25–45	6	80	[41–43]
Rifampicin	25–90	4	240 [#]	[44–47]
Tobramycin	63	8	120	[43,48,49]
Vancomycin	70	20	630–850 [#]	[50–52]

Note: Ranges are provided for achievable concentrations whenever multiple studies are identified. Data is presented from human patients except where indicated by [#], which denotes animal studies.

reduced by >8 logs compared to monotherapy, Fig. 2D). Importantly, 15 μM rifampicin is an achievable concentration systemically and in bone (Table 1). In additional experiments, various doses of halicin were tested in the absence and presence of 15 μM rifampicin. Halicin was substantially more potent in the presence of rifampicin against more-mature biofilms (Fig. 3A, $p = 0.006$). The combination of rifampicin with 125 μM halicin reduced viable bacteria number by >5 logs compared with monotherapy (Fig. 3A). Rifampicin also reduced the dose of halicin required to eradicate viable bacteria to undetectable levels in the more-mature biofilms to 250 μM (Fig. 3A), which is 8-fold less than the MBEC for halicin monotherapy against those biofilms (Supplementary Fig. 1B).

Rifampicin monotherapy rapidly induces resistance and is therefore primarily used clinically in combination

with a second antibiotic to mitigate resistance [25,26,31,59, 60]. To determine whether the addition of halicin reduces the emergence of rifampicin resistance, we performed MIC assays with bacteria from the biofilm sonicates indicated by the colored ellipses in Fig. 3B that overlay the data from Fig. 3A. Bacteria from biofilms that were not exposed to antibiotics (dark blue ellipse in Fig. 3B and dark blue symbols in Fig. 3C) had rifampicin and halicin MIC values equal to naïve bacteria. As expected, bacteria from biofilms treated with 15 μM rifampicin monotherapy (green ellipse in Fig. 3B) were highly resistant to rifampicin with no detectable inhibition of bacterial growth at all tested rifampicin concentrations (up to 64 X MIC, green symbols in Fig. 3C, $p < 0.0001$ compared to vehicle-treated biofilms). Most importantly, halicin prevented rifampicin resistance in biofilms when the combination therapy was sufficient to

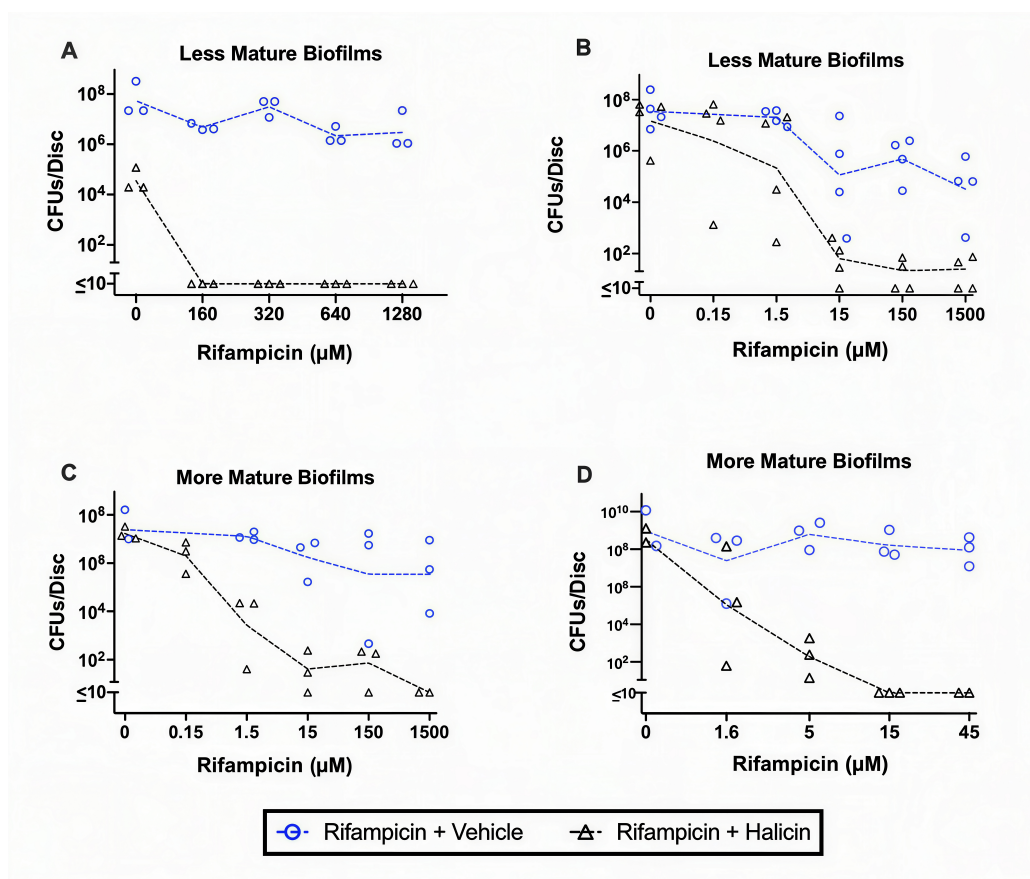


Fig. 2. Rifampicin is substantially more potent in the presence of halicin. Less-mature (A–B) or more-mature biofilms (C–D) were incubated for 20 hours with the indicated doses of rifampicin in combination with either vehicle (blue circles) or 250 μM halicin (black triangles). Final concentrations of vehicles in all wells were 2.5% DMSO (A), 2.5% Kolliphor-HS 15 and 2.5% DMSO (B–C) or 0.25% Kolliphor-HS 15 and 0.05% DMSO (D). Dotted lines connect geometric mean CFUs of $n = 3$ –4 independent experiments. Symbols denote the geometric mean for each group from an independent experiment, with 4 biofilms per group. p -values comparing rifampicin plus vehicle to rifampicin plus halicin were determined by mixed effects models (A–D). DMSO, dimethyl sulfoxide. The figure was designed using GraphPad Prism, version 10.3.1, GraphPad Software, San Diego, CA, USA.

partially reduce the number of viable bacteria within the biofilms (purple ellipse in Fig. 3B and purple symbols in Fig. 3C). In contrast, rifampicin resistance was not prevented in biofilms treated with combination therapy that had little to no effect on the number of viable bacteria (red ellipse in Fig. 3B and red symbols in Fig. 3C, $p < 0.0001$ compared to vehicle-treated biofilms). Halicin monotherapy did not induce resistance to rifampicin (light blue ellipse in Fig. 3B and light blue symbols in Fig. 3C) or to itself (Fig. 3D). Halicin resistance was also not induced in any of the other groups (Fig. 3D).

Clinically achievable levels of tobramycin (Table 1) were modestly but significantly more potent in the presence of 250 μM halicin against less-mature biofilms (Fig. 4A,B, $p < 0.008$). Clinically achievable levels of gentamicin (Table 1) were also modestly but significantly more potent in the presence of halicin with a 2.5% DMSO vehicle (Fig. 5A, $p = 0.011$), but not with a 2.5% Kolliphor-HS 15 vehicle (Fig. 5B, $p = 0.15$). In contrast to the re-

sults with less-mature biofilms, halicin did not significantly affect the potency of either aminoglycoside against more-mature biofilms (Figs. 4C and 5C).

Halicin did not detectably affect vancomycin potency against less-mature biofilms (Fig. 6, $p = 0.32$). Instead, the number of viable bacteria was similar with all doses of vancomycin $> 50 \mu\text{M}$ combined with halicin, reminiscent of the induction of persister bacteria [10,11]. These experiments included doses higher than the reported maximum concentrations of vancomycin systemically and in bone (Table 1) [50–52].

Cefazolin did not affect the number of viable cells in the less-mature biofilms in either the absence or presence of halicin (Fig. 7, $p = 0.39$). These experiments included cefazolin doses equivalent to the reported maximum achievable concentrations systemically and in bone (Table 1) [36–40]. Thus, we did not test higher concentrations of cefazolin in our biofilm assays.

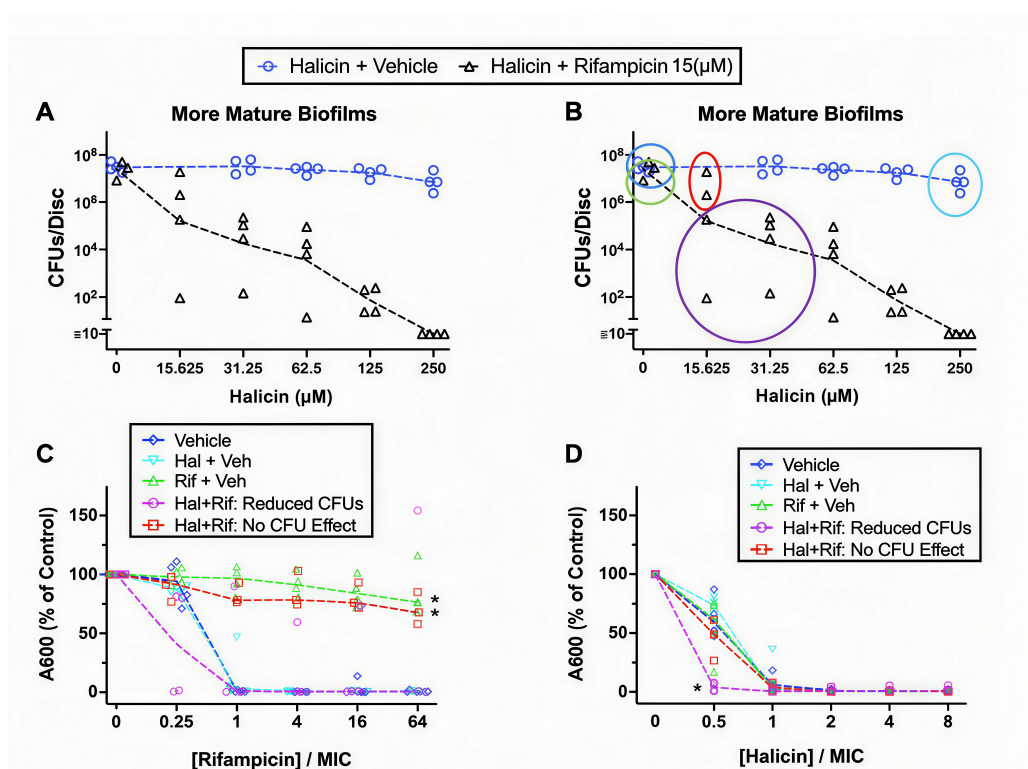


Fig. 3. Halicin is substantially more potent in the presence of rifampicin and prevents the emergence of rifampicin resistance. More-mature biofilms were incubated for 20 hours with the indicated doses of halicin with either vehicle (blue circles) or 15 μM rifampicin (black triangles) (A). The final concentration of vehicles in all wells was 2.5% Kolliphor-HS 15 and 2.5% DMSO. The resistance assays tested biofilm sonicates from all groups enclosed by the colored ellipses in (B), which overlays the data from (A). Colored ellipses in (B) match colors in (C,D): dark blue: vehicle treated biofilms, light blue: halicin monotherapy, green: rifampicin monotherapy, purple: combination therapy sufficient to partially reduce the CFUs in (B), and red: combination therapy that had little or no effect on CFUs in (B). To assess resistance, bacteria in the sonicates were exposed to the indicated concentrations of rifampicin (C) or halicin (D). Antibiotic concentrations are indicated on the x-axes of (C,D) as multiples of the MICs of naïve *S. aureus*-Xen36 (0.008 μM rifampicin and 25 μM halicin, see Fig. 1). Dotted lines connect geometric mean CFUs (A,B) or mean absorbances (C,D) of $n = 4$ independent experiments. Each symbol represents the geometric mean (A,B) or mean (C,D) value from an independent experiment, with 4 biofilms (A,B) or 2–6 sonicates (C,D) per group. p -values comparing effects of rifampicin plus vehicle to rifampicin plus halicin on CFUs were determined by mixed effects models (A). P values comparing resistance in sonicates from treated biofilms to sonicates from vehicle control biofilms were determined by 2-way ANOVA with Dunnett's multiple comparisons testing (C,D). MIC, minimum inhibitory concentration. *: $p < 0.0001$. The figure was designed using GraphPad Prism, version 10.3.1, GraphPad Software, San Diego, CA, USA.

Discussion

This study found that halicin can synergize with gentamicin, tobramycin, and cefazolin to inhibit planktonic *S. aureus*. However, the combination of halicin and rifampicin showed the greatest enhancement against both less-mature and more-mature biofilms. Biofilm maturity was confirmed in this study as the concentrations of antibiotics active against biofilms were substantially higher than the planktonic MICs [61–63]. To provide a clinical perspective of our results, we compared the antibiotic doses in our studies to achievable concentrations systemically and in bone (Table 1). Halicin reduced the effective dose of rifampicin against biofilms from $> 1500 \mu\text{M}$ to a level (15 μM) that is achievable systemically and in bone [44–47].

This is especially relevant as concentrations of rifampicin monotherapy substantially greater than those achievable did not reduce viable cell numbers. Our results show that different antibiotics have superior combinatorial activity with halicin against biofilms compared to planktonic cultures, further demonstrating the importance of studying biofilms to support translation to clinical studies. Although halicin's mechanism of action has not been completely defined, studies suggest that disruption of the proton motive force, binding to extracellular iron, induction of DNA damage, deoxygenation to a hydroxylamine by a bacterial nitroreductase, and/or covalent binding of a nitrotriazolone fragment to cysteine at the catalytic site of a protease may be involved [9,64,65].

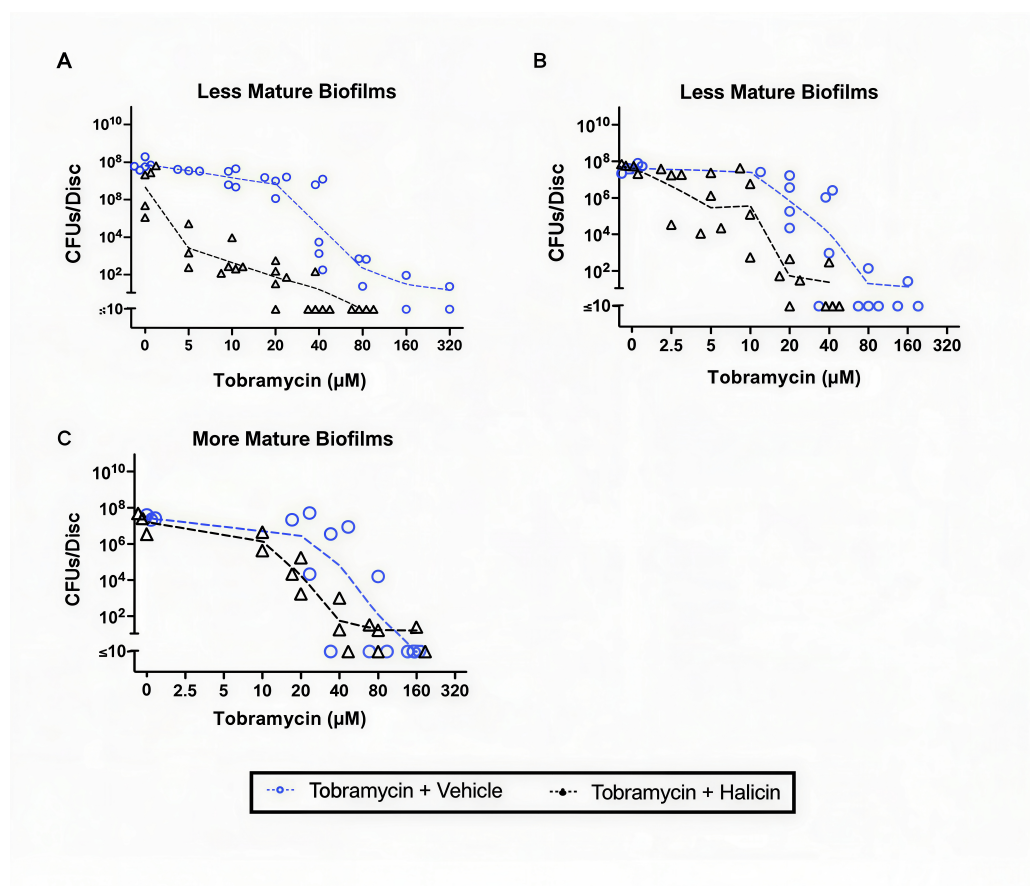


Fig. 4. Tobramycin is modestly more potent in the presence of halicin against less-mature biofilms. Less-mature (A,B) or more-mature biofilms (C) were incubated for 20 hours with the indicated doses of tobramycin in combination with either vehicle (blue circles) or 250 μM halicin (black triangles). Final concentrations of the vehicle in all wells were 2.5% DMSO (A) or 2.5% Kolliphor-HS 15 (B,C). Dotted lines connect the geometric mean CFUs of $n = 3\text{--}5$ independent experiments. Symbols denote the geometric mean for each group from an independent experiment, with 4 biofilms per group. p -values comparing tobramycin plus vehicle to tobramycin plus halicin were determined by mixed effects models (A–C). The figure was designed using GraphPad Prism, version 10.3.1, GraphPad Software, San Diego, CA, USA.

Rifampicin's stability and activity in anaerobic acidic biofilm environments likely account for its effect on biofilms in combination with halicin [29]. Rifampicin's ability to eradicate implant associated-biofilms when combined with other conventional antibiotics is well established in the literature [25,26]. The poor efficacy of rifampicin monotherapy on biofilms in our study is also consistent with the literature, as rifampicin monotherapy quickly induces resistance [28–31]. The resistance is likely due to the subtherapeutic doses achieved systemically and in bone by standard clinical doses of rifampicin monotherapy [66,67]. Halicin prevented the emergence of rifampicin resistance, similar to previously reported studies using rifampicin in combination therapy with conventional antibiotics [27,28]. However, rifampicin resistance can emerge despite combination with conventional antibiotics [68,69] and some combinations of other antibiotics can induce, rather than prevent, resistance [70,71]. Thus, preventing the emergence of antibiotic resistance with the addition of the novel antibiotic

halicin could lead to more efficacious bacterial eradication in animal models and in clinical medicine. Importantly, we also showed that halicin did not induce resistance to itself in bacterial biofilms. Those results are consistent with and extend previous studies showing that halicin does not induce resistance in planktonic cultures [9,18]. Rifampicin is well known to act primarily by inhibiting transcription and is one of the few conventional antibiotics that are effective against biofilms. Our finding that rifampicin is substantially more potent against biofilms when combined with halicin is therefore especially noteworthy. Understanding halicin's mechanism of action is a topic of current research, but it may include both disruption of the bacterial proton motive force and induction of DNA damage [9,65]. Although the molecular details require further study, our results suggest that one mechanism of halicin's combinatorial activity is the prevention of resistance to rifampicin. In that regard, prevention of resistance was shown to contribute to the combinatorial activity of pairs of conventional antibi-

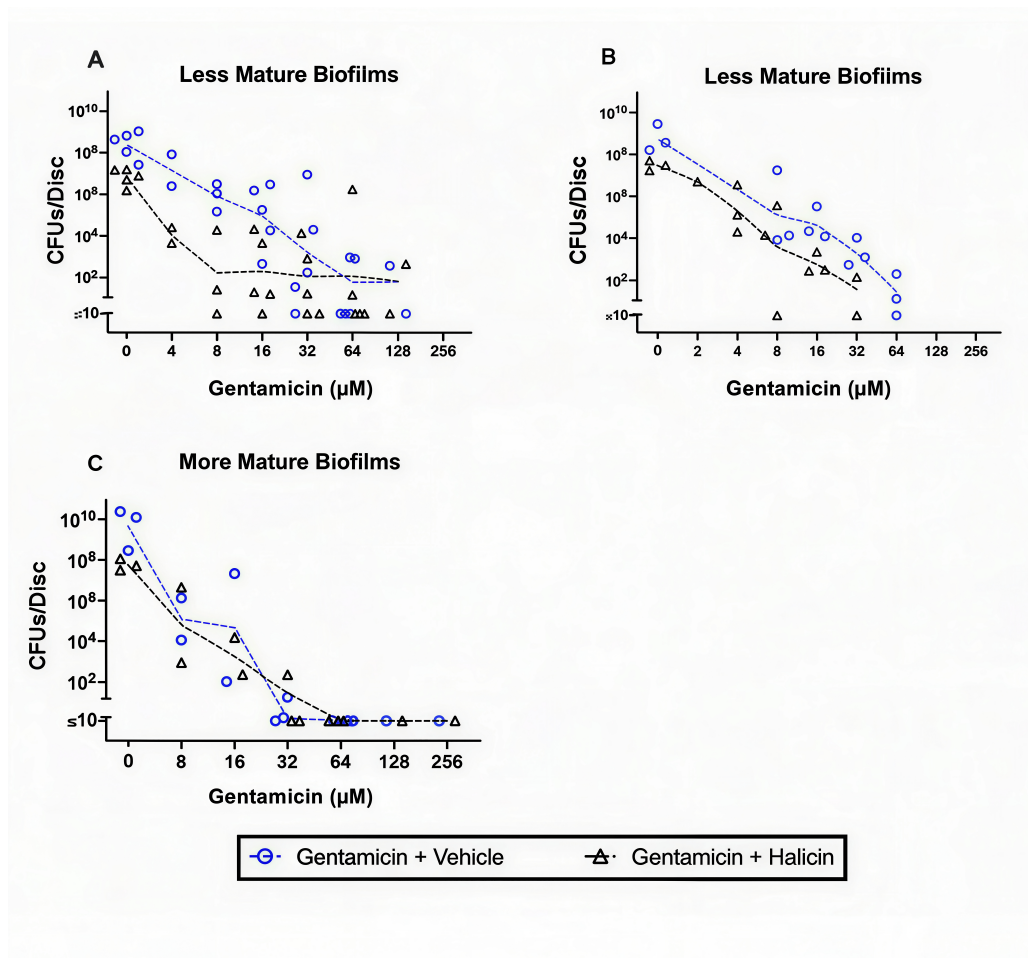


Fig. 5. The effect of halicin on the potency of gentamicin against less-mature biofilms depends on the vehicle that was used. Less-mature (A,B) or more-mature biofilms (C) were incubated for 20 hours with the indicated doses of gentamicin in combination with either vehicle (blue circles) or 250 μ M halicin (black triangles). Final concentrations of the vehicle in all wells were 2.5% DMSO (A) or 2.5% Kolliphor-HS 15 (B,C). Dotted lines connect geometric mean CFUs of $n = 3$ –5 independent experiments. Symbols denote the geometric mean for each group from an independent experiment, with 4 biofilms per group. p -values comparing gentamicin plus vehicle to gentamicin plus halicin were determined by mixed effects models (A–C). The figure was designed using GraphPad Prism, version 10.3.1, GraphPad Software, San Diego, CA, USA.

otics [72]. Other mechanisms of combinatorial activity include inhibition of sequential steps in a biosynthetic pathway and increased entry into the bacterial cells [72]. Future studies will be needed to determine which mechanism(s) are primarily responsible for the increase in rifampicin potency when combined with Halicin.

Halicin modestly increased the potency of the two aminoglycoside antibiotics in our study (gentamicin and tobramycin) against less-mature biofilms. However, the effect of adding halicin was largely lost against more-mature biofilms. Similar to the rifampicin combination results, the addition of halicin reduced the effective tobramycin dose against less-mature biofilms to concentrations that are achievable systemically and in bone [41–43]. Substantially decreasing the concentration of aminoglycosides needed for bacterial eradication would likely be clinically useful, given the side-effect profile of this antibiotic family at higher

doses [73].

Our finding of synergistic FICI values for combinations of halicin and aminoglycosides in planktonic *S. aureus* cultures are consistent with results using a different aminoglycoside (kanamycin) and *E. coli* in the original study of halicin as an antibacterial [9]. Those investigators also found that halicin and tetracycline were antagonistic. In contrast to the original study [9] other investigators reported combinatorial activity of halicin and doxycycline on both planktonic cultures and biofilms of gram-positive *Enterococcus* spp [14]. Further investigations are required to explain the discrepancy between that study and the results of the original study [9].

One of the strengths of our study was utilizing clinically-relevant titanium alloy substrates to grow *S. aureus*-Xen36 biofilms, as the biofilm substrate material can significantly impact antibiotic effectiveness [13,74–77].

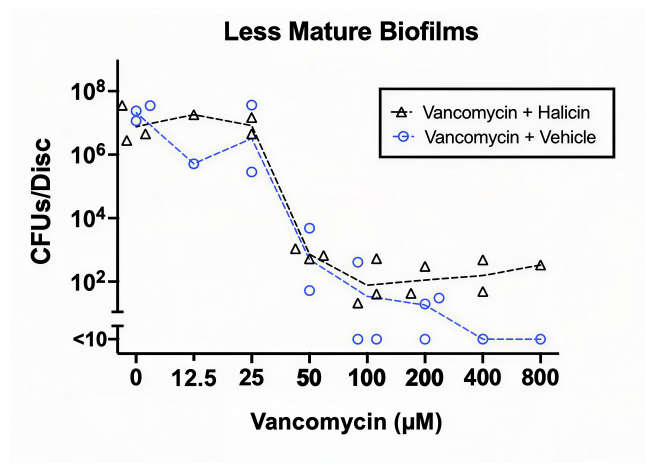


Fig. 6. Halicin does not detectably affect the potency of vancomycin. Less-mature biofilms were incubated for 20 hours with the indicated doses of vancomycin in combination with either vehicle (blue circles) or 250 μ M halicin (black triangles). The final concentration of the vehicle in all wells was 2.5% DMSO. Dotted lines connect geometric mean CFUs of $n = 3$ independent experiments. Symbols denote the geometric mean for each group from an independent experiment, with 4 biofilms per group. p -value comparing vancomycin plus vehicle to vancomycin plus halicin was determined by mixed effects models. The figure was designed using GraphPad Prism, version 10.3.1, GraphPad Software, San Diego, CA, USA.

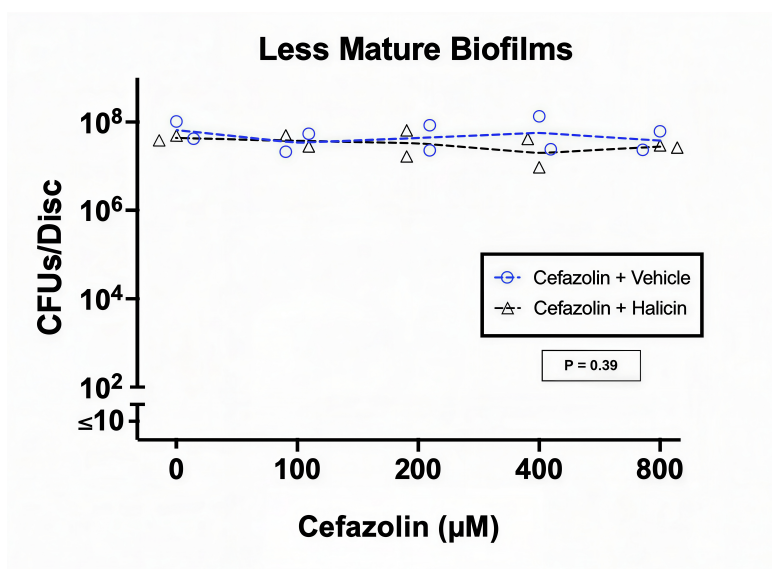


Fig. 7. Cefazolin does not alter the number of viable bacteria in less-mature biofilms with or without halicin. Less-mature biofilms were incubated for 20 hours with the indicated doses of cefazolin in combination with either vehicle (blue circles) or 250 μ M halicin (black triangles). The final concentration of the vehicle in all wells was 2.5% DMSO. Dotted lines connect geometric mean CFUs of $n = 2$ independent experiments. Symbols denote the geometric mean for each group from an independent experiment, with 4 biofilms per group. p -value determined by mixed effects model comparing cefazolin plus vehicle to cefazolin plus halicin. The figure was designed using GraphPad Prism, version 10.3.1, GraphPad Software, San Diego, CA, USA.

We also tested combinatorial therapy against biofilms of varying maturity, which increases the clinical translatability of our study, as more-mature biofilms are less susceptible to most antibiotics than are less-mature biofilms [61]. To increase the rigor of our biofilm experiments, we allowed biofilms to recover for 24 hours in broth after the antibiotic challenge, so that quiescent bacteria could shift back into a proliferative phenotype before CFU enumeration. Fi-

nally, we utilized antibiotics that are clinically relevant to the fields of infectious disease and orthopaedics and administered halicin using two separate vehicles, one of which is FDA approved with a safe toxicity profile [53–55].

This study is not without limitations. We tested only one strain of bacteria, specifically methicillin-sensitive *S. aureus*. However, halicin has previously shown broad-spectrum activity against planktonic and biofilm-residing

bacterial species, including multi-resistant strains [9,12–17]. Furthermore, clinically achievable levels of halicin are unknown and, therefore, cannot be compared to the effective doses in our study. A recent pharmacokinetic study demonstrated that halicin is poorly absorbed following oral administration, but higher levels might be achieved by intravenous or local administration [17]. Finally, while titanium alloy is a commonly utilized metallic surface in orthopaedic surgery and other surgical specialties, we did not examine biofilms grown on other clinically important substrates. However, halicin has robust activity against biofilms grown on a wide variety of substrates [12–16]. Those studies included silicone elastomer to mimic urinary and venous catheters as well as substrates clinically relevant to orthopedic infections (cobalt-chrome, ultra-high molecular weight polyethylene, devitalized muscle, and devitalized bone) [13,16].

Conclusions

Halicin synergized with gentamicin, tobramycin, and cefazolin against planktonic *S. aureus* and significantly increased the potency of rifampicin against less-mature and more-mature biofilms. The doses of conventional antibiotics in combination with halicin required to affect both planktonic and biofilm-residing *S. aureus* are all achievable systemically and in bone. Further, halicin prevented the emergence of rifampicin resistance, and halicin did not induce resistance to itself. The results of this *in vitro* study will drive future *in vivo* experiments utilizing combinations of halicin and rifampicin in small animal models of *S. aureus* biofilm infections.

Availability of Data and Materials

The datasets generated and analyzed during the current study are available from the corresponding author on request.

Author Contributions

JRS, AM, and AJW designed study, designed and conducted experiments, acquired and analyzed data, performed statistical analysis, prepared figures, and wrote and edited the manuscript; JES performed statistical analysis and provided statistical advice; DAW conducted experiments, acquired data, and edited the manuscript; SH designed experiments and edited the manuscript; RMN and EMG designed and supervised study, analyzed data, and edited the manuscript. All authors read and approved the manuscript. All authors declare no financial or non-financial competing interests.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgments

The authors would like to thank Polina Thang for their contributions to this project.

Funding

This project was aided by a grant from the Orthopaedic Research and Education Foundation with funding provided by the John T. Hocker Charitable Foundation in memory of John T. Hocker, MD (#22-068). This project was also supported by grants from the Foundation of Orthopaedic Trauma (#5022-3), the Orthopaedic Trauma Association (#7950), and the NIH (T32AR065971 and P30DK097512).

Conflict of Interest

The authors have no relevant conflicts of interest or funding sources not already stated in this manuscript.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.22203/eCM.v058a07>.

References

- [1] Costerton JW, Geesey GG, Cheng KJ. How bacteria stick. *Scientific American*. 1978; 238: 86–95. <https://doi.org/10.1038/scientificamerican0178-86>.
- [2] Hoiby N. *Pseudomonas aeruginosa* infection in cystic fibrosis. Relative prevalence of antibodies in serum against cathodic and anodic migrating *P. aeruginosa* antigens determined by immunoelectrophoretic methods. *Acta pathologica et microbiologica Scandinavica. Section C, Immunology*. 1977; 85: 149–152.
- [3] Novick RP, Geisinger E. Quorum sensing in staphylococci. *Annual Review of Genetics*. 2008; 42: 541–464. <https://doi.org/10.1146/annurev.genet.42.110807.091640>.
- [4] Uruén C, Chopo-Escuin G, Tommassen J, Mainar-Jaime RC, Arenas J. Biofilms as Promoters of Bacterial Antibiotic Resistance and Tolerance. *Antibiotics* (Basel, Switzerland). 2020; 10: 3. <https://doi.org/10.3390/antibiotics10010003>.
- [5] Yuan Y, Ren Y, Dijk M, Geertsema-Doornbusch GI, Atema-Smit J, Busscher HJ, *et al.* Phagocytosis and macrophage polarization on bacterially contaminated dental implant materials and effects on tissue integration. *European Cells & Materials* 2021; 41: 421–430. <https://doi.org/10.22203/eCM.v041a27>.
- [6] Kapadia BH, Berg RA, Daley JA, Fritz J, Bhav A, Mont MA. Periprosthetic joint infection. *Lancet* (London, England). 2016; 387: 386–394. [https://doi.org/10.1016/S0140-6736\(14\)61798-0](https://doi.org/10.1016/S0140-6736(14)61798-0).
- [7] Rowe SE, Beam JE, Conlon BP. Recalcitrant *Staphylococcus aureus* Infections: Obstacles and Solutions. *Infection and Immunity*. 2021; 89: e00694-20. <https://doi.org/10.1128/IAI.00694-20>.
- [8] Hogan S, Stevens NT, Humphreys H, O’Gara JP, O’Neill E. Current and future approaches to the prevention and treatment of staphylococcal medical device-related infections. *Current Pharmaceutical Design* 2015; 21: 100–113. <https://doi.org/10.2174/1381612820666140905123900>.
- [9] Stokes JM, Yang K, Swanson K, Jin W, Cubillos-Ruiz A, Donghia NM, *et al.* A Deep Learning Approach to Antibiotic Discovery. *Cell*. 2020; 180: 688–702.e13. <https://doi.org/10.1016/j.cell.2020.01.021>.

- [10] Waters EM, Rowe SE, O’Gara JP, Conlon BP. Convergence of *Staphylococcus aureus* Persister and Biofilm Research: Can Biofilms Be Defined as Communities of Adherent Persister Cells? *PLoS Pathogens*. 2016; 12: e1006012. <https://doi.org/10.1371/journal.ppat.1006012>.
- [11] Lewis K. Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology*. 2007; 5: 48–56. <https://doi.org/10.1038/nrmicro1557>.
- [12] Higashihira S, Simpson SJ, Collier CD, Natoli RM, Kittaka M, Greenfield EM. Halicin is Effective Against *Staphylococcus aureus* Biofilms *In Vitro*. *Clinical Orthopaedics and Related Research*. 2022; 480: 1476–1487. <https://doi.org/10.1097/CORR.0000000000002251>.
- [13] Higashihira S, Simpson SJ, Morita A, Suryavanshi JR, Arnold CJ, Natoli RM, *et al.* Halicin remains active against *Staphylococcus aureus* in biofilms grown on orthopaedically relevant substrates. *Bone & Joint Research*. 2024; 13: 101–109. <https://doi.org/10.1302/2046-3758.133.BJR-2023-0038.R2>.
- [14] Hussain Z, Pengfei S, Yimin L, Shasha L, Zehao L, Yifan Y, *et al.* Study on antibacterial effect of halicin (SU3327) against *Enterococcus faecalis* and *Enterococcus faecium*. *Pathogens and Disease*. 2022; 80: ftac037. <https://doi.org/10.1093/femspd/ftac037>.
- [15] van Gent ME, van der Reijden TJK, Lennard PR, de Visser AW, Schonkeren-Ravensbergen B, Dolezal N, *et al.* Synergism between the Synthetic Antibacterial and Antibiofilm Peptide (SAAP)-148 and Halicin. *Antibiotics* (Basel, Switzerland). 2022; 11: 673. <https://doi.org/10.3390/antibiotics11050673>.
- [16] Bouhrour N, van der Reijden TJK, Voet MM, Schonkeren-Ravensbergen B, Cordfunke RA, Drijfhout JW, *et al.* Novel Antibacterial Agents SAAP-148 and Halicin Combat Gram-Negative Bacteria Colonizing Catheters. *Antibiotics* (Basel, Switzerland). 2023; 12: 1743. <https://doi.org/10.3390/antibiotics12121743>.
- [17] Zhang M, Lin S, Han L, Zhang J, Liu S, Yang X, *et al.* Safety and efficacy evaluation of halicin as an effective drug for inhibiting intestinal infections. *Frontiers in Pharmacology* 2024; 15: 1389293. <https://doi.org/10.3389/fphar.2024.1389293/FULL>.
- [18] Wang S, Zhao K, Chen Z, Liu D, Tang S, Sun C, *et al.* Halicin: A New Horizon in Antibacterial Therapy against Veterinary Pathogens. *Antibiotics* (Basel, Switzerland). 2024; 13: 492. <https://doi.org/10.3390/antibiotics13060492>.
- [19] Grooters KE, Ku JC, Richter DM, Krinock MJ, Minor A, Li P, *et al.* Strategies for combating antibiotic resistance in bacterial biofilms. *Frontiers in Cellular and Infection Microbiology*. 2024; 14: 1352273. <https://doi.org/10.3389/fcimb.2024.1352273>.
- [20] Ventola CL. The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharmacy and Therapeutics*. 2015; 40: 277–283.
- [21] Uddin TM, Chakraborty AJ, Khusro A, Zidan BRM, Mitra S, Emran TB, *et al.* Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. *Journal of Infection and Public Health*. 2021; 14: 1750–1766. <https://doi.org/10.1016/j.jiph.2021.10.020>.
- [22] Coates ARM, Hu Y, Holt J, Yeh P. Antibiotic combination therapy against resistant bacterial infections: synergy, rejuvenation and resistance reduction. *Expert Review of Anti-Infective Therapy*. 2020; 18: 5–15. <https://doi.org/10.1080/14787210.2020.1705155>.
- [23] Doern CD. When Does 2 Plus 2 Equal 5? A Review of Antimicrobial Synergy Testing. *Journal of Clinical Microbiology* 2014; 52: 4124–4128. <https://doi.org/10.1128/JCM.01121-14>.
- [24] Mohamed NS, Wilkie WA, Remily EA, Nace J, Delanois RE, Browne JA. Antibiotic Choice: The Synergistic Effect of Single vs Dual Antibiotics. *The Journal of Arthroplasty*. 2020; 35: S19–S23. <https://doi.org/10.1016/j.arth.2019.11.002>.
- [25] Zimmerli W, Widmer AF, Blatter M, Frei R, Ochsner PE. Role of rifampin for treatment of orthopedic implant-related staphylococcal infections: a randomized controlled trial. *Foreign-Body Infection (FBI) Study Group. JAMA*. 1998; 279: 1537–1541. <https://doi.org/10.1001/jama.279.19.1537>.
- [26] Leijten B, Elbers JBW, Sturm PD, Kullberg BJ, Schreurs BW. Clindamycin-rifampin combination therapy for staphylococcal periprosthetic joint infections: a retrospective observational study. *BMC Infectious Diseases*. 2017; 17: 321. <https://doi.org/10.1186/S12879-017-2429-2>.
- [27] Gordon O, Lee DE, Liu B, Langevin B, Ordonez AA, Dikeman DA, *et al.* Dynamic PET-facilitated modeling and high-dose rifampin regimens for *Staphylococcus aureus* orthopedic implant-associated infections. *Science Translational Medicine*. 2021; 13: eabl6851. <https://doi.org/10.1126/scitranslmed.abl6851>.
- [28] Bayer AS, Lam K. Efficacy of vancomycin plus rifampin in experimental aortic-valve endocarditis due to methicillin-resistant *Staphylococcus aureus*: *in vitro-in vivo* correlations. *The Journal of Infectious Diseases*. 1985; 151: 157–165. <https://doi.org/10.1093/infdis/151.1.157>.
- [29] Zimmerli W, Sendi P. Role of Rifampin against Staphylococcal Biofilm Infections *In Vitro*, in Animal Models, and in Orthopedic-Device-Related Infections. *Antimicrobial Agents and Chemotherapy*. 2019; 63: e01746-18. <https://doi.org/10.1128/AAC.01746-18>.
- [30] Belden K, Zimmerli W, Lausmann C, Citak M, Zahar A. “When should rifampin be added to the regimen of antibiotics for management of patients with periprosthetic joint infections (PJIs) undergoing surgical treatment,” *Proceedings of the Second International Consensus Meeting on Musculoskeletal Infection*. 2018.
- [31] Achermann Y, Eigenmann K, Ledergerber B, Derksen L, Rafeiner P, Clauss M, *et al.* Factors associated with rifampin resistance in staphylococcal periprosthetic joint infections (PJI): a matched case-control study. *Infection*. 2013; 41: 431–437. <https://doi.org/10.1007/S15010-012-0325-7>.
- [32] El Belghiti I, Hammani O, Moustaoi F, Aghrouh M, Lemkhente Z, Boubrik F, *et al.* Halicin: A New Approach to Antibacterial Therapy, a Promising Avenue for the Post-Antibiotic Era. *Antibiotics* (Basel, Switzerland). 2025; 14: 698. <https://doi.org/10.3390/antibiotics14070698>.
- [33] Harrison JJ, Stremick CA, Turner RJ, Allan ND, Olson ME, Ceri H. Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. *Nature Protocols*. 2010; 5: 1236–1254. <https://doi.org/10.1038/nprot.2010.71>.
- [34] Odds FC. Synergy, antagonism, and what the checkerboard puts between them. *The Journal of Antimicrobial Chemotherapy*. 2003; 52: 1. <https://doi.org/10.1093/jac/dkg301>.
- [35] Dybvig DD, Dybvig M. Det tenkende mennesket: filosofi og vitenskapshistorie med vitenskapsteori. 2nd edn. Tapir Akademisk Forlag: Trondheim. 2003. Available at: <https://www.clinmicronow.org/doi/book/10.1128/9781683670438.CMPH> (Accessed: 29 April 2024).
- [36] Bhalodi AA, Housman ST, Shepard A, Nugent J, Nicolau DP. Tissue Pharmacokinetics of Cefazolin in Patients with Lower Limb Infections. *Antimicrobial Agents and Chemotherapy*. 2013; 57: 5679–5683. <https://doi.org/10.1128/AAC.01348-13>.
- [37] Douglas A, Udy AA, Wallis SC, Jarrett P, Stuart J, Lassig-Smith M, *et al.* Plasma and Tissue Pharmacokinetics of Cefazolin in Patients Undergoing Elective and Semielective Abdominal Aortic Aneurysm Open Repair Surgery. *Antimicrobial Agents and Chemotherapy*. 2011; 55: 5238–5242. <https://doi.org/10.1128/AAC.05033-11>.
- [38] Mannarino M, Montreuil J, Tanzer M, Hart A. Local tissue concentrations of cefazolin during total joint arthroplasty: a systematic review. *Canadian Journal of Surgery*. 2023; 66: E415–E421. <https://doi.org/10.1503/cjs.019621>.
- [39] Young SW, Zhang M, Freeman JT, Vince KG, Coleman B. Higher Cefazolin Concentrations with Intraosseous Regional Prophylaxis in TKA. *Clinical Orthopaedics and Related Research*. 2013; 471: 244–249. <https://doi.org/10.1007/s11999-012-2469-2>.
- [40] Paz E, Sanz-Ruiz P, Abenojar J, Vaquero-Martín J, Forriol F, Del

- Real JC. Evaluation of Elution and Mechanical Properties of High-Dose Antibiotic-Loaded Bone Cement: Comparative “*In Vitro*” Study of the Influence of Vancomycin and Cefazolin. *The Journal of Arthroplasty*. 2015; 30: 1423–1429. <https://doi.org/10.1016/j.arth.2015.02.040>.
- [41] Cobussen M, Stassen PM, Posthouwer D, van Tiel FH, Savelkoul PHM, Havenith T, *et al.* Improving peak concentrations of a single dose regime of gentamicin in patients with sepsis in the emergency department. *PLoS ONE*. 2019; 14: e0210012. <https://doi.org/10.1371/journal.pone.0210012>.
- [42] Torkington MS, Davison MJ, Wheelwright EF, Jenkins PJ, Anthony I, Lovering AM, *et al.* Bone penetration of intravenous flucloxacillin and gentamicin as antibiotic prophylaxis during total hip and knee arthroplasty. *The Bone & Joint Journal*. 2017; 99-B: 358–364. <https://doi.org/10.1302/0301-620X.99B3.BJJ-2016-0328.R1>.
- [43] Lizcano JD, Fernández-Rodríguez D, Goh GS, Demik DE, Hughes AJ, Parvizi J, *et al.* In Vivo Intra-Articular Antibiotic Concentrations at 24 Hours After TKA Fall Below the Minimum Inhibitory Concentration for Most Bacteria: A Randomized Study of Commercially Available Bone Cement. *The Journal of Bone and Joint Surgery American Volume*. 2024; 106: 1664–1672. <https://doi.org/10.2106/JBJS.23.01412>.
- [44] Wasserman S, Davis A, Stek C, Chirehwa M, Botha S, Daroowala R, *et al.* Plasma pharmacokinetics of high-dose oral versus intravenous rifampicin in patients with tuberculous meningitis: a randomized controlled trial. *Antimicrobial Agents and Chemotherapy*. 2021; 65: e0014021. <https://doi.org/10.1128/AAC.00140-21>.
- [45] Marsot A, Ménard A, Dupouey J, Muziotti C, Guilhaumou R, Blin O. Population pharmacokinetics of rifampicin in adult patients with osteoarticular infections: interaction with fusidic acid. *British Journal of Clinical Pharmacology*. 2017; 83: 1039–1047. <https://doi.org/10.1111/bcp.13178>.
- [46] Roth B. Penetration of parenterally administered rifampicin into bone tissue. *Chemotherapy*. 1984; 30: 358–365. <https://doi.org/10.1159/000238294>.
- [47] Qayoom I, Teotia AK, Panjla A, Verma S, Kumar A. Local and Sustained Delivery of Rifampicin from a Bioactive Ceramic Carrier Treats Bone Infection in Rat Tibia. *ACS Infectious Diseases*. 2020; 6: 2938–2949. <https://doi.org/10.1021/acinfecdis.0c00369>.
- [48] Beringer PM, Vinks AA, Jelliffe RW, Shapiro BJ. Pharmacokinetics of Tobramycin in Adults with Cystic Fibrosis: Implications for Once-Daily Administration. *Antimicrobial Agents and Chemotherapy*. 2000; 44: 809–813. <https://doi.org/10.1128/AAC.44.4.809-813.2000>.
- [49] Kawashima M, Torisu T. Study on tobramycin concentration in bone marrow. *The Japanese Journal of Antibiotics*. 1980; 33: 249–252. <https://doi.org/10.11553/antibiotics1968b.33.249>. (In Japanese)
- [50] Ghasemiyeh P, Vazin A, Zand F, Haem E, Karimzadeh I, Azadi A, *et al.* Pharmacokinetic assessment of vancomycin in critically ill patients and nephrotoxicity prediction using individualized pharmacokinetic parameters. *Frontiers in Pharmacology*. 2022; 13: 912202. <https://doi.org/10.3389/fphar.2022.912202>.
- [51] Graziani AL, Lawson LA, Gibson GA, Steinberg MA, McGregor RR. Vancomycin concentrations in infected and noninfected human bone. *Antimicrobial Agents and Chemotherapy*. 1988; 32: 1320–1322. <https://doi.org/10.1128/AAC.32.9.1320>.
- [52] Olsen Kipp J, Hanberg P, Slater J, Møller Nielsen L, Storgaard Jakobsen S, Stilling M, *et al.* Vancomycin bone and tissue concentrations following tibial intraosseous administration—evaluated in a porcine model. *Journal of Bone and Joint Infection* 2021; 6: 99–106. <https://doi.org/10.5194/jbji-6-99-2021>.
- [53] Stokes AH, Kemp DC, Faiola B, Jordan HL, Merrill CL, Hailey JR, *et al.* Effects of Solutol (Kolliphor) and cremophor in polyethylene glycol 400 vehicle formulations in Sprague-Dawley rats and beagle dogs. *International Journal of Toxicology*. 2013; 32: 189–197. <https://doi.org/10.1177/1091581813485452>.
- [54] Ali S, Kolter K. Kolliphor® HS 15 - An Enabler for Parenteral and Oral Formulations. 2019. Available at: <https://www.americanpharmaceuticalreview.com/Featured-Articles/358749-Kolliphor-HS-15-An-Enabler-for-Parenteral-and-Oral-Formulations/> (Accessed: 29 April 2024).
- [55] Ryoo HK, Park CW, Chi SC, Park ES. Development of propofol-loaded microemulsion systems for parenteral delivery. *Archives of Pharmacological Research*. 2005; 28: 1400–1404. <https://doi.org/10.1007/BF02977908>.
- [56] Madsen BK, Hilscher M, Zetner D, Rosenberg J. Adverse reactions of dimethyl sulfoxide in humans: a systematic review. *F1000Research*. 2019; 7: 1746. <https://doi.org/10.12688/f1000research.16642.2>.
- [57] Rubin LF. Toxicity of dimethyl sulfoxide, alone and in combination. *Annals of the New York Academy of Sciences*. 1975; 243: 98–103. <https://doi.org/10.1111/j.1749-6632.1975.tb25348.x>.
- [58] Verheijen M, Lienhard M, Schrooders Y, Clayton O, Nudischer R, Boerno S, *et al.* DMSO induces drastic changes in human cellular processes and epigenetic landscape *in vitro*. *Scientific Reports*. 2019; 9: 4641. <https://doi.org/10.1038/s41598-019-40660-0>.
- [59] O'Neill AJ, Cove JH, Chopra I. Mutation frequencies for resistance to fusidic acid and rifampicin in *Staphylococcus aureus*. *The Journal of Antimicrobial Chemotherapy*. 2001; 47: 647–650. <https://doi.org/10.1093/jac/47.5.647>.
- [60] Vall-Spinosa A, Lester W, Moulding T, Davidson PT, McClatchy JK. Rifampin in the treatment of drug-resistant mycobacterium tuberculosis infections. *The New England Journal of Medicine*. 1970; 283: 616–621. <https://doi.org/10.1056/NEJM197009172831202>.
- [61] Stewart PS. Antimicrobial Tolerance in Biofilms. *Microbiology Spectrum*. 2015; 3. <https://doi.org/10.1128/microbiolspec.MB-0010-2014>.
- [62] Chen X, Thomsen TR, Winkler H, Xu Y. Influence of biofilm growth age, media, antibiotic concentration and exposure time on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm removal *in vitro*. *BMC Microbiology*. 2020; 20: 264. <https://doi.org/10.1186/s12866-020-01947-9>.
- [63] Hickok NJ, Li B, Oral E, Zaat SAJ, Armbruster DA, Atkins GJ, *et al.* The 2023 Orthopedic Research Society’s international consensus meeting on musculoskeletal infection: Summary from the *in vitro* section. *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*. 2024; 42: 512–517. <https://doi.org/10.1002/jor.25774>.
- [64] Yang KS, Alex Kuo S-T, Blankenship LR, Geng ZZ, Li SG, Russell DH, *et al.* Repurposing Halicin as a potent covalent inhibitor for the SARS-CoV-2 main protease. *Current Research in Chemical Biology*. 2022; 2: 100025. <https://doi.org/10.1016/j.crchbi.2022.100025>.
- [65] Wang S, Zhao K, Chen Z, Sun C, Chen H, Wang Y, *et al.* SU3327: A multi-target compound targeting bacterial menaquinone and DNA. *International Journal of Antimicrobial Agents*. 2026; 67: 107679. <https://doi.org/10.1016/j.ijantimicag.2025.107679>.
- [66] Dheda K, Lenders L, Magombedze G, Srivastava S, Raj P, Arning E, *et al.* Drug-Penetration Gradients Associated with Acquired Drug Resistance in Patients with Tuberculosis. *American Journal of Respiratory and Critical Care Medicine*. 2018; 198: 1208–1219. <https://doi.org/10.1164/rccm.201711-2333OC>.
- [67] Bhattacharya G, Dey D, Das S, Banerjee A. Exposure to sub-inhibitory concentrations of gentamicin, ciprofloxacin and cefotaxime induces multidrug resistance and reactive oxygen species generation in methicillin-sensitive *Staphylococcus aureus*. *Journal of Medical Microbiology*. 2017; 66: 762–769. <https://doi.org/10.1099/jmm.0.000492>.
- [68] Riedel DJ, Weekes E, Forrest GN. Addition of rifampin to standard therapy for treatment of native valve infective endocarditis caused by *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. 2008; 52: 2463–2467. <https://doi.org/10.1128/AAC.00300-08>.
- [69] Donaldson AD, Chan RC, Gosbell IB. Community-acquired

methicillin-resistant *Staphylococcus aureus* in bone and joint infections: development of rifampicin resistance. *The Medical Journal of Australia*. 2006; 184: 196. <https://doi.org/10.5694/j.1326-5377.2006.tb00189.x>.

- [70] Dean Z, Maltas J, Wood KB. Antibiotic interactions shape short-term evolution of resistance in *E. faecalis*. *PLoS. Pathogens*. 2020; 16: e1008278. <https://doi.org/10.1371/journal.ppat.1008278>.
- [71] Hegreness M, Shores N, Damian D, Hartl D, Kishony R. Accelerated evolution of resistance in multidrug environments. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105: 13977–13981. <https://doi.org/10.1073/pnas.0805965105>.
- [72] Brennan-Krohn T, Kirby JE. When One Drug Is Not Enough: Context, Methodology, and Future Prospects in Antibacterial Synergy Testing. *Clinics in Laboratory Medicine*. 2019; 39: 345–358. <https://doi.org/10.1016/j.cll.2019.04.002>.
- [73] Grayson ML, Cosgrove SE, Crowe SM, Hope WW, McCarthy JS, Mills J, et al. Kucers' The Use of Antibiotics: A Clinical Review of Antibacterial, Antifungal, Antiparasitic and Antiviral Drugs. 7th edn. CRC Press: Boca Raton. 2018.
- [74] Naylor P, Myrvik QN, Gristina A. Antibiotic resistance of biomaterial-adherent coagulase-negative and coagulase-positive staphylococci. *Clinical orthopaedics and related research*. 1990; 126–133.
- [75] Gomes LC, Silva LN, Simões M, Melo LF, Mergulhão FJ. *Escherichia coli* adhesion, biofilm development and antibiotic susceptibility on biomedical materials. *Journal of Biomedical Materials Research Part A*. 2015; 103: 1414–1423. <https://doi.org/10.1002/jbm.a.35277>.
- [76] Badha V, Moore R, Heffernan J, Castaneda P, McLaren A, Overstreet D. Determination of Tobramycin and Vancomycin Exposure Required to Eradicate Biofilms on Muscle and Bone Tissue *In Vitro*. *Journal of Bone and Joint Infection*. 2019; 4: 1–9. <https://doi.org/10.7150/jbji.29711>.
- [77] Webb LX, Holman J, de Araujo B, Zaccaro DJ, Gordon ES. Antibiotic resistance in staphylococci adherent to cortical bone. *Journal of Orthopaedic Trauma*. 1994; 8: 28–33. <https://doi.org/10.1097/00005131-199402000-00007>.

Editor's note: The Scientific Editors responsible for this paper were Fintan Moriarty and Martin Stoddart.

Received: 25th March 2026; **Accepted:** 27th May 2026; **Published:** 31st July 2026