Halicin-Rifampicin Combinations are More Effective than Monotherapy in a Murine *S. aureus* Biofilm Model of Fracture Related Infection

Allison J. Wintring MD[1], Joash R. Suryavanshi MD[1], Akira Morita MD, PhD[1,2], Jason Minnich BS[1], Roman M. Natoli MD[1], PhD, Ed M. Greenfield PhD[1]
[1]Dept of Orthopaedic Surgery, Indiana University, Indianapolis, IN, [2]Dept of Orthopaedic Surgery, Yokohama City University, Yokohama, Japan
awintrin@iu.edu

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INTRODUCTION: Fracture-related infections (FRIs) remain one of the most challenging complications for orthopaedic surgeons to manage. A major contributor to their persistence is bacterial biofilm formation, which shields dormant bacteria from conventional antibiotics that primarily target actively dividing cells[1]. Eradicating bacteria within biofilms often requires higher antibiotic doses that are not clinically achievable. Halicin is a novel broad-spectrum antimicrobial active against *S. aureus*, the most frequently isolated pathogen in FRIs. It is effective against both drug-resistant and quiescent bacterial populations, demonstrating efficacy against mature (7-day-old) biofilms on orthopaedically relevant materials[2-5]. In vitro, halicin works synergistically with rifampicin, boosting its activity over 100-fold against mature *S. aureus* biofilms while also suppressing the development of rifampicin resistance[6]. Given these promising properties, we hypothesized that combination therapy with halicin and rifampicin would effectively treat *S. aureus* infections in a murine *in vivo* FRI model.

METHODS: Approval for this study was obtained from the IACUC before beginning animal procedures. Female C57BL/6J mice (8 weeks old, n=70, Jackson Laboratory) underwent right femoral fracture fixation using a stainless-steel intramedullary pin implant (25g needle) in a validated murine fracture model[7]. Before wound closure, mice received either sterile PBS (uninfected controls, n = 8) or 10^6 CFUs of bioluminescent Xen-36 *S. aureus* at the fracture site. Biofilms grew for 7 days at the surgical site; three mice were euthanized at this timepoint, and implants were collected and imaged using scanning electron microscopy (SEM) to characterize biofilm presence. Antibiotic treatment was initiated on postoperative day 7 for treatment groups. Infected mice were randomized into the following treatment cohorts: vehicle (infected control, n = 10), halicin (2 mg/kg, n = 12), rifampicin (10 mg/kg, n= 12), vancomycin (110 mg/kg, n = 10), and halicin/rifampicin (2 mg/kg + 10 mg/kg, n = 12). Treatments were administered for 14 days, followed by a 7-day washout period before euthanasia. Viable bacteria (colony-forming units, CFUs) were quantified at euthanasia in implant sonicates, homogenates of femurs, quadriceps, and spleens, and in blood. Metabolically active bacteria were monitored weekly using in vivo bioluminescent imaging. Fracture healing was evaluated using serial radiographs scored via the mRUST system by two blinded orthopaedic surgeons. Statistical analyses were performed using Kruskal–Wallis tests or 2-way ANOVA with multiple comparisons corrected using Dunn's or Dunnett's post hoc tests (GraphPad Prism 10.3.1).

RESULTS: Infections remained localized to the fracture site in all mice on bioluminescent imaging, and no bacteria were detected in spleen or blood samples. Biofilms were visualized on all pins that underwent SEM imaging. Consistent with biofilm formation, vancomycin had no significant effects on any of the outcome measures. The halicin/rifampicin combination was the only treatment that significantly ($p<0.001$) reduced CFUs on implants, femurs, or adjacent muscles and that eradicated bacteria from implants and muscle in all mice (Figure 1A-B & not shown). Halicin/rifampicin also eradicated bacteria in 3 of 12 femurs versus 1 of 12 femurs treated with either monotherapy (Figure 1B). By week 3, halicin/rifampicin and rifampicin alone reduced bacterial metabolic activity to uninfected control levels (~5 logs below infected controls, $p < 0.006$), whereas other treatment groups remained within 1 log of the infected controls (NS). At week 4, fracture healing (mRUST $\geq$ 12) occurred in all uninfected controls and 7 of 12 halicin/rifampicin-treated mice but in none of the infected controls or monotherapy groups.

DISCUSSION: In this murine FRI model, halicin/rifampicin combination therapy demonstrated superior efficacy against *S. aureus* biofilms compared with either monotherapy. The regimen eradicated biofilms from stainless-steel implants and surrounding muscles, and enhanced fracture healing in the presence of infection. These findings suggest that halicin/rifampicin may represent a promising novel therapeutic strategy for FRI. Ongoing studies include histomorphometric and uCT analyses to assess halicin's effects on fracture repair. As this work represents an initial evaluation in female mice, additional studies are warranted to investigate treatment effects in male cohorts.

SIGNIFICANCE/CLINICAL RELEVANCE: Combination halicin and rifampicin antibiotic drug therapy shows promising results in this *in vivo* study. Halicin is an investigational compound in the early stages of research and development; this study highlights its potential in eradicating orthopedic biofilm infections and further study is warranted.

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