The Novel Antibiotic Halicin Increases the Effects of Rifampicin on *S. aureus* Biofilms in a Murine Model of Fracture Related Infection

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INTRODUCTION:

Fracture-related infections (FRIs) are among the most challenging complications faced by orthopaedic surgeons. Bacterial biofilms, which protect quiescent bacteria, are key to infection persistence since many conventional antibiotics target proliferating cells. Increased antibiotic dosages are often required to eradicate biofilm-residing bacteria, elevating toxicity to the patient and contributing to antibiotic resistance. Halicin is a novel, broad-spectrum antimicrobial agent with potent activity against *S. aureus*, the most-common organism implicated in FRI. Halicin displays broad-spectrum activity against drug-resistant and quiescent bacterial populations; it has been proven effective against mature (7-day) biofilms formed on orthopaedically relevant substrates. Halicin also acts synergistically with rifampicin *in vitro*, enhancing rifampicin activity by over 100-fold against mature *S. aureus* biofilms and preventing the emergence of rifampicin resistance. Given these promising characteristics, we hypothesized that halicin/rifampicin combination therapy would successfully treat *S. aureus* infection in an *in vivo* murine FRI model.

METHODS:

Institutional Animal Care and Use Committee approval was obtained before beginning animal procedures. Eight-week-old female C57BL/6J (Jackson Laboratory) mice (n=70) underwent right femur fracture fixation with a stainless-steel implant in a well-characterized intramedullary pin-based fracture model. Mice were injected with sterile PBS (uninfected control, n=8) or 10^6 CFUs of bioluminescent *S. aureus-Xen36* at the fracture site prior to closure. Antibiotics were held for 7 days post-surgery to promote biofilm formation. Infected mice were then divided into treatment groups receiving no antibiotic (n=10, infected control), halicin (n=12, 2mg/kg), rifampicin (n=12, 10mg/kg), vancomycin (n=10, 110mg/kg), halicin/rifampicin (n=12, 2mg/kg + 10mg/kg), or halicin/vancomycin (n=5, 2mg/kg + 110mg/kg). Mice received antibiotics or vehicle control for 14 days, followed by a one-week washout period. We assessed infection severity using longitudinal bioluminescent imaging and by counting bacteria colony-forming units (CFUs) in the femur, quadriceps, spleen, and implant samples harvested at euthanasia. Fracture healing was monitored using longitudinal radiographs; randomized images were assessed by two blinded orthopaedic surgeons using the mRUST scoring system. Each implant was also assigned an integration score based on the difficulty of pin explantation at euthanasia. Statistical significance was determined by two-way ANOVA or Kruskal-Wallis testing, utilizing Dunn's or Dunnett's test for multiple comparisons as appropriate (Prism 10.3.1, GraphPad).

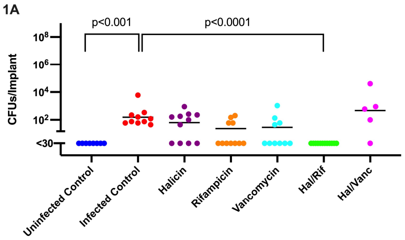
RESULTS:

Halicin/rifampicin was the only treatment that completely eradicated bacteria on the implant and in muscle samples from 100% of the mice (Figure 1A). Halicin/rifampicin also eliminated infection in 3 of 12 femur samples, while monotherapeutic treatment only eradicated infection in 1 of 24 femur samples (Figure 1B). Further, femur CFU counts were 3 logs lower in the rifampicin/halicin group than in the infected control group ($p=0.001$); monotherapeutic treatments did not statistically reduce femur CFUs ($p>0.08$, Figure 1B). Bioluminescent imaging confirmed similar luminescence levels in all infected groups in the early post-surgical period. At 3 weeks, the rifampicin monotherapy and halicin/rifampicin treatment groups displayed bioluminescence levels similar to the uninfected control group, approximately 5 logs less than the infected control group ($p<0.006$). In contrast, the remaining treatment groups' bioluminescence levels were within 1-log of the infected control group (ns). Bioluminescence was restricted to the fracture site and no bacterial colonies were detected in spleen or blood samples, confirming that infections remained localized to the thigh. Biofilms are typically characterized by resistance to vancomycin. Consistent with biofilm formation in our FRI model, vancomycin had a minimal effect on *S. aureus* CFU counts, both as monotherapy and when combined with halicin (Figure 1A-B). Radiographs at postoperative week 4 showed successful healing in the uninfected control group (mRUST score$\geq$12) and no successful healing in the infected controls ($p=0.002$). Monotherapeutic treatment did not increase healing ($p>0.9$). In contrast, halicin/rifampicin combination therapy facilitated successful healing in 7 of 12 mice (mRUST $\geq$12, $p=0.27$). All uninfected control implants were well-integrated at euthanasia while no infected control implants integrated ($p<0.0001$). In the halicin/rifampicin group, 21% of implants were well integrated while 32% showed intermediate integration ($p=0.013$). No other groups contained well-integrated implants.

DISCUSSION AND CONCLUSION:

Halicin/rifampicin combination treatment more effectively eradicated *S. aureus* biofilms in this murine FRI model than either monotherapeutic treatment group. Further, this treatment successfully eliminated biofilms on stainless-steel implants and improved fracture healing rates in the setting of infection. These results indicate halicin/rifampicin combination therapy may be a promising novel treatment for FRI. Scanning electron microscopy studies to characterize biofilm formation alongside histomorphometric and uCT studies to evaluate the effect of halicin on fracture healing are ongoing.

1A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Infected Control
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Rifampicin
Vancomycin
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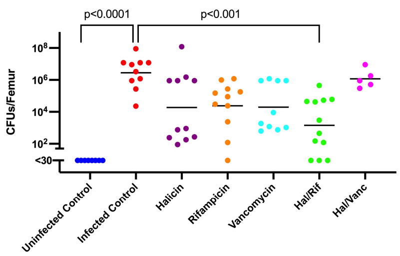
1B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Halicin
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Vancomycin
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