Effects of Hydroxybisphosphonate-Conjugated Sitafloxacin on Fracture Healing and Skeletal Growth in Mice

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Disclosures: JDN and EMS are paid consultants of BioVinc. FHE, SS are employees of BioVinc. This work was funded by BioVinc.

Introduction: Chronic bone infection is considered incurable due to bacterial colonization of the osteocyte-lacuno canalicular network (OLCN). The pharmacokinetic limitations of drug delivery to this compartment of bone render the infection resistant to standard of care antibiotics. Bone-targeted bisphosphonate-conjugated antibiotics have been developed to overcome this issue.[1] These target-and-release compounds are cleaved by acidic and/or enzymatic conditions at the bone-bacteria interface, releasing the active antibiotic and killing bacteria. Hydroxybisphosphonate-conjugated sitafloxacin (HBCS) has emerged as a leading candidate based on safety and efficacy results in mice and sheep.[2-4] However, due to the bisphosphonate component in HBCS, potential concerns about adverse effects on skeletal growth and bone healing remain. To this end, we tested the hypotheses that: 1) HBCS has similar effects to its bisphosphonate component, hydroxy-phenyl-ethane-hydroxy-bisphosphonate (HPHBP), on bone growth and fracture healing; and 2) HBCS has non-inferior adverse effects on fracture healing compared to the most potent bisphosphonate used in patients, zoledronic acid (ZA).

Methods: All in vivo experiments were performed according to IACUC-approved protocols. We utilized a closed, stabilized, mid-diaphyseal femur fracture model with 12-week-old female C57BL/6 mice. We used female mice in our study to reflect higher fracture incidence in women. Briefly, a stainless steel 25-gauge spinal needle was inserted into the intramedullary space of the femur, followed by three-point bending with an Einhorn device using a standardized force. The animals were injected i.p. with either: 1) PBS, or 2) ZA 0.1mg/kg (single dose), or 3) parental bisphosphonate (HPHBP) 3.0mg/kg/48hr until sacrifice, or 4) HBCS 3.0mg/kg/48hr until sacrifice. The fractures were radiographically assessed on day 0 and every 7 days post-fracture. At 2- and 3-week timepoints, the mice were euthanized via heart perfusion using lead chromate-based Microfil (MV120) under anesthesia to assess angiogenesis. The femurs were harvested and processed for μ-CT (n=5) and histological analysis (n=5). Femurs from the 4-week groups underwent biomechanical torsion testing (n=12). Statistical analyses were performed using one-way ANOVA.

Results: The μ-CT analyses of the fracture callus and vasculature at 2- and 3-weeks post-fracture revealed no drug effects on angiogenesis and minimal effects on callus that were limited to ZA and HBCS increased bone volume/total volume (BV/TV) vs. PBS at 3 weeks (**Fig. 1A, B**). Alcian blue staining confirmed similar amounts of soft callus in the fracture at 2 weeks, which was completely remodeled into bone by 3 weeks in all groups. Tartrate-resistant acid phosphatase (TRAP) staining confirmed the predicted increase in osteoclasts in the ZA group at 2 weeks, with no HBCS effects at this time point and decreases in $TRAP^+$ area in all drug treatment groups at 3 weeks. Dynamic histomorphometry confirmed that none of the drug treatments altered the mineral apposition rate and bone formation rate. At 4 weeks, ZA increased the polar moment of inertia concomitant with increased maximum torque (**Fig. 1C**). To assess the effects of HBCS on normal long bone growth, we extended the same studies to unfractured mice. ZA dramatically increased tibial growth plate length from unresorbed primary spongiosa, increased growth plate bone volume and BV/TV, and increased osteoclasts vs. PBS, consistent with prior reports (**Fig. 2**). In contrast, HBCS had no remarkable effects on growth plate appearance, osteoclasts, and bone volume but did increase BV/TV vs. PBS (**Fig. 2**).

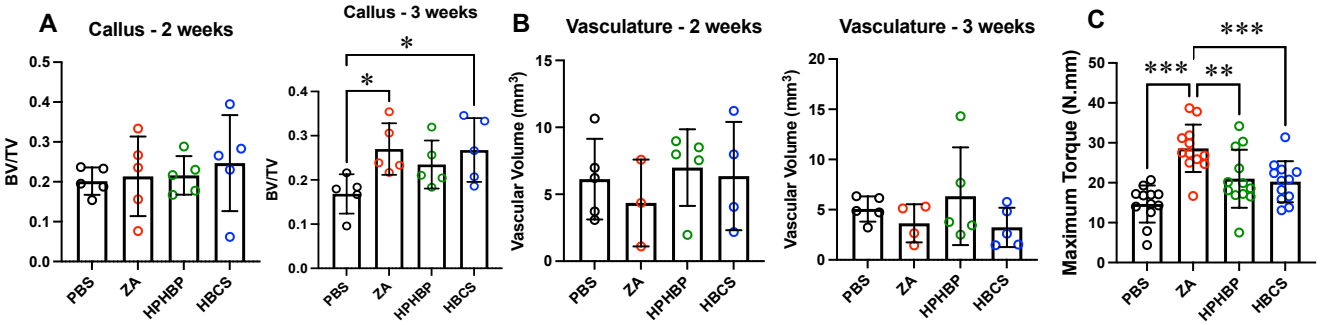

Figure 1. μCT assessment of (**A**) callus bone volume/total volume, and (**B**) vasculature at 2- and 3-weeks; (**C**) Maximum torque of femurs assessed at 4 weeks. *0.1<p<0.05, **p<0.05, ***p<0.01 by one-way ANOVA.

Discussion: HBCS is a promising bone-targeted antibiotic with unprecedented in vivo bactericidal effects within mature biofilm in bone marrow staphylococcus abscess communities (SACs) and within the OLCN. Based on this efficacy profile, we proposed a Phase 1 clinical trial in young, healthy adults being treated with external fixation frames for tibia fractures, as 30% of the pins are expected to become infected and could be harvested for analysis during conversion to internal fixation. While regulators favorably reviewed this trial design, potential concerns about HBCS inhibition of fracture healing in these patients were expressed, which prompted this study. Through our findings in this study, this concern about HBCS therapy is further abated on top of the pre-clinical and clinical evidence that bisphosphonates do not inhibit fracture healing.

Significance/Clinical Relevance: Here, we demonstrated that HBCS has no remarkable effects on fracture healing and skeletal growth in mice. Based on the extensive safety and efficacy data on bisphosphonates and sitafloxacin use in patients and promising preclinical data on HBCS with no evidence of unpredicted side-effects, initiation of clinical studies with this 1st in class bone infection-targeted antibiotic is warranted.

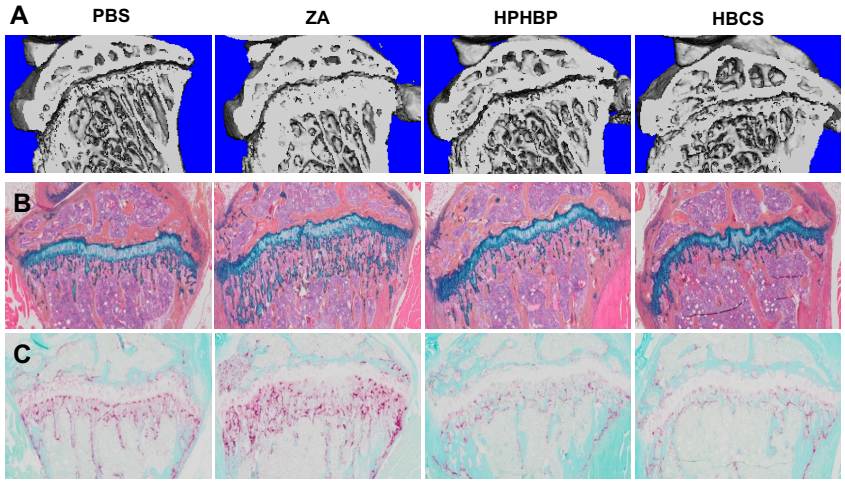

Figure 2. Representative images of (**A**) μCT reconstruction of growth plate bone, (**B**) Alcian Blue Hematoxylin/Orange G staining, and (**C**) TRAP staining of the tibias from the unfractured treatment groups.

References: [1] Adjei-Sowah E. et al. *Antibiotics* (2021); [2] Ren, Y. et al. *Front Cell Infect Microbiol* (2022); [3] Ren, Y. et al. *Bone Res* (2023); [4] Vanvelk N. et al. *Pharmaceuticals* (2025).

Acknowledgements: This work was supported by the NIH awards SBIR R44 AI125060, R21AR081050, P30 AR069655, and P50 AR072000.