Local Delivery of the Cationic Steroid Antibiotic CSA-90 Enables Osseous Union in a Rat Open Fracture Model of *Staphylococcus aureus* Infection

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Background: Treatment of infected open fractures remains a major clinical challenge. In this study, we investigated the novel broad-spectrum antibiotic CSA-90 (cationic steroid antibiotic-90) as an antimicrobial agent.

Methods: CSA-90 was screened in an osteoblast cell culture model for effects on differentiation and mineralization. Local delivery of CSA-90 was then tested alone and in combination with recombinant human bone morphogenetic protein-2 (rhBMP-2) in a mouse ectopic bone formation model (n = 40 mice) and in a rat open fracture model inoculated with pathogenic *Staphylococcus aureus* (n = 84 rats).

Results: CSA-90 enhanced matrix mineralization in cultured osteoblasts and increased rhBMP-2-induced bone formation in vivo. All animals in which an open fracture had been inoculated with *Staphylococcus aureus* and not treated with local CSA-90, including those treated with rhBMP-2, had to be culled prior to the experimental end point (six weeks) because of localized osteolysis and deterioration of overall health, whereas CSA-90 prevented establishment of infection in all open fractures in which it was used ($p \leq 0.012$). Increased union rates were seen for the fractures treated with rhBMP-2 or with the combination of rhBMP-2 and CSA-90 compared with that observed for the fractures treated with CSA-90 alone ($p = 0.04$).

Conclusions: CSA-90 can promote osteogenesis and be used for prevention of *Staphylococcus aureus* infection in preclinical models.

Clinical Relevance: Local delivery of CSA-90 represents a novel strategy for prevention of infection and may have specific benefits in the context of orthopaedic injuries.

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Ceragenins, or cationic steroid antibiotics (CSAs), are synthetically produced small-molecule chemical compounds that mimic the actions of cationic antibacterial peptides (CAPs)[1]. One of the best described CAPs is LL-37, a peptide derived from the C-terminal region of hCAP-18[2]. A broad range of bacteria are inhibited by LL-37, which is bactericidal via disruption of bacterial membranes[1]. CSAs are able to replicate the broad antibacterial activities of CAPs but are designed to have reduced cytotoxicity and improved in vivo stability compared with the endogenous peptides[3].

CSA-13 and the related compound CSA-90 have been shown to have antibacterial properties against a range of gram-positive

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and gram-negative bacteria associated with oral and respiratory tract infections[4]. The maximum bactericidal concentrations ranged between 0.35 and 44.8 mg/L and were no more than four times greater than the maximum inhibitory concentration, indicative of bactericidal action. Treatment with CSA-13 and CSA-90 was also associated with increased interleukin-8 (IL-8) production in cultured fibroblasts, suggesting a pleiotropic action where host immune processes may also be stimulated[2,4].

CSA-13 has been proposed as an antibacterial implant coating, and was found to impair bone necrosis in a model of methicillin-resistant *Staphylococcus aureus* infection[5]. Intriguingly, the mineral apposition rate was slightly increased in the noninfected controls treated with CSA-13 ($p < 0.08$), although the underlying mechanism was unclear. Bone morphogenetic proteins (BMPs) are important in bone formation and repair, and LL-37 is reported to increase BMP expression[2]. Subsequent screening of CSAs by Gianny Rossini and colleagues (Southwest Research Institute, San Antonio, Texas) revealed a more than sixfold increase in BMP-2 expression with CSA-90 treatment in cultured cells (unpublished data).

In the present study, we examined the potential pro-osteogenic and antimicrobial properties of CSA-90 with a number of standardized in vitro and surgical preclinical models[6-8].

Materials and Methods

Reagents

Recombinant human bone morphogenetic protein-2 (rhBMP-2) and porous collagen sheets were purchased from Medtronic (INFUSE Bone Graft Kit; Medtronic Australasia, North Ryde, NSW, Australia). CSA-90 (molecular weight = 851 g/mol) was produced by Dr. Paul Savage's laboratory at Brigham Young University (Provo, Utah) courtesy of N8 Medical (Columbus, Ohio)[4].

Cell Culture and Associated Assays

MC3T3-E1 pre-osteoblasts were cultured as previously described[6]. Osteogenesis was induced with 50 mg/L of ascorbic acid and 10 mM of β-glycerophosphate (Sigma-Aldrich, St. Louis, Missouri). Cells were treated with rhBMP-2 (50 ng/mL), alginate (500 μg/mL), and/or CSA-90 (0 to 50 μM) dissolved in sterile saline solution. A p-nitrophenyl phosphate assay was performed for alkaline phosphatase activity (Sigma-Aldrich) and normalized to day-4 cells grown in osteogenic media alone[9]. Mineralization was assessed by alizarin red-S staining (40 mM, pH 4.2) (LabChem, Pittsburgh, Pennsylvania)[6]. Assays were performed in triplicate with two independent repeats.

Animal Studies

Animals were purchased from the Animal Resources Centre (Canning Vale, Western Australia). Female C57BL6/J mice were operated on when they were eight weeks old, and male Wistar rats were operated on when they were nine weeks old. All animal studies were approved by the local animal ethics committee.

For surgical models, anesthesia was induced with ketamine (mice, 35 mg/kg; rats, 70 mg/kg) and xylazine (mice, 5 mg/kg; rats, 10 mg/kg) and maintained with inhaled isoflurane. Animals recovered on a heated pad and were given saline solution and buprenorphine (0.1 mg/kg) to manage dehydration and postoperative pain.

Ectopic Bone Formation Assay in the Mice

Collagen sponge discs were prepared with use of a sterilized biopsy punch (3 mm in diameter and 4 mm in height). Twenty minutes prior to implantation, 10 μL of treatment solution (Table I) was added. The scaffolds were introduced into a muscle pouch made in the hindlimb with use of a previously published surgical model[6,10]. Animals were killed three weeks postoperatively. Three mice were excluded because they failed to recover from anesthesia (one) or because an ectopic bone nodule fused to the adjacent femur (two). Two additional mice died during surgery.

TABLE I Study Design for Ectopic Bone Assay in Mice

Group	rhBMP-2 (μg)	CSA-90 (μg)	No. of Mice
1	10	None	9
2	10	25	9
3	10	250	9
4	None	250	8

Bacterial Culture

A patient-derived strain of *Staphylococcus aureus* (ATCC [American Type Culture Collection]-12600) was obtained from Simon Dingsdag (Westmead Millennium Institute for Medical Research, Westmead, NSW, Australia). Bacteria were grown overnight on LB (lysogeny broth) agar plates, and single colonies were picked for overnight culture in L (lysogeny) broth the day prior to surgical inoculation. Bacteria were quantified with use of an optical density (600-nm) measure with an optical density value of 1 treated as containing 1×10^9 bacteria/mL.

Femoral Fracture Model in the Rats

Collagen sponges ($16 \times 5 \times 4$ mm) were prepared at least twenty minutes prior to surgery with 90 μL of protein solution (Table II). An open femoral osteotomy with periosteal stripping was used to model an open fracture[11], modified to include infection[8]. Treated collagen sponges were placed circumferentially around the fracture site, and the wound was sutured closed.

Animals were monitored daily for health and had weekly radiographs performed with a Faxitron machine (Faxitron X-Ray, Wheeling, Illinois) and assessed by a veterinarian blinded to treatment. Animals showing declining overall health and evidence of septic nonunion (loss of body weight, low activity, poor coat condition, limping, and inflammation of the site, and/or substantial bone loss on radiographs) were killed at the veterinarian's instruction. These rats and those culled prematurely due to loss of intramedullary fixation had the fracture site swabbed and cultured to test for underlying infection. The remaining rats were killed at six weeks, and the femora were harvested.

Radiographic Analysis

Mouse ectopic bone nodules and rat femora were fixed in 4% paraformaldehyde and transferred to 70% ethanol. Rat fracture union was graded on three-week and six-week postoperative radiographs by an orthopaedic surgeon blinded to treatment group. Bone samples were scanned with a SkyScan 1174 compact micro-computed tomography (Micro-CT) scanner (SkyScan, Kontich, Belgium) with use of published settings[10]. The region of analysis was defined as the entire mouse ectopic bone nodule or the rat fracture callus. Outcomes included bone volume (in mm^3) with use of a threshold of 0.4 g/cm^3 of calcium hydroxyapatite, tissue volume (in mm^3), and bone tissue mineral density (in g/cm^3) as defined by Bouxsein et al.[12].

Histological Analysis of Bone Formation

Samples were fixed and decalcified in 0.34 M ethylenediaminetetraacetic acid (EDTA; pH 8.0) for four weeks. Mouse ectopic bone nodules were halved transaxially, and rat femora were halved sagittally. Then 5-μm paraffin sections were cut with use of a Leica RM2155 Microtome (Wetzlar, Germany) and stained with alcian blue/picrosirius red and for tartrate resistant acid phosphatase (TRAP). Stained sections were scanned with a ScanScope digital slide scanner

TABLE II Study Design for Open Femoral Fracture Study in Rats

Group	rhBMP-2 (μg)	CSA-90 (μg)	*Staphylococcus aureus*	No. of Rats
1	None	None	None	13
2	None	250	None	11
3	10	None	None	12
4	10	250	None	12
5	None	None	1×10^4 CFU	6
6	None	250	1×10^4 CFU	6
7	10	None	1×10^4 CFU	13
8	10	250	1×10^4 CFU	11

(Aperio Technologies, Vista, California). Representative samples were selected from the median bone volume values for each group.

Statistical Analyses

Statistical power calculations and analyses were performed with use of Graphpad Prism (La Jolla, California), and the cutoff for significance was set at $\alpha < 0.05$. Cell culture analyses were done with parametric statistics (analysis of variance [ANOVA] with a post-hoc t test) and show standard error. In vivo studies were powered to microCT bone volume (the mouse model) or fracture union (the rat model) based on means and variances from prior published work[10,11]. MicroCT data were analyzed with use of nonparametric statistical tests (Kruskal-Wallis) with post-hoc Mann-Whitney U tests comparing no-treatment controls with all test groups and comparing the rhBMP-2-only group with the rhBMP-2/CSA-90 group. Fracture union rates were compared by using a Fisher exact test, and 95% confidence intervals were calculated with the modified Wald method for proportions.

Sources of Funding

The cell culture and ectopic bone studies were performed as commissioned research for N8 Medical. The rat infected-fracture study was carried out with use of internal departmental funding.

Results

CSA-90 Promotes Matrix Mineralization

MC3T3-E1 cells were treated with a range of CSA-90 doses, with and without rhBMP-2 and also with and without alginate, which is purported to decrease toxicity of antimicrobial peptide LL-37[13]. Treatment with rhBMP-2 generated a potent effect on alkaline phosphatase activity, as a marker of pre-osteoblast differentiation. CSA-90 did not induce a comparable osteogenic response de novo (Fig. 1-A), but co-treatment with

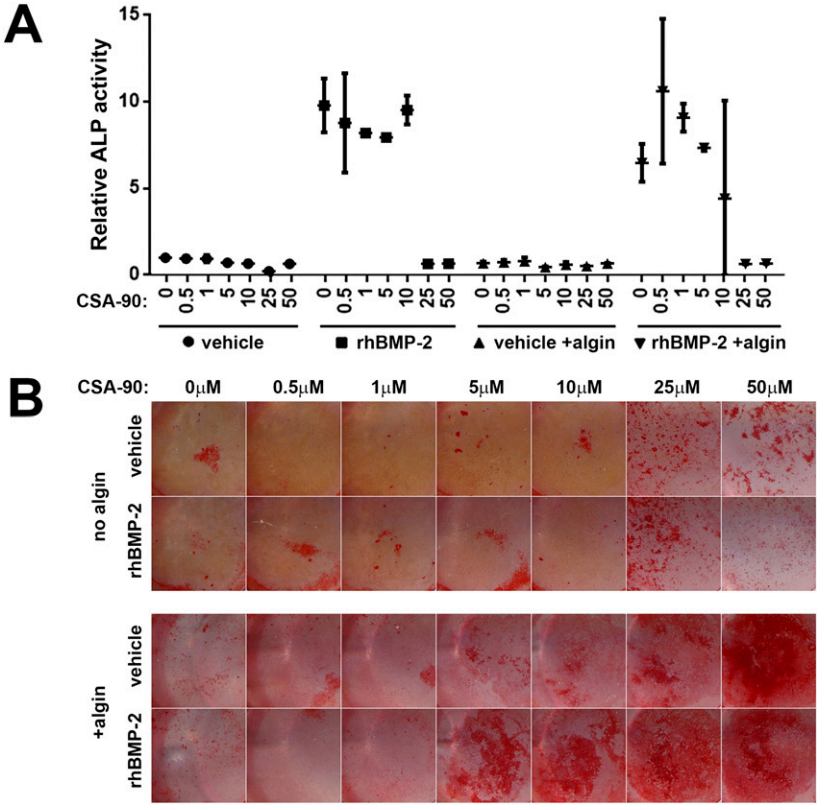

Fig. 1

Assays were performed on MC3T3-E1 pre-osteoblasts treated with or without rhBMP-2 (50 ng/mL), CSA-90 (0 to 50 μM), and sodium alginate (algin; 500 μg/mL) on day 4. Quantitative spectroscopic alkaline phosphatase (ALP) assays (**Fig. 1-A**) and alizarin red-S staining for matrix mineralization (**Fig. 1-B**) were carried out. CSA-90 enhanced rhBMP-2-induced matrix mineralization, and this was maximized in the presence of alginate. The error bars represent the standard error, and the symbols represent the mean.

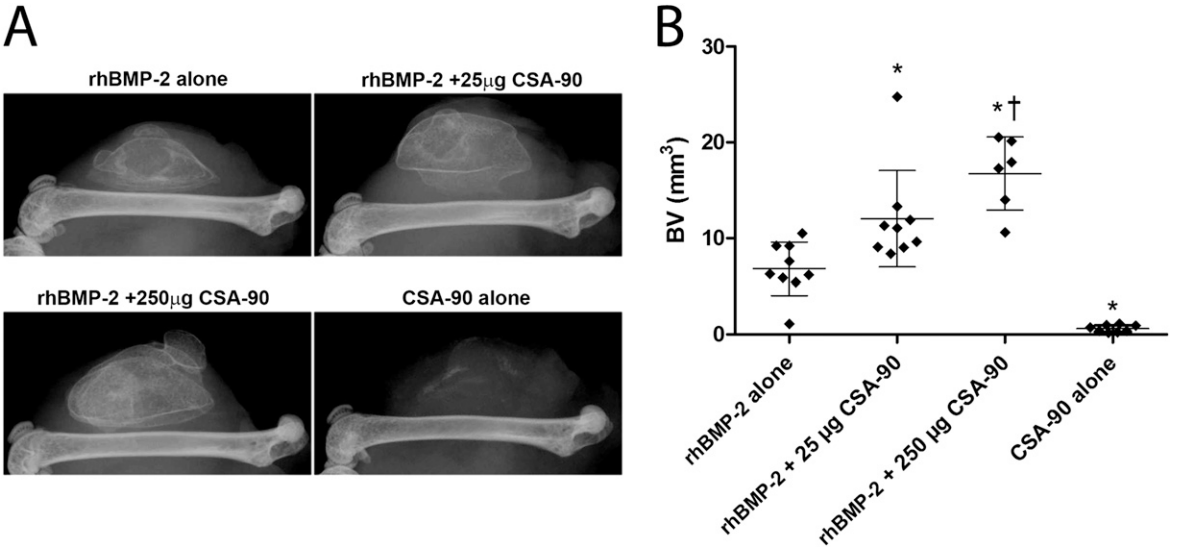

Fig. 2

Fig. 2-A Radiographs showing ectopic bone nodules in a murine model in which bone formation was induced with rhBMP-2 (10 μg). Increased bone was seen with local co-delivery of CSA-90 (25 μg and 250 μg). **Fig. 2-B** MicroCT assessment of bone volume (BV) showed that bone formation increased with increasing doses of CSA-90. A small amount of mineralized tissue was detected on radiographs and with microCT in the group treated with CSA-90 alone, but this was significantly less than was seen in the controls. *$P < 0.05$ for comparison with rhBMP-2 alone. †$P < 0.05$ for comparison with rhBMP-2 + 25 μg of CSA-90.

both rhBMP-2 and CSA-90 in the presence of alginate led to moderate increases in alkaline phosphatase activity over that seen with rhBMP-2 alone. This indicated some potentiation of rhBMP-2 activity by CSA-90. Doses of >25 μM of CSA-90 impaired the rhBMP-2-induced increases in alkaline phosphatase activity, but this was also associated with decreased cell viability (data not shown).

CSA-90 increased matrix mineralization measured with alizarin red-S staining in control and rhBMP-2-treated cells at day 10 (Fig. 1-B). This effect was most notable in rhBMP-2-treated cells, where co-treatment with 5 to 10 μM of CSA-90 produced robust mineralization at a time point at which mineralization was not seen with rhBMP-2 alone.

Co-Delivery of CSA-90 Increases Bone Volume in a Mouse Model of rhBMP-2-Induced Ectopic Bone Formation

Next, the capacity of CSA-90 to act in concert with rhBMP-2 to promote bone formation, or to generate bone de novo, was tested in a mouse ectopic bone formation model. In this model, 10 μg of rhBMP-2 delivered via an implanted collagen sponge led to ectopic bone nodules that were visible radiographically at three weeks (Fig. 2). The co-delivery of CSA-90 (25 or 250 μg) led to increased bone formation seen on radiographs (Fig. 2), and this was confirmed by microCT quantification of bone volume (Fig. 3). The increase in bone volume caused by local addition of CSA-90, which was 1.8-fold with 25 μg and 2.5-fold with 250 μg, was significant ($p < 0.05$).

Bone formation was negligible in the animals that received CSA-90 alone, although small areas of mineralized tissue were detected by microCT and radiography. Subsequent histological analysis indicated that this tissue did not have the normal morphology of ectopic bone pellets and instead resembled focal regions of connective-tissue hypermineralization.

In contrast, all of the rhBMP-2-treated groups showed a standard cortex-like shell surrounding marrow-like elements with islets of trabecular bone (Fig. 3). CSA-90 treatment led to the entire nodule being larger, rather than increased retention of internal trabecular-like elements. TRAP staining revealed no obvious alterations in osteoclasts, although bone nodules were highly heterogeneous, having undergone substantive remodeling.

CSA-90 Prevents Staphylococcus aureus *Infection and Improves rhBMP-2 Action in an Open Fracture Model*

A rat open fracture model was used to test the capacity of CSA-90 to promote bone healing in the context of bone infection and/or rhBMP-2 treatment. This model represents a challenge to normal bone repair processes and was previously reported to have a six-week union rate of ~50%[11]. A pilot study was performed with inoculation of the *Staphylococcus aureus* strain at 1×10^4 and 1×10^5 bacteria per fracture. The higher dose caused rapid degeneration of the animals' health and rapid osteolysis at the distal part of the femur/knee in the majority of animals (data not shown). Thus, the 1×10^4 bacterial dose was selected for the main study.

Surgery was carried out in eighty-four rats (Table II), and postsurgical assessment was performed by a veterinarian blinded to treatment. All *Staphylococcus aureus*-inoculated rats not treated with local CSA-90 showed poor health combined with a worsening radiographic score and/or substantial osteolysis, and all of these animals were killed by two weeks

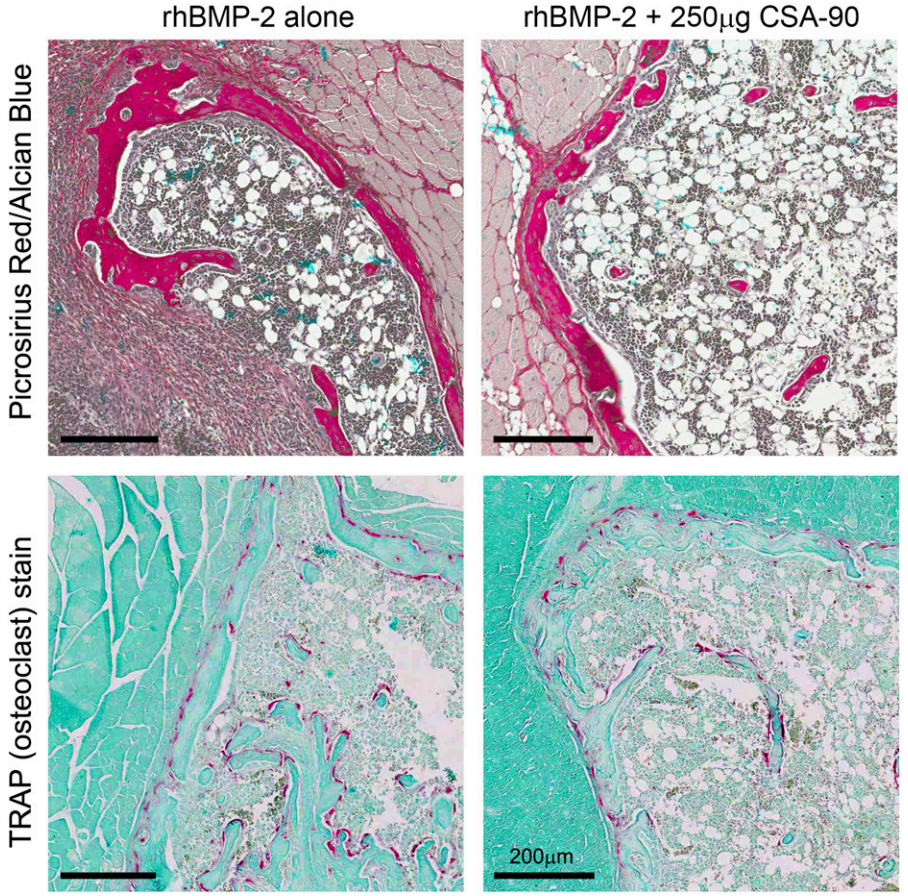

Fig. 3

Histological tissue cross sections through representative samples of ectopic bone nodules in animals treated with rhBMP-2 alone and with rhBMP-2 and 250 μg of CSA-90. Picrosirius red/alcian blue staining illustrates the larger size of the implants containing CSA-90 but that the internal structure is comparable between the groups. TRAP staining indicates comparable TRAP (osteoclast) staining between the groups. Scale bar = 200 μm.

(Fig. 4). The presence of infection was confirmed by overnight culture of a swab of the opened fracture site. There was a significant difference between the number of *Staphylococcus aureus*-infected animals killed in the group with no treatment or treated with rhBMP-2 alone and the numbers killed in all other groups (Fisher exact test; $p \leq 0.012$).

In contrast, rats inoculated with *Staphylococcus aureus* that received local CSA-90 exhibited overall good health and

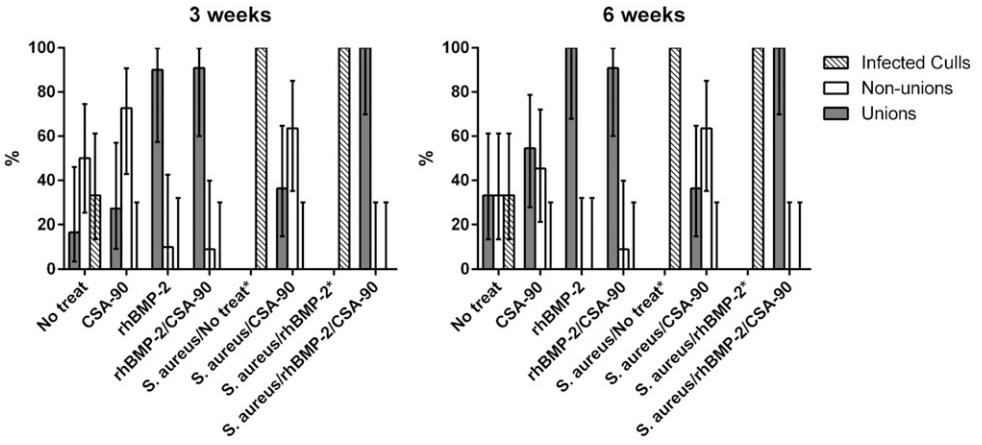

Fig. 4

Results in the *Staphylococcus aureus* fracture model at three weeks and six weeks. The graphs summarize the breakdown between confirmed infected culls (striped bars), radiographic nonunions (white bars), and radiographically evident unions (grey bars). Both early culls and union assessments were performed by an expert (a veterinarian and an orthopaedic surgeon, respectively) blinded to the treatment group. All animals inoculated with *Staphylococcus aureus* that did not receive CSA-90 were culled prior to three weeks (*). Error bars represent the 95% confidence intervals.

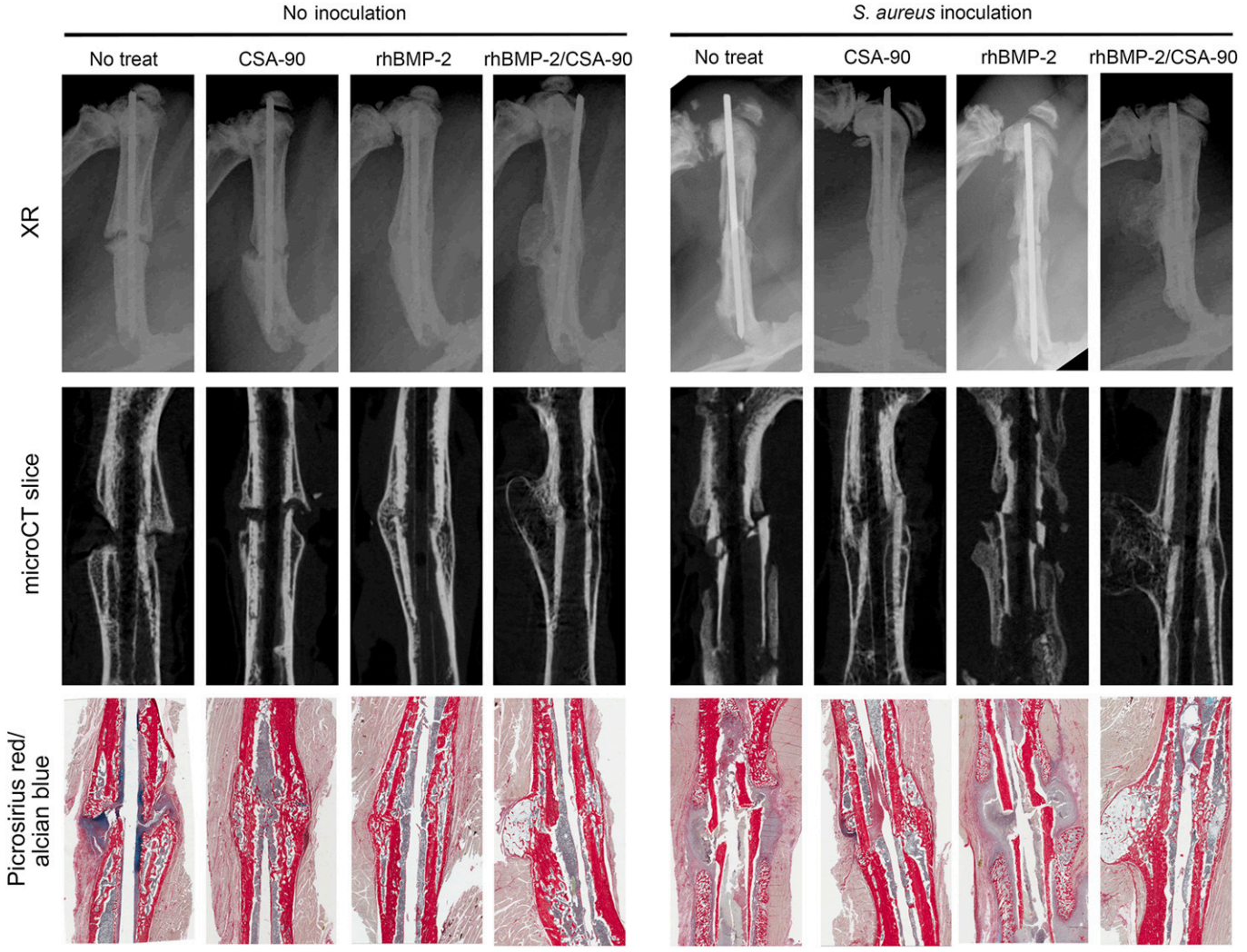

Fig. 5

Representative images of fractures at six weeks (less than two weeks for the *Staphylococcus aureus*-inoculated groups that received no treatment or rhBMP-2 only). The panels show radiographs (XR), microCT images of bisected femora through the fracture site, and picrosirius red/alcian blue-stained sections. Bone formation was promoted by rhBMP-2. Inflammatory tissue is seen around the fracture sites of the early infected callus.

normal bone healing, and those that received both rhBMP-2 and CSA-90 showed a higher rate of union compared with those treated with CSA-90 alone ($p = 0.04$). In fact, 100% of the initially infected open fractures in the group that received both rhBMP-2 and CSA-90 healed within three weeks.

In the groups not inoculated with *Staphylococcus aureus,* CSA-90 treatment alone resulted in no significant improvement in healing at three or six weeks ($p = 0.41$). However, treatment with rhBMP-2 alone or with rhBMP-2 and CSA-90 improved union rates ($p < 0.01$) at both time points.

These data are illustrated by representative fracture radiographs, microCT reconstructions of a computationally bisected femur, and histological tissue sections (Fig. 5). Superior net bone formation and union were seen in the rhBMP-2/CSA-90 groups. All images represent six-week specimens, except for those of the *Staphylococcus aureus*-inoculated untreated and rhBMP-2-treated groups, in which all rats were prematurely culled because of worsening infection. These specimens show no histological evidence of healing and abundant inflammatory tissue.

MicroCT analysis of the fracture calluses at six weeks showed a trend toward increased osseous callus (bone volume) with rhBMP-2 treatment, which was significantly different from that with CSA-90 alone ($p < 0.05$) (Fig. 6-A). The increase in callus tissue volume was more pronounced, with the rhBMP-2/CSA-90 group having a significant increase compared with the no-treatment controls ($p < 0.05$), even with *Staphylococcus aureus* inoculation (Fig. 6-B). Analysis of bone tissue mineral density, defined as the mineralization of the tissue designated as bone (>0.4 g/cm^3), revealed no significant hypomineralization or hypermineralization of the bone in the CSA-90-treated calluses (Fig. 6-C). Co-treatment with rhBMP-2 led to a small reduction in bone tissue mineral density (compared with fractures without rhBMP-2 treatment) of 10% to

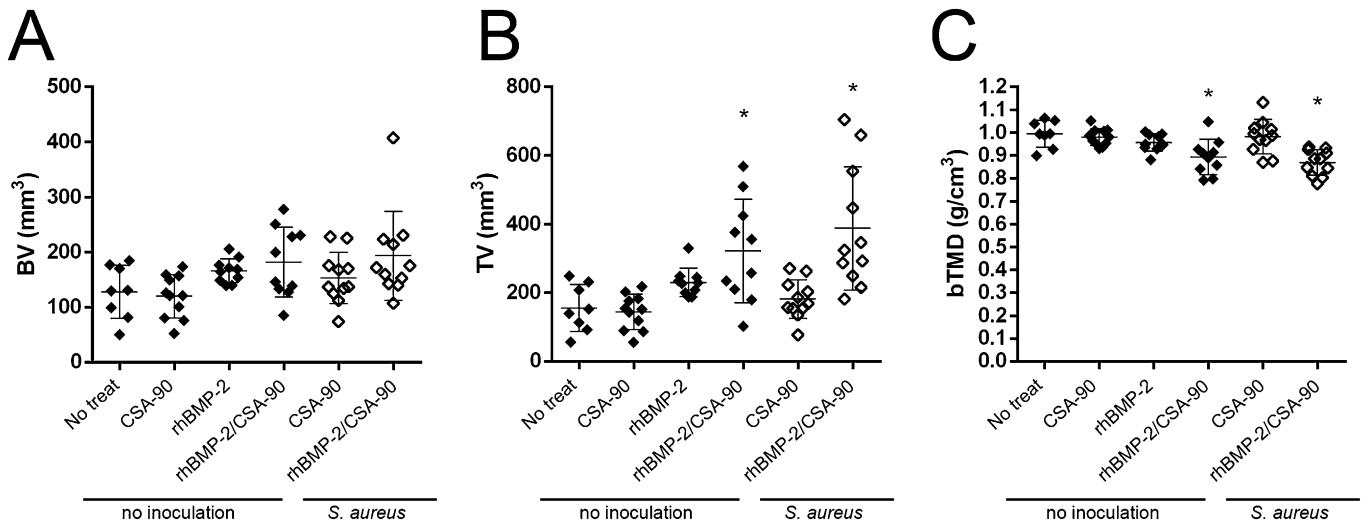

Fig. 6

Bone-healing three-dimensional quantification at the fracture callus at six weeks. **Fig. 6-A** Bone volume (BV, in mm^3). **Fig. 6-B** Tissue/callus volume (TV, in mm^3). **Fig. 6-C** Bone tissue mineral density (bTMD, in g/cm^3). *Significant changes versus the no-treatment group ($p < 0.05$).

15% ($p < 0.05$), which was of unknown functional releveance. Although not directly comparable, the infected bone from *Staphylococcus aureus*-inoculated, prematurely culled animals treated with rhBMP-2 and CSA-90 rhBMP-2 showed a reduction in bone tissue mineral density of 30% to 35% compared with the no-treatment controls ($p < 0.01$).

Discussion

In this study, we examined the broad spectrum antimicrobial CSA-90 in models of bone formation and orthopaedic infection. Bone and joint infections remain a major clinical challenge to prevent and treat in an orthopaedic setting, with high morbidity and cost[14]. Some cases of nonunion that are thought not to be infected may actually be infected but culture-negative[15]. Numerous studies have shown the benefits of preventative systemic antibiotic treatment for open fractures[16], although the type of antibiotic, timing of antibiotic administration, and timing of debridement and wound closure remain controversial[17].

We selected an open fracture model as we considered it most representative of fractures at risk for clinical infection[18-21]. Indeed, our standard procedure to minimize infection of rat open fractures is to provide a systemic fluoroquinolone antibiotic (Enrofloxacin, 25 mg/mL) in the drinking water postoperatively[11]. In this study, no such systemic antibiotic was provided, in order to test the local effects of CSA-90. This lack of systemic antibiotic protection likely underlies the 16% infection rate for rats that did not receive CSA-90 or *Staphylococcus aureus*. Moreover, the lack of infection from alternate pathogens in the CSA-90-treated animals may be representative of the broad antimicrobial protection offered by this agent.

When given in combination with rhBMP-2, CSA-90 increased bone volume in the mouse ectopic bone formation model, indicating its potential to augment bone growth. CSA-90 increased matrix mineralization in a culture model, but bone tissue mineral density was not significantly increased, indicating that CSA-90 promoted more normal quality bone rather than hypermineralized bone. The osteogenic benefits of CSA-90 in the fracture model were more modest, and the major improvements associated with CSA-90 were due to infection prevention. CSA-90 may be of particular applicability in combination with rhBMP-2, in terms of both maximizing BMP effects and infection prevention. While the initial trials of rhBMP-2 in the treatment of open tibial fractures seemed to suggest a potential benefit in terms of preventing infection[22], a more recent randomized controlled trial showed an increased risk of infection in rhBMP-2-treated open fractures[23]. Moreover, preclinical models have indicated that rhBMPs do not result in successful orthopaedic outcomes in the presence of infection[8].

One key limitation of our fracture study is that it addressed only the capacity of CSA-90 to be used as prophylaxis for infection at the time of surgery and not for treatment of an established or chronic infection. It is challenging to treat an infected nonunion, which is a major burden when it occurs[24]. Future experiments are required to address whether CSA-90 can be used in combination with debridement for established infections.

Another notable limitation is that we used only a single pathogen (*Staphylococcus aureus*) in our model. However, *Staphylococcus aureus* remains a particular challenge in orthopaedics, encompasses strains that are resistant to a range of conventional antibiotics, and has been recently shown to be able to conceal itself within host cells[25]. CSAs have been shown to be broadly effective against a range of pathogens[4], but future studies must address their efficacy for osteomyelitis.

In summary, this study demonstrated that local CSA-90 treatment is efficacious in preventing *Staphylococcus aureus*

infection in an open fracture model and has pro-osteogenic and antimicrobial properties. ■

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