OSTEOMYELITIS TREATMENT WITH NANOMETER-SIZED HYDROXYAPATITE PARTICLES AS A DELIVERY VEHICLE FOR A CIPROFLOXACIN- BISPHOSPHONATE CONJUGATE; NEW FLUOROQUINOLONE-BISPHOSPHONATE DERIVATIVES SHOW SIMILAR BINDING AFFINITY TO HYDROXYAPATITE AND IMPROVED ANTIBACTERIAL ACTIVITY AGAINST DRUG-RESISTANT PATHOGENS.

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ABSTRACT

Orthopaedic injuries comprise a majority of combat injuries seen in recent US military conflicts. The development and increased use of modern body armor, which has been correlated with decreased chest and upper abdominal injuries (Mabry et al., 2000), will likely continue to result in an increased relative rate of extremity injuries. These injuries commonly present with significant open bone fractures of the limbs and substantial contamination with bacteria from high energy trauma. Once bone infection is established, one fourth of open fractures will progress to osteomyelitis (OM) (Lew and Waldvogel, 1997; Wolski, 2004; Buxton et al., 2004).

We developed a novel local drug-delivery system consisting of ciprofloxacin-bisphosphonate conjugate (E41) bound to calcium phosphate (CP) bone particles. *In situ*, the conjugate dissociates from CP and maintains its antibacterial activity. This topical granulated antibiotic delivery system reduced infection rates in our *in vivo* OM model. Current studies contrast two CP homeostatic bone-substitute particles, nanometer-sized hydroxyapatite NanOss™ (Nan), and µ-sized tricalcium phosphate Skelite™ (TCP) *in vitro* for binding to E41, and *in vivo* for E41-CP blockade of rat OM.

E41's binding to both CPs were saturable, and maximal binding was similar. But affinity constants were different ($p \leq 0.01$), showing a higher E41 affinity for Nan. The amount of E41 able to saturate 50% of Nan was higher than TCP (ca. 30-fold). E41-CP concentrations were contrasted in our post-traumatic *S. aureus* acute rat OM model, with enumeration of *S. aureus* in infected tibiae (tibial load). Using the previously determined 95%-Infective inoculum, all untreated controls became infected (examined at 24 h); and both E41-CPs lowered tibial loads at 50% saturation: E41-Nan loads were lower than E41-TCP ($p \leq 0.01$). Sixteen of 19 E41-Nan tibiae were sterile, *vs.* 5 of 18 for E41-TCP ($p < 0.01$). Weak acids, e.g., lactic acid released c.a. thirty-fold more E41 from Nan (8.96 µg CB) than TCP (0.27 µg CB, $p \leq 0.05$). These results suggest E41 may be released from Nan *in situ* due to the low pH of wounds. We conclude that nanometer sized CP particles are comparable to, and perhaps superior than, µ-sized particles, as a local drug-delivery vehicle for bisphosphonate-containing antibiotics.

With the concept of treating open fracture wounds with targeted topical drug delivery consisting of an antibiotic-bisphosphonate bound to CP established, our attention has focused on the effectiveness of other fluoroquinolone-bisphosphonate combinations. Gatifloxacin-bisphosphonates (E43) and Moxifloxacin-hydroxybisphosphonate (E46) were contrasted to E41 for activity against methicillin-susceptible *S.aureus*, ciprofloxacin-resistant MRSA, ciprofloxacin-susceptible MRSA, *Acinetobacter baumannii* and other pathogens. E43 showed greater antimicrobial activity against several Gram-positive and Gram-negative pathogens, e.g., a 16-fold difference with MRSA. In *in vitro* binding studies binding was similar, and saturable. Maximal binding was: E41, 89.7 % ± 4.74, E43, 76.8 % ± 2.35 and E46, 95.9 % ± 0.13. K_d values were: E41, 1.35×10^{-8} mM (Nan), E43**,** 1.83×10^{-8} mM, and E46, 1.72×10^{-8} mM. Versus E41 and E46, E43 has improved activity against bone-pathogenic *S. aureus*, and other bacteria**,** and shows a similar binding affinity to Nan.

These investigations report an animal model mimicking severe trauma, for the prevention and treatment of OM, and the creation of antibiotic-bisphosphonate conjugates, now bound to nanometer size bone particles for the treatment of OM. These dry particles are ideal for topical treatment of open battlefield wounds, providing targeted drug delivery, and the prevention of disease from contaminated wounds. In addition, new flouroquinolone-bisphosphonates provise improved antibacterial activity against drug-resistant bacteria.

1. INTRODUCTION

We described a novel local drug-delivery system consisting of calcium phosphate (CP) containing particles bound to an osteoadsorptive bisphosphonate-

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
DEC 2008	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Osteomyelitis Treatment With Nanometer-Sized Hydroxyapatite Particles As A Delivery Vehicle For A Ciprofloxacin- Bisphosphonate Conjugate; New Fluoroquinolone-Bisphosphonate Derivatives Show Similar Binding Affinity To Hydroxyapatite And Improved Antibacterial Activity Against Drug-Resistant Pathogens.	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Department of Clinical Investigation, Eisenhower Army Medical Center, Fort Gordon, GA, USA 30905**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM002187. Proceedings of the Army Science Conference (26th) Held in Orlando, Florida on 1-4 December 2008

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**8**	

ciprofloxacin antibiotic conjugate, called E41 (Buxton et al., 2004). The carrier particle, a micron-sized tricalcium phosphate Skelite™ (TCP), acts as a resorbable bone filler, and has value for osseous repair of rat calvaria (Fleckenstein et al., 2006). E41-TCP performed well in comparison with TCP given alone in a post-traumatic acute OM rat tibial model (Buxton et al., 2005). Significant reductions in *S. aureus* tibial loads occurred. On a practical level, the E41-TCP slurry was easy to formulate and apply, lodged firmly into medullary bone defects, and was associated with only mild inflammation, even after 14 days. However, E41-TCP did not sterilize infected bone. We postulate not enough antibiotic was applied to bone.

Bisphosphonate-antibiotics are a family of "bone-seeking" conjugates of fluoroquinolone compounds, designed to concentrate within bone (Herczegh et al., 2002). The bisphosphonate moiety not only localizes E41 to the CP particle, but may impede osteoclastogenesis, a virulence factor for *S. aureus* bone pathology [Megji et al., 1998; Nair et al., 1995]. It also may localize the antibiotic to host bone after it is released (over time) within traumatized host bone (due to particle resorption or dissolution).

NanOss™ (Nan) Bone Filler was the first "nanotechnology" orthopedic medical device to receive clearance by the US Food and Drug Administration (Personal communication, Ed Ahn, Ph.D., Chief Science Officer, Angstrom Medica, 2005). Nanostructure processing was applied to derive resorbable hydroxyapatite bioceramics with ultrafine microstructures and significantly improved mechanical properties for orthopedic implant applications (Ahn et al., 2001).

This study contrasts E41 carried by Nan to TCP. Surface area difference may affect not only the amount of drug carried, but the amount of drug released *in situ*. By decreasing the bone-fill particle size, and using a constant volume for defects in traumatized tibiae, we increase total surface area, and potentially increase the amount of topically-applied E41 packable into the bony defect. In addition, second generation bisphosphonate-antibiotics with increased antimicrobial, and equivalent binding attributes represent improvements to the system.

2. MATERIALS AND METHODS

2.1 Materials

HP-ENC-41 (E41) conjugate (ElizaNor Biopharmaceuticals Inc., Princeton Junction, NJ) is the prototype of a new family of fluoroquinolone-bisphosphonates with high *in vitro* binding affinity for rat, porcine and human bone (Herczegh et al., 2002). Second generation compounds include a gatifloxacin (G)-bisphosphonate conjugate (E43), and a moxifloxacin (M)-hydroxybisphosphonate conjugate (E46).

2.2 Calcium phosphate (CP) particles

Calcium phosphates (CP) comprise the largest group of biominerals in vertebrates and have been classified into structural types; apatite, glaserite and sheet-containing compounds (Matthew et al., 2001). In this study, two commercially available CPs were contrasted as carrier particles for E41. Bisphosphonates have binding affinity to CP (Fleisch et al., 1968), and E41, E43 and E46 contain bisphosphonate moieties. However, the CPs differ with respect to particle size (nM vs μM), surface area, crystalline structures [apatite or glaserite type, (Matthew et al., 2001)], molecular weight, mean particle size, and porosity.

Skelite™ (TCP, Millenium Biologix, Kingston Ontario, Canada) acts as a synthetic resorbable bone filler. It is of the glaserite type and consists of micron-sized tricalcium phosphate [$Ca_3(PO_4)_2$] particles ranging from 125 – 212 μ in size. It has a m.w. of 310, and like Nan, is highly crystalline, thermally stable, and porous. Particles weigh 0.19 ng, with a mean pore size of 10.1Å and a surface area of 17 M^2/g. The particle has an α-tricalcium configuration of (ca. 70%), and hydroxyapatite (ca. 30%) (personal communication).

NanOss™ (Nan, Angstrom Medica, Inc, Woburn, MA), consists of nanometer-sized hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]. It is of the apatite type, and has a molecular weight **(**m.w.) of 1004, is highly crystalline, thermally stable, and has uniform morphology and particle size. Specifications include: crystallite size, 56 nm; particle size, 0.91 μm; surface area, 50 M^2/g; and a calcium phosphate ratio of 1.67.

2.3 *In vitro* experiments, E41 and CP

Binding of E41 to CP particles was measured *in vitro.* E41 was added to tubes containing washed and autoclaved CP particles (25 mg/2.5 ml), incubation followed (3 h, 37°C), tubes were centrifuged (5,104 ×g, 20 min, r.t.), and the supernatant removed for fluorescence measurement. Sample dilution was done if quenching of fluorescence was observed, i.e., high counts-per-second (cps) affecting the instrument's ability to measure changes in E41 levels. E41 cps were measured as "Total" fluorescence, determined from E41-tubes without CP particles, and "Free" cps, were determined using E41 supernatants from CP-containing tubes having E41 (unbound). "Percent Bound" (%B) was determined from "(Total – Free)/Total = Bound (x

100%)". From standard curves, the assay's limits of sensitivity were ca. 100 ng/ml.

Experiments were done to determine if binding was saturable, and the binding parameters; i.e., affinity constants (K_A), and maximal binding (B_{max}). To accomplish this, levels of E41 were held constant (2 µg/ml) and CP levels increased (0.1 to 100 mg).
Separately, dose response studies were done to determine CP particle capacity for E41, i.e., the maximum amount of E41 that can be carried by CP. To accomplish this, levels of CP were held constant (10 mg/ml) and E41 levels were sequentially increased. We chose to determine the 50%-binding capacity (BC_{50}), a point on the dose-response curve with maximum distance from (upper and lower) zero-order slopes. We measured E41-release from E41-CP particles in the presence of lactic acid (25 mM, pH 3.5). CP particles were exposed to E41 (BC_{50}), washed with alkaline biological buffer (AMPSO, pH 8.5), then dried (70°C, 24 h). Dried CP was added to centrifuge tubes (25 mg/2.5 ml), lactic acid added, and incubated (3 h, 37° C). Tubes were centrifuged and the supernatants scanned for fluorescence. Standard curves of E41 in 25 mM lactic acid were prepared and used to determine E41 released from Nan and TCP. Methylene diphosphonic acid (MDP) is the structural bisphosphonate moiety of E41. CP particles (40 mg/ml), E41 (2 µg/ml), and MDP (0.03 to 30 mM), were added simultaneously together, and then incubated (30 min, 37° C), centrifuged, and the supernatant read for E41-fluorescense (430 nm).

2.4 *In vivo* experiments, E41 and CP

A post-traumatic acute OM model (Buxton et al., 2005) was used to contrast the ability of E41-CP to eradicate bone infection. Briefly, adult rats received traumatic open tibial fracture with thermal injury and contamination by *Staphylococcus aureus* (Gustilo type III wound) (Gustilo et al., 1987). A longitudinal defect (6.8 mm × 1.7 mm × 3.0 mm) was created in the left tibiae by microdrilling, exposing the medullary cavity. The endosteal blood supply was disrupted with repeated passage of a handheld cautery device. *S. aureus* (ATCC strain 12598, Cowan 1 strain) were introduced into the medullary cavity using two inocula strength, one near the ID_{95}, and the other ca. 100-fold higher. After 10 min, the trough was pulsatile lavaged (100mL saline, 23.7 psi), and E41-Nan, or E41-TCP were packed into the medullary defect (ca. 40 mg) using a spatula. E41-CP was prepared as a hydrated paste (0.5 ml H_20/500 mg dried E41-CP), particles were washed once, and experiments performed using two concentrations of E41; the BC_{50} concentration, determined from *in vitro* dose-response experiments above (17mM and 0.62 mM for Nan and TCP, respectively); and 4 mM each. In addition, a weight measurement of the amount of CP applied to each tibial defect was done at the time of surgery. The musculature and skin were closed by simple continuous and interrupted sutures, respectively.

Animals were euthanized at 24 h. The physes and fibula were removed, and bone cylinders scraped free of adherent muscle and soft tissue. Bones were weighed, frozen in liquid nitrogen, and crushed to powder. Bone powder was added to PBS, vortexed (15 min), and serially-diluted. Aliquots were plated on growth medium (TSA agar), and incubated (24 h, 37°C). The next day colonies were enumerated, and results expressed as $\log_{10}$ colony forming units (cfu)/gm tibia. Limits of sensitivity were 100 cfu/gm.

Rats were divided into E41-Nan, E41-TCP, and no-treatment infection control groups. Two sets of experiments were done. Experiment one (61 rats) was done using a *S. aureus* inocula near the ID_{95} value [1,270 cfu, (Buxton et al., 2005)], and E41-CP particles were prepared from Cohort 1 results, and at 50%-saturation levels (BC_{50}). In the second experiment (20 rats), E41-CPs were prepared at an identical E41 concentration, (4 mM). Particles were washed extensively (ten times), and a higher *S. aureus* inoculum was used (87,000 cfu).

The three groups were compared by One Way Analysis of Variance (ANOVA), for *S. aureus* tibial loads, and by unpaired t test, for comparison between two groups. Chi Square and Fisher's Exact test were used for percent-infection contingency testing. Statistical significance was set at $p \leq 0.05$.

2.5 *In vitro* experiments, second generation bisphosphonate conjugates and CP

For minimal inhibitory concentration (MIC) experiments, two-fold serial dilutions of antibiotics and antibiotic-bisphosphonate conjugates (C, G, M, E41, E43, and E46; 512 to 0.25 µg/ml) were placed in U-bottomed microtiter plates containing 4% TSB. Bacteria (20 µL, ca. 10^7 cfu/ml), were added, plates were incubated (18h, 37° C), and wells examined for growth pellets. All bacteria were obtained from the ATCC, unless stated otherwise. Clinical isolates of C-resistant MRSA strain #1979 and C-sensitive MRSA strain #2043 were obtained from the Dwight David Eisenhower Army Medical Center, Ft. Gordon, GA. Binding parameters of Nan for E41, E43 and E46 were contrasted. For second generation bisphosphonate compounds, maximal binding (B_{max}) was determined using Nan particles prepared as above. To determine the K_d the levels of E41, E43 and E46 were held constant (4 µg/ml) and Nan levels increased (0.1 to 100 mg).

3. RESULTS

3.1 *In vitro* experiments

Saturation studies E41 binding to CP varied with CP-particle mass, in a saturable manner resembling the Michaelis-Menten model. Affinity constants, or K_A values, defined as the CP-particle molar concentration producing one-half binding, were: 7.57×10^8 M for Nan, and 1.65×10^6 M for TCP ($p \leq 0.01$, Figure 1). By contrasting K_A values, E41 showed a 22-fold higher affinity for Nan than for TCP. This was perhaps due to

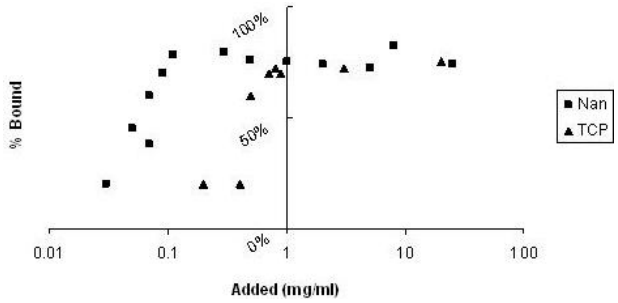

Fig. 1, E41 was held constant (8 µg/ml) and CP levels were increased. Following incubation, supernatants were used to measure E41-associated fluorescence. Binding expressed as "percent bound" (%B).

more binding sites on Nan, compared to TCP. Maximal binding (B_{max}) values, determined from *y*-intercepts of Eadie Hofstee plots (not shown), were similar; 74.6%, and 76.97% for Nan and TCP, respectively, p = NS. This suggested the intensity of E41 binding was similar for both the CPs. This is likely due to the fact that both particles primarily consist of calcium phosphate.

Dose Response studies The amount of E41 that can be carried by CP particles was determined. To accomplish this, CP-particle mass was held constant (10mg/mL), and E41 levels were increased. The amount of E41 able to saturate 50% of Nan was 17.5 mM. This compared to 0.62 mM for TCP, representing ca. a 28-fold difference. The carrying capacity determined, or binding capacity 50% (BC_{50}), has merit. On a sigmoidally-shaped curve, it is within the measurement's relevant range, and most accurate, as it is furthest away from decreasing slopes on upper and lower portions of the curve. Extrapolation of the curves to the x-axis (Figure 2) shows maximal saturation for CP-particles were near 55 mM and 1.5 mM of E41 respectively.

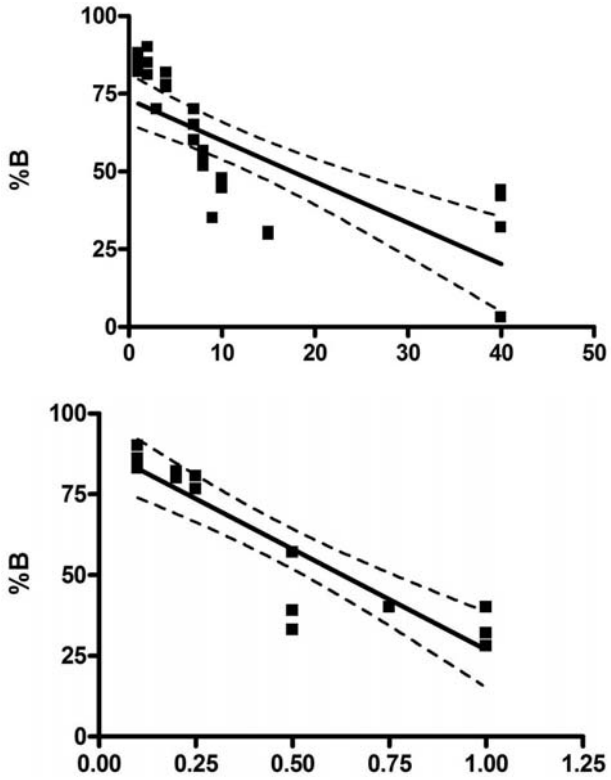

Fig 2, Levels of CP were held constant (10 mg/ml) and E41 levels were increased. BC_{50}% were 17.5, and 0.62 mM, for E41-Nan and E41-TCP, respectively. Top: E41-Nan, Lower: E41-TCP.

Acid release and competitive inhibition In the presence of lactic acid (25 mM), more E41 eluted from Nan than TCP ($p \leq 0.05$). The difference was 8.96 µg E41 for Nan *vs* 0.27 µg for TCP. The 33-fold difference was similar to the 26-fold difference seen above. These results also suggest acid, ca. pH 3.5, has the ability to release E41 from Nan *in situ*. MDP competitively inhibited E41's ability to bind Nan and TCP. An inverse relationship existed; as more MDP was added, more inhibition occurred. The MDP value showing 50% inhibition (Ki) was 1 mM for both Nan and TCP.

3.2 *In vivo* experiments

Antibacterial activity in an animal model The mean volume of the tibial defect was 23.4 ± 2.9 mm^3. Each tibia received 41 ± 8 mg of E41-CP, or ca. 1.75 mg / mm^3 of defect. *S. aureus* was the only bacteria identifiable in infected tibiae.

Results from experiment 1, using *S. aureus* at an inocula size near the ID_{95} value (1,270 cfu), and E41-CP particles prepared at BC_{50} levels, tibial loads were different among the three groups: Infection controls, 4.55 ± 0.235 log_{10} cfu/gm (n=24); E41-TCP, 2.72 ± 0.30 log_{10} cfu/gm (n=18); and E41-Nan, 0.52 ± 0.47 log_{10} cfu/gm (n=19)(ANOVA, F=42, $p \leq 0.01$, Mean ± SEM, Figure 3). E41-Nan produced lower tibial loads than E41-TCP ($p < 0.01$, t =4.0, 35 dof). Twenty-four of 24 Infection-controls yielded tibial loads at least one log higher than the original inoculum size. This suggested an active bone infection developing into acute osteomyelitis. Among E41-CP treatments, 16 of 19 tibiae were sterile in the E41-Nan group, *vs.* 5 of 18 for E41-TCP (Chi Sq = 12, $p \leq 0.01$).

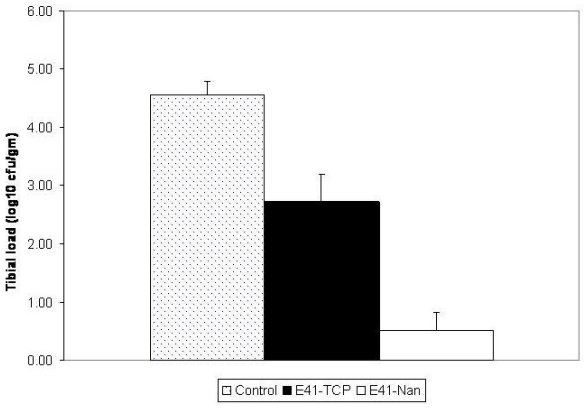

Figure 3. Tibial load (log_{10} cfu/gm) of *S. aureus* in treated and untreated rats. E41-Nan tibial loads were lower than E41-TCP ($p<0.01$). Mean ± SEM.

In experiment 2, several changes were made to place E41-CP particles under more stringent conditions. E41-CP particles were prepared at identical equimolar E41 concentrations (4 mM), and particles were washed extensively (10 times) the day before surgery. This was done to assure any observed antibacterial effect was attributable to E41 strongly bound to CP. Last, the inoculum of *S. aureus* was increased to 87,700 cfu. This increased the difficulty for complete eradication of bacteria. Again, tibial loads among the three groups were different (ANOVA, F=19, Mean ± SD, $p \leq 0.01$): Infection controls, 5.60 ± 0.44 log_{10} cfu/gm (n=9); E41-TCP, 2.82 ± 2.43 log_{10} cfu/gm (n=6); and E41-Nan, 0.57 ± 1.28 log_{10} cfu/gm (n=5). E41-Nan yielded marginally lower tibial loads ($p = 0.096$, t =1.85, dof = 9). One of 5, *vs* 4 of 6 were infected for E41-Nan and E41-TCP, respectively ($p = 0.17$, Fishers Exact).

3.3 *In vitro* experiments, second generation bisphosphonate conjugates and CP

Minimal inhibitory concentration studies E43 and E46 were contrasted to E41 for activity against MSSA, C-resistant MRSA, C-susceptible MRSA, *Acinetobacter baumannii* and other pathogens. Minimal inhibitory concentrations (MIC) were determined. E43 showed greater MIC activity against MRSA and MSSA, as well as *A. baumanni* (Table 1).

Release kinetics of bisphosphonates from Nan In *in vitro* binding studies, holding levels of either E41, E43 or E46 constant (4μg/mL), and increasing Nan levels as above, binding to CP was similar, and saturable. Maximal binding was: E41, 89.7 % ± 4.74, E43, 76.8 % ± 2.35 and E46, 95.9 % ± 0.13 (data not shown). K_d values were: E41, 1.35 x 10^{-8} mM (NanOss™), E43**,** 1.83 x 10^{-8} mM and E46, 1.72 X 10^{-8} (data not shown).

4. DISCUSSION

The idea to fill bony defects with antibiotic-containing carriers, i.e., biodegradable vehicles for local drug delivery, started with the introduction of the collagen-gentamicin sponge (Stemberger et al., 1997). Since then, CP and hydroxyapatite (HA) carriers have been added, and successfully used. Calcified bone contains three-fold more inorganic mineral than organic matrix, and the mineralized component consists mostly of osteoconductive calcium phosphate (CP).

CPs adsorb molecules on their surfaces (Ginebra et al., 2006). Exploited in HA chromatography, this property has aided in the purification of numerous proteins and other macromolecules. Most work to date in this field uses passively-coated antibiotics on CP [or HA (Ferraz et al., 2007; Kanellakopoulou et al., 2000; Shirtliff et al., 2002)]. Bisphosphonates bind strongly to these CPs (Fleisch et al., 1968; Lin, 1996). The discovery of bisphosphonates was a medical milestone in several bone diseases, e.g., osteoporosis and Paget's disease. Characterized by P-C-P bonds, they have been shown to inhibit osteoclastic resorption (Rodan and Fleisch, 1996) perhaps mediated through a concomitant action with osteoblasts (Sahni et al., 1993). The conjugation of antibiotics to bisphosphonates offers an opportunity to bind antibiotics more strongly to the CP carrier, perhaps maintaining high drug levels for longer periods than treatment with the parent antibiotic alone. In theory, the antibiotic negatively affects bacterial infection, and the bisphosphonate negatively affects host inflammatory-mediated bone resorption**.**

Table 1. MIC (μg/ml); MSSA = methicillin-susceptible *S. aureus*, MRSA = methicillin resistant *S. aureus*, C = ciprofloxacin, E41 = C-bisphosphonate, G = gatifloxcin, E43 = G-bisphosphonate, M = moxifloxacin, E46 = M-hydroxybisphosphonate, ATCC = American Type Culture Collection Type strains, EAMC-1 = Eisenhower Army Medical Center clinical isolate.

Bacteria	**Strain**	**C**	**E41**	**G**	**E43**	**M**	**E46**
MSSA	Cowan	0.25	8	0.25	0.5	4	4
MSSA	SMH	0.25	8	0.25	1	0.25	2
MRSA	C-susceptible	2	16	0.25	1	0.25	4
MRSA	C-resistant	>256	>256	64	>256	128	>256
A. baumannii	ATCC	0.5	16	0.25	1	0.25	64
A. baumannii	EAMC-1	64	>256	2	32	2	>256
Ps. aeruginiosa	ATCC	0.25	8	0.5	4	1	256
E coli	ATCC	0.25	0.25	0.25	0.25	0.25	2

Nanoparticles are excellent carriers for delivering drugs (Brigger et al., 2002). They protect drugs from degradation until they reach their target and provide sustained release. Nanoparticles, however, suffer from one major limitation, they are quickly removed from the blood, sometimes in minutes, rendering them ineffective in delivering drugs (Chambers and Mitragotri, 2007). Topical delivery of drug-containing particles may circumvent this problem.

High levels of teichoplanin (3%), passively bound to CP cement, were used to successfully treat chronic OM, caused by MRSA, in a rabbit model. Lazarettos *et al*, used single 2-mm femoral defects to implant *S. aureus*, with metal and bone wax added as foreign bodies (Lazarettos et al., 2004). The teichoplanin-CP combination totally eradicated femur loads of *S. aureus*, and reduced histologic scores examined two to six weeks after intramedullary packing. Our results are similar, in that we eradicated tibial infection caused by *S. aureus*, without (macroscopic) evidence of host toxicity, using high levels of ciprofloxacin-bisphosphonate bound to CP. The models' differ with respect to the level CP particle size, and the level of trauma. Our open fracture model focuses on acute OM, and has high level of mechanical and thermal trauma.

From these data, E41 showed binding affinity *in vitro* to both CP particles; μ-sized TCP and nanometer-sized Nan. The highest affinity was seen with Nan, suggesting E41 may be less readily released from Nan. And yet, the E41-Nan combination eradicated bone infection, perhaps surpassing E41-TCP in the antibacterial effect, examined 24 h after contamination.

E41 consists of methylene disphosphonic acid. This moiety is used as a bone-seeking agent in technetium bone scans (Okamoto, 1995). In previous work, E41 showed binding affinity to rat, human and porcine bone (Herczegh et al., 2002). Ciprofloxacin, also part of E41 as part of its structure, is a broad-spectrum, bactericidal fluoroquinolone widely used for the treatment of OM, with low toxicity (Lew and Waldvogel, 1997; Orhan et al., 2006). E41-TCP reduced *S. aureus* tibial loads and blocked experimental OM in rats (Buxton et al., 2004). However, the combination did not sterilize bone.

Nan has almost a three-fold higher total surface area *vs.* TCP, (50/17 M^2/g). These values were based on surface area assessments provided by the product's manufacturers. If it were possible to separate Nan into its individual nanosized crystallites, a 4,625-fold difference in particle size theoretically exists [Nan (56 nm) and TCP (259 μm)]. Ignoring CP porosity and considering surface parameters, the total amount of drug available for release, favors nanometer-sized particles.

Wounds are generally low in pH and oxygen (Dunn et al., 1993). And during the healing of acute wounds, a temporary acidosis occurs, resulting from the generation of organic acids, including lactic acid (Schneider et al., 2007). The increased demand for O_2, combined with a stasis of tissue perfusion, increases local pCO_2 levels in wounds (Hunt et al., 1967). Another contribution to the acidic milieu comes from pus. Immune system activation of macrophages, attracted to a wound, then killed, release cytoplasmic contents, including lactic acid. Concomitantly, infecting bacteria release lactic acid as well as other organic acids. From *in vitro* results, low levels (25 mM) of lactic acid promoted the release of ca. 30-fold more E41 from E41-Nan *vs* E41-TCP, prepared at BC_{50} levels. Also, E41 was released from both E41-CPs at pH 3.5. Perhaps wound acid levels *in vivo* played a role in the increased release and the antibacterial effect observed with E41-Nan. Studies are ongoing to answer this hypothesis.

While E41-Nan represents prophylaxis for OM, improvements to the model are currently underway. The second generation fluoroquinolone-conjugate E43

exhibits similar binding kinetics to Nan, with the increased benefit of enhanced antimicrobial performance against drug-resistant microorganisms of clinical interest such as MRSA and *A. baumannii*. Further study of this E43 is necessary to determine its value *in vivo*.

5. CONCLUSIONS

This study determined value of nano-sized versus μ-sized CP particles in the ability to bind more drug *in vitro*, and eradicate bone infection *in vivo*. We have shown these positive effects in the prevention of acute OM in a rat model. Future work will contrast E41-Nan *vs* parent antibiotic ciprofloxacin, passively adsorbed onto Nan, to confirm the added value of bound antibiotic to CP, and the role low wound pH plays in the long term release of antibiotic in the wound area. Several lower concentrations of E41-Nan will be used in dose-response studies with higher inocula of *S. aureus* to further assess the limits of the antibacterial effect(s) of E41-Nan.

ACKNOWLEDGMENTS

This research was funded by the Department of the Army, Award Number: W81XWHM-06-2-0013, and the U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD, 21702-5014, is the awarding and administering acquisition office. The content of the information does not necessarily reflect the position or the policy of the U.S. Army, and no official endorsement should be inferred. These data were presented in part at the American Chemical Society, Boston, MA, August 22, 2007.

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