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QUERY SHEET

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Gatifloxacin Biodegradable Implant for Treatment of Experimental Osteomyelitis: In Vitro and In Vivo Evaluation

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Osteomyelitis is an inflammatory bone disease caused by pyogenic bacteria. The advantages of localized biodegradable therapy for osteomyelitis include high local antibiotic concentration at the site of infection and obviation of the need for removal of the implant after treatment. The purpose of this study was to develop and evaluate a biodegradable implantable delivery system containing gatifloxacin (GAT) for the localized treatment of osteomyelitis, experimentally induced by methicillin resistant Staphylococcus aureus (MRSA). Implants, prepared by solvent casting technique, showed reasonable tensile strength. DSC examination indicated that GAT is present in an amorphous form in the implant. The in vitro release of GAT showed a profile characterized by an initial burst followed by a second stage of gradual delivery over 27 days. The in vivo release study revealed that GAT concentrations achieved during the first 3 weeks after implantation exceeded the MIC of GAT against MRSA by >100,000 times. Bacterial tibial bone count performed in rabbits tibia 2 and 4 weeks after implantation of GAT implant in infected bone indicated complete eradication of infection in all treated rabbits as indicated by the significant decrease in bacterial count. The results show that the proposed implant may have a promising role in the therapeutic approach to osteomyelitis.

Keywords: Gatifloxacin, Implant, MRSA, Osteomyelitis, Polycapro-lactone

Gatifloxacin can be either acute or chronic. Acute osteomyelitis can be successfully treated with antibiotics provided the disease is diagnosed early. In the treatment of chronic osteomyelitis, antibiotic therapy alone has not always yielded satisfactory results. Although the dose of antibiotic administered systemically is high, therapeutically effective drug concentrations are not always achieved at the site of infection. This is because bones are moderately perfused organs, and there is further reduction in the blood supply of the infected bone tissues (Dash and Suryanarayanan 1992). Thus the elevated antibiotic tissue levels necessary for treating chronic bone infections might be achieved by a local delivery system.

Several antibiotic implantable drug delivery systems have been developed for treatment of bone infection. The advantage of this approach is that an effective drug concentration is attained at the site of infection, while the systemic drug concentration remains very low (Gitelis and Brebach 2002). The main disadvantage is that the implants should be removed at the end of the treatment period. Implantable biodegradable polymer systems have a unique advantage in that the dosage form need not be removed from the body and thus saves cost and risk for the patient.

The use of fluoroquinolones for the treatment of osteomyelitis is now an established systemic therapeutic approach (Castro et al. 2003). Materials like tricalcium phosphate and hydroxyapatite as fluoroquinolone carriers for treating bone infection have been studied (Castro et al. 2005). In addition, biodegradable polymers such as poly(lactic acid) (Castro et al. 2003), polyurethanes (Schierholz et al. 1997) and cross-linked high amylase starch (Dévéaux et al. 2002 a, 2002b) have been tested and show potential effectiveness as quinolone carriers in local treatment of bone infection.

Gatifloxacin is an extended-spectrum fluoroquinolone with improved Gram-positive and anaerobe coverage compared with older agents such as ciprofloxacin. The methoxy substituent at its C-8 position ensures a greater inhibitory action on DNA gyrase and topoisomerase IV of Gram-positive bacteria and prevents the emergence of mutant strains in Staphylococcus aureus (Ince and Hooper 2001). Gatifloxacin has been used for a variety of clinical conditions including genitourinary infections (Liu and Mulholland 2005), lower respiratory tract infections (Grossman, Rotschafer, and Tan 2005), community acquired pneumonia (Noreddin, Hoban, and Zhanel 2005), acute bacterial exacerbations of chronic bronchitis (Martinez and Anzueto 2005), acute bacterial rhinosinusitis (Weckx et al. 2005), and recurrent acute otitis media (Elmwood Pediatric Group 2005).

Shirtliff, Calhoun, and Mader (2002a) reported equal efficacy of oral and parenteral GAT in treatment of experimental...
methicillin sensitive *Staphylococcus aureus*-induced osteomyelitis in rabbits. Although an implantable system for ciprofloxacin has been extensively studied (Overbeck et al. 1995; Ramchandani and Robinson 1998; Désévaux et al. 2002a, 2002b), gatifloxacin implantable system for treatment of bone infection has not been studied yet.

The objective of this study was to design an implantable GAT delivery system based on a biodegradable polymer, polycaprolactone (PCL), for site-specific delivery of GAT antibiotic to treat osteomyelitis experimentally induced in rabbits by methicillin-resistant *Staphylococcus aureus* (MRSA). As *Staphylococcus aureus* is the most common organism causing osteomyelitis, and as methicillin-resistant isolates of this bacterium are continuously on the rise (Munoz Bellido et al. 2002), it might well be warranted to expect increasing cases of osteomyelitis caused by MRSA. Hence, it was chosen as the study organism.

**MATERIALS AND METHODS**

Gatifloxacin was a generous gift from Bristol Myers Squibb Company (Princeton, NJ, USA). Polycaprolactone was purchased from Birmingham Polymer (Birmingham, AL, USA).

**Organism**

Several clinical isolates of MRSA as well as ATCC 33591 were used to induce osteomyelitis during the initial period of the study. The strain found to be most commonly associated with production of osteomyelitis was ATCC 33591 and was thus chosen as the study strain of MRSA.

**Study Animals**

New Zealand white female rabbits, 4–6 weeks of age and weighing 1–2 kg, were used in the study. Animals were kept in aluminum cages and food and water were provided *ad libitum*.

**Preparation of Implant**

PCL was dissolved in 10 ml dichloromethane. Gatifloxacin powder was dispersed in polymer solution in a ratio of 1:1 w/w of drug:polymer and stirred for 30 min. The dispersion was cast on a Teflon plate and dried overnight at 5°C. All the prepared implants were sterilized by gamma radiation.

**Differential Scanning Calorimetry (DSC)**

Samples of pure drug, polymer, and prepared implant were heated over the range of 25°C to 300°C with a heating rate 10°C/min.

**Tensile Strength**

Rectangular strips of 20 × 15 mm size were cut and strained to break at a constant crosshead speed of 20 mm/min using Instron 5842 (Instron, Canton, MA, USA) using the associated software “Merlin.” Tensile strength at breakpoint was measured. Five measurements were taken and the data were averaged to obtain a mean value.

**Minimum Inhibitory Concentration (MIC)**

MIC was determined for ATCC 33591 by the agar dilution method as described in the Clinical and Laboratory Standards Institute (CLSI) recommended methods using Mueller-Hinton agar and direct colony suspension, equivalent to a 0.5 McFarland standard and incubated at 35°C for 20 hr. Dilutions of GAT were started at 512 μg/L. The MIC was considered to be the lowest concentration of GAT that prevented visible growth (National Committee for Clinical Laboratory Standards 2003). The MIC was confirmed by the E-test (AB Biodisk, Solna, Sweden) performed according to the manufacturer’s instructions to expand the range of dilutions available.

**Sterility Test**

Sterility tests were performed for the sterilized membrane to assure sterility. Three samples of GAT implants sterilized by gamma radiation were each placed in 20 ml vials of tryptic soy broth and incubated at 37°C for 3 weeks. Subcultures were made onto blood agar after the 3 weeks for detection of any growth.

**In Vitro Drug Release**

Three weighed and sterilized pieces of antibiotic implants were placed each in 2 ml of PBS (pH 5.3) and incubated at 37°C for 24 hr. A predetermined amount of drug content was used to maintain the sink condition. The implant was removed, shaken free of excess PBS, transferred to fresh 2 ml aliquots of PBS after 2 hr, 4 hr, then every 24 hr for 2 weeks then every week for 2 more weeks. Samples of removed PBS were stored at −70°C until a microbiological disc diffusion assay was performed.

Disc diffusion assays were performed to determine the antibiotic concentration in the samples. An aliquot of 0.1 ml of overnight culture of *Bacillus subtilis* ATCC 6633 suspension was added per 100 ml of antibiotic agar medium one (Difco, Detroit, MI, USA). First, 5 ml of this seeded agar was aseptically pipetted into Petri dishes. Standard 2-fold serial dilutions of GAT were made in PBS (pH 5.3), producing standard concentrations ranging from 0.002 to 5 mg/ml. Second, 20 μl of each in vitro release sample and standard concentration were added to each of three sterile blank 6 mm filter paper discs placed on the seeded plates. The plates were incubated overnight at 37°C. The diameter of the zone of inhibition for each standard and in vitro release sample was measured. The unknown concentrations for the in vitro release samples were determined by comparing their respective zone size means to the standards (Mader, Calhoun, and Cobos 1997).

**In Vitro Inhibition of MRSA**

Test tubes containing Mueller-Hinton broth (Oxoid Ltd., London, UK) inoculated with 5 × 10⁶ CFU of MRSA and GAT implant (equivalent to 40 mg drug/10 ml broth) were incubated
at 37°C together with three control tubes without implants. The implants were washed and switched to a new set of inoculated tubes each day, and tubes were sampled for colony counts to determine bactericidal effect.

**In Vivo Release**

The local committee for animal studies in King Saud University had previously approved animal experiments. Rabbits were anesthetized using an intramuscular injection of 45 mg/kg ketamine (Tekam, Hikma Pharmaceuticals, Amman, Jordan) and 8 mg/kg xylazine (Rompun, Bayer, Germany). The left hind leg was shaved and disinfected. An incision of 2 cm was made on the medial side of the left leg just below the knee. Fascia and muscles were dissected to expose the tibia. A saw was used to make a 0.5 × 1 cm opening in the bone and the implant was inserted in a dose of 40 mg/kg then the site was sutured. Three rabbits were sacrificed at 1, 2, 3, and 4 weeks after implant insertion. The bones were extracted and the drug was assayed in the bones.

**Drug Assay in Bone**

Bones were prepared for assay by dissecting them free of all soft tissues and the implant was removed. Tibial diaphysis was broken down into small pieces, crushed, weighed and suspended in PBS (pH 5.3). Then 1 ml of buffer was used per 0.5 g bone. The crushed bone was shaken on a vortex mixer for 5 min. Antibiotic in the supernatant fluid was assayed by the described disc diffusion method mentioned in the In Vitro Drug Release section. Drug concentrations were determined from semilog standard curves (Shirtliff, Calhoun, and Mader 2001).

**Efficacy of Implant**

**Production of Osteomyelitis Model and Insertion of Implant**

The bacterium was grown overnight in tryptic soy broth and diluted in saline to a concentration of ~1 × 10⁷ CFU/ml. The animals were anesthetized as mentioned before in the In Vivo Release section. The left hind leg was shaved and an 18-gauge needle was inserted into the intramedullary cavity. Sodium morrhuate (scleromate; Glenwood, NJ, USA) (0.1 ml of a 5% [w/v] solution, 0.1 ml of MRSA (1 × 10⁶ CFU), and 0.2 ml of sterile saline were injected sequentially. Then a sterile stainless steel needle was inserted and left in the intramedullary cavity to act as a foreign body. A period of 2 weeks was given to allow for development of the osteomyelitis (Shirtliff and Mader 1999). Six animals were assigned per each study group (2 weeks treatment, 4 weeks treatment). Fully 12 control rabbits were assigned for each study period.

Surgical procedures were performed (Shirtliff and Mader 1999) 2 weeks after initiation of infection. Animals of both control and treatment groups were anesthetized and prepared as stated under In Vivo Release section. Bone marrow samples were taken, and implants were inserted in tibial bones of treatment groups in a dose of 40 mg/kg body weight to treat the infection as stated under In Vivo Release. Samples of bone marrow were weighed and emulsified in 1 ml saline. A 10 µL loopful was plated onto blood agar and mannitol salt agar plates. Colony counts were performed from any MRSA growth.

**Bone Cultures**

After the study period had elapsed (control, 2 and 4 weeks of treatment), the animals were euthanized and the left tibias were extracted and the implant was removed from bones of treatment group and bacterial cultures were performed. The tibias were treated as mentioned in the Drug Assay in Bone section. Fully 20 µl samples were spotted onto blood and mannitol salt agar plates in triplicates and incubated at 37°C for 24 hr. Colony-forming units were counted for each tibial sample and the bacterial count was calculated per gram bone (Shirtliff, Calhoun, and Mader 2002b).

**RESULTS AND DISCUSSION**

For the selected strain of *Staphylococcus aureus* used in the study, the MIC values for GAT determined by agar dilution and by E-test were 0.031 µg/ml and 0.023 µg/ml, respectively. The MIC value determined by E-test was lower due to extra gradings.

The results of sterility test indicated that no growth from any of the vials was detected indicating adequate sterility. DSC examination was performed to study the possible interaction between GAT and PCL. DSC thermograms revealed that GAT in its powder form produced an endotherm representing its melting point at 100°C (Figure 1). PCL melting point can be observed at 60°C. The implant of PCL and GAT exhibited a broad peak at 60°C, but GAT did not exhibit endotherm, suggesting that melting PCL and solubilization of GAT occurred simultaneously.

The measured tensile strength was 1 N indicating reasonable mechanical properties of the prepared implant.

**In Vitro Release Study**

The in vitro release profile of GAT revealed that 78% of drug content of implant was released at the end of the first day. The release showed a profile, characterized by an initial burst followed by a second stage of gradual delivery over 27 days (Figure 2). The high initial release or burst effect is a phenomenon frequently associated with drug delivery from monolithic-polymer controlled-release systems. The initial fast release is beneficial in certain drug administration strategies (Huang and Brazel, 2001). In local infection of bone, maintaining a high antibiotic concentration at the site of infection is highly appropriate for treating multibacterial infection and decreasing the development of rapid resistance of bacteria to the administered antibiotic, especially strains known to rapidly mutate into resistant variants such as MRSA, and consequently increases the effectiveness of treatment.

**In Vitro Inhibition Study**

Figure 3 shows the number of multiples of MRSA counts in Mueller-Hinton broth in the presence of GAT implant versus
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FIG. 1. DSC thermograms of gatifloxacin (GAT), polycaprolactone (PCL), and PCL-GAT implant.

FIG. 2. In vitro release profile of gatifloxacin from the prepared implant in PBS, pH 5.3 at 37°C.

FIG. 3. In vitro inhibition of MRSA in presence of implant versus control.
FIG. 4. In vivo release profile of gatifloxacin after insertion of the prepared implant (equivalent to 40 mg/kg) in rabbit’s tibia.

the control tubes without GAT implant and with the same MRSA inoculum. The number of bacteria in the control tubes multiplied by 70–76 times over the days. While in the presence of GAT implant, the MRSA count doubled only by 2–5 times. The decrease in the amount of drug released from one day to the following is probably the cause of the increase of MRSA counts from 2 times in the first set of tubes to 5 times after 4 days.

In Vivo Release Study

Figure 4 shows the local bone concentration of GAT per gram of bone tissue. Animals sacrificed after 1 week had average drug level of 5.2 mg/g. This exceeds by far (>100,000 times) the MIC of GAT for MRSA. After the second and third weeks the level averaged to 2.7 to 0.29 mg/g bone, respectively. Regarding therapeutic efficacy, antibiotic concentrations achieved during the first 3 weeks after implantation greatly exceeded the MIC of GAT against MRSA. However, after 4 weeks no drug was detected in the bones. Therefore, therapeutic coverage in the tibia is warranted for 3 weeks. The enhanced in vivo diffusion gradient resulted in more rapid drug release than that in vitro. This high drug concentration could very well shorten the period needed for the eradication of a chronic bone infection.

In Vivo Evaluation

Clinical evidence of infection including fever and loss of body weight over the first 2 weeks after induction of bone infection was observed in rabbits. The percentage mortality in control group (∼80%) was significantly higher than treatment group (∼8%). The gross pathology grading of the severity of osteomyelitis in rabbits was at 2+ level and characterized by soft tissue abscess and less than 10% widening of proximal tibial metaphysis for most rabbits and 3+ for some (>10% widening of the proximal tibial metaphysis) (Mader and Wilson 1983).

Changes in viable cell count in treated and control groups are presented in Figures 5–7. Changes in viable count in treated group were expressed as their log decrease on each time of sampling compared with the implanted inoculum. The developed biodegradable implant of GAT proved to be effective in the eradication of MRSA, as demonstrated by the significant decrease in cell count ($p \leq 0.01$) of the study group after 4 weeks of implantation. The time of 4 weeks was chosen on the basis of the recommended period of treatment by parenteral form of antibiotic in osteomyelitis, which is the gold standard for treatment of this condition. However, on observing the 2 weeks treatment group, bacterial sterility was achieved at the end of two weeks as indicated by the significant decrease in bacterial count ($p \leq 0.01$), (Figure 6). On the other hand, bacterial count

FIG. 5. Reduction in bacterial count 4 weeks after insertion of implant containing 40 mg/kg in rabbits experimentally infected with MRSA.

FIG. 6. Reduction in bacterial count 2 weeks after insertion of implant containing 40 mg/kg in rabbits experimentally infected with MRSA.
significantly increased ($p \leq 0.01$) in most rabbits or remained almost the same as the initial count as shown in Figure 7.

It seems that the high dose of GAT released from the implant after 1 week, as indicated by the in vivo release study, in the presence of a compromised blood flow at the implantation site due to infection was highly effective. By the end of the treatment period, the infection was eradicated and bone healing was almost complete as shown in Figure 8. This high dose of drug release also is beneficial in prevention of emergence of resistant isolates (Boos et al. 2001). Such an aggressive local treatment regimen has been reported by Ramanowski et al. (2005) to overcome resistance documented by in vitro testing and resulting in successful treatment experimentally.

Similar results were reported by Wei et al. (1991) with an oligomer of lactic acid carrying dideoxykanamycin B in rabbits as well as by Garvin et al. (1994) with polylactic/polyglycolide delivery system of gentamicin; however, these studies involved isolates susceptible to methicillin. Kanellakopoulou et al. (2000) have reported that the local treatment of experimental osteomyelitis caused by MRSA with a low molecular weight D-, L- dilactide polymer containing pefloxacin results in eradication of MRSA. Also, successful clinical data on osteomyelitis of the pelvis and of the hip caused by MRSA exist on two patients after application of methylmethacrylate beads impregnated with vancomycin (Ozaki et al. 1998). Similarly, Cevher et al. (2006) documented that implanted vancomycin-loaded chitosan microspheres were found to be more effective than the IM route for treatment of experimental osteomyelitis. As a consequence the application of PCL-GAT implant for the therapy of osteomyelitis by MRSA might be a novel therapeutic modality.

CONCLUSION

Gatifloxacin-polycaprolactone implant could be a promising delivery system for treatment of osteomyelitis caused by MRSA. As the threat of vancomycin-resistant *Staphylococcus aureus* is a reality (Munoz Bellido et al. 2002), the search for alternatives to vancomycin in treating MRSA is unleashed. Gatifloxacin has been shown to be attractively effective in vitro against our local MRSA isolates (>$99\%$ sensitive) (Baddour et al. 2005). In fact it is one of the most active fluoroquinolones marketed until quite recently (Jones et al. 2002).

Moreover, studies have indicated that it selects for 10–100-fold lower frequencies of less susceptible variants of

![FIG. 7. Bacterial count in control group 2 weeks postinfection.](image)

![FIG. 8. Infected rabbit's tibiae (a) untreated and (b) treated with gatifloxacin implant (40 mg/kg).](image)
GATIFLOXACIN BIODEGRADABLE IMPLANT

Staphylococcus aureus (Huczko et al. 2000; Takei et al. 2001; Fung-Tomc et al. 2001). However, proven clinical efficiency remains the ultimate measure of success of any treatment regimen. Thus, further studies using larger number of animals with osteomyelitis are required to confirm the effectiveness of localized PCL-GAT implants for the treatment of osteomyelitis before such a system could be tried clinically on humans.

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REFERENCES


