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Ex vivo efficacy of gemifloxacin in experimental keratitis induced by methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

In recent years, the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) strains has been observed in ocular infections. Resistance of MRSA to second- and third-generation fluoroquinolones has increased interest in the fourth-generation fluoroquinolones. In this study, the antibacterial activity of gemifloxacin against MRSA ocular isolates in vitro and in a modified ex vivo rabbit keratitis model was investigated. In vitro susceptibility test results indicated that the minimum inhibitory concentrations (MICs) of gemifloxacin were lower than the MICs of other fluoroquinolones, including moxifloxacin (MIC_{50} range, 0.016–0.032 μg/mL; MIC_{90} range, 0.047–0.094 μg/mL). Results from the ex vivo keratitis model showed a statistically significant decrease in MRSA counts (0.5–2 $\log_{10}$ CFU/g; $P < 0.05$) in corneas treated with 0.3% gemifloxacin every 30 min for 7 h. Moreover, the dose–response effect of different concentrations of gemifloxacin (3–3000 μg/mL) demonstrated that a dose of 30 μg/mL had the same efficacy as the highest dose of 3000 μg/mL against all *S. aureus* strains. Possibly, gemifloxacin reached a steady-state level in the cornea, as the fourth-generation fluoroquinolones have better anterior chamber penetration. This study demonstrated that 0.3% gemifloxacin ophthalmic solution may be an effective topical therapy for the treatment of MRSA keratitis. In addition, this reproducible, ethical and economic ex vivo infection model can be used as a mechanistically-based alternative to in vivo animal testing, bridging the gap between in vitro and in vivo results.

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1. Introduction

Bacterial ulcerative keratitis is a serious condition that requires an early diagnosis as well as immediate and adequate treatment. Failure to promptly eradicate the infectious agent may lead to serious complications including corneal scarring and permanent visual impairment or visual loss [1,2]. In the past, most cases of bacterial keratitis were associated with ocular trauma or ocular surface diseases. More recently, the widespread use of contact lenses and acute-onset endophthalmitis after cataract surgery have increased the incidence of bacteria-induced keratitis [3–6]. The common organisms involved in this pathology include *S. aureus*, *Staphylococcus epidermis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Serratia* spp. [7–9]. Among these, *S. aureus* is the predominant pathogen isolated from the majority of cases of keratitis [10–12]. Although β-lactams are not frequently used in ophthalmology, methicillin resistance is a key mechanism of resistance in staphylococci and is significantly associated with higher resistance rates to other non-β-lactam agents, contributing to the spread and persistence of multidrug-resistant strains in several settings. The rates of methicillin resistance among ocular staphylococci isolates are currently on the rise [13,14].

The fluoroquinolones have proven to be successful for treating keratitis following the introduction of ophthalmic solutions of ciprofloxacin and ofloxacin. However, antibiotic resistance of *S. aureus* and *P. aeruginosa* keratitis isolates to the second- and third-generation fluoroquinolones has increased interest in the fourth-generation fluoroquinolones for future coverage of bacterial keratitis [15–17]. Fourth-generation fluoroquinolones such as moxifloxacin have been shown to possess greater potency against Gram-positive organisms as well as activity against Gram-negative bacteria comparable with second- and third-generation fluoroquinolones (ciprofloxacin and levofloxacin). Fourth-generation fluoroquinolones achieving higher intraocular concentrations are less likely to develop resistance than earlier fluoroquinolones such as ofloxacin [1,2,18,19]. Among the fourth-generation fluoroquinolones, gemifloxacin was approved by the US Food and Drug Administration (FDA) in April 2003 for the treatment of mild-to-moderate community-acquired pneumonia and acute exacerbation of chronic bronchitis. Gemifloxacin acts by inhibiting bacterial DNA gyrase and

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topoisomerase IV and, compared with other fluoroquinolones, it possesses enhanced in vitro activity against *S. pneumoniae*, including isolates resistant to β-lactams, macrolides and ciprofloxacin, while retaining activity against Gram-negative and atypical pathogens [20]. Actually, gemifloxacin is undergoing development for topical ophthalmic therapy [21,22].

To date, numerous in vivo or in vitro models of bacterial keratitis have been used to study drug efficacy and absorption. However, these models show some disadvantages: in vivo studies are expensive and require elaborate maintenance procedures; and cell culture techniques fail to mimic corneal conditions such as wound healing, since they are composed of a single cell layer rather than the characteristic multilayered tissue [23,24]. Based on these observations, the aim of this study was (i) to assay the susceptibility of *S. aureus* ocular isolates [including methicillin-resistant *Staphylococcus aureus* (MRSA)] to gemifloxacin and other fluoroquinolones in vitro and (ii) to test the effectiveness of 0.3% gemifloxacin ophthalmic solution in a modified ex vivo rabbit model of MRSA-induced keratitis.

2. Materials and methods

2.1. Antimicrobial agent

Gemifloxacin mesylate powder was purchased from Cirex Pharmaceuticals Ltd. (Hyderabad, India). Gemifloxacin was dissolved in balanced salt solution (BSS).

2.2. In vitro susceptibility study

2.2.1. Micro-organisms and determination of minimum inhibitory concentrations (MICs)

A total of 24 *S. aureus* ocular isolates were used, collected from outpatients in three Italian hospitals. The bioassay was performed in parallel with American Type Culture Collection (ATCC) strains, including *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538 and *S. aureus* ATCC 43300. Identification of clinical isolates was conducted according to colony morphology, Gram stain and API systems (API; bioMérieux, Firenze, Italy). Strains were stored at −70 °C in Microbank™ vials (DID; Pro-Lab Diagnostics, Richmond Hill, ON, Canada) and single beads were removed from the cryovials and were used to directly inoculate Mueller–Hinton broth (Oxoid S.p.A., Milan, Italy). Antibiotic susceptibility of strains was characterised using the Etest method (PDM Epsilometer; AB BIODISK, Solna, Sweden). Etest strips of gemifloxacin, moxifloxacin, ciprofloxacin, levofloxacin and ofloxacin (range, 0.002–32 μg/mL) were tested according to the manufacturer's instructions. A resistance breakpoint at MIC ≥ 4.0 μg/mL for *S. aureus* was used [25].

2.3. Ex vivo study

2.3.1. Molecular characterisation of methicillin-resistant Staphylococcus aureus strains

The ocular isolates *S. aureus* 7786, *S. aureus* 815 and *S. aureus* 74CCH were used in the ex vivo keratitis model. In addition, *ATCC 43300* (*MRSA*) and *ATCC 6538* [methicillin-sensitive *S. aureus* (*MSSA*)] strains were used as controls. Methicillin resistance was determined by Etest for determining the MICs of oxacillin, by PCR for *mecA* gene detection and by PBP2′ Latex Agglutination Test (Oxoid S.p.A.) [26–28]. Briefly, for *mecA* gene detection, PCR amplification was carried out using two primers: 5′-AAA ATC GAT GGT AAA GGT TGG C-3′; and 5′-AGT TCT GCA GTA CCG GAT TTG C-3′). PCR was performed using *Taq* DNA Polymerase (QIAGEN, Milan, Italy) and related buffer. For amplification, the following was used: ca. 100 ng of bacterial DNA (determined spectrometrically), 0.25 μM of each primer, 200 μM dNTPs mixture (Sigma-Aldrich, Milan, Italy), 10 × PCR buffer providing a final concentration of 1.5 mM $MgCl_2$, Q-solution 5.0 × and 0.5 μL of *Taq* DNA Polymerase. DNA amplification was carried out for 40 cycles in 100 μL of reaction mixture as follow: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; with a final extension at 72 °C for 5 min. The PBP2′ Latex Agglutination Test was performed according to the manufacturer's instructions. The observed agglutination reaction was visually assessed and was compared with a positive (*S. aureus* ATCC 43300) and negative (*S. aureus* ATCC 6538) controls.

2.3.2. Preparation of inocula

Strains were cultured on tryptic soy agar (Oxoid S.p.A.) plus 5% sheep blood at 37 °C for 20 h. Then, a few colonies of each strain were washed three times with phosphate-buffered saline (PBS) (pH 7.4) to reach a density of 5×10^8 CFU/mL (V-1200-VWR; VWR, Milan, Italy) and were diluted to a final concentration of the inoculum (5×10^5 CFU/mL).

2.3.3. Rabbit globe harvest

Normal rabbit eyes, which were obtained from a local abattoir and were enucleated immediately following euthanisation, were washed with 1% povidone and were then rinsed with saline and submerged in tubes with 0.1% Dulbecco Modified Eagle Medium (DMEM) (DMEM/Ham's F-12; PAA Laboratories, Biolife, Milan, Italy). The tubes were placed in a container with ice for preservation and were transported to the laboratory and used immediately.

2.3.4. Staphylococcus aureus growth curve

The growth curves of *S. aureus* ATCC 6538 and *S. aureus* 7786 ocular isolate were assessed using a modified ex vivo keratitis model [29,30]. Enucleated eyes were randomly divided into two groups of 20 for each *S. aureus* strain. For each group, the eyes were injected intrastromally with 50 μL of the bacterial suspension (5×10^5 CFU/mL) using a 30 G needle. The groups were then divided into five subgroups (four eyes each) according growth curve times (0, 12, 24, 48 and 72 h from the insult). The sclerocorneal ring of each eye was excised using curved scissors and was placed on the corneal support in dishes containing 2.5 mL of DMEM as previously described [29,30]. Organ cultures were incubated at 37 °C in a humidified atmosphere of 6% CO_2. To moisten the epithelium, 100 μL of medium was added drop-wise to the surface of the corneal epithelium every 12 h. The culture medium in the dishes was changed every 24 h. At established fixed times, corneas without scleral ring were individually weighed and were homogenised in 2 mL of saline plus 0.1% peptone at 4 °C for 30 s. Then, 1 mL of homogenate was serially diluted (1:10 dilution) in saline plus peptone and was seeded in duplicate on tryptic soy agar plates. The results were reported to tissue weight and were expressed as CFU/g.

2.3.5. Treatment of Staphylococcus aureus keratitis

Staphylococcus aureus 7786, *S. aureus* 815, *S. aureus* 74CCH, *S. aureus ATCC 43300* and *S. aureus ATCC 6538* were used in the ex vivo model. Thirty enucleated eyes were randomly divided into five groups (six corneas/group) corresponding to each *S. aureus* strain. Each group was intrastromally injected with 50 μL of bacterial suspension (5×10^5 CFU/mL). At 20 h following the infection (late-phase infection), the eyes were divided into two treatment groups (three corneas/group) for each *S. aureus* strain. The first group was treated with 0.3% gemifloxacin ophthalmic solution, while the other was treated with BSS (control group). The treatment regimen consisted of 14 daily instillations (50 μL) every 30 min. The corneas were homogenised for standard colony counts at 1 h after the last

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