

Potential Antimicrobial Effects of Gatifloxacin on Periodontopathic Bacteria *in Vitro*

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Abstract

The aim of this study was to identify the potential antibacterial effects of gatifloxacin on periodontal pathogens including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia*. The minimum inhibitory concentrations (MIC) of gatifloxacin and its bactericidal effects were investigated. Gatifloxacin inhibited the growth of all three kinds of periodontopathic bacteria tested in broth. The MIC value of 2.5 nM was found to be the most effective in inhibiting *A. actinomycetemcomitans*. An adenosine triphosphate bioluminescence assay revealed that gatifloxacin exhibited bactericidal effects on the tested bacteria in a time-dependent manner. The safety of gatifloxacin in mammalian cells was evaluated by assessing the viability of normal human dermal fibroblast (NHDF) cells treated with gatifloxacin. Almost all NHDF cells survived after 2-d culture, while 81% of the cells survived after 4-d culture when treated with 1.0×10^3 nM gatifloxacin. These results indicate that gatifloxacin is a possible drug for local administration to prevent periodontal infection.

Keywords

Local Administration, Peri-Implantitis, Periodontopathic Bacteria, Antimicrobial Activity, Gatifloxacin

1. Introduction

The formation of microbial biofilm around dental implants is a major cause of peri-implantitis. Peri-implantitis, which is defined as “the inflammation around an implant with accompanying bone loss” usually causes implant failures. The presence of periodontopathic bacteria around titanium implants is a risk factor for peri-implantitis [1]-[3]. Anaerobic pathogens in the subgingival area are reported to be involved in the development of peri-implant bone defects [4]. Major etiologic bacteria causing human periodontal diseases include *Aggregatibacter actinomycetemcomitans* [5] and *Porphyromonas gingivalis* [6]. *Prevotella intermedia* is also associated with human periodontal diseases [7]. *A. actinomycetemcomitans* and two black-pigmented species, *P. gingivalis* and *P. intermedia*, are gram-negative strains which are often isolated from periodontal lesions [8]. These facts indicate that maintaining biofilm-free surfaces of a dental implant is critically important for preventing periodontitis and peri-implantitis.

In a previous study, comparatively large amounts of *A. actinomycetemcomitans* and *P. gingivalis* are reported to adhere to the titanium surfaces [9]. This suggests the potential risks of the adhesion of periodontopathic bacteria to titanium implants. In addition, our laboratory focuses on developing a defense system against bacterial adhesion on the surfaces of dental material [10]. The reduction of plaque accumulation has been emphasized in preventing periodontal disease such as peri-implantitis [11]. Antimicrobial materials may provide a new method of reducing the amount of oral bacteria involved in the development of oral diseases. In our previous study, antimicrobial peptides do not act as antigens against the host [12]. As part of our ongoing research for developing a defense system to prevent peri-implantitis, the antimicrobial peptide protamine is investigated. Although protamine strongly inhibits the growth of *P. gingivalis*, its antimicrobial effects are short-lived and sustained release is difficult to achieve [13]. Therefore, low-molecular weight drugs, which have more stable antibacterial effects, are needed. In the present study, the inhibitory effects of gatifloxacin on the growth of periodontopathic bacteria are examined.

Gatifloxacin is an antibiotic of the 4th generation fluoroquinolone drug family, and it has been frequently prescribed in the United States [14]. However, its adverse effects have often been profiled. In recent years, serious adverse effects including hypoglycemia and hepatotoxicity via systemic administration have led to the withdrawal or usage restriction of several fluoroquinolones [15] [16]. As for local administration, gatifloxacin is most commonly used as primary drug for various ocular infections [17].

This study aimed to investigate the antimicrobial effect of gatifloxacin on periodontopathic bacteria as well as the viability of mammalian cells in preventing periodontal infection as a local administration.

2. Materials and Methods

2.1. Materials

Gatifloxacin was obtained from LKT Laboratories, Inc. (St. Paul, MN). For the cell proliferation assay, gatifloxacin was solubilized in Dulbecco's modified essential medium (DMEM, Gibco, Grand Island, NY), which was then used as part of the culture medium described below. To evaluate minimum inhibitory concentration (MIC), the gatifloxacin was solubilized in the broth described below for liquid culture of bacteria.

2.2. Bacteria and Culture Conditions

For plate culture, the bacteria tested in this study were grown on plates containing tryptic soy agar (40 g·L⁻¹; Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 10% defibrinated horse blood (Nippon Bio-Test Laboratories Inc., Tokyo, Japan), hemin (5 g·L⁻¹; Sigma-Aldrich Corp., St. Louis, MO), and menadione (0.5 g·L⁻¹; Sigma-Aldrich Corp.) [18]. Preculture was performed in an anaerobic chamber (N₂: 80%, H₂: 10%, CO₂: 10%) at 37°C. For liquid culture, *P. gingivalis* ATCC 33277 and *P. intermedia* ATCC 25611, which were obtained from the American Type Culture Collection (Manassas, VA), were cultured in trypticase soy broth (30 g·L⁻¹; Becton, Dickinson and Company) supplemented with hemin (5 g·L⁻¹; Sigma-Aldrich Corp.) and menadione (0.5 g·L⁻¹; Sigma-Aldrich Corp.) in the anaerobic chamber at 37°C. *A. actinomycetemcomitans* 310a (kindly provided by Dr. H. Ohta, Ibaraki University) was cultured in Todd-Hewitt broth (30 g·L⁻¹; Becton, Dickinson Company) supplemented with yeast extract (10 g·L⁻¹; Becton, Dickinson and Company) in the anaerobic chamber at 37°C [19]. Each precultured colony was inoculated into a liquid broth and incubated for 2 to 4 days under the conditions described above.

2.3. Evaluation of Minimum Inhibitory Concentration

Broths containing gatifloxacin were used to determine MIC. Six serial concentrations of gatifloxacin were predetermined, ranging from 0 M to 25 nM for cultures of *A. actinomycetemcomitans* and *P. gingivalis*. Those of *P. intermedia* were predetermined from 0 M to 1.0×10^3 nM. Each bacterial strain was inoculated into the broth and then incubated for 3 - 7 days in an anaerobic chamber at 37°C. Minimum inhibitory concentration was defined as the lowest concentration of gatifloxacin that would inhibit visible growth of the microorganisms after incubation. To confirm the reliability of the data, the experiments were performed four times.

2.4. Antibacterial Activity of Gatifloxacin against Periodontopathic Bacteria

Periodontopathic bacteria were anaerobically grown at 37°C to the early stationary phase in the broth described above.

The harvested cells were washed once in aseptic broth and then resuspended in freshly aseptic broth containing sufficient concentrations of gatifloxacin. Bacterial cell suspensions were incubated at 37°C and examined for bacterial cell viability after 6, 24, and 48 h.

Bacterial cell viability was determined by adenosine triphosphate bioluminescence assay using the BacTiter-Glo Microbial Cell Viability Assay kit (Promega, Madison, USA). Specifically, a volume of BacTiter-Glo reagent equal to the volume of each bacterial suspension was added and briefly mixed. The luminescence of the solution was then recorded by using the Gene Light Model GL-210A luminometer (Microtec Co., Ltd., Funabashi, Japan). The value obtained was expressed as the ratio to that at the start of incubation. The results were expressed as the mean \pm SD of three experiments.

2.5. Mammalian Cell Viability Assay

Normal human dermal fibroblast (NHDF) adult donor cells derived from connective tissue (Takara Bio Inc., Shiga, Japan) were used in this study. The culture medium consisted of MEM α (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS French origin; Biowest, Nuaille, France) and antibiotics. Cells subcultured at 37°C in a humidified atmosphere with 5% CO₂ were suspended in MEM α at a concentration of 2.5×10^7 cells·L⁻¹. An aliquot of 1×10^{-4} L of cells was placed in the wells of a 96-well plate and then incubated for 4 h at 37°C in a humidified atmosphere with 5% CO₂. The medium was replaced with medium containing gatifloxacin at concentrations adjusted by stepwise dilution. The culture medium was refreshed every two days. After completion of cultivation, cell viability was determined by using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) every 24 h for 4 days. Briefly, a 10- μ L aliquot of CCK-8 solution was added to each well. The cells were then incubated for 3 to 4 h. Absorbance was measured at 450 nm by using a SpectraMax M5e Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA). The experiments were performed in triplicate. The data were expressed as the survival rate.

2.6. Statistical Analysis

Statistical analyses were conducted by using computer software (Prism, version 5.0c; GraphPad Software, San Diego, CA, USA) for comparison across the experimental conditions. Statistical comparisons were performed using an unpaired t-test or Kruskal-Wallis test followed by Dunn's post-hoc test.

3. Results and Discussion

3.1. Inhibitory Effect of Gatifloxacin against Periodontopathic Bacteria

As shown in **Table 1**, gatifloxacin showed an inhibitory effect on the growth of all the periodontopathic bacteria tested, with the inhibitory effect greatest on the growth of the *A. actinomycetemcomitans* strain (**Table 1(a)**). The MIC value for *A. actinomycetemcomitans* was 2.5 nM, and that for *P. gingivalis* was 25 nM, while that for *P. intermedia* required a higher concentration at 7.5×10^2 nM (**Table 1(b)**). These findings suggest that gatifloxacin exhibits selective inhibitory action against the growth of periodontopathic bacteria. To further investigate its potential to inhibit the growth of periodontopathic bacteria, the bactericidal activity of gatifloxacin was assessed. As shown in **Figure 1**, gatifloxacin showed bactericidal activity in a time-dependent manner against all bacteria tested. A highly significant difference was observed in the inhibitory action between cultivation time

Table 1. Effect of gatifloxacin on growth of periodontopathic bacteria and minimum inhibitory concentration (MIC) of the reagent. (a) *A. actinomycetemcomitans* 310a and *P. gingivalis* ATCC33277; (b) *P. intermedia* ATCC 25611. Each mathematical symbol means state of bacterial growth. ++: well grown, +: little growth, and -: no bacterial suspension was seen.

(a)							
Bacterial strain	0 μ M	0.0025 μ M	0.0075 μ M	0.0125 μ M	0.0175 μ M	0.025 μ M	MIC
<i>A. a.</i> 310a	++	-	-	-	-	-	2.5 nM
<i>P. g.</i> ATCC33277	++	++	++	+	+	-	25 nM

(b)							
Bacterial strain	0 μ M	0.01 μ M	0.025 μ M	0.05 μ M	0.75 μ M	0.1 μ M	MIC
<i>P. i.</i> ATCC25611	++	++	+	+	-	-	0.75 μ M

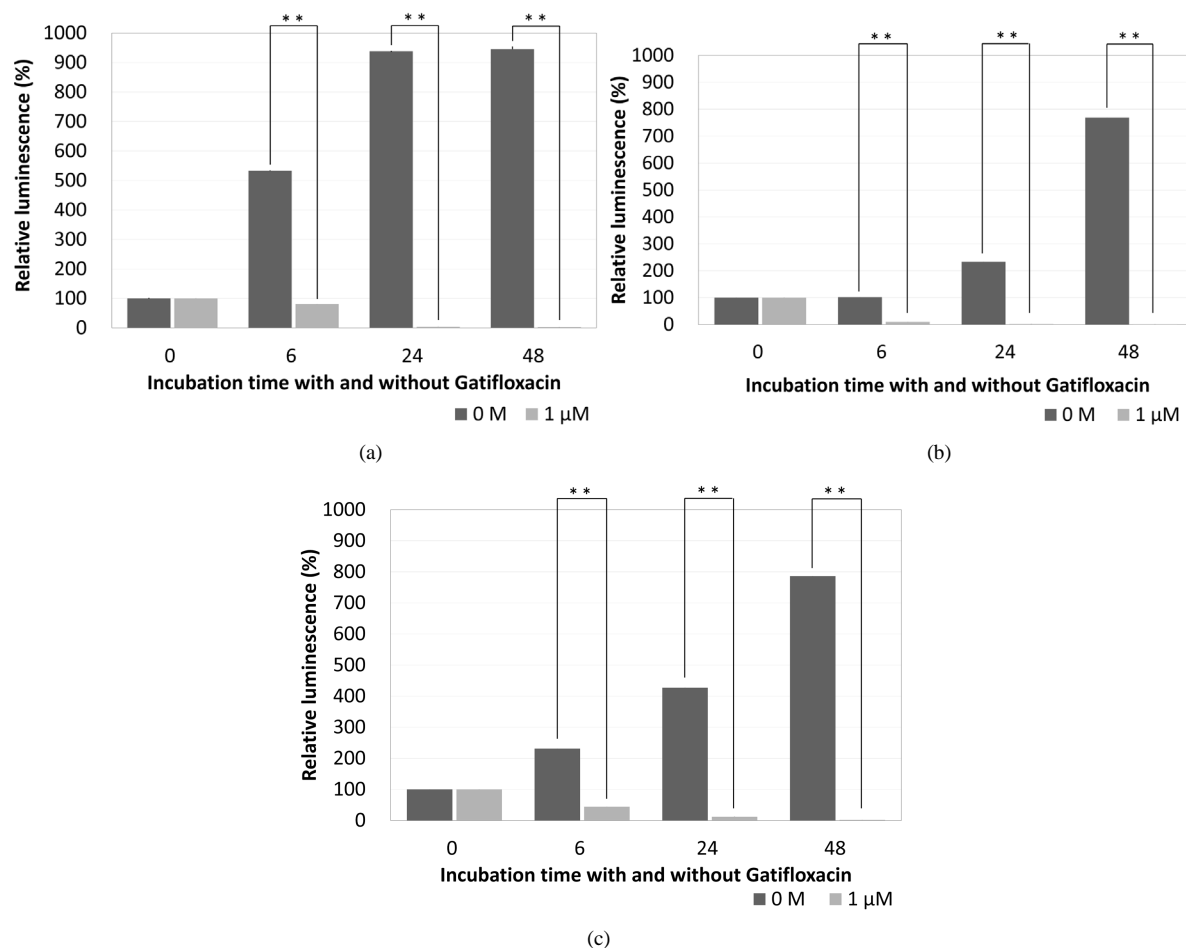


Figure 1. Influence of gatifloxacin on cell viability of periodontopathogens. (a) *A. actinomycetemcomitans* 310a; (b) *P. gingivalis* ATCC 33277; (c) *P. intermedia* ATCC 25611. Left bars show ratio of cell numbers after time of incubation (0, 6, 24, 48 h) to numbers of initial cells at 0 M of gatifloxacin. Right bars show ratio of cell numbers after time of incubation to numbers of initial cells at 1.0×10^3 nM of the drug. Standard deviation is expressed by bar. Asterisks (**) indicate a value of $p < 0.01$, which was considered to be statistically significant.

and drug concentration using the unpaired t-test against all bacteria tested. The number of viable bacterial cells decreased significantly as the bacteria was exposed to gatifloxacin ($p < 0.01$) during the time tested. At 1.0×10^3 nM, higher than the MIC, gatifloxacin reduced the number of *A. actinomycetemcomitans* cells after 48 hours to less than 3% of that at the start of cultivation. (Figure 1(a)). It also inhibited the growth of an even larger number

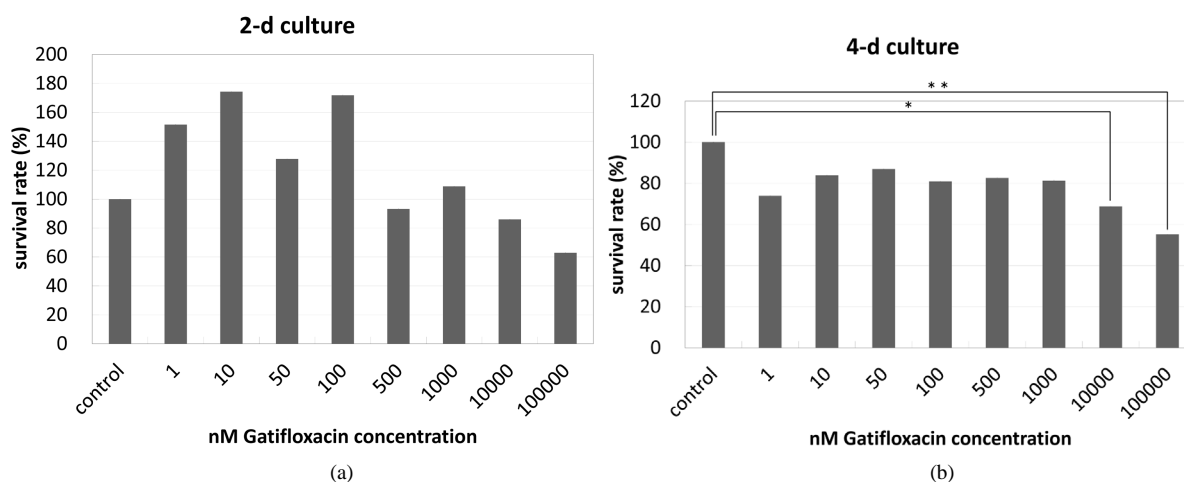


Figure 2. Survival rate of NHDF cells after 2-d (a) and 4-d (b) incubation to control (0 M), which was defined as 100%, shown at each concentration of gatifloxacin. Standard deviation is expressed by bar. Asterisks indicate p-values; **: $p < 0.01$, and *: $p < 0.05$, which were considered to be statistically significant. (a) Growth of NHDF cells after 2-d culture. (b) Growth of NHDF cells after 4-d culture.

of *P. intermedia* (to less than 2%) and *P. gingivalis* cells (to less than 1%) (Figure 1(b) and Figure 1(c)). These data support the possibility that exposure to gatifloxacin suppresses bacterial growth over time, indicating that the drug may exert an inhibitory effect on the growth of periodontopathic bacteria.

3.2. Cell Viability Assay in Mammalian Cells

As shown in Figure 2(a), gatifloxacin had no effect on cell proliferation of NHDF cells in concentrations of 1.0×10^3 nM or less after 2-d culture. Almost all seeded NHDF cells survived after 2-d culture (Figure 2(a)), and even 81% of the cells survived after 4-d culture (Figure 2(b)), at 1.0×10^3 nM, which concentration was higher than the MIC of bacteria tested. A highly significant difference was observed in the survival rate between 1.0×10^4 nM and control ($p < 0.05$), and between 1.0×10^5 nM and control ($p < 0.01$).

The results of antibacterial activity and cell viability assays suggested that gatifloxacin had bactericidal effects on *A. actinomycetemcomitans* and *P. gingivalis* at low concentrations, while it did not damage mammalian cells. The drug also exhibited anti-bacterial effects on *P. intermedia* at 7.5×10^2 nM, while approximately 100% of mammalian cells survived after 2-d incubation, as well as 82% after 4-d incubation. This suggested that this drug would be harmless at an MIC of 7.5×10^2 nM, also without exception in the case of *P. intermedia* in mammalia, especially under conditions of short-term local administration. In this study, we found that gatifloxacin had an inhibitory effect on periodontal pathogens. Further investigation was necessary, however, to elucidate the properties of this local administration drug, using the sustained release complexes established at our laboratory [20]. Moreover, the immobilization methods employed on surfaces of dental materials must be examined with reference to this drug. Of interest, also, are the functional mechanisms involved in its inhibition of the growth of periodontopathic bacteria. One of our goals is to develop a defense system against peri-implantitis. We believe that the application of gatifloxacin onto dental implants will offer advantages in the prevention of periodontal diseases such as peri-implantitis, thereby improving oral care.

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