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Original Article

Bactericidal activity and post-antibiotic effect of ozenoxacin against *Propionibacterium acnes*

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ABSTRACT

Ozenoxacin, a novel non-fluorinated topical quinolone, is used for the treatment of acne vulgaris in Japan. We investigated bactericidal activity and post-antibiotic effect (PAE) of ozenoxacin against *Propionibacterium acnes*, a major causative bacterium of acne vulgaris. The minimum inhibitory concentrations (MICs) of ozenoxacin against 3 levofloxacin-susceptible strains (MIC of levofloxacin; ≤4 μg/mL) and 3 levofloxacin-resistant strains (MIC of levofloxacin; ≥8 μg/mL) ranged from 0.03 to 0.06 μg/mL and from 0.25 to 0.5 μg/mL, respectively. These MICs of ozenoxacin were almost the same or lower than nadifloxacin and clindamycin. The minimum bactericidal concentrations (MBCs) of ozenoxacin against the levofloxacin-susceptible and -resistant strains were from 0.06 to 8 μg/mL and from 0.5 to 4 μg/mL, respectively. These MBCs were lower than those of nadifloxacin and clindamycin. In time-kill assay, ozenoxacin at 1/4, 1 and 4 times the respective MIC against both levofloxacin-susceptible and -resistant strains showed a concentration-dependent bactericidal activity. Ozenoxacin at 4 times the MICs against the levofloxacin-susceptible strains showed more potent and more rapid onset of bactericidal activity compared to nadifloxacin and clindamycin at 4 times the respective MICs. The PAEs of ozenoxacin at 4 times the MICs against the levofloxacin-susceptible strains were from 3.3 to 17.1 h, which were almost the same or longer than nadifloxacin and clindamycin. In contrast, the PAEs were hardly induced by any antimicrobial agents against the levofloxacin-resistant strains.

The present findings suggest that ozenoxacin has a potent bactericidal activity against both levofloxacin-susceptible and -resistant *P. acnes*, and a long-lasting PAE against levofloxacin-susceptible *P. acnes*.

1. Introduction

Acne vulgaris is one of the most common inflammatory skin diseases, characterized by seborrhea, non-inflammatory comedones, inflammatory papules, pustules or nodules in the pilosebaceous units, affecting adolescents and often persisting or occurring in adulthood [1–4]. The pathogenesis of acne is multifactorial and includes an increase in sebum production by androgen-mediated hyperactive sebaceous gland, follicular hyperkeratinization, hypercolonization of *Propionibacterium acnes*, a common anaerobic Gram-positive commensal of normal skin, and release of inflammatory mediators [5,6]. Multiple acne therapies such as topical retinoids, antimicrobials and benzoyl peroxide, oral antimicrobials, and oral isotretinoin are available worldwide, depending on the severity, type and area of acne lesions [2–4]. However, it has been concerned an expansion of antimicrobial-resistant *P. acnes* [7–11].

Ozenoxacin, a novel non-fluorinated quinolone, has demonstrated a broad antimicrobial spectrum against both Gram-positive and -negative organisms including major pathogenic bacteria of acne vulgaris and superficial skin infections such as *P. acnes, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pyogenes* [12–17]. Recently, 2% ozenoxacin-containing lotion

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(Zebiax® Lotion 2%, Maruho Co., Ltd., Osaka, Japan) has demonstrated a good therapeutic effect in patients with acne vulgaris and superficial skin infections in Japan [7,18]. Moreover, in the Japanese Guideline for the Treatment of Acne Vulgaris 2016, ozenoxacin-, nadifloxacin- and clindamycin-containing topical pharmaceuticals are strongly recommended for the treatment of inflammatory papules [19].

Most quinolones have been reported to show the bactericidal activity and post-antibiotic effect (PAE) against both Gram-positive and -negative bacteria [20–24]. Nevertheless, there are insufficient data about the bactericidal activity and PAE of quinolones including ozenoxacin against *P. acnes*.

In the present study, therefore, we investigated the *in vitro* antimicrobial activity especially bactericidal kinetics and PAE of ozenoxacin against *P. acnes* isolated from patients with acne vulgaris and compared to the existing antimicrobial agents for the topical treatment of acne vulgaris.

2. Materials and methods

2.1. Organisms

Bacterial strains used in the present study were *P. acnes* ATCC11827 and 5 clinical isolates (*P. acnes* No. 1, 2, 9, 14 and 19), which were selected by susceptibility to antimicrobial agents among 20 clinical isolates recovered from Japanese patients with acne vulgaris during a period from 2012 to 2013. All clinical isolates were used according to the Ethical Guidelines for Epidemiological Research (issued on 1st December 2007, partial revision), and patient's information was unlikable anonymized.

2.2. Antimicrobial agents

Antimicrobial agents used were as follows: ozenoxacin (Toyama Chemical Co., Ltd., Tokyo, Japan), nadifloxacin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), levofloxacin (LKT laboratories, Inc., St. Paul, MN, U.S.A.) and clindamycin (Sigma–Aldrich Co., LLC., St. Louis, MO, U.S.A.).

2.3. Determination of minimum inhibitory concentration (MIC)

MIC was determined by broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) standard methods [25,26]. Prior to MIC assay, *P. acnes* strains were pre-cultured on anaero columbia agar with rabbit blood (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) at 35 °C for 3–4 days under anaerobic condition using AnaeroPack® system (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). Brucella broth (Nippon Becton Dickinson Company, Ltd.) supplemented with 5% lysed horse blood (Nippon bio-test laboratories INC., Tokyo, Japan), 5 μg/mL hemin (Sigma–Aldrich Co., LLC.) and 1 μg/mL vitamin K1 (Sigma–Aldrich Co., LLC.) (5% LHB brucella broth) was used as a culture medium for the MIC measurement. Ozenoxacin, nadifloxacin and levofloxacin were dissolved in 0.1 N sodium hydrate (Nacalai tesque, INC., Kyoto, Japan) and diluted by sterile water. Clindamycin was dissolved in sterile water. Two-fold dilution of antibiotics and a final bacterial concentration of 1.5×10^6 CFU/mL were placed in each well and was cultured at 35 °C under anaerobic condition. MIC was determined after 46–48 h incubation. Based on the MIC of levofloxacin, the strains were defined as being levofloxacin-susceptible (MIC: ≤4 μg/mL) and -resistant (MIC: ≥8 μg/mL) [26]. The quality control ranges of ozenoxacin, nadifloxacin, levofloxacin and clindamycin against *Bacteroides fragilis* ATC25285 were defined by three repeated MIC measurements as follows: from 0.03 to 0.25 μg/mL, from 0.5 to 4 μg/mL, from 0.5 to 2 μg/mL, and from 0.5 to 2 μg/mL, respectively. The MICs of the antimicrobial agents against quality control strain were confirmed to meet the defined ranges in all experiments.

2.4. Determination of minimum bactericidal concentration (MBC)

The MBCs were determined after the MIC assay according to Clinical Microbiology Procedures Handbook 3rd edition [27]. Briefly, 10 μL of *P. acnes* suspension was removed from all wells of 96-well microplates showing no visible bacterial growth, serially diluted and then plated onto anaero columbia agars with rabbit blood. After anaerobic incubation at 35 °C for 3–4 days, the numbers of colonies on the agar plates were counted. The MBCs were defined as the lowest concentration showing ≥99.9% killing of the initial inoculum of the MIC assay.

2.5. Time-kill assay

Prior to the time-kill assay, *P. acnes* strains were pre-cultured anaerobically with Gifu Anaerobic Medium broth (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) at 35 °C for 3 h. The bacterial suspension (approximately 1.5×10^6 CFU/mL, as final concentration) and 5% LHB brucella broth containing ozenoxacin at 1/4, 1, 4, 16 or 64 times the MIC were added into wells of 96-well microplates, and incubated anaerobically at 35 °C for 1, 2, 4, 6 and 24 h. In another set of experiment, the bacterial suspension of *P. acnes* (approximately 1.5×10^6 CFU/mL, as final concentration) and 5% LHB brucella broth containing ozenoxacin, nadifloxacin, levofloxacin or clindamycin at 4 times the respective MICs were added into wells of 96-well microplates, and incubated anaerobically at 35 °C for 3, 6, 24 and 48 h. After the respective incubations, 100 μL of the bacterial suspension was removed from each well, serially diluted by saline and then plated onto anaero columbia agars with rabbit blood. After anaerobic incubation at 35 °C for 3–4 days, the numbers of colonies on the agar plates were counted.

2.6. Determination of PAE

The PAE was determined by the method of Craig et al. [21]. *P. acnes* strains were pre-cultured anaerobically with Gifu Anaerobic Medium broth at 35 °C for 3 h. The bacterial suspension of *P. acnes* (approximately 1.5×10^6 CFU/mL, as final concentration) and 5% LHB brucella broth containing ozenoxacin, nadifloxacin, levofloxacin or clindamycin at 4 times the respective MIC were added into wells of 96-well microplates, and incubated anaerobically for 2 h. Thereafter, the bacterial suspension was centrifuged (10,000 rpm for 1 min at room temperature) to wash with saline followed by the culture medium. The precipitate of bacteria was re-suspended in the culture medium, and further cultured anaerobically at 35 °C for 1, 2, 3, 6, 9, 12, 15, 18, 21 and 24 h. After the respective incubations, 100 μL of the bacterial suspension was removed from each well, serially diluted in saline and then plated onto anaero columbia agar with rabbit blood. After anaerobic incubation at 35 °C for 3–4 days, the numbers of colonies on the agar plates were counted. The PAE was determined using the equation: $PAE = T - C$, at where T is the time required for the test strain count to increase to 10-fold ($1 \times \log_{10}$) above the count observed immediately after removal of the antimicrobial agent and C is the time required for the control strain count to increase to 10-fold. Both T and C were calculated using the viable counts between 0 and 15 h because the viable counts of all control strains were increased to 10-fold by 15 h after removal of the control medium.