Besifloxacin, a novel fluoroquinolone antimicrobial agent, exhibits potent inhibition of pro-inflammatory cytokines in human THP-1 monocytes

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Received 25 June 2007; returned 10 August 2007; revised 20 August 2007; accepted 23 September 2007

Objectives: This study was conducted to evaluate the anti-inflammatory effects of besifloxacin, a novel fluoroquinolone under clinical evaluation for treatment of ophthalmic infections.

Methods: Cytokine expression in human THP-1 monocytes was stimulated by lipopolysaccharide (LPS), and Luminex technology was used to determine the effect of besifloxacin on LPS-induced cytokine expression. Moxifloxacin, a marketed fluoroquinolone used in ophthalmic infections, was used as the control.

Results: LPS induced measurable cytokine expression for 14 of the 16 cytokines assayed. Besifloxacin significantly inhibited LPS-stimulated cytokine production in a dose-dependent manner, with a comparable [granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1β, IL-8, interferon-inducible protein (IP-10), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α)] or better [granulocyte CSF (G-CSF), IL-1α, IL-1 receptor antagonist (IL-1ra) and IL-6] potency compared with moxifloxacin. A significant inhibitory effect of besifloxacin was observed at 0.1 mg/L for IL-1α, at 1 mg/L for G-CSF, IL-1ra and IL-6 and at 30 mg/L for GM-CSF, IL-12p40, IL-1β, IL-8, IP-10, MCP-1 and MIP-1α.

Conclusions: Besifloxacin acts as an anti-inflammatory agent in monocytes in vitro; this attribute may enhance its efficacy in ocular infections with an inflammatory component and warrants further investigation.

Keywords: antibiotics, lipopolysaccharide, moxifloxacin, ophthalmic infections, Luminex

Introduction

Fluoroquinolones are synthetic, broad-spectrum antimicrobial agents commonly used to treat a variety of infections.1 Later-generation fluoroquinolones such as moxifloxacin and gatifloxacin appear to possess improved safety and efficacy profiles compared with previous agents, particularly for topical administration in conditions such as bacterial conjunctivitis.2 In addition to their antimicrobial properties, recent studies have suggested that certain fluoroquinolones, including ciprofloxacin,3 alatrofloxacin,4 grepafloxacin,5 gemifloxacin6 and moxifloxacin7 may modulate cytokine synthesis and release from immune and inflammatory cells in some experimental systems. Although the clinical significance of these data is currently unclear, such observations have led to speculation that these agents may not only afford antimicrobial activity but also may be beneficial in suppressing infection-associated tissue inflammation and destruction.8,9

Besifloxacin \( \{7\-\[(3R)\-3\-aminohexahydro-1H-azepin-1-yl\]-8\-chloro-1-cyclopropyl-6\-fluoro-1,4\-dihydro-4\-oxo-3\-quinolinecarboxylic acid\} \) (Figure 1) is a novel fluoroquinolone being developed by Bausch & Lomb for the topical treatment of ophthalmic infections. Besifloxacin is anticipated to have a number of advantages over available therapies based on its structure, unique usage profile and antimicrobial properties.10 However, to date, there are no published studies investigating the anti-inflammatory properties of this novel molecule. Therefore, the objective of the present investigation was to characterize the effect of besifloxacin on cytokine expression in human THP-1 monocytes in vitro. To help place these data into context with previous publications, moxifloxacin was also employed in this investigation for comparison purposes.

Materials and methods

Reagents

RPMI 1640 medium, fetal bovine serum (FBS) and penicillin-streptomycin solution were obtained from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) and dialysed FBS were obtained...
from Hyclone (Logan, UT, USA). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was purchased from Sigma (St Louis, MO, USA). Alamar Blue solution was obtained from Biosource (Camarillo, CA, USA). Moxifloxacin hydrochloride and besifloxacin hydrochloride (BOL-303224-A; >95% purity) were prepared by or for Bausch & Lomb (Rochester, NY, USA). A human 16-cytokine multiplex Luminex kit was obtained from Linco Research (St Charles, MO, USA). All reagents were of the highest available purity. All doses and concentrations described below are listed as free base equivalents. For moxifloxacin and besifloxacin, stock solutions were prepared at a concentration of 30 mg/mL in DMSO and were stored frozen until use.

**Cells and cell treatments**

Human THP-1 monocytes (ATCC TIB 202) were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified incubator with 5% CO₂. THP-1 cells were pre-cultured in RPMI 1640 medium containing 10% dialysed FBS for 24 h. Cells were seeded in 24-well plates in RPMI 1640 medium containing 2% dialysed FBS and treated with vehicle (0.1% DMSO), 0.1, 1, 10 or 30 mg/L moxifloxacin, 0.1, 1, 10 or 30 mg/L besifloxacin, 10 µg/mL LPS + 0.1, 1, 10 or 30 mg/L moxifloxacin, or 10 µg/mL LPS + 0.1, 1, 10 or 30 mg/L besifloxacin for 18 h. Each treatment was performed in triplicate and appropriate dilutions were prepared to deliver a constant amount of the vehicle to each well.

**Multiplex Luminex**

Samples were analysed using multiplex bead technology, which utilizes microspheres as the solid support for immunoassays and allows the analysis of all cytokines from each sample.16 Sixteen cytokines were measured according to the manufacturer’s instructions. Briefly, 50 µL aliquots of medium samples were incubated with antibody-coated capture beads overnight at 4°C. Washed beads were further incubated with biotin-labelled anti-human cytokine antibodies for 2 h at room temperature followed by incubation with streptavidin–phycoerythrin for 30 min.

**Data analysis and statistics**

All cytokine concentrations (pg/mL) were expressed as means ± SD. Statistical analysis comparing the effects of treatment across groups was performed using a one-way ANOVA with a Dunnett’s post hoc comparison test using either vehicle control or LPS treatment as references. For all assays, *P* ≤ 0.05 was predetermined as the criterion of statistical significance.

**Results**

Human THP-1 monocytes were treated with LPS, moxifloxacin, besifloxacin, LPS plus moxifloxacin or LPS plus besifloxacin. In no instance did any of the treatments produce a statistically significant effect on cellular metabolic activity as measured by the Alamar Blue assay (data not shown). The overall results from the studies determining cytokine levels in the culture medium from these various treatment groups are summarized in Table 1. Substantial levels of 14 out of the 16 cytokines were inhibited by either moxifloxacin or besifloxacin.

**Table 1. Summary of inhibition of LPS-stimulated cytokine production by moxifloxacin and besifloxacin in human THP-1 monocytes**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Inhibited by moxifloxacin (mg/L)</th>
<th>Inhibited by besifloxacin (mg/L)</th>
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<tr>
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<td>0.1 1 10 30</td>
<td>0.1 1 10 30</td>
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<tr>
<td>Fractalkine</td>
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<td>G-CSF</td>
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<td>VEGF</td>
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‘X’ signifies significant inhibition at a particular concentration.

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**Zhang and Ward**

**Figure 1. Two-dimensional chemical structure of besifloxacin (7-[(3R)-3-aminohexahydro-1H-azein-1-yl]-8-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid).**

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range of the standard curve, an appropriate dilution was performed to ensure that the concentration was in the linear portion of the curve.

**Cellular metabolic function**

Cellular metabolic competence was determined by an Alamar Blue assay.12 Briefly, after removal of medium, cells were incubated with 1:10 diluted Alamar Blue solution for 3 h at 37°C in a humidified incubator with 5% CO₂. The plate was read fluorometrically by excitation at 530–560 nm and emission at 590 nm. Relative fluorescence units were used to determine the cell viability.

**Summary of inhibition of LPS-stimulated cytokine production by moxifloxacin and besifloxacin in human THP-1 monocytes**

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‘X’ signifies significant inhibition at a particular concentration.
Inhibition of pro-inflammatory cytokines by besifloxacin

cytokines in the assay were detectable in culture media from THP-1 monocytes, with all cytokines except epidermal growth factor (EGF) and interleukin (IL)-7 affected. Exposure of THP-1 monocytes to 10 μg/mL LPS for 18 h resulted in a significant increase in 13 out of the 14 detectable cytokines; the amount of vascular endothelial growth factor (VEGF) in THP-1 monocyte culture medium also increased, but the increase did not attain statistical significance.

Both moxifloxacin and besifloxacin significantly inhibited LPS-induced cytokine production in THP-1 monocytes. For moxifloxacin, a significant inhibitory effect was observed at 1 mg/L for IL-12p40, at 10 mg/L for IL-1 receptor antagonist (IL-1ra) and IL-6 and at 30 mg/L for granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1α, IL-1β, IL-8, interferon-inducible protein (IP-10) and macrophage inflammatory protein-1α (MIP-1α) (Table 1). For besifloxacin, a significant inhibitory effect was observed at 0.1 mg/L for IL-1α, at 1 mg/L for G-CSF, IL-1ra and IL-6 and at 30 mg/L for GM-CSF, IL-12p40, IL-1β, IL-8, IP-10, monocyte chemotactic protein-1 (MCP-1) and MIP-1α. Table 1). Neither moxifloxacin nor besifloxacin altered LPS-stimulated production of RANTES (‘regulated upon activation, normal T cell expressed, and secreted’) or fractalkine.

The cytokines detected in this study were divisible into three different response groups. The first group includes those cytokines for which these fluoroquinolones had no significant efficacy (RANTES and fractalkine; data not shown). The second group of cytokines includes GM-CSF, IL-1β, IL-8, IP-10, MCP-1 and MIP-1α. For these cytokines, both moxifloxacin and besifloxacin had comparable effects after LPS stimulation (Figure 2). The third group of cytokines includes those for which besifloxacin demonstrated either better (G-CSF, IL-1α, IL-1ra, IL-6 and VEGF) or less (IL-12p40) potency than moxifloxacin (Figure 3).

Discussion

As with other infectious conditions, ophthalmic infections are commonly associated with or followed by ocular inflammation, manifest by light sensitivity, blurred vision, pain, redness of the eye and floaters. Consequently, any anti-inflammatory activity embodied in ophthalmic anti-infective agents would likely be beneficial. Previously, it has been reported that other fluoroquinolones such as moxifloxacin significantly inhibit cytokine production in human THP-1 monocytes and peripheral blood mononuclear cells stimulated in vitro by LPS. In general, the previous work has been performed with a fairly limited panel of cytokines, including IL-1β, IL-6, IL-8 and TNF-α. In these studies, the inhibitory effect was seen at drug concentrations of 10 μg/mL or below, a concentration that may well be achievable in monocytes under clinical circumstances. However, not all fluoroquinolones are associated with such anti-inflammatory activity. For instance, in an in vivo veterinary medicine study, marbofloxacin was demonstrated not to have significant anti-inflammatory activity. Furthermore, trovafloxacin has actually been associated with increased pro-inflammatory cytokine expression in an LPS-challenge rat study. Therefore, the anti-inflammatory properties of fluoroquinolones cannot be assumed to be equivalent across the class.

The present data further support the observation of variation in anti-inflammatory properties based on fluoroquinolone structure. With besifloxacin, significant cytokine inhibitory effects were observed at very low concentrations. For example, a significant inhibitory effect of besifloxacin was seen as low as 0.1 mg/L on IL-1α and at 1 mg/L on G-CSF, IL-1ra and IL-6. These data may be compared with MIC values of besifloxacin, which are in the order of 0.006–0.2 mg/L for ophthalmic clinical isolates of various organisms. Although clinical pharmacokinetic data are not yet available for besifloxacin, these concentrations are well below the predicted ocular concentrations following topical administration. Therefore, it is reasonable to assume that there may be clinical benefit resulting from this cytokine inhibition profile, although this must be further investigated clinically.

Whereas the mechanisms of antibacterial activity of fluoroquinolones in prokaryotic and eukaryotic cells have been extensively investigated in vitro and in vivo, the mechanisms underlying their anti-inflammatory effect have not been fully elucidated. The results from the current study indicate that the production of a variety of cytokines is altered by both moxifloxacin and besifloxacin in monocytes. Available data are lacking on the precise cascade of intracellular processes leading to stimulatory or inhibitory effects of fluoroquinolones on cytokines, chemokines and other components of the immune system, and it is unclear at present whether these effects are mediated by nuclear factor κB (NF-κB) or other transcription factors. However, it has been reported that moxifloxacin inhibits mitogen-activated protein kinase (MAP kinase) and NF-κB activation in monocytes and cystic fibrosis epithelial cells. Other possible mechanisms include the effects of quinolones on intracellular cyclic AMP, protein kinase A and phosphodiesterases and the role of quinolone–topoisomerase II interactions leading to a eukaryotic equivalent of the bacterial SOS response with its ensuing intracellular events. Until these molecular mechanisms are further elucidated, it is not possible to further speculate on the rationale behind the diverse structure–activity relationship for this phenomenon within the fluoroquinolone class, but this is a research area that bears further consideration.

Although this study only examined the anti-inflammatory effects of besifloxacin in THP-1 stimulated by LPS, other studies have looked at the anti-inflammatory effects of moxifloxacin and other quinolones on human peripheral blood monocytes, human respiratory epithelium, in vivo lung infections and bronchial CF cells. In addition, other stimuli were used in the former studies, including IFN/IL-1β, Aspergillus and Candida. Data from these studies and the current study support a broad-ranged anti-inflammatory effect of certain quinolones.

In summary, the present study demonstrates that besifloxacin, a novel ophthalmic fluoroquinolone, may have anti-inflammatory effect through its capacity to inhibit cytokine production by monocytes, with improved potency against several cytokines compared with moxifloxacin. Although the clinical significance of this observation is not currently understood, this attribute may enhance its efficacy in ocular infections with an inflammatory component and warrants further investigation. The present study also highlights the diversity of anti-inflammatory properties of structurally variant fluoroquinolones and suggests the need for further mechanistic research in this area.
Figure 2. Effect of moxifloxacin and besifloxacin on LPS-simulated GM-CSF, IL-1β, IL-8, IP-10, MCP-1 and MIP-1α production in THP-1 monocytes. Cells were treated with 10 μg/mL LPS and the indicated concentrations of moxifloxacin or besifloxacin for 18 h. Cytokine expression in THP-1 conditioned media was determined using Luminex technology. Data are means ± SD, n = 3. *P ≤ 0.05 versus control; **P ≤ 0.05 versus LPS.
Figure 3. Effect of moxifloxacin and besifloxacin on LPS-stimulated G-CSF, IL-1α, IL-1α, IL-6, VEGF and IL-12p40 production in THP-1 monocytes. Cells were treated with 10 μg/mL LPS and the indicated concentrations of moxifloxacin or besifloxacin for 18 h. Cytokine expression in THP-1 conditioned media was determined using Luminex technology. Data are means ± SD, n = 3. *P ≤ 0.05 versus control; **P ≤ 0.05 versus LPS.
Acknowledgements

We thank Karl VanDerMeid, Yong-Qing Lin, Kristina Getman and Rezarta Ajazi for their technical assistance.

Funding

All funding for this work was provided by Bausch & Lomb.

Transparency declarations

Both J.-Z. Z. and K. W. W. are of Bausch & Lomb employees and own Bausch & Lomb stock.

References