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Levonadifloxacin, a Novel Benzoquinolizine Fluoroquinolone, Modulates Lipopolysaccharide-Induced Inflammatory Responses in Human Whole-Blood Assay and Murine Acute Lung Injury Model

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ABSTRACT Fluoroquinolones are reported to possess immunomodulatory activity; hence, a novel benzoquinolizine fluoroquinolone, levonadifloxacin, was evaluated in lipopolysaccharide-stimulated human whole-blood (HWB) and mouse acute lung injury (ALI) models. Levonadifloxacin significantly mitigated the inflammatory responses in an HWB assay through inhibition of proinflammatory cytokines and in the ALI model by lowering lung total white blood cell count, myeloperoxidase, and cytokine levels. The immunomodulatory effect of levonadifloxacin, along with promising antibacterial activity, is expected to provide clinical benefits in the treatment of infections.

KEYWORDS fluoroquinolone, lipopolysaccharide, acute lung injury, immunomodulatory, cytokine, myeloperoxidase

Levonadifloxacin is a novel benzoquinolizine fluoroquinolone active against respiratory Gram-positive and Gram-negative pathogens, including methicillin- and quinolone-resistant *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and *Moraxella catarrhalis* and atypical pathogens (1–7). Additionally, it exhibits clinically relevant activity against quinolone-susceptible Gram-negative species, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas*, and *Acinetobacter* (7). Enabling preclinical and clinical features of levonadifloxacin, such as potent bactericidal action, enhancement of activity in an acidic environment, intracellular activity, and excellent lung pharmacokinetics, demonstrates promising potential for treating respiratory infections such as community-acquired bacterial pneumonia (CABP) and hospital-acquired bacterial pneumonia (HABP) (4, 8). Because of growing antibiotic resistance, the rate of treatment failure in CABP and HABP is on the rise and persistently calls for new effective antibiotics (9–11). In hospitalized patients with pneumonia or sepsis, the overproduction of proinflammatory cytokines by host immune cells in response to toxins secreted by bacteria is responsible for acute lung injury (ALI). In the past, several antibacterial drugs, including fluoroquinolones, exhibited good clinical outcome in infected patients due to immunomodulatory activity (12–14). Nonclinical *in vitro* and *in vivo* studies have also revealed immunomodulatory activity of fluoroquinolones (15). Considering these findings, the anti-inflammatory potential of levonadifloxacin was evaluated using clinically relevant *ex vivo* (lipopolysaccharide [LPS]-stimulated human whole-blood [HWB] assay) and *in vivo* (LPS-induced ALI in mice) models (16, 17).

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For *ex vivo* study, heparinized HWB was collected from healthy adults after receipt of written informed consent. For *in vivo* study, healthy male Swiss albino mice kept under standard laboratory conditions were used. All of the experimental protocols were approved by the Institutional Review Board of Wockhardt Ltd., India. HWB was isolated from five individual healthy donors for the cytokine assessment. The freshly collected HWB from each donor was diluted 1:3 with sterile RPMI 1640 containing L-glutamine and 25 mM HEPES. Each diluted HWB sample was stimulated with an LPS concentration of 10 ng/ml for the cytokine (interleukin 6 [IL-6], tumor necrosis factor α [TNF-α], and IL-1β) assay and a concentration of 50 ng/ml for the granulocyte-macrophage colony-stimulating factor [GM-CSF] assay. The test drugs were subsequently added to the samples, which were incubated for 6 and 48 h to assess cytokine and GM-CSF levels, respectively. The collected samples were then analyzed using enzyme-linked immunosorbent assay (ELISA) kits (R & D systems, USA). Levonadifloxacin and levofloxacin concentrations for the study were selected from the initial dose-response study (data not shown). Based on these results, levonadifloxacin effective concentrations of 15 and 30 μg/ml, which correspond with the plasma therapeutic maximum concentration (C_{max}) range, were selected for subsequent comparative study with levofloxacin (8). Levofloxacin concentrations of 25 and 100 μg/ml were selected because their cytokine inhibitory effects were comparable with those of levonadifloxacin concentrations of 15 and 30 μg/ml, respectively.

In the ALI model, mice ($n = 6$ per time point) were subcutaneously administered vehicle (for saline and LPS control), levonadifloxacin 200 mg/kg, and dexamethasone 10 mg/kg and, after 1 h, were anaesthetized and treated intranasally with LPS solution (100 μg/mouse) or normal saline. After 12 and 24 h of LPS or saline administration, mice were euthanized; the trachea was cannulated with a catheter and instilled with normal ice cold saline (0.6 ml) to collect bronchoalveolar lavage (BAL) fluid. The BAL fluid samples were centrifuged, and the supernatant was transferred in separate tubes leaving behind the cell pellets. The supernatant was analyzed for cytokines using ELISA kits and myeloperoxidase (MPO) activity using the *o*-dianisidine method (18). The cell pellet was suspended in saline, stained with trypan blue dye, and counted with a hemocytometer using a microscope. For histopathological assessment, a separate set of animals were sacrificed after 24 h of LPS challenge, and then their lungs were perfused, excised, and stored in 10% formalin. The lungs were embedded in paraffin, sliced into 4-μm sections, and stained with hematoxylin and eosin. The pathological changes were observed via microscope and scored by assigning different grades, as described previously (19).

Statistical analyses were performed using the GraphPad prism (version 5). The paired *t* test was used for the *ex vivo* study and one-way analysis of variance followed by Dunnett's test for the *in vivo* study to compare drug treatment groups with the LPS control group.

In the HWB assay, LPS evoked a significant increase in cytokine levels at 6 h and GM-CSF at 48 h, indicating that this experimental setting is feasible for studying the early phase of endotoxemia. Levonadifloxacin inhibited TNF-α, IL-6, IL-1β, and GM-CSF production in a concentration-dependent manner. The absolute levels of IL-6, TNF-α, IL-1β, and GM-CSF after incubation of HWB with LPS or LPS plus levonadifloxacin 15 and 30 μg/ml and levofloxacin 25 and 100 μg/ml are depicted in Table 1. Levonadifloxacin 15 and 30 μg/ml exhibited significant inhibition of TNF-α, IL-6, and IL-1β that was comparable to that with levofloxacin concentrations of 25 and 100 μg/ml, respectively. The GM-CSF was significantly inhibited by levonadifloxacin 30 μg/ml, which was comparable to levofloxacin 100 μg/ml.

These findings were further confirmed in LPS-induced ALI in mice, where levonadifloxacin at a clinically relevant dose significantly countered the inflammatory responses. Intranasal treatment of LPS in mice produced progressive elevation of total white blood cell (WBC) count, cytokines, and MPO in the BAL fluid, with peak effect observed mostly at 24 h (Fig. 1). An increase in total WBC count in the BAL fluid of LPS-treated mice indicated inflammation because most of the cells were neutrophils. Neutrophil accu-

TABLE 1 Effect of levonadifloxacin and levofloxacin on proinflammatory cytokine release in LPS-stimulated HWB samples

Treatment	Release (pg/ml) of cytokines[a]:			
	IL-6[b]	TNF-α[b]	IL-1β[b]	GM-CSF[c]
Vehicle control, LPS + dextrose	11,170.5 ± 1,773.1	4,371.6 ± 743.0	4,414.8 ± 892.6	15.4 ± 4.0
Levofloxacin 25 μg/ml	9,093.7 ± 1,684.6**	3,393.7 ± 659.7***	1,964.5 ± 506.1**	11.7 ± 3.7
Levofloxacin 100 μg/ml	5,209.5 ± 1,236.7***	1,986.9 ± 456.0**	214.3 ± 90.9**	4.5 ± 1.3*
Vehicle control, LPS + 1% arginine	10,446.9 ± 1,657.8	4,402.6 ± 743.7	4,182.0 ± 815.9	14.4 ± 3.8
Levonadifloxacin 15 μg/ml	8,430.3 ± 1,746.7***	3,146.9 ± 686.2**	2,615.2 ± 567.3**	10.8 ± 2.5
Levonadifloxacin 30 μg/ml	5,714.7 ± 1,415.4***	2,183.8 ± 465.4**	1,438.9 ± 392.7**	9.2 ± 2.3*

[a]Values are means ± SEM; $n = 5$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus respective control calculated by paired t test.
[b]LPS 10 ng/ml was used to estimate cytokine level.
[c]LPS 50 ng/ml was used to estimate cytokine level.

mulation at the site of infection plays a vital role in the progression of ALIs because neutrophils are responsible for releasing free radicals, inflammatory mediators, and proteases (20). Levonadifloxacin significantly lowered the total WBC count compared with that in the LPS control group. To further confirm these findings, MPO, an indirect marker of neutrophil accumulation, was measured in BAL fluid. MPO is a peroxidase enzyme expressed in neutrophil granulocytes and is released on stimulation by proinflammatory factors and oxidative stress. Excessive release of MPO is reported to cause tissue damage (21, 22). In this study, levonadifloxacin significantly reduced MPO activity. It is widely recognized that excessive secretion of proinflammatory cytokines are implicated in the pathogenesis of ALI. TNF-α, IL-1β, and IL-6 are proinflammatory cytokines released during the early phase of ALI by macrophages and monocytes. They are responsible for triggering an inflammatory cascade and instigating neutrophil recruitment at the site of infection (23, 24). Hence, effective therapies in controlling the release of these cytokines may significantly impede the progression of lung damage. LPS-induced lung injury in mice is also associated with higher levels of cytokines. The levonadifloxacin groups in this study exhibited significant reductions in TNF-α, IL-1β, and IL-6 levels in BAL fluid compared with those in the LPS control group. Furthermore,

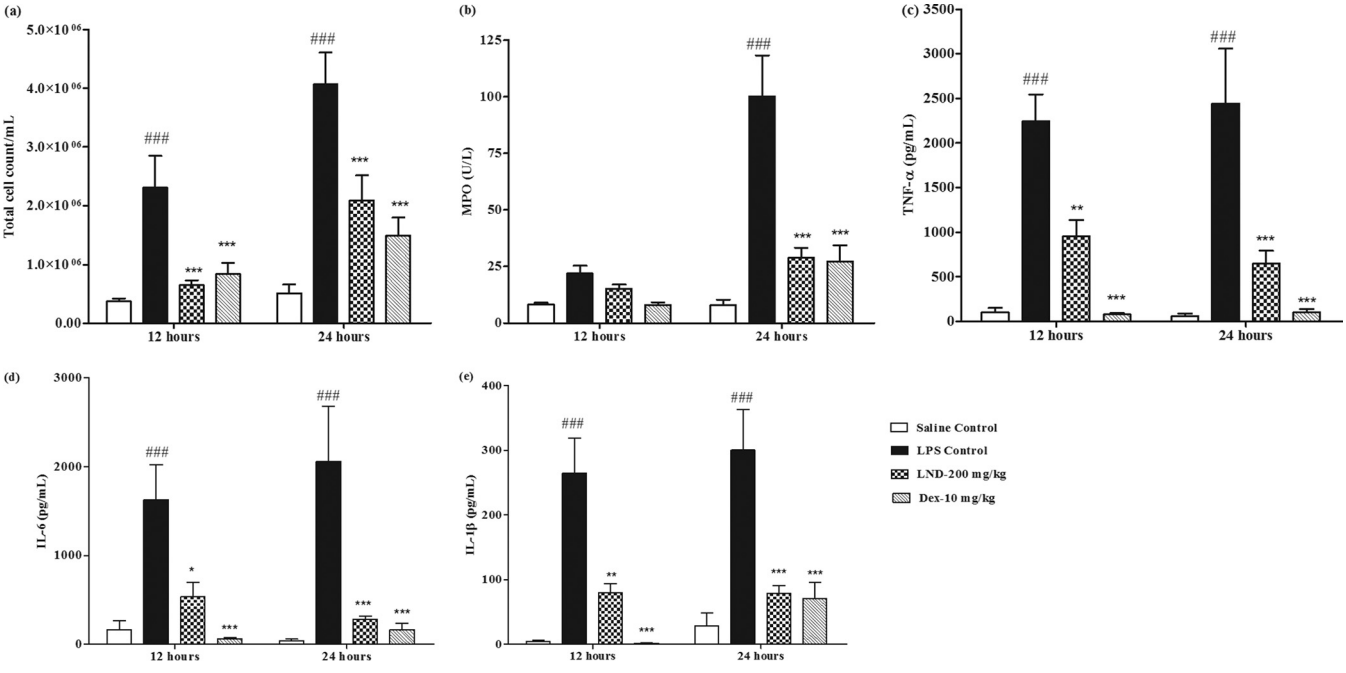

FIG 1 Effect of levonadifloxacin (LND) and dexamethasone (Dex) on total cell count (a) and MPO (b), TNF-α (c), IL-6 (d), and IL-1β (e) levels in mouse BAL fluid ($n = 6$). Statistical significance for LPS versus saline control, $P < 0.001$ (###); statistical significance for LPS versus levonadifloxacin and dexamethasone, $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

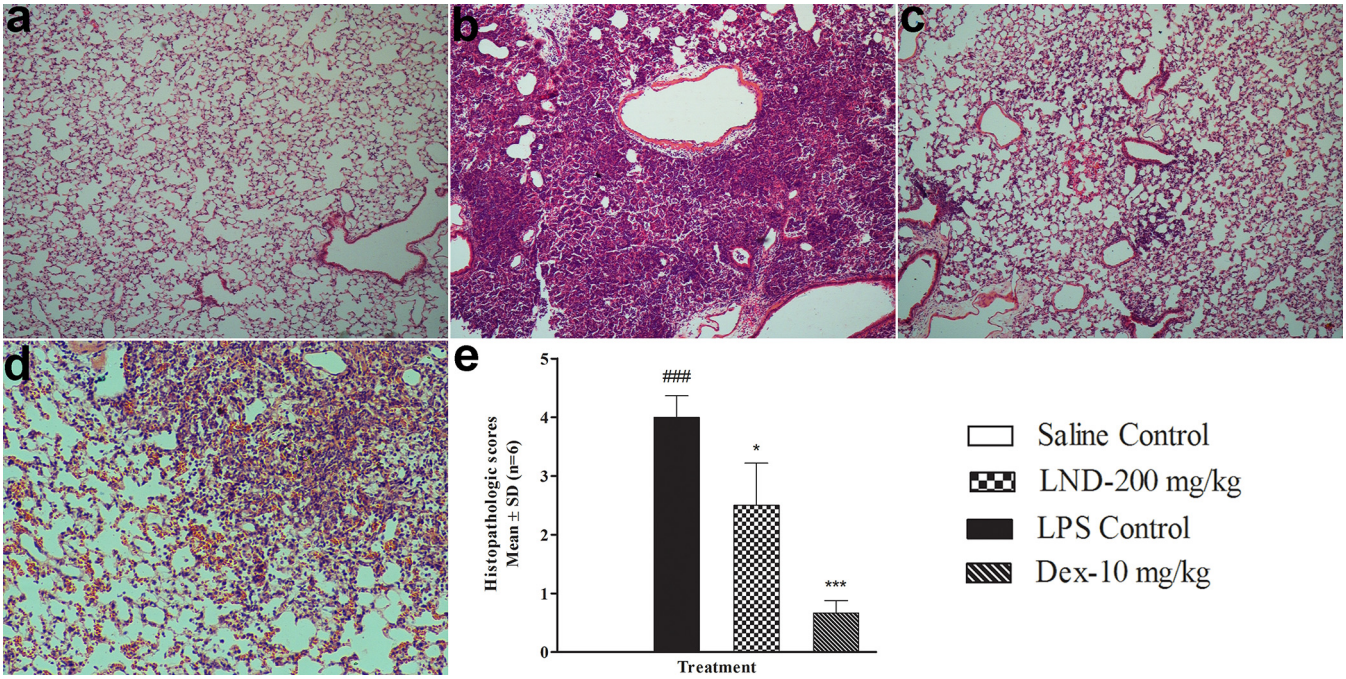

FIG 2 Effects of levonadifloxacin (LND) and dexamethasone (DEX) on the histological changes in lung tissue in mice with LPS-induced ALI. Illustrative histological changes in lungs observed in saline control (a), LPS control (b), dexamethasone 10 mg/kg + LPS (c), and levonadifloxacin 200 mg/kg + LPS (d). (e) The comparative lung histopathological scores are presented as means ± standard deviation ($n = 6$). Statistical significance for LPS versus saline control, $P < 0.001$ (###); statistical significance for LPS versus levonadifloxacin and dexamethasone, $P < 0.05$ (*) and $P < 0.001$ (***).

this immunomodulatory finding was corroborated using histopathological evidence that suggested minimal infiltration of neutrophils into the lung tissue by levonadifloxacin after an LPS challenge (Fig. 2). As expected, dexamethasone demonstrated significant anti-inflammatory activity in this model (Fig. 1 and 2).

Structurally, levonadifloxacin is different from other marketed fluoroquinolones. It is a benzoquinolizine, which is a novel subclass of the quinolone class of antibiotics with spectral coverage against Gram-positive bacteria, especially quinolone-resistant and methicillin-resistant *Staphylococcus aureus* (MRSA). Being a novel molecule with structural modifications, it may have enhanced potential to alter anti-inflammatory activity compared with levofloxacin. The production and release of a number of pro- and anti-inflammatory cytokines were reported in the systemic circulation of all patients with severe pneumococcal pneumonia. Treatment with levofloxacin exhibited clinical stability in a short time, in parallel with lower levels of cytokines, such as TNF-α, IL-6, and IL-1β (25). Based on these observations, our *ex vivo* findings with levonadifloxacin in HWB predict an enhanced inhibitory effect on proinflammatory cytokines in patients. Levonadifloxacin's immunomodulatory effect, along with its antibacterial activity, is expected to provide clinical benefits in the treatment of severe respiratory infections. These findings need to be confirmed in future clinical studies.

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We have no conflicts of interest to declare.

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