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Research Article

Effect of Linezolid on Human Phagocytic Cell Functions in Response to Gram-Negative Pathogens: *In vitro* and *Ex vivo* Studies

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- Phagocytosis
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Abstract

Linezolid has a wide spectrum of activity against gram-positive organisms, but no clinical activity against gram-negative organisms. In a phase 3 study comparing linezolid with vancomycin in patients with intravascular catheter-related infections, a mortality imbalance occurred in patients with gram-negative bacterial infections. The hypothesis that this imbalance might be related to immunosuppressive effects of linezolid (impeding the ability of phagocytic cells to respond to gram-negative infections) was tested using *in vitro* and *ex vivo* approaches. Human whole blood was incubated with linezolid in vitro. Phagocytosis of *Escherichia coli* (*E.coli*) particles, cytokine (interleukin-1 receptor antagonist [IL-1ra], IL-8) secretion and mRNA synthesis by neutrophils in response to lipopolysaccharide (LPS) stimulation, were measured. Subsequently, in an *ex vivo* study, linezolid or placebo was administered to healthy subjects before measurements of phagocytosis of *E. coli* particles by neutrophils and monocytes, and cytokine (IL-1ra, IL-1β, IL-6, IL-8, and tumor necrosis factor-α) secretion by total white blood cells in response to LPS stimulation. Linezolid and placebo group comparisons were based on individual baseline-corrected values. Linezolid had no effect on phagocytosis of *E.coli* particles by neutrophils or monocytes *in vitro* or *ex vivo*. Small to moderate decreases in IL-1ra and IL-8 secretions from neutrophils were observed at high linezolid concentrations (≥16.5 μg/mL) in vitro, but cytokine gene expression was not affected. Linezolid had no significant effect on secretion of 5 cytokines evaluated *ex vivo*. There is no strong evidence suggesting that linezolid inhibits phagocytic cell functions in response to gram-negative pathogensat clinically relevant concentrations.

ABBREVIATIONS

LPS: Lipopolysaccharide; MFI: Mean Fluorescence Intensity; ELISA: Enzyme-Linked Immunosorbent Assay; RT-PCR: Real Time-Polymerase Chain Reaction

INTRODUCTION

Linezolid is an oxazolidinone antibiotic with a wide spectrum of activity against gram-positive organisms (e.g., staphylococci, enterococci, bacteria resistant to methicillin and vancomycin), but has no clinical activity against gram-negative organisms [1]. In a phase 3 study comparing linezolid with vancomycin in patients with intravascular catheter-related infections, a mortality imbalance (linezolid group, 21.5%; vancomycin group, 16.0%) was observed at 12 weeks post treatment [2]. Further analysis revealed that much of this imbalance occurred in patients with Gram-negative pathogens or those who had negative culture results at baseline, rather than the primary analysis population with gram-positive pathogens, and less than one-half of patients in both groups received potentially effective treatment [2]. Additional analyses to assess mortality imbalance factors, such as synergy and antagonism analyses using organisms isolated from study patients, effect on cytokine production, and effect on neutrophil function, have shown no adverse effect with linezolid [2]. Overall, the mechanism of this imbalance was unclear based on the analyses of the data from this clinical study. Several studies

were conducted to test various hypotheses. One hypothesis was that linezolid may increase the virulence of gram-negative pathogens and studies using murine models of septicemia and pulmonary infection were conducted [3]. Results of these studies showed that linezolid did not increase the virulence of gram-negative pathogens [3].

Several antibiotics have previously been shown to have immunomodulatory effects on innate immune cells [4-8], which play a major role in the clearance of both Gram-positive and gram-negative bacteria. Innate immune cells include neutrophils, the first responders to sites of bacterial infection, as well as monocytes and macrophages. Important functions of innate immune cells, which lead to destruction of pathogens, include the phagocytosis of bacteria, production of reactive oxygen species (oxidative burst), chemotaxis, antigen presentation, and production of cytokines leading to recruitment and activation of other immune cells.

Investigators have studied the *in vitro* effects of linezolid on human phagocytic cell function in response to exposure to gram-positive organisms. Linezolid, at therapeutic or supratherapeutic concentrations (up to 160 μg/ml), had no effect on the phagocytosis of gram-positive organisms by purified human neutrophils [9], or by a mixture of neutrophils and monocytes [10].

Other *in vitro* studies have assessed phagocyte function following exposure to lipopolysaccharide (LPS), a major cell wall component of gram-negative bacteria. A study using purified human monocytes showed that linezolid decreased cytokine (interleukin-1 receptor antagonist [IL-1ra], IL-1β, IL-6, and tumor necrosis factor [TNF]-α) secretion in a concentration-dependent manner [11]. In another study with whole blood, linezolid treatment resulted in a statistically significant decrease in mRNA levels of IL-1β, IL-6, IL-8, and TNF-α in total white blood cell, and a significant reduction in cytokine secretion of IL-6 but not IL-8 or TNF-α [12].

Because of the previous findings of linezolid effects on *in vitro* cytokine secretion [11], it was hypothesized that the imbalance in the phase 3 study might have been related to the immunosuppressive effects of linezolid on innate immune cells in response to gram-negative pathogens. Therefore, we aimed to further test direct effects of linezolid on the function of phagocytes of the innate immune system in response to gram-negative pathogens, by measuring phagocytosis and cytokine secretion. An *in vitro* study was conducted to assess the effect of linezolid on phagocytosis of *Escherichia coli* (*E.coli*) particles (representative of gram-negative bacteria) by human neutrophils and monocytes in whole blood. We also measured cytokine (IL-1ra and IL-8) secretion and corresponding cytokine mRNA synthesis by purified human neutrophils in response to LPS stimulation. Subsequently, to more closely reflect the *in vivo* situation, we conducted a study in which linezolid or placebo was administered to healthy male subjects before *ex vivo* measurements of the phagocytosis of *E. coli* particles by neutrophils and monocytes, and cytokine (IL-1ra, IL-1β, IL-6, IL-8, and TNF-α) secretion in whole blood samples stimulated with LPS.

MATERIALS AND METHODS

In vitro studies

Study design: Heparinized whole blood samples for phagocytosis and cytokine assays were obtained from 5 healthy subjects. Three linezolid concentrations (8.25, 16.5, and 33 μg/mL) were selected based on the range observed in subjects receiving the recommended dosing regimen (600 mg twice daily) [1] and were evaluated in all assays. The phagocytosis assay was performed in duplicate for samples from each subject; the cytokine secretion assay was performed in triplicate for samples from each subject, and the cytokine gene expression assay was performed once for samples from each subject. The *in vitro* studies using samples from human participants were conducted in accordance with Pfizer and United States federal guidelines.

Phagocytosis assay: Phagocytosis was evaluated using the Bioparticles Phagocytosis Kit (Life Technologies). This kit uses *E. coli* particles labeled with a pH-sensitive fluorescent dye. Heparinized whole blood samples were incubated with or without linezolid at 37°C for 18 h. Aliquots of whole blood were incubated with *E. coli* particles for 15 min on ice (control) or at 37°C, and processed according to kit instructions. Samples were analyzed using flow cytometry. The proportions of neutrophils or monocytes that were fluorescent and mean fluorescence intensity (MFI) of the *E. coli* particles in each cell population were determined. Net phagocytosis was calculated by subtracting the MFI of samples incubated on ice from those incubated at 37°C. White blood cell viability was measured by trypan blue exclusion.

Neutrophil purification for cytokine secretion and gene expression assays: Neutrophils were purified from heparinized whole blood samples by dextran sedimentation followed by Ficoll purification and hypotonic lysis to remove the remaining erythrocytes [13]. Cell viability was assessed using trypan blue exclusion. Purity and activation state of the neutrophil preparations were measured using anti-CD66b and anti-CD62L as granulocyte and activation markers, respectively [14]. Cytokine secretion and gene expression assays were done with preparations that met the acceptance criteria of >95% live cells, ≥99.5% pure neutrophils, and ≥95% unactivated neutrophils [14,15].

Cytokine secretion in response to lipopolysaccharide stimulation: Purified neutrophils were plated at 5 x 10^5 cells per well (24-well plates) in Roswell Park Memorial Institute (RPMI) 1640 media with 10% low-endotoxin fetal bovine serum. Cells were pretreated with or without linezolid for 30 min. Saline (vehicle control) or LPS (026:B6; final concentration of 1 μg/mL) was added to cells and incubated at 37°C for a total of 24 h. Cell-free supernatants were stored frozen at –80°C. IL-1ra and IL-8 concentrations in supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) following manufacturer's instructions. Net cytokine secretion was calculated by subtracting the values of vehicle-treated samples from those of LPS-treated samples.

Gene expression in response to lipopolysaccharide stimulation: A minimum of 5 x 10^6 purified neutrophils was cultured per treatment condition. Treatment of the cells with linezolid and LPS was the same as for the cytokine secretion

assay, except that the total incubation time was 3 h. After incubation, cells were collected by centrifugation and frozen at –80°C for real-time polymerase chain reaction (RT-PCR) analysis.

Total RNA was extracted using Tri Reagent (Molecular Research Center) according to the manufacturer's instructions. After reverse transcription, quantitative RT-PCR was performed in triplicate. PCR primer and probe sequences for the amplification of secreted IL-1ra (sIL-1ra), intracellular IL-1ra (icIL-1ra), and IL-8 mRNA were previously published [15]. The fluorophore/quencher combination for the probes was FAM/MGB and the conditions for PCR were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The mRNA levels of the cytokines were normalized to β–actin (Life Technologies) mRNA levels using standard primers and probes.

Statistical analysis: Arithmetic mean and standard deviation (SD) were reported for all data. For cytokine secretion analysis, data were analyzed using a 2-way analysis of variance model with the GLM procedure (SAS v9.2; SAS Institute Inc.). The effect of linezolid on cytokine secretion in response to LPS was tested for a linear trend across the 4 concentrations (including the control). To determine which concentrations of linezolid significantly decreased cytokine secretion, post hoc comparisons between linezolid-treated and control groups were conducted using a Dunnetts' adjusted, 2-sided Student's t-test (SAS v9.2). P values <0.05 were considered statistically significant. For gene expression analysis, because of the variability and skewness of the cytokine mRNA response, the values were log-transformed for analysis. The effect of linezolid on gene expression in response to LPS was tested for trends across the 4 concentrations using a 2-way factorial model with the MIXED procedure in SAS v9.2.

Ex vivo studies

Study design: This was an open-label, randomized, placebo-controlled study in 18 healthy male subjects, conducted at a Pfizer Clinical Research Unit (CRU; New Haven, CT, USA). The *ex vivo* study was approved by an independent institutional review board (IntegReview, Austin, TX, USA). Written informed consent was obtained before the subjects entered the study.

Twelve subjects were randomized to receive linezolid (Zyvox®, Pfizer Inc) 600 mg administered orally twice daily for 4.5 days; the other 6 subjects received placebo. A placebo group was included to help determine whether any changes in phagocytic cell functions observed in this study were specific to linezolid administration. Three plasma samples were collected to confirm linezolid exposure: at 1 h post the morning dose (peak concentration) on day 4; at 1 h and 12 h post the morning dose (peak and trough concentrations) on day 5. Heparinized blood samples for phagocytic cell function tests were collected at 4 occasions: baseline samples were collected on days 0 and 1 (before linezolid or placebo administration), and post-baseline samples were collected at 1 h post the morning dose on days 4 and 5 (at steady state). Routine safety laboratory tests were performed throughout the study. The *ex vivo* phagocytic cell function assays and safety laboratory tests were performed at the clinical laboratory of the CRU.

Phagocytosis assay: Phagocytosis by neutrophils and monocytes in whole blood was measured as per the *in vitro* assay, with the exception that the *ex vivo* assay was performed immediately following blood sample collection.

Cytokine secretion by total white blood cells in response to lipopolysaccharide stimulation: LPS (final concentration of 100 ng/mL) or an equal volume of saline (vehicle control) was added to heparinized whole blood (containing neutrophils, monocytes, and lymphocytes) immediately following sample collection and incubated at 37°C for 24 h. Plasma was collected following centrifugation and frozen at –80°C until assay.

The concentration of IL-1ra was measured using the same assay kit as for the *in vitro* assay above, while IL-1β, IL-6, IL-8, and TNF-α concentrations were measured using a 4-plex electrochemiluminescence assay (MesoScale Discovery) according to manufacturer's instructions. Calculation of net cytokine secretion was the same as that for the *in vitro* assay.

Linezolid measurement: Plasma samples were analyzed for linezolid concentration using a validated liquid chromatography tandem mass spectrometricmethod at WuXi AppTec (Shanghai, China), which was similar to a previously published method [16]. The dynamic range of the assay for linezolid was 0.1 to 50 µg/mL. Pharmacokinetic parameters (i.e., peak and trough concentrations) were obtained directly from the observed data.

Statistical analysis: A sample size of 18 subjects (12 on linezolid, 6 on placebo) was selected for this *ex vivo* study. Based on the intra subject variability's (coefficient of variation) of IL-8, IL-1ra, and phagocytosis (0.39, 0.20, and 0.14, respectively) from our *in vitro* study, with this sample size, a minimum of 30% difference in changes for IL-8 (the most variable parameter) could be detected with a precision of 0.2587.

Arithmetic mean and SD were reported for all data. For comparisons between linezolid and placebo groups, individual values of net phagocytosis (neutrophils and monocytes) and net cytokine (IL-1ra, IL-1β, IL-6, IL-8, and TNF-α) secretion were natural log-transformed then averaged for baseline (mean of days 0 and 1) and post-baseline (mean of days 4 and 5). Individual average baseline values were subtracted from their corresponding average post-baseline values, which were noted as baseline-corrected values. These baseline-corrected values were analyzed using an analysis of covariance model with treatment as a fixed effect and baseline as a covariate via the MIXED procedure in SAS v9.2. The adjusted mean differences (linezolid – placebo) and corresponding 90% confidence intervals (CIs) were exponentiated to provide estimates of the ratio of adjusted geometric means (linezolid/placebo) and 90% CIs for the ratios. Lack of effect was established if the 90% CIs on the geometric mean ratios for the parameters were contained in the equivalence acceptance interval (80 to 125%).

RESULTS

In vitro phagocytosis

Overnight incubation of whole blood with vehicle or linezolid had no effect on white blood cell viability (≥97.8% viable). Linezolid had no effect on phagocytosis by either neutrophils or monocytes as measured by both the percentage of cells that had

phagocytosed *E. coli* particles and by the fluorescence intensity of the cells (Figure 1).

In vitro cytokine secretion by neutrophils in response to lipopolysaccharide stimulation

Linezolid significantly inhibited IL-8 response to LPS treatment when linear trend across all individuals was analyzed (P=0.001). At concentrations of 33 and 16.5 µg/mL, linezolid significantly decreased IL-8 secretion by 37% (P<0.001) and 29% (P=0.001), respectively. At linezolid 8.25 µg/mL, the decrease was not significant (15%; P=0.089) (Figure 2, A-E).

Similarly, there was a decreasing trend in IL-1ra secretion with increasing linezolid concentration (P=0.003), but the decrease was statistically significant only at linezolid 33 µg/mL (12% decrease; P=0.009) (Figure 2, F-J). The lower linezolidconcentrations (8.25 and 16.5 µg/mL) did not result in significant decreases in IL-1ra secretion.

In vitro cytokine gene expression by neutrophils in response to lipopolysaccharide stimulation

Unlike cytokine secretion, the relative quantities (RQ) of mRNA for IL-8, sIL-1ra, or icIL-1ra did not show any significant changes in the presence of linezolid at any concentration (Figure 3).

Ex vivo study – Subject demographics

The mean (range) age and weight of 18 healthy male subjects were 30.9 (22, 41) years and 82.2 (57.9, 104) kg, respectively. The majority (10/18) of subjects were black.

Linezolid concentrations in human subjects

The mean ± SD for linezolid peak concentration was 15.1 ± 3.0 µg/mL on day 4 and 14.2 ± 3.4 µg/mL on day 5, and that for linezolid trough concentration on day 5 was 6.6 ± 2.1 µg/mL. Similar peak concentrations on days 4 and 5 indicated steady state was reached by day 4.

Ex vivo phagocytosis

Net phagocytosis and percent phagocytosing neutrophils and monocytes are summarized in (Table 1). Intersubject variability in phagocytosis for both monocytes and neutrophils was low, <22% for all groups (data on file).

For both monocytes and neutrophils, the 90% CIs for adjusted geometric mean ratios between linezolid and placebo for baseline-corrected net phagocytosis were within the equivalence acceptance range (80 to 125%; Table 2). This indicates that linezolid had no effect on the phagocytosis of E. coli bioparticles by neutrophils or by monocytes.

In general, the average percent of phagocytosing monocytes was >80% except for the baseline samples in the placebo group (75%). The average percent of phagocytosing neutrophils was >95% for both groups. Linezolid also had no effect on the percent of phagocytosing monocytes or neutrophils as demonstrated by

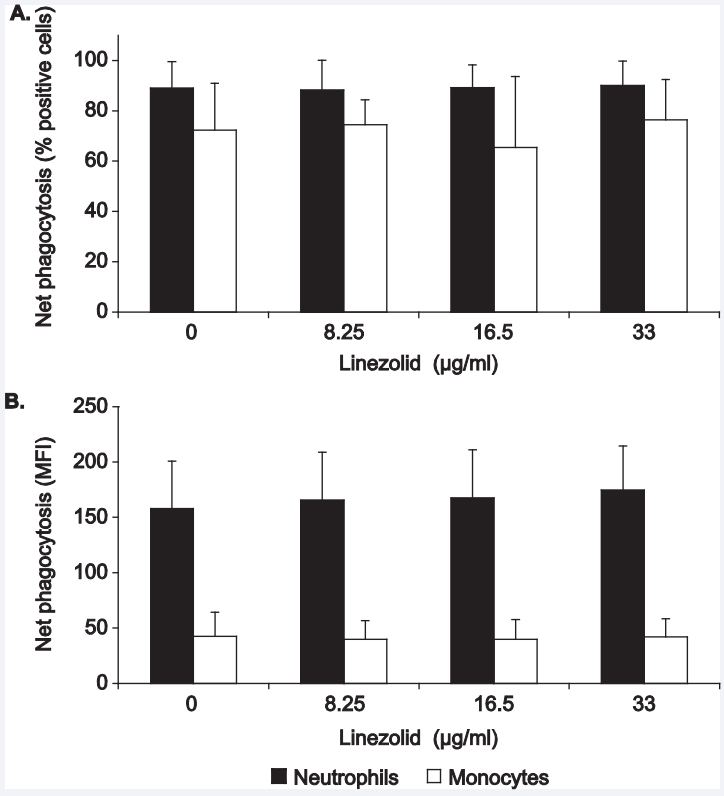

Figure 1 Effect of linezolid on *in vitro* net phagocytosis of *E. coli* particles by neutrophils or monocytes. (A) The percent of cells that have phagocytosed *E. coli* particles; (B) mean fluorescence intensity (MFI) of cells that have phagocytosed *E.coli* particles. Net phagocytosis = incubation at 37°C – incubation on ice. Data are presented as mean + SD (error bar).

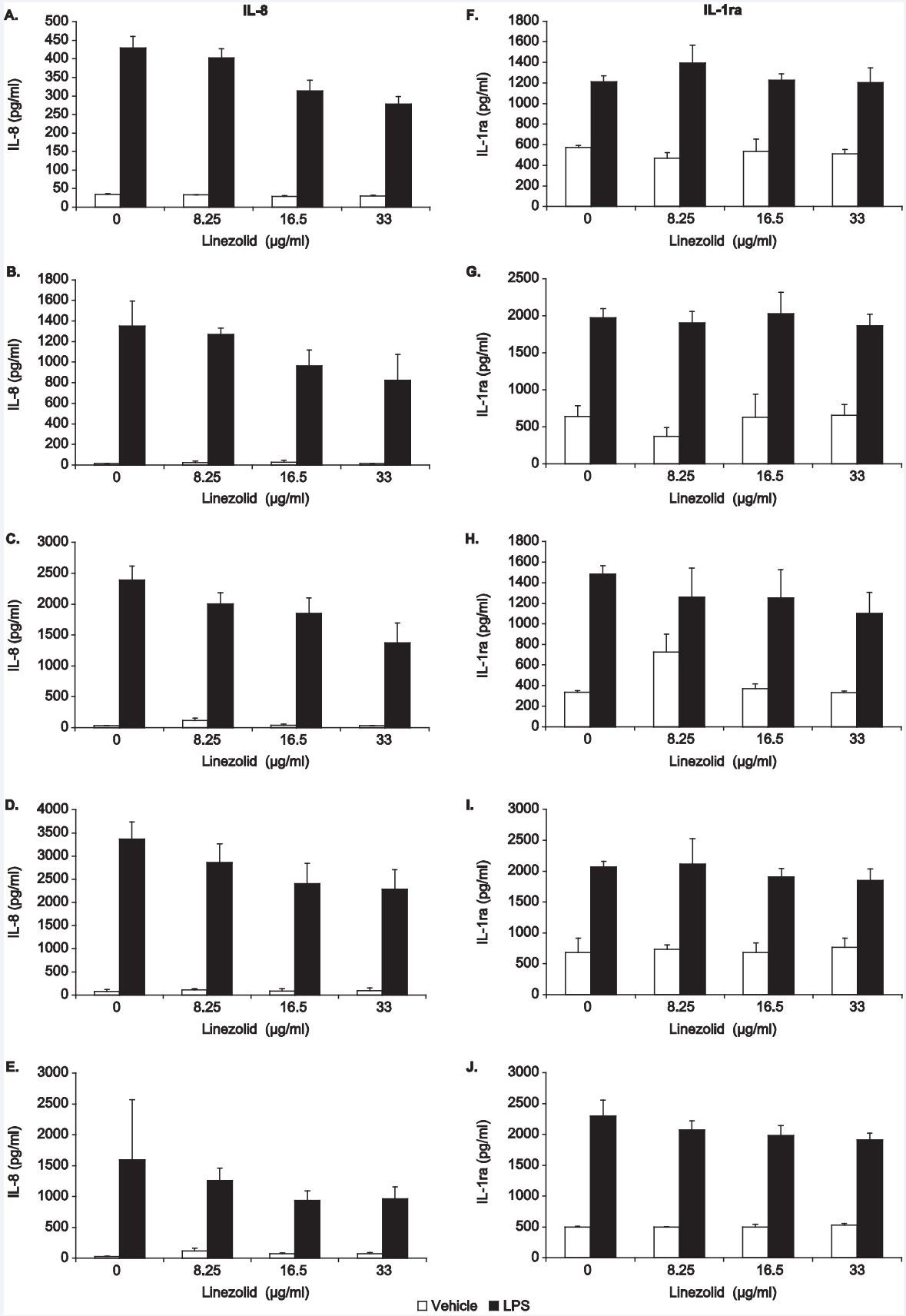

Figure 2 Effect of linezolid on *in vitro* cytokine secretion by purified neutrophils in response to LPS. (A-E) IL-8 secretion from 5 individuals and (F-J) IL-1ra secretion from the same 5 individuals. Data are presented as mean + SD (error bar).

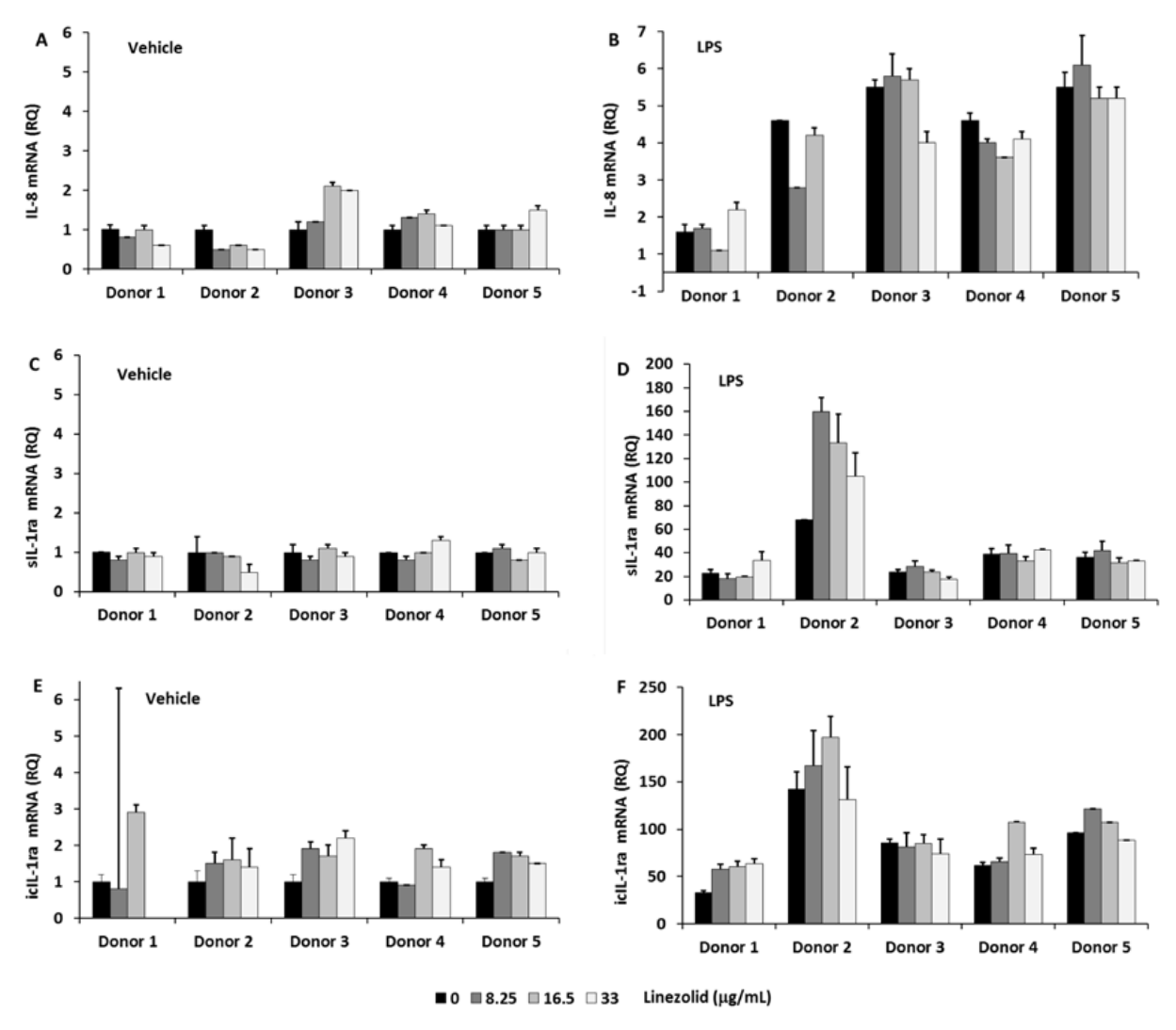

Figure 3 Effect of linezolid on *in vitro* gene expression of IL-8 (A,B), sIL-1ra (C,D) and icIL-1ra (E,F) from purified neutrophils in response to vehicle (control) (left panel) or LPS stimulation (right panel). Data are presented as relative quantity (RQ) of mRNA at each dose of linezolid (0, 8.25, 16.5 and 33 µg/mL). Note that levels of mRNA (at 33 µg/mL of linezolid) from Donor 1 (icIL-1ra without LPS) and Donor 2 (IL-8 with LPS) were below detection. Data are presented as mean + SD (error bar).

Table 1: *Ex vivo* net phagocytosis by monocytes and neutrophils in linezolid- (600 mg orally twice daily) or placebo-treated healthy subjects.

		Net phagocytosis[a]				Net percent phagocytosing cells[b]			
		Monocytes		Neutrophils		Monocytes		Neutrophils	
		Linezolid	Placebo	Linezolid	Placebo	Linezolid	Placebo	Linezolid	Placebo
		(*n* = 12)	(*n* = 6)	(*n* = 12)	(*n* = 6)	(*n* = 12)	(*n* = 6)	(*n* = 12)	(*n* = 6)
Baseline[c]	Mean	2660.1	3013.3	3047.8	3159.4	84.2	75.2	96.0	96.2
	SD	534.9	480.4	654.8	468.6	6.4	12.0	2.1	1.1
Post-baseline[c] (steady state)	Mean	2632.5	3034.8	3019.7	3032.8	82.7	82.9	96.0	96.6
	SD	476.4	431.8	558.9	370.2	7.4	10.0	3.2	1.4
% change from baseline	Mean	-0.4	1.0	0.0	-3.5	–1.5	7.7	–0.01	0.3
	SD	8.1	3.5	7.9	7.8	6.5	7.0	1.9	1.7

[a]Phagocytosis was measured as MFI. Net phagocytosis = phagocytosisat 37°C – phagocytosis on ice.
[b]Net percent phagocytosing cells = % phagocytosing cells at 37°C – % phagocytosing cells on ice.
[c]Data on days 0 and 1 were combined for baseline; data ondays 4 and 5were combined for post-baseline.

the 90% CIs of the corresponding ratios between linezolid and placebo within the equivalence range (80 to 125%; Table 2).

Ex vivo cytokine secretion in response to lipopolysaccharide stimulation

Net cytokine secretion in response to LPS stimulation in whole blood is summarized in Table 3. Intersubject variability in cytokine secretion was high, especially for IL-8 (range, 87 to 117%). This large variability in cytokine secretion over the course of the study was also reflected in the mean percent change from baseline for the placebo group (range, 7 to 36%).

As shown in Table 4, although not all of the 90% CIs for adjusted geometric mean ratios between linezolid and placebo for the cytokines (IL-1ra, IL-1β, IL-6, IL-8, and TNF-α) met the equivalence acceptance criterion (80 to 125%), all of the CIs included 100%. This indicates that the effect of linezolid on cytokine secretion in whole blood was not statistically significant.

DISCUSSION

These studies were conducted to further investigate the underlying mechanism for the mortality imbalance observed in the phase 3 clinical study of linezolid in patients with intravascular catheter-related infections described earlier [2], and to address concerns of potential immunosuppressive effects of linezolid raised following previous *in vitro* findings on cytokine secretion [11].

Recognition of gram-negative pathogens by cells of the innate immune system is largely dependent on the binding of LPS to receptors on the innate immune cells. LPS is the major component of the outer membrane of all gram-negative bacteria. It is recognized that other bacterial components can activate host cells but LPS, found in all gram-negative bacteria, is the most potent microbial product to elicit an immune response [17]. This makes it reasonable to use a single species such as *E. coli*, to represent gram-negative bacteria.

In our *in vitro* studies, linezolid had no effect on the phagocytosis of *E. coli* particles by neutrophils or monocytes or on cytokine (IL-1ra and IL-8) gene expression, but induced small to moderate decreases in IL-1ra (12%) and IL-8 (up to 37%) secretions from purified human neutrophils in response to LPS stimulation. The biological consequences of these magnitudes of decreases in secretion of these cytokines by neutrophils are unknown, since other cell types also produce these cytokines in response to stimulation [18-20], and the possibility exists that secretion by other cell types may compensate for neutrophil-specific decreases. For example, IL-1ra can be produced by monocytes, macrophages, and hepatocytes [18] and IL-8 can be produced by monocytes, endothelial cells, fibroblasts, and epithelial cells [19]. In addition, monocytes have been shown to release approximately 5- to 70-fold more IL-8 than neutrophils following stimulation with LPS [20].

In vitro assays with purified cell populations have several limitations: cell-cell interactions are absent and methods required to isolate specific populations of cells may affect their function [14]. Also, daily peaks and troughs of linezolid concentrations experienced during oral administration are not present under

Table 2: Statistical summary of treatment comparisons for *ex vivo* net phagocytosis and percent of phagocytosing cells.

	Cell type	Adjusted geometric mean[a]		Ratio (linezolid/placebo) of adjusted mean[b]	90% CI for ratio[b]
		Linezolid	Placebo		
Net phagocytosis	Monocytes	0.9854	1.0250	96.13	90.84, 101.74
	Neutrophils	0.9894	0.9666	102.36	96.34, 108.76
% phagocytosing cells	Monocytes	0.9937	1.0830	91.75	85.16, 98.84
	Neutrophils	0.9998	1.0032	99.67	97.96, 101.41

[a]Values were baseline corrected.
[b]Ratios and 90% CIs are expressed as percentages.

Table 3: *Ex vivo* net cytokine secretiona in response to LPS stimulation in the presence of linezolid (600 mg orally twice daily) or placebo in healthy subjects.

		IL-1ra (pg/ml)		IL-1β (pg/ml)		IL-6 (pg/ml)		IL-8 (pg/ml)		TNF-α (pg/ml)	
		Linezolid	Placebo	Linezolid	Placebo	Linezolid	Placebo	Linezolid	Placebo	Linezolid	Placebo
		(*n* = 12)	(*n* = 6)	(*n* = 12)	(*n* = 6)	(*n* = 12)	(*n* = 6)	(*n* = 12)	(*n* = 6)	(*n* = 12)	(*n* = 6)
Baseline[b]	Mean	46,200	51,600	10,400	10,400	87,300	71,400	60,900	81,000	4410	3610
	SD	18,600	12,700	5440	7730	48,500	45,900	52,900	94,800	2870	2930
	% CV[c]	40	25	52	75	55	64	87	117	65	81
Post-baseline (steady state)[b]	Mean	41,900	54,000	11,700	11,200	85,100	76,100	68,900	93,100	5100	4260
	SD	16,800	17,700	6150	8100	46,300	48,700	63,600	105,000	3020	3360
	% CV	40	33	53	72	54	64	92	113	59	79
% change from baseline	Mean	-8.62	7.08	15.0	23.6	-0.854	13.9	19.1	36.4	25.4	26.8
	SD	10.2	28.9	26.1	39.8	14.2	24.8	37.9	62.6	33.8	19.8

[a]Net cytokine secretion = cytokine secretion (LPS-stimulated) –cytokinesecretion (vehicle-treated).
[b]Data on days 0 and 1 were combined for baseline; data ondays 4 and 5were combined for post-baseline.
[c]%CV, percent coefficient of variation.

Table 4: Statistical summary of treatment comparisons for *ex vivo* net cytokine secretions in response to LPSstimulation.

Cytokine	Adjusted geometric mean[a]		Ratio (linezolid/ placebo) of adjusted mean[b]	90% CI for ratio[b]
	Linezolid	Placebo		
IL-1ra	0.9078	1.0434	87.01	73.25, 103.36
IL-1β	1.1415	1.1587	98.51	79.65, 121.84
IL-6	0.9994	1.0844	92.17	79.88, 106.35
IL-8	1.1683	1.2333	94.73	69.96, 128.27
TNF-α	1.2458	1.2154	102.50	84.27, 124.67

[a]Values were baseline corrected.
[b]Ratios and 90%CIs are expressed as percentages

in vitro conditions. Immune responses are highly dependent on cell-cell interactions, and isolated neutrophils and monocytes may respond differently in the absence of other immune cells, including lymphocytes [21]. Linezolid-induced decreases in cytokine secretion from isolated neutrophils or monocytes may not be representative of an *in vivo* response. Because of the limitations of *in vitro* evaluation, we subsequently conducted the *ex vivo* study to better reflect the *in vivo* situation.

Only young male subjects were enrolled in the *ex vivo* study in order to minimize intersubject variability. Since the longitudinal variability in values between sample collection time points has not been established for the phagocytic cell function assays, each subject provided 2 baseline samples (served as their own control) and 2 post-baseline samples to improve the precision of the measurements.

The *ex vivo* phagocytosis results were consistent with the phagocytosis results in vitro. Linezolid showed no effect on phagocytosis by neutrophils or monocytes.

Also, linezolid had no statistically significant effect on *ex vivo* cytokine secretion in whole blood in response to LPS stimulation. As expected, the quantities of cytokines released by total white blood cells in the *ex vivo* study were much higher than those observed in the *in vitro* studies. Since cell-cell interactions occur in whole blood, cytokines released by cells would stimulate other cells to release cytokines, resulting in even higher quantities of cytokines. The higher levels of cytokines observed in the *ex vivo* study may more accurately represent the immune response to an *in vivo* exposure to gram-negative pathogens than those observed in the *in vitro* studies with isolated cells. However, the *ex vivo* study also has its own limitations compared with the *in vivo* situation. The measurement of phagocytosis and cytokine secretion in response to LPS in whole blood was still performed *in vitro* although the healthy subjects were exposed to the clinical dose of linezolid for 5 days mimicking the clinical use of linezolid in patients. With the currently available techniques, it is challenging to measure the phagocyte function *in vivo* directly.

Linezolid peak (median [range]: 15 [6 to 21] μg/mL) and trough concentrations (median [range]: 7 [4 to 11] μg/mL) obtained in the *ex vivo* study were within the range observed in previous clinical studies [1, 22, 23], which confirmed that the phagocytosis and cytokine secretion results from this study were obtained at clinically relevant linezolid concentrations. Given the limited sample size (n = 12), preliminary assessment showed no correlation between linezolid concentrations and cytokine changes. This is not unexpected since there were no significant differences in changes of cytokine secretions between linezolid-treated group and placebo-treated group. Note that linezolid concentration data were not collected in the aforementioned phase 3 study and the correlation between linezolid concentrations and mortality imbalance could not be assessed.

The small to moderate decreases in cytokine (IL-1ra and IL-8) secretions were observed when exposed to constant linezolid concentrations of 16.5 and 33 μg/mL in vitro, which were much higher than what was observed in the *ex vivo* study. The possibility of *in vivo* immunosuppression at these high linezolid concentrations (if occurred) cannot be ruled out completely.

Grüger et al. recently reported that linezolid decreased human neutrophil function *in vitro* [24]. Specifically, linezolid transiently decreased the phagocytosis of a specific *E. coli* strain (U12987) by human neutrophils. A statistically significant decrease was observed at linezolid concentrations of 20 and 50 μg/mL following a 15-min incubation with *E. coli*, but phagocytosis returned to control levels when incubation time was increased to 30 min. Reasons for this unexpected finding were not provided. The phagocytosis of another gram-negative strain, *Pseudomonas aeruginosa* (*P. aeruginosa*; strain 014121), was slightly decreased by linezolid, most apparent after 30 min with 50 μg/ml of linezolid. In addition, the phagocytosis of a different *E. coli* strain (ATCC 25922) was not impaired by linezolid. Importantly, linezolid at concentrations up to 50 μg/mL, did not impair the killing of either *E. coli* U12987 or *P. aeruginosa.* It is noted that the evaluated concentration of 50 μg/ml was much higher than the clinically relevant concentration of linezolid. Transient decreases in phagocytosis that return to normal levels by 30-min of incubation without concomitant decreases in the killing of those bacteria strains are not likely to be clinically relevant. In addition, linezolid impairment of phagocytosis in response to gram-negative pathogens would not be expected to be strain specific.

CONCLUSION

In summary, our studies showed that linezolid had no effect on phagocytosis by neutrophils and monocytes *in vitro* or *ex vivo*. Small to moderate decreases in IL-1ra and IL-8 secretions from purified neutrophils were observed at high linezolid concentrations (≥16.5 μg/mL) in vitro, but gene expression of these cytokines was not affected. More importantly, linezolid had no significant effect on the secretion of cytokines (IL-1ra, IL-1β, IL-6, IL-8, and TNF-α) evaluated *ex vivo*. There is no strong evidence suggesting that linezolid inhibits phagocytic cell functions in response to gram-negative pathogensat clinically relevant concentrations.

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Conflict of Interest

S.C. and P.L. are employees of Pfizer Inc.T.T.K. was an employee of Pfizer Inc. during the preparation of this manuscript.

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