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Increased activity of linezolid in combination with rifampicin in a murine pneumonia model due to MRSA

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Objectives: The chloramphenicol/florfenicol resistance gene *cfr*, which mediates cross-resistance to linezolid and other classes of antimicrobial agents, represents a global therapeutic challenge due to its dissemination among MDR nosocomial pathogens, including MRSA. This study aimed to compare the efficacy of the linezolid/rifampicin combination in a murine pneumonia model caused by *cfr*-positive and *cfr*-negative clinical MRSA strains.

Methods: Synergistic activity between linezolid and rifampicin was evaluated by chequerboard and time–kill assays. Pharmacokinetic profiles in plasma and epithelial lining fluid (ELF) as well as the therapeutic efficacy of linezolid alone and in combination with rifampicin were investigated in a murine pneumonia model. The E_{max} Hill equation was used to model the dose–response relationship.

Results: Increased susceptibility of the study MRSA strains to linezolid was observed with the rifampicin combination (MIC decreased 2- to 16-fold versus linezolid alone). The combination had synergistic activity (fractional inhibitory concentration index $\leq$0.5) against all *cfr*-positive MRSA isolates. Linezolid demonstrated excellent pulmonary penetration with an ELF/*f*plasma AUC ratio of 2.68$\pm$0.17. The addition of rifampicin significantly improved the efficacy of linezolid in the pneumonia model due to *cfr*-positive and *cfr*-negative MRSA strains. The *f*AUC/MIC targets of linezolid associated with stasis, 1 $\log_{10}$ kill and 2 $\log_{10}$ kill were 15.9, 38.8 and 175 in plasma, and 43.5, 108 and 415 in ELF, respectively. Importantly, the linezolid *f*AUC/MIC targets in both plasma and ELF were 2.4–6.7 times lower in combined linezolid/rifampicin therapy versus linezolid monotherapy ($P < 0.005$).

Conclusions: Combination of linezolid with rifampicin significantly improved the efficacy of linezolid in the murine pneumonia model caused by MRSA strains in the presence and absence of the *cfr* gene.

Introduction

Nosocomial pneumonia is a leading cause of life-threating infections, and is responsible for the high mortality of hospital-acquired infections.[1] MRSA is responsible for up to 40% of all nosocomial pneumonias.[2] In addition, MRSA pneumonia is associated with a significant rapid decline in lung function, increased morbidity and worse survival as compared with MSSA.[3]

The *cfr* gene, which confers phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A ('PhLOPSA') resistance, has now been found worldwide in various bacterial species, especially in MDR nosocomial pathogens such as MRSA and *Enterococcus*.[4] Over the past decades, the number of reported cases due to linezolid-resistant MRSA associated with the *cfr* gene has significantly increased in humans.[5] Although glycopeptide antibiotics (e.g. vancomycin and teicoplanin) have long been the standard treatment for MRSA infections, recent American Thoracic Society guidelines suggest that oxazolidinones may be preferred over glycopeptides for MRSA pneumonia given their significantly better clinical cure rates and lower incidence of renal failure.[6] However, rapid emergence of the *cfr* gene among MRSA isolates suggests that patients may have limited antibiotic options. Specifically, vancomycin-intermediate and linezolid-resistant MRSA strains harbouring the *cfr* gene have been recently detected,[7] posing a further serious challenge to public health. Therefore, combinations of linezolid with other antimicrobial

Table 1. Genotype summary of study MRSA isolates, MICs of oxacillin and rifampicin alone, MICs of linezolid in the presence of 0–0.25 mg/L rifampicin and FICIs

		MIC (mg/L)						
				LZD				
MRSA isolate	Genotype comments	OXA_{alone}	RIF_{alone}	LZD_{alone}	$+RIF_{0.06}$	$+RIF_{0.13}$	$+RIF_{0.25}$	FICI[a]
161400	ST764; *spa*-type t1084	32	0.5	1	0.25	0.25	0.13	0.5
161813	ST764; *spa*-type t1084	32	1	0.5	0.25	0.13	0.13	0.75
161837	ST764; *spa*-type t1084	16	1	0.5	0.13	0.13	0.06	0.375
161402	ST764; *spa*-type t1084; *cfr*	128	1	8	2	2	1	0.375
161429	ST764; *spa*-type t1084; *cfr*	128	0.5	8	2	1	0.5	0.5
161494	ST764; *spa*-type t1084; *cfr*	128	1	4	1	1	0.5	0.5
126250	ST764; *spa*-type t899; *cfr*	16	8	8	2	1	0.5	0.5

OXA, oxacillin; RIF, rifampicin, LZD, linezolid.
[a]Interpreted as synergy (FICI $\leq$ 0.5), no interaction (0.5 < FICI $\leq$ 4) or antagonism (FICI > 4).

agents may represent a particularly interesting treatment alternative.

The goals of the current study were to investigate the antimicrobial activities of linezolid when combined with rifampicin and to define combinational dosing strategies to successfully treat infections due to linezolid-resistant MRSA carrying the *cfr* plasmid in a murine pneumonia model.

Materials and methods

Bacterial strains and culture conditions

Seven clinical MRSA isolates were used for this study (Table 1). Five of the seven strains (161813, 161837, 161429, 161494 and 126250) were isolated from sputum specimens of hospitalized patients with pulmonary infections at the Third Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China), and the remaining two strains (161400 and 161402) were collected from mechanically ventilated inpatients with pneumonia at Guangdong Second Traditional Chinese Medical Hospital (Guangzhou, China). All MRSA were identified by MALDI-TOF MS (Axima-Assurance-Shimadzu) and the *cfr* gene was confirmed by PCR.[4] The clonal relationship of these strains was determined by MLST and results were analysed using an MLST database (http://www.mlst.net). *spa* typing based on the detection of the *spa* gene of the X-region among MRSA isolates was carried out according to a method described previously.[8] MRSA strains were grown in Mueller–Hinton broth or Mueller–Hinton agar (Difco Laboratories, Detroit, MI, USA).

Antimicrobial agents

Analytical-grade linezolid and rifampicin powders for *in vitro* studies were purchased from Sigma-Aldrich Co. (Shanghai, China) and reconstituted according to the manufacturer's recommendations. Linezolid and rifampicin for *in vivo* studies were obtained commercially from Haosen Pharmaceutical Group (Jiangsu, China) and Shuangding Pharmaceutical Company (Shenyang, China).

In vitro fractional inhibitory concentration index (FICI) assay

The *in vitro* anti-MRSA activity of the linezolid/rifampicin combination was evaluated using a standard chequerboard test (linezolid range 0.06–64 mg/L; rifampicin range 0.13–16 mg/L).[9] An FICI of $\leq$0.5 indicates a synergistic effect between linezolid and rifampicin.

In vitro time–kill curves

Time–kill experiments were conducted to further characterize the synergistic activity of the linezolid/rifampicin combination as previously described.[10] In brief, we used an initial inoculum of $\sim 10^6$ cfu/mL logarithmic-phase MRSA cells in the presence of linezolid (4 or 8 mg/L) with/without rifampicin (0.25 mg/L) and compared time–kill for the combination versus that for each individual agent. The linezolid concentrations were chosen to simulate the average steady-state serum concentration (C_{ss}; 6.1–9.8 mg/L) at the clinically recommended dose in humans, i.e. 600 mg twice a day.[11] Similarly, the concentration of rifampicin was chosen in order to mimic the free plasma peak concentration (fC_{max} 0.30 $\pm$ 0.07 mg/L) achieved with a recommended human clinical dose (150 mg).[12,13] Synergy was defined as achieving a $\geq$2 $\log_{10}$ cfu/mL reduction at 24 h with the combination compared with the most active individual drug on its own.[14] At least three independent experimental runs were performed.

Murine pneumonia model

Mice were maintained in accordance with the National Standards for Laboratory Animals in China (GB 14925-2010). All animal studies were conducted in accordance with SCAU Institutional Animal Welfare and Ethics guidelines. The animal use procedures were approved by the Animal Research Committees of SCAU. Six-week-old, specific-pathogen-free, female ICR mice (25–27 g; obtained from Guangdong Medical Lab Animal Center, Guangzhou, China) were used in the experiments. Neutropenia was induced by two doses of cyclophosphamide injected intraperitoneally on 4 days (150 mg/kg) and 1 day (100 mg/kg) prior to infection.[10] Lung infections were induced by administration of 50 μL of bacterial suspension ($10^{7.5–8.0}$ cfu/mL of logarithmic-phase MRSA cells) into the trachea through a pre-inserted tracheal tube in mice anaesthetized with an isoflurane/oxygen gas mixture.[15]

Pharmacokinetic (PK) studies

PK studies were performed in neutropenic lung-infected mice following administration of single-dose linezolid (2.5, 10, 40 and 160 mg/kg) or rifampicin (1.25 and 5 mg/kg), or a combination of the two (40 mg/kg linezolid + 1.25 mg/kg rifampicin), by oral gavage. Animals (three mice per group and per timepoint) were humanely sacrificed at 10, 15, 30 and 45 min and 1, 3, 6, 9 and 12 h post-dose. Immediately, concomitant samples of plasma and bronchoalveolar lavage (BAL) fluids were collected from each animal using our previously reported technique.[15] In brief, blood was collected by retro-orbital puncture and plasma was separated by

centrifugation at 3000 **g** for 10 min. BAL was performed using 0.5 mL of sterile 0.9% saline for two cycles and the fluids were immediately aspirated and pooled per animal. The BAL fluid was centrifuged to remove blood, macrophages and cellular debris, and supernatant was then collected for urea and drug concentration assay as we previously described.[15]

Concentrations of linezolid and rifampicin in plasma and BAL fluids were determined by an LC-MS/MS method.[16,17] The limit of quantification in both plasma and BAL samples was 0.001 mg/L. The recoveries of linezolid and rifampicin were >90% and relative standard deviations for both intraday and interday were <8.7% at all tested concentrations. All PK parameters were estimated using Phoenix WinNonlin 6.1 (Pharsight, St Louis, MO, USA). Non-compartmental and one-compartment models were explored. A linear extrapolation was used to estimate PK parameters for dose levels that were not directly determined in this study. Protein-binding values of 30% and 43% were used to determine the free drug fractions of linezolid and rifampicin, respectively, in mice.[18,19]

Drug concentrations in pulmonary epithelial lining fluid (ELF) were calculated from BAL concentrations by urea correction methodology using the following formula: $[drug]_{ELF} = [drug]_{BAL\ fluids} \times ([urea]_{plasma}/[urea]_{BAL\ fluids})$.[15] Urea concentrations in BAL and plasma samples were determined using a commercial urea assay kit (MLBIO Biotechnology, Shanghai, China). The assay was linear with an R^2 of >0.997 for both plasma and BAL fluid urea concentrations over the range of 0.05–3.0 mg/dL. The intraday and interday coefficients of variations for the urea assay of the quality control samples were within 4.3% and 7.2%, respectively. The penetration of each drug into the ELF space was calculated by comparing ELF/*f*plasma AUC ratios.[15]

In vivo efficacy and dose–response relationships

Neutropenic mice were infected with each of the seven MRSA strains as described above. Two hours after pulmonary challenge, mice were randomized to receive: (i) no therapy (control); (ii) linezolid at 1.25, 2.5, 5, 10, 20, 40 or 80 mg/kg, orally twice a day; (iii) rifampicin at 1.25 or 5 mg/kg, orally twice a day; or (iv) a combination of linezolid (1.25–80 mg/kg) with rifampicin (1.25 or 5 mg/kg), orally twice a day. Linezolid doses were selected to vary the effect from maximal to no efficacy. The rifampicin doses (1.25 and 5 mg/kg) were chosen in order to mimic the PK profiles of the recommended human clinical doses (150 and 600 mg, respectively).[12,20] Treatments lasted for 1 day. Control mice were sacrificed at 2 h post-infection (representing bacterial density in target tissue at the beginning of treatment) or at 24 h post-treatment, while treated mice were euthanized after 24 h of therapy. At sacrifice, infected lungs were aseptically removed, homogenized and quantitatively cultured. Four mice were utilized for each treatment and control group. Data were expressed as mean change in $\log_{10}$ cfu/lung ($\pm$SD, $n = 4$) compared with the bacterial burden at the start of therapy.

PK/pharmacodynamic (PD) relationship analysis

The AUC/MIC ratio was used as a PK/PD index to predict treatment efficacy for linezolid alone or in combination with rifampicin.[18] The correlation between treatment efficacy and AUC/MIC ratio was evaluated by a sigmoid E_{max} model using the Phoenix WinNonlin identification module: $E = (E_{max} \times C^N)/(EC_{50}{}^N + C^N)$, where E is the effector, in this case, the $\log_{10}$ change in cfu per lung, E_{max} is maximum effect, C is the PK/PD index being examined, EC_{50} is the C value required to achieve 50% of the E_{max} and N is the slope of the dose–response curve. In addition, free drug (defined as unbound drug) concentrations were used for the determination of the *f*AUC/MIC ratios that are required to achieve each endpoint (e.g. net stasis, 1 $\log_{10}$ kill or 2 $\log_{10}$ kill) taking protein binding into consideration.[15,18] Analysis of variance (ANOVA) or Mann–Whitney *U*-test was used to determine whether the differences in PD targets needed for stasis and killing were significant between monotherapy and combination therapy.

Results

In vitro susceptibility and time–kill curves

Three of the four *cfr*-positive MRSA strains were resistant to linezolid (MICs = 8 mg/L), while one *cfr*-positive and all three study *cfr*-negative MRSA strains were susceptible to linezolid (Table 1). All MRSA strains were susceptible to rifampicin, except for 126250, which had an MIC of 8 mg/L. Interestingly, the combination of linezolid with rifampicin reduced the linezolid MICs 2- to 16-fold in all study strains. In addition, a concentration-dependent effect of rifampicin on linezolid MIC was observed (higher rifampicin concentration correlated with lower linezolid MICs; Table 1). Significantly, synergistic effects of the linezolid/rifampicin combination were observed in six of seven clinical MRSA isolates, with FICIs ranging from 0.375 to 0.5 (Table 1).

The *in vitro* time–kill activities of linezolid at 4 or 8 mg/L alone and in combination with 0.25 mg/L rifampicin against MRSA strains 161400 (*cfr*-negative) and 161402 (*cfr*-positive) are shown in Figure 1. Both linezolid concentrations tested had bacteriostatic activity against the *cfr*-negative strain (Figure 1a), whilst rifampicin alone caused the strain to regrow by 24 h. Of note, the addition of 0.25 mg/L rifampicin to linezolid significantly improved the *in vitro* antimicrobial activity as compared with linezolid alone ($P < 0.05$) and showed rapid killing of the *cfr*-negative MRSA strain at 9 h of incubation.

For the *cfr*-positive MRSA strain 161402, linezolid alone at clinically achievable C_{ss} of 4–8 mg/L had no bacteriostatic activity (Figure 1b). However, the combination of linezolid with 0.25 mg/L rifampicin resulted in a synergistic effect with >4.0 $\log_{10}$ cfu/mL reduction as compared with linezolid alone ($P < 0.05$; Figure 1b), against the *cfr*-positive MRSA strain at 9 h of incubation.

PK profiles of linezolid and rifampicin

After oral administration, the C_{max} of linezolid was observed in plasma and ELF within approximately 30 min and 1 h, respectively, in a dose-dependent manner [Table 2 and Figure S1 (available as Supplementary data at *JAC* Online)]. AUCs were linear for both plasma and ELF concentration measurements, with R^2 of 0.993 and 0.986, respectively (Figure S1). The elimination half-life ($t_{1/2}$) of linezolid was ~1 h in plasma and ~3–5 h in ELF ($P < 0.005$ for $t_{1/2}$ in plasma versus ELF), while C_{max} was significantly higher in plasma versus ELF ($P < 0.05$; Table 2). Similarly, rifampicin had a significantly longer $t_{1/2}$ and lower C_{max} in ELF versus in plasma ($P < 0.03$ ELF versus plasma; Table 2). Of note, linezolid demonstrated very good pulmonary distribution as the mean penetration ratio into the ELF (i.e. ELF/*f*plasma AUC ratio) was 2.68±0.17. However, a poor penetration into the ELF was observed for rifampicin, with a ELF/*f*plasma AUC ratio of 0.91 (Table 2). In the murine pneumonia model, the plasma $t_{1/2}$ of linezolid was relatively short as compared with the $t_{1/2}$ in humans (~5 h),[21] whereas the ELF/*f*plasma AUC ratios for both drugs were similar to those achieved in humans (3.19 for linezolid and 1.28 for rifampicin).[22,23] In addition, although linezolid concentrations in plasma and ELF in mice that received linezolid/rifampicin combination therapy were slightly lower than linezolid alone at several timepoints (Figure S1), the differences did not reach statistical significance ($P > 0.47$; Table 2). Similarly, no significant difference in PK profiles was observed

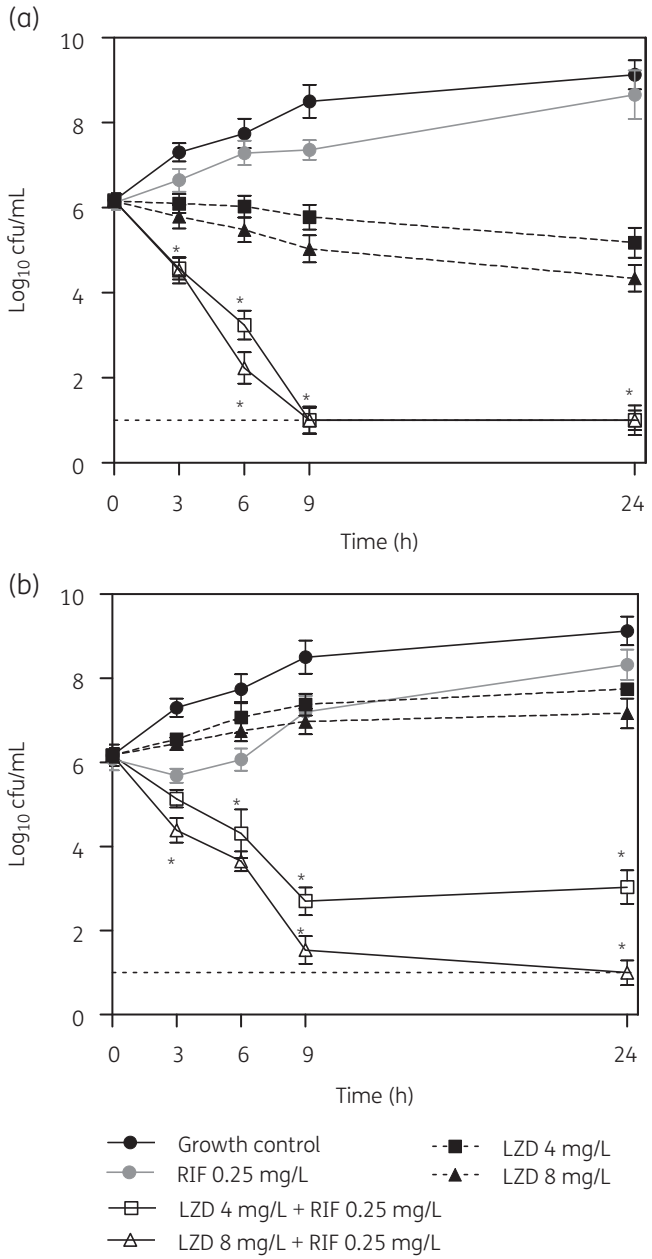

Figure 1. *In vitro* time–kill curves of linezolid alone at 4 or 8 mg/L, rifampicin alone at 0.25 mg/L and linezolid/rifampicin combination against *cfr*-negative MRSA strain 161400 (a) and *cfr*-positive strain 161402 (b). *$P < 0.05$ linezolid/rifampicin combinations versus linezolid alone. LZD, linezolid; RIF, rifampicin.

between rifampicin alone and in combination with linezolid ($P > 0.16$; Figure S1).

Dose–efficacy relationships of linezolid alone and in combination with rifampicin

The dose–efficacy relationships of linezolid alone and in combination with rifampicin in the study MRSA strains are shown in Figure 2. Higher linezolid doses were required to achieve similar efficacy outcomes against *cfr*-positive MRSA strains versus *cfr*-negative MRSA strains. For instance, with linezolid monotherapy at 80 mg/kg twice a day, a 2–3 $\log_{10}$ cfu/lung MRSA density reduction was observed in animals infected with *cfr*-negative strains, while a <1 $\log_{10}$ cfu/lung reduction was seen in animals infected with *cfr*-positive strains ($P < 0.001$; Figure 2a). Interestingly, the combination of linezolid with rifampicin significantly increased linezolid efficacy in the pneumonia model caused by both *cfr*-negative and *cfr*-positive MRSA strains (Figure 2b and c). Specifically, neither linezolid (40 mg/kg twice a day) nor rifampicin (1.25 or 5 mg/kg twice a day) resulted in a significant reduction in bacterial burden in lung in the murine pneumonia model caused by *cfr*-positive MRSA strains. However, the combination of linezolid with rifampicin resulted in a significant reduction in MRSA density in lung ($\sim$2 $\log_{10}$ cfu/lung) as compared with linezolid monotherapy ($P < 0.01$; Figure 2d).

The relationships between linezolid *f*AUC/MIC ratios in plasma and in ELF and the efficacy of linezolid alone and in combination with rifampicin against *cfr*-negative and *cfr*-positive MRSA strains are shown in Figure 2(e and f). Similar to the dose–response relationships as mentioned above, therapeutic outcomes correlated significantly with the linezolid *f*AUC/MIC index in both plasma and in ELF ($R^2 > 0.946$), suggesting that *f*AUC/MIC is a suitable predictor for the treatment outcome in the pneumonia model. In addition, the combination treatment significantly enhanced the efficacy of linezolid in the murine pneumonia model, with lower EC_{50} values versus linezolid monotherapy ($P < 0.005$; Table S1). The *f*AUC/MIC magnitudes necessary to produce stasis and killing activities are presented in both plasma and ELF for *cfr*-negative and *cfr*-positive MRSA strains (Table 3). Across all study MRSA strains, target values of the linezolid *f*AUC/MIC ratio in plasma required to achieve a net stasis, 1 $\log_{10}$ kill and 2 $\log_{10}$ kill were 15.9, 38.8 and 175, respectively, for linezolid monotherapy, whereas they were 3.05, 9.62 and 26.1, respectively, for linezolid and rifampicin (5 mg/kg twice a day) combination therapy ($P < 0.005$). Importantly, similar correlations of *f*AUC/MIC targets and treatment outcomes between plasma and ELF were observed, with significantly lower target values in the combination treatment versus linezolid alone ($P < 0.005$).

Discussion

Treatment of nosocomial pneumonia caused by MRSA is a growing clinical problem due to the ability of MRSA strains to develop antibiotic resistance via multiple mechanisms, including the acquisition of antibiotic resistance genes.[24] The recent emergence of a new mobile linezolid resistance determinant, i.e. the *cfr* gene, represents a major therapeutic challenge, especially when the strains are MDR.[25] In such cases, the combination of linezolid with existing antimicrobials may represent a potential treatment alternative. Thus, our current studies focus on the combination of linezolid with rifampicin against MRSA strains, including linezolid-resistant MRSA harbouring the *cfr* gene, both *in vitro* and in a murine pneumonia model.

In the present study, we demonstrated that MRSA strains carrying the *cfr* gene had 4- to 16-fold higher linezolid MICs versus strains without the *cfr* gene. Importantly, the combination of linezolid with rifampicin significantly enhanced the susceptibility of the study MRSA isolates to linezolid regardless of the *cfr* gene. The *in vitro* time–kill studies further demonstrated a synergistic effect of the linezolid/rifampicin combination at clinically achievable

Table 2. PK parameters in plasma and ELF, and ELF/plasma penetration ratios of linezolid and rifampicin after oral administration of a single dose of linezolid, rifampicin or linezolid/rifampicin in the murine pneumonia model

		Plasma			ELF			ELF/plasma AUC ratio	
Antimicrobial(s)	Dose (mg/kg)	$t_{1/2}$[a] (h)	C_{max} (mg/L)	AUC[b] (mg·h/L)	$t_{1/2}$ (h)	C_{max} (mg/L)	AUC[b] (mg·h/L)	total	free[c]
Linezolid	2.5	0.77	0.86	0.90	2.94	0.41	1.53	1.70	2.43
	10	0.89	5.37	7.59	3.89	2.48	15.5	2.04	2.92
	40	0.96	26.9	47.7	3.78	17.6	90.9	1.91	2.72
	160	1.05	111.6	212.1	4.93	92.7	395.8	1.87	2.67
	mean±SD	0.91±0.10	NA	NA	3.89±0.71	NA	NA	1.88±0.12	2.68±0.17
Rifampicin	1.25	3.28	0.57	3.48	15.9	0.18	1.97	0.56	0.97
	5	3.83	4.32	27.7	17.4	1.12	13.2	0.48	0.84
	mean±SD	3.56±0.27	NA	NA	16.7±0.75	NA	NA	0.52±0.04	0.91±0.07
Linezolid/rifampicin[d]	40 (linezolid)	0.92	25.2	43.1	3.87	17.8	84.7	1.97	2.81
	1.25 (rifampicin)	2.91	0.69	3.96	14.8	0.21	1.89	0.47	0.82

$t_{1/2}$, elimination half-life; C_{max}, peak concentration in plasma and ELF; AUC, area under the concentration–time curve; NA, not applicable.
[a]$P < 0.005$ linezolid $t_{1/2}$ in ELF versus in plasma.
[b]AUCs were linear for both plasma and ELF concentration measurements, with R^2 of 0.993 and 0.986, respectively.
[c]The protein binding for linezolid and rifampicin in murine plasma was 30% and 43%, respectively, and in ELF was negligible.[18,19]
[d]$P > 0.16$ for PK parameters of 40 mg/kg linezolid and 1.25 mg/kg rifampicin in combination versus linezolid and rifampicin alone.

concentrations against MRSA strains. In agreement with our study, results from other studies have demonstrated that the addition of rifampicin to tedizolid (a novel oxazolidinone) significantly improved the killing activity against *Staphylococcus aureus* isolates.[26] As well as *S. aureus*, synergistic effects for the combination of linezolid with rifampicin were also observed in *Staphylococcus epidermidis* and *Enterococcus faecalis*.[27,28] In addition, *in vitro* additive activity between linezolid and rifampicin was also reported.[29] Taken together, these results suggest that the addition of rifampicin could significantly enhance linezolid activity against MRSA isolates.

The treatment option most frequently recommended for nosocomial pneumonia due to MRSA is a prolonged course of vancomycin.[6] However, as reported in two large multicentre trials of linezolid and vancomycin for patients with hospital-acquired pneumonia, linezolid was found to have a significant association with higher clinical cure, lower mortality and absence of renal comorbidities, especially for patients with ventilator-associated pneumonia due to MRSA.[24] This advantage may be due to the higher penetration of linezolid into the ELF compared with vancomycin (ELF/fplasma AUC ratio of 2.68 versus 0.74).[30] However, for linezolid-resistant MRSA strains harbouring the *cfr* plasmid, alternative combination therapy is recommended to improve therapeutic efficacy.[24] In the present study, combination therapy with linezolid/rifampicin exerted significantly increased *in vivo* efficacy as compared with linezolid monotherapy in the murine pneumonia model due to *cfr*-positive MRSA strains. Similarly, Tsaganos *et al.*[31] showed enhanced efficacy of linezolid plus rifampicin compared with that of linezolid alone in an experimental model of *S. aureus* endocarditis. More importantly, the linezolid/rifampicin combination was shown to be superior versus vancomycin, linezolid or daptomycin monotherapy for the treatment of implant-associated osteomyelitis due to *S. aureus* biofilm infections.[32]

It should be noted that the existing linezolid-based combinations with other antibiotics (e.g. tigecycline, glycopeptides and aminoglycosides) against staphylococci generally failed to capitalize on one of the key advantages of oxazolidinones (the almost 100% oral bioavailability),[23] since these agents would still require intravenous administration. Therefore, the combination of linezolid with orally bioavailable antistaphylococcal agents may allow the possibility of an earlier discharge from the hospital. In addition, some case reports showed a reduction of linezolid concentration in serum when combined with rifampicin, which might be due to enzymatic induction by rifampicin.[33,34] However, rifampicin has demonstrated a protective effect against the development of linezolid-resistant staphylococci.[35] These results suggest that a slight decrease in linezolid serum concentration under the influence of rifampicin probably will not reduce the therapeutic efficacy of the combination. In fact, our current results demonstrated no significant changes in plasma and ELF concentrations of linezolid and rifampicin when these were used as monotherapy or in combination. Taken together, our findings suggest that the linezolid/rifampicin combination might be another choice for the treatment of invasive infections due to linezolid-resistant MRSA strains.

Importantly, numerous factors may contribute to the treatment failure of drug combinations, including antibiotic resistance, drug PK variability and insufficient exposure due to inadequate dose level.[6] Thereby, determining the PD targets associated with optimal antimicrobial exposure is necessary in order to address these shortfalls. The animal model-based PK/PD investigation provided a useful approach for adjusting the antibiotic dosing regimens to prevent treatment failure. In the current study, we reported significantly lower PD targets of linezolid when combined with rifampicin at clinically used dose levels. The estimated linezolid PD targets will be useful in establishing a dose regimen to optimize linezolid-based combination therapy for respiratory

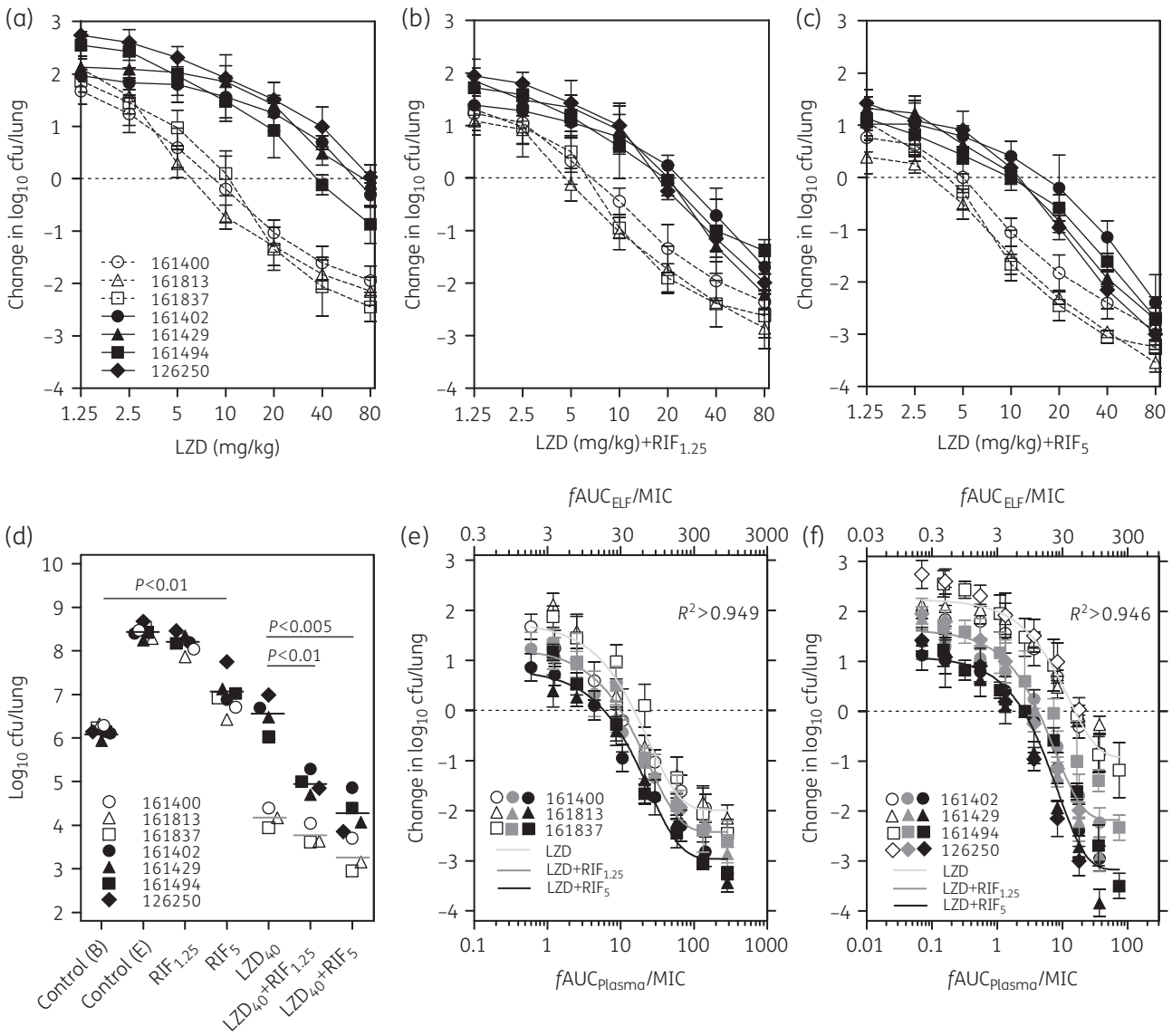

Figure 2. Dose–response relationships of linezolid alone and in combination with rifampicin in the murine pneumonia model caused by study MRSA strains. Mice received one of a series of 2-fold increasing doses of linezolid alone (a) or in combination with rifampicin at 1.25 mg/kg (b) or 5 mg/kg (c), twice a day, over a 24 h treatment period. Each symbol represents the mean $\log_{10}$ change from 0 h (beginning of antibiotic treatment) to 24 h (24 h after antibiotic treatment) in bacterial burdens from four mice ($n = 4$/group/timepoint). Bacterial densities of each MRSA strain in the lungs of mice treated with linezolid (40 mg/kg twice a day) and rifampicin (1.25 or 5 mg/kg twice a day) alone or in combination (open symbols represent *cfr*-negative strains and filled symbols represent *cfr*-positive strains) (d). Each symbol represents the bacterial count determined for each MRSA strain and the horizontal lines represent the mean organism densities for each control and treatment group. Control (B) and (E) represent the untreated control groups at the 0 and 24 h timepoints, respectively. Correlations between the changes in $\log_{10}$ cfu/lung at 24 h post-treatment and *f*AUC/MIC ratios in plasma and ELF following monotherapy or combination therapy against *cfr*-negative (e) and *cfr*-positive (f) MRSA strains. Each symbol represents the mean $\log_{10}$ change from 0 to 24 h in bacterial burdens from four mice. Data points below the horizontal broken line represent killing and points above the line represent growth. R^2 represents the coefficient of determination. LZD, linezolid; RIF, rifampicin.

infections due to MRSA. Based on the previously reported *f*AUC/MIC target (~220) for rifampicin,[19] either 600 or 900 mg of rifampicin orally (*f*AUC/MIC of 29.1 or 32.9) was unable to achieve killing of *cfr*-positive MRSA strains when accounting for 80% plasma protein binding in humans.[13,20] However, when combined with rifampicin, administration of the recommended dosing regimen of linezolid (600 mg twice a day) orally to humans was estimated to be effective against *cfr*-positive MRSA strains whilst achieving a mean *f*AUC/MIC of 21.5 that exceeded the combinatorial PK/PD requirement of linezolid (*f*AUC/MIC of 14.3 for 2 $\log_{10}$ kill) identified herein. In fact, the human-equivalent doses of linezolid (80 mg/kg twice a day) and rifampicin (5 mg/kg twice a day) in combination in mice

Table 3. Modelling estimates of linezolid *f*AUC/MIC targets in plasma and ELF associated with stasis, 1 $\log_{10}$ kill and 2 $\log_{10}$ kill of *cfr*-positive (*cfr*+) and *cfr*-negative (*cfr*−) MRSA isolates in lungs of mice ($n = 4$) for linezolid monotherapy and co-dosed with rifampicin at 1.25 or 5 mg/kg twice a day

	Target values of linezolid *f*AUC/MIC ratio								
				linezolid/rifampicin combination[a]					
	linezolid monotherapy			linezolid + 1.25 mg/kg rifampicin			linezolid + 5 mg/kg rifampicin		
Organism	stasis	1 $\log_{10}$ kill	2 $\log_{10}$ kill	stasis	1 $\log_{10}$ kill	2 $\log_{10}$ kill	stasis	1 $\log_{10}$ kill	2 $\log_{10}$ kill
Target values of linezolid $fAUC_{plasma}$/MIC in plasma									
MRSA^{cfr-}	14.1±5.27	35.4±9.17	175±54.9	7.85±1.41	23.1±2.09	75.2±1.92	4.36±0.56	14.2±1.41	41.9±4.17
MRSA^{cfr+}	17.3±1.50	49.2±0.00	NA	4.36±1.31	11.5±5.15	29.6±16.1	2.07±0.63	6.19±2.48	14.3±5.85
mean ± SD	15.9±3.97	38.8±9.95	175±54.9	5.85±2.20	16.5±7.05	49.1±25.7	3.05±1.29	9.62±4.49	26.1± 14.6
Target values of linezolid $fAUC_{ELF}$/MIC in ELF									
MRSA^{cfr-}	39.9±14.8	100±25.1	415±67.3	22.1±4.10	65.9±5.85	211±6.85	12.0±1.73	40.1±3.87	118±11.12
MRSA^{cfr+}	46.2±3.56	132±0.00	NA	11.7±3.82	31.6±14.1	78.8±42.5	5.92±1.84	17.1±6.71	38.7±15.7
mean±SD	43.5±10.5	108±25.7	415±67.3	16.1±6.47	46.3±20.5	136±73.2	8.53±3.50	27.0±12.7	72.8± 41.8

*f*AUC/MIC, free drug (non-protein-bound) AUC/MIC ratio; NA, not achieved.
[a]$P < 0.005$ linezolid/rifampicin combinations versus linezolid monotherapy.

resulted in ∼3 $\log_{10}$ kill activity against all MRSA strains regardless of the presence of the *cfr* gene. Moreover, this combinatorial PK/PD property has its advantages because of their similar half-lives (∼5 h) in humans and the relatively lower risk of nephrotoxicity compared with vancomycin.[20,21,24] Therefore, our findings suggest that the current clinical dose of linezolid in combination with rifampicin is likely to be efficacious and well-tolerated therapy in patients with *cfr*-positive MRSA pneumonia.

The mechanism of the enhanced anti-MRSA activity of the combination of rifampicin and linezolid is not fully understood, but is postulated to be associated with a synergistic inhibition of protein synthesis by different mechanisms of action.[36] It is well known that linezolid inhibits bacterial protein synthesis by binding to the 23S ribosomal RNA in the catalytic site of the 50S ribosome.[29] Rifampicin also inhibits protein synthesis, but in a prior step, by targeting the β subunit of RNA polymerase that blocks the transcriptional process.[36] Therefore, the potential synergistic effect of the linezolid/rifampicin combination can plausibly be ascribed to interference with both RNA transcription and protein translation.

There are several limitations to this study that should be noted. For example, we only used one genotype of an MRSA clone (ST764) that is associated with the increasing prevalence of hospital-acquired MRSA infections in Asia.[37] In addition, we only tested a limited number of *cfr*-positive MRSA isolates and only one oxazolidinone in the current study. Future studies should confirm the reproducibility of these results in larger collections of MRSA strains and with other oxazolidinones. Moreover, based on our current findings, although the benefit of adding rifampicin to linezolid was observed in the pneumonia model, further investigation is warranted to examine the effectiveness of this combination in other clinically relevant animal models, such as those for skin and soft tissue, bacteraemia, and infective endocarditis due to MRSA.

In summary, this study revealed the increased *in vitro* and *in vivo* anti-MRSA activities of linezolid in combination with rifampicin. These results indicate that the linezolid/rifampicin combination may be an appealing therapeutic option against serious MRSA invasive infections, including pneumonia due to linezolid-resistant MRSA strains.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Table S1 are available as Supplementary data at *JAC* Online.

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