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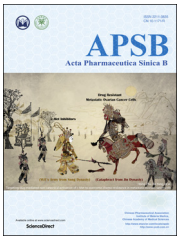

ORIGINAL ARTICLE

In vitro and *in vivo* activity of D-serine in combination with β-lactam antibiotics against methicillin-resistant *Staphylococcus aureus*

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KEY WORDS

MRSA;
D-Serine;
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Abstract As D-amino acids play important roles in the physiological metabolism of bacteria, combination of D-amino acids with antibiotics may provide synergistic antibacterial activity. The aim of the study was to evaluate *in vitro* and *in vivo* activity of D-serine alone and in combination with β-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA) strains, and to explore the possible sensitization mechanisms. The activity of D-serine, β-lactams alone and in combinations was evaluated both *in vitro* by standard MICs, time–kill curves and checkerboard assays, and *in vivo* by murine systemic infection model as well as neutropenic thigh infection model. An *in vitro* synergistic effect was demonstrated with the combination of D-serine and β-lactams against MRSA standard and clinical strains. Importantly, the combinations enhanced the therapeutic efficacy in the animal models as compared to β-lactam alone groups. Initial mechanism study suggested possible revision of D-alanine-D-alanine residue to D-alanine-D-serine in peptidoglycan by adding of D-alanine in the medium, which may cause decreased affinity to PBPs during transpeptidation. In conclusion, D-serine had synergistic activity in combination

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with β-lactams against MRSA strains both *in vitro* and *in vivo*. Considering the relatively good safety of D-serine alone or in combination with β-lactams, D-serine is worth following up as new anti-MRSA infection strategies.

1. Introduction

Staphylococcus aureus (*S. aureus*) is one of the most important clinical organisms among Gram-positive bacteria. It is a leading cause of skin and soft tissue infections (SSTIs), bacteremia and infective endocarditis[1,2]. Methicillin-resistant *S. aureus* (MRSA) strain has been a heavy burden worldwide and caused a range of serious infections with poor clinical outcomes[3–5]. However, grim scenario of drug discovery for new antibiotics has been presented for the last few decades as pharmaceutical companies lack interest in this field, owing to difficulty in recouping drug discovery costs from antibiotics which developed resistance within a decade or so[6]. Therefore, there is a critical need to develop new treatment strategies against these MRSA life-threatening infections.

D-Amino acids play important roles in bacterial physiology[7,8]. D-alanine (D-Ala) and D-glutamate (D-Glu) are components of bacterial peptidoglycan[9]. D-amino acids could also influence peptidoglycan composition, amount and strength, both *via* their incorporation into the polymer and by regulating enzymes that synthesize and modify it[8,10,11]. Up to date, researches mainly focused on the effects of D-amino acids on biofilm, finding that D-amino acids could not only prevent biofilm formation, but also disrupt existing biofilms[12–15]. In addition, D-amino acids were also able to enhance the activity of rifampin against biofilm formation in *S. aureus*, and to increase the efficacies of colistin, ciprofloxacin and amikacin against *Pseudomonas aeruginosa*[16,17]. However, less studies focused on the effects of D-amino acids on planktonic bacteria, except Tong et al.[18] showed that the application of D-cysteine (D-Cys), D-aspartic acid (D-Asp) and D-Glu could significantly improve the antibacterial activity of nisin against planktonic bacteria of *S. mutans*.

D-Serine (D-Ser) was reported to be able to replace D-Ala residue of peptidoglycan stem peptides and increase susceptibility of methicillin in MRSA[19]. In order to determine whether D-Ser can improve susceptibility of β-lactams against MRSA strains, we investigated the activity of D-Ser, β-lactams (*e.g.*, oxacillin and meropenem) alone and in combination against MRSA strains, including clinical and standard isolates, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Bacterial strains

Three standard MRSA strains, 17 clinical MRSA strains and 1 standard methicillin-susceptible *S. aureus* (MSSA) strain, 18 clinical MSSA strains, randomly selected from our *S. aureus* strain collection from hospitals in China during 2005–2013 were included in the current study. MLST was performed as described by Enright et al.[20] previously. The seven housekeeping gene sequences were compared with known alleles from the MLST database (https://pubmlst.org/saureus/), and the allelic profiles and ST types were determined based on the database. The polymorphic X region of *spa* gene was amplified as previously described[21], and the *spa* type was determined by submitting the sequencing data to the *S. aureus* type database (http://spaserver.ridom.de). The genotypic features of the isolates are shown in Supporting Information Table S1.

2.2. Antibiotics, D-amino acids and culture medium

D-Amino acids were purchased from Sigma–Aldrich (St. Louis, MO, USA). The stock solutions were prepared in water (for *in vitro* experiments) or 0.85% NaCl (for *in vivo* experiments), and sterilized by filtration after adjusting pH to 7.0. Antibiotics were purchased from National Institute for Food and Drug Control (National Institutes for Food and Drug Control, Beijing, China).

2.3. Laboratory animals

CD-1 (ICR) mice (female, 18–20 g for systemic infection model, and 24–26 g for neutropenic thigh infection model) were purchased from Vital River Laboratories (Beijing, China). All animals were housed under controlled humidity (30%–70%), temperature (22 ± 3 °C) and a 12-h light-dark cycle. Animals had free access to food and water during the study. All the animal studies complied with the ARRIVE guidelines, and all experiments were approved by Animal Research Committee of the Institute of Medicinal Biotechnology (Beijing, China).

2.4. Minimal inhibitory concentration (MIC) determination

MICs were determined by broth microdilution method as recommended[22]. The final inoculum in each well was about 5×10^5 CFU/mL. The microtiter plates were incubated at 35 °C for 24 h, and the results were recorded by naked eyes.

2.5. Checkerboard assay

Seventeen clinical MRSA isolates and 3 standard MRSA isolates (*S. aureus* ATCC 33591, ATCC 43300 and N315) were used. The test concentrations were D-Ser: 0, 2.5, 5, 10, 20, 40, 80, and 100 mmol/L (The concentrations of D-Ser for MIC determination were 62.5, 125, 250, 500, 1000, and 2000 mmol/L); oxacillin (OXA): 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 mg/L and meropenem (MEM): 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 mg/L. The combination effect of D-Ser with OXA or MEM was determined by calculating the fractional inhibitory concentration index (FICI) using the concentration

combinations with highest combination effects (Eq. (1)):

$$\mathrm{FICI} = (\text{MIC of drug A in the combination}/\text{MIC of drug A alone}) + (\text{MIC of drug B in the combination}/\text{MIC of drug B alone}). \quad (1)$$

The antimicrobial combination was defined to be synergistic when the FICI was ≤ 0.5; indifferent when $0.5 < \mathrm{FICI} < 4$; antagonistic when $\mathrm{FICI} \geq 4$[23]. The experiments were performed in duplicate on different days.

2.6. Time–kill curves

Time–kill curve assays were performed with standard MRSA ATCC 43300 and N315, as well as clinical MRSA isolates (0603 and 0850), according to method described by Lu et al.[24] with minor modifications. Briefly, an overnight culture of each isolate was diluted with 3 mL CAMH broth to a final concentration of $\sim 10^6$ CFU/mL. Then D-Ser (at 20 mmol/L), antibiotics (at the lowest concentrations that can show synergistic effects when combined with D-Ser) alone and in combinations were added. Viable cell counts were determined at 0, 2, 4, 6, 8 and 24 h after incubation at 35 °C by plating 10 μL serial diluted samples onto MH agar plates in triplicate. The results were recorded as $\log_{10}$ CFU/mL. Synergy was defined as ≥ 2 $\log_{10}$ CFU/mL decrease at 24 h incubation in the combination treatment in comparison to single antibiotic alone exposure[23].

2.7. Murine systemic infection model

Representative MRSA strains, ATCC 43300, N315 and 0850, were used in this model. After intraperitoneal injection of 0.5 mL bacterial suspension (100% minimum lethal dose) in 5% mucin, mice were administered with D-Ser, antibiotics alone or in combinations through subcutaneous injection at 1 and 6 h after infection (8–10 animals/group). The doses of OXA were 2.5, 5, and 10 mg/kg for N315 strain and 5, 10, and 25 mg/kg for ATCC 43300; the doses of MEM were 0.5, 1, and 2.5 mg/kg for N315 and 25, 50, and 100 mg/kg for 0850. Animal deaths were recorded for 7 days and the survival rates were calculated.

2.8. Murine neutropenic thigh infection model

The experiment was carried out according to previously described methods with some modifications[25,26]. Briefly, CD-1 (ICR) female mice were rendered neutropenic by intraperitoneally dosed with cyclophosphamide on day 4 (150 mg/kg) and day 1 (100 mg/kg) prior to infection[27]. The right thighs were then infected intramuscularly with 100 μL of overnight cultures of MRSA N315 ($4–7 \times 10^5$ CFU per thigh). Mice then received: (1) no treatment (control group); (2) D-Ser alone at 4 mmol/kg (administrated at 2, 10, 18, 26, 34 and 42 h post-infection); (3) OXA alone at 20 or 50 mg/kg (administrated at the same time-points as D-Ser); (4) OXA+D-Ser 4 mmol/kg; (5) MEM alone at 25 or 50 mg/kg (administrated at 2, 8, 14, and 20 h after infection); (6) MEM+D-Ser 4 mmol/kg. The OXA and MEM doses were chosen according to previous reports[28,29]. Mice were sacrificed at 24 h for MEM groups and 48 h for OXA groups after infection. Right thigh muscles were then aseptically excised, homogenized, serially diluted and plated on MH agar plates for CFU counts. Bacterial colony counts were expressed as mean $\log_{10}$ CFU/thigh (±SEM).

2.9. The docking assay

Penicillin-binding protein 2a (PBP2a), a protein exists in MRSA strains, can lead to resistance by its low affinity with β-lactams but normal affinity to natural substrate as well as existing activity on catalyzing cell wall synthesis. As we speculated that the effect of D-Ser might be related with PBP2a, we compared the binding activity of natural substrate with PBP2a as well as changed substrate after adding D-Ser with PBP2a by docking assay. Using crystal structure of PBP2a (PBD entry: 1VQQ) as receptor, we compared the binding activity of D-Ala-D-Ala and D-Ala-D-Ser by Discovery Studio 4.5. The binding modes for dipeptides towards the binding site of PBP2a were generated by MOE (Molecular Operating Environment) version 2009.10. The docking was performed through the "Dock" module and scored using LibDock scoring system. The higher the score, the better affinity of the dipeptide with the protein.

2.10. Statistical analysis

Statistical analysis was performed by SPSS v16.0. P values were calculated using one-way ANOVA to compare the differences between each pair of groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. In vitro activity of D-Ser in combinations against MRSA and MSSA strains

The MICs of 12 different β-lactams alone and in combination with D-Ser at 20 mmol/L against MRSA ATCC 43300 are summarized in Table 1. The MICs of β-lactams against ATCC 43300 were from 8 to > 1024 mg/L. Interestingly, the MICs of the tested β-lactams against the studied MRSA strain were significantly reduced (8 to >128-folds) with addition of D-Ser at 20 mmol/L. MEM (meropenam, usually not used alone in MRSA infection) and OXA (oxacillin, traditionally considered "inactive" against MRSA) were then chosen as the representative antibiotics in further evaluation for the purpose of reusing them.

In contrast, D-Ser showed very limited sensitization effect on oxacillin and meropenem against MSSA strains. MICs of the

Table 1 MICs of β-lactams in combination with D-Ser against MRSA ATCC43300.

Antibiotics	MIC (mg/L) at D-Ser of		Fold reduced
	0 mmol/L	20 mmol/L	
Cefepime	32	2	16
Cefuroxime	16	1	16
Cephalothin	16	0.25	64
Cefixime	>1024	8	>128
Ceftazidime	64	8	8
Cefotaxime	64	1	64
Ceftriaxone	128	2	64
Ampicillin	16	2	8
Oxacillin	16	0.125	128
Penicillin	16	2	8
Meropenem	8	0.125	64
Ertapenem	8	0.125	64

antibiotics against the 19 MSSA strains were generally 2–4 folds reduced (Supporting Information Table S2).

3.2. Checkerboard assay

The checkerboard assay was conducted using D-Ser and the representative β-lactams (MEM and OXA) against all studied MRSA strains and the results are summarized in Table 2. D-Ser alone showed minor antibacterial activity with MICs of 500–2000 mmol/L. OXA and MEM alone had weak inhibition effects on bacterial growth, as MICs can be as high as 1024 and 128 mg/L respectively. However, when combined with D-Ser, the MICs of OXA and MEM against the MRSA strains were reduced in a concentration dependent manner of D-Ser. MICs of ≤ 0.25 mg/L for OXA and ≤ 0.06 mg/L for MEM were observed when combined with 100 mmol/L D-Ser against the studied MRSA strains (data not shown). FICIs were calculated using the concentration combinations with highest combination effects, that is 1/256–1/32 MIC of OXA or 1/256–1/2 MIC of MEM in combination with 10–100 mmol/L D-Ser. FICIs were 0.024–0.216 and 0.018–0.580 for OXA/D-Ser combination and MEM/D-Ser combination respectively. According to the results, synergistic effects existed in 20 (OXA/D-Ser combination) and 19 (MEM/D-Ser combination) MRSA strains.

3.3. Time–kill curve analysis of OXA/D-Ser and MEM/D-Ser

As shown in Fig. 1, OXA and MEM alone at sub-MIC levels showed modest bactericidal activity. However, the OXA/D-Ser and MEM/D-Ser combinations demonstrated enhanced bactericidal activities against all tested MRSA strains, with viable cell counts significantly reduced by 2.97–3.36 and 2.31–3.96 $\log_{10}$ CFU/mL respectively at 24 h compared with the corresponding OXA and MEM alone groups. These data suggest synergistic bactericidal activities of the combinations.

3.4. OXA/D-Ser and MEM/D-Ser combinations enhanced animal survival rates in murine systemic infection model

Generally, the combination of OXA or MEM with D-Ser increased animal survival rates as compared to OXA and MEM alone groups in a concentration dependent manner of D-Ser (Fig. 2). Combination of D-Ser (4 mmol/kg) with OXA significantly increased the animal survival rates, from 0% to 62.5% (OXA at 2.5 mg/kg), 62.5% to 100% (OXA at 5 mg/kg), 75% to 100% (OXA at 10 mg/kg) for MRSA N315 strain infection (Fig. 2A), and from 10% to 40% (OXA at 5 mg/kg), 10% to 50% (OXA at 10 mg/kg), 10% to 100% (OXA at 25 mg/kg) for MRSA ATCC43300 strain infection (Fig. 2B). The combination of D-Ser (4 mmol/kg) with MEM also significantly increased animal survival rates, from 0% to 60% (MEM at 0.5 mg/kg), 10% to 80% (MEM at 1 mg/kg), 50% to 100% (MEM at 2.5 mg/kg) for MRSA N315 strain infection (Fig. 2C), and from 0% to 40% (MEM at 25 mg/kg), 0% to 80% (MEM at 50 mg/kg), 10% to 100% (MEM at 100 mg/kg) for MRSA 0850 strain infection (Fig. 2D). D-Ser alone at 1, 2 and 4 mmol/kg had no protection on animals with 100% mortality due to tested MRSA strain infections (data not shown).

3.5. In vivo antibacterial activity of OXA/D-Ser and MEM/D-Ser combinations in murine neutropenic thigh infection model

As shown in Fig. 3A, D-Ser alone at 4 mmol/kg didn't reduce colony counts in thigh *vs.* the control group, OXA alone at

Table 2 MICs and FIC indexes of D-Ser with β-lactam antibiotics against MRSA strains.

Strains	OXA/D-Ser			MEM/D-Ser		
	MIC in single use (mg/L)/(mmol/L)	MIC in combination (mg/L)/(mmol/L)	FIC index	MIC in single use (mg/L)/(mmol/L)	MIC in combination (mg/L)/(mmol/L)	FIC index
MRSA 0501	512/500	4/40	0.088	128/500	4/40	0.111
MRSA 0516	1024/2000	4/40	0.024	64/2000	1/40	0.036
MRSA 0520	256/2000	2/40	0.028	16/2000	0.25/100	0.066
MRSA 0533	512/2000	4/40	0.028	32/2000	0.25/40	0.028
MRSA 0603	256/500	2/10	0.028	16/500	0.25/40	0.096
MRSA 0616	64/500	1/100	0.216	4/500	2/40	0.580
MRSA 0623	512/2000	8/40	0.036	32/2000	0.25/20	0.018
MRSA 0629	512/1000	4/40	0.048	32/1000	0.5/20	0.036
MRSA 0637	512/2000	8/40	0.036	32/2000	0.125/40	0.024
MRSA 0826	1024/1000	8/40	0.048	32/1000	0.25/100	0.108
MRSA 0832	512/1000	2/40	0.044	32/1000	1/40	0.071
MRSA 0836	512/500	16/20	0.071	32/500	1/40	0.111
MRSA 0844	1024/2000	4/40	0.024	32/2000	1/40	0.051
MRSA 0845	512/2000	8/40	0.036	32/2000	0.25/100	0.058
MRSA 0848	512/2000	4/40	0.028	32/2000	1/40	0.051
MRSA 0850	512/500	2/40	0.084	32/500	2/20	0.103
MRSA 0852	512/1000	8/40	0.056	32/1000	0.25/100	0.108
ATCC 33591	256/1000	8/20	0.051	32/1000	2/20	0.083
ATCC43300	64/500	0.25/20	0.044	4/500	0.06/40	0.095
MRSA N315	64/500	0.5/40	0.088	8/500	0.25/40	0.111

The test concentrations were D-Ser: 0, 2.5, 5, 10, 20, 40, 80, and 100 mmol/L; oxacillin (OXA): 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 mg/L and meropenem (MEM): 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 mg/L. We found that with the increase of D-Ser concentration, the MICs of the antibiotics decreased further, MICs of oxacillin and meropenem were as low as ≤ 0.25 mg/L and ≤ 0.06 mg/L with 100 mmol/L D-Ser.

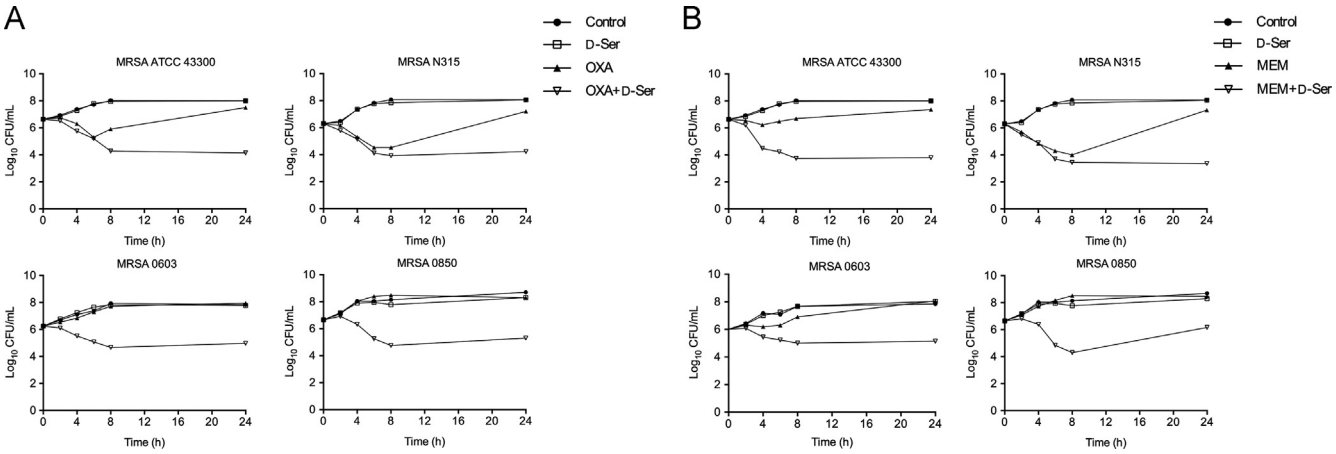

Figure 1 Time–kill curves against MRSA strains ATCC43300, N315, 0603 and 0850 for combination of D-Ser (20 mmol/L, equivalent to 1/25 MIC) with OXA (Panel A) and MEM (Panel B). For Panel A, the OXA doses were: 1/32 MIC for MRSA ATCC 43300, 1/4 MIC for MRSA N315, 1/32 MIC for MRSA 0603, 1/8 MIC for MRSA 0850. For Panel B, the MEM doses were: 1/4 MIC for MRSA ATCC43300, 1/2 MIC for MRSA N315, 1/2 MIC for MRSA 0603, 1/2 MIC for MRSA 0850.

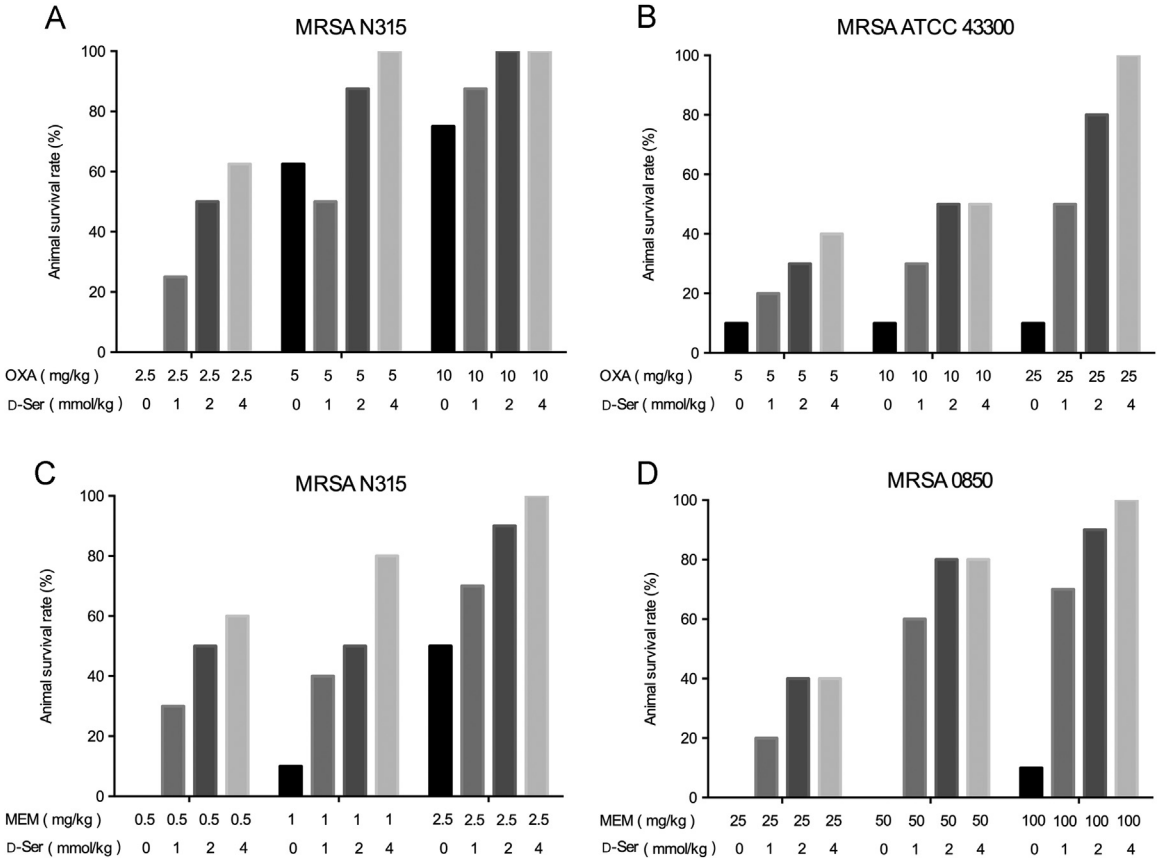

Figure 2 Animal survival rates of OXA, MEM alone and in combination with D-Ser in murine systemic infection model. Panel A: OXA ± D-Ser against MRSA N315; Panel B: OXA ± D-Ser against MRSA ATCC 43300; Panel C: MEM ± D-Ser against MRSA N315; Panel D: MEM ± D-Ser MRSA 0850. The infection doses were 7.91×10^3 CFU per mouse for panel A, 6.5×10^5 CFU per mouse for panel B, 1.87×10^4 CFU per mouse for panel C and 1.2×10^5 CFU per mouse for panel D.

20 mg/kg had weak activity in curing thigh infection as CFU counts had no significant difference *vs*. the untreated control group ($P > 0.05$). However, combination of D-Ser at 4 mmol/kg significantly enhanced the antibacterial activity of OXA ($P < 0.001$ *vs*. untreated control and D-Ser alone groups, and $P < 0.05$ *vs*. OXA alone group). The antibacterial activity of OXA/D-Ser and MEM/D-Ser combinations was then evaluated in detail. As shown in Fig. 3B, OXA alone at 20 mg/kg or 50 mg/kg were ineffective in curing thigh infections, with CFU counts similar to the control group ($P > 0.05$). Addition of D-Ser at 4 mmol/kg significantly enhanced the antibacterial activity of OXA, with CFU counts significantly reduced compared to OXA alone groups ($P < 0.001$). With addition of

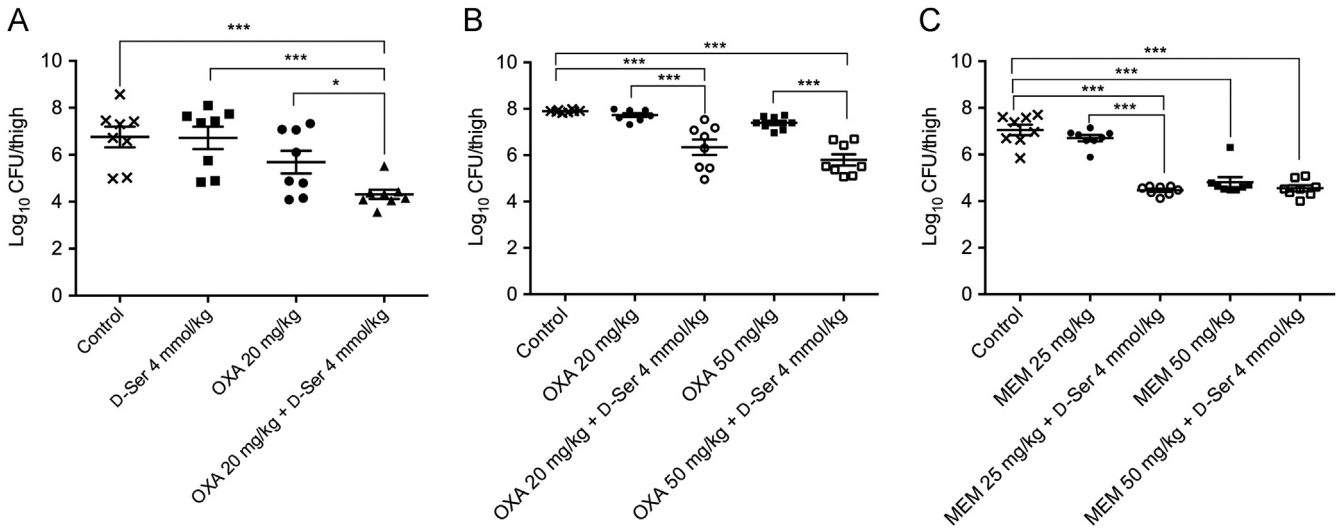

Figure 3 Efficacy of OXA, MEM alone and in combination with D-Ser in murine neutropenic thigh infection model caused by MRSA N315. Infection doses: 4.0×10^5 CFU per thigh for panel A, 7.0×10^5 CFU per thigh for panel B and 6.3×10^5 CFU per thigh for panel C. Oneway-ANOVA test was used for statistical analysis. $^{*}P < 0.05$, $^{***}P < 0.001$.

4 mmol/kg D-Ser, 50 mg/kg OXA group showed further enhanced antibacterial activity than 20 mg/kg OXA group, with CFU counts of 1.85×10^6 CFU per thigh *vs.* 8.79×10^6 CFU per thigh respectively ($P > 0.05$). As shown in Fig. 3C, MEM alone at 25 mg/kg was ineffective in this animal model, while the addition of D-Ser (4 mmol/kg) significantly enhanced the antibacterial activity ($P < 0.001$ *vs.* control or MEM alone). MEM alone at 50 mg/kg was effective in reducing MRSA counts, while addition of D-Ser (4 mmol/kg) showed a little further effect, with CFU counts of 2.86×10^5 CFU per thigh *vs.* 4.79×10^4 CFU per thigh respectively ($P > 0.05$). Notably, with addition of 4 mmol/kg D-Ser, 50 mg/kg MEM group showed no further enhanced antibacterial activity than 25 mg/kg MEM group ($P > 0.05$).

3.6. *Effects of D-Ala and D-Glu on the sensitization effect of D-Ser*

As shown in Supporting Information Table S3, in the absence of D-Ala or D-Glu, 20 mmol/L D-Ser significantly sensitized the antibacterial activity of OXA or MEM, with MICs decreased from 16- to >32-fold. However, with the adding of 20 mmol/L D-Ala in the medium, the sensitization effect of D-Ser almost disappeared, while adding of D-Glu at concentration as high as 80 mmol/L didn't show any obvious effect.

3.7. *The docking results of dipeptides with PBP2a*

As shown in Supporting Information Table S4, the LibDock_Score of D-Ala-D-Ala and D-Ala-D-Ser were 70.9502 and no poses respectively, hence we predicted that D-Ala-D-Ala had a good affinity with PBP2a, while D-Ala-D-Ser had no (or very weak) affinity with the same protein.

3.8. *The sensitization activity of D-Ser with β-lactam antibiotics with relatively selective affinities for different PBPs*

Cefaclor and cephradine are representative compounds which have relatively selective affinities for PBP3, while cefoxitin for PBP4. As shown in Supporting Information Table S5, the level of sensitization activity of D-Ser varied with β-lactam antibiotics. Addition of 40 mmol/L D-Ser could enhance the antibacterial activity of all three cephalosporins, with MICs reduced by 2–128 times. Notably, MICs of cefoxitin in combination with 40 mmol/L D-Ser were reduced to larger degrees than cefaclor and cephradine. The reduced folds of cefoxitin with addition of 40 mmol/L D-Ser were generally 8–16 times larger than cefaclor, and 4–8 times larger than cephradine.

4. Discussion

MRSA has the ability to cause a range of serious problems since its first emergence and it now still maintains a high infection rate worldwide. β-Lactam antibiotics, widely used in the treatment of infections caused by *S. aureus*, have a limited effect in treating infections caused by MRSA strains. This highlights the need of development of novel treatments. Research institutes, including our laboratory, have been searching for novel targets[30], potential alternatives to antibiotics[31], as well as effective combinations of antibiotics with agents such as manuka honey, isoliquiritigenin, plant essential oils and plant extracts[24,30–34].

D-Ser was reported to be able to increase the susceptibility of MRSA strain to methicillin by replacing D-Ala residue of the peptidoglycan stem peptides[19]. In order to determine the usability of D-Ser as sensitizer for β-lactams against MRSA strains, we evaluated the *in vitro* and *in vivo* antibacterial activity of D-Ser with different β-lactams (especially OXA and MEM) against different MRSA strains, including standard and clinical isolates. The MIC results demonstrated that D-Ser had sensitization effects with all tested β-lactams. Checkerboard assay with D-Ser/OXA or D-Ser/MEM combinations further confirmed the synergistic effects of the combinations in clinical MRSA isolates. The synergistic bactericidal activity of the combinations was then demonstrated by time–kill curve analysis. More importantly, the *in vivo* antibacterial activity of the combinations was manifested in murine systemic infection and neutropenic thigh infection models. Notably, in murine neutropenic thigh infection model, with addition of

4 mmol/kg D-Ser, the antimicrobial activity of OXA at 20/50 mg/kg and MEM at 25/50 mg/kg showed no significant difference. Considering the potential resistance development in future, the lowest effective concentration is recommended, and more precise data are needed for future preclinical as well as clinical studies.

Besides, D-Ser also had sensitization activity in combination with ceftaroline. Ceftaroline, currently used as treatment of critical *S. aureus* infections, received FDA approval several years ago, now facing challenges that non-susceptible *S. aureus* is on the rise worldwide[35,36]. We studied and found that addition of 40 mmol/L D-Ser could enhance the antibacterial activity of ceftaroline against MRSA strains, with the MICs reduced by 2–16 times (data not shown). The results suggested that D-Ser might have a promising application in future.

β-Lactams inhibit bacterial growth by competing with D-Ala-D-Ala termini to bind with active sites of PBPs and interfering with cell wall assembly[37]. Methicillin resistance of *S. aureus* is associated with PBP2a[38], which can mediate cell wall assembly with low *β*-lactam affinity[39]. Our results demonstrated that the sensitization effect of D-Ser in MSSA (Supporting Information Table S2) was much weaker than in MRSA strains (Table 1), suggesting the possible involvement of PBP2a in D-Ser sensitization in MRSA.

It was reported that addition of D-Ser could replace D-Ala in D-Ala-D-Ala termini of the peptidoglycans, and result in sensitization of the bacteria to methicillin[19]. In consistent with this report, our study showed that the sensitization effect of D-Ser on OXA and MEM against MRSA could be abolished with addition of the same concentration of D-Ala, while addition of D-Glu with even higher concentration had no effect (Supporting Information Table S3). Herein, the sensitization mechanism of D-Ser on *β*-lactams against MRSA may be related to the replacement of D-Ala residue in the peptidoglycans and the formation of D-Ala-D-Ser. D-Ala-D-Ser may not be recognized by PBPs, especially PBP2a, and result in unnormal transpeptidation process as well as cell wall assembly, leading to increased sensitivity of MRSA to *β*-lactams. Results by Thorsing et al.[40] also demonstrated that the exposure to thioridazine can lead to sensitization of MRSA strain to *β*-lactams through change of intracellular amino acids, which lead to unnormal peptidoglycan precursor formation. To verify the hypothesis that we made on PBP2a, docking study between protein and dipeptides (D-Ala-D-Ala or D-Ala-D-Ser) was conducted. The binding activity of D-Ala-D-Ala and D-Ala-D-Ser to PBP2a predicted by the dock module (Supporting Information Table S4) showed that compared with D-Ala-D-Ala, D-Ala-D-Ser had no detectable affinity to PBP2a.

PBP2a cannot accounts for the full mechanism of resistance present in MRSA strains, though it dose play an important role. Researches had been done that different *β*-lactam antibiotics had relatively selective affinities for PBPs[41]. Sieradzki et al.[42] found that cephradine, a relatively selective inhibitor of PBP3, was effective in reducing methicillin resistance, indicating that PBP3 might participate in methicillin resistance through some cooperative functioning with PBP2a. However, recent researches through genome sequencing and molecular genetic studies revealed that PBP4 could mediate *S. aureus* resistance of *β*-lactam antibiotics[43,44]. Our results (Supporting Information Table S5) demonstrated that although D-Ser had sensitization activity with all three antibiotics, D-Ser in combination with cefoxitin, a relatively selective inhibitor of PBP4, had a more profound sensitization activity than relatively selective inhibitors of PBP3, *i.e.*, cefaclor and cephradine. We hypothesize that several PBPs might participate in *β*-lactam resistance, while PBP4 might act primarily as an efficient transpeptidase in addition to PBP2a.

D-Ser is well known for its nephrotoxicity due to oxidative stress caused by hydrogen peroxide, a byproduct of D-amino acid oxidase (DAAO)-mediated metabolism of D-Ser[45,46]. However, in our initial safety experiment in mice, D-Ser alone and in combination with MEM or OXA didn't demonstrate obvious toxicity. Administration of D-Ser 10, 20, and 40 mmol/kg, MEM 100 mg/kg+D-Ser 4 mmol/kg or OXA 100 mg/kg+D-Ser 4 mmol/kg didn't cause any death in mice over 7 days. No significant body weight change was observed between treatment and control groups during the 7 days experiment period. And there was also no significant difference of urea nitrogen and creatinine in serum between the treatment and control groups at 24 and 48 h after dosing (Supporting Information Fig. S1). Co-administration of DAAO inhibitors with D-Ser was believed to be able to minimize its metabolism by DAAO, and hence to improve D-Ser bioavailability and reduce nephrotoxic effects. Indeed, oral administration of DAAO inhibitor CBIO in conjunction with D-Ser enhanced the plasma and brain levels of D-Ser in rats compared to the oral administration of D-Ser alone[47]. Hence, we expected that co-administration of DAAO inhibitors with D-Ser+antibiotic combinations can reduce the effective dose of D-Ser and the possible toxicity caused by oxidation of D-Ser.

5. Conclusions

In summary, bacterial resistance is an increasingly serious problem. While new antibiotic development is time-consuming, combination therapy of bioactive molecules with existing antibiotics is a good way to solve this conflict. Considering the great *in vitro* and *in vivo* activity as well as relatively good safety of OXA/D-Ser and MEM/D-Ser combinations against MRSA, D-Ser/*β*-lactam combinations may have the potential to be new treatment strategies against MRSA infections.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2019.01.017.

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