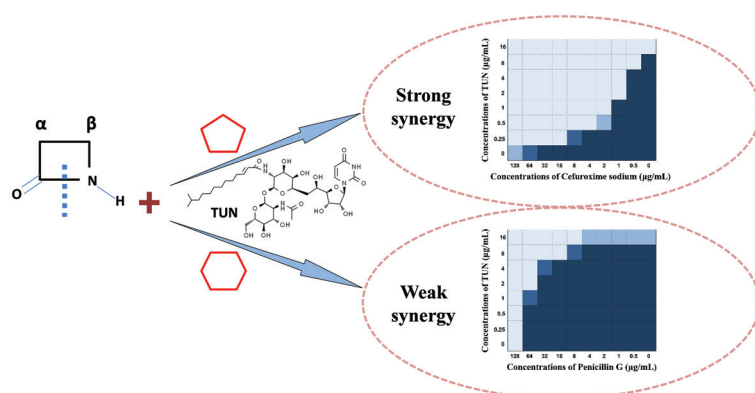




ORIGINAL ARTICLE

Tunicamycin and β -Lactams with Five Membered Ring Structures Have Stronger Synergistic Effects Against Gram-Positive Bacteria

Graphical Abstract



Highlights

- TUN restored the bactericidal activity of specific cephalosporins or penicillins against methicillin-resistant *S. aureus*.
- TUN showed better synergy (FIC index < 0.5) with β -lactam antibiotics with a five-membered ring on the group adjacent to the acetamino group, including cephalosporins or penicillins.
- The effective synergistic concentration of TUN was much lower than the toxic concentration; therefore, TUN has excellent potential for development.

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Brief Statement

This study demonstrated a novel synergistic strategy combining tunicamycin (TUN) with specific β -lactam antibiotics containing a five-membered ring structure. Both drugs affect bacterial cell wall synthesis. This strategy exhibited better synergistic effects against methicillin-resistant *Staphylococcus aureus* (MRSA). Crucially, these effects occurred at non-cytotoxic doses of TUN. Our findings might provide a preclinical foundation for structurally optimized β -lactam/TUN combinations against multidrug-resistant gram-positive infections.

Tunicamycin and β -Lactams with Five Membered Ring Structures Have Stronger Synergistic Effects Against Gram-Positive Bacteria

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Abstract

Objective: The prevalence of antimicrobial resistance is increasing among gram-positive bacteria, particularly the resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) to β -lactam antibiotics.

Methods: Checkerboard minimum inhibitory concentration (MIC) assays, time-killing assays, enzyme inhibition assays, hemolysis tests, and cytotoxicity analyses were performed to confirm the synergistic effects of tunicamycin (TUN) in combination with β -lactam antibiotics at levels without potential cytotoxicity.

Results: TUN at $\leq 1 \mu\text{g/mL}$ decreased the MICs of cephalosporins or penicillins against MRSA strain USA300, which became sensitive. TUN showed synergistic effects with cefuroxime sodium (FIC index ≤ 0.19) against tested gram-positive bacterial strains of both human clinical and animal origin. Time-killing assays indicated that the combination treatment eliminated MRSA 3–9 h after inoculation. Furthermore, we confirmed the better synergy of specific β -lactam antibiotics at potentially cytotoxicity-free concentrations in combination with TUN against β -lactam-resistant gram-positive microorganisms.

Conclusion: This study provides an experimental basis for combination treatment with TUN and β -lactam antibiotics with specific chemical structures for the clinical treatment of gram-positive bacterial infections.

Key words: Tunicamycin, β -lactam antibiotics, methicillin-resistant *Staphylococcus aureus*, anti-infection

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INTRODUCTION

The escalating mortality rate attributable to antimicrobial-resistant bacterial infections has garnered substantial public health concern and emerged as a critical research focus among global disease control and prevention agencies [1]. Resistant bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA); vancomycin-resistant *Enterococcus* spp.; and several multidrug-resistant *Enterobacteriaceae*, such as

New Delhi metallo- β -lactamase-positive *Enterobacteriaceae* and mobile colistin resistance-positive *Enterobacteriaceae* [2,3]. MRSA is among the most concerning gram-positive bacteria and has been listed as “high priority” by the World Health Organization [4]. However, the resistance of other gram-positive bacteria, such as *Listeria monocytogenes*, is also increasing [5,6]. Therefore, novel drugs or treatment options are urgently needed to address serious gram-positive pathogen infections.

Antimicrobial resistance restricts clinically available therapeutic alternatives, and consequently increases infection-associated morbidity and mortality rates. Critically, bacterial pathogens often concurrently bear multiple resistance mechanisms against specific antibiotics. The main resistance mechanisms of gram-positive pathogens, such as MRSA, to β -lactam antibiotics involve the initial production of penicillinases (including genome-encoded and plasmid-carried enzymes) and the production of a mutant penicillin-binding protein (PBP2a) encoded by the *mecA* gene [7,8]. MRSA has broad-spectrum resistance to β -lactam antibiotics, including mostly penicillins and cephalosporins [4,9]. However, β -lactams and β -lactamase inhibitors are generally the preferred drugs for treating gram-positive bacterial infections [10].

Tunicamycin (TUN) has antibacterial activity against gram-positive bacteria [11,12]. TUN has been reported to inhibit polyprenyl phosphate N-acetylhexosamine 1-phosphate transferase (PPHP), which initiates the biosynthesis of bacterial peptidoglycan and catalyzes an early step in eukaryotic protein N-glycosylation [13]. Consequently, TUN leads to cellular death through blocking the assembly of bacterial cell wall polysaccharides. Both TUN and β -lactam antibiotics affect the integrity of the bacterial cell wall but have different binding sites. Herein, we explored the synergistic effects of TUN and specific β -lactam antibiotics against severe antibiotic-resistant *Staphylococcus aureus* (*S. aureus*) infections *in vitro*, to lay a foundation for future clinical trials.

MATERIALS AND METHODS

Bacterial strains, culture media, and chemicals

The bacterial strains used in this study, including standard strains (purchased from the American Type Culture Collection (ATCC)) and human clinical or animal origin isolates (collected in Shandong and Jilin, China), are listed in Table 1. All bacteria were stored in sterile 40% glycerol stocks at -80°C and subcultured on agar plates at 37°C before use.

TUN was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. All antibiotics were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Unless otherwise stated, the chemicals and reagents used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Enzyme inhibition assays

Nitrocefin can be used to detect all types of β -lactamase activity [14]. We detected the effect of TUN on the activity of β -lactamase produced by MRSA. In brief, various concentrations of TUN (0–4 $\mu\text{g/mL}$) were mixed in 2 mL culture medium containing 1×10^8 CFU/mL bacterial cells, which were then cultivated for 6 h at 37°C . Subsequently, culture supernatants were collected by centrifugation at 12,000 rpm for 5 min at 4°C ; 75 μL

phosphate-buffered saline and 100 μL of each culture supernatant were mixed with 25 μL nitrocefin; and the mixture was incubated at 37°C for 30 min. The change in absorbance at 492 nm was determined in 96-well plates at room temperature. In addition, various concentrations of TUN were separately coincubated with untreated culture supernatants.

Minimum inhibitory concentration assays, checkerboard minimum inhibitory concentration assays, and fractional inhibitory concentration index determination

The minimum inhibitory concentration (MIC) values for all bacterial isolates were quantitatively assessed with a standardized serial two-fold dilution method, in strict accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) [15,16]. Briefly, two treatments were performed in parallel in the wells of a microplate: one treatment comprised the tested antibiotics (concentrations of 0.5–128 $\mu\text{g/mL}$) without TUN, whereas the other treatment comprised the tested antibiotics (concentrations of 0.5–128 $\mu\text{g/mL}$) plus TUN (0.5 $\mu\text{g/mL}$). For checkerboard MIC tests, 1 μL of bacterial cells was inoculated in 200 μL LB broth (final bacterial concentration of 5×10^5 CFU/mL per well) containing two-fold dilutions of TUN (ranging from 0–64 $\mu\text{g/mL}$ in each column) and two-fold dilutions of β -lactam antibiotics (0–128 $\mu\text{g/mL}$ in each row), then grown for 24 h at 37°C in ambient air. The lowest concentrations of TUN or β -lactam antibiotics resulting in clear cultures were visually determined as the MIC values of TUN or β -lactam antibiotics.

The fractional inhibitory concentration (FIC) index and the significance of the FIC index were calculated as previously described:

$$\text{FIC index} = (\text{MIC value of TUN alone} / \text{MIC value of TUN in combination}) + (\text{MIC value of antibiotic alone} / \text{MIC value of antibiotic in combination}).$$
 FIC index values ≤ 0.5 were considered to indicate synergistic effects; values of 0.5–4 (inclusive) were considered to indicate an absence of interaction; and values > 4 were classified as antagonistic.

Time-killing assays

A time-killing study was performed on two typical MRSA strains [17]. Briefly, overnight cultures of *S. aureus* strains were grown to exponential phase, and 1 μL 1×10^8 CFU/mL bacterial cells were inoculated in the wells of a 96-well plate in LB medium supplemented with TUN (0.5 $\mu\text{g/mL}$), β -lactam antibiotics, β -lactam antibiotics combined with TUN, or solvent control. The concentrations of antibiotics used in the experiments were based on the checkerboard MIC results. Subsequently, the mixed cultures were continuously cultured at 37°C , and samples from each group of mixed cultures were removed and coated onto resistant LB agar plates (methicillin for *S. aureus*) with the ten-fold serial dilution method, to detect

TABLE 1 | MIC values of combination therapies with TUN and various antibiotics, for each tested bacterial isolate.

Species	Source	MIC of TUN (μg/mL)		MIC of cefuroxime sodium (μg/mL)		FIC index
		Alone	Combination	Alone	Combination	
<i>S. aureus</i> ATCC25904	American Type Culture Collection (ATCC)	21.30 ± 9.24	0.50 ± 0.00	53.33 ± 18.48	1.67 ± 0.58	0.06 ± 0.01
<i>S. aureus</i> ATCC29213	American Type Culture Collection (ATCC)	10.70 ± 4.62	0.50 ± 0.00	2.67 ± 1.15	0.17 ± 0.07	0.11 ± 0.02
<i>S. aureus</i> USA400	American Type Culture Collection (ATCC)	16.00 ± 0.00	0.50 ± 0.00	13.33 ± 4.62	0.67 ± 0.29	0.08 ± 0.02
<i>S. aureus</i> 252	American Type Culture Collection (ATCC)	21.30 ± 9.24	0.50 ± 0.00	42.67 ± 18.48	1.67 ± 0.58	0.07 ± 0.02
<i>S. aureus</i> 8325-4	Presented by Professor Timothy J. Foster	8.00 ± 0.00	0.50 ± 0.00	1.67 ± 0.58	0.21 ± 0.07	0.19 ± 0.00
<i>S. aureus</i> ST2032	Collected in Shandong, China ^a	16.00 ± 0.00	0.50 ± 0.00	42.67 ± 18.48	1.33 ± 0.58	0.07 ± 0.02
<i>S. aureus</i> ST2064	Collected in Shandong, China ^a	16.00 ± 0.00	0.50 ± 0.00	32.00 ± 0.00	2.00 ± 0.00	0.09 ± 0.00
<i>S. aureus</i> JL02	Collected in Jilin, China ^b	21.33 ± 9.24	0.50 ± 0.00	53.33 ± 18.48	2.00 ± 0.00	0.07 ± 0.01
<i>L. monocytogenes</i> L96	Presented by Dr. Masao Mitsuyama	16.00 ± 0.00	0.50 ± 0.00	16.00 ± 0.00	0.25 ± 0.00	0.05 ± 0.00
<i>S. suis</i> ZY05719	Presented by Dr. Hongjie Fan	5.33 ± 2.31	0.50 ± 0.00	10.67 ± 4.62	0.42 ± 0.14	0.14 ± 0.05
<i>A. baumannii</i> ATCC19606	American Type Culture Collection (ATCC)	85.33 ± 36.95	4.00 ± 0.00	53.33 ± 18.48	53.33 ± 18.48	1.05 ± 0.02
<i>S. typhimurium</i> SL1344	Derived from the virulent strain SL1344	16.00 ± 0.00	0.50 ± 0.00	6.67 ± 2.31	6.67 ± 2.31	1.03 ± 0.00
<i>E. coli</i> Z1487	Collected in Zhejiang, China ^c	256.00 ± 0.00	4.00 ± 0.00	426.67 ± 147.80	426.67 ± 147.80	1.02 ± 0.00
<i>K. pneumoniae</i> K7	Collected in Jilin, China ^b	256.00 ± 0.00	4.00 ± 0.00	512.00 ± 0.00	426.67 ± 147.80	0.85 ± 0.29

All MICs were determined in triplicate, and MIC values are reported as mean ± SD, n = 3. The FICI values less than 0.5 are indicated in bold.

^aObtained from porcine samples in Shandong, China. ^bObtained from the First Hospital of Jilin University in Jilin, China. ^cObtained from human intra-abdominal fluid in Zhejiang, China.

the number of bacteria in each group at various times (0, 1, 3, 6, 9, and 12 h). The number of colonies on the agar plates was recorded after incubation at 37°C for 24 h.

Cytotoxicity analysis

TUN has been reported to have potential toxicity, but whether TUN is cytotoxic at low concentrations required further study. We used mouse monocyte macrophage J774A.1 cells (J774, ATCC), human lung epithelial cells (A549, ATCC), African green monkey SV40 transformation cells (COS-1, ATCC), and mouse peritoneal macrophages to confirm whether TUN (0, 1, 2, 4, 8, or 16 µg/mL) had potential toxicity toward various cells at the combined concentrations. All cells were seeded into

96-well plates at 5×10^4 cells/well and incubated at 37°C in CO₂ incubators overnight. Next, the cells were incubated with various concentrations of TUN for 6 h at 37°C. Subsequently, the 96-well plate was centrifuged at 1000 rpm for 10 min, and the supernatants were collected and used to confirm the cytotoxicity of TUN through lactate dehydrogenase (LDH) activity detection (Roche, Mannheim, Germany). Wells of Triton X-100 treated cells served as a positive control, and wells of untreated cells served as a negative control.

Statistical analysis

SPSS version 19.0 and GraphPad Prism 7 were used to analyze the experimental data and prepare the graphics.

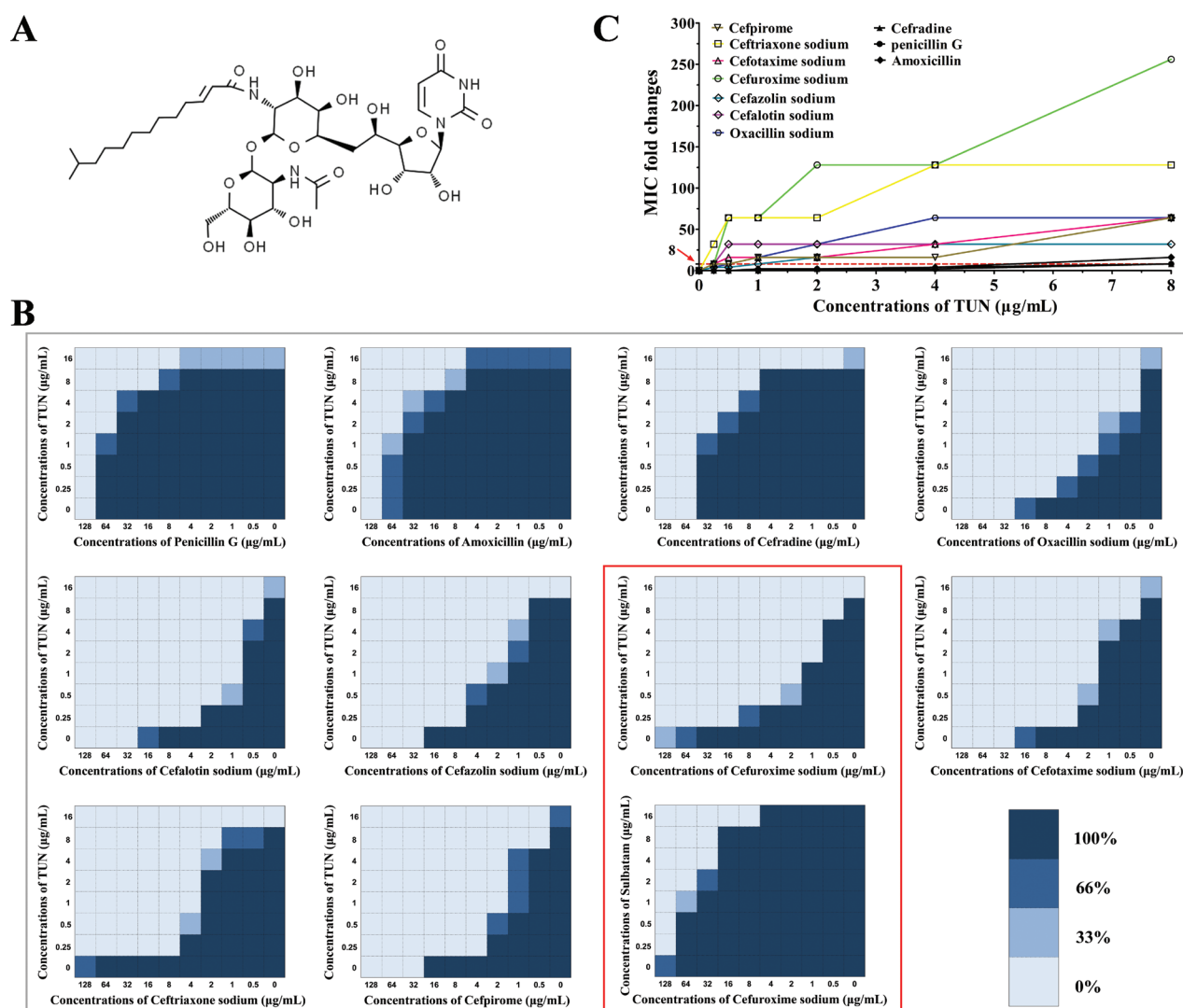


FIGURE 1 | (A) Chemical structure of TUN. (B) Checkerboard MIC analysis indicating the combined effects of TUN and β-lactam antibiotics against MRSA strain USA300. Cefuroxime sodium combined with TUN had better effects than cefuroxime sodium combined with sulbactam (delineated by a red solid line). (C) All MIC fold changes for β-lactam antibiotics with TUN. Colored lines indicate statistically significant MIC decreases for β-lactam antibiotics, whereas black lines indicate non-significant MIC decreases for β-lactam antibiotics. The red dashed horizontal line indicates the threshold for an 8-fold MIC decrease.

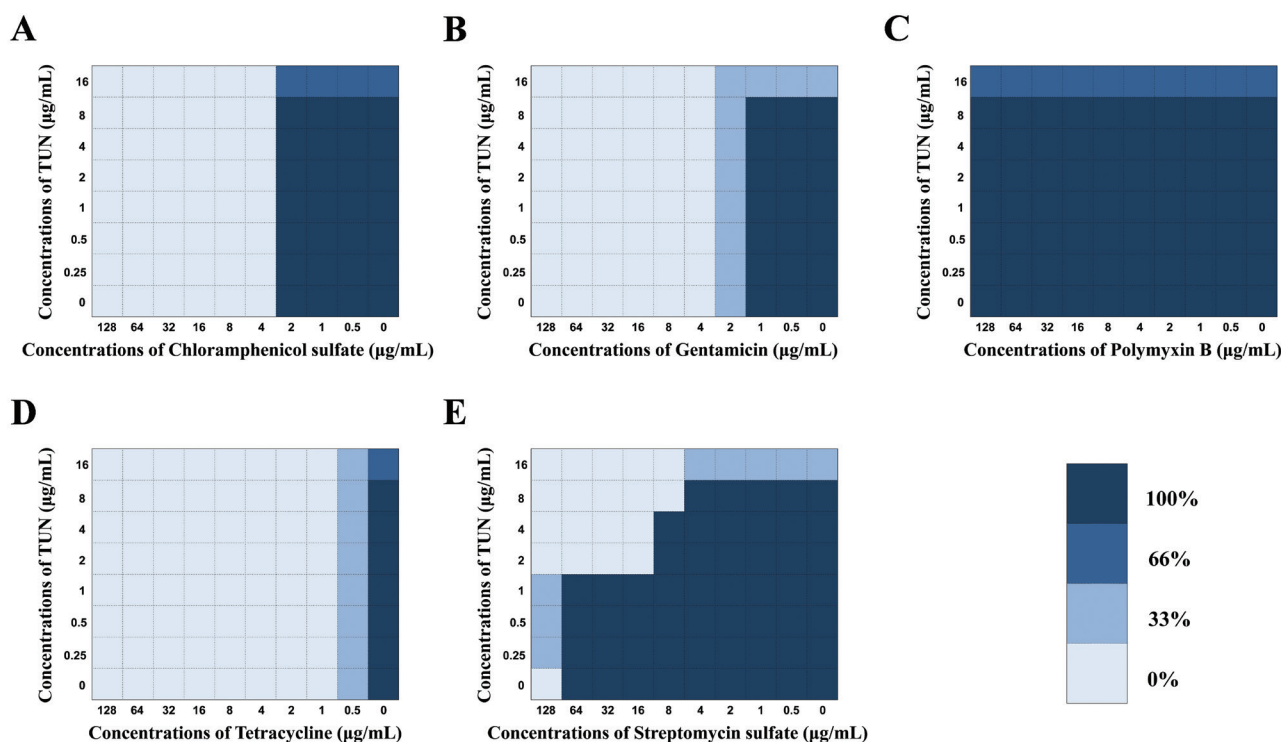


FIGURE 2 | Checkerboard MIC analysis showing the combined effects of TUN and other types of antibiotics against MRSA strain USA300. (A) Chloramphenicol sulfate. (B) Gentamicin. (C) Polymyxin B. (D) Tetracycline. (E) Streptomycin sulfate.

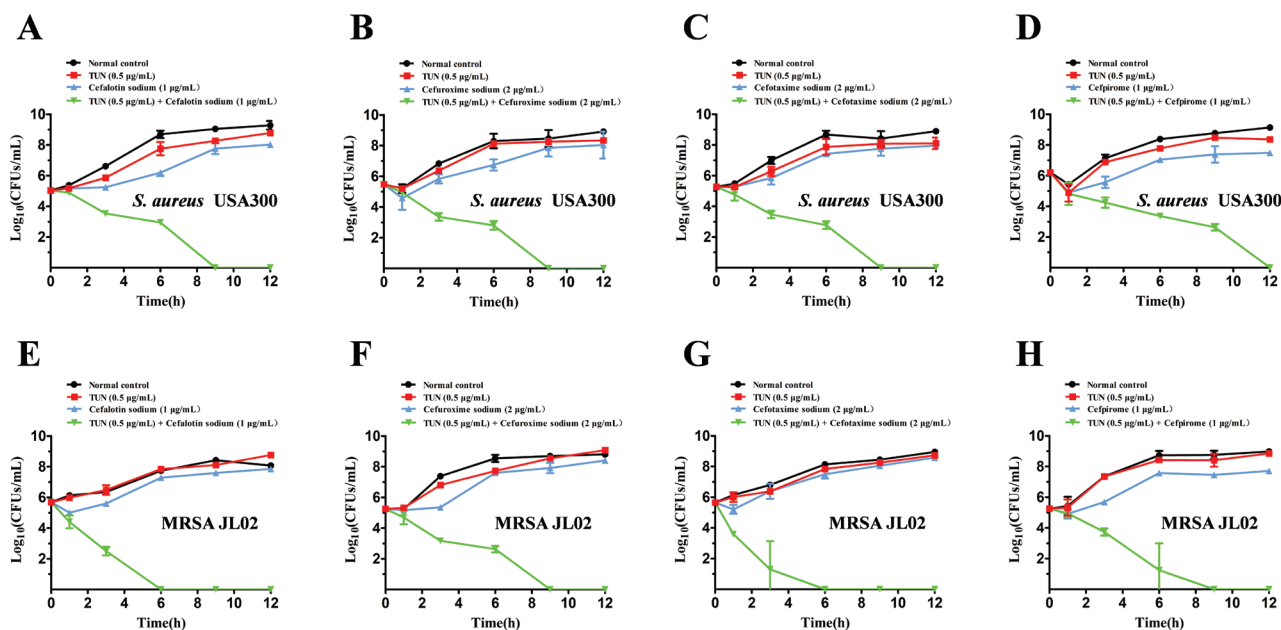


FIGURE 3 | Time-killing assays with various combinations of TUN and cephalosporins (first to fourth generation), compared with single treatments or control treatments (medium alone) against *S. aureus* USA300 (A, B, C, and D) and MRSA JL02 (E, F, G, and H). Values represent the averages of three independent experiments ($n = 3$).

Data are reported as mean $\pm$ SD ($n \geq 3$ independent experiments), and the SD calculations incorporated both technical and biological variability components.

Two-tailed P values less than 0.05 (*) or 0.01 (**) were considered statistically significant or highly statistically significant, respectively.

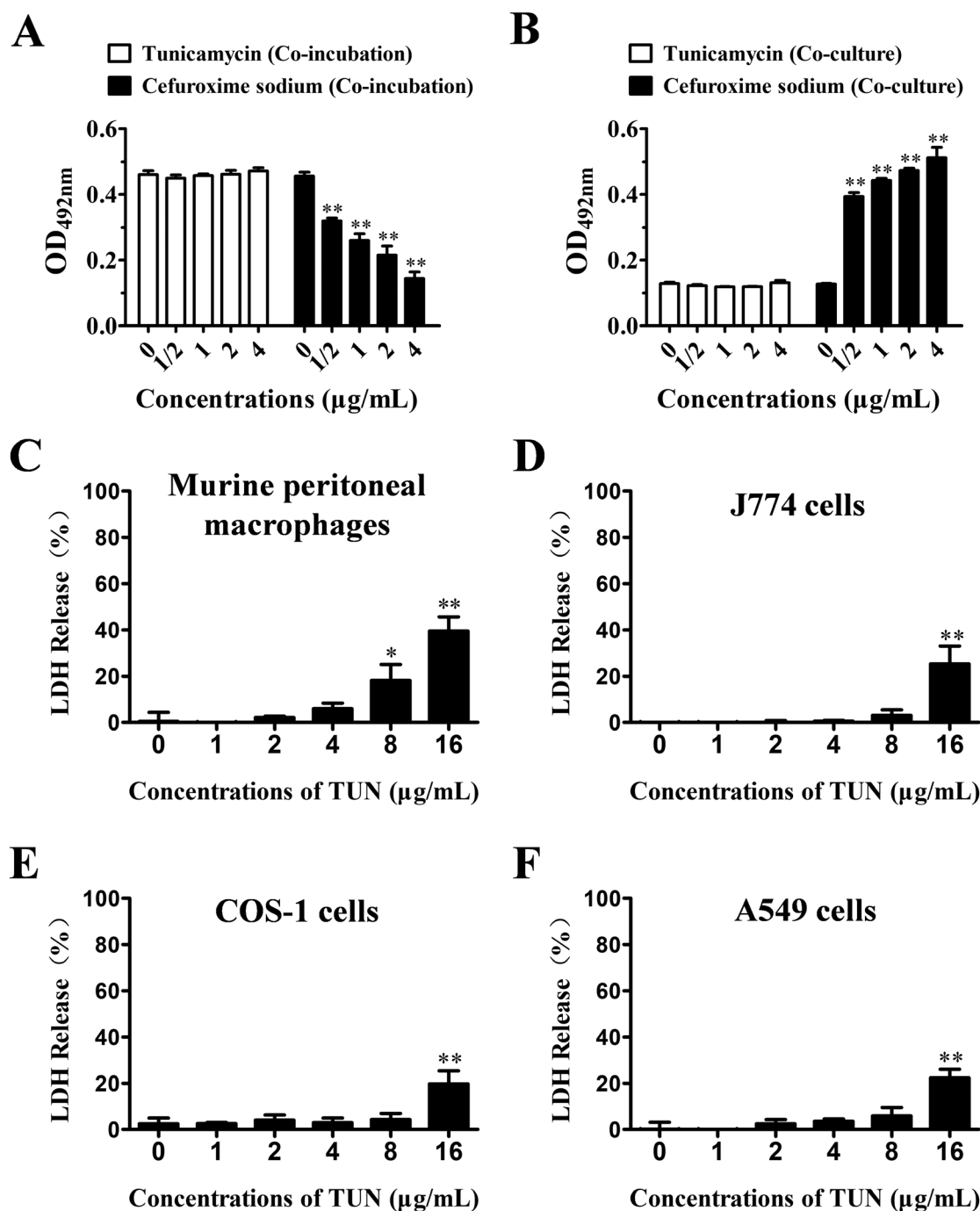


FIGURE 4 | Activity of β -lactamases, detected with enzyme inhibition assays after co-culture (A) or co-incubation (B) with various concentrations of TUN (white) and cefuroxime sodium (black). The cytotoxicity of TUN (0–16 μ g/mL) was detected with LDH release assays performed on supernatants from murine peritoneal macrophages (C), J774 cells (D), COS-1 cells (E), and A549 cells (F). Experiments were performed as three biologically independent experiments, and mean $\pm$ SD is shown; * indicates $P < 0.05$, ** indicates $P < 0.01$.

RESULTS

TUN restores the antibacterial activity of specific β -lactam antibiotics against gram-positive microorganisms

MRSA is among the most common and serious gram-positive bacteria encountered in clinical practice. To determine synergistic effects between TUN (Fig 1A) and

β -lactams, and provide guidance for clinical applications, we first examined the typical MRSA strain USA300. Checkerboard MIC assays indicated that TUN, at relatively low concentrations of 0.5–2 μ g/mL, showed better synergy with most cephalosporins, except cefradine or penicillins, against MRSA strain USA300 (Fig 1B and 1C), whereas no synergy was observed with any other tested antibiotics (except streptomycin sulfate)

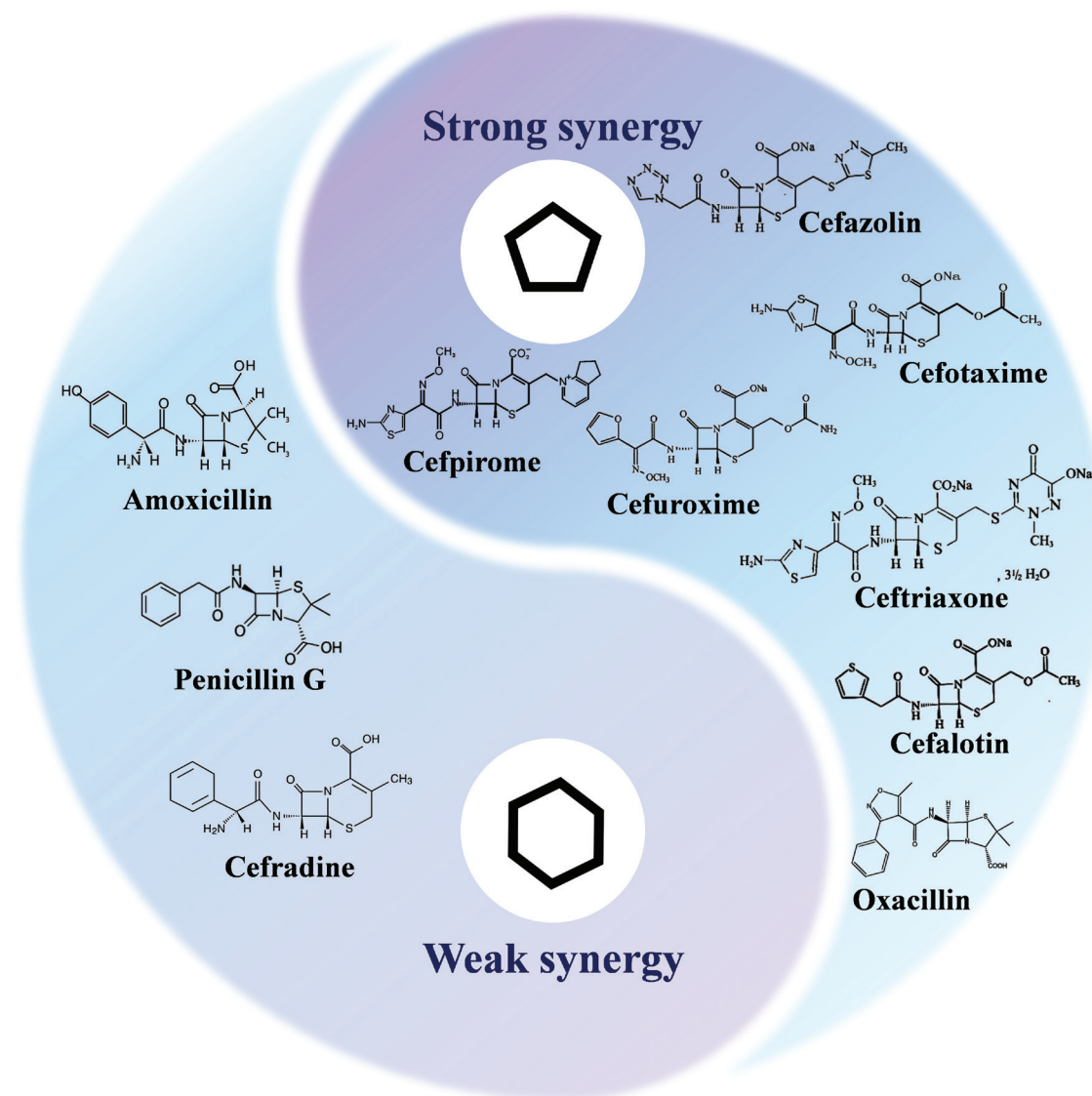


FIGURE 5 | Relationship between β -lactam antibiotic structure and synergy with TUN. The β -lactam antibiotics with a five-membered ring on the group adjacent to the acetamino group showed strong synergy in combination with TUN. In contrast, poor synergy with TUN was observed for β -lactam antibiotics with a six-membered ring.

(Fig 2A–E). All tested gram-positive strains showed MIC fold changes of 8–128 at a TUN concentration of 0.5 $\mu\text{g/mL}$ (FIC index from 0.05 ± 0.00 to 0.19 ± 0.00), whereas the gram-negative strains showed an MIC fold change ≤ 2 (Table 1). The MICs of TUN were 8–32 $\mu\text{g/mL}$ for all tested gram-positive strains. Therefore, TUN increased the antibacterial activity of cephalosporins against most gram-positive bacteria.

In addition, we evaluated the bactericidal effect of TUN in combination with cephalosporin through time-killing assays. TUN or cefuroxime sodium alone had little effect on MRSA growth. However, as expected, combination treatment completely killed the tested isolates after at least 12 h (Fig 3A–3H).

TUN does not affect β -lactamase activity

β -lactamase inhibition assays were performed to determine the effect of TUN on enzymatic activity. As expected, the enzymatic activity of the culture supernatant increased with cefuroxime sodium treatment, in a dose-dependent manner, when the MRSA strain USA300 were cocultured with TUN but not untreated culture supernatant of MRSA strain USA300 co-incubated with TUN (Fig 4A and 4B). These results suggested that cefuroxime sodium treatment induced the expression of β -lactamase in MRSA. However, the absorbance values of the MRSA strain USA300 culture supernatant at $\text{OD}_{492\text{nm}}$ remained unchanged after co-culture or co-incubation with TUN (Fig 4A and 4B). Therefore, TUN

treatment affected neither the expression nor the catalytic activity of β -lactamase.

TUN has no potential cytotoxicity below the effective synergistic concentrations

The cytotoxicity of TUN was preliminarily assessed with LDH assays. TUN, at concentrations less than 4 $\mu\text{g/mL}$ for 6 h, scarcely exhibited cytotoxicity toward cells from various sources (Fig 4C–4F). Therefore, our results confirmed that TUN restored the antimicrobial activity of the main β -lactams against common gram-positive bacteria, when applied at a concentration of 0.5 $\mu\text{g/mL}$, which was far below its cytotoxic concentration.

DISCUSSION

Antibiotics can protect humans and animals against bacterial infection. β -lactam antibiotics, which are among the most frequently used antibiotics in human clinical and livestock farming worldwide, have been widely applied over several decades, because of their advantages of various dosage forms, low cost, and minimal adverse effects [18]. Cephalosporins of the first to the fourth generation act primarily on gram-positive bacteria and have antibacterial activity against several gram-negative bacteria. However, the threat of resistance to β -lactam antibiotics is continually evolving with the emergence of bacterial resistance [19]. Our results also indicated that TUN showed better synergistic effects with cefquinome, a cephalosporin extensively used in veterinary medicine to treat bovine respiratory disease and cow mastitis [20].

Herein, TUN showed better synergistic effects with certain cephalosporins and penicillins with specific chemical structures. TUN had a better synergistic effect with β -lactam antibiotics whose structures contained a five-membered ring on the group adjacent to the acetylamino group. In contrast, when the chemical group was a six-membered ring, poor synergistic effects were observed (Fig 5). TUN is likely to exert enhanced synergistic antibacterial activity by stabilizing the acyl-enzyme intermediate formed after opening of the five-membered ring in β -lactam antibiotics, thereby prolonging the binding time to penicillin-binding proteins. In contrast, β -lactam antibiotics containing a six-membered ring exhibited suboptimal synergy with TUN, because of structural incompatibility. Hence, we speculated that temocillin and cefoxitin, depending on whether their chemical structures contain a five-membered ring, might have better synergistic effects with TUN. Because carbapenems and monocyclic β -lactam antibiotics are used primarily for gram-negative bacterial infections [21], we did not study them herein. Whether the synergistic mechanism of TUN in combination with β -lactam antibiotics might be identified through chemical structure analysis is worthy of further exploration.

No commercial TUN is currently in clinical use. Although structurally modified TUN (nontoxic “TUN-X,X”

analogues of TUN) have not been reported to have diminished bioactivity [22,23], the toxicity of analogues of TUN to different cells is greatly attenuated. TUN toxicity studies have indicated that combined treatment with TUN and β -lactam antibiotics is effective and feasible for treating infections with severe drug-resistant bacteria such as MRSA [24].

MRSA, a common pathogenic bacterium encountered in medical and veterinary clinical infections, is categorized as health-care-associated MRSA, community-associated MRSA, or livestock-associated MRSA, according to the source and association with hospital, habitation zone and Livestock farm [25]. Our findings indicated that the MICs of most cephalosporins against MRSA were 16–256 $\mu\text{g/mL}$, and the MICs of specific cephalosporins and enzyme-resistant penicillin were 2 $\mu\text{g/mL}$ or lower when 0.5 $\mu\text{g/mL}$ TUN was added. Furthermore, toxicity analysis and cytotoxicity assays indicated that toxicity occurred at concentrations much higher than 0.5 $\mu\text{g/mL}$ TUN. In addition, the synergistic effects of TUN and cephalosporins were unchanged with prolonged MIC observation times (equivalent MIC at 36 h and 24 h). These findings might potentially support expanded clinical use of TUN for bacterial infections.

In conclusion, we observed better synergy between TUN and specific β -lactam antibiotics at cytotoxicity-free concentrations against β -lactam-resistant gram-positive microorganisms. Further studies on TUN clinical treatment are warranted. Our results provide a compatibility strategy for the clinical use of TUN combined with β -lactam antibiotics.

DATA AVAILABILITY STATEMENT

All data included in this study are available from the corresponding author on request.

ETHICS STATEMENT

Procedures in all mice used to obtain peritoneal macrophages were conducted according to protocols approved by the Ethics Review Committee of Animal Experimentation at Ningxia University (No. NXU-24-F-020) and in compliance with the principles of the Animal Welfare Act.

AUTHOR CONTRIBUTIONS

YLZ and QHL designed the project; XYYB, JJX, FH, and XTY performed experiments; HRL and KG analyzed the data and prepared the figures; YLZ provided resource support; and YLZ and XYYB drafted and revised the manuscript. All authors reviewed, revised, and approved the final report.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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