ZTW-41, a Potent Indolizinoquinoline-5,12-Dione Derivative Against Drug-Resistant *Staphylococci* and *Enterococci* Bacteria

Chong-Fa Lai,[1,2,*] Wei-Bin Xiao,[2,*] Hua-Cheng Yan,[3] Hui Yang,[4] Lu-Xia Wang,[2] Hui Guan,[2] Qiu-ju Peng,[3] Zhi-Rong Deng,[3] Jian-Wen Chen,[4] Lin-Kun An,[4,5] and Lei Shi[1,2]

ZTW-41, an indolizino uinoline-5,12-dione derivative, was investigated or antibacterial activity against Gram-positive bacteria, especially methicillin-resistant *Sa h cc a e* (MRSA). In our study, the MIC_{90}s (minimum inhibitory concentrations) o ZTW-41 against MRSA (MRSA, =200), methicillin-sensitive *S. a e* (MSSA, =100), *E e c cc faeca i* (*E. faeca i* , =32), and *E e c cc faeci* (*E. faeci* =32) were 0.25, 0.25, 0.125, and 8 µg/mL, respectively, whereas the MBC_{90}s (minimum bactericidal concentrations) were 2, 1, 1, and >32 µg/mL, respectively. ZTW-41 maintained its potency at di erent pH levels (range 5–9) and in starting inoculum size up to 10^7 CFU/mL. The presence o human serum (25–75%) increased ZTW-41 MICs by two- to eight old. Time-kill curves showed that ZTW-41 had bactericidal activity against MRSA, MSSA, and *E. faeca i* strains within 8 hours, and rebound growth occurred a ter 8 hours except at higher multiples o the MIC (4× and 8×). In the acute toxicity study, no mortality or signs o toxicity was noted in mice a ter 14 days o observation at doses <50 mg/kg. ZTW-41 exhibited good selectivity indices (SIs) ($SI = IC_{50}/MIC_{90}$) ranging rom 1.12 to 71.76 against clinical isolates, demonstrating excellent therapeutic selectivity in MRSA, MSSA, and *E. faeca i* strains. Moreover, the *i* e ficacy (e ective dose $[ED]_{50}$ = 6.59 mg/kg) o ZTW-41 was ound comparable with vancomycin. Collectively, our avorable results supported ZTW-41 as a promising investigational candidate or treating drug-resistant bacteria in ection.

Keywords: ZTW-41, indolizino uinoline-5,12-dione derivatives, Gram-positive bacteria, MRSA, antibacterial activity

Introduction

OVER THE LAST DECADE, multidrug-resistant bacteria have posed serious threats to global public health, and represent a leading cause o morbidity and mortality globally.[1,2] Among them, methicillin-resistant *Sa h cc a e* (MRSA) is an in amous pathogen and rises as a significant clinical concern as it can cause li e-threatening in ections such as septic shock, endocarditis, and severe pneumonia, which brings great challenge to human beings.[3] In recent years, the rocketing incidence o MRSA in ections has been ound in the community and hospitals.[4] According to the Centers or Disease Control and Prevention (CDC),[5] >2 million people su er rom antibiotic-resistant in ections every year, and ~23,000 people die rom these in ections in the United States. It is estimated that 80,461 invasive MRSA in ections and >11,000 related deaths occurred in 2011 in the United States. Based on the monitoring results rom China Antimicrobial Surveillance Network (CHINET), the prevalence o methicillin-resistant strains was 35.3% in *S. a e* in Chinese hospitals in 2017, which represented predominant pathogens in multiple in ectious diseases or its high resistance rates to most o antimicrobial agents.[6] At the same time, hospital-ac uired MRSA has become increasingly resistant to the limited available antibiotics,[7] and ew new drugs are introduced into market,[8] which means that the world is going to lack e ective antibiotics. There ore, the current situation puts orward an austere challenge, and highlights the need or the development o more potent antibiotics as alternative therapy against MRSA.[8,9]

[1]Southern Medical University, Guangzhou, China.
[2]General Hospital o Southern Theatre Command, Guangzhou, China.
[3]Center or Disease Control and Prevention o Guangzhou Military Command, Guangzhou, China.
[4]School o Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China.
[5]Guangdong Provincial Key Laboratory o New Drug Design and Evaluation, Guangzhou, China.

In the previous research, 9-bromo-substituted indolizino uinoline-5,12-dione derivatives showed excellent antibacterial activity against Gram-positive bacteria, especially MRSA.[10] According to structure–activity relationship studies o these derivatives,[10,11] the structure o 9-bromo-substituted indolizino uinoline-5,12-dione is essential or the antibacterial activity, and ester analogs with a longer side chain and amino terminal group at 6-position play a vital role in its improved water solubility. Among many derivatives, F1 ▶ ZTW-41 (Fig. 1) is the most promising candidate compound or its potent anti-MRSA activity and satis actory water solubility.[10] The mechanism o the active compound was to inhibit the activity o DNA gyrase and DNA topoisomerase IV.[12] This dual targeting is believed to help reduce the selection o resistant mutants and enhances potency against bacteria.[13]

However, the *i* ▶ pharmacodynamics properties o ZTW-41 were unknown, and the *i* ▶ e ficacy in animal model remained to be evaluated. The objective o the present research was to investigate the *i* ▶ activity o ZTW-41 against Gram-positive isolates, and the mouse sepsis protection model was used to evaluate *i* ▶ e ficacy. In addition, the cytotoxicity in HepG2 cells and the acute toxicity study o ZTW-41 were primarily assessed in mice. The plasma protein binding rates were also determined in rat and human plasma.

Materials and Methods

Bacterial strains and antimicrobials

Minimum inhibitory concentrations (MICs) were determined against 364 clinical isolates, including MRSA (MRSA, =200), methicillin-sensitive *S. a e* (MSSA, =100), *E e c cc faeca i* (*E. faeca i* , =32), and *E e c cc faeci* (*E. faeci* =32). These strains were collected rom General Hospital o Southern Theatre Command rom 2015 to 2018. All o the isolates had been identified by VITEK®

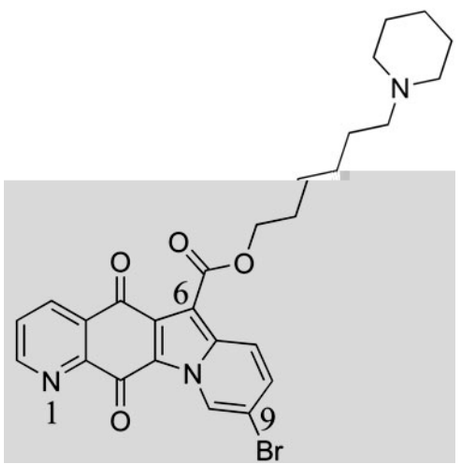

FIG. 1. The structure o ZTW-41.

Minimum inhibitory concentration

The MICs were determined by standardized broth microdilution recommended by CLSI guidelines.[14] The test ◀ AU7 medium or these strains was cation-adjusted Mueller-Hinton broth (CAMHB) except *S. a e* were grown in CAMHB supplemented with 2% NaCl when the susceptibility o oxacillin was determined. Exponential-phase organisms were generated to the turbidity o 0.5 McFarland standard (Densimat; BioMerieux, SA, France) and urther diluted in CAMHB to target a final cell density o $\sim 5 \times 10^5$ cells/mL. The plate was incubated at 37°C or 16–20 hours, ◀ AU8 except the incubation time was 24 hours when the MIC o oxacillin and vancomycin was tested. The MIC was defined as the lowest concentration o antimicrobial agent with no visible growth. Experiments were per ormed with biological triplicates. Susceptibility and resistance to these antibiotics were determined using CLSI breakpoints.

For evaluating the pH e ect on MIC, CAMHB was adjusted to pH 5, 7, or 9 with appropriate volumes o HCl or NaOH. Inoculum e ects were tested in the starting inoculum size rom 10^3 to 10^8 CFU/mL. And the e ect o serum ◀ AU9 concentration on MICs was estimated by supplementing CAMHB with 25%, 50%, and 75% (vol/vol) human serum (Sigma-Aldrich, China).

Minimum bactericidal concentration

The minimum bactericidal concentrations (MBCs) o ZTW-41, vancomycin, ampicillin, and oxacillin were determined. An ali uot 100 µL content rom the wells con- ◀ AU10 taining concentrations higher than or e ual to the MIC was plated onto blood agar by the sur ace viable-count method. The colonies were counted a ter incubating or 24 hours at 37°C. The MBC was defined as the lowest drug concentration that reduced viable organism counts by ≥3 log10 in ◀ AU11 24 hours.[15]

Time-kill kinetics

For time-kill kinetics, ZTW-41 was tested against ATCC 43300, ATCC 29213, ATCC 29212, and each clinical isolate o MRSA, MSSA, and *E. faeca i* at concentrations o 0.5×, 1×, 2×, 4×, and 8× o the MIC. At time 0, 2, 4, 8, and 24 hours, an ali uot o 100 µL rom all ZTW-41 con- ◀ AU12 centration tubes was sampled; serially 10- old dilutions o the suspension in sterile PBS were plated on blood agar and incubated at 37°C or 24 hours. Dilution was expected to minimize the carryover e ect o antibiotics. Colonies were enumerated, and results were expressed as $\log_{10}$ CFU/mL. Time-kill experiments were done in triplicates. Growth ◀ AU13 control was done with no antibiotic added to the studied

strain. The lower limit o bacterial detection utilized was 2 log10 CFU/mL. Bacterial concentrations <2 log10 CFU/mL were counted as 2 log10 CFU/mL. Bactericidal and bacteriostatic activities were defined as a ≥3 $\log_{10}$ CFU/mL decrease and 0 to <3 log10 CFU/mL decrease in bacterial counts a ter 24 hours o incubation, respectively.

Mammalian cytotoxicity

Cell Counting Kit-8 (CCK-8) was used to determine the cytotoxicity o ZTW-41. In brie , HepG2 cells (ATCC HB-8065) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% etal bovine serum. The cells were incubated at 37°C and 5% CO_2 or 24 hours, and the medium was replaced with resh medium containing di erent concentrations o ZTW-41 or vancomycin. A ter 2 hours o incubation, the A490 nm (OD_{490}) was measured using a Thermo Multiskan GO Microplate Reader. IC_{50} values were determined rom biological triplicates. The selectivity index (SI) was calculated using the IC_{50} and MIC data or ZTW-41 and vancomycin.

Mouse sepsis protection model

All the studies on animals ollowed the Guidelines or the Care and Use o Laboratory Animals. Kunming mice (18–22 g) were purchased rom Animal Experimental Center, Guangdong Academy o Medical science. All the animals were acclimatized under controlled conditions (temperature 25°C ± 2°C, humidity 40–60%, 12-hours light/dark cycle) with standardized diet or ∼7 days. All the mice were asted or 12 hours be ore the experiments with ree access to water.

The mouse sepsis protection model in mice was per ormed as previously described.[16] In brie , Kunming strain-derived male mice were in ected intraperitoneally with 0.5 mL o MRSA 43300 suspension (1.25×10^8 CFU/kg), a concentration that achieves 100% mortality within 72 hours a ter in ection. At 6 hours, 1, 2, 3, 4, 5, and 6 days postin ection, mice (10/group) received ZTW-41 at single intraperitoneal doses o 2.5, 5, 10, and 20 mg/kg. Control mice were dosed with vehicle or vancomycin at a dose o 10 mg/kg. Mortality was observed daily or 7 days postin ection. The median e ective dose (ED_{50}) and 95% confidence limits were determined by GraphPad Prism 6.0 so tware.

Acute toxicity study

Acute toxicity test was carried out according to the Organization or Economic Co-operation and Development (OECD) guidelines or Testing o Chemicals.[17] A total o 20 mice weighing between 24 and 30 g were randomly divided into 5 experimental groups o 4 mice each. A ter an overnight ast, ZTW-41 was administered intraperitoneally to each treatment group at single doses o 25.6, 32, 40, and 50 mg/kg, respectively. The control group was treated with the same volume o normal saline. All animals were observed or the mortality and changes in general behavior or 24 hours a ter treatment. Symptoms o toxicity such as hypoactivity, piloerection, breathing di ficulty, tremors, and convulsion were evaluated a ter administration o the various doses o ZTW-41. During the remaining experimental period, the animals were monitored and observed at least once daily or the postdosing period o 14 days. Body weights were measured at the initiation o treatment, and on days 2, 7, and 14 a ter administration. All results were expressed as mean ± standard deviation. The statistical signi icance o di erences between means was calculated using one-way ANOVA, ollowed by Dunnett's test or multiple comparisons with the control group.

Plasma protein binding

The protein binding o ZTW-41 in rat and human plasma was per ormed using a rapid e uilibrium dialysis (RED) kit (Thermo Fisher Scientific) with LC-MS/MS analysis.[16] Drugs were added to rat or human plasma to achieve final concentrations o 2,000 ng/mL. Plasma solutions (200 µL) were placed into the sample chamber and 350 µL o PBS into the adjacent chamber. The plate was incubated at 37°C on an orbital shaker (1.5 g approx.) or 4 hours. Then, ali uots (50 µL) o the samples rom both sides were processed, and 50 µL o blank plasma or PBS was added to each sample. Five hundred microliters o methanol containing 1 µg/mL carbamazepine IS was added and centri uged at 12,800 *g* or 5 minutes be ore LC-MS/MS analysis. Experiments were per ormed with six biological replicates. The percentage o the compound bound to protein was calculated as the ormula given below according to test compound concentration in protein-containing (PC) compartment and in protein- ree (PF) compartment:

$$\%\ \text{Plasma protein binding} = \frac{\text{PC} - \text{PF}}{\text{PC}}.$$

Results

MIC and MBC determinations

ZTW-41 was ound to be highly active against a panel o standard Gram-positive pathogens, and the MIC values o ZTW-41 evaluated or ATCC29213, ATCC 43300 (MRSA), ATCC 29212 (*E. faecali*), and ATCC 700699 (VISA) were 0.25, 0.25, 0.031, and 0.25 µg/mL, respectively (Table 1), ◀T1

Table 1. Summary of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Values (µg/mL) for ZTW-41 and Comparators Against Gram-Positive ATCC Strains

Standard bacterial strain	*ZTW-41*		*Vancomycin*		*Oxacillin*	
	MIC	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*
ATCC 29213	0.25	0.5	0.5	1	0.25	0.25
ATCC 43300	0.25	0.5	1	2	32	64
ATCC 29212	0.031	0.25	2	>64	1	>64
ATCC 700699	0.25	8	8	32	>128	ND

ATCC 29213: MSSA; ATCC 43300: MRSA; ATCC 29212: *Enterococcus faecalis*; ATCC 700699: VISA.

MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; ND, not detected; VISA, vancomycin-intermediate *Staphylococcus aureus*.

which were comparable with or more active than vancomycin and oxacillin. However, Gram-negative bacteria were less susceptible than Gram-positive bacteria. The MIC o ZTW-41 against *E. c 'i* (ATCC 25922) was 4 μg/mL, and the MICs against *A. ba a ii* (ATCC 19606), *P. ae gi a* (ATCC 27853), and *K. e iae* (ATCC 700603) were all >16 μg/mL (data not shown).

In previous work, MRSA was susceptible to ZTW-41 (=100), with an MIC_{50} value o 0.063 μg/mL and an MIC_{90} value o 0.125 μg/mL, 16- old more active than vancomycin.[10] The potent antibacterial activity o ZTW-41 was urther confirmed in a number o clinical strains in the present work, as shown in Table 2. Overall, ZTW-41 was the most potent agent against 364 Gram-positive bacteria tested compared with vancomycin, oxacillin, ciprofloxacin, levofloxacin, moxifloxacin, linezolid, and ampicillin. All MRSA and MSSA clinical strains in our study were highly susceptible to ZTW-41, with MICs ranging between 0.031 and 0.5 μg/mL, which were e ual to or lower than comparator agents. The $MIC_{50/90}$ values or ZTW-41 to MRSA were 0.125 and 0.25 μg/mL, respectively. And the $MIC_{50/90}$ values or MSSA were 0.063 and 0.25 μg/mL, respectively. For *E. faeca 'i* tested, the $MIC_{50/90}$ values were 0.063 and 0.125 μg/mL, and $MIC_{50/90}$ values against *E. faeci* were 0.063 and 8 μg/mL, respectively.

An important eature o ZTW-41 is its strong bactericidal property. An antibiotic was considered to show bactericidal activity against a particular strain when the MBC/MIC ratio was ≤4.[18] ZTW-41 showed excellent bactericidal activity to ATCC 29213 and ATCC 43300, with MBC values o 0.5 μg/mL against them. In addition, ZTW-41 showed bacteriostatic activity against ATCC 29212 and ATCC 700699 as the MBC/MIC values were 8 and 32, respectively (Table 1). As or clinical strains, the MBC_{90} values o ZTW-41 or MRSA and MSSA were 0.5 and 0.25 μg/mL, respectively, while those o vancomycin were both 2 μg/mL (Table 3). For *E. faeca 'i* and *E. faeci e e ZTW-41* MBC_{50} *'* *e e 0.25 a d 4* μg/mL, respectively. ZTW-41 was bactericidal against 80.5% (161/200) o MRSA isolates and 90.0% (90/100) o MSSA isolates, exhibiting an MBC/MIC ratio ≤4, when vancomycin were 71.5% (143/200) and 72.0% (72/100), respectively. ZTW-41 was bactericidal against 46.9% (15/32) o *E. faeca 'i* , while vancomycin and ampicillin were 6.3% (2/32) and 37.5% (12/32), respectively (data not shown).

Various cultural conditions or bacteria (such as pH, starting inoculum size, and human serum) may a ect the MIC values.[19] In our study, the MICs o ZTW-41 against ATCC 29213 or ATCC 43300 were slightly a ected by pH (5–9) or inoculum size (10^3 to 10^7 CFU/mL), a similar

Table 2. Summary of Minimum Inhibitory Concentration Values (μg/mL) for ZTW-41 and Comparators Against Clinical Isolates

S ecie	*N . f i 'la e*	*A ibi ic*	*MIC (μg/ L)*			
			Ra ge	MIC_{50}	MIC_{90}	*S%*
MRSA	200	ZTW-41	0.031–0.5	0.125	0.25	—
		Vancomycin	0.25–2	0.5	1	100
		Oxacillin	4 to >128	>128	>128	0
		Ciprofloxacin	0.5–8	2	8	39.0
		Levofloxacin	0.12–8	2	8	45.0
		Moxifloxacin	0.12–8	1	8	44.5
		Linezolid	1–4	2	2	100
MSSA	100	ZTW-41	0.031–0.25	0.063	0.5	—
		Vancomycin	0.25–1	0.5	1	100
		Oxacillin	0.063–2	0.25	1	100
		Ciprofloxacin	0.25–8	0.5	2	88.0
		Levofloxacin	0.12–2	0.125	0.5	92.0
		Moxifloxacin	0.125–1	0.25	0.5	98.0
		Linezolid	1–4	2	2	100
E e c cc faeca 'i	32	ZTW-41	0.016–0.25	0.063	0.125	—
		Vancomycin	0.5–4	1	2	100
		Ampicillin	0.5 to >128	1	4	94.8
		Ciprofloxacin	0.5–8	1	8	62.5
		Levofloxacin	0.25–8	1	8	62.5
		Moxifloxacin	0.25–16	0.5	8	—
		Linezolid	2	2	2	100
E e c cc faeci	32	ZTW-41	0.016 to >32	0.063	8	—
		Vancomycin	0.5–128	0.5	64	87.5
		Ampicillin	0.5 to >128	>128	>128	12.5
		Ciprofloxacin	1–16	8	8	6.25
		Levofloxacin	1–16	8	8	3.13
		Moxifloxacin	0.5–16	8	8	—
		Linezolid	1–2	2	2	100

Interpretation based on CLSI breakpoints (M100-S25) or vancomycin, oxacillin, ampicillin, ciprofloxacin, levofloxacin, Moxifloxacin, and linezolid.

MIC_{50}/MIC_{90}, minimum inhibitory concentration reached by 50% or 90% o the strains; respectively; S, susceptible.

Table 3. Summary of Minimum Bactericidal Concentration Values (μg/mL) for ZTW-41 and Comparators Against Clinical Isolates

Species	No. of Isolates	Antibiotic	MBC (μg/mL) Range	MBC_{50}	MBC_{90}
MRSA	200	ZTW-41	0.063–8	0.5	2
		Vancomycin	0.25–32	2	8
MSSA	100	ZTW-41	0.063–2	0.25	1
		Vancomycin	0.5–8	2	4
		Oxacillin	0.25–4	1	2
Enterococcus faecalis	32	ZTW-41	0.063–2	0.25	1
		Vancomycin	1 to >128	64	>128
		Ampicillin	2 to >128	16	>128
Enterococcus faecium	32	ZTW-41	0.063 to >32	4	>32
		Vancomycin	1 to >128	>128	>128
		Ampicillin	4 to >128	>128	>128

T4 ▶ behavior to that observed with vancomycin (Table 4). However, the MICs increased 16- old in 10^7 CFU/mL inoculum size compared with standard condition in ATCC 29212, which suggested an inoculum e ect o ZTW-41 in this strain. The presence o human serum increased ZTW-41 MICs by two- to eight old. In comparison, vancomycin was more stable in the presence o human serum, with the MIC values changing by one- to two old (Table 4).

Time-kill studies

Our *i* v time-kill experiments suggested that ZTW-41 had bactericidal activity against MRSA, MSSA, and *E. faecalis* strains. For ATCC 43300, ZTW-41 demonstrated significantly killing activity at concentrations o 4×and F2 ▶ 8×MIC in 24 hours (Fig. 2). Bactericidal activity occurred in the presence o 1×and 2×MIC within 8 hours, and rebound growth occurred a ter 8 hours. The results or MRSA clinical isolate 408235 were identical to that o ATCC 43300. Compared with MRSA, ZTW-41 exhibited better bactericidal activity against ATCC 29213 and achieved F3 ▶ bacterial killing at 2×, 4×, 8×MIC in 24 hours (Fig. 3). It could be observed that the isolates regrew at 0.5×and 1×MIC in which a remarkable increase in growth occurred rom 4 to 24 hours time point. The curves or MSSA clinical isolate 616033 were similar to that o ATCC 29213. With respect to ATCC 29212, ZTW-41 showed bactericidal activity in 4×and 8×MIC in 4 hours, and no isolates were F4 ▶ observed in 8×MIC over 24 hours (Fig. 4). Regrowth was observed a ter 8 hours when exposed to 0.5×, 1×, 2×, and 4×MICs o ZTW-41. As or *E. faecalis* clinical isolate 306002, the same results were obtained as ATCC 29212.

Mammalian cytotoxicity

It is critically significant to assess the potential cytotoxicity o a test agent as drug toxicity is an important aspect in new drug development. The *i* v cytotoxicity o ZTW-41 was investigated in HepG2 cells (Fig. 5). The IC_{50} values o ◀F5 ZTW-41 and vancomycin were 8.97 and 12.22 μg/mL, respectively. The inhibition rates o ZTW-41 and vancomycin were 80.3% and 67.8%, respectively, when tested at the highest concentration (100 μg/mL). The SIs o ZTW-41 and vancomycin were determined as an estimate o a therapeutic window. ZTW-41 possessed good SIs ranging rom 1.12 to 71.76, while vancomycin showed much lower SIs ranging rom 0.19 to 12.22 (Table 5). The highest value o 71.76 was ◀T5 determined or ZTW-41 against *E. faecalis* . Considering SI, ZTW-41 was a more avorable antibiotic than vancomycin.

Mouse sepsis protection model

Given the *i* v activity along with the desirable therapeutic selectivity, the *i* vivo e icacy o ZTW-41 against MRSA was determined in the mouse septicemia model. Figure 6 illustrates the survival rate o mice treated with ◀F6 ZTW-41 and vancomycin in experimental septicemia model. As shown in Fig. 6, ZTW-41 demonstrated dose-dependent e ects on survival in this model in ected with

Table 4. Effect of pH, Inoculum Serum, and Serum Concentration on the Minimum Inhibitory Concentrations of ZTW-41 and Vancomycin Against *Staphylococcus aureus* and *Enterococcus faecalis*

Micro ga i	C d	MIC (μg/mL) de he i dica ed c di i H 5	H 7	H 9	Sa i g i c (CFU/mL) 10^3	10^5	10^7	H a e 0	25%	50%	75%
ATCC 29213	ZTW-41	0.5	0.25	0.5	0.031	0.25	0.5	0.25	0.5	1	1
	Vancomycin	2	0.5	2	0.5	0.5	2	0.5	0.5	0.5	0.5
ATCC 43300	ZTW-41	0.5	0.25	0.25	0.25	0.25	0.5	0.25	0.25	0.5	0.5
	Vancomycin	2	1	2	1	1	2	1	0.5	1	1
ATCC 29212	ZTW-41	0.25	0.031	0.063	0.031	0.031	0.5	0.031	0.25	0.25	0.25
	Vancomycin	2	2	4	1	2	4	2	2	2	4

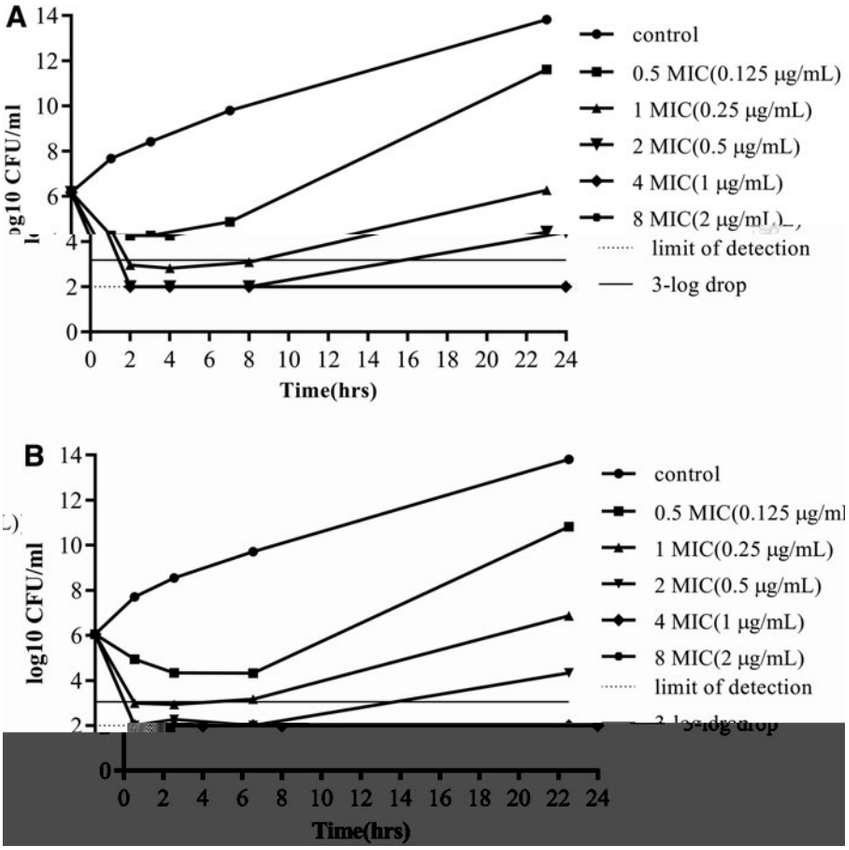

FIG. 2. Time-kill curves or ZTW-41 against ATCC 43300 **(A)** and clinical MRSA isolate **(B)**. MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *S. a h ʔ c cc a e .*

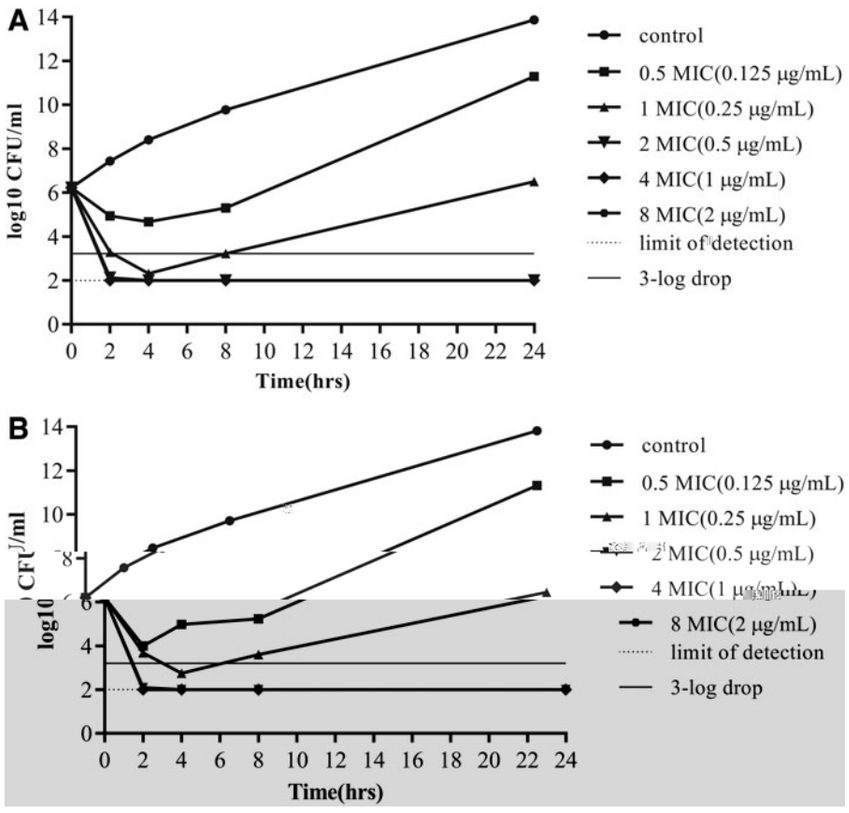

FIG. 3. Time-kill curves or ZTW-41 against ATCC 29213 **(A)** and clinical MSSA isolate **(B)**. MSSA, methicillin-sensitive *S. a e .*

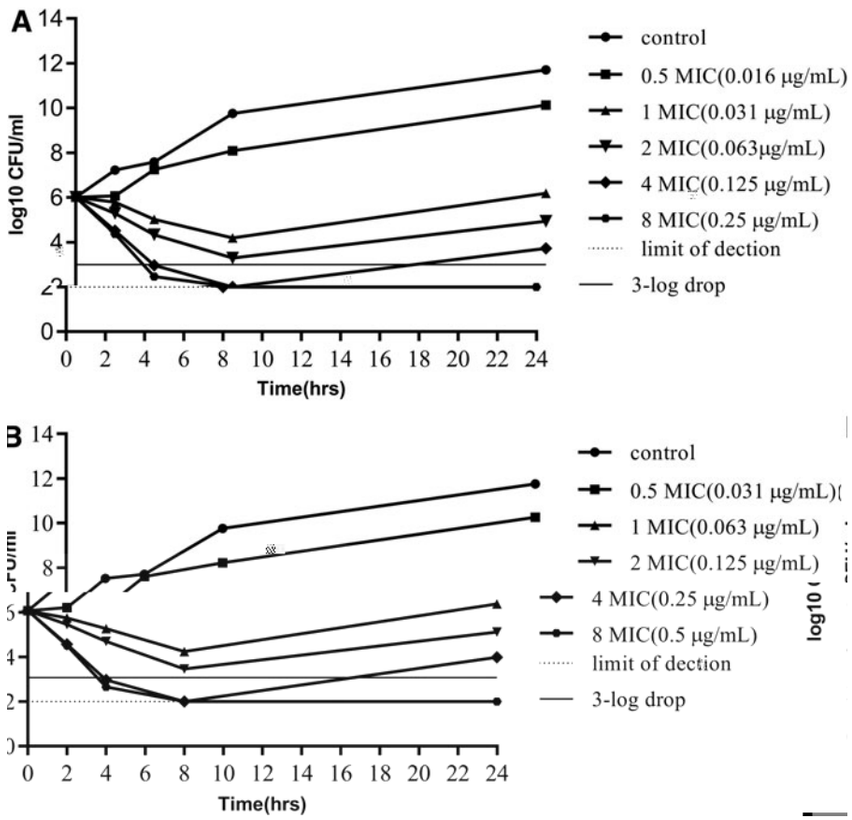

FIG. 4. Time-kill curves or ZTW-41 against ATCC 29212 **(A)** and clinical *E e c cc faeca i* isolate **(B)**.

AU14► MRSA 43300. The treatment o ZTW-41 at 10 mg/kg resulted in a survival rate o mice comparable with vancomycin at 10 mg/kg. Moreover, up to 90% o mice survived >7 days when ZTW-41 was administered at the highest doses o 20 mg/kg. As control, injection with vehicle alone led to 100% mortality in this model within 7 days postinection. In the septicemia model, the ED_{50} o ZTW-41 was 6.59 mg/kg. The ED_{50} values indicated that ZTW-41 was highly e icacious in treating mice in ected with MRSA.

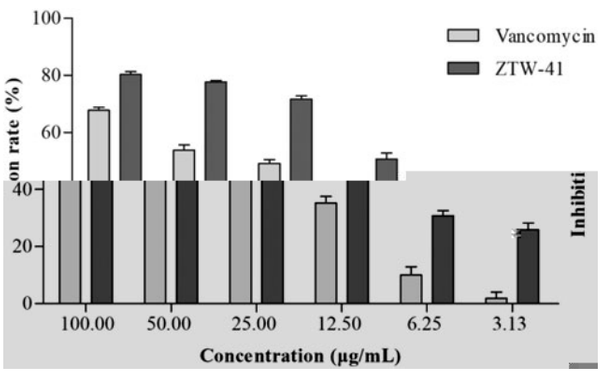

FIG. 5. Cytotoxicity and selectivity index o the ZTW-41 and vancomycin. The cytotoxicity o ZTW-41 against HepG2 cells. HepG2 cells were incubated with di erent concentrations o ZTW-41 and vancomycin, and CCK-8 was used to determine cell viability. Graphs represent mean o three independent experiments ± SD (mean ± SD). IC_{50} values were calculated rom the means o three independent experiments. CCK-8, Cell Counting Kit-8; SD, standard deviation.

Acute toxicity study

The changes in the body weights in each ZTW-41 treatment group are shown in Fig. 7. Overall, ZTW-41 treatment ◄F7 decreased motor activity in each group within 30 minutes a ter administration, and then mice gradually returned to normal. At doses <50 mg/kg, ZTW did not produce any death or signs o toxicity in mice throughout the 14 days

TABLE 5. CYTOTOXICITY AND SELECTIVITY INDEX OF THE ZTW-41 AND VANCOMYCIN

S ecie	*A ibi ic*	MIC_{90} *(μg/ L)*	IC_{50} *(μg/ L)*	IC_{50}/MIC_{90}
MRSA	ZTW-41	0.5	8.97	17.94
	Vancomycin	1	12.22	12.22
MSSA	ZTW-41	0.25	8.97	35.88
	Vancomycin	1	12.22	12.22
E e c cc faeca i	ZTW-41	0.125	8.97	71.76
	Vancomycin	2	12.22	6.11
E e c cc faeci	ZTW-41	8	8.97	1.12
	Vancomycin	64	12.22	0.19

SI was calculated or ZTW-41 and vancomycin taking into account the MIC_{90} against clinical isolates and the IC_{50} on HepG2 cell line ($SI = IC_{50}/MIC_{90}$) by the CCK-8 assay.

CCK-8, Cell Counting Kit-8; SI, selectivity index.

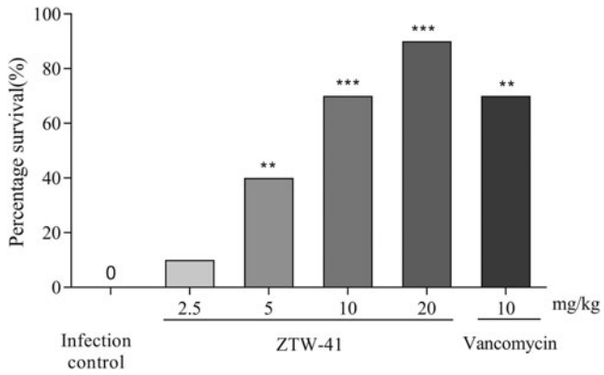

FIG. 6. ZTW-41 is e ficacious in septicemia protection model. ZTW-41 is e ficacious in septicemia protection model. Survival is depicted 7 days a ter in ection (10 mice/group). * <0.05, ** <0.01, *** <0.001, compared with in ection control group (determined by nonparametric log-rank test).

study period. Moreover, the treatment groups did not show remarkable changes in body weight during the experimental period compared with the control group.

Plasma protein binding

Plasma protein binding was assessed by the RED approach and determined as the percentage o bound drug. The protein binding rates o propranolol in rat and human plasma were assessed as the uality control, and our results were in agreement with those reported in the re erence, 87.73% and 89.73%, respectively.[20] The protein binding rates o ZTW-41 were 97.87% and 97.80%, respectively (Table 6). No significant statistical di erence was observed or plasma protein binding o ZTW-41 in rat and human plasma.

Discussion

Antibiotic resistance in bacteria has weakened the e ectiveness o drugs that people have been relying on or nearly a century, and ew new drugs have entered clinical trials

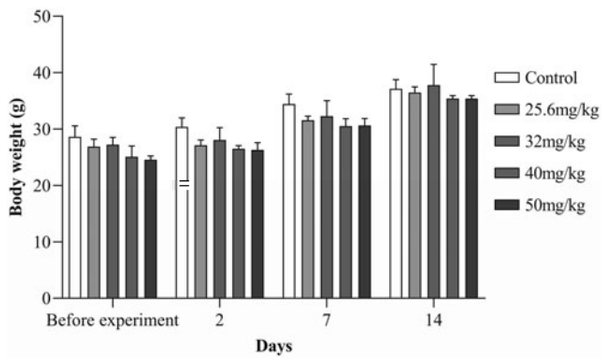

FIG. 7. The acute toxicity study o ZTW-41. Changes in the body weights in mice treated with ZTW-41 in the acute toxicity study in 14-day treatment. Data are represented as the mean ± SD (4 mice/group). * <0.05, ** <0.01, compared with control group (determined by one-way ANOVA).

TABLE 6. PLASMA PROTEIN BINDING OF ZTW-41 AT CONCENTRATION OF 2,000 NG/mL ($N=6$, MEAN ± STANDARD DEVIATION)

	% 'a a ei bi di g	
C d	Ra 'a a	H a 'a a
ZTW-41	97.87 ± 0.20	97.80 ± 0.21
Propranolol	87.73 ± 0.58	89.73 ± 0.63

rom laboratories.[21] It is estimated that only two distinctly new antibiotic classes have been introduced into the market over the past 30 years[8]: the oxazolidinone linezolid in 2000 and the cyclic lipopeptide daptomycin in 2003. However, showing up resistance to some o these new antibiotics results in a growing demand or new antibiotics, pushing development o novel products and new chemical classes o antibiotics.[22–24]

ZTW-41 is an indolizino uinoline-5,12-dione derivative that possesses potent antibacterial activity against Gram-positive bacteria. Similar to most anti-MRSA drugs, such as vancomycin and other newly approved antibiotics, such as dalbavancin[25] and delafloxacin,[26] the antimicrobial spectrum o ZTW-41 is limited to Gram-positive bacteria. In our study, ZTW-41 was significantly active against MRSA and MSSA. The strong antimicrobial activity o ZTW-41 was consistent with data reported by Yang a '.[10] Although vancomycin has generally been used as the first choice or treating MRSA in ections,[27] vancomycin-intermediate *S. a e* [28] and vancomycin-resistant *S. a e* [29] are rising up and associated with vancomycin treatment ailure in recent years. Notably, the MIC value or ZTW-41 to ATCC 700699 (VISA) was 0.25 µg/mL, 32- old more potent than vancomycin, while the MBC value was 8 µg/mL, 4- old more potent than vancomycin. These results showed the potential o ZTW-41 to cure these di ficult-to-treat in ections. Despite a similar mechanism to that o uinolones, ZTW-41 remained active to those uinolone-resistant bacteria (Table 2). The lack o cross-resistance to present available uinolones and other cell wall-inhibiting antibiotics added important values to ZTW-41, which may find application in patients in ected by drug-resistant bacteria. Besides, our research first evaluated the activity o ZTW-41 against *E. faeca 'i* and *E. faeci* . ZTW-41 was the most potent agent against *E. faeca 'i* and *E. faeci* compared with vancomycin, ampicillin, ciprofloxacin, levofloxacin, Moxifloxacin, and linezolid, exhibiting MIC_{50} values o both 0.063 µg/mL against these two strains. In particular, the activities o ZTW-41 against *E. faeci* were generally lower than that o *E. faeca 'i* , which were similar to vancomycin and ampicillin. O note, there were two *E. faeci* strains showing MIC values >32 µg/mL, suggesting ZTW-41-resistant phenotype in these strains. ZTW-41 showed comparatively potent activity against ATCC 29213 and ATCC 43300 even at high inoculum level, except an inoculum e ect was observed in ATCC 29212. When evaluated at di erent pH levels, MICs o ZTW-41 did not show significant fluctuation.

Although moderate shi t in MIC was observed in the presence o human plasma, ZTW-41 maintained potent antibacterial activity against these strains, showing 2- to 16-

old more potent than vancomycin. A possible explanation or this shi t, to some extent, may be attributed to the binding to serum albumin, which could decrease the concentration o the ree drug.[30] The e ect o human serum on the antibacterial activities was paralleled to our protein binding results, which correlated with the significantly high levels o protein binding o ZTW-41.

The bactericidal index is an important eature to assess the bactericidal activity o antibiotics, representing the total bacterial killing over a drug concentration range.[31,32] The bactericidal activity o ZTW-41 discussed in our study was presented in MBC results and time-kill curves. There were no colonies calculated at 4×and 8×MICs or MRSA and MSSA and 8×MIC or *E. faeca'i* in time-kill assays, which demonstrated the significant killing potential o ZTW-41. The rapid bactericidal activity o ZTW-41 against Gram-positive bacteria made it an ideal candidate or prevention and treatment o in ections caused by these important bacteria. However, it should be noted that, in spite o reductions in the viable CFU/mL in time-kill experiments, there was evidence o re-

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Address correspondence to: ◀AU21
Lei Shi ◀AU22
Ge e a H a f S he The e C a d G a g h ◀AU23
Chi a

E- ai: lucyshi622._921@163.com

Li -K A
Ge e a H a f S he The e C a d G a g h
Chi a

E- ai: lssalk@mail.sysu.edu.cn

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