Original Article

Thai Journal of Pharmaceutical Sciences

Time-kill study of the *in vitro* antimicrobial activity of tedizolid against methicillin-resistant *Staphylococcus aureus*

Tanawat Nunart[1], Tanitta Chatsuwan[2], Wanchai Treyaprasert[1]

[1]Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand, [2]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, Bangkok, Thailand

Corresponding Author:
Wanchai Treyaprasert, Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. E-mail: twanchai@chula.ac.th

Received: Aug 10, 2016
Accepted: Dec 21, 2016
Published: Jan 15, 2017

Keywords:
Methicillin-resistant *Staphylococcus aureus*, tedizolid, time-kill curve experiments

ABSTRACT

Objective: To evaluate the *in vitro* antimicrobial activity of tedizolid against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) by time-kill curve experiments. **Materials and Methods:** Four bacterial strains were used in methicillin susceptibility tests including MRSA strain H0340, H5086, H6023, and H7515, obtained from Department of Microbiology at King Chulalongkorn Memorial Hospital. The minimum inhibitory concentrations (MICs) were determined by macrodilution method. **Results:** The time-kill curves were conducted using H5086 strain, showing the highest MIC of 0.25 μg/ml. The time-kill studies also showed that tedizolid exhibited bacteriostatic activity at all concentrations (from 0.25× to 16× MIC) with a reduction in growth of <3 $\log_{10}$ colony forming units (CFU)/ml. Furthermore, the compound appeared to have a maximum effect at the concentration of 8× MIC with 1.8 $\log_{10}$ CFU/ml reduction from the initial inoculum at 12 h. **Conclusion:** Tedizolid is potentially a good antimicrobial activity for MRSA with bacteriostatic activity.

INTRODUCTION

The emergence of antimicrobial resistance is the major public health problem worldwide, resulting in community and hospital-acquired infected therapeutic failures which are associated with prolonged hospitalization and the increase in the rate of mortality. *Staphylococcus aureus*, a Gram-positive bacterium with the most common cause of infections, has worldwide progressively developed to be multidrug-resistant in Thailand [1]. It is because increase in the prevalence of methicillin-resistant *S. aureus* (MRSA) leads to more complicated infection management. Although the potent antimicrobial agents, such as vancomycin and linezolid, have been developed and in use, the resistance has been observed [2,3]. The new therapeutic options thus are necessary for solving this issue.

Tedizolid, the active moiety of an ester prodrug tedizolid phosphate, is the second-generation of oxazolidinone-based antibiotic approved by US Food and Drug Administration in June 2014 for the treatment of acute bacterial skin and skin structure infections. Mechanistically, the antimicrobial inhibits the first step of proteins synthesis by binding to the 23S ribosomal RNA of the 50S subunit that prevents the bacterial translation. Tedizolid has demonstrated the superior *in vitro* activity to linezolid, the first marketed oxazolidinone, against aerobic Gram-positive bacteria even MRSA strains that are not susceptible to linezolid, daptomycin, or vancomycin [4-7]. Furthermore, tedizolid provides desirable pharmacokinetic characteristics with high bioavailability (90%), extensive penetration and a safety profile, i.e., less hematological toxic when compares with linezolid. Protein binding of tedizolid to human plasma proteins is approximately 70-90. Pharmacokinetic data have suggested a linear relationship between C max (1.8-4.5 mg/l) and dosage (200, 300, and 400 mg), with a half-life of 8.4-10.2 h, following once daily administration. The AUC of tedizolid is approximately 23.8-30.50 μg/h/ml [4].

The minimum inhibitory concentration (MIC) is a routinely static *in vitro* parameter used to describe the

antimicrobial activity against specified microorganisms. The MIC corresponds to the total antimicrobial effect at a single time point over an incubation period with the static concentration of antimicrobial agent. It does not provide any information on the time dependency of the bacterial growth rates as well as the antimicrobial effect. An identical MIC value may result in many different combinations of growth and kill rates. As the systemic exposure of the drug after administration is time-dependent, MIC might not be suitable for antimicrobial activity assessment. The alternative approach is the time-kill curves assay which provides more detailed and dynamic information. It exhibits both time and concentration dependency of activity against microorganism [8]. In a previous study, the *in vitro* activities of the investigational oxazolidinone RWJ-416457 and the first-in-class representative linezolid have been determined in time-kill curve experiments [9].

The objective of this study was to investigate the *in vitro* antimicrobial activity of tedizolid against the clinical isolate of MRSA by time-kill curve experiments covering the full effective concentration range.

MATERIALS AND METHODS

Bacterial Strains and Media

The blood isolates of *S. aureus* (H0340, H5086, H6023, and H7515) were collected from hospitalized patients who admitted at King Chulalongkorn Memorial Hospital and were kept at the Department of Microbiology, Faculty of Medicine of Chulalongkorn University, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. All strains were stored at −80°C in the sterile glycerol.

For MICs determination and time-kill curve experiments, the inoculum was prepared from colonies plated onto Muller-Hinton agar (MHA: Oxoid Ltd, Basingstoke, Hampshire, England) which were incubated at 35°C for 18 h. Colonies from overnight growth were collected and suspended as necessary in 0.9% sterile saline solution to an optical density of 0.5 McFarland scale (Remel Microbiology Products, Lenexa, KS, USA) to obtain a suspension of 1×10^8 colony forming units (CFU)/ml. Bacteria were grown at 35°C in Cation-adjusted Mueller-Hinton broth (CAMHB: Fluka, Bucsh, Switzerland) which were prepared the day before, according to the manufacturer's instructions, to produce a final concentration of bacterial inoculum approximately 5×10^5 CFU/ml.

Antimicrobial Agent

Tedizolid powder was purchased from MedChem Express, Princeton, NJ, USA. Tedizolid stock solutions were freshly prepared daily before each experiment by dissolving tedizolid powder in sterile dimethyl sulfoxide and diluted with CAMHB to the desired concentrations.

Methicillin Susceptibility Test

Methicillin susceptibility tests were determined by the disk diffusion method using cefoxitin disk for each of *S. aureus* isolate in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline [10]. A 0.5 McFarland standard turbidity suspension of each clinical *S. aureus* isolate was plated onto MHA plate and then applied to the 30 μg cefoxitin disk. An inhibition zone size was measured using transmitted light after the incubation of MHA plate at 35°C for 24 h. An isolate which exhibited the diameter of inhibition zone ≤21 millimeters was considered as methicillin-resistant (*mecA*-positive), whereas that of ≥22 millimeters was reported as methicillin sensitive (*mecA*-negative). *S. aureus* American Type Culture Collection (ATCC) 25923 was served as a control strain which the diameter of inhibition zone should be between 23 and 29 mm (*mecA*-negative).

Determination of the MICs

The MIC value was defined as the lowest concentration of antimicrobial agent that completely inhibits the growth of microorganism approximately 5×10^5 CFU/ml as detected by visual inspection after 18-20 h of the incubation period.

The MICs of tedizolid for MRSA strains were determined by a modified broth macrodilution method [11], according to the CLSI guideline [10]. The determinations were repeated at least two times in duplicate on separate occasions for each strain with *S. aureus* ATCC 29213 which was served as the quality control strain. The determination was performed with flat bottom 24-well plates (Corning Incorporated, NY, USA) by serial 2-fold dilutions to produce the final concentration of tedizolid from 0.015 to 8 μg/ml. Positive controls (with bacteria and without antibiotic) and negative controls (without bacteria and without antibiotic) were performed simultaneously. Susceptibility breakpoint to tedizolid was defined as a MIC ≤0.5 μg/ml [10]. Results of MICs determinations were used to study the test concentrations for time-kill curve experiments.

Static Time-kill Curve Experiments

A previously described dynamic model was used in the study [11]. Briefly, a one-compartment *in vitro* infection model was used to evaluate the antimicrobial activity of constant tedizolid concentrations for 24 h. The model consisted of a 75 ml vented cap tissue culture flask with canted neck (Corning Incorporated, NY, USA), containing 30 ml of CAMHB. An aliquot of the suspension (100 μl) of initial inoculum (equivalent to 0.5 McFarland scale) was added to *in vitro* model and incubated with shaking for 2 h before addition of tedizolid to achieve the logarithmic phase of bacterial growth.

Time-kill curve experiments were performed on selected MRSA; strain with the highest MIC value for tedizolid was employed. Tedizolid concentrations used were based on the previous MIC determination included 0.25×, 0.5×, 1×, 2×, 4× 8×, and 16× MIC, covering the full range of tedizolid antimicrobial activity. The *in vitro* models were placed in shaking incubator at 35°C and were run simultaneously with the growth control (without antibiotic). Time-kill curves experiments were performed in triplicate.

Bactericidal activity and bacteriostatic activity were defined as ≥3 $\log_{10}$ and <3 $\log_{10}$ reduction of total count of CFU/ml, respectively, in comparison with the initial inoculum after 24 h of incubation. Moreover, regrowth was defined as ≥2 $\log_{10}$ increase of viable count of CFU/ml after ≥6 h [12].

Bacterial Quantification

Inoculum (20 μl) for viable counts were collected from the *in vitro* model at 0, 1, 2, 4, 6, 8, 12, 16, 20, and 24 h. Bacterial survival was determined by an adapted droplet-plate method [11], using 96-well microtiter plates (Corning Incorporated, NY, USA) for serial 10-fold dilutions of inoculum in 0.9% sterile saline solution that was plated with 5 × 10 μl droplets in duplicate of the chosen dilution onto MHA plates. The total colonies were quantified and presented as the average number of CFU/ml value at each time point after 18-20 h of incubation at 35°C. Bacterial quantification data were exhibited as a function of time for each concentration studied.

RESULTS

Methicillin Susceptibility Test

H0340, H5086, H6023, and H7515 are clinical isolates of *S. aureus*. The results of the methicillin susceptibility test using cefoxitin disk are shown in Table 1. It can be seen that all strains were identified as MRSA strains according to methicillin susceptibility testing method with the diameter of the inhibition zone ≤21 millimeters (*mecA*-positive) and with the diameter of inhibition zone between 23 and 29 mm (*mecA*-negative) of the control strain (*S. aureus* ATCC 25923) [10].

Determination of MICs

The MIC values of H0340, H5086, H6023, and H7515 strains were found to be 0.125, 0.25, 0.125, and 0.0625 μg/ml, respectively. Four MRSA clinical isolates were susceptible to tedizolid according to MIC breakpoints of ≤0.5 μg/ml. In addition, the quality control strains *S. aureus* ATCC 29213 with MIC of 0.25 μg/ml was within the standard range of 0.25-1 μg/ml [10].

Time–kill Curve Experiments

Based on the highest MIC value, MRSA H5086 strain (MIC = 0.25 μg/ml) was selected in time–kill study. The range of tedizolid concentrations from 0.25- to 16-fold of MIC (0.0625 to 4 μg/ml) was tested in the time-kill study. The patterns of antimicrobial activity of tedizolid against MRSA H5086 strain at the different times of MIC were presented in Figure 1.

As shown in Figure 1, the growth control (without antibiotic) displayed exponential growth between 2 and 6 h until reaching the stationary phase at 8 h of inoculation period with an increased growth by 7.5 $\log_{10}$ CFU/ml as compared with the initial inoculum.

At the concentrations within minimum inhibition including 0.25×, 0.5×, and 1× MIC, tedizolid exhibited the inhibitory effect for 4, 6, and 8 h, respectively. After the inhibition period, tedizolid at 0.25× MIC produced the bacterial growth in a similar pattern to that of control group and reached the maximum concentration of bacteria at 12 h of inoculation with an increased regrowth by 7.2 $\log_{10}$ CFU/ml from the initial inoculum. Whereas at the concentration of 1× MIC, tedizolid showed the slow regrowth of bacterium with an increase in viable counts of 3.4 $\log_{10}$ CFU/ml at 24 h compared with the initial count.

For the concentrations of 2× and 4× MIC, the efficient bacterial killing concentration, tedizolid exhibited the inhibitory effect for 16 and 24 h, respectively. Tedizolid concentration of 4× MIC exhibited the bacteriostatic activity at 4 h with a decrease in viable counts of −0.3 $\log_{10}$ CFU/ml compared with the initial count and a slightly increasing viable counts were observed at the end of inoculation. Unlike the concentration of 2× MIC, the regrowth occurred with an increase in 2.7 $\log_{10}$ CFU/ml from the initial inoculum at 24 h.

At the concentrations of maximum bacterial killing, 8× and 16× MIC, tedizolid did not exhibit any bactericidal effect. Both concentrations revealed the bacteriostatic manner which the inhibition of bacterial growth was achieved at each of the time period after 2 h of inoculation. The maximal decreased mean of −1.8 $\log_{10}$ CFU/ml occurred at 12 h for both concentrations. At 24 h the viable counts exhibited reduction of −0.7 and −0.8 $\log_{10}$ CFU/ml from the initial count for tedizolid concentration of 8× and 16× MIC, respectively. The results of the maximum killing curves indicated that tedizolid showed a maximum bacterial killing effect at the concentration of 8× MIC.

DISCUSSION

The MIC is frequently represented as the *in vitro* threshold concentration that is measured the efficacy of antimicrobial agent to the specified microorganism. Moreover, the MIC indicates only a single point estimate or a snapshot time of the net result on growth and kill of the antimicrobial effect. In addition, MIC is considered as all-or-none concentration effect relationship. However, although very popular, this approach

Table 1: The results of methicillin susceptibility test using cefoxitin disk

MRSA strains	Diameter of inhibition zone (mm)	*mecA*
H0340	13	Positive
H5086	18	Positive
H6023	6	Positive
H7515	6	Positive
ATCC 25923	26	Negative

MRSA: Methicillin-resistant *Staphylococcus aureus*, ATCC: American Type Culture Collection

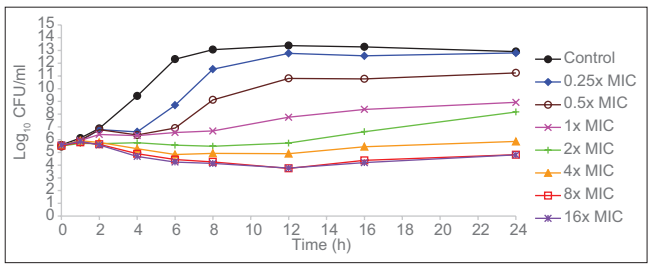

Figure 1: Time-kill curves of tedizolid against methicillin-resistant *Staphylococcus aureus* H5086 strain. Mean change ($\log_{10}$ colony forming units/ml) from the initial inoculum concentration after exposed with tedizolid concentrations during time-kill study

present several limitations, time-kill curves are alternative approach which provides more dynamic information to evaluate the concentration effect relationship over the time of antimicrobial agent. By this approach, the antimicrobial effects of various concentration profiles can be directly compared over the wide range of concentrations [8,13].

This is the first study on the *in vitro* activity of tedizolid against MRSA clinical isolates from Thai patients. For MIC determinations, tedizolid was shown to have an excellent activity on MRSA clinical isolates under test. The resultant MICs range was 0.0625-0.25 μg/ml which was consistent with those of previously reported [4,7,14,15].

The bacterial time-kill curve of tedizolid was determined by *in vitro* infection model. As expected, the time-kill curves for the control and the lowest concentration of 0.25× MIC groups produced the bacterial regrowth exponentially until reaching the stationary phrase. The significant regrowth over 24 h of inoculation did not occur with any concentration of tedizolid greater than 2× MIC. The maximum antimicrobial effect of tedizolid was attained at concentration of 8× MIC. Like linezolid, the first member of the oxazolidinones, tedizolid demonstrated time-dependent effect, and bacteriostatic activity against MRSA with less than a 3-$\log_{10}$ reduction in growth after 24 h as compare with the initial inoculum. Our results were consistent with the previous study in neutropenic murine model [16].

The tested concentrations of tedizolid in this study were covering full effective concentration range. With pharmacokinetic data, the doses of 200 mg intravenous tedizolid allowed the average maximum serum concentration (C_{max}) of 3.1 μg/ml [17]. Taking the average protein binding of tedizolid about 90% [18,19], the unbound tedizolid maximum concentration would be 0.31 μg/ml, that is, between the concentrations of 1× MIC and 2× MIC of this time-kill study. Thus, it is deduced that this average concentration of 0.31 mg/L may be sufficient to inhibit the growth of MRSA. This is consistent with that the free drug concentration exceeds the MIC for time-dependent antibiotic. In addition, *in vivo* studies may be warranted to fully access the killing kinetic of tedizolid in the presence of a competent immune system. Clinical studies, as well as pharmacokinetic/pharmacodynamics analyses, will be necessary to prove in future study.

In conclusion, our time-kill curves study revealed that tedizolid exhibited the excellent potency with *in vitro* bacteriostatic activity against MRSA strain. This characterization of tedizolid could be useful in the treatment of MRSA infections.

ACKNOWLEDGMENT

This study was partially supported by "CU GRADUATE SCHOOL THESIS GRANT" from Chulalongkorn University.

REFERENCES

1. National Antimicrobial Resistance Surveillance Center (NARST). Result of Antimicrobial Resistance Surveillance; 2012. Available from: http://www.narst.dmsc.moph.go.th/antibiotrend.pdf. [Last cited on 2015 Aug 17].
2. Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother 1997;40:135-6.
3. Tsiodras S, Gold HS, Sakoulas G, Eliopoulos GM, Wennersten C, Venkataraman L, *et al.* Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. Lancet 2001;358:207-8.
4. Livermore DM, Mushtaq S, Warner M, Woodford N. Activity of oxazolidinone TR-700 against linezolid-susceptible and - Resistant staphylococci and enterococci. J Antimicrob Chemother 2009;63:713-5.
5. Shaw KJ, Poppe S, Schaadt R, Brown-Driver V, Finn J, Pillar CM, *et al. In vitro* activity of TR-700, the antibacterial moiety of the prodrug TR-701, against linezolid-resistant strains. Antimicrob Agents Chemother 2008;52:4442-7.
6. Brown SD, Traczewski MM. Comparative *in vitro* antimicrobial activities of torezolid (TR-700), the active moiety of a new oxazolidinone, torezolid phosphate (TR-701), determination of tentative disk diffusion interpretive criteria, and quality control ranges. Antimicrob Agents Chemother 2010;54:2063-9.
7. Yum JH, Choi SH, Yong D, Chong Y, Im WB, Rhee DK, *et al.* Comparative *in vitro* activities of torezolid (DA-7157) against clinical isolates of aerobic and anaerobic bacteria in South Korea. Antimicrob Agents Chemother 2010;54:5381-6.
8. Mueller M, de la Peña A, Derendorf H. Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: Kill curves versus MIC. Antimicrob Agents Chemother 2004;48:369-77.
9. Schmidt S, Sabarinath SN, Barbour A, Abbanat D, Manitpisitkul P, Sha S, *et al.* Pharmacokinetic-pharmacodynamic modeling of the *in vitro* activities of oxazolidinone antimicrobial agents against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 2009;53:5039-45.
10. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 24th Informational Supplement. CLSI Document M100-S24. Wayne, PA: CLSI; 2014.
11. Treyaprasert W, Schmidt S, Rand KH, Suvanakoot U, Derendorf H. Pharmacokinetic/pharmacodynamic modeling of *in vitro* activity of azithromycin against four different bacterial strains. Int J Antimicrob Agents 2007;29:263-70.
12. Clinical and Laboratory Standards Institute. Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guidelines M26-A. Wayne, PA: CLSI; 1999.
13. Schmidt S, Schuck E, Kumar V, Burkhardt O, Derendorf H. Integration of pharmacokinetic/pharmacodynamic modeling and simulation in the development of new anti-infective agents - Minimum inhibitory concentration versus time-kill curves. Expert Opin Drug Discov 2007;2:849-60.
14. Betriu C, Morales G, Rodríguez-Avial I, Culebras E, Gómez M, López-Fabal F, *et al.* Comparative activities of TR-700 (torezolid) against staphylococcal blood isolates collected in Spain. Antimicrob Agents Chemother 2010;54:2212-5.
15. Prokocimer P, De Anda C, Fang E, Mehra P, Das A. Tedizolid phosphate vs linezolid for treatment of acute bacterial skin and skin structure infections: The ESTABLISH-1 randomized trial. JAMA 2013;309:559-69.
16. Keel RA, Tessier PR, Crandon JL, Nicolau DP. Comparative efficacies of human simulated exposures of tedizolid and linezolid against *Staphylococcus aureus* in the murine thigh infection model. Antimicrob Agents Chemother 2012;56:4403-7.
17. Bien P, Prokocimer P, Munoz KA, Bethune C. Absolute Bioavailability of TR-701 FA and Pharmacokinetics After Single and Multiple Dose Intravenous Administration in Healthy Adult Subjects. In: 50th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 12; 2010. p. 12-5.
18. Sahre M, Sabarinath S, Grant M, Seubert C, Deanda C, Prokocimer P, *et al.* Skin and soft tissue concentrations of

tedizolid (formerly torezolid), a novel oxazolidinone, following a single oral dose in healthy volunteers. Int J Antimicrob Agents 2012;40:51-4.
19. Housman ST, Pope JS, Russomanno J, Salerno E, Shore E, Kuti JL, *et al.* Pulmonary disposition of tedizolid following administration of once-daily oral 200-milligram tedizolid phosphate in healthy adult volunteers. Antimicrob Agents Chemother 2012;56:2627-34.