

In Vitro Activity of Taurine-5-Bromosalicylaldehyde Schiff Base Against Planktonic and Biofilm Cultures of Methicillin-Resistant *Staphylococcus aureus*

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Staphylococcus aureus is a major human pathogen, implicated in both community and hospital acquired infections. The therapy for methicillin-resistant *Staphylococcus aureus* (MRSA) infections is becoming more difficult because of multidrug resistance and strong biofilm-forming properties. Schiff bases have attracted attention as promising antibacterial agents. In this study, we investigated the *in vitro* activity of taurine-5-bromosalicylaldehyde Schiff base (TBSSB) against MRSA. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) were determined using a microtiter broth dilution method. TBSSB effectively inhibited planktonic MRSA, with an MIC of 32 µg/ml. The time-kill curve confirmed that TBSSB exhibited bactericidal activity against MRSA. TBSSB was also found to significantly inhibit MRSA biofilm formation at 24 h, especially at 1×MIC and sub-MIC levels. Furthermore, scanning electron microscopy and transmission electron microscopy showed remarkable morphological and ultrastructural changes on the MRSA cell surface, due to exposure to TBSSB. This study indicated that TBSSB may be an effective bactericidal agent against MRSA.

Keywords: Taurine-5-bromosalicylaldehyde Schiff base, methicillin-resistant *Staphylococcus aureus*, biofilm, electron microscopy

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are not only a major healthcare-associated pathogen but may also be a community-acquired pathogen [11, 15]. MRSA presents a significant threat to public health because of its multidrug resistance and strong biofilm-forming properties. Biofilms are communities of surface associated with microorganisms embedded in a self-produced extracellular polymeric matrix. They have an impact on the treatment of indwelling medical device infections by preventing the penetration of immunological factors and antibiotics, which often results in persistent and difficult-to-treat infections [5].

The increasing drug resistance among bacterial pathogens

has created an urgent need for the development of new antimicrobial agents with novel and more efficient mechanisms of action. Schiff base complexes come to the forefront as novel therapeutic agents owing to their antifungal, anticancer, and antibacterial activities [3, 17, 20]. Schiff base, named by the chemist Hugo Schiff, is a functional group that contains a double bond between carbon and nitrogen. Schiff bases are some of the most widely used organic compounds and have also been shown to exhibit a broad range of biological activities, including antibacterial, antiproliferative, antifungal, anti-inflammatory, and antiviral properties [19].

Taurine, a sulfur-containing β-amino acid, is the most abundant free amino acid in many mammal tissues [2]. Taurine plays an important role in many physiological

processes: modulation of neurotransmitter and hormone release regulation of cardiovascular responses and neuronal excitability [1, 9]. As for chemical synthesis, taurine has primary amino groups on its side chain and is widely used as an organic ligand. It is also reported that salicylaldehyde derivatives, with one or more haloatoms in the aromatic ring, showed a variety of biological activities, like antibacterial and antifungal activities [21]. Schiff base complexes can be synthesized from the condensation of primary amines and active carbonyl groups. These investigations led us to suspect that taurine-salicylaldehyde Schiff base would possess potential antimicrobial properties, and so we designed and synthesized the novel taurine-5-bromosalicylaldehyde Schiff base (TBSSB) (Fig. 1), the structure of which was characterized by IR spectral and single-crystal X-ray diffraction methods [8].

In the present study, we have investigated the antimicrobial activity of TBSSB against planktonic MRSA, and we also examined the effect of the compound on the formation of MRSA biofilm. Morphological and ultrastructural changes were examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to clarify the antimicrobial mechanisms.

Materials and Methods

Bacterial Strains and Growth Media

Methicillin-resistant *Staphylococcus aureus* ATCC 43300 was used in this study. MRSA ATCC 43300, which was often used for quality control for drug-resistant strains in clinic, has resistance to β -lactam antibiotics (especially to oxacillin). Media used in this study were cation-adjusted Mueller-Hinton broth for minimum inhibitory concentration (MIC), and tryptic soy agar for minimum bactericidal concentration (MBC) and colony counts. Tryptic soy broth supplemented with 1% glucose was used for biofilm production.

Preparation of Schiff Base

Taurine-5-bromosalicylaldehyde Schiff base (TBSSB) was prepared as described previously [8]. In brief, equimolar quantities (1 mmol) of 5-bromosalicylaldehyde, potassium hydroxide, and taurine were dissolved in methanol (10 ml), and the mixture was stirred for 5 h at 40°C. After standing for approximately 3 days, the yellow single crystals were separated by filtration and recrystallized by addition of methanol. The novel Schiff base complex was characterized by IR spectral and single-crystal X-ray diffraction methods.

Antimicrobial Susceptibility Testing

MIC and MBC determinations. MIC and MBC values for TBSSB were determined using a microtiter broth dilution method

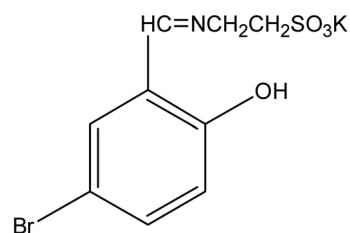


Fig. 1. Taurine-5-bromosalicylaldehyde Schiff base (TBSSB). Potassium 2-[[1-(5-bromo-2-hydroxyphenyl)-meth-(Z)-ylidene]-amino] ethanesulfonic acid.

as recommended by the Clinical and Laboratory Standards Institute [4]. MRSA ATCC 43300 was cultured in cation-adjusted Mueller-Hinton broth (CAMHB) for 24 h at 37°C, and diluted to give a final concentration of 5×10^5 colony-forming units (CFU)/ml. This suspension was added to each well of a 96-well culture microtiter plate (Nunc 167008). Two-fold serial dilutions of the compound were added to the suspension; the optical density of the culture was measured at 595 nm using a microplate reader (Multiskan Ascent 354; Thermo Labsystems) after the incubation of 24 h at 37°C. The MIC was defined as the lowest concentration of the compound that produced complete inhibition of visible growth. The MBC was determined at the end of the incubation period by removing two 10 μ l samples from each well in which there was no visible growth and plating the samples onto tryptic soy agar. Resultant colonies were counted after overnight incubation at 37°C. The MBC was defined as the lowest concentration of antimicrobial that produced at least 99.9% killing of the initial inoculum. All MIC and MBC determinations were made in triplicate.

High-inoculum MIC determinations. Since a high cell density of about 5×10^7 CFU/ml is needed to observe electron microscopy images, the MIC for the compound was also determined with high inoculation doses. High-inoculum MIC was determined by the broth microdilution technique as previously described, with the following modification [4]. MRSA was diluted to give a final concentration of approximately 5×10^7 CFU/ml, instead of 5×10^5 CFU/ml. The other steps were the same as the above description. The MIC was defined as the lowest concentration of the compound that produced complete inhibition of visible growth.

Time-Kill Assay

The time-kill kinetic studies against MRSA were performed using the broth macrodilution method [14]. In brief, bacterial suspensions were diluted to 1×10^6 CFU/ml. Drug concentrations were adjusted to 0.5×MIC, 1×MIC, 2×MIC, and 4×MIC. Cultures were incubated at 37°C for 0, 2, 4, 6, 8, 12, and 24 h. Samples were serially diluted in PBS with the appropriate dilutions and spread-plated onto tryptic soy agar. The plates were incubated anaerobically at 37°C for 48 h followed by enumeration of the colonies. Killing curves were constructed by plotting the \log_{10} CFU/ml versus time

over a 24 h time period. Bactericidal activity was defined as a $\geq 3\text{-log}_{10}\text{CFU/ml}$ decrease from the initial inoculum. All assays were performed in duplicate in three independent experiments.

Inhibition of biofilm formation. Biofilm attachment assays were performed as previously described with some modifications [22]. Biofilm formation was performed in the wells of a sterile 96-well flexible vinyl (PVC) microplate (Costar 2595). MRSA was cultured in TSB-glucose overnight and diluted to give a final concentration of approximately $1 \times 10^5 \text{CFU}/200 \mu\text{l}$. Two-fold serial dilutions of the compound were prepared, with the final concentration ranging from $1/8 \times \text{MIC}$ to $1 \times \text{MIC}$. The plates were incubated for 24 h at 37°C . The positive control wells contained only MRSA in TSB-glucose. The negative control was TSB-glucose. After the incubation, the wells of the plates were washed three times with $250 \mu\text{l}$ of physiological buffered saline (PBS) solution to remove unattached bacteria, and then air dried. A $200 \mu\text{l}$ volume of 99% methanol was added per well for 15 min for fixation. Wells were stained with $200 \mu\text{l}$ of 0.1% crystal violet for 5 min. The excess stain was rinsed off with distilled water and the wells were air dried. Stain was resolubilized in $200 \mu\text{l}$ of 95% ethanol with shaking in an orbital shaker for 30 min, and the absorbance at 595 nm of each well was measured by a microplate reader. The optical density was measured at 595 nm (OD_{595}) and was considered as an index of bacterial adherence and biofilm formation. The experiment was performed in triplicate, results were averaged, and the standard deviation (SD) was calculated.

Electron Microscopy

The electron microscopy was performed according to the procedure previously described with little modification [10]. MRSA was cultured in MHB for 24 h at 37°C and was diluted to give a final concentration of approximately $5 \times 10^7 \text{CFU/ml}$. The compound was added into the culture and the final concentration was $1 \times \text{MIC}$ for high inoculum and $2 \times \text{MIC}$ for high inoculum. After incubation for 4 h, bacteria were harvested by centrifugation and washed twice with 0.1 M PBS (pH 7.2).

Scanning Electron Microscopy

The bacterial were fixed with 2.5% glutaraldehyde, washed, and postfixed with 0.2% OsO_4 . The samples were dehydrated with a graded ethanol series. After critical point drying and gold coating, the samples were examined with a scanning electron microscope (JEOL JSM-6510LV).

Transmission Electron Microscopy

Bacterial cells were fixed with 2.5% glutaraldehyde for 4 h at room temperature; after washing twice with PBS, the samples were further fixed with 1% OsO_4 for 1 h and then dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 10 min each. Finally, the samples were embedded in epoxy resin. Thin sections of the specimens were cut with an ultramicrotome and the sections were double-stained with saturated uranyl acetate and lead citrate. The ultrathin

sections were viewed and photographed using a transmission electron microscope (JEM-2000EX) at 120 kV.

Statistical Analysis

Results are expressed as the mean and standard deviation (SD). Differences amongst groups were compared by analysis of variance, and the Student's *t*-test was used to calculate the significance of the difference between the different groups. Difference of *p* values < 0.05 was considered statistically significant.

Result

Antimicrobial Susceptibility Testing

The *in vitro* activities of the TBSSB against MRSA ATCC 43300 planktonic cells were determined. In addition to normal inoculums ($5 \times 10^5 \text{CFU/ml}$), the high-cell density inoculum ($5 \times 10^7 \text{CFU/ml}$) was included to facilitate the observation of the electron microscopic examination. The MIC of TBSSB was $32 \mu\text{g/ml}$ at standard inoculation cell density, while the MIC value was increased 4-fold when the high inoculum was $5 \times 10^7 \text{CFU/ml}$. The MBC value was two times greater than the MIC values.

Time-Kill Assay

In the present study, time-kill experiments were used to assess the activity of TBSSB on the MRSA ATCC 43300. The advantage lies in that killing curves provide a dynamic picture of antimicrobial action. The results of the time-kill kinetic studies are shown in Fig. 2. The results revealed that the killing curves exhibited a dose- and time-dependent pattern. TBSSB significantly inhibited bacterial growth compared with control cultures, where the reduction was $\geq 3\text{-log}_{10}\text{CFU/ml}$ (99.9%) at a TBSSB concentration of $2 \times \text{MIC}$.

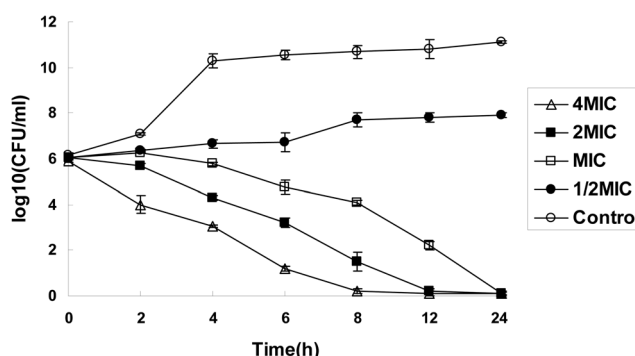


Fig. 2. Time-kill curves for MRSA ATCC 43300 by TBSSB. TBSSB-free growth control (○); $1/2 \times \text{MIC}$ TBSSB (●); $1 \times \text{MIC}$ TBSSB (□); $2 \times \text{MIC}$ TBSSB (■); $4 \times \text{MIC}$ TBSSB (△). Values are means of three independent experiments \pm standard deviations.

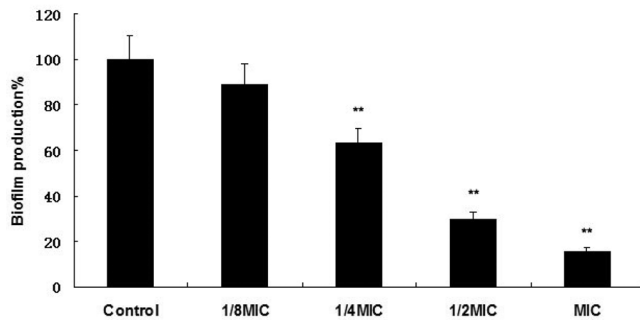


Fig. 3. Inhibition of MRSA ATCC 43300 biofilm formation by TBSSB.

Control group indicates MRSA cultures without TBSSB, accepted as 100%. Different concentrations of TBSSB (1/8~1×MIC) were added in the culture of 1×10^6 CFU/200 μ l MRSA in TSB-glucose. The plates were incubated for 24 h at 37°C. Results are expressed as the mean and standard deviation. Differences of *p* values <0.05 were considered statistically significant.

The bactericidal endpoints were reached after 6 h of incubation at a TBSSB concentration of 2×MIC or after 4 h of incubation at the concentration of 4×MIC.

Inhibition of biofilm formation. In this study, the ability of TBSSB to prevent biofilm formation was also assessed. As shown in Fig. 3, TBSSB exhibited significant inhibition against MRSA ATCC 43300 biofilm formation at 24 h. Exposure to 8 μ g/ml TBSSB (1/4×MIC) resulted in inhibition of biofilm formation, and the inhibition increased gradually with the increase of concentration of TBSSB.

Scanning Electron Microscopy

In the control samples of MRSA ATCC 43300, the cells looked a normal round shape and had a smooth cell surface (Fig. 4A). After incubation with 1×MIC for high inoculum of TBSSB, some bacteria had multiple dents and had burst with deep craters in their cell wall (Fig. 4B). After treatment with 2×MIC of TBSSB, we observed a rough and damaged surface, depression, rupture lines, and hole formation. Lysed cells and cell debris were also observed (Fig. 4C).

Transmission Electron Microscopy

A better view and understanding of the cellular morphology degenerations can be observed through the

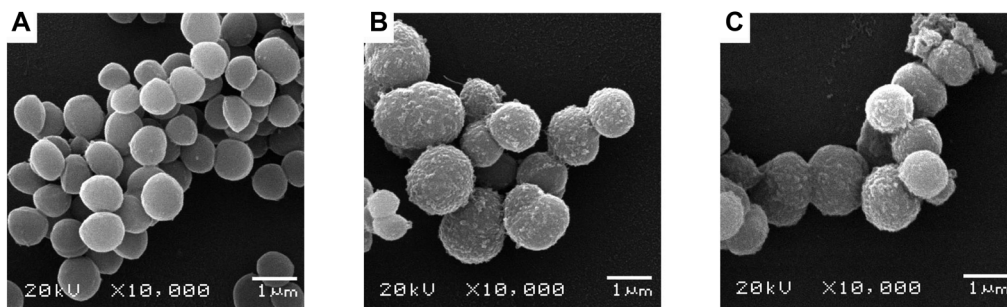


Fig. 4. Scanning electron micrographs of MRSA ATCC 43300.

(A) Untreated MRSA ATCC 43300; (B) treated with 1×MIC TBSSB; (C) treated with 2×MIC TBSSB. SEM magnification, $\times 10,000$. Bar indicates 1 μ m.

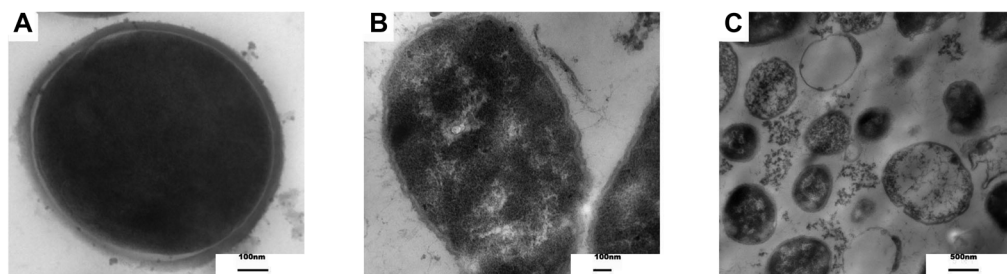


Fig. 5. Transmission electron micrographs of MRSA ATCC 43300.

(A) Untreated MRSA ATCC 43300; (B) treated with 1×MIC TBSSB; (C) treated with 2×MIC TBSSB. TEM magnification: (A), (B) $\times 80,000$, bar indicates 100 nm; (C) $\times 15,000$, bar indicates 500 nm.

TEM results. As indicated in Fig. 5A, untreated MRSA ATCC 43300 showed an intact cell shape with homogeneous cell wall and symmetrical cell membranes. After incubation with 1×MIC for high inoculum, we observed the well-defined membranes inclusions were apparent or even completely disrupted (Fig. 5B). At 2×MIC for high inoculum, a critical alteration of the outer membrane was seen. We also observed many lysed cells, with cytoplasmic contents leaking out (Fig. 5C).

Discussion

The rising number of infections caused by bacterial isolates resistant to conventional antibiotics has led to an intense search for novel antimicrobial agents. Schiff bases have been pointed to as promising antibacterial agents [7]. In our previous studies, we designed and synthesized a series of Schiff base compounds, which include benzohydrazide forms Schiff base and taurine forms multiple types of Schiff base compounds [12, 13, 23, 24]. Taurine forms Schiff base has especially aroused common concern, because taurine is indispensable to human beings and plays an important role in physiological functions. In the present study, we investigated the *in vitro* activities of TBSSB against MRSA ATCC 43300 planktonic cells (5×10^5 CFU/ml (standard inoculum) or 5×10^7 CFU/ml (high inoculum)). We found that planktonic MRSA cells were susceptible to TBSSB. It was shown that TBSSB exhibited higher antibacterial activity than lots of similar compounds, because the MIC of most Schiff bases was higher than 50 µg/ml, whereas the MIC of TBSSB was 32 µg/ml [24]. The study on structure-activity relationships of these Schiff base derivatives indicated that the sulfonic acid group of taurine seemed to play an important role in the antimicrobial activity. Although the determination of MIC is still the gold standard for determining the activities of antimicrobial agents, these techniques do not provide any information about the time course of the antibiotic activities. This limitation can be overcome with time-kill experiments. The results of the time-kill curve demonstrated that TBSSB was rapidly bactericidal against MRSA within 6 h. In addition to high antimicrobial activity, TBSSB showed a low cytotoxicity (data not shown). Its *in vitro* cytotoxicity was screened by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using the L929 murine cell line (recommended by the International Standard ISO 10993:2009 for evaluation of cytotoxic activities) [16]. The cytotoxicity (IC_{50}) was determined to establish a selectivity index ($SI = IC_{50}/MIC$; SI values higher than 10 could be considered

being sufficient; others are poor). The SI for TBSSB was >10, which indicated that TBSSB was a selective and less toxic antimicrobial agent. In addition, TBSSB in combination with conventional antibiotics is a promising novel therapeutic agent that could be used to overcome the development and spread of antibiotic-resistant microorganisms.

Staphylococci, especially MRSA strains, are the most frequent cause of biofilm-associated infections, especially catheter- or artificial graft-related infections [18]. A biofilm is a microbial community that is attached to abiotic surfaces and produces extracellular polysaccharides. Since bacteria in biofilms exhibit elevated resistance to both antibiotics and the host defence systems, the treatment of biofilm-associated infections is extremely difficult [6]. In this study, TBSSB was also found to significantly inhibit MRSA biofilm formation at 24 h, especially at sub-MIC ($1/4 \times MIC$) levels ($p < 0.01$). This indicates that, in addition to inhibiting the growth of planktonic bacteria, TBSSB may be capable of inhibiting the adherence of cells to the host surface, thus disrupting the initial step in the formation of mature biofilms. It is generally known that inhibition of mature biofilms is very difficult and requires achievement of very high concentrations of antimicrobial agents, where the inhibition of biofilm formation during the early stages seems more applicable. Thus, TBSSB appears to be a good candidate for treating biofilm-associated MRSA infections. It could be especially attractive for topical treatment of catheter-related or prosthetic joint infections. Animal experiments and *in vivo* studies are required to validate the clinical applicability of TBSSB to prevent medical device-associated MRSA infections in the future.

It is doubtless that bacterial cell membrane integrity is necessary for many essential functions, and its disturbance can directly or indirectly cause metabolic dysfunction and cell death. Observing alterations in the bacterial membrane integrity by electron microscopy can help to gain insight into the compound action, by revealing not only cell surface effects but also intracellular alterations. In the present study, SEM images clearly indicated severe staphylococcal membrane perturbation, and several dents, holes, and craters were seen on the surface of the bacteria. Furthermore, the loss of cell wall and the presence of cells without cytoplasmic content were clearly visible from TEM images of TBSSB-treated MRSA cells. The result further confirmed the bactericidal activity of TBSSB against MRSA, being indicative of a mechanical rupture of the membrane permeability.

In conclusion, this study evaluated the antimicrobial activity of TBSSB against MRSA. TBSSB exhibited bactericidal

activity against planktonic MRSA and substantially attenuated its ability to form biofilms. Morphological and ultrastructural changes were observed by electron microscopic images, which indicated the mechanism of TBSSB may be associated with the destruction of membrane integrity. This study suggests that TBSSB may have a potential clinical application in treating MRSA infections.

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