INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an organism that represents a worldwide threat due to its ability to acquire resistance to most antibiotics (Aqil *et al*., 2006; Gibbons, 2004). It is one of the most common human pathogens that is responsible for a wide variety of infections, many of which can be life-threatening (Couto *et al*., 2008). Methicillin-resistant *Staphylococcus aureus* is often found commensally associated with skin, skin glands, and mucous membranes, particularly in the nose of healthy individuals (Plata *et al*., 2009; Short *et al*., 2005; Schito, 2006). Methicilin resistance was first identified in 1961 among nocosomial isolates of S.aureus and till the last 10 years, was mainly restricted to the nocosomial setting (Drago *et al*., 2007).

Sanguinarine is a benzophenanthridine alkaloid derived from the root of *Sanguinaria canadensis*. It is known to perform a wide spectrum of biological activities. The aim of this study is to examine the antimicrobial actions of sanguinarine against methicillin-resistant *Staphylococcus aureus* (MRSA). Sanguinarine antimicrobial activity was assessed by broth dilution method; its mechanism of action was investigated by bacteriolyis, detergent or ATPase inhibitors and transmission electron microscopy were used to monitor the survival characteristics and the changes in bacteria morphology. The activity of sanguinarine against MRSA strains ranged from 3.12 to 6.25 μg/ml, while the minimum inhibitory concentrations of the two reference strains are 3.12 μg/ml and 1.56 μg/ml. The treatment of the cells with sanguinarine induced the release of membrane-bound cell wall autolytic enzymes, which eventually resulted in lysis of the cell. The OD<sub>600</sub> of the suspensions treated with the combination of Tris-(hydroxymethyl) aminomethane and Triton X-100 with sanguinarine were reduced to 40% and 8%, respectively. Transmission electron microscopy of MRSA treated with sanguinarine showed alterations in septa formation. The predisposition of lysis and the altered morphology seen by transmission electron microscopy suggest that sanguinarine compromises the cytoplasmic membrane.

Key words: Sanguinarine, Methicillin-resistant *Staphylococcus aureus*, Transmission electron microscopy, Cell wall

The mechanism of action of sanguinarine against methicillin-resistant *Staphylococcus aureus*

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ABSTRACT — Sanguinarine is a benzophenanthridine alkaloid derived from the root of *Sanguinaria canadensis*. It is known to perform a wide spectrum of biological activities. The aim of this study is to examine the antimicrobial actions of sanguinarine against methicillin-resistant *Staphylococcus aureus* (MRSA). Sanguinarine antimicrobial activity was assessed by broth dilution method; its mechanism of action was investigated by bacteriolyis, detergent or ATPase inhibitors and transmission electron microscopy were used to monitor the survival characteristics and the changes in bacteria morphology. The activity of sanguinarine against MRSA strains ranged from 3.12 to 6.25 μg/ml, while the minimum inhibitory concentrations of the two reference strains are 3.12 μg/ml and 1.56 μg/ml. The treatment of the cells with sanguinarine induced the release of membrane-bound cell wall autolytic enzymes, which eventually resulted in lysis of the cell. The OD<sub>600</sub> of the suspensions treated with the combination of Tris-(hydroxymethyl) aminomethane and Triton X-100 with sanguinarine were reduced to 40% and 8%, respectively. Transmission electron microscopy of MRSA treated with sanguinarine showed alterations in septa formation. The predisposition of lysis and the altered morphology seen by transmission electron microscopy suggest that sanguinarine compromises the cytoplasmic membrane.
ine (Singla et al., 2010). Many benzylisoquinoline alkaloids have been reported to show therapeutic properties and to act as novel medicines. Alkaloids are naturally occurring secondary metabolites, low molecular weight, and nitrogen containing compounds that are found in more than 20% of plant species (Singla et al., 2010). Studies of sanguinarine have also shown some plaque and gingivitis reduction (Ciancio, 1992; Grenby, 1995), killing of animal cells through its action on the Na⁺-K⁺-ATPase transmembrane protein (Pitts and Meyerson, 1981). If applied to the skin, sanguinarine kills cells and may destroy tissue. In turn, the bleeding wound may produce a massive scab. In plants, sanguinarine is synthesized from dihydrosanguinarine through the action of Dihydrobenzophenanthridine oxidase (Howell, 1972).

Although the in vitro antimicrobial activity of sanguinarine has been reported, less is known about its mechanism of action. In this study, we tried to evaluate its mechanism of action against MRSA.

**MATERIALS AND METHODS**

**Bacterial strains and culture medium**

MRSA (ATCC 33591) and MSSA (ATCC 25923) strains (American Type Culture Collection, Manassas, VA, USA) were commercially purchased and the other 15 clinical isolates used in the study were obtained from different patients at the Wonkwang University Hospital (Iksan, South Korea). Before use, all bacteria were stored in 30% glycerol and frozen at -70°C. The bacteria were cultured in Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) (Difco Laboratories, Baltimore, MD, USA) and incubated at 37°C for 20 hr.

**Antimicrobial agents**

The following chemicals were all purchased from Sigma-Aldrich Co. (St. Louis, MO, USA): Sanguinarine (SN), ampicillin (AC), ciprofloxacin (CP), Triton X-100 (TX), Sodium azide (NaN₃), Tris-(hydroxymethyl) aminomethane (TRIS), N,N'-Dicyclohexylcarbodiimide (DCCD) glutaraldehyde, paraformaldehyde, sodium cacodylate, osmium tetroxide, uranyl acetate, Spurr’s resin and toluidine blue.

**Determination of the mecA gene**

Detection of the mecA gene in the MRSA strains was performed by Polymerase Chain Reaction (PCR) amplification. Prior to the DNA extraction, frozen bacteria were subcultured twice onto MHA plates. For rapid extraction one to five bacterial colonies were suspended in 300 μl of cell lysis buffer and heated at 100°C for 20 min. After centrifugation at 12,000 RPM for 10 min, 2 μl of the supernatant was used for the DNA extraction. PCR reactions were performed using a MRSA Primer Mix Kit (Genotek Co., Daejeon, Korea). The PCR amplification consisted of 30 cycles (94°C, 60 sec; 55°C, 60 sec; 72°C, 60 sec). The primers used in this study were as follows: mecA-forward primer; 5’-ATGAGATTCAGCATGTTTC-3’, reverse primer; 5’ TGGATGACAGTACCTGAGCC-3’ and femA-forward primer; 5’ CATGATGGCGAGATACAGG-3’, reverse primer; 5’-CGCTAAAGGGTACTAACACACGG-3’. The final PCR products were separated on 2% agarose gel.

**Determination of the minimum inhibitory concentration (MICs)**

The MIC was performed by the microdilution broth method (CLSI, 2006). Serial two fold dilutions of SN, AC and CP were prepared in sterile 96-well micro plates and microtube with concentrations ranging between 1/2 by using MHB. The MRSA suspensions were adjusted to the 0.5 McFarland standards (approximately 1 × 10⁶ CFU/ml). Final inoculums were adjusted to the 10⁶ CFU/spot. The MHB was supplemented with serial AC and CP concentrations ranging from 0.19 to 5,000 μg/ml, and with SN at concentrations from 0.52 to 1,000 μg/ml. The data were reported as MICs, the lowest concentration of AC, CP and SN inhibiting visible growth after 24 hr of incubation at 37°C (Choi et al., 2010).

At the end of incubation period, the well plates were visually examined for turbidity. Cloudiness indicates that bacterial growth has not been inhibited by the concentration of antimicrobial agent contained in the medium.

**Bacteriolysis**

Bacteriolysis was carried out following the modified method (Carson et al., 2002). Suspensions of MRSA were prepared as described above and treated with sanguinarine at concentrations equivalent to 1/2 MIC, MIC and 2MIC. The suspensions were mixed and OD₆₂₀nm was measured to detect cell lysis as indicated by the decrease in OD. Samples were taken 3 min after the experiment was set up (corresponding to exposure time 0) and the test tubes were thereafter incubated at 37°C. Additional samples were taken for analysis after 12 and 24 hr, respectively.

**Antibacterial activity with detergents or ATPase-inhibitors**

To elucidate whether antibacterial activity of sanguinarine was associated with the altered membrane permeability or the action of multidrug-resistant pumps, anti-
bacterial susceptibility of sanguinarine was examined in the presence of detergent or ATPase-inhibiting agents. To increase the permeability of the outer membrane, the concentration of sanguinarine, as a fractional inhibitory concentration (FIC) determined in a combination assay with other therapeutic agents, was added to bacterial cells in the presence of 0.001% Triton X-100, and 125 μg/ml Tris, respectively. NaN³ and N₂,N-dicyclohexylcarbodiimide (DCCD) were used as inhibitors of ATPase (Linnett et al., 1979; Jung and Lee, 2008). The antibacterial susceptibility of sanguinarine, in the presence of 0.005% NaN³ and 125 μg/ml DCCD, was also carried out at the same condition.

**Inoculum preparation for electron microscopy**

One milliliter of an 18 hr MHB culture (10⁶ CFU ml⁻¹) was added to 10 ml of MHB containing 1 ml of sanguinarine at MIC concentration. The control containing only MHB and MRSA was also set up. The tubes were incubated at 37°C for 8 hr after which bacteria cell were collected by centrifugation.

**Transmission electron microscopy (TEM)**

The pellets were fixed and further processed as previously described (Kim and Park, 2007). Ultrathin sections (~60 nm in thickness) were mounted on bare copper grids and stained with 2% uranyl acetate and Reynolds’ lead citrate each for 7 min. The specimens were examined with an energy-filtering transmission electron microscope (LIBRA 120; Carl Zeiss, Oberkochen, Germany) operated at an accelerating voltage of 120 kV. Transmitted electron signals were recorded using a 4k X 4k slow-scan charge-coupled device camera (Ultrascan 4000 SP; Gatan, Pleasanton, CA) attached to the electron microscope (Kim et al., 2009).

**RESULTS**

**Determination of the mecA gene and resistance pattern**

Table 1 shows the results of the mecA gene determination and the resistance pattern towards ampicillin and oxacillin.

**MIC determinations**

The activity of sanguinarine (SN) against MRSA strains using the micro dilution technique is reported in Table 2. The MIC ranged from 3.12 to 6.25 μg/ml, while MIC of the two reference strains are 3.12 μg/ml for ATCC 25923 and 1.56 μg/ml for ATCC 33591. The clinical isolated strains showed MIC values ranging from 31.25 to 250 μg/ml for ampicillin and 125-1,000 μg/ml for ciprofloxacin.

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**Table 1.** Determination of the mecA gene of the s. aureus strains used in the experiment

<table>
<thead>
<tr>
<th>S. aureus strain</th>
<th>Class</th>
<th>mecA gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>β-Lactamase activity</th>
<th>Antibiotic resistance pattern&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25923&lt;sup&gt;c&lt;/sup&gt;</td>
<td>MSSA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ATCC 33591&lt;sup&gt;c&lt;/sup&gt;</td>
<td>MRSA</td>
<td>+</td>
<td>+</td>
<td>Am, Ox</td>
</tr>
<tr>
<td>Clinical isolates&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPS-1</td>
<td>MRSA</td>
<td>+</td>
<td>+</td>
<td>Am, Ox</td>
</tr>
<tr>
<td>DPS-2</td>
<td>MRSA</td>
<td>+</td>
<td>-</td>
<td>Am, Ox</td>
</tr>
<tr>
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<td>MRSA</td>
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<td>DPS-14</td>
<td>MRSA</td>
<td>+</td>
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<td>Am, Ox</td>
</tr>
<tr>
<td>DPS-15</td>
<td>MRSA</td>
<td>+</td>
<td>+</td>
<td>Am, Ox</td>
</tr>
</tbody>
</table>

<sup>a</sup> +, positive; -, negative.  
<sup>b</sup> AM, ampicillin; OX, oxacillin.  
<sup>c</sup> American Type Culture Collection, AC, ampicillin; CF, ciprofloxacin.  
<sup>d</sup> DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital.
Effect of sanguinarine on MRSA cell wall

The results obtained from the measurement of OD 620 nm (Fig. 1) indicated that treated cells with one-half the MIC had no significant effect on the growth of bacteria. In contrary, the bacteria grew over time in the absence or presence of sanguinarine 1/2 MIC. Fig. 1 also shows the results obtained when MRSA was treated with sanguinarine at MIC and two times MIC (2MIC). A large reduction of OD value is observed at 24 hr compared to OD620 nm measured at 12 hr.

Antibacterial activity with detergents or ATPase-inhibitors

To investigate the effects of enhanced membrane permeability on the activity of sanguinarine using detergents, the antibacterial activity of sanguinarine under increased membrane permeability was examined using 125 μg/ml Tris, and 0.001% Triton X-100 (TX). Tris and Triton X-100 are membrane-permeabilizing agents which can increase the permeability of the outer membrane (Irvin et al., 1981). Modest reductions in the OD600 of sanguinarine-treated suspensions were noted in combination with Tris and TX when measured 4 hr later (Figs. 2 and 3). When compared to SN alone, the OD600 of the suspensions treated with the combination of Tris and TX with SN were reduced to 40% and 8% respectively, when they were read again 12 hr later.

The bacterial viability in the presence of sanguinarine with 0.005% NaN3 as a metabolic inhibitor which can decrease ATP levels by disrupting electrochemical proton gradients in bacterial environment was investigated (Swallow et al., 1990). Sanguinarine in combination with NaN3 and DCCD decreased the viability of MRSA strains. Treatment with ATPase inhibitors agents reduced the OD600 slightly (Figs.4 and 5). However, when measured 12 hr later, the combination of NaN3 and DCCD with SN (0.39 μg/ml) did not reduced the viability of MRSA.

TEM of MRSA

The control cells had normal morphology of S. aureus

Table 2. MIC (μg/ml) of sanguinarine and antibiotic on clinical isolates and a reference strain

<table>
<thead>
<tr>
<th>S.aureus</th>
<th>SN</th>
<th>AC</th>
<th>CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolates (n = 15)*</td>
<td>3.12-6.25</td>
<td>31.25-250</td>
<td>125-1000</td>
</tr>
<tr>
<td>ATCC 25923*</td>
<td>3.12</td>
<td>0.06</td>
<td>3.9</td>
</tr>
<tr>
<td>ATCC 33591*</td>
<td>1.56</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>
*American Type Culture Collection, AC= ampicillin; CP= ciprofloxacin; SN= sanguinarine; ▲ = clinical isolates from Wonkwang University Hospital.
with distinct septa (Fig. 6A). However, MRSA cells treated with sanguinarine showed few or distorted septa (Fig. 6B). Distinct septa formation was rarely discerned in the treated cells. It was common to observe more ghost cells, degenerated cells, and less cell division in sanguinarine-exposed cells (Fig. 6C) than the control.

**DISCUSSION**

MRSA is one of the main causes of hospital-and community-acquired infections which can result in increasing antibiotic resistance. In the United States alone, more than 70% of hospital-acquired infections are antibiotic-resistant pathogens (Otto et al., 2010). Sanguinarine has been shown to possess a broad spectrum of *in vitro* activity against a wide variety of microorganisms including...
fungal, yeasts, and phages (Dzink and Socransky, 1985). The research presented here documents an understanding into the mechanism of action of sanguinarine.

The MIC assay is considered to be the standard for determining the susceptibility of microorganisms to antibacterials (Bala et al., 2005). *In vitro* results show that the MIC of sanguinarine, ampicillin and ciprofloxacin are within the range that has been reported by other researchers against MRSA (Dzink and Socransky, 1985; Kwon et al., 2007; Choi et al., 2010).

Antibacterial agents inhibit bacterial growth through a variety of complex mechanisms, including inhibition of cell wall synthesis, disruption of cell membranes, inhibition of nucleic acid synthesis and protein synthesis, an inhibition of nucleic acid metabolism (Al-Habib et al., 2010). The analysis of Fig. 1 suggests that it is possible that the treatment of the cells with sanguinarine might induce the release of membrane-bound cell wall autolytic enzymes, which may eventually result in lysis (Ifesan et al., 2009). Sanguinarine exhibits bactericidal activity against MRSA as determined by OD_{620 nm} possible due to weakening of the cell wall. This hypothesis is confirmed when sanguinarine was used in combination with detergents (Tris and Triton X-100) responsible for bacterial increased membrane permeability. MRSA is killed at slower rate with membrane permeabilization. Figs. 2 and 3 summarize the membrane permeability data determined by OD_{600 nm}. After a 12 hr treatment, MRSA viability or OD value decreased by 40% and 50%, when exposed to the combination of sanguinarine and TRIS compared to sanguinarine alone and the control. In contrast, the combination of sanguinarine with ATPase inhibitors (NaN3 and DCCD) has no effect on the OD value (Figs. 4 and 5). It may indicate that the primary mechanism is not through ATPase inhibitors. These results are in line with published studies showing the slightly resistance of *S. aureus* to NaN3 (Lichstein et al., 1944) compared to gram-negative bacteria.

On the other hand, the alkaloid berberine is a plant based compound with similar chemical classification as sanguinarine. It is a potentially excellent antimicrobial agent, because it accumulates in cells driven by membrane potential and hits two immutable target, the membrane and DNA (Ball et al., 2006). It can be assumed that these two compounds may have similar site of action bacteria.

TEM observations seemed to be well correlated with the effect of sanguinarine on the cell wall experiment. Sanguinarine showed good and rapid bactericidal activity within 12 hr as indicated by OD_{520 nm}. It is known that when bacteria are exposed to antibiotics at low concentrations, changes in bacterial morphology, ultrastructure, biochemistry, and multiplication rate have been observed (Lorian, 1978). Therefore, the effect of antibiotics on the morphology and ultrastructure of bacteria can be classified according to the target where the alteration occurs (Ifesan et al., 2009). Antibiotics treatment is known to induce other cellular changes, such as reduced cell wall integrity (Al-Habib et al., 2010). TEM of *S. aureus* treated with sanguinarine showed structural alterations such as aberrant septa and cell disruption. Accumulation of hydrophobic cations in the membrane causes leaks (Davidson et al., 1977).

While TEM analysis provides useful insight into the mechanism of sanguinarine, the resulting images are observational only. Other techniques must be used in tandem to verify the observations generated from the TEM images (Otto et al., 2010). The predisposition of lysis and the altered morphology seen by TEM suggest that sanguinarine compromises the cytoplasmic membrane. In this study, we provide evidence that the antibacterial action of sanguinarine results in (i) lysis of cells and (ii) deformation of the septa in MRSA.

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**REFERENCES**


Mechanism of action of sanguinarine


