Synergistic Effects of the Combination of 20-Hydroxyecdysone with Ampicillin and Gentamicin Against Methicillin-Resistant Staphylococcus aureus

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The emergence of methicillin-resistant Staphylococcus aureus (MRSA) has led to an urgent need for the discovery and development of new antibacterial agents. As part of an ongoing investigation into the antibacterial properties of natural products, 20-hydroxyecdysone (20E), isolated from the roots of Achyranthes japonica Nakai, was found to be active against MRSA, either alone or in combination with ampicillin (AM) or gentamicin (GM), via checkerboard assay. This study investigated the antibacterial activity of 20E, which exhibited poor antibacterial activity (MIC= 250–500 µg/ml) against MRSA tested. The combined activity of AM or GE plus 20E against MRSA resulted in fractional inhibitory concentrations (FICs) ranging from 4.00 to 0.031 µg/ml, respectively. Meanwhile, the FIC index ranged from 0.16–4.50, indicating a marked synergistic relationship between AM, GE, and 20E against MRSA. Time-kill assays also showed a remarkable decrease between the combination and the more active compound. Therefore, this study demonstrated that AM, GE, and 20E can act synergistically in inhibiting MRSA in vitro.

Keywords: Achyranthes japonica, 20-hydroxyecdysone, methicillin-resistant Staphylococcus aureus, synergism

Methicillin-resistant Staphylococcus aureus (MRSA) is known to be one of the most common pathogens of chronic otitis media (COM) and skin infection. It can cause not only middle ear infection but also other complications in the inner ear and brain, such as sensorineural hearing loss (SNHL) [6, 12, 21]. Conductive hearing loss due to middle ear disorders can be treated by surgery. COM, however, may be associated with a functional damage to the inner ear. SNHL due to COM has been found to be significant by some authors [7, 12, 21]. Those considering SNHL as a consequence of COM assumed inflammatory mediators as the substances that can cause the function of the inner ear to deteriorate [10, 13]. The SNHL can develop from inflammatory mediators, histamine, endotoxins, and free radicals [8, 10, 13–15].

Achyranthes japonica (A. japonica) has been widely used as the basis of oriental medicines or folk remedies [27]. A. japonica extracts inhibit cytochrome P450 drug metabolizing enzymes, influence immune regulation, lessen mutagenic effects, quell acute and subacute inflammations, inhibit tumor development, and influence rheumatoid arthritis [3]. Although the biological activities of A. japonica have been intensively researched, there have been no reports

Fig. 1. Chemical structure of 20-hydroxyecdysone isolated from Achyranthes japonica.
of the antibacterial effects of *A. japonica* on MRSA. In the course of our ongoing project on the detection of bioactive compounds from medicinal plants, the n-BuOH-soluble extract of roots of *A. japonica* was found to have the compound that have an antibacterial activity (20-hydroxyecdysone, Fig. 1). In the present study, we investigated the antimicrobial activity of 20-hydroxyecdysone (20E) and the synergistic effect of the mixture of ampicillin (AM) or gentamicin (GE) with 20E against MRSA with the exotoxin gene in COM.

**MATERIALS AND METHODS**

**Isolation of 20E**

The plant materials, roots of *A. japonica*, were collected in Jinan, Korea, in December 2007. The plant was identified and authenticated by Prof. Hong-Jun Kim at the College of Oriental Medicine, Woosuk University. A voucher specimen (No. JS10903) has been deposited at the Department of Herbolology, College of Oriental Medicine, Woosuk University, Chonbuk, Republic of Korea. Air-dried and coarsely ground roots of *A. japonica* (770 g) were extracted with MeOH at room temperature to afford 73.3 g of residue. The MeOH extracts were suspended in water and then partitioned with CHCl₃, EtOAc, and n-BuOH, successively. A 15 g n-BuOH fraction was subjected to silica gel column chromatography over a silica gel (300 g, 3.0×70 cm) eluting with a CHCl₃–MeOH (7:1→1:1) gradient system. The fractions were combined based on their TLC pattern to yield subfractions designated as A1—A6. Subfraction A5 (1.08 g) was finally purified by prep-HPLC (JAI908, JAIGEL-W252 column) eluting with MeOH (flow rate: 3.5 ml/min; UV at 254 nm), which was finally purified by prep-HPLC (JAI908, JAIGEL-W252 column) eluting with MeOH at room temperature to afford 0.63 g of residue. The MeOH extracts were suspended in water and then partitioned with CHCl₃, EtOAc, and n-BuOH, successively. A 15 g n-BuOH fraction was subjected to silica gel column chromatography over a silica gel (300 g, 3.0×70 cm) eluting with a CHCl₃–MeOH (7:1→1:1) gradient system. The fractions were combined based on their TLC pattern to yield subfractions designated as A1—A6. Subfraction A5 (1.08 g) was finally purified by prep-HPLC (JAI908, JAIGEL-W252 column) eluting with MeOH (flow rate: 3.5 ml/min; UV at 254 nm), which afforded 20E (38 mg). The chemical structure of 20E is shown in Fig. 1.

**Preparation of Bacterial Strain**

Five MRSA strains that induced bearing loss were clinical isolates from Wonkwang University Hospital (Iksan, Korea), and the standard strain was *Staphylococcus aureus* (S. aureus) ATCC 33591. The MRSA strains were defined on the basis of the occurrence of the mecA gene and of their resistance to ampicillin and oxacillin, according to the 2009 guidelines of the Clinical and Laboratory Standards Institute [5]. After culturing all strains on Mueller–Hinton agar (MHA), all bacteria were resuspended in Mueller–Hinton broth (MHB) to give 10⁶ colony-forming units/ml; the resuspended bacteria were then incubated.

**Detection of mecA Gene**

Detection of the mecA gene in the MRSA strains was performed by PCR (polymerase chain reaction) amplification. For rapid extraction, one to five bacterial colonies were suspended in 300 l of cell lysis buffer and heated at 100°C for 20 min. After centrifugation at 12,000 rpm for 10 min, 2 l of the supernatant was used for the DNA extraction. PCR reactions were performed using a MRSA Primer Mix Kit (Genotek Co., 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'-ATGAGATT AGGGCATCTTC-3'; reverse primer, 5'-TGGATGACTGCTACC TGAGCC-3' [23]. The final PCR products were separated on 2% agarose gel. AM and GE were commercially purchased from Sigma Chemical Co. (U.S.A.).

**Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)**

The MIC test was performed by the microdilution broth method, following the recommendations of the CLSI (2006) [4]. Serial 2-fold dilutions of AM, GE, 20E, AM plus 20E, and GE plus 20E were prepared in sterile 96-well microplates. The *S. aureus* suspensions were adjusted to the 0.5 McFarland standards (approximately 1×10⁶ CFU/ml). Final inocula were adjusted to the 10⁵ CFU/ml. The MIC range was supplemented with serial AM and GE concentrations ranging from 0.25 to 1,024 µg/ml, and with serial 20E concentrations from 0.24 to 1,000 µg/ml. The data were reported as MICs, the lowest concentration of AM and GE inhibiting visible growth after 16 h of incubation at 37°C [25]. For the study of MBCs, after the MICs were read, approximately 100 ml of the culture mixture was removed from each well of the MIC plate (i.e., from each antimicrobic concentration and from controls) and transferred onto MHA. These plates were incubated for 24 h at 37°C. The MBCs were determined by using colony counts and all of the MBCs were read at 24 h as the lowest concentration of antimicrobic to produce 99% kill [1].

**Kinetics of Bacterial Killing**

The kinetics of the bacterial killing curve were evaluated by using MRSA [20], in order to study the combined effects of time and antimicrobial agent concentration on the bacterial growth. For this assay, a standard inoculum of approximately 10⁶ CFU/ml of an overnight culture was used. All agents were used at MIC concentration. Combinations of AM or GE plus 20E were also evaluated. A test plate containing only MHB was inoculated and served as the control. Counts of viable strains were carried out at different intervals up to 16 h at 37°C. The rate and extent of killing were determined by plotting viable colony counts (CFU/ml) against time in MHA. In this paper, all experiments were independently repeated three times.

**Combination Effects of 20E with AM and GM**

The antibacterial effects that resulted from combining AM and GM agents were assessed by the checkerboard test [16]. FIC indexes were calculated using the following formula: FIC indexes=(MIC<sub>Dru g A</sub> in combination/MIC<sub>Dru g A</sub> alone)+(MIC<sub>Dru g B</sub> in combination/MIC<sub>Dru g B</sub> alone). In brief, the MRSA cells (1×10⁶/ml) were inoculated into a MHB and dispensed at 0.01 ml/well in 96-well microtiter plates. MICs were determined by a serial 2-fold dilution of 20-hydroxyecdysone and/or antibiotics. After 16 h of incubation at 37°C, the minimal compound concentration that prevented the growth of a given test organism was determined and was defined as the MIC. The MIC values were determined by three independent assays and the FIC indexes were calculated from both the MIC indexes of 20E and antibiotics. FIC indexes were synergy ≤0.5; partial synergy FIC >0.5 but <1; additive FIC=1.0; indifferent effect when values were >1 and <4 and antagonistic when values were ≥4.0 [19].

**RESULTS AND DISCUSSION**

This study investigated the antimicrobial activity of 20E against 5 MRSA strains and the standard strain of *S.*
Synergistic Effect of 20-Hydroxyecdysone against MRSA

In the checkerboard test results, AM or GE in combination with 20E showed good antimicrobial activity against the MRSA strains. The MICs of AM or GM ranged from 128 to 256 µg/ml and 256 to 512 µg/ml, respectively. The MIC of 20E was 250 to 500 µg/ml. However, in assessment of synergy, the AM plus 20E combinations exhibited markedly lowered MICs and the FICs of AM plus 20E were 0.125–4.000 µg/ml. The FICI of its combination ranged from 0.16 to 0.31 µg/ml in all strains except for strain ENT 9 (Table 1).

Table 1. Checkerboard assay of 20-hydroxyecdysone and ampicillin against ENT MRSA.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Agent</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt;/MBC&lt;sup&gt;b&lt;/sup&gt; (µg/ml)</th>
<th>FIC (µg/ml)</th>
<th>FICI&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>Ampicillin</td>
<td>256/256</td>
<td>64.0/512</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>ATCC 33591</td>
<td>20-Hydroxyecdysone</td>
<td>250/250</td>
<td>31.3/250</td>
<td>0.125</td>
<td>0.19</td>
</tr>
<tr>
<td>ENT2 (TSST)</td>
<td>Ampicillin</td>
<td>256/256</td>
<td>16.0/32.0</td>
<td>0.125</td>
<td>0.19</td>
</tr>
<tr>
<td>ENT3 (TSST, sec)</td>
<td>Ampicillin</td>
<td>256/256</td>
<td>7.80/15.6</td>
<td>0.125</td>
<td>0.19</td>
</tr>
<tr>
<td>ENT6 (etb)</td>
<td>Ampicillin</td>
<td>256/256</td>
<td>3.90/15.6</td>
<td>0.125</td>
<td>0.19</td>
</tr>
<tr>
<td>ENT9 (sec)</td>
<td>Ampicillin</td>
<td>256/256</td>
<td>16.0/64.0</td>
<td>0.125</td>
<td>0.19</td>
</tr>
<tr>
<td>ENT19 (TSST, sea, sec)</td>
<td>Ampicillin</td>
<td>256/256</td>
<td>15.6/15.6</td>
<td>0.125</td>
<td>0.19</td>
</tr>
</tbody>
</table>

<sup>a</sup>MIC required to inhibit 90% of strains.
<sup>b</sup>MBC required to kill 90% of strains.
<sup>c</sup>The checkboard test was performed as previously described [15]. MICs and MBCs of 20-hydroxyecdysone with ampicillin against ENT MRSA are indicated.
<sup>d</sup>FIC indexes were synergy ≤0.5; partial synergy FIC >0.5 but <1; additive FIC=1.0; indifferent effect when values were >1 and <4 and antagonistic when values were ≥4.0 [18].

<em>aureus</em> ATCC 33591. In the checkerboard test results, AM or GE in combination with 20E showed good antimicrobial activity against the MRSA strains. The MICs of AM or GM ranged from 128 to 256 µg/ml and 256 to 512 µg/ml, respectively. The MIC of 20E was 250 to 500 µg/ml. However, in assessment of synergy, the AM plus 20E combinations exhibited markedly lowered MICs and the FICs of AM plus 20E were 0.125–4.000 µg/ml. The FICI of its combination ranged from 0.16 to 0.31 µg/ml in all strains except for strain ENT 9 (Table 1). However, the FICs of GE were 0.125–2.000 µg/ml and its FICI ranged from 0.16 to 0.38, detecting against ENT 2, 3, 19 (Table 2). Fig. 2 and 3 show the results of the time-kill curves against bactericidal activities of AM and GM, two

Table 2. Checkerboard assay of 20-hydroxyecdysone and gentamicin against ENT MRSA.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Agent</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt;/MBC&lt;sup&gt;b&lt;/sup&gt; (µg/ml)</th>
<th>FIC (µg/ml)</th>
<th>FICI&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>Gentamicin</td>
<td>512/512</td>
<td>128/128</td>
<td>0.250</td>
<td>0.50</td>
</tr>
<tr>
<td>ATCC33592</td>
<td>20-Hydroxyecdysone</td>
<td>250/250</td>
<td>62.5/62.5</td>
<td>0.250</td>
<td>0.19</td>
</tr>
<tr>
<td>ENT2 (TSST)</td>
<td>Gentamicin</td>
<td>256/256</td>
<td>32/32</td>
<td>0.125</td>
<td>0.38</td>
</tr>
<tr>
<td>ENT3 (TSST, sec)</td>
<td>Gentamicin</td>
<td>256/256</td>
<td>15.6/15.6</td>
<td>0.125</td>
<td>0.63</td>
</tr>
<tr>
<td>ENT6 (etb)</td>
<td>Gentamicin</td>
<td>256/256</td>
<td>128/128</td>
<td>0.125</td>
<td>2.50</td>
</tr>
<tr>
<td>ENT9 (sec)</td>
<td>Gentamicin</td>
<td>256/512</td>
<td>512/512</td>
<td>2.000</td>
<td>0.16</td>
</tr>
<tr>
<td>ENT19 (TSST, sea, sec)</td>
<td>Gentamicin</td>
<td>256/256</td>
<td>15.6/31.3</td>
<td>0.031</td>
<td>0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup>MIC required to inhibit 90% of strains.
<sup>b</sup>MBC required to kill 90% of strains.
<sup>c</sup>The checkboard test was performed as previously described [15]. MICs and MBCs of 20-hydroxyecdysone with gentamicin against ENT MRSA are indicated.
<sup>d</sup>FIC indexes were synergy ≤0.5; partial synergy FIC >0.5 but <1; additive FIC=1.0; indifferent effect when values were >1 and <4 and antagonistic when values were ≥4.0 [18].
commonly used antibiotics worldwide. In the result, for 20E alone, the rate of killing CFU/ml decreased after 4 h, whereas the combination of the 20E and AM or GM increased the rate of killing before 4 h and after 16 h. Thus, the drug combinations exhibited a bactericidal effect. MRSA is very dangerous, and produces serious medical problems because it causes the most common infectious diseases and often acquires multidrug resistance. Thus, many researchers are studying natural products that could be used as antibiotics against MRSA [26]. Many researchers are studying 20E, the steroid hormone that triggers the major developmental transitions in *Drosophila*, and it provides a model system for defining the developmental and molecular mechanisms of steroid signaling. Moreover, it has been reported that 20E prevented lipid synthesis [10], and that the effect of 20E on triglyceride lipase activity was significantly decreased [2]. However, there is no previous report on the inhibitory effects of 20E on MRSA in the literature. GM inhibits protein synthesis and AM inhibits cell wall synthesis in bacteria [24, 25]. Some studies have demonstrated that impermeability of the bacterial plasma membrane plays an important role in resistance to aminoglycoside [18]. It was suggested that the change of plasma membrane by unstable lipid synthesis is increased antibacterial activity. In other words, when 20E interacts with the membrane, this raises the unstable state of the plasma membrane. The mechanism by which such combination achieves synergy to help the antimicrobial effect of GM and of AM against cells, after the partial disruption of the cell membrane, is through the action of 20E. Testing mechanisms of the action of 20E on MRSA are warranted for further studies. In addition, further

**Fig. 2.** *In vitro* bactericidal activities of ampicillin (AM) plus 20-hydroxyecdysone (20E) as time-kill curve’s versus six MRSA stains. Symbol: ■, control; ●, ampicillin; ▲, 20-hydroxyecdysone; ▼, AM+20E. Sa, *S. aureus* 33591; ENT, clinical isolated strains from COM.
studies with large sample scales are needed to conclude the statistical correlated significance of MRSA from clinical specimens and antibiotics.

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