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New activity for old drug: *In vitro* activities of vitamin K₃ and menadione sodium bisulfite against methicillin-resistant *Staphylococcus aureus*

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To discover new lead compounds against methicillin-resistant *Staphylococcus aureus* (MRSA), trimethylhydroquinone, vitamins K₁, K₂, K₃ and menadione sodium bisulfite were targeted for anti-MRSA assay. Their anti-MRSA activities were evaluated by agar diffusion method, and their minimum inhibitory concentrations (MICs) were determined by broth microdilution method. The results showed that trimethylhydroquinone, vitamin K₃ and menadione sodium bisulfite presented obvious anti-MRSA activity, and their MICs against MRSA ATCC 33592 and three clinical MRSA isolates were successively 16 to 32, 8 to 16 and 16 µg/ml. Vitamins K₁ and K₂ showed no anti-MRSA activity when the test discs respectively carried 1024 µg of them. These indicated that the anti-MRSA activity would disappear when the methyl of vitamin K₃ was substituted by alkyl that contained four isopentenyl units, and vitamin K₃ probably has an ancillary effect on the treatment of MRSA infection.

Key words: Vitamin K₃, menadione sodium bisulfate, trimethylhydroquinone, methicillin-resistant *Staphylococcus aureus* (MRSA).

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious threat to public health because of its resistance to multiple antibiotics (Payne, 2008; Taubes, 2008). To overcome resistance, various efforts at discovery of novel antibiotics were carried forward, such as classic screening methods, chemical modification of known antimicrobials, improving antimicrobial activity of known compounds, hybrid agents and new targets (Moellering, 2011). Moreover, it is worth noticing that discovering anti-MRSA compounds from known compounds and drugs should be an economical and effective method (Berger, 2007; Chong and Jr Sullivan, 2007).

Based on the structure-activity analysis of anti-MRSA natural products (Saleem et al., 2010; Gibbons, 2004), some phenols and quinones contained hydrophobic groups were deduced to present potential anti-MRSA activity. So, three potential anti-MRSA compounds as trimethylhydroquinone (compound 1) (Figure 1), α-tocopherol and phloroglucinol were selected for anti-MRSA assays. The results showed that compound 1 presented obvious anti-MRSA activity with daptomycin as a positive control, while α-tocopherol and phloroglucinol
showed no activity against MRSA (Data was not reported). Since the skeleton structures of vitamins K1 (compound 2), K2 (compound 3), K3 (compound 4) and menadione sodium bisulfate (compound 5) were a little similar to that of 1 (Figure 1), these four compounds may also show anti-MRSA activities. To prove this and discover new anti-MRSA activity for old drugs, compounds 2 to 5 were further targeted for anti-MRSA assay, and in vitro activities of them against three clinical isolates of MRSA, two reference strains as MRSA ATCC 33592 and methicillin-susceptible S. aureus (MSSA) ATCC 25923 were evaluated.

MATERIALS AND METHODS

Reagents

Compounds 1, 2, 4 and 5 were purchased from Aladdin Industrial Corporation (Shanghai, China), and compound 3 and daptomycin (Purity of 90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds avoided light, and were stored at 4°C. Mueller-Hinton Agar (MHA) and Mueller-Hinton broth (MHB) used for anti-MRSA assay were purchased from Qingdao Hope Biotechnology Co., Ltd. (Qingdao, China) and were used for the measurement of anti-MRSA activities. The water used was freshly distilled, deionized and purified with Milli-Q plus equipment (Millipore, Bedford, MA). All other solvents and reagents used were of analytical grade. A top pipette 20 to 200 μl and an eight channels pipette 50 to 300 μl were purchased from Dragon Laboratory Instruments Limited (Beijing, China). Ninety six-well plates were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Strains

Two reference strains as MRSA ATCC 33592 (gentamycin and methicillin-resistant) and MSSA ATCC 25923 were purchased from ATCC (American Type Culture Collection, USA). Three clinical MRSA isolates as MRSA 01, MRSA 02 and MRSA 03 were obtained from Clinical Laboratory of the Second Affiliated Hospital, Sun Yat-sen University, Guangzhou, China (Yuan et al., 2012). These three MRSA isolates were identified by PBP2a Latex Agglutination Kit test, and were respectively resistant to erythromycin and methicillin (MRSA 01), methicillin (MRSA 02) and gentamycin and methicillin (MRSA 03). All these MRSA and MSSA strains were sub-cultured with MHB at 35°C for 24 h prior to anti-MRSA assay.

Inhibition zones

The inhibition zone of compounds 1 to 5 against all MRSA strains were performed using agar diffusion method described as Clinical and Laboratory Standards Institute standard with a little modification (CLSI, 2012). Compounds 2 to 4 were respectively dissolved in ethyl acetate to obtain sample solutions with the concentrations of 20.48, 20.48 and 5.12 mg/ml, and compounds 1, 5 and daptomycin were respectively dissolved in purified water to obtain sample solutions with the concentrations of 2.56 mg/ml, 5.12 mg/ml and 64 μg/ml. Daptomycin solution and ethyl acetate were respectively used as a positive control and a solvent control. Diluted inoculums (100 μl, 0.5 of McFarland standard) of MRSA ATCC 33592 and MSSA ATCC 25923 were respectively spread on MHA plates using sterile cotton swab. Next, each sample solution was circularly dropped on a 10 mm diameter disc with pipette and dried in the clean bench until 50 μl of it was carried. After this, each disc was placed on the surface of MHA plates, and then incubated at 35°C for 18 h. All tests were repeated twice, and the test plates stood in dark to avoid the degradation of compounds 1 to 5 in the culture process. The results were described as the diameters (in mm) of inhibition zones, and expressed as mean value ± standard deviation (SD).

Minimum inhibitory concentrations (MICs)

The MICs of compounds 1, 4 and 5 against all MRSA strains were determined by broth microdilution method described as CLSI standard with a little modification (CLSI, 2012). The tests were
Table 1. Diameters of inhibition zones of 1 to 5 against two reference strains \((n = 3)\).

<table>
<thead>
<tr>
<th>Reference strain</th>
<th>Diameters of inhibition zones (mm)*</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Daptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA ATCC 33592</td>
<td></td>
<td>20.3</td>
<td>-**</td>
<td>-**</td>
<td>-**</td>
<td>-</td>
<td>21.0 ± 1.7</td>
</tr>
<tr>
<td>MSSA ATCC 25923</td>
<td></td>
<td>21.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>21.7 ± 1.5</td>
</tr>
</tbody>
</table>

*The diameter of all discs was 10 mm, and the amount of 1, 2, 3, 4, 5 and daptomycin were respectively 128, 1024, 1024, 256, 256 and 32 μg. **Indicated that there was no inhibition zone.

Table 2. Minimum inhibitory concentrations of 1, 4 and 5 against MRSA strains.

<table>
<thead>
<tr>
<th>MRSA strain</th>
<th>Minimum inhibitory concentrations (μg/ml)</th>
<th>1</th>
<th>4</th>
<th>5</th>
<th>Daptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA ATCC 33592</td>
<td></td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>MRSA 01</td>
<td></td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>MRSA 02</td>
<td></td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>MRSA 03</td>
<td></td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>1</td>
</tr>
</tbody>
</table>

performed on 96-well plates in duplicate, and daptomycin was used as a positive control. The sample solution of compound 4 was prepared with dimethyl sulphoxide (DMSO) and MHB, and the initial well contained 5% DMSO in the 96-well plate. So, 5% DMSO aqueous solution was used as a solvent control. Briefly, 100 μl MHB was added into each well. 100 μl each compound solution (256 μg/ml) was respectively added into column 1, and was mixed with pipette. Then starting with the concentration of 128 μg/ml for each compound, two-fold dilution was followed. Subsequently, 100 μl diluted inoculum (0.5 of McFarland standard) was added to each well. Finally, the test plates were incubated at 35°C for 24 h, and also stood in dark to avoid the degradation of compounds 1, 4 and 5 in the culture process. The microbial growth was observed by adding 20 μl of 2.0 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). When the microbial growth in the well of solvent and growth controls was good (Color change from yellow to blue purple), the MICs of samples were determined as the lowest concentration where bacterial growth was visibly inhibited (no obvious color change from yellow).

RESULTS

As the most important member of vitamin K2 homologs, compound 3 (menaquinone-4, MK-4) was used as vitamin K2 in this research. The results (Table 1) indicated that compounds 1, 4 and 5 presented obvious activity against MRSA ATCC 33592 and MSSA ATCC 25923 with respective inhibition zones of 20.3 to 21.3, 30.0 to 30.3 and 29.0 to 30.0 mm, and those for daptomycin were 21.0 to 21.7 mm (the reference for the inhibition zones of 30 μg daptomycin against MSSA ATCC 25923 were 18 to 23 mm). While compounds 2 and 3 showed no anti-MRSA activity. Further, the minimum inhibitory concentrations (MICs) of compounds 1, 4 and 5 against all four MRSA strains were respectively 16 to 32, 8 to 16 and 16 μg/ml, and those of daptomycin were 0.5 to 1 μg/ml (the reference for MICs of daptomycin against MRSA ATCC 33592 was less than 1 μg/ml) (Table 2).

DISCUSSION

Vitamin K1 (compound 2) known as phylloquinone, is biosynthesized by plants. Menaquinone-4 (compound 3, MK-4) was the most important member of vitamin K2 homologs called menaquinones, has several subtypes, and is biosynthesized by animals and bacteria. While compound 4 is a synthetic form of vitamin K. Considering that compound 4 is insoluble in water, compound 5 is usually its application form. Meanwhile, vitamin K3 can be converted to vitamin K2 in vivo (Shearer and Newman, 2008). After the anti-MRSA activity of compound 1 was discovered, their similar skeleton structures to compound 1 deduced that compounds 2 to 5 may also show anti-MRSA activity. Based on these facts, a hypothesis that anti-MRSA compounds would be found everywhere (in plant, animal, human and bacteria) was put forward if compounds 2 to 4 were proved to be active against MRSA. So, the anti-MRSA assays of compounds 2 to 5 were further evaluated with daptomycin used as a positive control. The results showed that the anti-MRSA activity of compound 4 was basically equal to that of compound 5 by comparing their molar concentrations calculated from their MICs, while it was about twofold stronger than that of compound 1. Many new bioactivities of vitamin K3 (compound 4 or 5) and its derivatives were discovered recently, such as anti-cancer (Matzno et al., 2008; Tomasetti et al., 2012; Tanahashi et al., 2011), anti-Alzheimer's disease (Huy, et al., 2013), anti-allergic and anti-inflammatory effects (Kohli et al., 2011; Chinzei...
et al., 2011). Here, the obvious antibacterial activity especially anti-MRSA activity of vitamin K₃ (compound 4 or 5) was first discovered by us, which showed that vitamin K₃ probably have an ancillary effect on the treatment of MRSA infection except for being used as vitamin K supplement and potential cancer treatment.

Moreover, it is unfortunate to find that compounds 2 and 3 have no anti-MRSA activity when the test discs, respectively carried 1024 µg of them, which showed that the anti-MRSA activity would disappear when the C₂ methyl of compound 4 was substituted by alkyl that contained four isopentenyl units. This raises the following questions: (1) What is the reason? and (2) how do we interpret another obvious and thoughtful fact that vitamin K₃ has obvious toxicity and aforementioned bioactivities, while vitamin K₂ has no toxicity and bioactivities except as a supplement of vitamin K? It maybe very worthy to further research the molecular mechanism and the structure-activity and structure-toxicity relationships involved the alkyl side-chain substituted at C₂ position of vitamin K₃.

Conclusions

Conclusively, in vitro activities of vitamin K₃, menadione sodium bisulfate and trimethylhydroquinone against MRSA were determined. The results indicated that these three compounds presented obvious anti-MRSA activity, and showed that vitamin K₃ or menadione sodium bisulfite probably has an ancillary effect on the treatment of anti-MRSA infection.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

MRSA, Methicillin-resistant Staphylococcus aureus; MIC, minimum inhibitory concentration.

REFERENCES