Novel antiseptic compound OPB-2045G shows potent bactericidal activity against methicillin-resistant \textit{Staphylococcus aureus} and vancomycin-resistant \textit{Enterococcus} both \textit{in vitro} and \textit{in vivo}: a pilot study in animals

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There is a need for new compounds to effectively treat methicillin-resistant \textit{Staphylococcus aureus} (MRSA) and vancomycin-resistant \textit{Enterococcus} (VRE). The novel monobiguanide compound 1-(3,4-dichlorobenzyl)-5-octylbiguanide gluconate (OPB-2045G) has potential bactericidal activity. We sought to elucidate the potency of OPB-2045G bactericidal activity against MRSA and VRE compared to those of chlorhexidine digluconate (CHG) and povidone iodine (PVP-I). \textit{In vitro} bactericidal activity was analysed using minimum bactericidal concentration (MBC) as the index. The \textit{in vivo} bactericidal efficacy of OPB-2045G was examined by determining MRSA and VRE contamination of the normal dorsal skin of mice following removal of hair. After a 3 min treatment period, the MBC of OPB-2045G was lower than that of CHG and PVP-I against standard strains and clinical isolates. Additionally, in our \textit{in vivo} mouse model, the \textit{in vivo} bactericidal activity of 1.5 % OPB-2045G (a clinically relevant dose) was higher than that of 0.5 % CHG and equivalent to that of 10 % PVP-I against MRSA. Similarly, the \textit{in vivo} bactericidal activity of OPB-2045G was higher than that of 0.5 % CHG and 10 % PVP-I against VRE. OPB-2045G showed more potent bactericidal activity against MRSA and VRE both \textit{in vitro} and \textit{in vivo} compared to CHG and PVP-I, indicating that OPB-2045G may provide better protection against health care-associated infections caused by these pathogens.

INTRODUCTION

Prevention of health care-associated infection requires a multifaceted approach involving infection control, antibiotic prophylaxis and antisepsis treatment (Kramer et al., 2010). The emergence of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) and vancomycin-resistant \textit{Enterococcus} (VRE) underscores the importance of using appropriate antiseptic agents for the prevention of infection (Jones, 2001). However, while advances in most treatment strategies have led to the development of newer, more effective drugs, the two most commonly used skin antiseptic compounds, chlorhexidine digluconate (CHG) and povidone iodine (PVP-I), were both developed prior to 1960 (Garnes et al., 1959; Paulson, 2003). CHG is one of the most effective antiseptics because it has broad-spectrum antibacterial activity, persistent efficacy and residual activity. Moreover, it is compatible with most materials and is safe for use in humans. However, one study reported three clinical MRSA isolates that survived treatment with CHG (Kobayashi et al., 1989). Additionally, another studied showed that CHG may not be sufficient to eradicate VRE (Kampf et al., 1999). As exposure to CHG becomes more widespread, reports of adverse reactions, ranging from mild irritant contact dermatitis to life-threatening anaphylaxis, are also increasing (Silvestri & McEnery-Stonelake, 2013).

PVP-I is a broad-spectrum microbicide with potency to inactivate not only bacteria, but also several viruses (Sauerbrei & Wutzler, 2010). However, PVP-I may not function well in the presence of organic materials, such as blood or pus, because the bactericidal activity of PVP-I can be rapidly neutralized by these materials (Zamora et al., 1985). Additionally, PVP-I treatment must be avoided in pregnant or lactating mothers as PVP-I can induce...
transient hypothyroidism in the fetus or newborn (Ito, 2000). Therefore, development of novel antiseptic agents is critical for preventing the acquisition of incurable infections.

The compound 1-(3,4-dichlorobenzyl)-5-octylbiguanide (OPB-2045), the precursor to 1-(3,4-dichlorobenzyl)-5-octylbiguanide gluconate (OPB-2045G), was synthesized in 1997 (Tsubouchi et al., 1997). OPB-2045G is 100 times more soluble than OPB-2045 and exhibits an excellent safety profile in humans. In this study, we investigated the bactericidal activity of OPB-2045G against MRSA and VRE in vitro and in vivo.

METHODS

Test substance and antiseptics. OPB-2045G (Fig. 1) was synthesized in the Pharmaceutical Technology Department of Otsuka Pharmaceutical Factory. The CHG used for in vitro tests (Hibitane gluconate solution) was obtained from Dainippon Sumitomo Pharma, while that used for in vivo tests (Sterilcon W solution) was obtained from KENEI Pharmaceutical. PVP-I (Isodine solution) was obtained from Meiji Seika Kaisha.

Test organisms. MRSA ATCC33591 and VRE ATCC 51575 were obtained from American Type Culture Collection. Thirty clinical isolates of MRSA and Enterococcus faecalis were supplied by Kotobiken Medical Laboratories and Mitsubishi Chemical Medience Corporation, respectively.

Experimental animals. Male ICR mice (6–7 weeks old) were obtained from Japan SLC. All animals were housed in a pathogen-free environment at our laboratory and received sterile food and water. Animal experiments were conducted after a review by the Animal Experiment Committee in accordance with Guidelines on Animal experiments were conducted after a review by the Animal Experiment Committee in accordance with Guidelines on Animal Experiments of our institute.

In vitro evaluation of bactericidal activity. The minimum bactericidal concentrations (MBCs) were determined using the microdilution technique (Oishi et al., 2004). Test solutions were prepared containing 11 different concentrations of each drug (6.8–6950 μg OPB-2045G ml⁻¹, 4.9–5000 μg CHG ml⁻¹, 48.8–50 000 μg PVP-I ml⁻¹), diluted in water. A 50 μl aliquot of each challenge strain containing MRSA ATCC33591 (5.2 × 10⁶ c.f.u. ml⁻¹), VRE ATCC 51575 (6.4 × 10⁶ c.f.u. ml⁻¹), 30 different clinical isolates of MRSA (1.3 × 10⁶ to 5.9 × 10⁷ c.f.u. ml⁻¹), or 30 different clinical isolates of E. faecalis (1.1 × 10⁷ to 7.1 × 10⁷ c.f.u. ml⁻¹) was mixed with 50 μl of each drug, at various concentrations as described above for 30, 60 or 180 s. After the exposure, 10 μl of the reaction mixture was transferred to an inactivation plate containing 200 μl of the soybean–casein lecithin polysorbate (SCDLP) digest broth with neutralizer (0.1 % lecithin and 7 % polysorbate 80). The inactivation plate was incubated at 35 °C for over 20 h until the growth of test bacteria was observed in the well in which sterile distilled water had been added as a reaction solution. After the incubations were complete, the MBC of each combination (bacteria, time, drug type and concentration) was determined visually based on the turbidity of the growth media.

In vivo evaluation of bactericidal activity. Mice were anaesthetized with pentobarbital (Dainippon Pharmaceutical) (approximately 3.3 mg pentobarbital per animal) via the intraperitoneal route. Dorsal skin was shaved with hair clippers, disinfected with cotton soaked in ethanol and allowed to dry for over 30 min. The test area (3.5 cm²) on the back of each mouse was exposed to 10 μl of one bacterial solution (approximately 3 × 10⁸ c.f.u. ml⁻¹), and the solution was allowed to dry for over 30 min. The test substance (10 μl) was dropped on the bacterial-contaminated application sites (3.5 cm²) and spread using sterile inoculation loop without occlusion. Nothing was applied to the animals in the control group. After 30 s, 3 min or 10 min following application of the test materials, the skin of the application site (full-thickness skin) of each treated animal was removed under anaesthesia using sterile forceps and scissors and placed in 10 ml of chilled SCDLP culture medium in order to neutralize the bactericidal activity of the test substance. The skin of untreated animals was recovered using the same protocol. A portion of the SCDLP medium was placed in agar and the agar was cultured overnight at 35 °C for approximately 38 h. Viable bacteria in the agar were quantified.

Statistical analysis. In each treatment period, one-way ANOVA and two-tailed Dunnett’s multiple comparison test was performed on the log of the number of survived bacteria among 1.5 % OPB-2045G group, 0.5 % CHG group and 10 % PVP-I group, using the 1.5 % OPB-2045G group as the reference group. The dependent variable was the test substance. The level of significance was 5 %.

Data were compiled using Microsoft Excel 2003 and EXSAS 7.6 (CAC) was used as the statistical analysis software. This software analysed data on Microsoft Excel 2003 by SAS 9.1.5 for Windows (SAS Institute) and the results were output to Microsoft Excel 2003.

RESULTS

In vitro bactericidal activity against MRSA ATCC33591 and VRE ATCC 51575

The MBCs of OPB-2045G, CHG and PVP-I against MRSA ATCC33591 and VRE ATCC 51575 are shown in Table 1. The MBC of OPB-2045G was lower than that of CHG and

Table 1. Minimum bactericidal concentrations of OPB-2045G, CHG and PVP-I against standard strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Compound</th>
<th>MBC (μg ml⁻¹)</th>
<th>30 s</th>
<th>1 min</th>
<th>3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA</td>
<td>OPB-2045G</td>
<td>434</td>
<td>109</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>ATCC33591</td>
<td>CHG</td>
<td>2500</td>
<td>1250</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PVP-I</td>
<td>391</td>
<td>391</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td>VRE ATCC5175</td>
<td>OPB-2045G</td>
<td>54</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHG</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PVP-I</td>
<td>50 000</td>
<td>50 000</td>
<td>391</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Chemical structure of OPB-2045G.
equivalent to that of PVP-I against MRSA ATCC33591. For VRE ATCC 51575, the MBC of OPB-2045G was lower than that of CHG and PVP-I.

In vitro bactericidal activity against clinical isolates of MRSA and E. faecalis

The MBC ranges of OPB-2045G, CHG and PVP-I against clinical isolates of MRSA and E. faecalis are shown in Table 2. After a 3 min treatment, OPB-2045G completely killed all clinical isolates of MRSA and E. faecalis when used at a concentration of 217 \( \mu \text{g} \) OPB-2045G \( \text{m}^{-1} \). In contrast, CHG was relatively ineffective, with 24 out of 30 clinical strains of MRSA surviving after a 3 min exposure to 0.5 % CHG (5000 \( \mu \text{g} \) CHG \( \text{m}^{-1} \)). PVP-I was effective at 1563 \( \mu \text{g} \) PVP-I \( \text{m}^{-1} \), killing all clinical isolates after a 3 min treatment.

In vivo bactericidal activity against MRSA ATCC33591 and VRE ATCC 51575

Next, we analysed the in vivo activity of the antiseptics. After only a 30 s treatment, 1.5 % OPB-2045G showed bactericidal activity on mouse skin contaminated with MRSA. Also, the bactericidal activity of 1.5 % OPB-2045G was higher than that of 0.5 % CHG and equivalent to that of 10 % PVP-I based on the 95 % confidence interval after treatment times of 30 s, 3 min and 10 min (Table 3). Importantly, 1.5 % OPB-2045G also showed in vivo bactericidal activity against VRE after only 30 s of treatment, and the bactericidal activity of 1.5 % OPB-2045G was higher than that of 0.5 % CHG and 10 % PVP-I based on the 95 % confidence interval after treatment times of 30 s, 3 min and 10 min (Table 4).

DISCUSSION

MRSA and VRE represent major problems in healthcare settings worldwide. Many trials have been performed in an attempt to develop methods for prevention of MRSA and VRE infections (Backman et al., 2011; Simor et al., 2013; De Angelis et al., 2014; Magill et al., 2014; Morioka et al., 2014). From the results of current multistate prevalence surveys of healthcare-associated infections in the USA, we

<table>
<thead>
<tr>
<th>Strain</th>
<th>Compound</th>
<th>30 s</th>
<th>1 min</th>
<th>3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA of 30 strains</td>
<td>OPB-2045G</td>
<td>217 to &gt;3475</td>
<td>217 to 869</td>
<td>54 to 217</td>
</tr>
<tr>
<td></td>
<td>CHG</td>
<td>2500 to &gt;5000</td>
<td>2500 to &gt;5000</td>
<td>2500 to &gt;5000</td>
</tr>
<tr>
<td></td>
<td>PVP-I</td>
<td>781 to 1563</td>
<td>781 to 1563</td>
<td>195 to 781</td>
</tr>
<tr>
<td>E. faecalis of 30 strains</td>
<td>OPB-2045G</td>
<td>54 to 434</td>
<td>27 to 217</td>
<td>14 to 109</td>
</tr>
<tr>
<td></td>
<td>CHG</td>
<td>625 to &gt;5000</td>
<td>313 to &gt;5000</td>
<td>156 to 5000</td>
</tr>
<tr>
<td></td>
<td>PVP-I</td>
<td>781 to 50000</td>
<td>391 to 3125</td>
<td>195 to 1563</td>
</tr>
</tbody>
</table>

Table 2. Minimum bactericidal concentrations of OPB-2045G, CHG and PVP-I against clinical isolates

<table>
<thead>
<tr>
<th>Treatment period</th>
<th>Test substance</th>
<th>Survived bacteria (n=7)</th>
<th>95 % CI of the mean log of surviving bacteria</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Log c.f.u. per 3.5 cm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 s</td>
<td>Control</td>
<td>5.53 ± 0.19</td>
<td>5.35 to 5.71</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.5 % OPB-2045G</td>
<td>0.64 ± 0.69</td>
<td>0.01 to 1.28</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.5 % CHG</td>
<td>3.88 ± 0.58</td>
<td>3.34 to 4.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10 % PVP-I</td>
<td>2.10 ± 1.05</td>
<td>1.13 to 3.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3 min</td>
<td>Control</td>
<td>5.56 ± 0.06</td>
<td>5.51 to 5.62</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.5 % OPB-2045G</td>
<td>0.30 ± 0.37</td>
<td>−0.05 to 0.65</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.5 % CHG</td>
<td>2.32 ± 0.32</td>
<td>2.03 to 2.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10 % PVP-I</td>
<td>0.33 ± 0.57</td>
<td>−0.20 to 0.85</td>
<td>NS</td>
</tr>
<tr>
<td>10 min</td>
<td>Control</td>
<td>5.54 ± 0.13</td>
<td>5.42 to 5.66</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.5 % OPB-2045G</td>
<td>0.10 ± 0.26</td>
<td>−0.14 to 0.34</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.5 % CHG</td>
<td>1.94 ± 0.62</td>
<td>1.37 to 2.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10 % PVP-I</td>
<td>0.10 ± 0.26</td>
<td>−0.14 to 0.34</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Significant difference versus 1.5 % OPB-2045G of each treatment period. CI, Confidence interval; NS, not significant; –, not applicable.

Table 3. Bactericidal activities of OPB-2045G, CHG and PVP-I against MRSA ATCC33591 in the mouse contaminated skin model
know that MRSA is the second most common pathogen causing healthcare-associated infections (Magill et al., 2014). The Enterococcus species are also quite common, reported as the fifth most frequent pathogen causing healthcare-associated infections (Magill et al., 2014). Both CHG and PVP-I have long been used in clinical setting and are excellent antiseptics, possessing broad-spectrum antimicrobial activity (Digison, 2007). However, these antiseptics have only minimal bactericidal activity against MRSA and VRE in ex vivo tests using direct treatment on the skin (Messager et al., 2001). Here, we investigated the effects of OPB-2045G, CHG and PVP-I both in vitro and in vivo. Our data indicate that OPB-2045G may have applications as a novel antiseptic in the treatment of MRSA and VRE.

In this study, CHG and PVP-I were not sufficient to eradicate MRSA and VRE in our contaminated skin mouse model. The effectiveness of antiseptics is usually investigated by in vitro tests. However, results from in vitro tests might overestimate the bactericidal activity of antiseptics (Maillard et al., 1998). Because of this, in vivo human trials should only be used for analysis of treatment efficacy for non-pathogenic bacteria due to ethical reasons (ASTM, 2013). Therefore, we used a contaminated skin mouse model for evaluation of the in vivo bactericidal activity of OPB-2045G against MRSA and VRE. Our data demonstrated that 1.5% OPB-2045G almost completely eradicated both MRSA and VRE when used for only 30 s in this model. The results of in vitro tests were similar, with OPB-2045G showing more potent bactericidal activity than CHG and PVP-I against both standard strains and clinical isolates. Alcohol supplemented with 0.5% CHG was more effective than 0.5% CHG and PVP-I against MRSA (Sakuragi et al., 1995). CHG–alcohol is superior to PVP-I for cleansing to prevent surgical-site infection after clean-contaminated surgery (Darouiche et al., 2010). In the future, we will compare alcohol supplemented either with CHG or with OPB-2045G.

In conclusion, our data support OPB-2045G as a component that may have potential clinical applications for preventing healthcare-associated MRSA and VRE infections. The safety of 1.5% OPB-2045G has already been confirmed in a clinical trial in Japan. Therefore, OPB-2045G may be an important agent in the fight against healthcare-associated infections.

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The authors' affiliations were based on the information at the time this manuscript was prepared. This study was conducted by the authors, at Otsuka Pharmaceutical Factory. This study was funded by Otsuka Pharmaceutical Factory. The authors declare no conflict of interests.

**REFERENCES**


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**Table 4. Bactericidal activity of OPB-2045G, CHG, and PVP-I against VRE ATCC 51575 in the mouse contaminated skin model**

<table>
<thead>
<tr>
<th>Treatment period</th>
<th>Test substance</th>
<th>Survived bacteria (n=7) (Log c.f.u. per 3.5 cm²)</th>
<th>95% CI of the mean log of surviving bacteria</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 s</td>
<td>Control</td>
<td>5.57±0.35</td>
<td>5.25 to 5.90</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.5% OPB-2045G</td>
<td>0.30±0.37</td>
<td>–0.05 to 0.65</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.5% CHG</td>
<td>4.45±0.43</td>
<td>4.06 to 4.85</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10% PVP-I</td>
<td>4.59±0.36</td>
<td>4.26 to 4.92</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3 min</td>
<td>Control</td>
<td>5.57±0.27</td>
<td>5.32 to 5.82</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.5% OPB-2045G</td>
<td>0.44±0.59</td>
<td>–0.10 to 0.99</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.5% CHG</td>
<td>2.82±0.99</td>
<td>1.90 to 3.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10% PVP-I</td>
<td>2.95±1.03</td>
<td>2.00 to 3.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10 min</td>
<td>Control</td>
<td>5.67±0.06</td>
<td>5.61 to 5.73</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.5% OPB-2045G</td>
<td>0.20±0.53</td>
<td>–0.29 to 0.69</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.5% CHG</td>
<td>2.90±0.65</td>
<td>2.30 to 3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10% PVP-I</td>
<td>2.40±0.27</td>
<td>2.15 to 2.65</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Significant difference versus 1.5% OPB-2045G of each treatment period. CI, Confidence interval; –, not applicable.


