Mechanism of Action of Dipropropofol and Synergistic Action with Other Antibacterial Agents in Vitro

Masahiro Ogata, Takao Kunikane, Masako Seki, Kentaro Oka, Shiro Urano, Sachiko Seki, Yasuhide Seki, and Toyoshige Endo

Aomori University; 2–3–1 Kohbata, Aomori-shi, Aomori 030–0943, Japan; b Kyoritsu University of Pharmacy; 1–5–30 Shibakoen, Minato-ku, Tokyo 105–8512, Japan; and c Shibaura Institute of Technology; 3–8–14 Shibaura, Minato-ku, Tokyo 108–8548, Japan. Received February 15, 2005; accepted May 6, 2005

During the past decade, nosocomial infections caused by methicillin-resistant Staphylococcus aureus (MRSA) in hospitals have become a serious clinical problem.1) The glycopeptide antibiotic, vancomycin, has been used for the treatment of infections due to MRSA.2) However, vancomycin resistant Staphylococcus aureus (VRS) has recently been isolated.3) The MIC results for vancomycin, teicoplanin, and oxacillin of the resistant strains increased to >128 μg/ml, 32 μg/ml, and >16 μg/ml, respectively, by the broth microdilution method. These isolates were reported to contain the vanA, vancomycin resistance gene from enterococci, which is consistent with the glycopeptide MIC profiles.3,4) Therefore, there is a clear need for new antibiotic regimens with strong bactericidal activity against MRSA. From this viewpoint, an alternative to the development of new classes of agents could be the combined use of well-known compounds.5)

This study was initiated to investigate the mode of action of dipropofol (Fig. 1), a dimer of propofol, having antimicrobial activity against Gram-positive bacteria, including MRSA and vancomycin resistant Enterococci (VRE) at low concentrations (2–4 μg/ml) as described in our previous paper.6) Its mode of action was estimated to be different from other antimicrobials because it has a different type of chemical structure from other antibiotics and has higher bactericidal activity compared to other phenols (MIC >100 μg/ml). Based on this estimation, we evaluated combinational effect of dipropofol with other classes of antimicrobial agents against Gram-positive and Gram-negative strains in vitro.

MATERIALS AND METHODS

Media and Antibiotics Trypticase soy broth and agar were purchased from Nippon Becton Dickinson Co. (Tokyo, Japan). Muller–Hinton broth and agar were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Carbencillin and ampicillin were obtained from Fujisawa Pharmaceutical Co. (Osaka, Japan). Vancomycin, norfloxacin, erythromycin and tetracycline were from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Rifampicin and chloramphenicol purchased from were Wako Pure Chemical Co. (Osaka, Japan), kanamycin from Takeda Pharmaceutical Co. (Osaka, Japan), and l-[G-3H]glutamic acid, [methyl-3H]thymidine, [5,6-3H]uridine, and l-[4,5-3H]leucine were purchased from Amersham Biosciences Co. (Piscataway, U.S.A.). Dipropofol was synthesized by a method reported previously.7)

Strains Vancomycin resistant Enterococcus faecium, vanA type, was kindly supplied by Dr. K. Hiramatsu of Juntendo University, Tokyo. S. aureus 209P and E. coli JCM5491 (ATCC25922) were standard strains in our laboratories.

Time–Kill Curve Assay Growth inhibition of S. aureus 209P by dipropofol was measured with Trypsitcose soy broth (TSB). In 49 ml of the medium, 0.5 ml of a stationary culture of S. aureus 209P was added, and the medium was incubated at 37°C with shaking. After 0, 2, 4, 6, 8, and 10 h of incubation, dipropofol (10 μg/ml) was added. A 1 ml portion of the culture was sampled at 60-min intervals. The turbidity of the culture was measured at 600 nm with a Hitachi 220A spectrophotometer (Hitachi Seisakusho Co. Ltd., Japan).8)

Assay for Syntheses of Cellular Macromolecules S. aureus 209P, cultivated in TSB overnight at 37 °C, was harvested, and washed with fresh medium by centrifugation at 3000 rpm for 15 min at 4°C. The bacterial cells were suspended at 2–3×10^9 cells in casamino acid medium (l-cysteine–HCl 50 mg/ml, KH_2PO_4 4.5 mg/l, sodium citrate 3.5 g/l, dextrose 2.5 g/l, MgSO_4 40 mg/l, thiamine-HCl 10

Fig. 1. Structure of Dipropofol

* To whom correspondence should be addressed. e-mail: ogata-ms@aomori-u.ac.jp © 2005 Pharmaceutical Society of Japan
mg/ml, niacinamide 10 mg/ml and casein acid hydrolysate; Vitamin free (CAA) 100 mg/l). In the incorporation experiments the cell suspension was incubated in a medium containing dipropofol (2 μg/ml) and a radioactive substrate at 0.2 μCi/ml of [methyl-3H]thymidine, [5,6-3H]uridine, l-[4,5-3H]leucine, or l-[2-3H]glutamic acid. Samples taken at 30-min intervals were placed into an equal volume of 0.2 mM NaN3–saline, and acid-precipitable counts were collected on Whatman GF/C glass fiber disks. After washing with 0.2 mM NaN3–saline and then with ethyl ether, the disks were immersed in an organic counting scintillant and counted using a liquid scintillation system LSC-3500 (Aloka Co. Japan).\(^9\)

*B. subtilis* PCI219 was cultivated in Davis minimal medium (K₂HPO₄ 7 g/l, KH₂PO₄ 2 g/l, MgSO₄ 0.1 g/l, (NH₄)₂SO₄ 1 g/l, sodium citrate 0.5 g/l and glucose 2 g/l) for 18 h at 37 °C, and the cells were harvested and then washed by centrifugation. Radioactivity was measured by the method mentioned above.

\[
\text{incorporation rate (%)} = \frac{A \text{ (control)} - A \text{ (control+sample)}}{A \text{ (control)}} \times 100
\]

where A represents cmp/ml.

**Combination Study and Synergy Determination** The in vitro combinational effect of dipropofol with other antibiotics was examined by the checkerboard titration method. Overnight cultures of test strains, grown in 10 ml of Mueller–Hinton broth at 37 °C, were diluted 10⁴-fold with fresh Mueller–Hinton broth, followed by the addition of about 5 x 10⁴ CFU onto 20-ml Mueller–Hinton agar layers which contained dipropofol (0.39 μg/ml) and other antibiotics at concentrations ranging from 0 to 100 μg/ml. After 18 h of incubation at 37 °C, the minimum inhibitory concentrations (MICs) in every combination were determined according to the method of the Japan Society of Chemotherapy.\(^10\)

The fractional inhibitory concentration (FIC) for each component was calculated based on the following formula: FIC index = (MIC of drug A, tested in combination)/(MIC of drug A, tested alone) + (MIC of drug B, tested in combination)/(MIC of drug B, tested alone). The interaction was defined as synergistic if the FIC index was <0.5, additive if the FIC index was >0.5 to 4, and antagonistic if the FIC index was >4.\(^11\)

**RESULTS AND DISCUSSION**

In order to determine whether dipropofol acts bacteriostatically or bactericidally, the following experiments were carried out using *S. aureus* 209P as a test organism by the turbidimetric method. Dipropofol (10 μg/ml, 5×MIC) was added to the medium during the logarithmic growth phase. The turbidity (optical density value) was decreased by the addition of dipropofol and the reaction was determined to be bactericidal (Fig. 2).

Successively, the effect of dipropofol (2 mg/ml, MIC of *S. aureus*)\(^9\) on the syntheses of biomacromolecules was tested with *S. aureus* 209P and *B. subtilis* PCI219.\(^9\) As shown in Fig. 3, the incorporation of ¹H-thymidine, a precursor of DNA synthesis, was inhibited about 40% and 20% in *S. aureus* and *B. subtilis*, respectively, after 60 min and the incorporation ¹H-uridine, a precursor of RNA synthesis, was depressed about 50% in both strains. From these results, it was concluded nucleic acid synthesis could not be the primary target of dipropofol. On the other hand, the incorporation of ³H-leucine, which is a precursor of protein, was markedly decreased 30 min and 60 min in *S. aureus* and *B. subtilis*, respectively, after the addition of dipropofol (2 μg/ml). In addition, dipropofol blocked the incorporation of glutamate within 30 min and 60 min of addition in *S. aureus* and *B. subtilis*, respectively. Dipropofol did not the direct chemical reaction with the glutamate (data not shown). These results indicated that the mechanism of action of dipropofol was mediated by the inhibition of protein synthesis or amino acid incorporation. However, glutamate is an essential amino acid for the biosynthesis of several amino acids such as glutamine, arginine, proline, asparagine, aspartic acid, isoleucine and lysine, and it is a constituent of peptidoglycan, and it participates in nucleic acid biosynthesis. However, more detailed studies and discussion are needed in order to determine the precise mode of action of dipropofol.

Based on the discussion above, a mode of action could be hypothesized for dipropofol, which is a dimeric phenol compound having strong antibacterial and antioxidant activities,\(^6,7\) and some synergistic action could be expected with other antibiotic substances. The data were analyzed using the FIC (fractional inhibitory concentration) index (FIC index was <0.5).\(^11\) The FIC index is the most frequently used method to determine interactions between antimicrobial drugs. In subsequent experiments, the combined effects between dipropofol and nine other antibiotics (carbenicillin, ampicillin, vancomycin, kanamycin, norfloxacin, erythromycin, tetracycline, rifampicin, and chloramphenicol) were examined. As shown in Table 1, a synergistic effect between
Dipropofol and rifampicin was observed in *S. aureus* 209P. The MIC value of rifampicin decreased from 0.78 μg/ml to <0.005 μg/ml in combination with dipropofol (0.39 μg/ml, 1/4×MIC) (FIC index: <0.26). The synergism against VRE (vanA type) was only confirmed in the combination between dipropofol and rifampicin, and the MIC value of rifampicin was decreased from 0.39 μg/ml to <0.005 μg/ml in combination with dipropofol (0.39 μg/ml, 1/4×MIC) (FIC index: <0.27). However, no synergistic effect was observed in *E. coli* JCM5491, which is the bacterium recommended to validate the control of the sensitivity test, between dipropofol and the nine antibiotics. It appears that dipropofol has a different mechanism of action than rifampicin (an inhibitor of RNA polymerase), and inhibits the growth of *S. aureus* strain synergistically. No positive synergistic activity was observed with other seven antibiotics, when used carbenicillin, ampicillin and vancomycin (inhibitors of cell wall biosynthesis), norfloxacin (an inhibitor of DNA gyrase), and kanamycin (an inhibitor of protein synthesis), erythromycin, tetracycline and chloramphenicol (inhibitors of protein synthesis different from kanamycin). However, whether the inhibition of protein synthesis or amino acid incorporation by dipropofol is the primary site of action or a secondary one remains a problem. In the present study, the combination of dipropofol and rifampicin to treat VRE highlights novel drug targets and has importance in the design of new therapeutic regimes against resistant pathogens.

In summary, dipropofol is a new type antibiotic with bactericidal activity against Gram-positive strains. The mechanism of action of dipropofol appears to be mediated by its inhibition of protein synthesis or amino acid incorporation. Also, the action of dipropofol is synergistic when used in combination with rifampicin against VRE. The details of the mechanism of action are currently being studied using a cell free enzyme system and at the gene level.

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**REFERENCES**


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**Table 1. MICs (μg/ml) of Dipropofol and in Combination with Other Antibiotics**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Staphylococcus aureus 209P</th>
<th>Enterococcus faecium (vanA)</th>
<th>Escherichia coli JCM5491</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>+ dipropofol</td>
<td>MIC</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>0.39</td>
<td>0.39</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.09</td>
<td>0.19</td>
<td>6.25</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.78</td>
<td>0.39</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>1.56</td>
<td>0.78</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0.39</td>
<td>0.39</td>
<td>3.31</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.19</td>
<td>0.19</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.09</td>
<td>0.09</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.78</td>
<td>&lt;0.005</td>
<td>0.39</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>6.25</td>
<td>0.78</td>
<td>50</td>
</tr>
<tr>
<td>Dipropofol</td>
<td>1.56</td>
<td>—</td>
<td>1.56</td>
</tr>
</tbody>
</table>

a) Dipropofol (0.39 μg/ml) was added.

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