Potentiation of Antimicrobial Activity of Aminoglycosides by Carnosol from *Salvia officinalis*

Kumiko Horiuchi, Sumiko Shiota, Teruo Kuroda, Tsutomu Hafano, Takashi Yoshida, and Tomofusa Tsuchiya*

*Department of Molecular Microbiology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University; †Department of Pharmacognosy, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University; Tsushima, Okayama 700–8530, Japan; and ‡Laboratory of Pathogenic Microbiology, School of Pharmacy, Shijitsu University, Nishigawara, Okayama 703–8516, Japan.

Received August 31, 2006; accepted November 20, 2006; published online November 27, 2006

We found that a crude extract from *Salvia officinalis* (sage) reduced the minimum inhibitory concentrations (MICs) of aminoglycosides in vancomycin-resistant enterococci (VRE). We isolated the effective compound from the extract and identified it as carnosol, one of diterpenoids. Carnosol showed a weak antimicrobial activity, and greatly reduced the MICs of various aminoglycosides (potentiated the antimicrobial activity of aminoglycosides) and some other types of antimicrobial agents in VRE. Carnosic acid, a related compound, showed the similar activity. The effect of carnosol and carnosic acid with gentamicin was synergistic.

Key words: carnosol; carnosic acid; aminoglycoside; vancomycin-resistant enterococci (VRE)

The infections caused by vancomycin-resistant enterococci (VRE) are very serious problems because of the resistance against not only vancomycin but also many types of antimicrobials. Although enterococci are less virulent, they cause nosocomial infections. VRE was noted from the U.K. and France at first, and then they have become one of the most important nosocomial pathogens worldwide. In the case of the United States, the percentage of VRE in enterococci isolated at intensive care units (ICUs) was 0.4% in 1989, but it was raised up to 28.5% in 2003. Of the strains belonging to the enterococcal species, *Enterococcus faecalis* and *E. faecium* are common isolates from human. Among the clinical isolates of enterococcal species, *E. faecalis* accounts for 80—90% and the rest is mainly *E. faecium*. The strains of VRE are mainly *E. faecalis* and *E. faecium*. In the case of the treatment for infections caused by enterococci, it is one of the most popular methods to use aminoglycosides together with inhibitors of cell wall synthesis such as β-lactams although enterococci are intrinsically resistant to aminoglycosides. Nonetheless, in the cases of infection by highly resistant enterococci against aminoglycosides (for example, MIC of gentamicin is equal or more than 500 μg/ml, or MIC of streptomycin is equal or more than 2000 μg/ml), use of this treatment is limited and it is very difficult to treat patients in such cases. Because many strains of enterococci, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus faecalis* NCTC 12201, *faecalis* FA2-2, and *Serratia marcescens* NUSM8905 were kindly provided by Dr. Y. Ike (Gunma University) and Dr. Y. Arakawa (National Institute of Infectious Diseases, Tokyo), respectively. MRSA OM481 and OM584 were clinically isolated in Okayama University hospital. *Pseudomonas aeruginosa* PAO1 was also used in this study.

**Materials and Methods**

**Bacterial Strains** Four VRE strains, *E. faecium* FN-1, *E. faecium* BM4147, *E. faecalis* NCTC 12201, *faecalis* FA2-2, and *Serratia marcescens* NUSM8905 were determined in cation-supplemented Mueller-Hinton broth (CSMHB), Mueller-Hinton broth (Difco Laboratories) supplemented with CaCl₂ (50 μg/ml) and MgSO₄ (25 μg/ml), by a microdilution method.

**Drug Susceptibility Testing** The MICs of antimicrobial agents with VRE, MRSA, *S. marcescens* or *P. aeruginosa* were determined in cation-supplemented Mueller-Hinton broth (CSMHB), Mueller-Hinton broth (Difco Laboratories) supplemented with CaCl₂ (50 μg/ml) and MgSO₄ (25 μg/ml), by a microdilution method. To isolate the compound that showed the highest effect on the reduction of the MIC of aminoglycoside against VRE, 850 g of dried, ground leaves of *Salvia officinalis* were extracted with 81 of 70%...
acetone at room temperature and filtered. The extract was concentrated and dried by using a rotary evaporator, and 300 g of sample was obtained. A half of the sample was dissolved with water and extracted with ethyl acetate (AcOEt). The AcOEt extract was concentrated by using the rotary evaporator and dried by using N$_2$ gas and 70 g of sample was obtained. This AcOEt extract was subjected to column chromatography over DIAION HP-20 (Mitsubishi-Kagaku Co.) and eluted with aqueous methanol (70, 80, 90, 100%) in a stepwise manner. At the final step, it was eluted with 100% acetone. The fraction eluted with 90% methanol showed the highest activity to reduce the MIC of arbekacin against VRE strains, E. faecium FN-1 and E. faecalis FA2-2. One gram of this 90% methanol fraction was further subjected to chromatography over Sephadex LH-20 (Amersham Biosciences Co.) by using 100% ethanol as a solvent. Active fractions were collected and 200 mg of the dried material from these fractions were separated by preparative high-pressure liquid chromatography (HPLC) on Inertsil ODS-3 (25 cm, GL science Co.) using 70% acetonitrile as an eluant. After that, active fraction was purified by HPLC on Inertsil ODS-3 (1 by 25 cm, GL science Co.) using 60% acetonitrile as an eluant. Twenty milligrams of the active compound was isolated and its structure was determined by $^{1}$H-, $^{13}$C-NMR and MS spectrum.

**Materials** The leaves of S. officinalis, carnosic acid, antimicrobial agents and chemicals used in this study were purchased from commercial sources. Arbekacin was obtained from Meiji Seika Co. (Tokyo, Japan).

**RESULTS AND DISCUSSION**

**Isolation and Identification of the Effective Compound** We have been trying to find compounds that potentiate activity of antimicrobial agents against VRE and other multidrug resistant bacteria. We found that the extract from sage leaves showed such effect, and greatly reduced MICs of aminoglycosides in VRE. In the presence of 1/8 MIC of 70% acetone extract from sage, the MIC of arbekacin was reduced to 1/32 in VRE. The AcOEt extract of 70% acetone extract showed the highest activity, and the presence of 1/8 MIC of this fraction reduced the MIC of arbekacin to 1/16. Then, AcOEt extract was subjected to column chromatography over DIAION HP-20. The 90% methanol fraction that showed the highest activity was further subjected to chromatography over Sephadex LH-20 by using 100% ethanol as a solvent. Addition of 1/8 MIC of the most effective fraction reduced the MIC of arbekacin to 1/8. This fraction was subjected to HPLC and we isolated an effective compound, and identified it as carnosol, one of diterpenoids. It is likely that other compound(s) that show additive effect on the reduction of MIC of arbekacin (Table 1). The MIC values regarding S. aureus were almost same as those reported by other group.

It should be noted that other activities of carnosol and carnosic acid, for example inhibition of pancreatic lipase activity, antioxidant activity and anti-inflammatory activity, have been reported.

**Effects of Carnosol and Carnosic Acid on VRE** When carnosol (16 µg/ml) or carnosic acid (8 µg/ml) was added to the growth medium, the MICs of several aminoglycosides for VRE were greatly reduced compared with MICs measured in the absence of these compounds (Table 2). Concentrations of carnosol and carnosic acid added to the medium were lower than 1/4 of their MICs. These two compounds reduced the MIC values of almost all aminoglycosides tested about 8- to 128-fold, or more. Thus, carnosol and carnosic acid greatly potentiated the antimicrobial activity of aminoglycosides on VRE. The FIC index was calculated for the enhanced antibacterial effect of arbekacin by carnosol or carnosic acid. The FIC index for arbekacin in combination with carnosol or carnosic acid ranged from 0.19 to 0.38 in the enterococcal strains. These results indicate that the antibacterial effect of arbekacin plus carnosol or carnosic acid is synergistic. They also potentiated activity of other antimicrobial agents, such as ethidium bromide, erythromycin and tetracycline, on some VRE strains (Table 2). However they did not affect the activities of β-lactam (ampicillin) or fluoroquinolone (norfloxacin) (data not shown).

**Effects of Carnosol and Carnosic Acid on Other Drug-Resistant Bacteria** We also examined the effects of carnosol and carnosic acid on the activity of aminoglycosides on MRSA, P. aeruginosa and S. marcescens. Carnosol and carnosic acid potentiated the antimicrobial activity of aminoglycosides on MRSA although their activity was weaker than that observed with VRE (Tables 2, 3). We also found that antimicrobial activity of ethidium bromide on MRSA was potentiated by carnosol or carnosic acid (Table 3). Both carnosol and carnosic acid have been reported to potentiate...
might be specific to some strains of potentiation activities with tetracycline and erythromycin acid on the MIC of erythromycin in these cells. Thus, their haps we could not detect the effect of carnosol or carnosic showed high resistance level regarding erythromycin, per-
cycline (Table 3). Since MRSA strains used in our study carnosic acid were ineffective on 
carnosol, 16m
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
carnosic acid >2048 >2048 
carnosol >2048 >2048 
E. faecalis FA2-2, they do not possess these enzymes. If carnosol and carnosic acid inhibit these modification enzymes, at least AAC(6</code>

Table 3. MICs of Various Antimicrobial Agents for MRSA in the Absence or Presence of Carnosol or Carnosic Acid

<table>
<thead>
<tr>
<th>Antimicrobial/Compound</th>
<th>MIC (μg/ml)</th>
<th>E. faecium FN-1</th>
<th>E. faecium BM4147</th>
<th>E. faecalis NCTC 12201</th>
<th>E. faecalis FA2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arbekacin – carnosol</td>
<td>64</td>
<td>64</td>
<td>&gt;128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>+carnosol</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>+carnosic acid</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Gentamicin – carnosol</td>
<td>&gt;8192</td>
<td>64</td>
<td>&gt;8192</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>+carnosol</td>
<td>1024</td>
<td>8</td>
<td>512</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>+carnosic acid</td>
<td>1024</td>
<td>4</td>
<td>128</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Streptomycin – carnosol</td>
<td>&gt;8192</td>
<td>256</td>
<td>&gt;8192</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>+carnosol</td>
<td>2048</td>
<td>64</td>
<td>1024</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>+carnosic acid</td>
<td>1024</td>
<td>64</td>
<td>1024</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td><strong>Other antimicrobials</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethidium bromide –</td>
<td>128</td>
<td>16</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>+carnosol</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>+carnosic acid</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Erythromycin – carnosol</td>
<td>&gt;2048</td>
<td>2</td>
<td>&gt;2048</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>+carnosol</td>
<td>&gt;2048</td>
<td>2</td>
<td>&gt;2048</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>+carnosic acid</td>
<td>2</td>
<td>2</td>
<td>&gt;2048</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>Tetracycline – carnosol</td>
<td>64</td>
<td>128</td>
<td>128</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>+carnosol</td>
<td>16</td>
<td>128</td>
<td>64</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>+carnosic acid</td>
<td>16</td>
<td>64</td>
<td>32</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

a) The concentrations added to the medium are lower than 1/4 of their MICs (carnosol, 16 μg/ml; or carnosic acid, 8 μg/ml).

the activity of tetracycline and erythromycin against S. aureus. However, we observed only a small effect with tetracycline (Table 3). Since MRSA strains used in our study showed high resistance level regarding erythromycin, perhaps we could not detect the effect of carnosol or carnosic acid on the MIC of erythromycin in these cells. Thus, their potentiation activities with tetracycline and erythromycin might be specific to some strains of S. aureus. Carnosol and carnosic acid were ineffective on P. aeruginosa and S. marcescens (data not shown), thus, it seems that these com-
ounds are not effective on gram-negative bacteria. Although the effects of carnosol and carnosic acid are not so specific to VRE, their antimicrobial spectrum seems to be narrow.

**Possible Mechanism of Action** There are several mechanisms that confer aminoglycosides-resistance to enterococci. Carnosol and carnosic acid are thought to inhibit one or some of these mechanisms. The first mechanism of the resistance is related to aminoglycoside-modifying enzymes. To investigate the effect of carnosol or carnosic acid on these enzymes, we used two types of VRE strains. One type is E. faecium FN-1 and E. faecalis NCTC12201, they possess at least 2 aminoglycoside-modifying enzymes, AAC(6’)/APH(2”) and APH(3’). Another type is E. faecium BM4147 and E. faecalis FA2-2, they do not possess these enzymes. If carnosol and carnosic acid inhibit these modification enzymes, AAC(6’)/APH(2”) and/or APH(3’), they should not affect the MICs of aminoglycosides with E. faecium BM4147 and E. faecalis FA2-2 that do not possess these modification enzymes. However, as shown in Table 2, carnosol and carnosic acid greatly reduced the MICs of aminoglycosides with all of the four strains. These results suggest that the effect of carnosol and carnosic acid on antimicrobial activity of aminoglycosides is not related to aminoglycoside-modifying enzymes, at least AAC(6’)/APH(2”) and APH(3’). The second mechanism is the low aminoglycoside permeation into enterococcal cells. It is known that enterococci are relatively impermeable to aminoglycosides. It seems possible that carnosol or carnosic acid affects (increases) cell membrane permeability and then aminoglycosides can easily enter into the cells in the presence of carnosol or carnosic acid. Carnosol and carnosic acid potentiated antimicrobial activity not only of aminoglycosides but also of other antimicrobials such as ethidium bromide, erythromycin and tetracycline (Table 2). In MRSA, these compounds also reduced MICs of aminoglycosides. These results support the view that carnosol and carnosic...
acid increase permeability of cell membrane in gram-positive bacteria. It is likely that presence of outer membrane in gram-negative bacteria hinders permeation of aminoglycosides and others into cells. The third possible mechanism is the involvement of drug efflux pumps. The mechanism for resistance against ethidium seems to be due to the efflux pumps. EfrAB, a multidrug efflux pump in E. faecalis, could be related to resistance against many drugs such as arbekacin, ciprofloxacin, doxycycline and ethidium bromide.31) Thus, it seems also possible that carnosol and carnosic acid inhibit such drug efflux pump(s). We do not have experimental data for or against this possibility at the present time.

Antimicrobial drugs effective for treatment of patients infected with VRE or MRSA are limited. Aminoglycosides are one of the important antimicrobials for the treatment of such patients. Thus, it is important and valuable to find compounds that potentiate antimicrobial activity of aminoglycosides on VRE and MRSA. Carnosol and carnosic acid could be candidates or lead compounds for such type of drugs.

Acknowledgments We thank Dr. Manuel Varela of Eastern New Mexico University for critically reading the manuscript. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES