In vitro activity of carvacrol against staphylococcal preformed biofilm by liquid and vapour contact

Antonia Nostro,1 Andreana Marino,1 Anna Rita Blanco,2 Luigina Cellini,3 Mara Di Giulio,3 Francesco Pizzimenti,1 Andrea Sudano Roccaro2 and Giuseppe Bisignano1

Correspondence Antonia Nostro atnostro@pharma.unime.it
1Pharmaco-Biological Department, University of Messina, Messina, Italy
2SIFI Pharma SpA, Catania, Italy
3Department of Biomedical Sciences, School of Pharmacy, University ‘G. d’Annunzio’, Chieti, Italy

Carvacrol is an important component of essential oils and recently has attracted much attention as a result of its biological properties, such as a wide spectrum of antimicrobial activity. The aim of this study was to evaluate the effect of carvacrol in liquid and vapour phase on preformed biofilms of Staphylococcus aureus and Staphylococcus epidermidis by determining biofilm biomass and cultivable cell numbers, and by using epifluorescence and scanning electron microscopy. Carvacrol was able to reduce biofilm biomass and cell viability more effectively when used with liquid contact rather than with vapour phase. The efficacy of treatment with carvacrol vapour was found to be dependent on exposure time. The predominance of red fluorescence using a LIVE/DEAD BacLight Viability kit (Molecular Probes) and the partially destroyed biofilm architecture as determined by microscopy in treated samples provided evidence for the efficacy of carvacrol. The findings of this investigation suggest a potential application for carvacrol in the inactivation of staphylococcal biofilms.

INTRODUCTION

In recent decades, an increase in the number of staphylococcal infections has been observed worldwide. This has become a clinical and therapeutic problem, as these micro-organisms are resistant to many antimicrobial agents and are a common cause of biofilm-related infections associated with the widespread use of implanted medical devices (O’Gara & Humphreys, 2001; Vuong & Otto, 2002). Poor antimicrobial penetration, nutrient limitation, adaptive stress responses, phenotypic variability and persister-cell formation all contribute towards making biofilm eradication difficult (Hoyle & Costerton, 1991; Mah & O’Toole, 2001).

Carvacrol (5-isopropyl-2-methylphenol) is one of the most common components of essential oils. Approved as a safe food additive in the USA and Europe (Center for Food Safety and Applied Nutrition, 2006; Commission of the European Communities, 1999), carvacrol has attracted considerable attention as a result of its wide-spectrum antimicrobial activity, which has been the subject of several investigations (Ben Arfa et al., 2006; Chami et al., 2005; Dorman & Deans, 2000; Jeong et al., 2008; Kordali et al., 2008; Ultee et al., 1998). In particular, Staphylococcus aureus and Staphylococcus epidermidis, including meticillin-resistant strains, have been shown to be susceptible to carvacrol (Nostro et al., 2004).

It has recently been reported that carvacrol is able to inhibit the growth of preformed biofilm and to interfere with biofilm formation (Knowles & Roller, 2001; Knowles et al., 2005; Nostro et al., 2004). Moreover, a higher antimicrobial activity of carvacrol has been shown using the vapour form compared with solution contact (Burt et al., 2007a; Inouye et al., 2000, 2001a, b, 2003; López et al., 2007).

The antibacterial activity of carvacrol has been attributed to its hydrophobic nature, the presence of a free hydroxyl group and a delocalized electron system. Carvacrol acts on the cytoplasmic membrane, with considerable effects on the structural and functional properties of the membrane itself, which becomes increasingly permeable to protons and ions and loses its integrity (Ben Arfa et al., 2006; Helander et al., 1998; Lambet et al., 2001; Ultee et al., 1999). Carvacrol has also been shown to inhibit ATPase (Gill & Holley, 2006) and to induce Hsp60 in bacteria (Burt et al., 2007b).

In this study, we investigated the effect of carvacrol in liquid and vapour forms on the viability and cell...
morphology of *S. aureus* and *S. epidermidis* preformed biofilms. To do this, the biofilm biomass and number of cultivable cells in preformed biofilm were measured. Viability staining and ultrastructural observations were also performed.

**METHODS**

**Essential oil component and bacterial strains.** Carvacrol (≥ 97 % pure) was purchased from Sigma-Aldrich. The bacteria used in this study were *S. aureus* 815 isolated from an ocular infection belonging to our private collection and the reference strain *S. epidermidis* ATCC 35984, a known slime producer. The strain was stored at –70 °C in Microbanks (Pro-Lab Diagnostics); a single head was removed from the cryovials and inoculated directly into tryptic soy broth (TSB; Oxoid). All reagents were purchased from Sigma-Aldrich unless otherwise specified in the text.

**Experimental design.** *S. aureus* 815 and *S. epidermidis* ATCC 35984 were grown as biofilms in 35 mm diameter polystyrene plates (Costar; Corning) as reported previously (Nostro et al., 2007). Briefly, the culture was grown overnight in 10 ml TSB with 1 % (w/v) glucose (TSBG), diluted in growth medium to 5 × 10⁵ c.f.u. ml⁻¹, and 1 ml was dispensed into polystyrene plates. After 24 h incubation at 37 °C, the planktonic cells were gently removed and the biofilm was washed three times with PBS. One set of plates was then treated with 1 ml TSBG with 1 % (v/v) carvacrol (liquid contact); a second set of plates was treated with 1 ml TSBG and a paper disc (1 cm diameter) soaked with carvacrol (1 %, v/v, air space) was fixed on the cover of the plate (vapour contact). Controls received 1 ml TSBG. All of the plates were sealed immediately after inoculation with Parafilm and reincubated at 37 °C for 3 and 24 h. After the incubation, the viability and morphology of the remaining biofilm were evaluated by using the various investigative methods reported below.

**Biofilm biomass and cultivable cell measurements.** The supernatant of treated plates (liquid and vapour contact) was removed and plated onto solid tryptic soy agar (TSA; Oxoid) followed by incubation for 48 h at 37 °C. The remaining biofilm was then scraped into 1 ml PBS, transferred to a new tube, vortexed for 5 min and subjected to (i) biofilm biomass evaluation by measuring the OD₄₉₂ and (ii) evaluation of cell count estimated as c.f.u. ml⁻¹ by plating diluted samples onto TSA followed by incubation for 48 h at 37 °C. Furthermore, to determine whether carvacrol treatment prevented the regrowth of micro-organisms embedded in the biofilm, after 3 and 24 h exposure to carvacrol (liquid and vapour contact), the medium was removed, the biofilm was washed with PBS and new TSBG was added. Plates were reincubated at 37 °C overnight and the biofilm biomass and the number of cultivable cells were evaluated as described above.

**Staining with 2,3,5-triphenyltetrazolium chloride (TTC).** The *S. epidermidis* biofilm from treated plates (liquid and vapour contact) was scraped into 1 ml PBS, transferred to a new tube, vortexed for 5 min and stained with TTC (1 %, w/v) for 15 h at 37 °C. The presence of a red-stained sediment indicated the reduction by live bacteria of colourless TTC to red insoluble formazan crystals.

**LIVE/DEAD BacLight Viability kit.** The *S. epidermidis* biofilm of each treated plate (liquid and vapour contact) was rinsed once with PBS and stained by using a LIVE/DEAD BacLight Viability kit (Molecular Probes). The solution (1 ml) containing SYTO 9 and propidium iodide (1:1) was added to the plates. The plates were incubated at room temperature for 15 min in the dark. After incubation, residual stain was removed. The images were observed using a fluorescence microscope (Reichert) equipped with a halogen lamp, Neoplan 100/1.25 oil objective and 1713 filter cube (fluorescein; 490/510/520 nm).

**Scanning electron microscopy.** The *S. epidermidis* biofilm of each treated plate (liquid and vapour contact) was fixed in 2 % glutaraldehyde in 0.1 M PBS for 2 h at 4 °C and post-fixed for 1 h at 4 °C in 1 % osmium tetroxide in the same buffer. After thorough washing with PBS, samples were dehydrated through a series of ethanol solutions (30–100 %). Specimens were mounted on aluminium stubs with conductive carbon cement, allowed to dry and then coated with a gold film. Samples were observed by using an S-400 scanning electron microscope (Hitachi).

**Statistical analysis.** One-way analysis of variance and Dunnet’s post-test were used to evaluate the differences between the means of the results for the control and treated samples. A P value of <0.05 was considered significant.

**RESULTS**

In this study, we investigated *S. aureus* and *S. epidermidis* biofilms after exposure to carvacrol (1 % concentration) in liquid and vapour phase, by using different investigative methods. As reported in Table 1, *S. aureus* and *S. epidermidis* biofilms treated with carvacrol by liquid contact demonstrated decreased optical density measure-

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Strain</th>
<th>Control</th>
<th>Carvacrol in liquid contact</th>
<th>Carvacrol in vapour contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><em>S. aureus</em> 815</td>
<td>0.293 ± 0.063</td>
<td>0.287 ± 0.054</td>
<td>0.296 ± 0.036</td>
</tr>
<tr>
<td>3</td>
<td><em>S. aureus</em> 815</td>
<td>0.272 ± 0.056</td>
<td>0.283 ± 0.069</td>
<td>0.275 ± 0.045</td>
</tr>
<tr>
<td></td>
<td><em>S. epidermidis</em> ATCC 35984</td>
<td>0.302 ± 0.063</td>
<td>0.198 ± 0.070</td>
<td>0.287 ± 0.060</td>
</tr>
<tr>
<td>24</td>
<td><em>S. aureus</em> 815</td>
<td>0.298 ± 0.054</td>
<td>0.212 ± 0.067</td>
<td>0.274 ± 0.048</td>
</tr>
<tr>
<td></td>
<td><em>S. epidermidis</em> ATCC 35984</td>
<td>0.345 ± 0.042</td>
<td>0.180 ± 0.035*</td>
<td>0.200 ± 0.040*</td>
</tr>
</tbody>
</table>

*P <0.05, showing the statistical difference between the carvacrol-treated and control groups.*
Table 2. Quantitative measurement of cultivable cells in biofilms after treatment with carvacrol (1 %, v/v)

Data are given as log c.f.u. cm⁻² and are the means ± SD of three independent experiments carried out in triplicate.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Strain</th>
<th>Sample</th>
<th>Control</th>
<th>Carvacrol in liquid contact</th>
<th>Carvacrol in vapour contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>S. aureus 815</td>
<td></td>
<td>8.72 ± 0.55</td>
<td>8.68 ± 0.95</td>
<td>8.65 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>S. epidermidis ATCC 35984</td>
<td></td>
<td>8.26 ± 0.76</td>
<td>8.29 ± 0.19</td>
<td>8.37 ± 0.72</td>
</tr>
<tr>
<td>3</td>
<td>S. aureus 815</td>
<td></td>
<td>8.64 ± 0.72</td>
<td>2.35 ± 0.34*</td>
<td>5.52 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>S. epidermidis ATCC 35984</td>
<td></td>
<td>8.47 ± 0.32</td>
<td>2.94 ± 0.43*</td>
<td>6.26 ± 0.44</td>
</tr>
<tr>
<td>24</td>
<td>S. aureus 815</td>
<td></td>
<td>9.53 ± 0.91</td>
<td>2.42 ± 0.36*</td>
<td>3.83 ± 0.41*</td>
</tr>
<tr>
<td></td>
<td>S. epidermidis ATCC 35984</td>
<td></td>
<td>9.85 ± 1.05</td>
<td>3.09 ± 0.28*</td>
<td>4.06 ± 0.32*</td>
</tr>
</tbody>
</table>

*P <0.001, showing the statistical difference between the carvacrol-treated and control groups.

ments after 3 h incubation with a more evident effect for S. aureus, whereas biofilms treated with carvacrol by vapour contact displayed optical density values decreasing only after prolonged incubation (24 h). These results were consistent with cell number determination (Table 2). After a short period (3 h) of carvacrol treatment by liquid contact, a major reduction (>6 log c.f.u. for S. aureus and >5 log c.f.u. for S. epidermidis) was observed in biofilm aggregated cells and this effect was similar for a 24 h exposure, suggesting that the carvacrol in liquid contact mainly acted on the biofilm during the first 3 h, after which no further antibacterial activity was exhibited. In contrast, inhibition as a result of vapour contact was less effective. After a 3 h treatment, the inhibition of bacterial cell number was about 3 log units for S. aureus and about 2 log units for S. epidermidis. A higher biofilm inhibition was obtained with the vapour contact only after 24 h of incubation, when a 4 log unit reduction in bacterial cell number was obtained for both strains.

The results of regrowth experiments showed that, upon reincubation in broth at 37 °C for 24 h, the controls displayed regrowth of S. epidermidis embedded in the biofilm, whereas the samples that had been exposed to carvacrol for 3 and 24 h retained almost the same number of cells with no or low levels of bacterial regrowth (Fig. 1).

As carvacrol was able to reduce biofilm biomass and cultivable cell numbers of S. aureus and S. epidermidis biofilms, it was of interest to examine whether it had an effect on biofilm viability and the biofilm architecture of S. epidermidis, which is known to be a high slime producer. The efficacy of 24 h carvacrol treatment (liquid and vapour contact) on S. epidermidis biofilm viability evaluated by TTC and a LIVE/DEAD BacLight Viability kit was shown by the absence or low levels of red-stained deposit after TTC staining (data not shown) and the prevalence of red fluorescence after LIVE/DEAD BacLight Viability staining (Fig. 2). These results were further substantiated by scanning electron microscopy (Fig. 3), which showed that the dense matrix architecture of the biofilm and the typical cell morphology observed in controls (Fig. 3a) became partially destroyed with a damaged cell surface after treatment with carvacrol in both liquid (Fig. 3b) and vapour (Fig. 3c) form.

**DISCUSSION**

The discovery of new antimicrobial compounds that are also active against biofilms is a relevant achievement. Recently, scientific interest in the biological properties of carvacrol has increased markedly and a large number of papers on its in vitro antimicrobial activity have been published (Ben Arfa et al., 2006; Chami et al., 2005; Dorman & Deans, 2000; Kordali et al., 2008; Lambert et al., 2001; Nostro et al., 2004; Ultee et al., 1998). However, only a small proportion has focused on its effects on bacterial biofilm. Knowles & Roller (2001) first reported the treatment of bacterial dried films with carvacrol on
stainless-steel surfaces. In a later study, these authors observed that, although complete eradication of the entire population was not achieved, carvacrol affected the viability and protein concentration of the quasi-steady state (at 12 days) of dual-species biofilms (\textit{S. aureus} and \textit{Salmonella enterica} serovar Typhimurium) (Knowles et al., 2005). Interestingly, Perez-Conesa et al. (2006) demonstrated that surfactant-encapsulated carvacrol was highly effective against \textit{Escherichia coli} and \textit{Listeria monocytogenes} cells aggregated in a biofilm. In particular, carvacrol-loaded micelles (0.3–0.7 %) inhibited the solid/air biofilm interface within 3 h of exposure.

We have shown previously that carvacrol in a liquid state inhibits biofilm formation and is effective against preformed biofilms of \textit{S. aureus} and \textit{S. epidermidis} (Nostro et al., 2007). Here, we reported the effects of carvacrol on the viability of cells embedded in a matrix to form a biofilm and on the cell morphology of a typical biofilm architecture.

Some authors have addressed the mode of action of carvacrol (Ben Arfa et al., 2006; Helander et al., 1998; Lambert et al., 2001; Ultee et al., 1999). Carvacrol interacts with the lipid bilayer of bacterial cytoplasmic membranes causing loss of integrity and collapse of the proton motive force, resulting in leakage of cellular material such as ions, ATP and nucleic acids. The extent of membrane damage induced by carvacrol can be related to its intrinsic hydrophobicity, determined experimentally by the partition coefficient in octanol/water (log $P_{o/w}$ equal to 3.64; Griffin et al., 1999). In addition, the presence of the hydroxyl group and a delocalized electron system seem to contribute to the antimicrobial activity. Ultee et al. (2002) proposed that this particular structure of carvacrol would allow the compound to act as a transmembrane carrier of monovalent cations by exchanging its hydroxyl proton for a potassium ion, thereby reducing the gradient across the cytoplasmic membrane.

Even though carvacrol is often considered a hydrophobic compound, it has been reported to possess a relative hydrophilicity, having a water solubility of $830 \pm 10$ p.p.m. (Griffin et al., 1999). Although its mechanism of action on biofilms remains unclear, the amphipathic nature of

\textbf{Fig. 2.} Fluorescence microscopic images. \textit{S. epidermidis} ATCC 35984 biofilms after 24 h treatment with carvacrol were stained by using a LIVE/DEAD BacLight Viability kit. (a) Biofilm control; (b) biofilm after treatment with carvacrol by liquid contact (1 %, v/v); (c) biofilm after treatment with carvacrol by vapour contact (1 %, v/v, air space). Live cells are indicated by green fluorescence whilst cells with damaged membranes are indicated by red fluorescence. Magnification, \times 1000.
carvacrol could account for the observed effects. Hence, we hypothesize that its relative hydrophilicity may allow the diffusion of carvacrol through the polar polysaccharide matrix, whilst the prevalent hydrophobic properties of this compound could lead to specific interactions with the bacterial membrane with considerable effects on its structural and functional properties such that it would lose its integrity. The differences observed between *S. epidermidis*

Fig. 3. Scanning electron micrographs of *S. epidermidis* ATCC 35984 biofilm. (a) Biofilm control; (b) biofilm after treatment with carvacrol by liquid contact (1%, v/v); (c) biofilm after treatment with carvacrol by vapour contact (1%, v/v, air space). Two different magnifications for each group are shown (left and right panels)
and S. aureus could be due to dissimilar diffusion through the polymeric matrix, caused by the different composition of the matrix itself (Izano et al., 2008).

A second important result of this study was the anti-biofilm effect of carvacrol in the vapour state. The higher potency of oil vapours compared with liquid phase has recently been reported, but only on planktonic cells (Inouye et al., 2000, 2001a, b, 2003). Some investigations demonstrated that the higher efficacy of vapours was probably due to the combined effect of direct deposition of carvacrol on micro-organisms together with an indirect effect via adsorption through the agar medium (Inouye et al., 2001a).

Conversely, the accumulated carvacrol vapour in the medium, rather than the vapour concentration in the air, was found to be mainly responsible for the inhibition of Salmonella enterica serotype Enteritidis (Burt et al., 2007a). In addition, the vapour exposure time affected bacterial inhibition, having a greater influence when longer incubation periods were used (Burt et al., 2007a; Weissinger et al., 2001).

The present study shows for the first time the activity of carvacrol vapour on staphylococcal biofilms, evaluating their indirect effect after absorption into the medium. We demonstrated that the higher efficacy of vapours was probably due to the combined effect of direct deposition of carvacrol on micro-organisms together with an indirect effect via adsorption through the agar medium (Inouye et al., 2001a).

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REFERENCES


