Intracellular, biofilm-inhibitory and membrane-damaging activities of nimbolide isolated from *Azadirachta indica* A. Juss (Meliaceae) against meticillin-resistant *Staphylococcus aureus*

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*Staphylococcus aureus* is a leading aetiological agent of nosocomial- and community-acquired infectious diseases worldwide. The public health concern regarding staphylococcal infections is inflated by the increasing occurrence of multidrug-resistant strains, e.g. multidrug- and meticillin-resistant *S. aureus* (MDR MRSA). This study was designed to evaluate the intracellular killing, membrane-damaging and biofilm-inhibitory activities of nimbolide isolated from *Azadirachta indica* against MDR MRSA. In *vitro* antibacterial activity of nimbolide was determined by performing MIC, minimal bactericidal concentration (MBC) and time-kill kinetic studies. Bacterial membrane-damaging activity was determined by membrane perturbation and scanning electron microscopy (SEM) examination. Biofilm-inhibitory activities were determined by SEM. Cellular drug accumulation and assessments of intracellular activities were performed using Vero cell culture. SEM revealed that nimbolide caused significant membrane damage and lysis of the *S. aureus* cells. The biofilm structure was disrupted, and the biofilm formation was greatly reduced in the presence of nimbolide as examined by SEM. The level of accumulation of nimbolide in Vero cells incubated for 24 h is relatively higher than that of ciprofloxacin and nalidixic acid (C<sub>c</sub>/C<sub>e</sub> for nimbolide > ciprofloxacin and nalidixic acid). The viable number of intracellular *S. aureus* was decreased [reduction of ~2 log<sub>10</sub> c.f.u. (mg Vero cell protein)<sup>−1</sup>] in a time-dependent manner in the presence of nimbolide (4 × MBC) that was comparable to that of tetracycline and nalidixic acid. The significant intracellular, biofilm-inhibitory and bacterial membrane-damaging activities of nimbolide demonstrated here suggested that it has potential as an effective antibacterial agent for the treatment of severe infections caused by MDR MRSA.

INTRODUCTION

*Staphylococcus aureus* is a leading aetiologic agent of nosocomial- and community-acquired infectious diseases worldwide. *S. aureus* causes several human diseases, ranging from minor skin and soft tissue infections to more severe conditions such as toxic shock syndrome, glomerulonephritis, pneumonia, meningitis, endocarditis, osteomyelitis and septicemia (Archer, 1998; Lowy, 1998; Kumar & Chopra, 2013). The public health concern regarding staphylococcal infections is exaggerated by the increasing occurrence of...
multiple-antibiotic-resistant strains such as meticillin-resistant
*S. aureus* (MRSA) and glycopeptide-insensitive *S. aureus* (Zetola et al., 2005). Despite the introduction of several anti-MRSA drugs in recent years, vancomycin still remains the gold standard for anti-MRSA therapy (Kumar & Chopra, 2013). However, the appearance of vancomycin-resistant strains suggests the possibility of going back to the pre-antibiotic period when 80% of bloodstream infections were fatal (de Lencastre et al., 2007; Zetola et al., 2005). Fluoroquinolones are broad-spectrum and potent antimicrobial agents that have been widely used for bacterial infections including *S. aureus* infections. However, extensive clinical use of these has led to an increase of resistant organisms, and a high prevalence of fluoroquinolone resistance among *S. aureus* isolates has been reported (Guirao et al., 2001). Resistance to quinolones has been a problem ever since nalidixic acid was introduced into clinical medicine more than 40 years ago. For a time, the greater potency of the fluoroquinolones, compared with that of older quinolones, permitted complacency regarding their use, but successful treatment outcomes led to increased use, which, in turn, led to an increasing rate of resistance (Jacoby et al., 2005). Since quinolone resistance is developing comparatively rapidly in this group of *S. aureus* and quinolone resistance seems to be already frequent among MRSA (Kumar & Chopra, 2013; Witte & Grimm, 1992), searching for newer drugs should be in parallel with a constant monitoring of staphylococcal resistance patterns in order to avoid selection and spread of multidrug-resistant *S. aureus*.

Natural products have been used for centuries as traditional remedies to combat a variety of ailments, including infectious diseases (Rios & Recio, 2005). *Azadirachta indica* A. Juss (Meliaceae), locally known as neem, is well known in India and its neighbouring countries as well as in African countries as one of the most versatile medicinal plants having a broad spectrum of biological activities (Biswas et al., 2002; Okpaniyi & Ezeukwu, 1981). We have also demonstrated previously the antibacterial, antiexcretory and antihaemorrhagic activities of *A. indica* in a mouse model (Thakurta et al., 2007). Neem, a versatile medicinal plant, is the unique source of various types of compounds having diverse chemical structure. Although a large number of compounds have been isolated from various parts of neem, few of them have been extensively studied for biological activity (Biswas et al., 2002). One of these compounds is nimbolide, a tetrnortriterpenoid limonoid, which was isolated first from leaves and flowers of neem. Nimbolide has been shown to possess various biological activities (Biswa et al., 2002). Although antimalarial activity of nimbolide has been studied previously (Rochanakij et al., 1985; Khalid et al., 1989) and anticancer activity of nimbolide has been studied extensively (Gupta et al., 2010; Roy et al., 2007), antibacterial activity of nimbolide has not been well established. In the present study, we demonstrated the intracellular bactericidal activity and *in vitro* membrane-damaging and biofilm-inhibitory activities of nimbolide isolated from *A. indica* against multidrug- and meticillin-resistant *S. aureus* (MDR MRSA).

### METHODS

**Plant material.** Nimbolide was isolated, purified and identified from leaves of *A. indica* A. Juss (Meliaceae) as described in the Supplementary file.

**Nimbolide.** \([(4\alpha,5\alpha,6\alpha,7\alpha,15\beta,17\alpha)-7,15:21,23-Diepoxy-6-hydroxy-4,8-dimethy1-1-oxo-18,24-dionor-11,12-secochola-2,13,20,22-tetraene-4,11-dicarboxylic acid ϕ-lactone methyl ester]: white to off-white powder; \(^1\)H NMR see Table S1, available in the online Supplementary Material; \(^13\)C NMR see Table S1; QTOF MS m/z 489.06 [M+Na]^+ (calculated for C_{27}H_{34}O_{27}, 266.52).

**Bacterial strains.** The strains *S. aureus* PCS111 (clinical isolate) and *S. aureus* ATCC 25923 [meticillin-susceptible *S. aureus* (MSSA)] were used in this study. The strains were stored in 15% glycerol stock at ~70°C. Strain PCS111 was a Gram-positive, catalase-positive coccus, mannitol salt agar-positive, positive in Staphaurex Plus (Oxoid) latex agglutination test and tube coagulase-positive and showed a strong DNase activity. Since MIC of meticillin was 32 µg ml\(^{-1}\) against this strain (Table 1), following the breakpoints published by CLSI (2009) [meticillin resistant (R), \(\geq 16\) µg ml\(^{-1}\)], it was designated MRSA. Since this strain was resistant to meticillin as well as to nalidixic acid (R, \(\geq 32\) µg ml\(^{-1}\)), ciprofloxacin (R, \(\geq 4\) µg ml\(^{-1}\)) and streptomycin (R, \(\geq 16\) µg ml\(^{-1}\)) (Table 1) according to the CLSI (2009) breakpoints, it was designated MDR MRSA (Merlino et al., 2002).

**Antibacterial activity.** The MICs and minimal bactericidal concentrations (MBCs) of nimbolide and reference antibiotics (meticillin, streptomycin, tetracycline, nalidixic acid and ciprofloxacin) were determined using broth microdilution as previously (Thakurta et al., 2007) following the methods recommended by CLSI (2009) and NCCLS (1999). A final inoculum of 1 x 10^8 c.f.u. ml^{-1} was used, and the concentrations of drugs tested were 1 to 512 µg ml^{-1}.**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Streptomycin</th>
<th>Nalidixic acid</th>
<th>Ciprofloxacin</th>
<th>Tetracycline</th>
<th>Meticillin</th>
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<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
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<td>256</td>
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<td>32</td>
<td>128</td>
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ND, Not determined. All experiments were performed in triplicate.

Table 1. MICs and MBCs (both in micrograms per millilitre) of reference antibiotics and nimbolide against *S. aureus*.
Time--kill kinetic study. Cultures of bacteria (\(1 \times 10^6\) c.f.u. ml\(^{-1}\)) in Mueller--Hinton broth (MHB; Hi-Media) were incubated separately in the absence (control) and presence of drug (32 µg ml\(^{-1}\)) for a period of 24 h at 37°C. Samples of the bacterial cultures were removed at 0, 2, 4, 8, 12 and 24 h intervals, and viable counts were determined as follows: the samples were serially diluted in sterile 10 mM PBS (pH 7.2), and 100 µl aliquots were plated onto nutrient agar. The plates were incubated at 37°C for 24 h, and survival counts were recorded and expressed as c.f.u. ml\(^{-1}\). The surviving log\(_{10}\) c.f.u. ml\(^{-1}\) was plotted against time (h). Experiments were performed in triplicate.

Selection of resistant mutants in vitro. The selection for resistant mutants in vitro was performed following the procedure described previously (Acharyya et al., 2015). The strain of S. aureus was grown and maintained in MHB in the presence of sublethal doses of nimbolide (2 µg ml\(^{-1}\)) for 37°C. Consecutive subcultures of the bacterium were exposed to sublethal doses of nimbolide for a period of 8 weeks, and the bacteria periodically (at every 2 week interval) were restested to determine any increase in MIC following the method described above.

Determination of mammalian cell toxicity. Toxicity of test compound was assayed on Vero cells and human red blood cells as described previously (Acharyya et al., 2015) following the previously described methods (De La Fuente et al., 2006; Situ & Bobek, 2000).

Morphological assay of bacterial cells using scanning electron microscopy (SEM) examination. Cultures of bacteria in MHB (around \(1 \times 10^6\) c.f.u. ml\(^{-1}\)) were incubated separately in the absence (control) and presence of nimbolide at 1× MBC (32 µg ml\(^{-1}\)) and meticillin (32 µg ml\(^{-1}\)) for a period of 12 h at 37°C. Samples of the bacterial cultures were removed at different time intervals and harvested (1500 g, 10 min, 4°C). The cell samples were fixed with 2.5% glutaraldehyde overnight at 4°C, washed with sodium phosphate buffer (0.1 M, pH 7.2), air dried and sputter coated with platinum (10 nm) by Q150T ES turbo-pumped sputter coater (Quorum Technologies). Finally, the morphology of the bacterial cells was observed under SEM (ZEISS EVO-MA 10). The SEM observation was done under the following analytical conditions: EHT, 15.00 kV; WD, 8.0 mm; signal A, SE1.

Membrane perturbation assay. The membrane-perturbation assay was performed using propidium iodide (PI) fluorescence detection as described previously (Tsao et al., 2002). The bacteria (2×10^6 c.f.u. ml\(^{-1}\)) collected from the exponential phase were cultured with nimbolide (1× and 4× MIC) at 37°C. At various intervals, the bacteria were collected and stained with PI (10 µg ml\(^{-1}\)) for 10 min, and after washing the cells, dye penetration was measured by the presence of fluorescence (excitation at 535 nm and emission at 560 nm). Experiments were performed in triplicate.

Biofilm susceptibility assay using 96-well polystyrene tissue culture plate. The effect of nimbolide on biofilm formation by bacteria was examined by the microdilution method (Wei et al., 2006) using 96-well polystyrene flat-bottom microtitre plate (Tarsen). The inoculum concentration was \(1 \times 10^6\) c.f.u. ml\(^{-1}\) in each well, and drugs were used at MBC (32 µg ml\(^{-1}\)), 1× MIC (8 µg ml\(^{-1}\)) and 0.5 MIC (4 µg ml\(^{-1}\)). After incubation at 37°C for 24 h, absorbance at 595 nm was recorded to assess the culture growth. After aspirating out the culture supernatant, the wells were washed with PBS (pH 7.2) to remove planktonic cells. The biofilm was fixed with methanol for 15 min and air dried at room temperature followed by staining with 0.1% (w/v) crystal violet for 10 min. The wells were thoroughly washed with water until the negative control wells appear colourless. To each of the crystal-violet-stained wells, 200 µl of 95% ethanol was added. Absorbance at 595 nm was recorded using a microplate reader (Bio-Rad). Experiments were performed in triplicate.

Biofilm susceptibility assay using SEM. Coverslips placed in the wells of 12-well microtitre plate (Tarsen) were incubated with bacteria (around \(1 \times 10^6\) c.f.u. ml\(^{-1}\)) for 24 h in brain--heart infusion broth (Hi-Media) separately in the absence (control) and presence of nimbolide at 1× MBC (32 µg ml\(^{-1}\)). Samples were washed extensively up to six times in PBS, fixed with 2.5% glutaraldehyde overnight at 4°C and washed with sodium phosphate buffer (0.1 M, pH 7.2), air dried and sputter coated with platinum. The SEM observation was done as described above.

Determination of cellular antibiotic accumulation. Vero cells (African green monkey kidney cells) were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum in an atmosphere of 95% air/5% CO\(_2\) as described previously (Bag et al., 2008). Accumulation studies were performed as described previously (Barcia-Macay et al., 2006; Carryn et al., 2002). Cells were incubated with antibiotics for 24 h. Antibiotic concentration was measured by microbiological assay (by agar diffusion method) using Bacillus subtilis ATCC 6623 as test organism. Experiments were repeated five times.

Cell infection and assessment of intracellular activities of antibiotics. All experiments were performed with Vero cell line. The cells were maintained as described earlier. The cells (5×10^6 cells ml\(^{-1}\)) were infected using a fresh inoculum of S. aureus (2.5×10^8 c.f.u. ml\(^{-1}\)) that had been incubated for 1 h at 37°C and washed extensively to remove non-firmly adherent bacteria (four successive sedimentations at 1300 r.p.m. followed by gentle resuspension in pre-warmed sterile PBS), yielding an average infection index of one bacterium per four Vero cells (as determined by counting the numbers of c.f.u.) (Barcia-Macay et al., 2006; Carryn et al., 2002). The cells were thereafter incubated in fresh medium (with/without antibiotic) for up to 24 h. To ensure the absence of extracellular bacteria, the culture medium that contained cells with firmly adherent S. aureus and that had not been exposed to antibiotics after the washing procedure described here was incubated at 37°C for 48 h; no bacterial growth was detected. After 24 h incubation, the cells were collected by centrifugation, washed with ice-cold sterile PBS and lysed in distilled water. The lysates were then plated on nutrient agar at appropriate dilutions for determination of the number of viable bacteria by counting of the colonies (determination of the numbers of c.f.u.) and used for total cell protein measurement (Carryn et al., 2002). All results are expressed as the number of c.f.u. per milligram of cell protein. Experiments were performed in triplicate.

RESULTS

In vitro antibacterial activity

To evaluate the antimicrobial activity of nimbolide, the MICs and the MBCs were determined. Nimbolide evoked significantly high inhibitory activity against MDR MRSA with much lower MIC and MBC values than reference antibiotics (Table 1).

Nimbolide did not show cytotoxicity to human erythrocytes and Vero cells (after 24 h incubation) at concentrations of up to 4× MBC (128 µg ml\(^{-1}\)) (results not shown).

Time--kill kinetic study

Nimbolide and meticillin at a concentration of 32 µg ml\(^{-1}\) showed an –2.5- to 4.5-log reduction in viable cell number (c.f.u.) within 24 h for S. aureus PCS111 and S. aureus ATCC 25923, compared to the untreated control (Fig. 1).
Selection of resistant mutants in vitro

After successive subculturing of *S. aureus* in the presence of sublethal doses of nimbolide (2 and 4 µg ml\(^{-1}\)) for a period of 8 weeks, the bacteria did not show any increase in MIC, and MIC remained the same (i.e. 8 µg ml\(^{-1}\)).

Bacterial membrane-damaging activity determined by SEM experiments

In the control samples of *S. aureus* PCS111 (Fig. 2a, b) and *S. aureus* ATCC 25923 (Fig. 2g) in MHB medium, the cells seemed round and intact with normal smooth surfaces. After 4 h incubation with nimbolide at MBC (32 µg ml\(^{-1}\)), morphological changes including surface roughness, multiple blisters and bubble formation on their surface, partial deformation, depression and dent formation were observed for *S. aureus* PCS111 (Fig. 2c, d) and *S. aureus* ATCC 25923 (Fig. 2h) cells. After 8 h incubation, significant membrane damage, bursting of cells and some lysed cells were found for nimbolide-treated PCS111 (Fig. 2e) and ATCC 25923 (Fig. 2f) cells. In addition, in membrane perturbation assay, it was found that PI uptake increased with time after treatment with nimbolide in a dose-dependent manner (Fig. 3).

Biofilm-inhibitory activity

The effect of nimbolide on biofilm formation by bacteria was examined by 96-well polystyrene flat-bottom microtitre plate assay. Nimbolide showed an inhibitory effect on the formation of biofilm produced by *S. aureus* strains PCS111 (Fig. 4a) and ATCC 25923 (Fig. 4b) in a dose-dependent manner. It exhibited significant (*P*<0.05) inhibitory activity against biofilm formation at the concentrations of 8 and 32 µg ml\(^{-1}\) for the strain PCS111. In addition, it significantly (*P*<0.05) inhibited the biofilm formation by the strain ATCC 25923 at the concentrations of 4 to 32 µg ml\(^{-1}\).

SEM analysis was carried out to visualize the detailed architecture of biofilm and the effect of nimbolide on biofilm formation. For the untreated controls, biofilm formation on the coverslips that consisted of nearly uniform, multilayered thick clusters of cells was observed for both *S. aureus* PCS111 (Fig. 5a, b) and *S. aureus* ATCC 25923 (Fig. 5d, e). The formation of the biofilm was prevented or greatly reduced in the presence of nimbolide at a concentration of 32 µg ml\(^{-1}\). In nimbolide-treated samples of *S. aureus* PCS111 (Fig. 5c) and *S. aureus* ATCC 25923 (Fig. 5f), the biofilm structure was disrupted, and a significant part of the coverslip surface was cleared of bacterial cells and there were only small clusters of cells scattered on the surface.

Comparison of antibiotic accumulation inside Vero cells

Table 2 shows the levels of accumulation of ciprofloxacin, tetracycline, nalidixic acid and nimbolide in Vero cells incubated for 24 h in the presence of the drugs at 1× MBC. The level of accumulation of nimbolide and tetracycline is relatively higher than that of ciprofloxacin and nalidixic acid, since the \(C_c/C_e\) ratio is higher for nimbolide and tetracycline than that of ciprofloxacin and nalidixic acid.

Intracellular activity

Intracellular activity of nimbolide and other reference antibiotics at 4× MBC is shown in Fig. 6. One hour postinfection, reference antibiotic or nimbolide was added to *S. aureus*—
infected Vero cells, and viability of intracellular bacteria was measured at different time points (0, 2, 5 and 24 h) of incubation. The viable number of intracellular bacteria was decreased in a time-dependent manner in the presence of the reference drugs or nimbolide. Nimbolide reduced the cell number [reduction of ~2 log_{10} c.f.u. (mg Vero cell protein)^{-1}] of intracellular *S. aureus* within 24 h at 37°C. These observations confirmed the intracellular activity of nimbolide.

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**Fig. 2.** SEM micrographs of untreated *S. aureus* PCS111 (a, b) and *S. aureus* ATCC 25923 (g); the cells are round and intact with normal smooth surfaces. After 4 h incubation with nimbolide at MBC (32 µg ml^{-1}), morphological changes including surface roughness, partial deformation, depression and dent formation are observed for *S. aureus* PCS111 (c, d) and *S. aureus* ATCC 25923 (h) cells. After 8 h incubation, significant membrane damage, bursting of cells and some completely lysed cells are found for nimbolide-treated PCS111 (e) and ATCC 25923 (i, j) cells and for meticillin (32 µg ml^{-1})-treated PCS111 (Fig. 2f) and ATCC 25923 (Fig. 2k) cells. Morphological changes are indicated by arrows.
SA-1
SA-4
strains such as MRSA, vancomycin-resistant MRSA, etc. (Zetola et al., 2005; Huang & Yousefa, 2014). The bacterial cytoplasmic membrane is indispensable to bacterial life, as it contains one-third of the proteins in a cell and is the place for many vital processes (Huang & Yousefa, 2014; Hurdle et al., 2011). Therefore, these antimicrobial agents including nimbolide may interfere with numerous cellular functions by damaging the cell membrane (Huang & Yousefa, 2014; Hurdle et al., 2011). Furthermore, in our study, after successive subculturing of S. aureus in the presence of sublethal doses of nimbolide for a period of 8 weeks, the bacteria did not show any increase in MIC indicating that the exposure to nimbolide for this long period did not produce any resistance against nimbolide. It has been suggested earlier that unlike other conventional antibiotics, membrane-active agents are less likely to mediate antibiotic resistance in treated bacteria (Huang & Yousefa, 2014; Hurdle et al., 2011). Since nimbolide clearly damages bacterial cytoplasmic membranes and S. aureus treated with nimbolide
did not show any nimboide resistance, in terms of the challenges of antibacterial resistance, nimboide could be a potential therapeutic agent for the treatment of emerging infectious diseases caused by S. aureus.

S. aureus biofilms exhibit increased resistance to antimicrobial agents and are widely concerned in many persistent and chronic infections that are difficult to eradicate (Costerton et al., 1999). S. aureus biofilms are characteristically dense and highly hydrated clusters of bacterial cells, adhered to a surface and enclosed in a matrix composed of exopolysaccharides such as polysaccharide intercellular adhesin (Sing et al., 2010; Hall-Stoodley et al., 2004). This matrix, also termed slime or extracellular polymeric substance, gives stability to the biofilm structure, facilitates adherence and trapping of nutrients and also plays a significant role in the pathogenesis of biofilm-associated infections, as well as the observed antibiotic resistance (Sing et al., 2010; Hall-Stoodley et al., 2004). In addition, bacteria protected within biofilms can be up to 1000 times more resistant to antibacterial agents than their planktonic counterparts (Gupta et al., 2013; Luppens et al., 2002). Mechanisms contributing to high antimicrobial resistance in biofilms are thought to

![Fig. 5. SEM micrographs of biofilm formation by S. aureus PCS111 (a, b, c) and S. aureus ATCC 25923 (d, e, f) in the presence or absence of nimboide. (a, b, d, e) In the absence of nimboide. (c, f) In the presence of nimboide at MBC (32 µg ml⁻¹).](image-url)
include physical or chemical diffusion barriers to anti-
microbial penetration into the biofilm, slow growth of the bio-
film owing to nutrient limitation, activation of the general
stress response and the emergence of a biofilm-specific phe-
notype (Mah & O’Toole, 2001; Davies, 2003). A recent
study showed that several antibiotics (oxacillin, cefotaxime
and vancomycin) had reduced penetration throughout S.
*aureus* and *Staphylococcus epidermidis* biofilms (Sing et al.,
2010; Archer et al., 2011) resulting in development of resis-
tance to these antibiotics. In addition, the biofilm mode of
growth is now recognized as a major mediator of infection,
with an estimated 80% of all infections caused by biofilms
(Archer et al., 2011). Furthermore, it has been estimated
that biofilms are associated with 65% of nosocomial infec-
tions (Mah & O’Toole, 2001). In the present study, it was
found that nimbolide significantly inhibited the biofilm for-
mation by *S. aureus* in a dose-dependent manner as deter-
mined by microtitre plate assay. To confirm these results,
SEM examination was performed. SEM findings revealed
that the biofilm structure was disrupted and the biofilm for-
mation was prevented or greatly reduced for both of the
strains PCS111 (MDR MRSA) and ATCC 25923 (MSSA) in
the presence of nimbolide. Therefore, observed inhibitory
activity of nimbolide against biofilm formation by *S. aureus*
in this study could be useful to treat the *S. aureus*-infected
patients.

Intracellular survival of pathogenic micro-organisms, such
as *S. aureus*, may result in recurrent infections, and antibi-
otic treatment will not be successful if penetration ability
of the drug into cells is poor (Nielsen et al., 1997). The fluoro-
quinolones such as ciprofloxacin was associated with
the isolation of MRSA and thereby exposure to quinolones
is a significant risk factor for the isolation of MRSA, but not
MSSA (Weber et al., 2003). Here, we have demonstrated
the significant penetration and accumulation of nimbolide
inside Vero cells and its intracellular killing activity against
MDR MRSA, which could be effective for the treatment of
infected patients. The C/C0 ratios for ciprofloxacin, tetracy-
cline and nimbolide were 26.15, 58.40 and 56.13, respec-
tively, demonstrating that ciprofloxacin, tetracycline and
nimbolide were accumulated up to 26.15-, 58.40- and
56.13-fold, respectively, inside Vero cells. Hence, it can be
suggested following the analysis of Barcia-Macay et al.
(2006) that ciprofloxacin, nimbolide and tetracycline
achieved high levels (26.15–58.40-fold) of accumulation.
Penetration of antibiotics in effective concentrations inside
the cells is one of the important criteria to kill intracellular
pathogens (Kadurugamuwa & Breveridge, 1998). In addi-
tion, intracellular killing activity of nimbolide was compar-
able to that of nalidixic acid and tetracycline.

In short, nimbolide showed significant penetration of and
accumulation inside Vero cells, and the intracellular killing
activity of nimbolide against MRSA was comparable to that
of tetracycline and nalidixic acid. To our knowledge, this is
the first report of intracellular killing, biofilm-inhibitory
and bacterial membrane-damaging activities of nimbolide.
In addition, the exposure of MRSA to nimbolide for a long
period did not show up any nimbolide resistance. The signif-
ificant intracellular bactericidal activity and *in vitro* bac-
tericidal, membrane-damaging and biofilm-inhibitory
activities of nimbolide demonstrated here suggest that it
could be potentially considered as an effective antibacterial
agent for the treatment of severe infections caused by MDR
MRSA, particularly those resistant or with reduced suscepti-
bility to most of the recommended antibiotics. However,

![graph](image-url)

**Fig. 6.** Influence of time on the rate and the extent of the activi-
ties of the antibiotics against intracellular *S. aureus* upon incuba-
tion at a fixed extracellular concentration (4× MBC). The graph
shows the change in the number of c.f.u. from time 0 (log10 c.f.u.:
mean±SD; n=3) in Vero cells (intracellular) per milligram of cell
protein. ♦ Intracellular growth in the absence (control) of antibi-
otic. ■, △, ×, Intracellular growth in the presence of nimb-
olide, nalidixic acid, tetracycline and ciprofloxacin, respectively.

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Table 2. Cellular accumulation of antibiotics in Vero cells after
24 h incubation

<table>
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<th>Antibiotic</th>
<th>Antibiotic added (µg ml⁻¹)</th>
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<tr>
<td>Nimbolide</td>
<td>32</td>
<td>56.13±0.80</td>
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MBCs of streptomycin, nalidixic acid, ciprofloxacin, tetracycline
and nimbolide were 256, 256, 128, 32 and 32 µg ml⁻¹, respectively,
against *S. aureus*. Antibiotic concentration was determined by agar
diffusion assay against *B. subtilis*.

*The C/Cₐ ratio (mean±SD; n=5) was calculated from the drug con-
tents per milligram of cell protein by using a conversion factor of 5 µl
mg⁻¹ of cell protein. Cₐ intracellular concentration; Cₑ extracellular
concentration.

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[Table 2: Cellular accumulation of antibiotics in Vero cells after
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</tbody>
</table>

MBCs of streptomycin, nalidixic acid, ciprofloxacin, tetracycline
and nimbolide were 256, 256, 128, 32 and 32 µg ml⁻¹, respectively,
against *S. aureus*. Antibiotic concentration was determined by agar
diffusion assay against *B. subtilis*.

*The C/Cₐ ratio (mean±SD; n=5) was calculated from the drug con-
tents per milligram of cell protein by using a conversion factor of 5 µl
mg⁻¹ of cell protein. Cₐ intracellular concentration; Cₑ extracellular
concentration.
the in vitro antimicrobial and biofilm-inhibitory assays used in this study are basically initial screens for nimbolide. Studies are ongoing to explicate the molecular mechanism of action of nimbolide in biofilm inhibition and in antibacterial activity other than membrane-damaging activity, which might be useful for the exploration of a novel strategy in controlling biofilm-mediated infections.

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