Evaluation of activity of recombinant lysostaphin against isolates of meticillin-resistant *Staphylococcus aureus* from Indian hospitals

In the past decade, treatment of staphylococcal infections has become more challenging due to the emergence of multidrug resistant/meticillin-resistant *Staphylococcus aureus* (MRSA) strains, community-associated (CA)-MRSA strains such as USA300 evolving resistance to non-β-lactams, reduced clinical response to vancomycin, and spread of resistance to alternative compounds, such as linezolid, daptomycin and mupirocin (Hill et al., 2010; Mangili et al., 2005; Jones et al., 2007). Therefore, new anti-staphylococcal agents without cross-resistance to currently available antibacterial drugs are required urgently. Lysostaphin is a 27 kDa zinc metalloproteinase produced by *Staphylococcus simulans* (Schindler & Schuhardt, 1964) which cleaves pentaglycine cross-bridges of the *S. aureus* cell wall leading to its lysis (Browder et al., 1965). Development of recombinant (r)-lysostaphin (Recsei et al., 1987) made it possible to explore this molecule as an effective therapeutic agent, as evident from various studies conducted between 1999 and 2007 (Patron et al., 1999; Dajcs et al., 2000; Kokai-Kun et al., 2003; Yang et al., 2007). Motivated by the initial success of preclinical studies of r-lysostaphin produced at the laboratory scale, efforts have been made to produce r-lysostaphin at a commercial scale by improving its activity and yield at the downstream processing stages. One such formulation of r-lysostaphin (BSYX-L210) obtained from *Lactococcus lactis* has entered into phase I and II clinical trials (McCoy, 2004).

The present study is, to the best of our knowledge, the first report describing the activity of mature purified r-lysostaphin obtained from the 765 bp gene of *S. simulans* cloned and expressed in *Escherichia coli* BL21, which is considered a safe expression host as far as pharmaceutics/therapeutics are concerned. Further, this mature lysostaphin is free from preprolysostaphin and prolysostaphin (Khatri & Sharma, 2005).

The r-lysostaphin obtained by this method was evaluated against clinical isolates of MRSA from various hospitals in India and epidemic *S. aureus* strains of global origin. As the prevalence of MRSA and CA-MRSA is increasing worldwide, we advocate the development of r-lysostaphin as an effective alternative treatment for the emerging MRSA threat.

MRSA isolates (n=66) collected from different hospitals across India were provided by the Sir Dorabji Tata Centre for Research in Tropical Diseases, Bangalore to Bharat Biotech International Ltd (BBIL), Hyderabad. *S. aureus* ATCC 29213 and *Staphylococcus epidermidis* ATCC 12228 were obtained from the American Type Culture Collection. *S. aureus* strains mupr-1 and lysor-1 were laboratory-generated mupirocin and lysostaphin mutants, respectively (Mirza et al., 2011). Epidemic strains of *S. aureus* (n=14) were provided by Dr Knut Ohslen (University of Würzburg, Germany) to BBIL. MRSA 562, MRSA P57 and MRSA P6 were chosen for minimum biofilm eradication concentration (MBEC) assay after screening 20 MRSA strains for biofilm formation as described by Christensen et al. (1985). Muller–Hinton (MH) broth (Becton Dickinson) supplemented with calcium (25 μg ml⁻¹), magnesium (12.5 μg ml⁻¹) and 0.1 % BSA was used for all susceptibility and kill kinetic studies. MH agar was used for the disc diffusion assay. Tryptic soy agar (TSA; Becton Dickinson) was used for colony counts. R-lysostaphin (5 mg ml⁻¹) was obtained from a Good Manufacturing Practice facility of BBIL. Vancomycin and linezolid were purchased from Aldrich.

The disc diffusion assay was performed on different *S. aureus* isolates, *S. aureus* ATCC 29213, *S. aureus* mupr-1 and *S. aureus* lysor-1 (CLSII, 2009b). Twofold dilutions of r-lysostaphin ranging from 2 to 0.0035 μg ml⁻¹ in 0.1 % BSA-supplemented MH broth were tested in U-bottom 96-well microtitre plates. The minimum bactericidal concentration (MBC) assay was performed as a follow-up to the MIC assay with little modification to CLSI (2009b). A 10 μl aliquot of 10 μg proteinase K ml⁻¹ in PBS was added to each well to stop the activity of residual r-lysostaphin and 10 μl mixed content was plated on TSA supplemented with 2 % NaCl from wells showing no visual growth. The MIC of r-lysostaphin for MRSA isolates ranged from 0.007 to 0.25 μg ml⁻¹ (Table 1). MIC₅₀ and MIC₉₀ values were found to be 0.06 and 0.25 μg ml⁻¹, respectively. *S. aureus* lysor-1 was found to be resistant to r-lysostaphin (MIC>64 μg ml⁻¹), whilst the MIC for *S. aureus* mupr-1 was found to be 0.06 μg ml⁻¹. The MBC for the tested isolates ranged from 0.06 to 0.5 μg ml⁻¹. The MBC at which 90 % of the strains tested were susceptible (MBC₉₀) was 0.5 μg ml⁻¹. No significant reduction in bacterial counts for *S. aureus* lysor-1 was observed even at 100 μg ml⁻¹. To further assess the use of r-lysostaphin as an effective therapeutic agent against globally emerging resistant strains, *S. aureus* EMRSA-15, ST22, FU2339, ST239, ANS 46, MW2, ST5, BK2464, ST45 (USA600), HDE228, Mu50, USA300, Newman and COWAN were subjected to comparative MICs of vancomycin and linezolid to r-lysostaphin. All the epidemic strains of *S. aureus* were found to be sensitive to sterile filter paper discs with 10 μl of 5 mg r-lysostaphin ml⁻¹ (from the stock solution prepared in phosphate buffer). All 66 MRSA isolates used in this study were found to be sensitive to r-lysostaphin at 50 μg disc⁻¹ with zone diameters ranging from 13 to 20 mm (Table 1).

The disc diffusion assay was performed on 66 MRSA isolates, *S. aureus* ATCC 29213, *S. aureus* mupr-1 and *S. aureus* lysor-1 (CLSII, 2009a). r-Lysostaphin discs (50 μg disc⁻¹) were used for the study. These discs were prepared by impregnating 6 mm sterile filter paper discs with 10 μl of 5 mg r-lysostaphin ml⁻¹ (from the stock solution prepared in phosphate buffer). All 66 MRSA isolates used in this study were found to be sensitive to r-lysostaphin at 50 μg disc⁻¹ with zone diameters ranging from 13 to 20 mm (Table 1). No zone of inhibition was observed for *S. aureus* lysor-1, whilst *S. aureus* mupr-1 was found to be susceptible to r-lysostaphin.

The MIC of r-lysostaphin was determined by micro broth dilution assay (CLSI, 2009b). Twofold dilutions of r-lysostaphin ranging from 2 to 0.0035 μg ml⁻¹ in 0.1 % BSA-supplemented MH broth were tested in U-bottom 96-well microtitre plates. The minimum bactericidal concentration (MBC) assay was performed as a follow-up to the MIC assay with little modification to CLSI (2009b). A 10 μl aliquot of 10 μg proteinase K ml⁻¹ in PBS was added to each well to stop the activity of residual r-lysostaphin and 10 μl mixed content was plated on TSA supplemented with 2 % NaCl from wells showing no visual growth. The MIC of r-lysostaphin for MRSA isolates ranged from 0.007 to 0.25 μg ml⁻¹ (Table 1). MIC₅₀ and MIC₉₀ values were found to be 0.06 and 0.25 μg ml⁻¹, respectively. *S. aureus* lysor-1 was found to be resistant to r-lysostaphin (MIC>64 μg ml⁻¹), whilst the MIC for *S. aureus* mupr-1 was found to be 0.06 μg ml⁻¹. The MBC for the tested isolates ranged from 0.06 to 0.5 μg ml⁻¹. The MBC at which 90 % of the strains tested were susceptible (MBC₉₀) was 0.5 μg ml⁻¹. No significant reduction in bacterial counts for *S. aureus* lysor-1 was observed even at 100 μg ml⁻¹. To further assess the use of r-lysostaphin as an effective therapeutic agent against globally emerging resistant strains, *S. aureus* EMRSA-15, ST22, FU2339, ST239, ANS 46, MW2, ST5, BK2464, ST45 (USA600), HDE228, Mu50, USA300, Newman and COWAN were subjected to comparative MICs of vancomycin and linezolid to r-lysostaphin. All the epidemic strains of *S. aureus* were found to be sensitive to...
r-lysostaphin with MICs ranging from 0.0156 to 0.0625 μg ml⁻¹. It is noteworthy that vancomycin-, fusidic acid- and mupirocin-resistant strains (Mu50, Fu2339 and ST239, respectively) were also found to be sensitive to r-lysostaphin. r-Lysostaphin was also found to be two- to eightfold better than the reference standard lysostaphin (Sigma) against seven isolates out of a total of 11 MRSA strains tested.

Time kill kinetic studies were performed as per CLSI (1999). *S. aureus* ATCC 29213 was used as the test organism in this study. Final concentrations of r-lysostaphin ranging from 0.015 (MIC) to 0.12 (8 × MIC) μg ml⁻¹ were tested in individual flasks containing 20 ml MH broth. Kill curves were constructed by plotting log₁₀c.f.u. ml⁻¹ against time over 8 h. The bactericidal activity (≥3 log reduction in c.f.u.) of r-lysostaphin for *S. aureus* ATCC 29213 was observed within 1 h of exposure to all of the tested concentrations. There was a concentration-dependent killing; however, regrowth was observed after 4 h at the lowest concentration of 0.015 μg ml⁻¹. The higher concentrations of 0.06 and 0.12 μg ml⁻¹ exhibited rapid killing within 10 min of exposure.

The MBEC assay was performed on three MRSA isolates (MRSA 562, MRSA P57 and MRSA P6) using the method described by Christensen et al. (1985). The sessile inoculum of ~2–6 × 10⁸ c.f.u. ml⁻¹ was obtained in 20 h biofilms developed in wells of flat-bottom microtitre plates. The mean c.f.u. ml⁻¹ obtained from untreated sessile growth formed by each test isolate was used as the reference inoculum for determining the percentage reduction in sessile growth of the same isolate using the formula: 100–[(c.f.u. of treated well/c.f.u. of reference inoculum) × 100]. MBEC was defined as the minimum concentration of r-lysostaphin required to reduce sessile growth by ≥ 99.9 %. r-Lysostaphin was successful in eradicating ≥99.9 % of sessile growth of *S. aureus* within the range 8–16 μg ml⁻¹.

Biofilms of *S. aureus* ATCC 29213 for scanning electron microscopy (SEM) were developed on electron microscopic plastic sheets (1 cm²) in 24-well tissue culture plates (Nunc). Biofilms (24 h) were treated with 4, 8 and 16 μg r-lysostaphin ml⁻¹. Samples were then fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4 °C for 1 h, followed by the addition of 1 % OsO₄ for 2 h at 4 °C. After fixation, samples were dehydrated in acetone and dried by a using critical point drier with liquid CO₂. SEM observations are presented in Fig. 1. The untreated sessile cells were seen embedded in a glycocalyx matrix (Fig. 1a). There was thinning of the glycocalyx matrix after the treatment with 4 μg ml⁻¹, but sessile cells could be seen (Fig. 1b). However, at 8 and 16 μg ml⁻¹ there was a complete eradication of sessile and planktonic cells along with the extracellular matrix (Fig. 1c, d).

The in vivo efficacy of r-lysostaphin topical formulations was tested on the murine model of skin infection as described by Mirza et al. (2011). Based on the result of release kinetics of r-lysostaphin from the formulation, topical formulations of 60, 100, 120 and 150 μg g⁻¹ were evaluated. Groups under treatment were observed for the physical and pathological conditions of wounds by quantifying bacterial load on 1 cm² skin patches. Wound healing and reduction in bacterial load was observed for the entire group under treatment except for the placebo on each consecutive day. The initial bacterial c.f.u. load in the skin patch of the placebo-treated control group on day 1 was 6.3 × 10⁸ c.f.u. ml⁻¹, which was maintained steadily at 1.8 × 10⁸ c.f.u. ml⁻¹ on day 5, whilst a

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**Table 1.** Anti-staphylococcal activity of r-lysostaphin against MRSA isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC₅₀ (μg ml⁻¹)/MBC (μg ml⁻¹)</th>
<th>Disc diffusion zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC₅₀</td>
<td>MIC₉₀</td>
</tr>
<tr>
<td>MRSA (n=66)</td>
<td>0.06/–</td>
<td>0.25/0.5</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 29213</td>
<td>0.02</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. aureus</em> lyso⁻¹</td>
<td>&gt;64</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. aureus</em> mup⁻¹</td>
<td>0.06</td>
<td>NA</td>
</tr>
</tbody>
</table>

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**Fig. 1.** SEM revealed that r-lysostaphin eradicates *S. aureus* biofilm. (a–d) Biofilms (24 h) were exposed to r-lysostaphin at 4, 8 and 16 μg ml⁻¹ (b–d), whilst untreated biofilm served as control (a). Complete eradication of sessile and planktonic cells along with the extracellular matrix was observed in biofilms treated with r-lysostaphin at 8 and 16 μg ml⁻¹ (c, d, respectively), whilst sessile cells were observed in biofilms treated with 4 μg ml⁻¹ (b). The untreated control shows sessile cells in the glycocalyx matrix (a).
Fig. 2. In vivo efficacy of r-lysostaphin topical formulations against MRSA 15187 in the Swiss albino mice dermal infection model. Data presented are normalized from experiments performed three times and the results are shown as mean ± SD. Placebo control (no r-lysostaphin) and r-lysostaphin at 60, 100, 120 or 150 μg g⁻¹ as indicated.

>3 log reduction in bacterial load was observed in the group treated with 120 and 150 μg g⁻¹ cream (Fig. 2).

This study reports the detailed evaluation of the in vitro activity of r-lysostaphin against MRSA isolates obtained from various hospitals across India and 14 epidemic strains, including emerging CA-MRSA. The in vitro activity of r-lysostaphin was found to be comparable with the other recently reported r-lysostaphin (von Eiff et al., 2003; Yang et al., 2007); in addition, it showed better activity than the reference standard lysostaphin (Sigma). Further, the indigenous r-lysostaphin was also found to be potent in eradicating established staphylococcal biofilms, which is in agreement with earlier reports (Walencka et al., 2005). The rapid bactericidal activity and biofilm eradication ability of r-lysostaphin makes it a potential life-saving agent in the case of life-threatening situations arising due to biofilm-associated infection of S. aureus.

r-Lysostaphin has been found to be an effective agent in various in vivo studies. Topical application has been reported to clear nasal colonization and keratitis (Dajcs et al., 2000). It has also been reported to clear infection in a model of systemic infection and endocarditis without any adverse effects (Patron et al., 1999). Similarly, in our model of topical skin infection, it has been found to bring 3 log reductions in bacterial load and sterilized the surface of the skin. Results from our study recommend the topical use of r-lysostaphin on open-wound MRSA infections. The reproducibility of results with r-lysostaphin developed on a commercial scale has now opened the door for developing formulations by skin infections caused by S. aureus.

Acknowledgements

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Ashwani Kumar,¹,² Inshad Ali Khan,¹ Parduman Raj Sharma,² Sumathy K² and Krishna M. Ella²

¹Clinical Microbiology Division, Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India
²R & D Department, Bharat Biotech International Ltd, Genome Valley, Shameerpet Mandal, Hyderabad, India
³Cancer Pharmacology Division, Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India

Correspondence: Inshad Ali Khan
(inshad@rediffmail.com or iakhan@iiim.res.in)


Correspondence


