Rifabutin liposomes as an effective strategy to overcome antibiotic resistance of *Staphylococcus aureus* infections

J.O. Pinho¹, S.N. Pinto², M. Ferreira¹,³, S.I. Aguiar³, M.M. Gaspar¹

1 – Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, University of Lisboa, Portugal
2 – iBB-Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, University of Lisboa, Portugal
3 – Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisboa, Portugal

PRC Grant number MMG-2021-092/1-1

12th September, Heidelberg, Germany
2022
THE DISEASE

*Staphylococcus aureus* infections

- Major public health problem: high morbidity and mortality;
- Vancomycin (VCM) is the gold-standard antibiotic used in clinic;
- Emergence of methicillin- and VCM-resistant strains;
- Biofilm-forming bacteria are difficult to treat;
- Treatment often fails against these infections.

---

Repurposing RFB against *S. aureus* infections

• Belongs to the antibiotic class rifamycins (RFB, rifampin, rifapentine);
• Rifamycins are known for their use against tuberculosis;
• RFB inhibits bacterial DNA-dependent RNA polymerases;
• RFB is clinically used for the treatment of mycobacterial infections.

Molecular formula: $C_{46}H_{62}N_4O_{11}$

$\text{MW} = 847.02$
THE NANOSYSTEM

Liposomes

- Improve the safety profile of loaded RFB;
- Change the biodistribution profile;
- Enhance RFB delivery to infection sites;
- Promote biofilm internalization and in situ release of RFB.

RFB-loaded liposomes as therapeutic strategy against S. aureus infections

Experimental Outline

- Susceptibility of *S. aureus* to antibiotics
- RFB-loaded liposomes
- Planktonic and biofilm interaction studies
- Liposome-biofilm interaction studies
- *In vivo* evaluation of RFB formulations antibacterial effect
- Systemic MRSA infection
- Confocal microscopy
### RESULTS

Susceptibility of *S. aureus* to free antibiotics

<table>
<thead>
<tr>
<th></th>
<th>S. aureus strain</th>
<th>MIC (ng/ml)</th>
<th>MBIC&lt;sub&gt;50&lt;/sub&gt; (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RFB</td>
<td>VCM</td>
</tr>
<tr>
<td>Methicillin-sensitive (ATCC®25923™)</td>
<td>MSSA</td>
<td>3 ± 1</td>
<td>1291</td>
</tr>
<tr>
<td>Methicillin-resistant (Clinical isolates)</td>
<td>MRSA-1</td>
<td>9 ± 4</td>
<td>901</td>
</tr>
<tr>
<td></td>
<td>MRSA-2</td>
<td>12 ± 1</td>
<td>1875</td>
</tr>
</tbody>
</table>

**RFB**: rifabutin  
**VCM**: vancomycin  
**MIC**: minimum inhibitory concentration  
**MBIC<sub>50</sub>**: minimum biofilm inhibitory concentration

RFB showed superior antibacterial activity

**Experimental details:**
Planktonic and biofilm bacteria inoculum were seeded at 0.5×10<sup>6</sup> and 1×10<sup>6</sup> CFU/mL, respectively. MIC was assessed by broth microdilution method followed by turbidity measurement (OD<sub>570nm</sub>). MBIC<sub>50</sub> was determined by the broth microdilution method, performed in mature biofilms (24 h old), followed by MTT assay.
RESULTS

RFB-loaded liposomes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Lipid composition (molar ratio)</th>
<th>Loading capacity (μg/μmol)</th>
<th>I.E. (%)</th>
<th>Ø (nm) (PdI)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFB-LIP1</td>
<td>DMPC:DMPG (80:20)</td>
<td>36 ± 5</td>
<td>51 ± 7</td>
<td>101 (&lt;0.1)</td>
<td>-21 ± 3</td>
</tr>
<tr>
<td>RFB-LIP2</td>
<td>DPPC:DPPG (80:20)</td>
<td>23 ± 2</td>
<td>32 ± 8</td>
<td>118 (&lt;0.1)</td>
<td>-13 ± 2</td>
</tr>
<tr>
<td>RFB-LIP3</td>
<td>DMPC:DMPG:DSPE-PEG (65:30:5)</td>
<td>42 ± 8</td>
<td>55 ± 3</td>
<td>102 (&lt;0.1)</td>
<td>-5 ± 1</td>
</tr>
<tr>
<td>RFB-LIP4</td>
<td>DPPC:DPPG:DSPE-PEG (65:30:5)</td>
<td>39 ± 3</td>
<td>45 ± 7</td>
<td>100 (&lt;0.1)</td>
<td>-4 ± 2</td>
</tr>
</tbody>
</table>

Experimental details:
I.E.(%): incorporation efficiency; Ø: mean size; PdI: polydispersity index; DMPC: dimyristoyl phosphatidyl choline; DMPG: dimyristoyl phosphatidyl glycerol; DSPE-PEG: distearoyl phosphatidyl ethanolamine covalently linked to poly(ethylene glycol)2000; DPPC: dipalmitoyl phosphatidyl choline; DPPG: dipalmitoyl phosphatidyl glycerol.

RFB was successfully loaded in liposomes
RESULTS
Susceptibility of MRSA-1 to RFB formulations

**Experimental details:**
Planktonic and biofilm bacteria inoculum were seeded at 0.5x10^6 and 1x10^6 CFU/mL, respectively.
MIC was assessed by broth microdilution method followed by turbidity measurement (OD_570 nm). MBIC_{50} was determined by the broth microdilution method, performed in mature biofilms (24 h old), followed by MTT assay. Empty liposomes (LIP3 and LIP4) with the corresponding lipid composition were tested at the same lipid concentrations. RFB-LIP3: DMPC:DMPG:DSPE-PEG and RFB-LIP4: DPPC:DPPG:DSPE-PEG.

<table>
<thead>
<tr>
<th>RFB formulation</th>
<th>MIC (ng/mL)</th>
<th>MBIC_{50} (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free RFB</td>
<td>9 ± 4</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>RFB-LIP3</td>
<td>13 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>RFB-LIP4</td>
<td>13 ± 1</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

RFB antibacterial activity was preserved after incorporation in liposomes.
RESULTS
Liposome-biofilm interaction

RFB liposomes were internalized within biofilm, exerting antibacterial effect.

Experimental details:
MRSA-1 biofilms after 4 h incubation with RFB-loaded liposomes labelled with rhodamine (Rho) (RFB-LIP3 and RFB-LIP4). Untreated MRSA biofilm was used as a control. Biofilms were stained with the nucleic acid-binding dye (SYTO 9) (green).
RESULTS
Systemic MRSA-1 murine model

Infection induction (t = day 0)
Systemic MRSA infection induction was performed in male Balb/c mice.

Endpoint (t = day 7)
Mice were sacrificed and collected organs and blood were homogenized and serially diluted for CFU counting.

Growth index was calculated as the difference between the log10 CFU at end of treatment and the log10 CFU at beginning of treatment. RFB-LIP4: DPPC:DPPG:DSPE-PEG. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs Control group.

RFB liposomes exerted the highest antibacterial effect, compared to Control and Free RFB.
CONCLUSIONS

- Superior antibacterial activity of RFB compared with VCM, the gold-standard antibiotic in clinical practice;
- RFB incorporated in long circulating liposomes preserved its antibacterial activity against MRSA;
- RFB liposomes were internalized within biofilm and reduced biofilm formation;
- Preliminary preclinical results confirmed the high antibacterial effect of RFB formulations;
- Compared to control, mice treated with RFB liposomes showed significantly lower bacterial burden in major organs, as demonstrated by the reduced growth index values;

RFB liposomes as promising therapeutic approach against *S. aureus* infections.
ACKNOWLEDGEMENTS

People involved in this work:

M. Manuela Gaspar, PhD  
FFUL

Sandra I. Aguiar, PhD  
CIISA, FMV

Sandra N. Pinto, PhD  
iBB, IST

Jacinta O. Pinho, PhD  
FFUL

Magda Ferreira, MSc  
FFUL/FMV

Financial support:

Phospholipid Research Center Grant number MMG-2021-092/1-1

FCT Contract DL57/2016/CP1438/CT0002

FCT Projects UIDB/04138/2020 and UIDP/04138/2020

University of Lisboa PhD grant BD/2018
Rifabutin liposomes as an effective strategy to overcome antibiotic resistance of *Staphylococcus aureus* infections

J.O. Pinho¹, S.N. Pinto², M. Ferreira¹,³, S.I. Aguiar³, M.M. Gaspar¹

1 – Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, University of Lisboa, Portugal
2 – iBB-Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, University of Lisboa, Portugal
3 – Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisboa, Portugal

PRC Grant number MMG-2021-092/1-1

12th September, Heidelberg, Germany
2022