Workshop - Recent advances in the use of phospholipid excipients

Advancing the treatment of infectious diseases - the key role of liposomes as delivery systems

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CRS 2024 Annual Meeting AND Exposition
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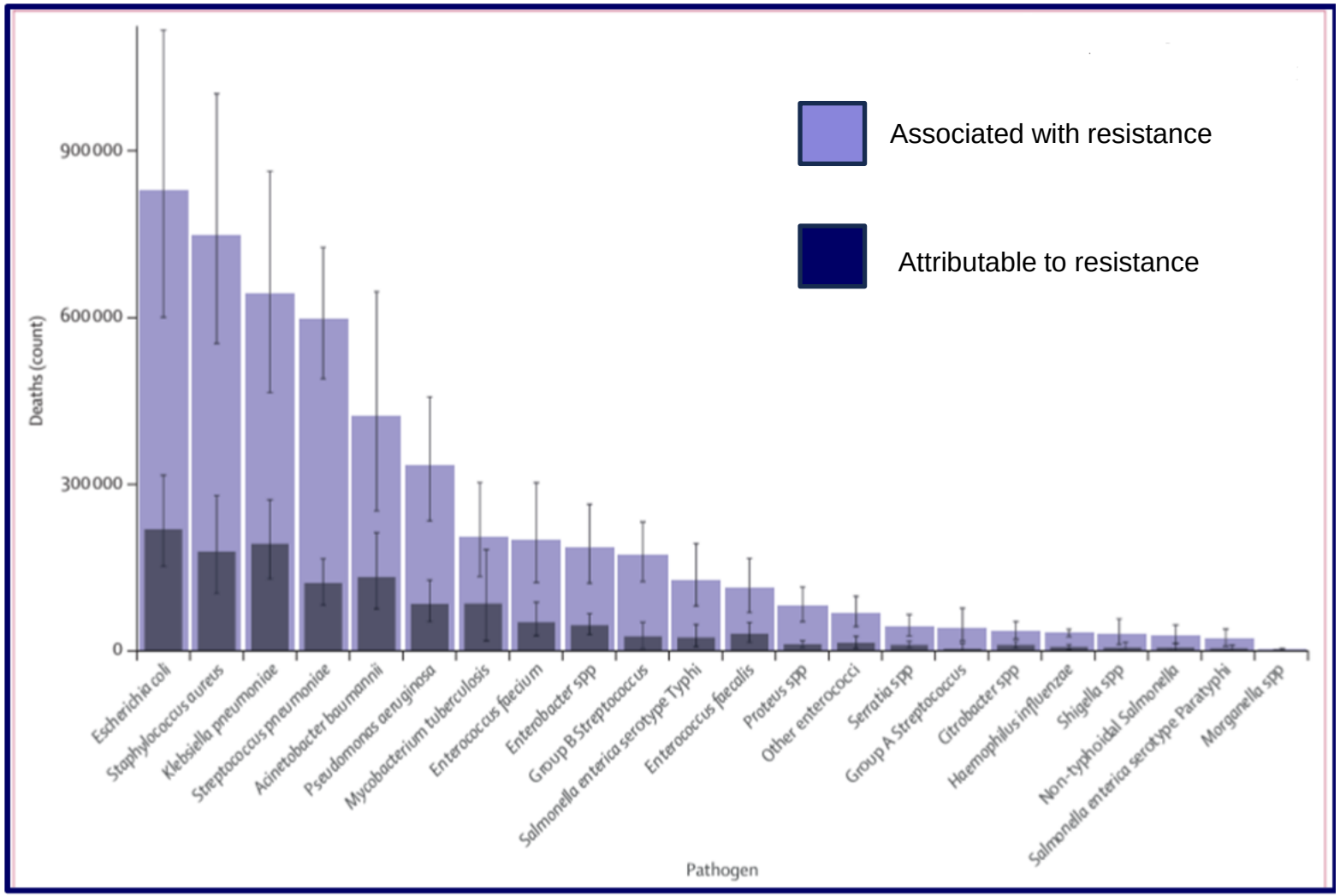
INTEGRATING
Delivery Science
ACROSS DISCIPLINES

Main topics

- ✓ *Infectious diseases worldwide - MRSA*
- ✓ *Drawbacks associated to conventional therapy*
- ✓ *Rifabutin / Drug delivery Systems*
- ✓ *In vitro and in vivo studies*

Worldwide deaths associated with bacterial antimicrobial resistance, 2019



Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis, Antimicrobial resistance collaborators, 2022, https://doi.org/10.1016/S0140-6736(21)02724-0

Staphylococcus aureus

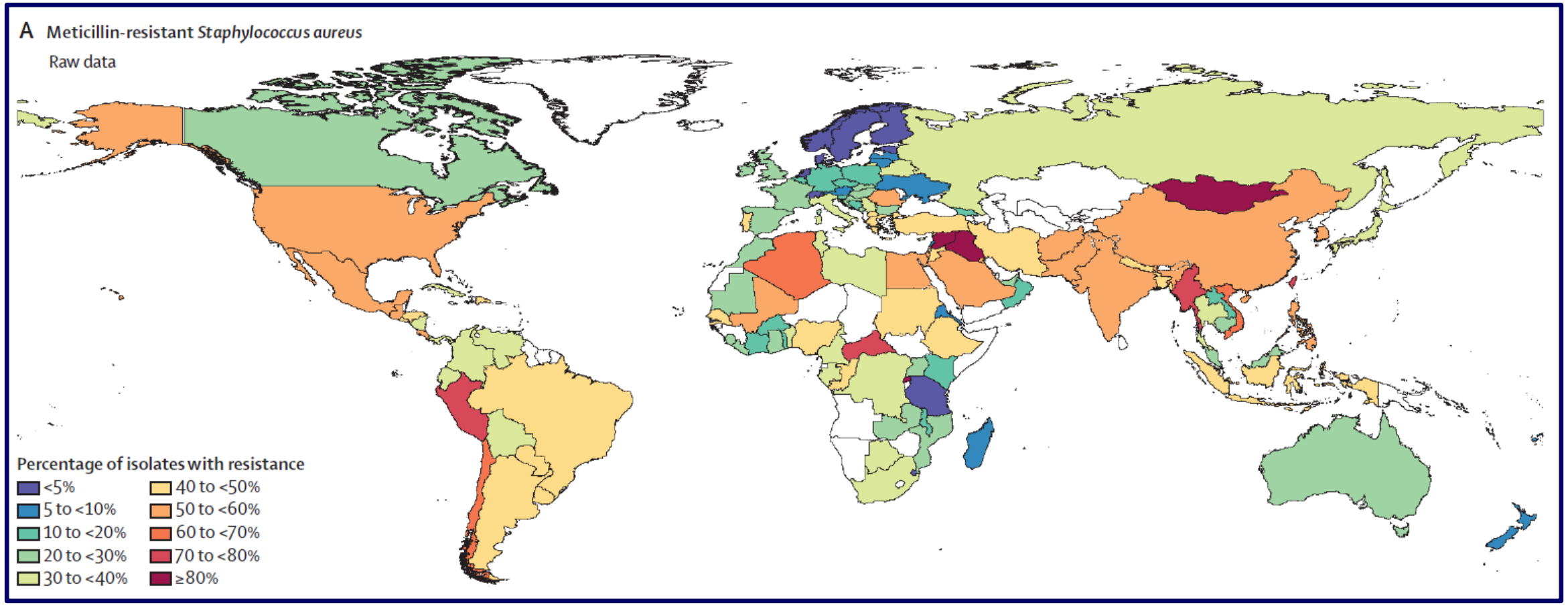
Staphylococcus aureus - a gram-positive bacteria present on skin and in mucous membranes in healthy people (usually harmless).

When the cutaneous and mucosal barriers are disrupted (chronic skin conditions, wounds or surgical interventions, medical devices, venous catheters), *S. aureus* may originate systemic infections: infective endocarditis, osteomyelitis, bacteremia.

Associated with poor clinical outcomes, high rates of morbidity and mortality

Emergence of resistance strains – MRSA – major public health

Percentage of isolates with resistance, 2019



Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis, Antimicrobial resistance collaborators, 2022, https://doi.org/10.1016/S0140-6736(21)02724-0

Management of MRSA infections

Vancomycin (VCM) is the **gold-standard antibiotic** used in clinic (Daptomycin, Linezolid)

Limitations of Conventional Therapy

- Low accumulation at infected sites;
- Prolonged treatments (MRSA *vs* MSSA);
- High doses;
- Severe side effects;
- Emergence of methicillin- and vancomycin-resistant strains.

Alternative therapeutic strategies for the management of MRSA infections

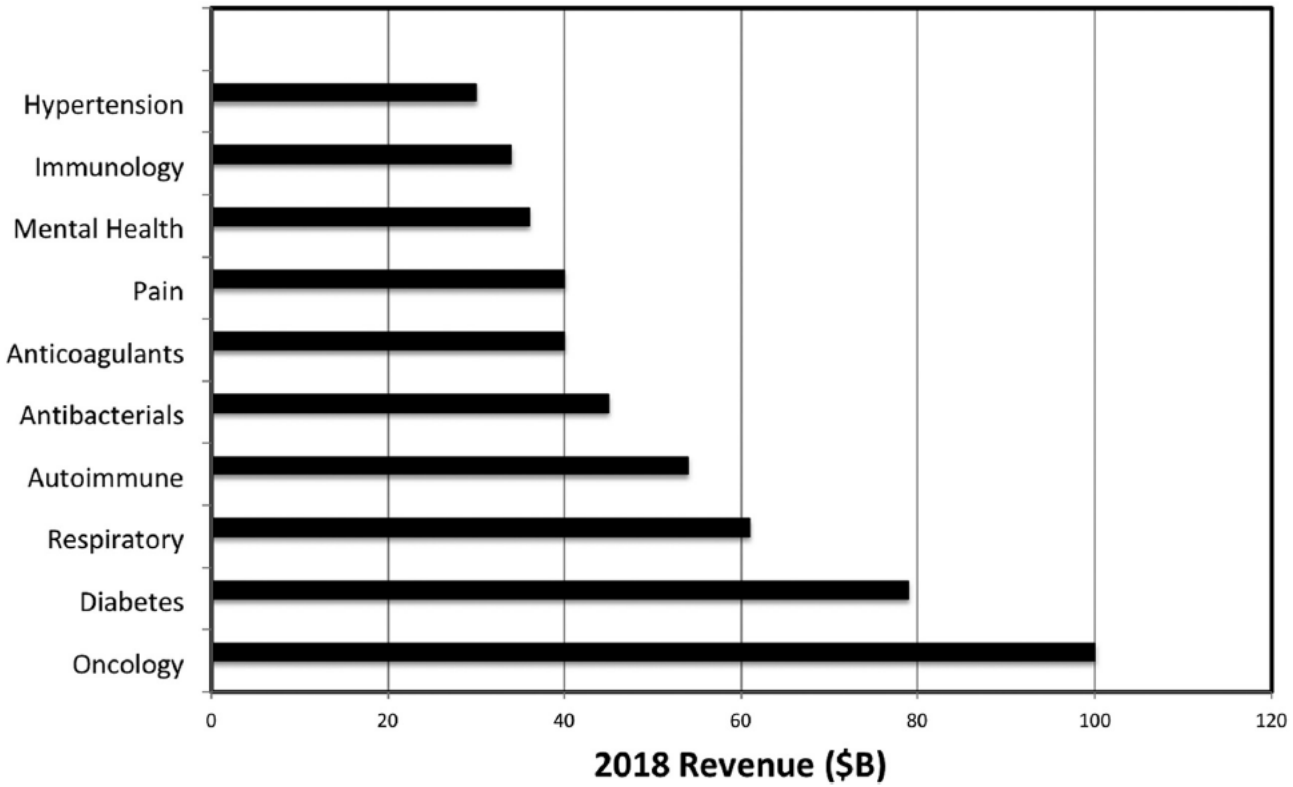
Discovery of new antimicrobial compounds

Expensive and long process;

Low levels of success.

Repurposing existing antimicrobial compounds

Pharmaceutical Therapeutic Area Values



Antibacterial drug market is large, but not as large as markets for oncology, inflammation, and diabetes.

Shlaes DM. 2020. The economic conundrum for antibacterial drugs. Antimicrob Agents Chemother 64:e02057-19.

Rifabutin

- Belongs to the class of rifamycins;
- With a broad spectrum of antimycrobial activity (*M. tuberculosis*, *M. leprae*, *M. avium,* staphyloccocccal invasive infections*);*
- Allows more regimen options for HIV patients (antiretroviral therapies) than RFP;
- **Rifabutin** is as weaker induction of CYP450 enzymes than RFP resulting in reduced drug–drug interactions;
- Higher activity than RFP (strains resistant to RFP).

MW : 847 g/mol

Log P: 4.218

Abad *et al*, 2020, https://doi.org/10.1093/jac/dkaa061

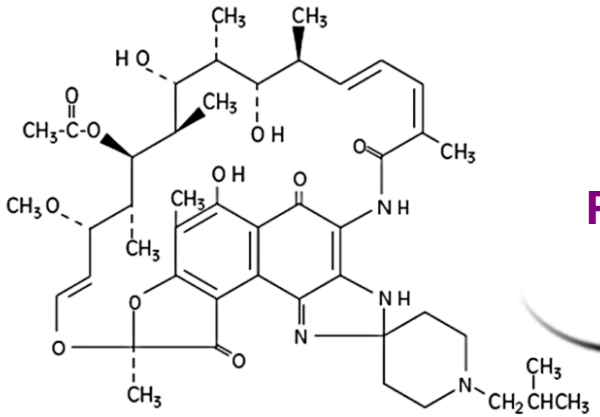
Proposed Strategy

Repurposing an existing antimicrobial compound

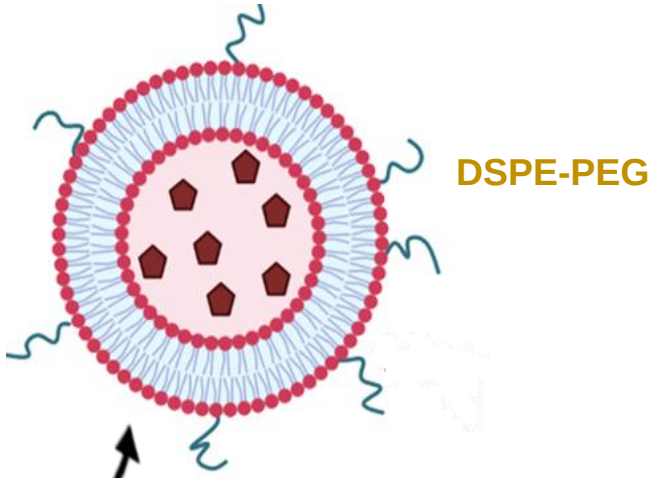
Associate to a nanotechnological platform

Increase antibiotic penetration and accumulation at infected sites

Rifabutin



RFB



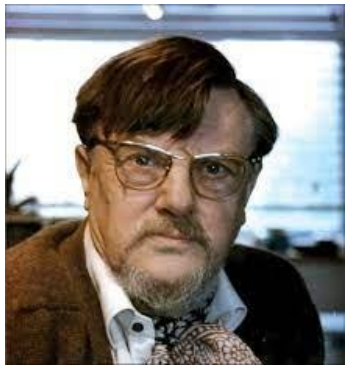
Active loading
Ammonium sulphate gradient

Liposomes as drug delivery systems – several requirements

- High loadings (lipid composition);
- Stability in buffer, physiological media, lyophilized form;
- Cellular internalization in macrophages, *in vitro* activity;
- Accumulation in affected organs after administration;
- High therapeutic activity (devoid of toxic effects).

Since 1970´s liposomes as drug delivery systems have proved to be safe and efficient

Alec Bangham



Observation and discovery of first liposomes

Gregory Gregoriadis

Introduced liposomes as drug delivery systems

Bangham, A. D., and Horne, R.W. (**1964**) Negative staining of phospholipids and their structural modification by surface active agents as observed in the electron microscope. *J. Mol. Biol* **8**, 660–668

Gregoriadis, G. and Ryman, B.E. Fate of protein-containing liposomes Injected into rats: an approach to the treatment of storage diseases. Eur. J. Biochem. **1972**, 24, 485–

pharmaceutics

MDPI

Article

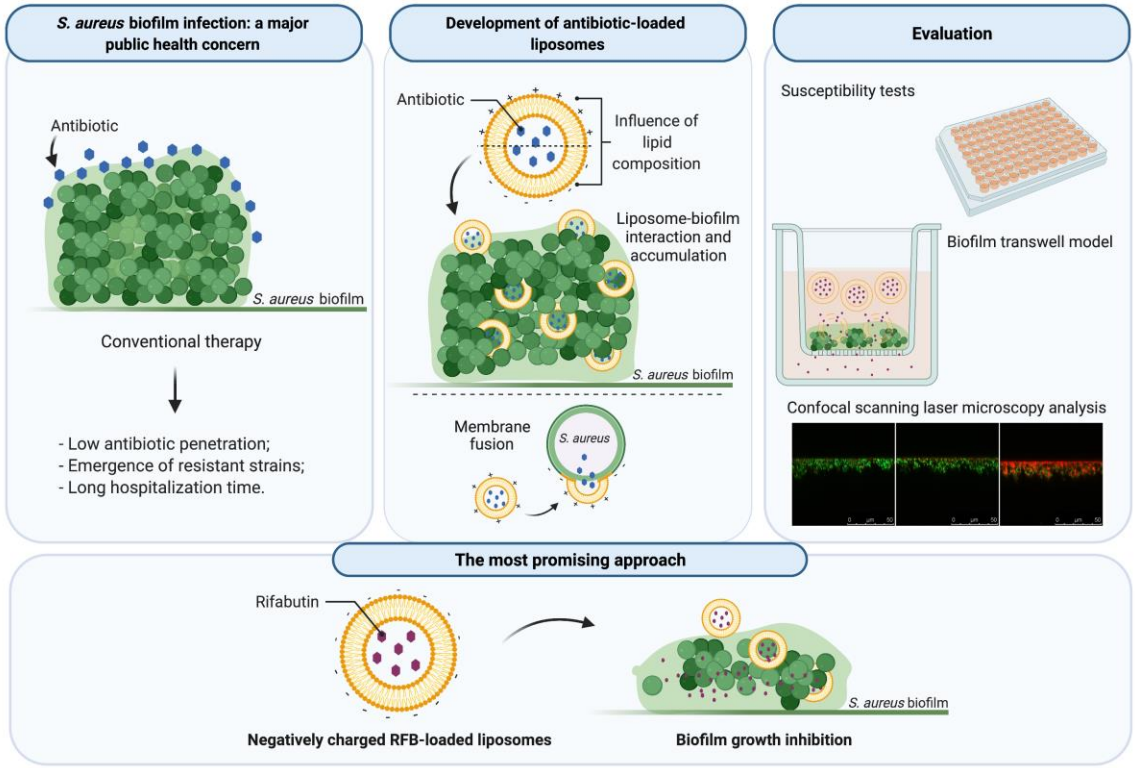
Liposomes as a Nanoplatform to Improve the Delivery of Antibiotics into *Staphylococcus aureus* Biofilms

Magda Ferreira [1,2], Sandra N. Pinto [3], Frederico Aires-da-Silva [1], Ana Bettencourt [2], Sandra I. Aguiar and Maria Manuela Gaspar [2,*]

MSSA strain (RFB, LEV, VCM)

- Validation of superior antibacterial effect of RFB over the other antibiotics (MIC, MBIC).
- Microscopic assessment of the interaction of RFB liposomes within *S. aureus* biofilm.

RFB liposomes <u>without</u> long blood circulating properties



Ferreira et al, 2021, Pharmaceutics, 13, 321

pharmaceuticals

MDPI

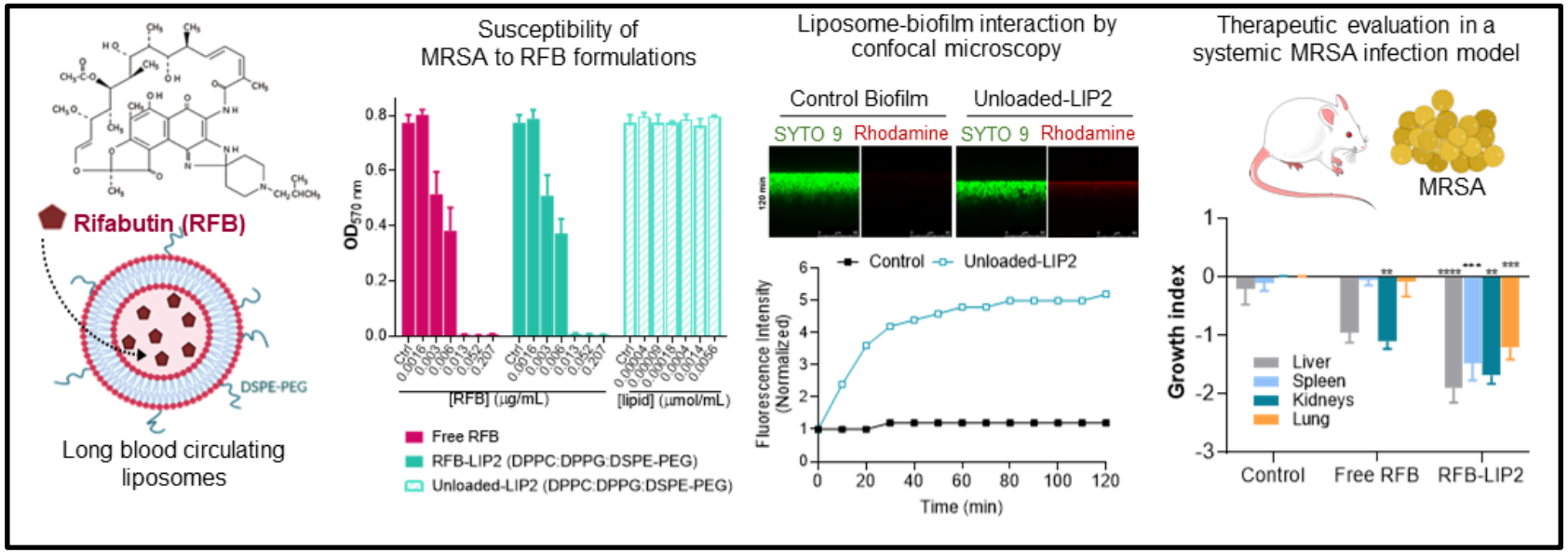
Article

Liposomal Rifabutin—A Promising Antibiotic Repurposing Strategy against Methicillin-Resistant *Staphylococcus aureus* Infections

Jacinta O. Pinho [1], Magda Ferreira [1,2,3], Mariana Coelho [1], Sandra N. Pinto [4], Sandra I. Aguiar [2] and Maria Manuela Gaspar [1,5,*]

MRSA clinical strains

- Antibacterial effect of RFB;
- Microscopic assessment of the interaction of RFB liposomes within *S. aureus* biofilm;
- *In vivo* evaluation in a systemic MRSA infection model.



RFB liposomes <u>with</u> long blood circulating properties

Pinho et al, 2024, Pharmaceuticals, https://doi.org/10.3390/ph17040470

Validate the antibacterial effect of Rifabutin against a commercial MRSA strain

A commercial MRSA strain isolated from blood resistant to gentamicin and methicillin

MRSA-ATCC 33592

RFB loaded liposomes - with and without long blood circulating properties

Formulation	Lipid composition (molar ratio)	(RFB/Lip)f (µg/µmol)	I.E. (%)	Ø (nm) (PdI)	Zeta Pot. (mV)
RFB-LIP1	DMPC:DMPG:DSPE-PEG (65:30:5)	53 ± 5	59 ± 9	108 ± 8 (<0.1)	-5 ± 1
RFB-LIP2	DPPC:DPPG:DSPE-PEG (65:30:5)	47 ± 3	57 ± 7	100 ± 4 (<0.1)	-4 ± 2
RFB-LIP3	DMPC:DMPG (70:30)	56 ± 3	63± 6	108 ± 9 (<0.1)	-24 ± 3
RFB-LIP4	DPPC:DPPG (70:30)	46 ± 6	57 ± 5	116 ± 4 (<0.1)	-23 ± 3

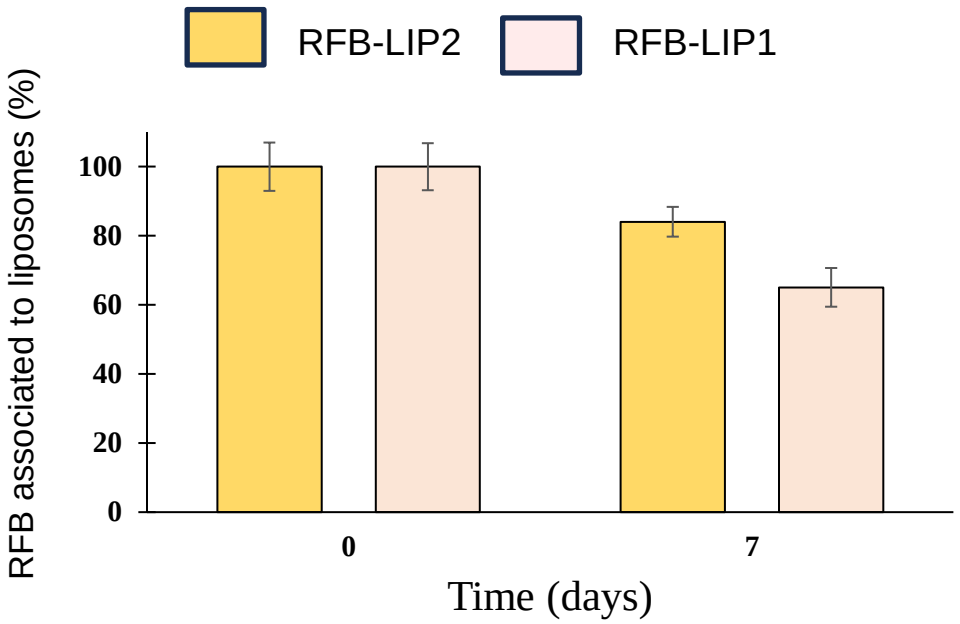
Experimental details:

RFB was loaded in pre-formed empty liposomes following the establishment of an ammonium sulphate gradient between the intraliposomal and extraliposomal media [Gaspar MM, *et al*. 2008. Int. J. Antimicrob. Agents, 31, 37-45].

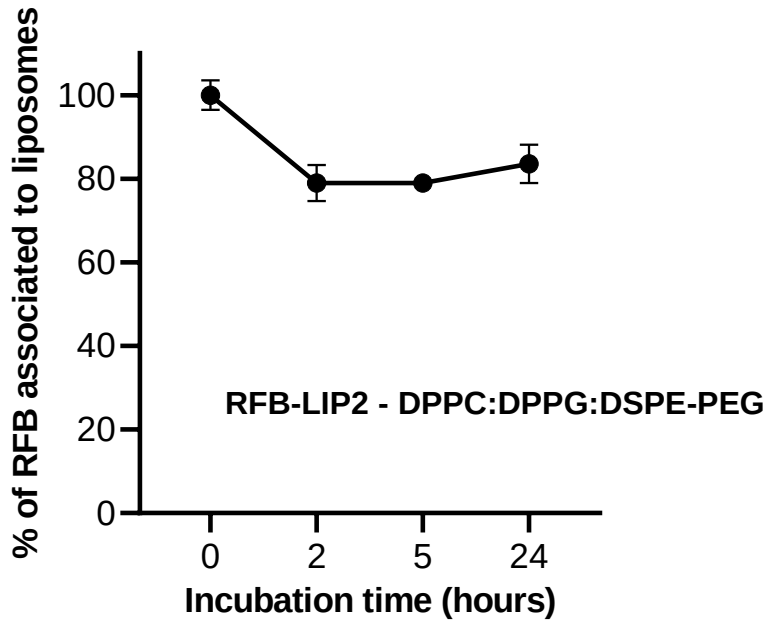
Initial lipid concentration: 30 µmol/mL; Initial RFB concentration: 100 nmol/µmol lipid; I.E.(%): incorporation efficiency; ø: mean size; PdI: polidispersity index; DMPC: dimyristoyl phosphatidyl choline; DMPG: dimyristoyl phosphatidyl glycerol; DSPE-PEG: distearoyl phosphatidyl ethanolamine covalently linked to poly(ethylene glycol)2000; DPPC: dipalmitoyl phosphatidyl choline; DPPG: dipalmitoyl phosphatidyl glycerol.

RFB loaded liposomes with long blood circulating properties

Stability in suspension, 4ºC



Stability in human plasma, 37ºC



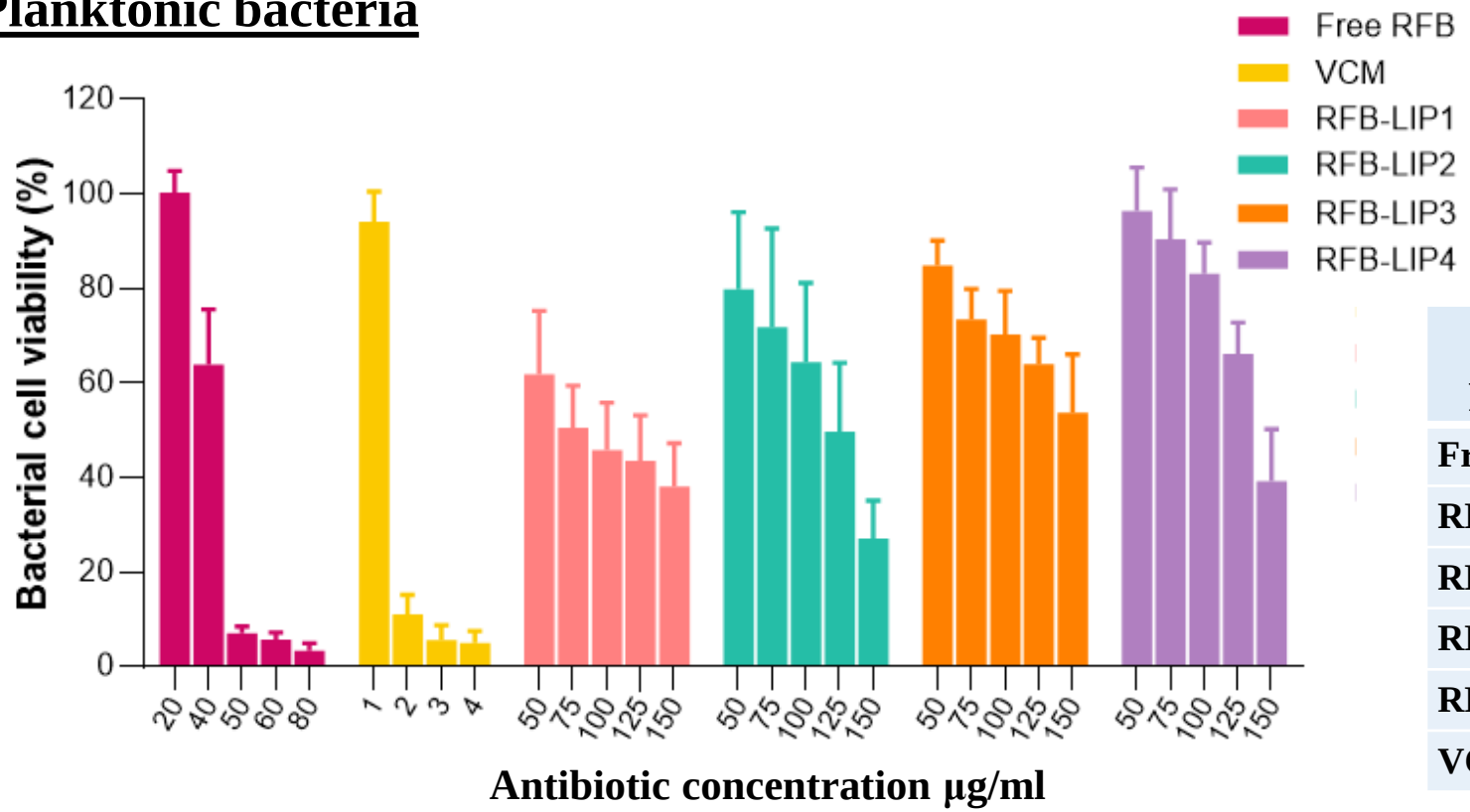
RFB-LIP1 - DMPC:DMPG:DSPE-PEG / RFB-LIP2 - DPPC:DPPG:DSPE-PEG

More rigid phospholipid mixture, **RFB-LIP2, DPPC:DPPG:DSPE-PEG** prooved to **be stable either in suspension (4ºC) or in human plasma (37ºC). Preservation of mean size and surface charge.**

Susceptibility of MRSA-ATCC 33592 to RFB formulations *vs* VCM

A commercial MRSA strain isolated from blood resistant to gentamicin and methicillin.

Planktonic bacteria



Compound / Formulation / Lipid composition	MIC_{50} (µg/ml)
Free RFB	41.5 ± 1.2
RFB-LIP1 (DMPC:DMPG:DSPE-PEG)	69.3 ± 16.9
RFB-LIP2 (DPPC:DPPG:DSPE-PEG)	91.8 ± 10.4
RFB-LIP3 (DMPC:DMPG)	151.1 ± 12.8
RFB-LIP4 (DPPC:DPPG)	138.9 ± 6.9
VCM	1.5 ± 0.1

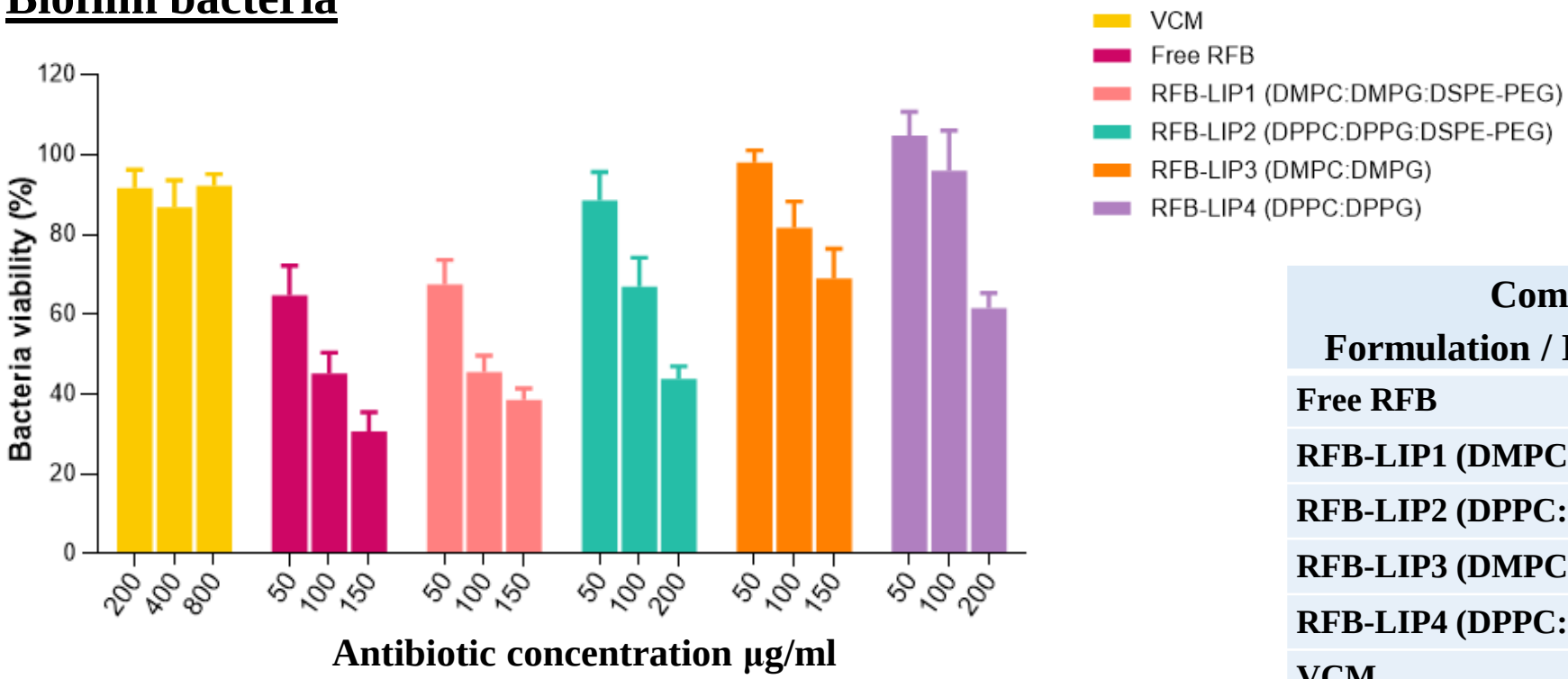
Experimental details:

Planktonic bacteria inoculum was seeded at $5x10^5$ CFU/mL Then samples were added at concentrations under study and incubated for 24 h. MIC_{50} was assessed by MTT assay and defined as the antibiotic concentration that inhibits 50% of bacterial growth related to negative control. Antibiotic concentrations tested: RFB (20 – 150 µg/mL) and VCM (1– 4 µg /mL).

Susceptibility of MRSA-ATCC 33592 to RFB formulations *vs* VCM

A commercial MRSA strain isolated from blood resistant to gentamicin and methicillin.

Biofilm bacteria



Compound / Formulation / Lipid composition	$MBIC_{50}$ (µg/ml)
Free RFB	**80.8 ± 9.8**
RFB-LIP1 (DMPC:DMPG:DSPE-PEG)	**93.5 ± 6.9**
RFB-LIP2 (DPPC:DPPG:DSPE-PEG)	**173.8 ± 10.4**
RFB-LIP3 (DMPC:DMPG)	**> 150**
RFB-LIP4 (DPPC:DPPG)	**> 200**
VCM	**>800**

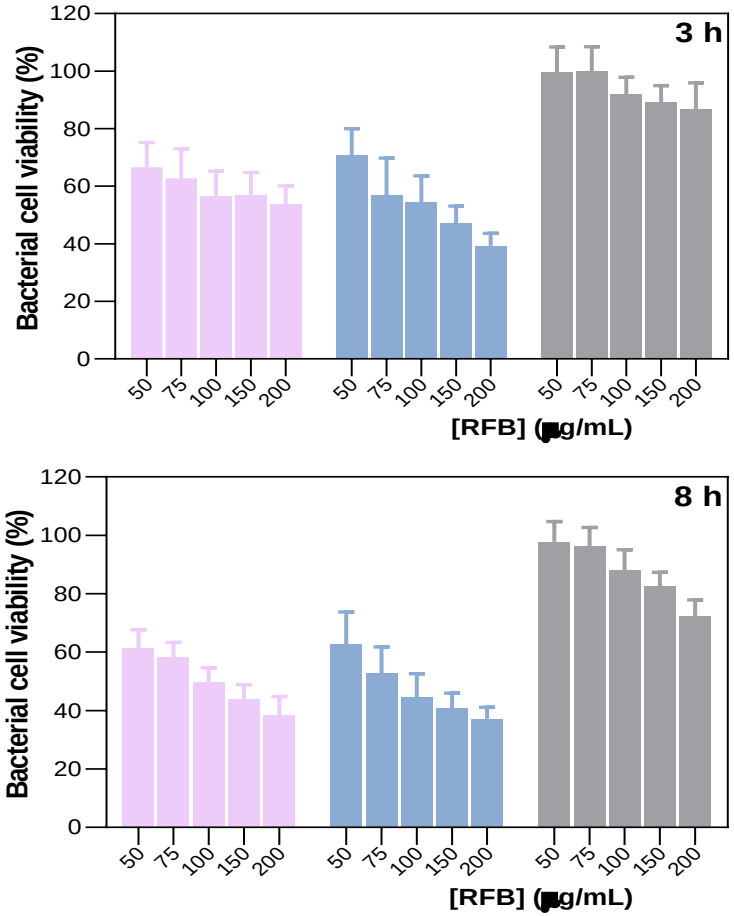
Experimental details:

Biofilm bacteria inoculum were seeded at $5x10^5$ CFU/mL. 24 h after antibiotics were added. Concentrations under study: RFB (50–200 µg /mL) and VCM (200–800 µg /mL). $MBIC_{50}$ was determined by MTT assay being defined as the antibiotic concentration that inhibits 50% of bacterial growth related to negative control.

Susceptibility of MRSA-ATCC 33592 to RFB formulations

A commercial MRSA strain isolated from blood resistant to gentamicin and methicillin.

Biofilm bacteria - Influence of the presence of DSPE-PEG at liposome surface



Free RFB | RFB-LIP2 - DPPC:DPPG:DSPE-PEG | RFB-LIP4 - DPPC:DPPG

$MBIC_{50}$ (μg/mL) 3 and 8 h after incubation with RFB formulations

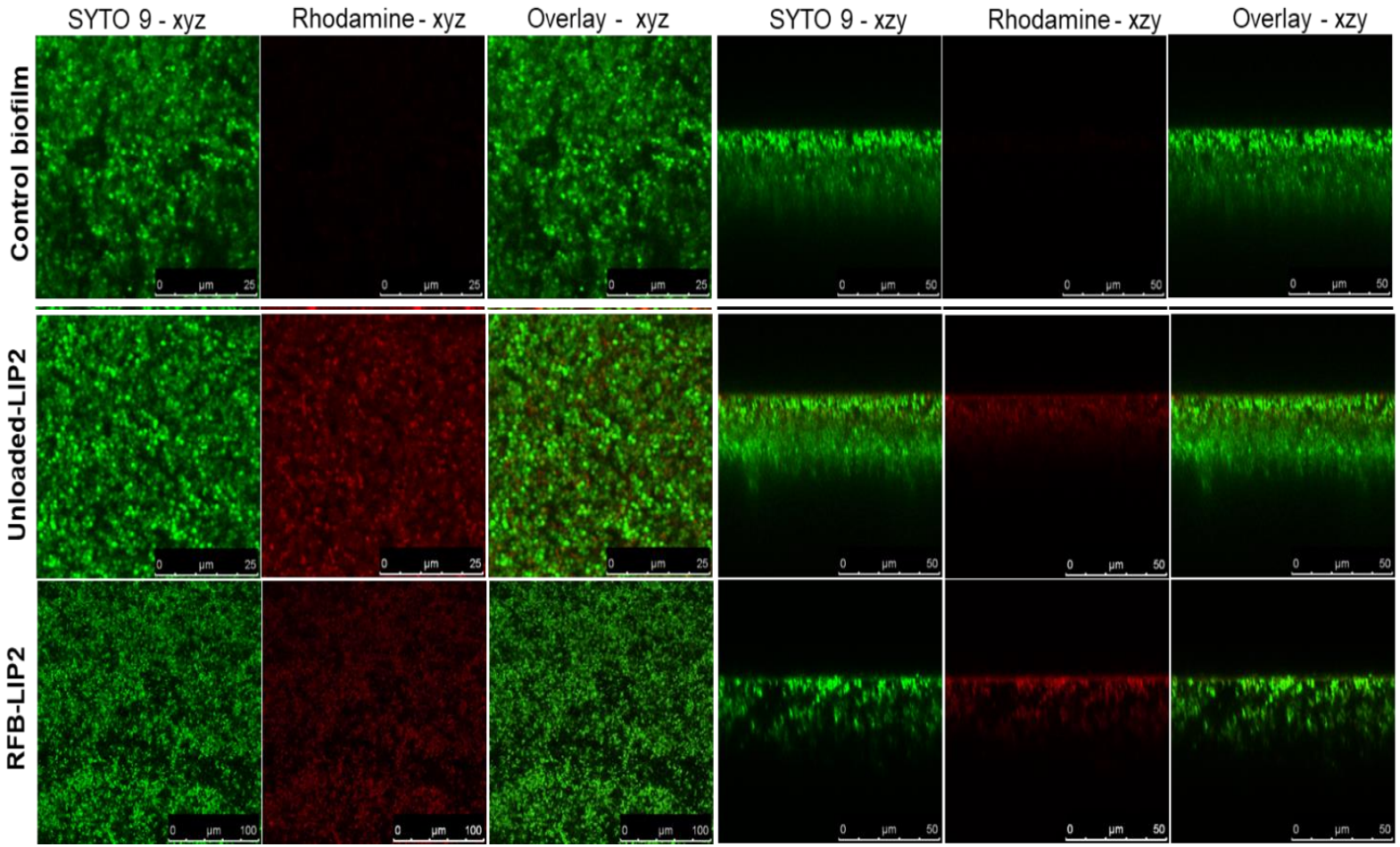
	3h	8h
Free RFB	>200	106 ± 11
RFB-LIP2 - DPPC:DPPG:DSPE-PEG	135 ± 9	97 ± 14
RFB-LIP4 - DPPC:DPPG	>200	>200

The presence of **DSPE-PEG** at liposome surface enhances the **interaction with bacteria cell wall, pegylated RFB nanoformulation** exhibited **higher anti-biofim** effect than **non pegylated nanoformulation.**

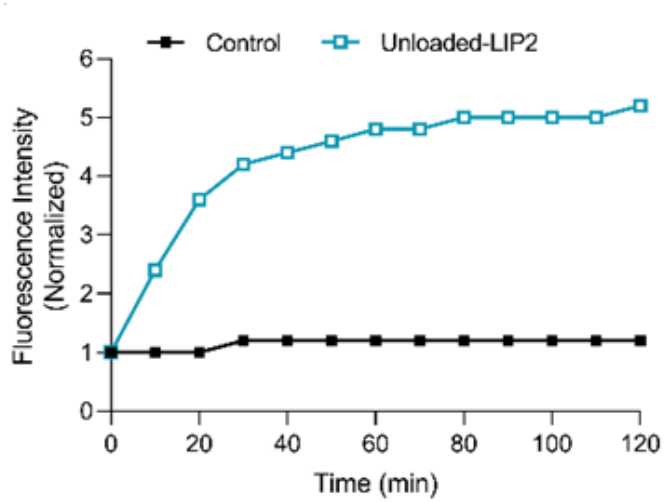
Experimental details: Biofilm bacteria inoculum were seeded at $5x10^5$ CFU/mL. 24 h after antibiotics were added. Concentrations under study: RFB (50–200 μg/mL). Bacterial cell viability was evaluated 3, 5 and 8 h after RFB formulations added by MTT assay.

Interaction of liposomes with *S. aureus* biofilm by microscopy

24 h incubation with rhodamine-labelled liposomes



Unloaded LIP2 rhodamine-labelled liposomes

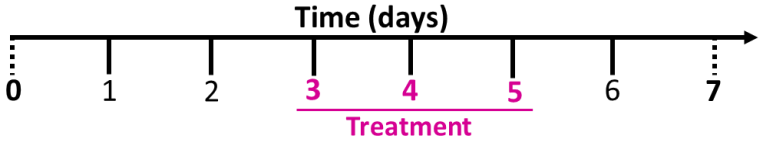


Liposomes internalization within the biofilm structure - monitored during 120 min and the fluorescence intensity was normalized in relation to time 0 - a rapid event (a sharp increase in fluorescence intensity in the first 30 min, followed by a steady phase up to 120 min).

Representative images of mature MRSA biofilms after 24 h incubation with rhodamine-labelled liposomes Lipid concentration of 1.5 µmol/mL. Biofilms were stained with the green dye SYTO 9 at 3 µM. Untreated biofilm - Control biofilm. Left panels - xyz plane, right panels - xzy orthogonal plane. Lipid composition: DPPC:DPPG:DSPE-PEG (RFB-LIP2 and Unloaded-LIP2).

Antibacterial effect in a systemic MRSA murine model of infection

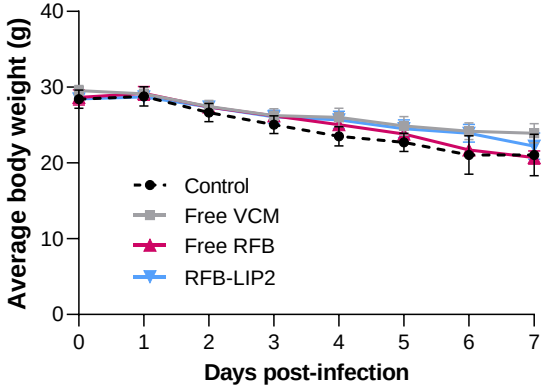
Infection induction (t = day 0)
Systemic MRSA infection induction was performed in male Balb/c mice. Animals received an i.v. injection of **MRSA-ATCC 33592 1x10^7 CFU/mouse**



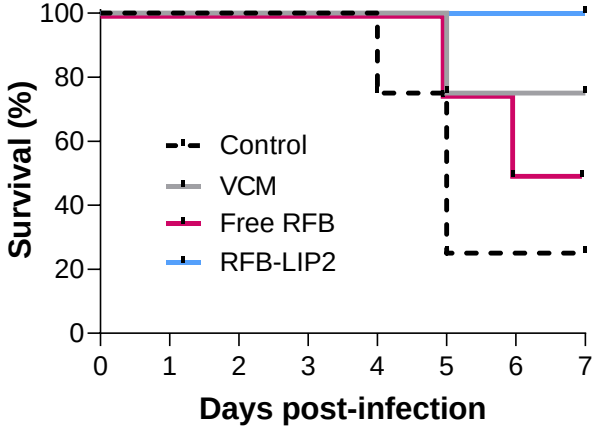
Mice received 3 i.v. injections of VCM and RFB formulations (Free RFB, RFB-LIP2) at 40 and 20 mg/kg body weight, respectively.

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

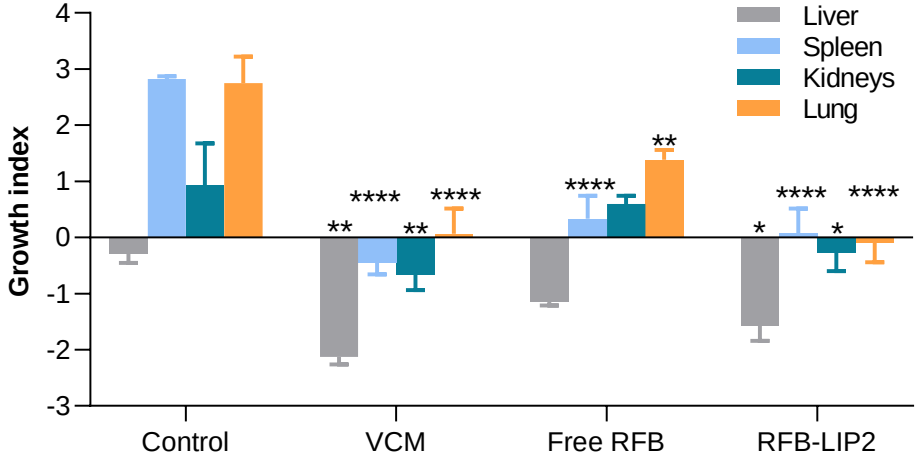
Average Body Weight



Survival



Therapeutic Effect (Growth Index)

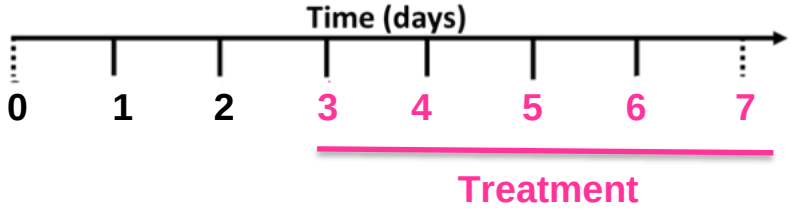


RFB liposomes exerted the highest antibacterial effect, with 100% survival

RFB-LIP2: DPPC:DPPG:DSPE-PEG.
Results are expressed as mean ± SEM (n≥4). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 *versus* Control group.
Growth Index was determined as the difference between the log_{10}CFU at the end of the protocol and the log_{10}CFU at the beginning of treatment

Antibacterial effect in a systemic MRSA murine model of infection

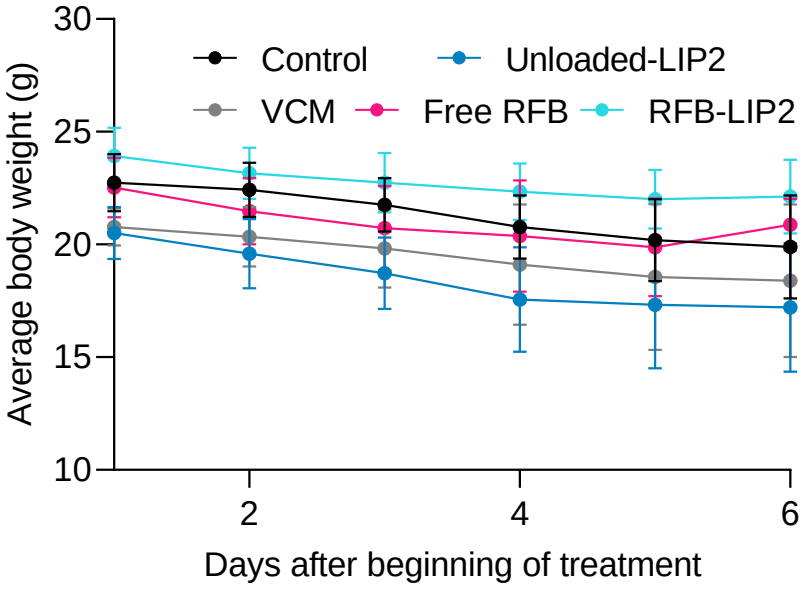
Infection induction (t = day 0)
Systemic MRSA infection induction was performed in male Balb/c mice. Animals received an i.v. injection of **MRSA-ATCC 33592 5x10^6 CFU/mouse**



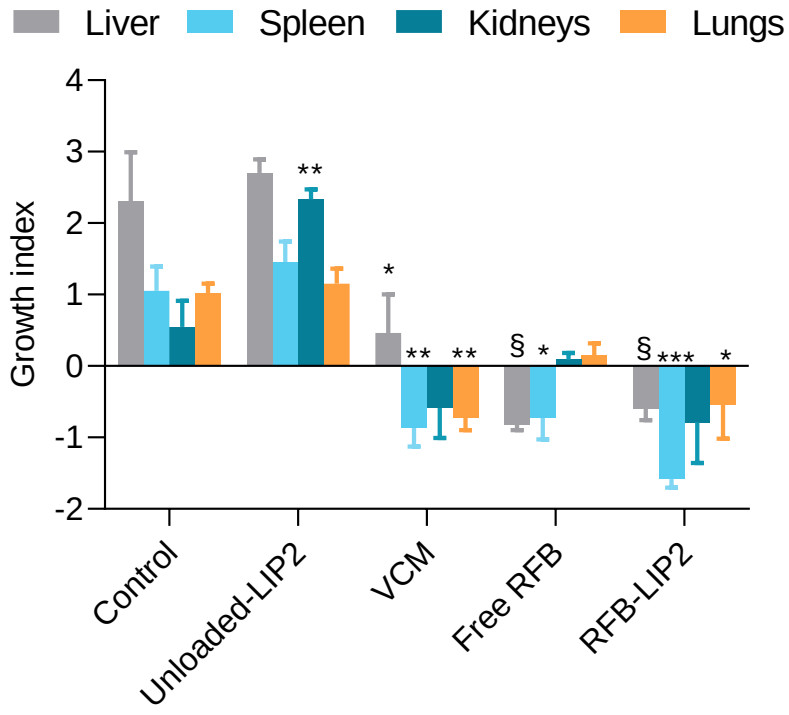
Endpoint (t = day 8)
Mice were sacrificed and collected organs were homogenized and serially diluted for CFU counting.

Mice received 5 i.v. injections of VCM and RFB formulations (Free RFB, RFB-LIP2) at 20 mg/kg body weight, respectively.

Average Body Weight



Therapeutic Effect (Growth Index)



RFB liposomes exerted the highest antibacterial effect

RFB-LIP2: DPPC:DPPG:DSPE-PEG. Growth Index was determined as the difference between the log_{10}CFU at the end of the protocol and the log_{10}CFU at the beginning of treatment

Conclusions

- ✓ *In vitro* and *in vivo* studies were performed using a high virulent commercial MRSA strain (isolated from blood and resistant to gentamicin and methicillin);
- ✓ **RFB**, the repurposed antibiotic, **exhibited therapeutic potential against MRSA infections**: the antibacterial activity of RFB against biofilms of commercial MRSA strain ATCC®33592™ was superior to VCM, the gold-standard antibiotic in clinical use;
- ✓ RFB incorporated in long blood circulating liposomes preserved its antibacterial activity against MRSA;
- ✓ In two systemic MRSA murine models of infection, **RFB formulations** showed superior antibacterial effect compared to VCM resulting in a **100% survival** ;
- ✓ Ongoing work: labelling of RFB with isotopes to further perform biodistribution studies.

The team

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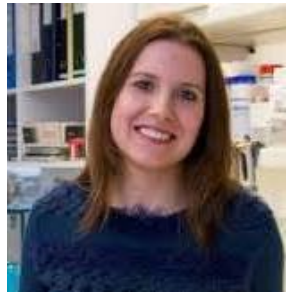



Phospholipid Research Center

PRC awardee

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