A novel synthetic antimicrobial peptide for the cure of Gram-negative infections. Mechanism of action, efficacy in vivo, toxicity, biodistribution and resistance selection

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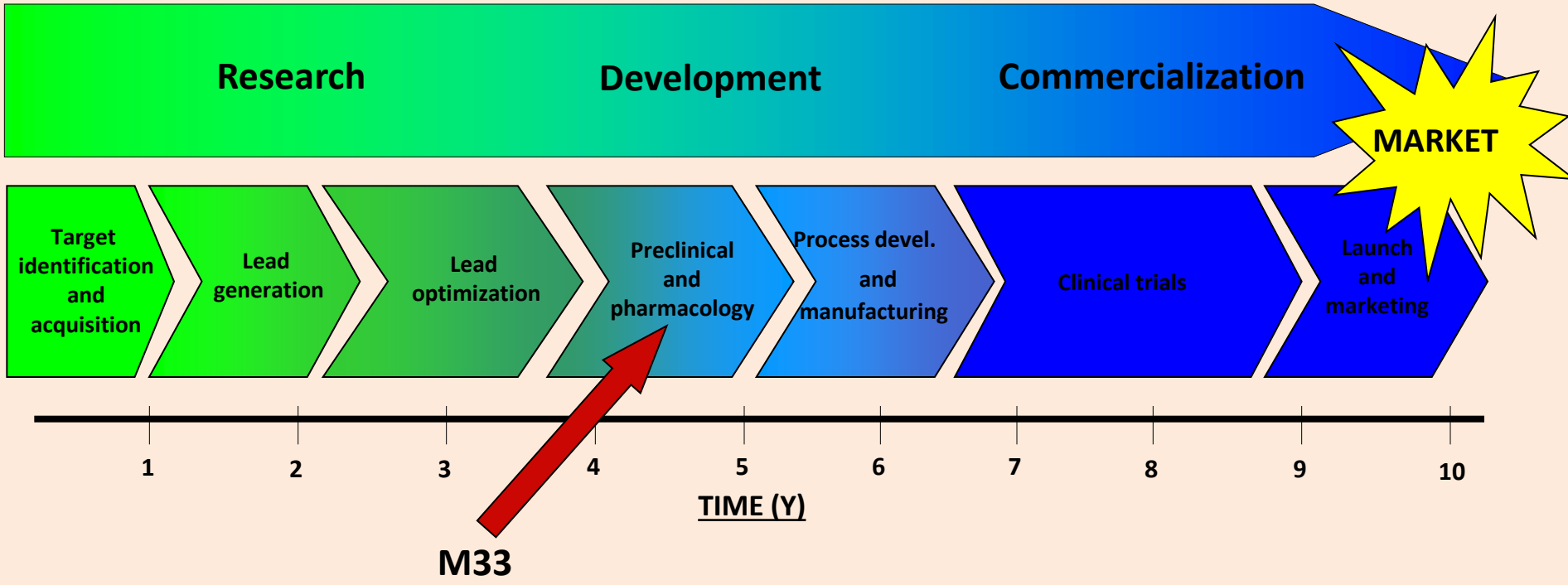
The new AMP M33

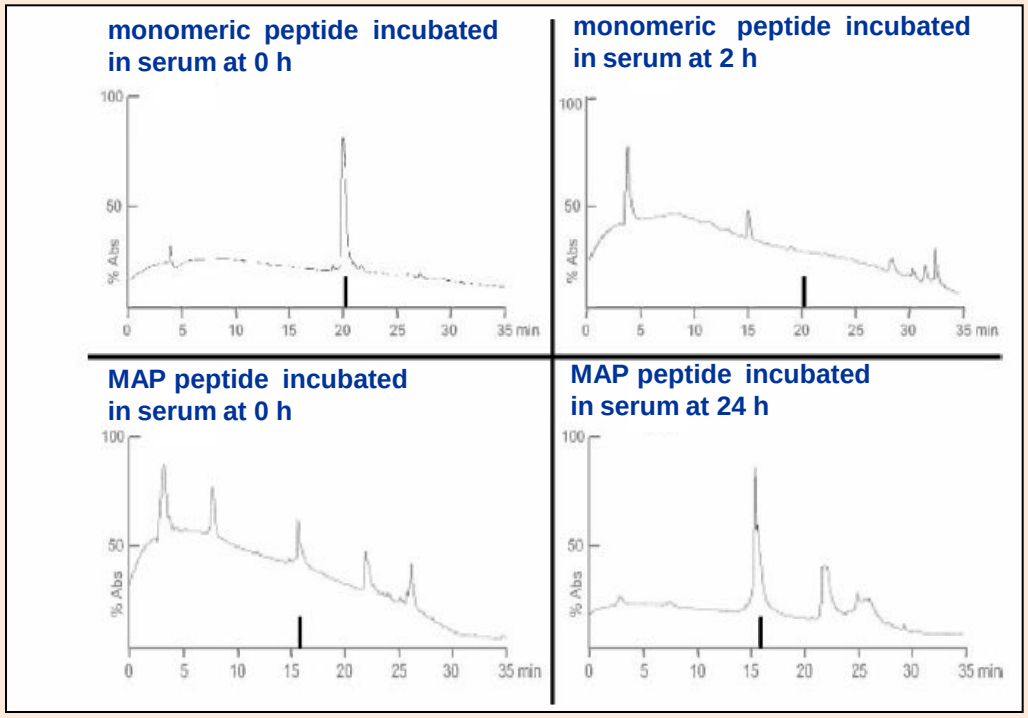
M33 is a novel <u>non natural</u> antimicrobial peptide discovered at the University of Siena. It is under development for the set up of a new antibacterial drug.

PIPELINE OF DRUG DEVELOPMENT FROM LAB TO MARKET



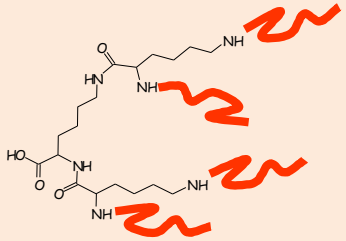
Technology

Tetrabranched (MAP) peptides acquire high resistance to protease activity making these molecules good candidates for in vivo use.



HPLC profiles of monomeric and tetrabranched peptides incubated in serum

Monomeric peptide



Tetrabranched peptide

Bracci et al., 2002, Biochemistry-US

Bracci et al., 2003, J Biol Chem

Lozzi et al., 2003, Chem Biol

Pini et al., 2005, Antimicrob Agents Chemother

Pini et al., 2006, Biochem J

Falciani et al., 2007, Mol Cancer Therapeut

Falciani et al., 2007, Chem Biol Drug Des

Pini et al., 2007, J Pept Sci

Pini et al., 2008, Cur Prot Pept Sci

Falciani et al., 2009, Exp Opin Biol Ther

Pini et al., 2010, FASEB J

Falciani et al., 2010, Curr Cancer Drug Targets

Falciani et al., 2010, ChemMedChem

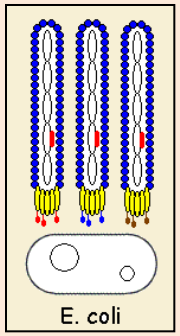
Falciani et al., 2011, ChemMedChem

Pini et al., 2012, AminoAcids

Falciani et al., 2012, PLOS One

Falciani et al., 2013, J Med Chem

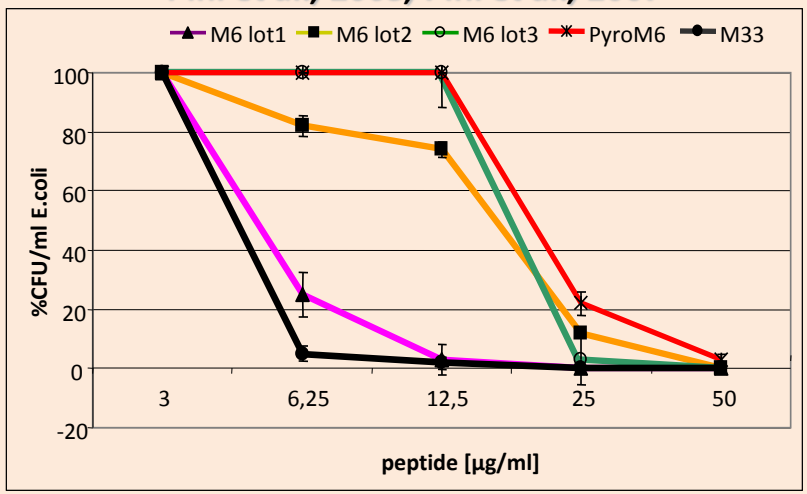
Identification and optimization of new antimicrobial peptides



phage library selection

QKKIRVRLSA (M6)

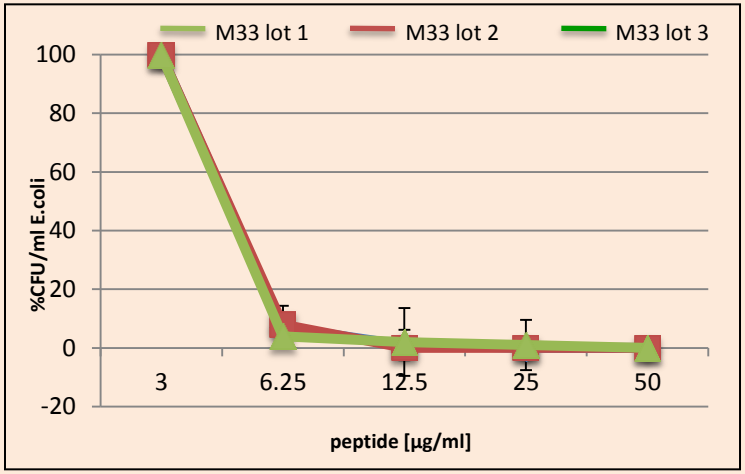
Pini et al., 2005; Pini et al., 2007



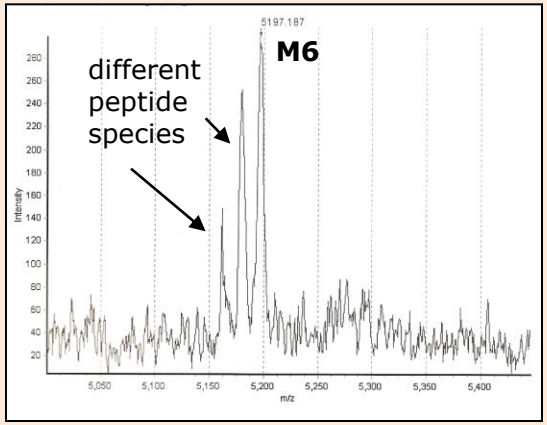
Antimicrobial activity

KKIRVRLSA (M33)

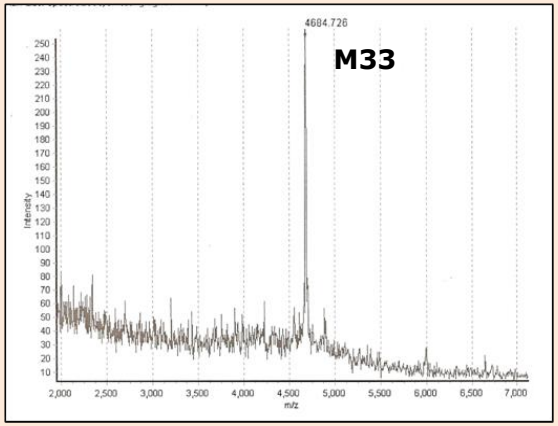
Pini et al., 2010; Pini et al., 2012



Antimicrobial activity



HPLC profile



HPLC profile

Antimicrobial activity

Pini et al., 2010, FASEB J; Pini et al., 2012, AminoAcids

MICs (μM) of M33 in comparison with polymyxin B against bacterial strains representative of several pathogenic species, including MDR strains of clinical origin

Species and strains	Resistance[a]	MIC (μM)	
		M33	Polymyxin B
Pseudomonas aeruginosa ATCC 27853	Reference strain, wild type	1.5	1.5
P. aeruginosa PAO-1	Reference strain, wild type	1.5	1.5
P. aeruginosa VR-143/97	FQ^r AG^r ESC^r NEM^r (MBL/VIM-1)	1.5	1.5
P. aeruginosa SC-MDr03-06[b]	FQ^r AG^r ESC^r NEM^r	3	1.5
P. aeruginosa SC-VMr04-05[b]	FQ^r AG^r ESC^r NEM^r	3	1.5
P. aeruginosa SC-DMr05-04[b]	FQ^r AG^r ESC^r NEM^r	1.5	1.5
P. aeruginosa SC-BGr12-02[b]	FQ^r AG^r ESC^r NEM^r	1.5	1.5
P. aeruginosa EF-OBG6-1[b]	FQ^r AG^r ESC^r NEM^r (MBL/IMP-13)	1.5	0.7
P. aeruginosa SC-MDm03-02[b,c]	FQ^r AG^r ESC^r NEM^r	3	1.5
P. aeruginosa SC-GMm03-05[b,c]	FQ^r AG^r ESC^r NEM^r	1.5	1.5
P. aeruginosa SC-CNm03-07[b,c]	FQ^r AG^r ESC^r NEM^r	0.3	0.7
Klebsiella pneumoniae ATCC 13833	Reference strain, wild type	1.5	0.7
K. pneumoniae 7086042	FQ^r AG^r ESC^r NEM^r (MBL/VIM-1)	3	1.5
K. pneumoniae C8-27	FQ^r AG^r ESC^r ETP^r (ESBL/CTX-M-15)	1.5	0.7
K. pneumoniae FIPP-1	FQ^r AG^r ESC^r NEM^r (MBL/KPC-3)	3	1.5
Escherichia coli ATCC 25922	Reference strain, wild type	1.5	0.7
E. coli W03BG0025	FQ^r AG^r ESC^r (ESBL/CTX-M-15)	0.7	0.7
Enterobacter aerogenes W03BG0067	AG^r ESC^r (ESBL/SHV-5)	1.5	0.7
Enterobacter cloacae W03AN0041	ESC^r (ESBL/SHV-12)	1.5	0.7
Acinetobacter baumannii RUH 134	Reference strain, European clone II	1.5	1.5
A. baumannii RUH 875	Reference strain, European clone I	3	1.5
A. baumannii MR157	FQ^r AG^r ESC^r NEM^r (OXA/OXA-58)	3	1.5
Staphylococcus aureus ATCC 29213	Reference strain, PEN^r	6	96
S. aureus 3851	MR VAN^i	6	96

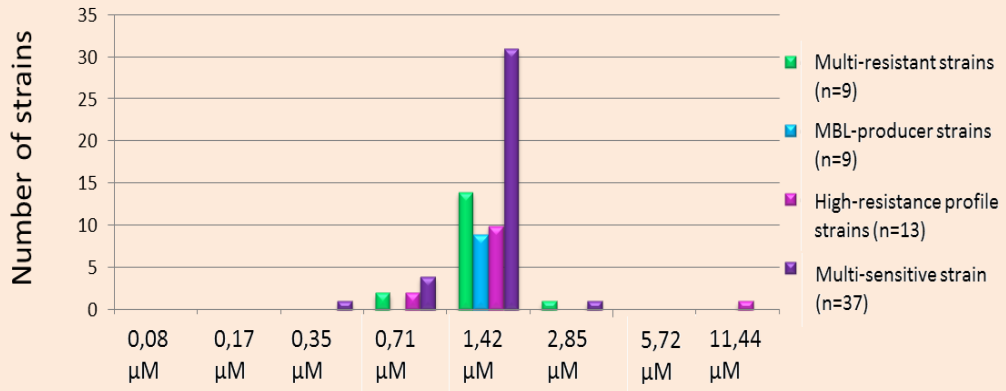
***a*Tested strains included either reference strains (indicated) or clinical isolates (mostly showing an MDR phenotype); relevant resistance traits and resistance mechanisms are indicated:_FQr, resistant to fluoroquinolones; AGr, resistant to aminoglycosides (gentamicin, amikacin, and/or tobramycin); ESCr, resistant to expanded-spectrum cephalosporins; NEMr, resistance to carbapenems (imipenem and/or meropenem), ETPr resistance to ertapenem; ESBL, extended spectrum β-lactamase; MBL, metallo-β-lactamase; OXA, oxacillinase; MR methicillin-resistant; PENr resistance to penicillin; VANi, vancomycin-intermediate;**

***b*Clinical isolates from Cystic Fibrosis patients**

***c*Mucoid phenotype**

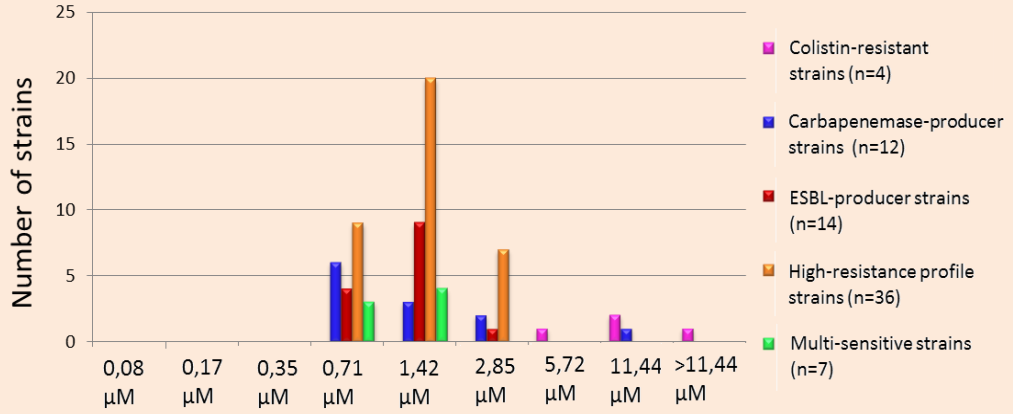
MIC 50 and 90 for *P. aeruginosa* and *K. pneumoniae*

Specie batterica	MIC 50	MIC 90
Pseudomonas aeruginosa, 76 strains	1,4 µM	1,4 µM
Klebsiella pneumoniae, 73 strains	1,4 µM	2,8 µM



MIC value distribution with correlation to ***P. aeruginosa*** bacterial resistance profile.

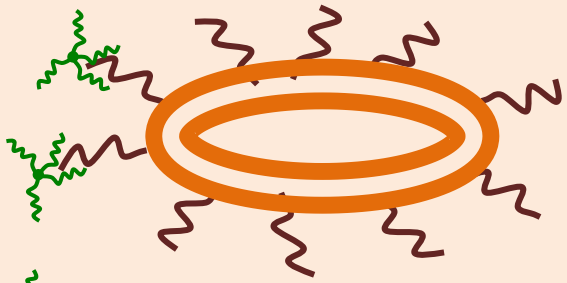
MIC value distribution with correlation to ***K. pneumoniae*** bacterial resistance profile.



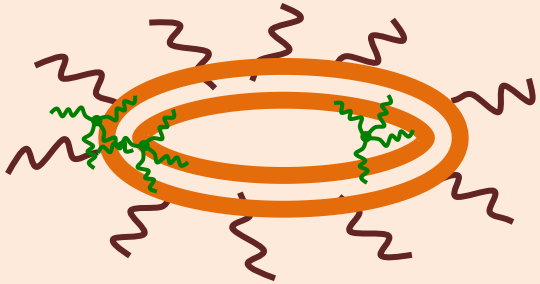
Mechanism of action

TWO STEPS MECHANISM

1- LPS and LTA recognition and binding

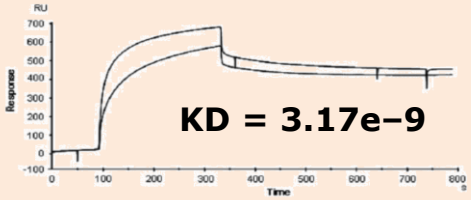


2 – Bacteria membrane is crossed and impaired



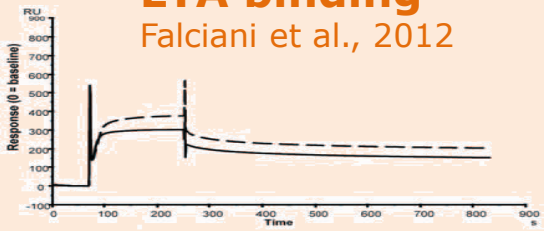
LPS binding

Pini et al., 2007



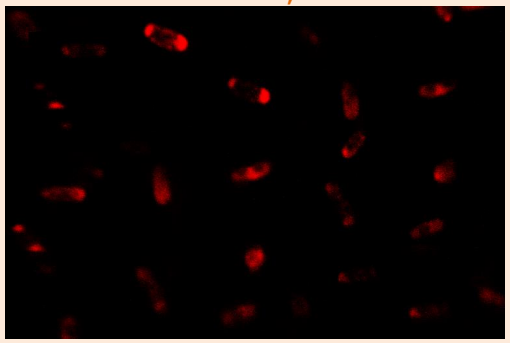
LTA binding

Falciani et al., 2012



Confocal laser microscopy

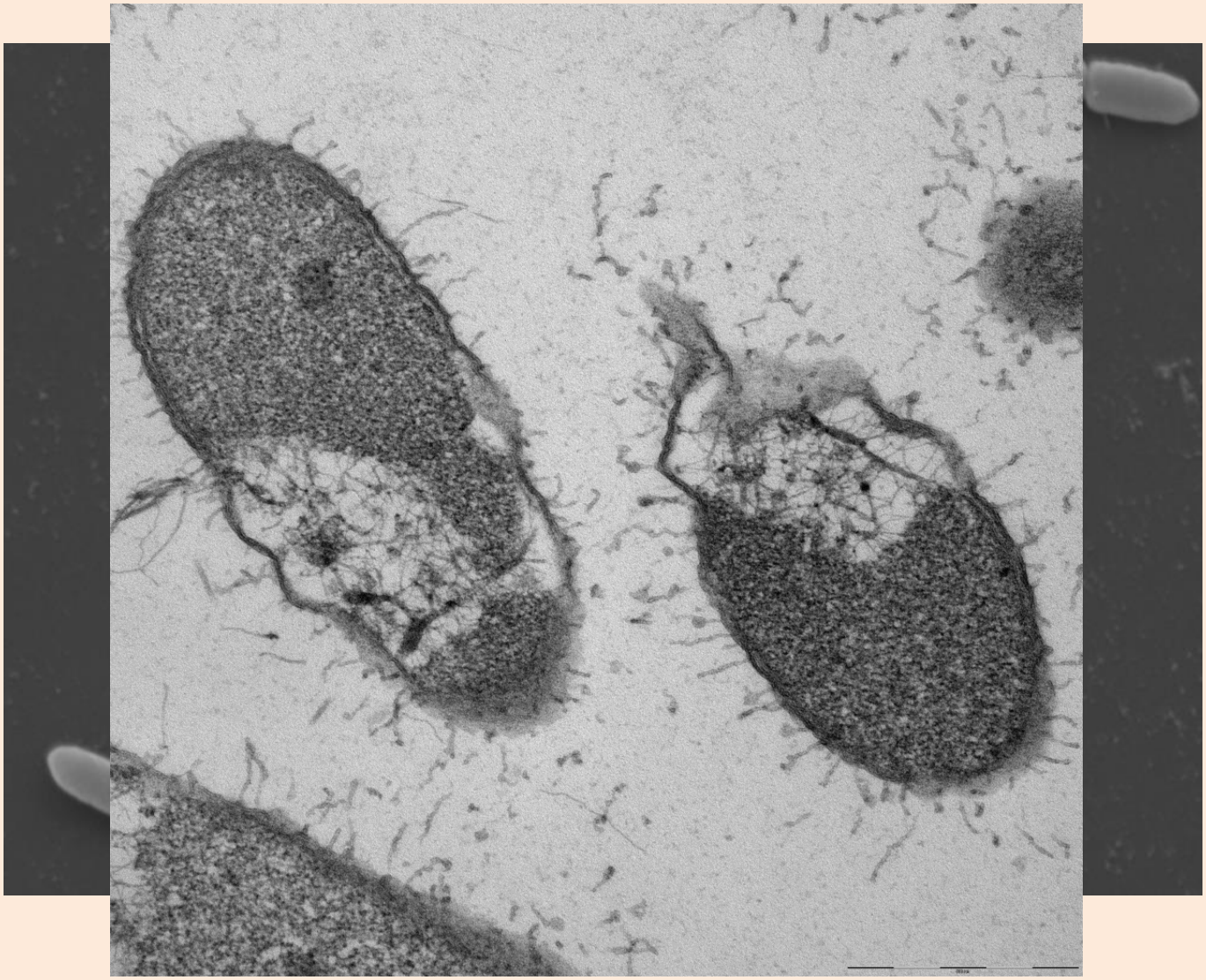
Pini et al., 2007



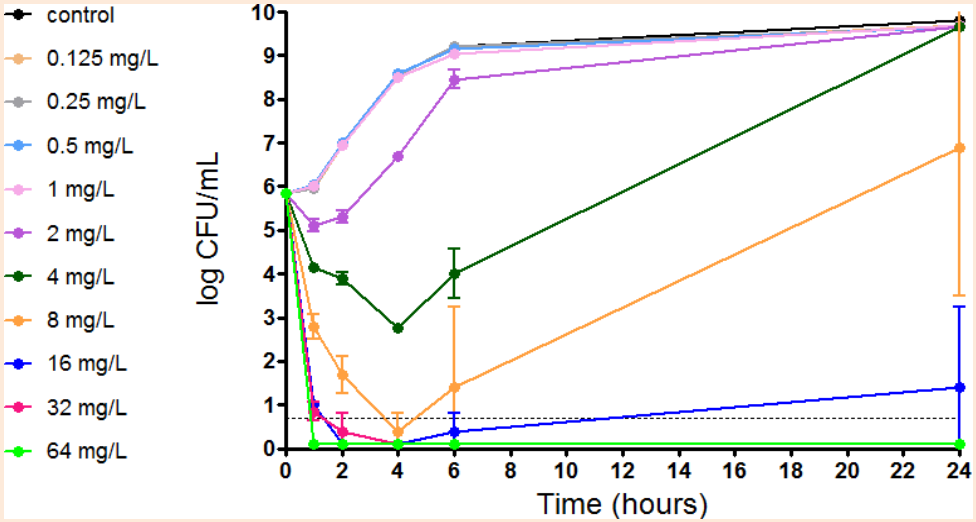
CLSM experiments showed that rhodamine-labelled M33 is able to enter the cells within 5 minutes from incubation

Transmission Electron Microscopy

***Pseudomonas aeruginosa* incubate with M33 1.5 µM (MIC value)**

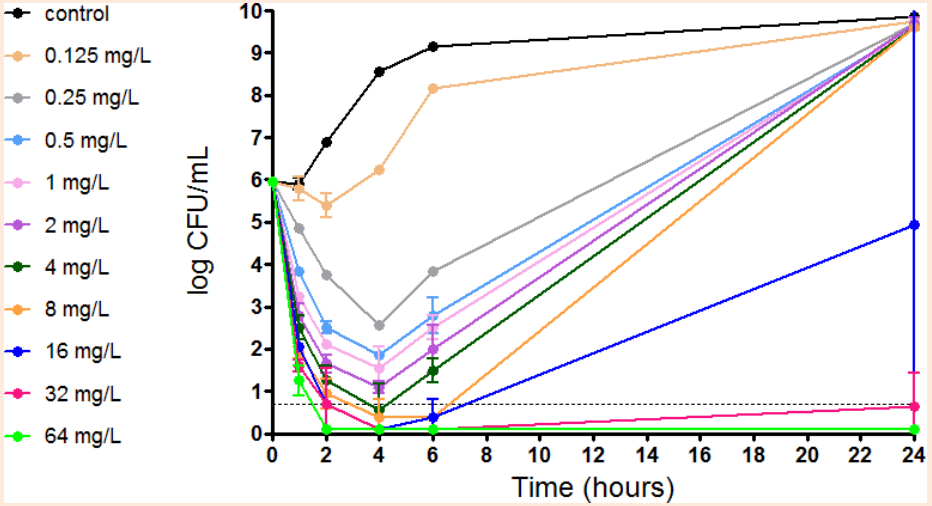


Time Killing Kinetics



Time Kill Kinetics of a *K. pneumoniae*-ESBL resistant isolate and M33. M33 shows <u>concentration-dependent</u> killing activity.

Time Kill Kinetics of a *K. pneumoniae*-ESBL resistant isolate and Colistin. M33 shows <u>concentration-dependent</u> killing activity.



<u>M33-resistant mutants were not found, while Colistin resistant mutants were found at the same time</u>

Bacterial resistance to M33

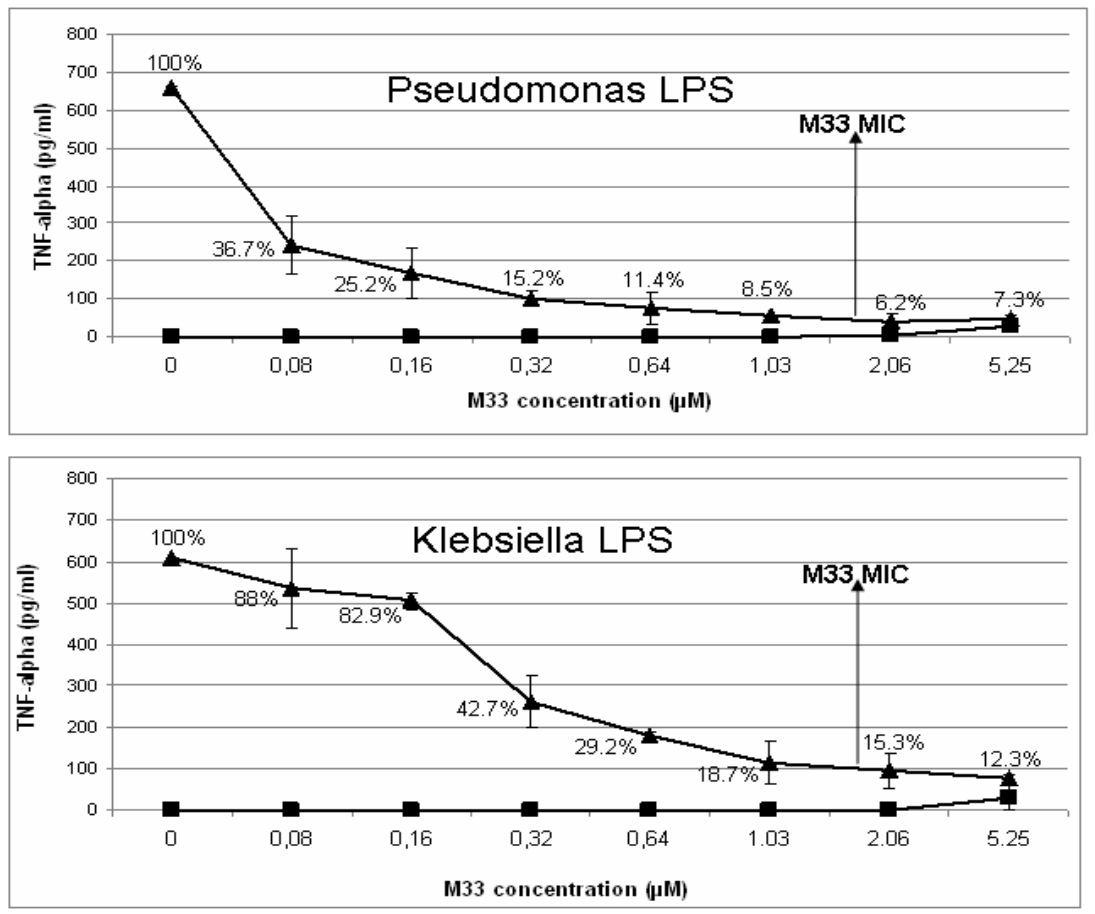
Manuscript in preparation

M33-resistant mutants selection was attempted in vitro using the M33-susceptible (MIC 0.35 µM) and colistin-susceptible (MIC 0.15 µM) *K. pneumoniae* strain KKBO-1 (Cannatelli et al., 2013) by plating cells on M33-containing medium. Colistin-containing plates were also used as control for the selection of colistin-resistant mutants. With this approach, colistin-resistant mutants were selected at a frequency of approximately 1×10^{-7}, while no mutant strains could be selected for M33 using an inoculum up to 5×10^{9} CFU (i.e. selection frequency of resistant mutants was $< 5 \times 10^{-9}$). Results of these experiments suggested a significantly lower M33 propensity for resistance selection with respect to colistin (at least 500 fold lower for M33).

Frequency of colistin-resistant clones	Frequency of M33-resistant clones
$\underline{1 \times 10^{-7}}$	$\underline{< 5 \times 10^{-9}}$

Neutralization of LPS

Pini et al., 2010, FASEB J



Inhibition of TNF-α release by LPS neutralization. Raw 264.7 (mouse leukaemic monocyte macrophage cells) were incubated with LPS from *P. aeruginosa* and *Klebsiella pneumonie* and M33. Triangles indicates incubation with LPS and different concentrations of M33. Squares indicates incubation with M33 only.

Anti-inflammatory activity

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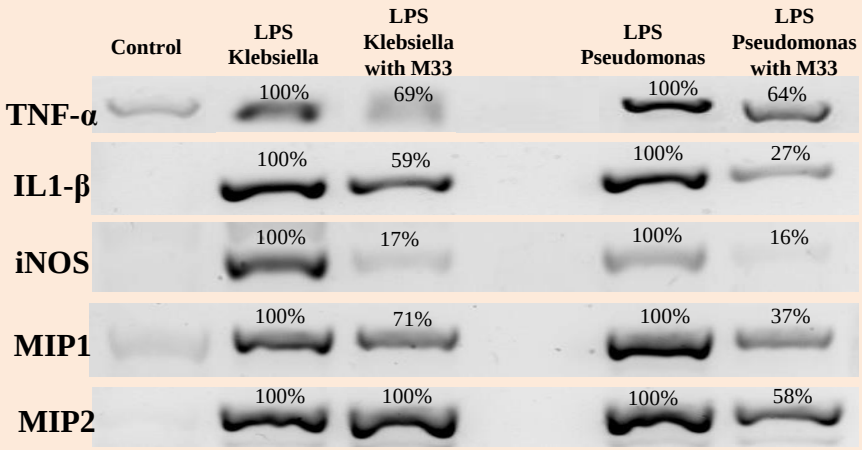
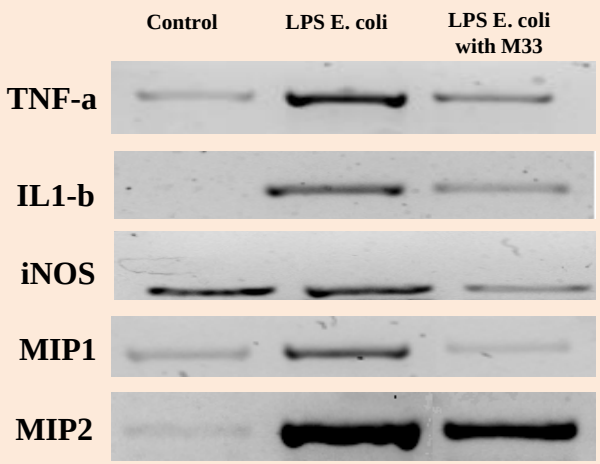
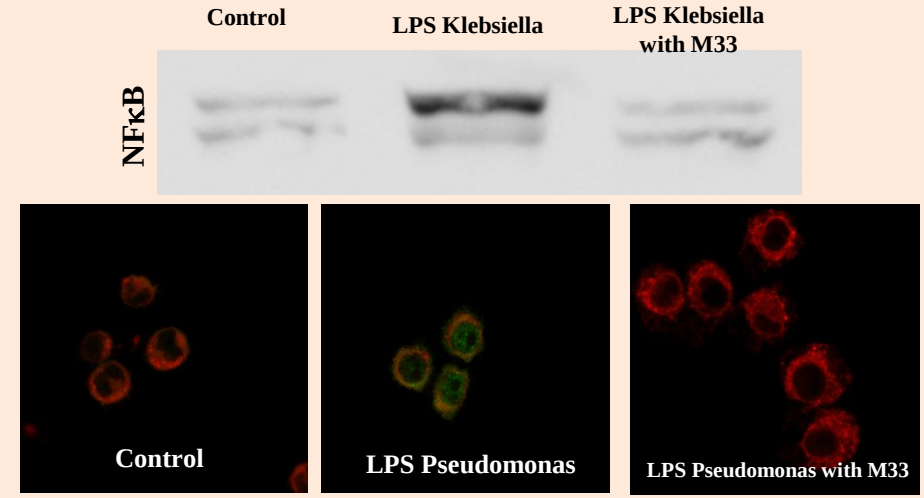
Gene expression (P. aeruginosa or K. pneumoniae)



Gene expression (E. coli)



NFkB Protein production



Cells incubated with LPS or with LPS and M33

Control = cells not incubated. LPS Pseudomonas = cells stimulated with LPS and producing **NFkB** (green signal). LPS Pseudomonas with M33 = cells incubated with LPS and M33 where the green signal is disappeared

- **TNF-α** is the most important cytokine involved in systemic inflammation and is implicated in acute phase reaction
- **IL1-beta** is an important mediator of the inflammatory response, and is involved in a variety of cellular activities
- **iNOS** is a proximate cause of septic shock
- **MIP1** and **MIP2** are among the major factors produced by macrophages after they are stimulated with bacterial endotoxins
- **NF-κB** is involved in cellular responses to several stimuli including bacterial or viral antigens.
- **Cox-2** is an enzyme that acts to speed up the production prostaglandins that play a key role in in promoting inflammation.

Wound Healing

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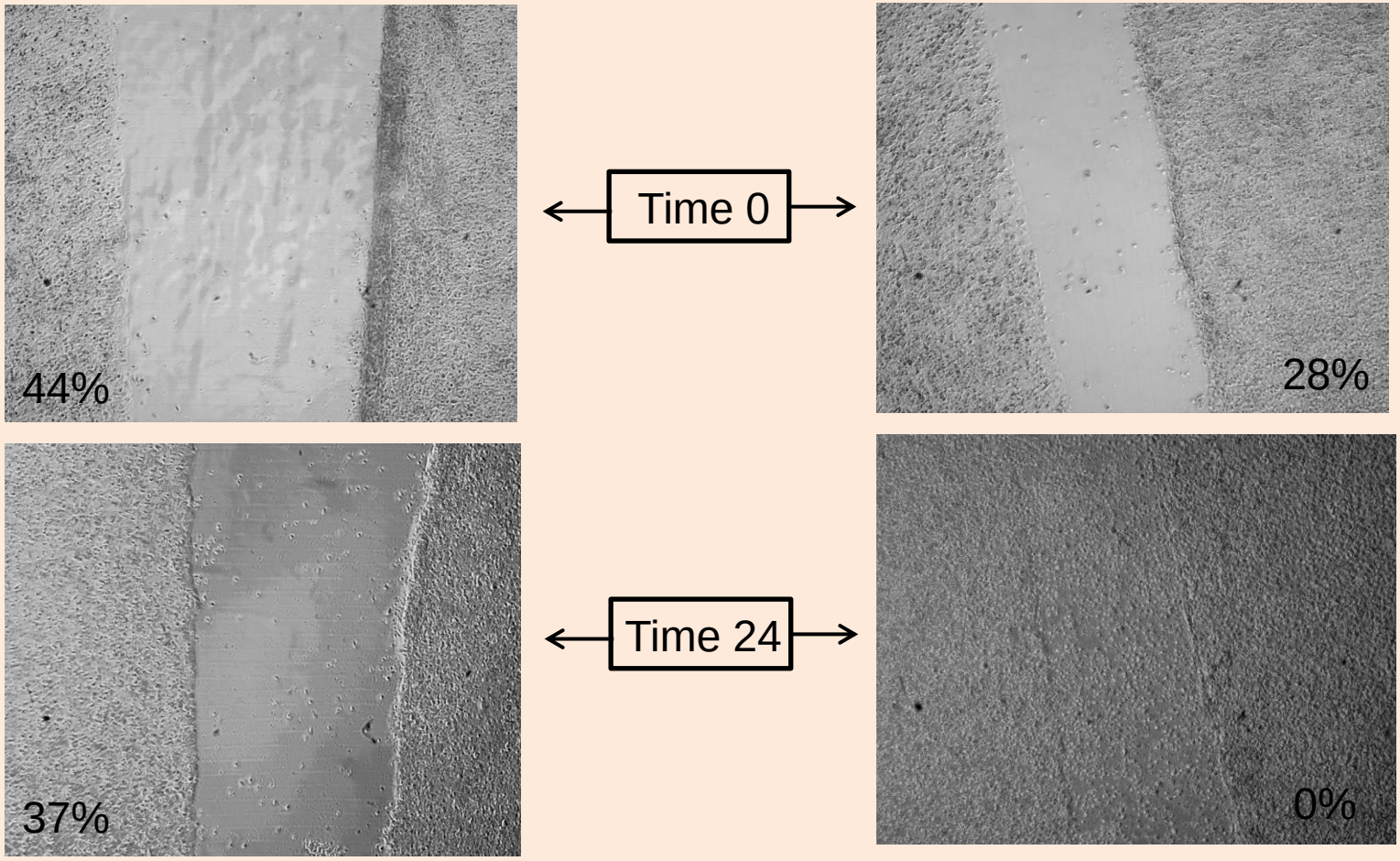
Keratinocyte culture with wound in the cell carpet and treatment with M33

Control culture

Culture treated with M33



In vivo activity – The sepsis animal model

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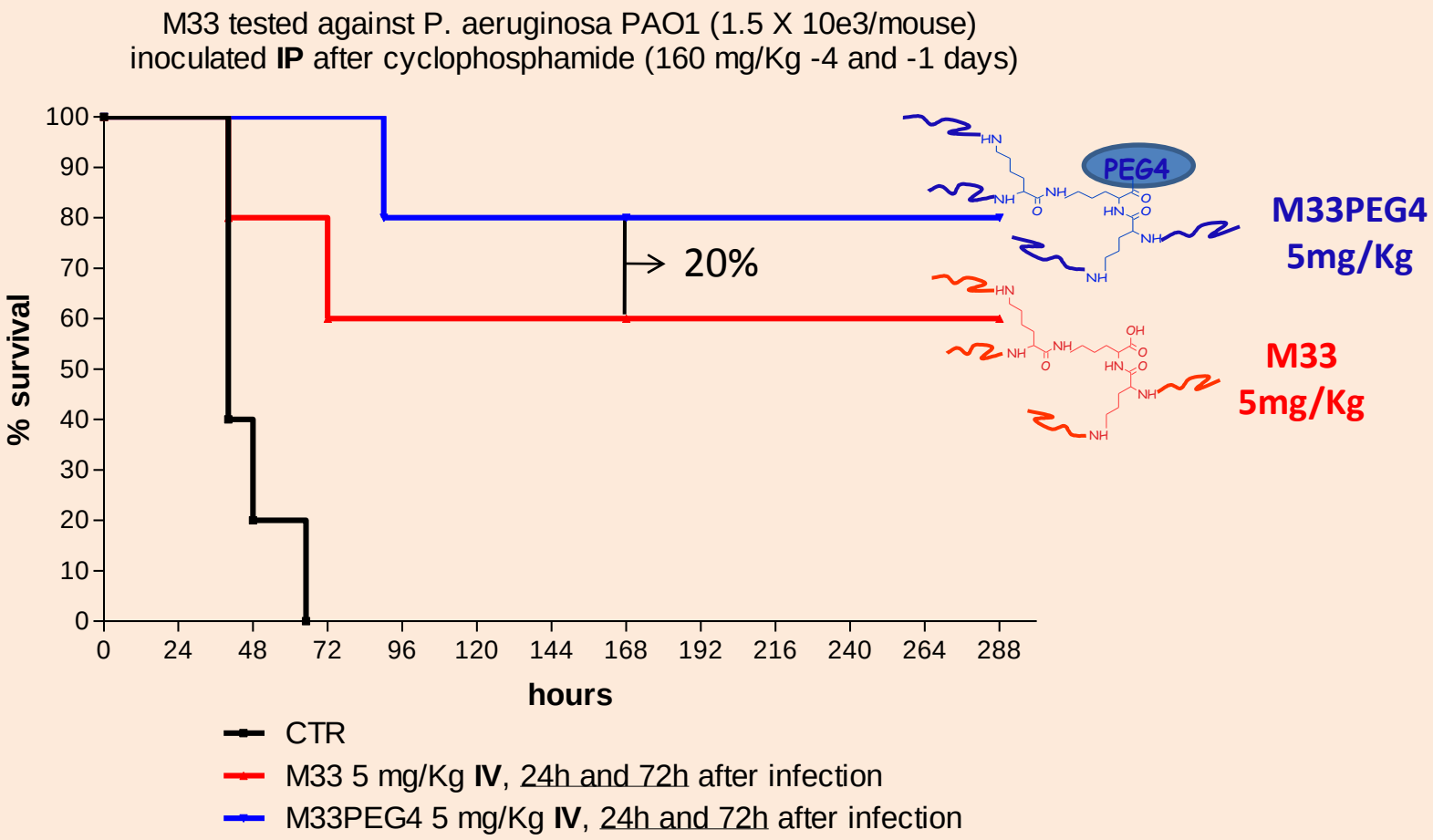
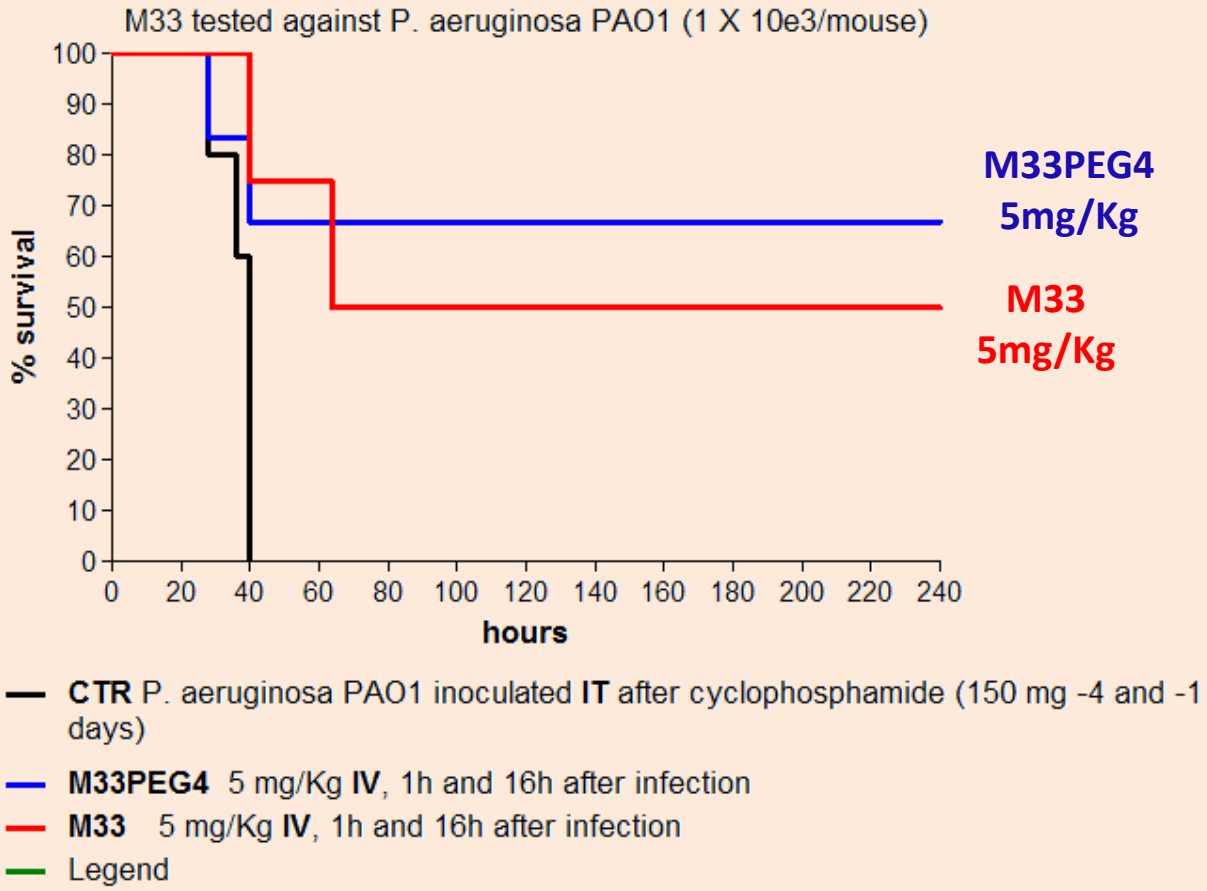
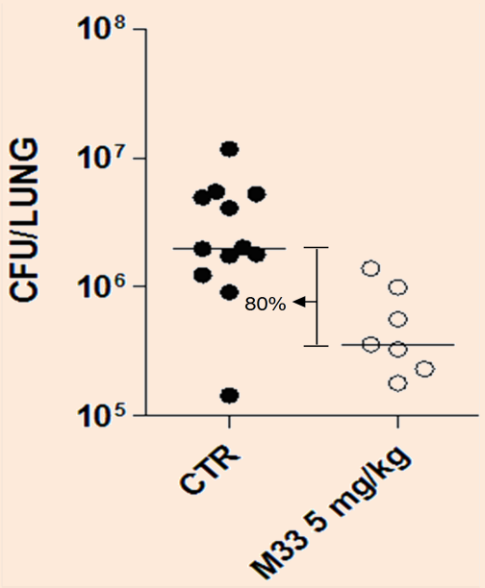


In vivo activity – The lung infection model

Manuscript in preparation





Number of CFU present in lungs of animals infected IT with *P. aeruginosa* and then treated IT with M33

In vivo activity – Plasma clearance

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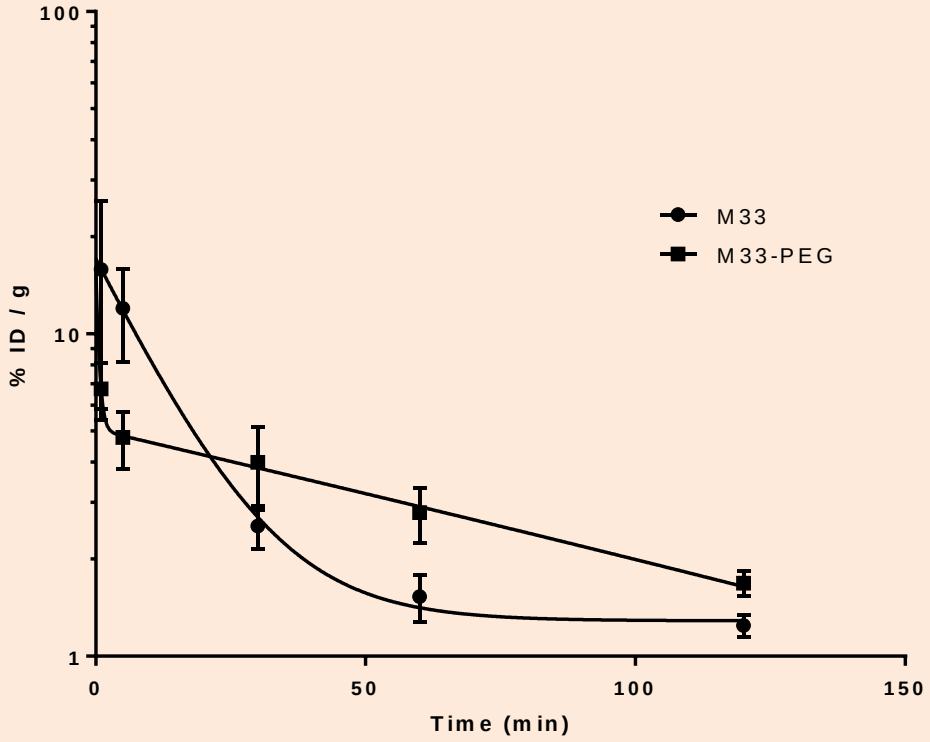
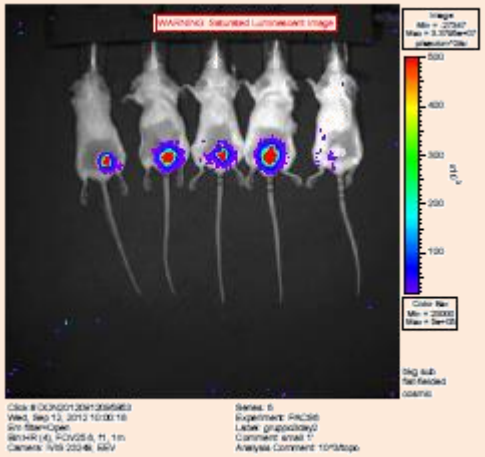


Concentration of [^{125}I]SET-M33L and [^{125}I]SET-M33L-PEG in plasma, expressed as % ID/g, at different time points after administration of the labeled species.
Calculated half-life is around 10 min for M33 and 70 min for M33-PEG

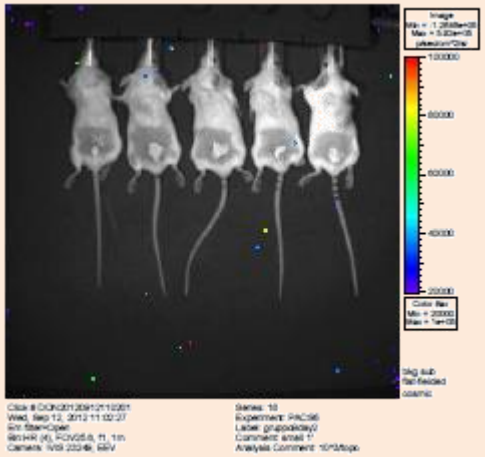
In vivo activity – The skin infection model

Manuscript in preparation

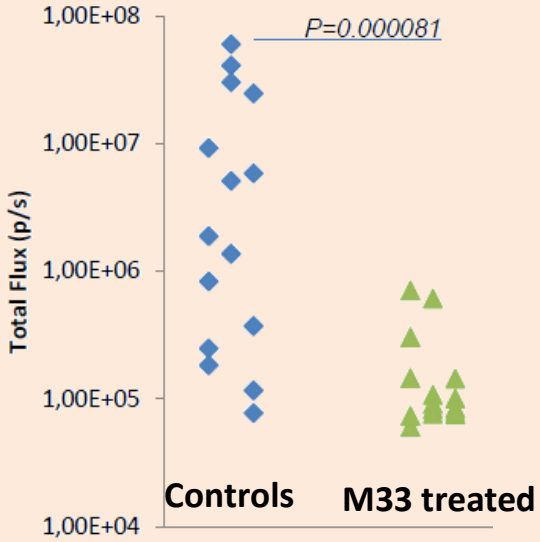
Animals abraded and infected with P. aeruginosa. Then treated with M33 in cream 1 day after infection



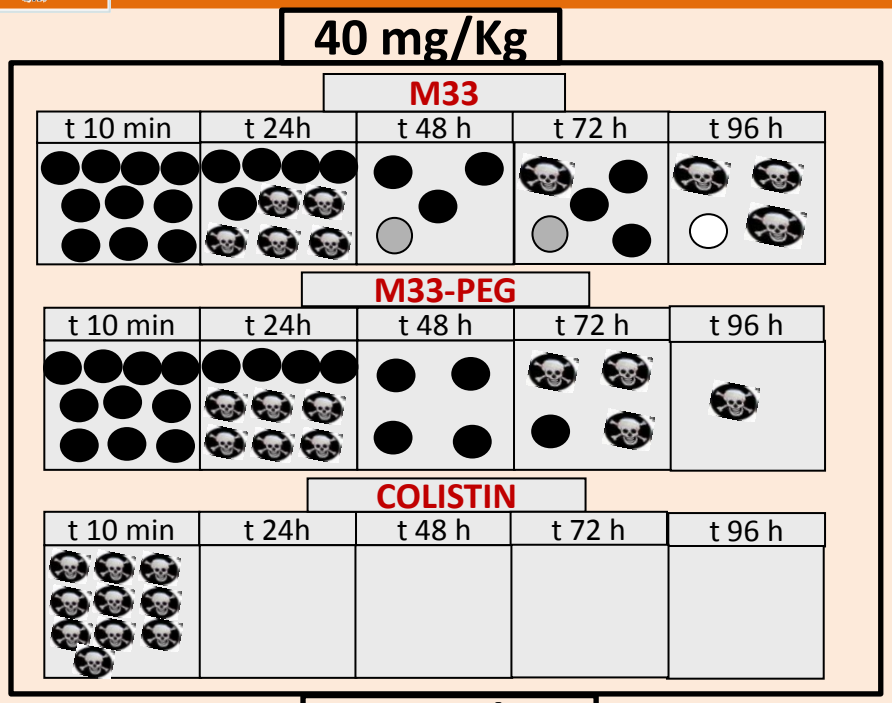
Controls

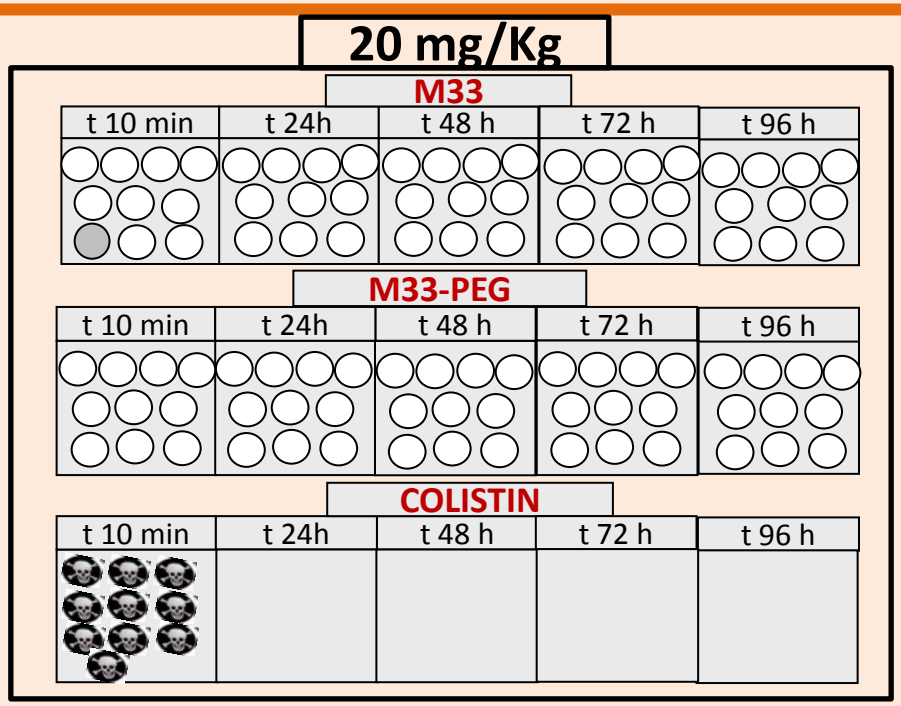


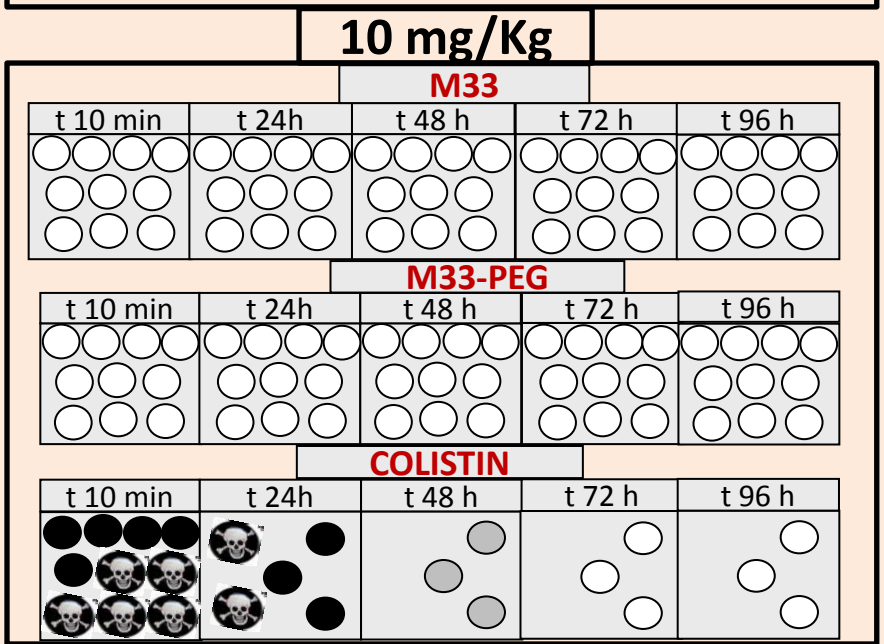
M33 treated



Preliminary acute toxicity IV

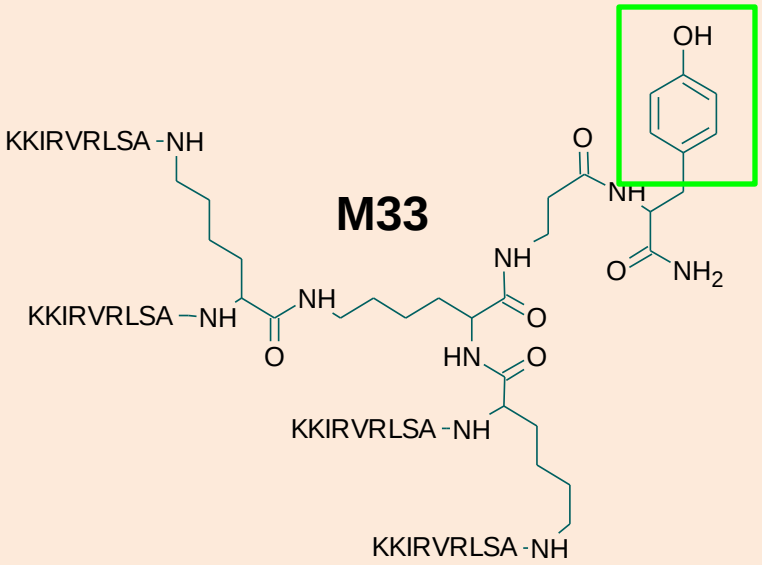


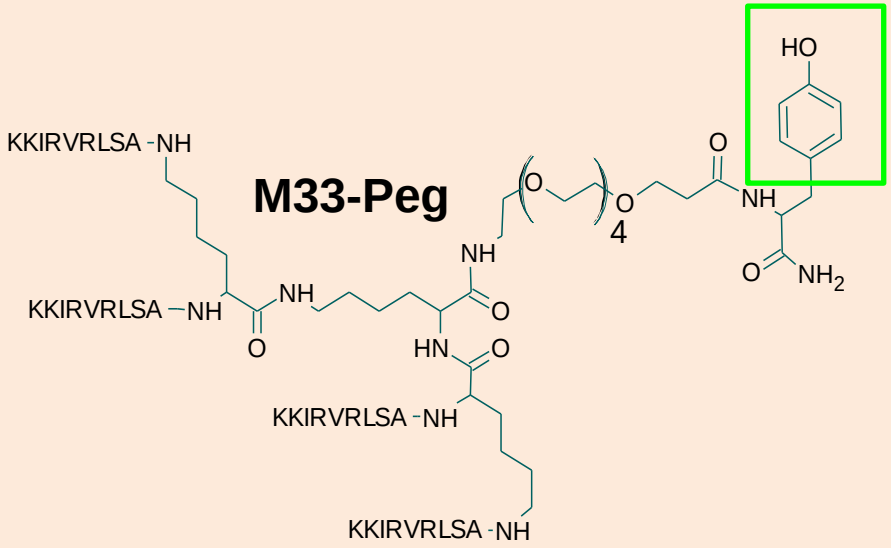


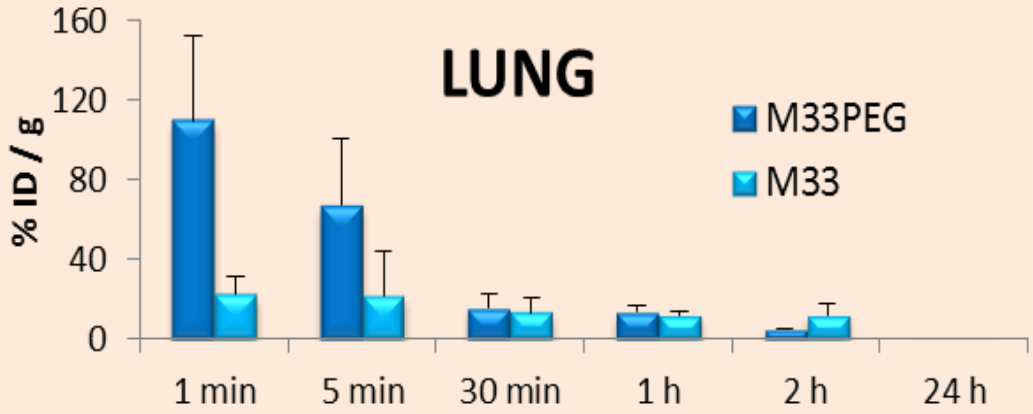


Preliminary biodistribution

Synthesis of M33 and M33-Peg with tyrosine for iodination (^{125}I) and preliminary biodistribution studies in rodents







In progress

Preclinical Development

- Bioanalytical method set up
- GLP production of M33
- Pharmacokinetics and biodistribution
- Safety pharmacology in rodents and non rodents

Research and back up molecules

- Animal model of K. pneumoniae infection and M33 treatment
- Conjugation with nanoparticles and formulation for delivery in lungs
- Broadening spectrum of activity using M33 with D-aminoacids
- Preliminary efficacy and toxicity with M33-D

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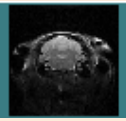

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