Université de Poitiers

PHAR2
PHARmacologie des agents anti-infectieux et antibioRésistance

Inserm
La science pour la santé
From science to health

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Enhancement of amphotericin B activity against *Cryptococcus neoformans* using lipid nanoparticles

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Cryptococcus neoformans

A yeast causing life-threatening infection

- Cryptococcosis can affect the lungs but mainly the brain (meningitidis)
- 150 000 cases/years, immunocompromised patients (HIV, hematological malignancies, immunosuppressive therapies)

- Treatments: First line, liposomal Amphotericin B (L-AmB) + 5-fluorocytosine (5-FC)

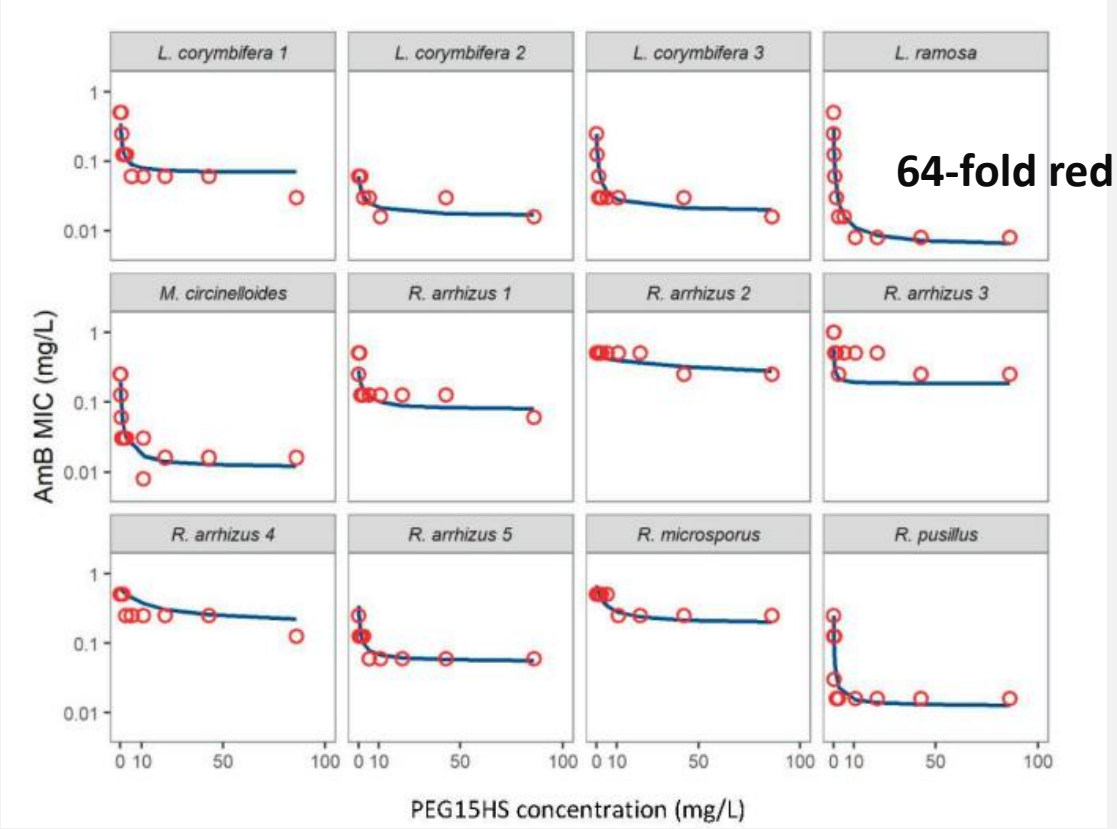
➢ Despite this treatment, the mortality rate is still high, around 30%.

➢ Access to this expensive treatment is limited in some of the countries where the incidence of cryptococcosis is high

→ Need for a new formulation of AmB more effective against *Cryptococcus neoformans* and more affordable than L-AmB.

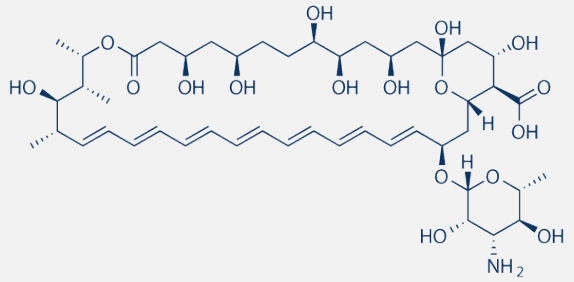
How to improve AmB efficacy?

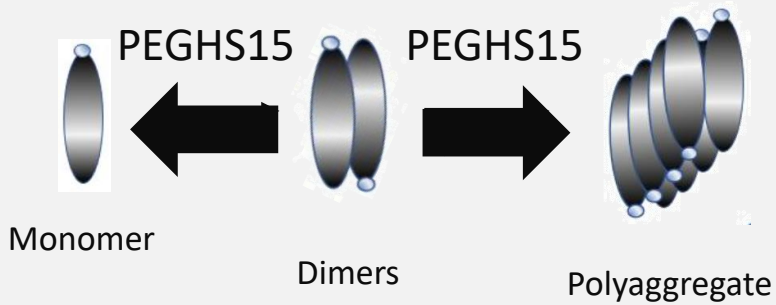
- A nonionic surfactant (PEGHS15) has previously been shown to increase the efficacy of AmB against *Mucorales* without increasing its hemolytic properties.



64-fold reduction

Amphotericin B (AmB) MICs (mg/L) versus Solutol® HS15 concentrations (mg/L) for twelve *Mucorales* strains. Circles represent the AmB MICs determined during one checkerboard experiment and the solid lines the individual AmB MICs predicted by the Emax model based on three checkerboard experiments

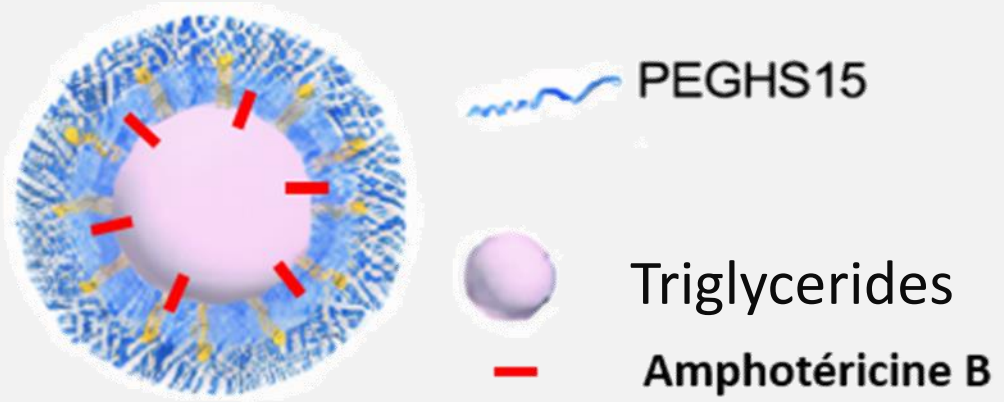




Brunet *et al*, Improved In Vitro Anti-Mucorales Activity and Cytotoxicity of Amphotericin B with a Pegylated Surfactant, J. Fungi 2022, 8, 12

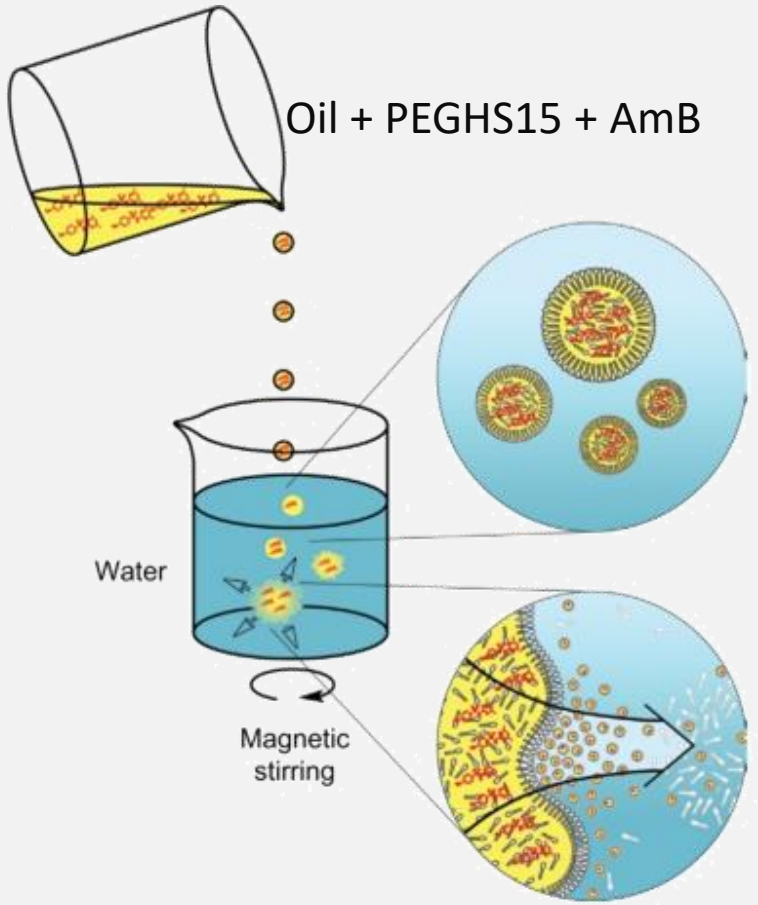
Why using nanoparticles to formulate AmB?

- Nanoparticles aim to combine PEGHS15 and AmB in a precise ratio and to achieve the same pharmacokinetics for both molecules (by bringing the two molecules together at the site of infection).
- Other lipophilic antifungal agents can be loaded into their lipid core.
- Potentially obtain a system that targets fungi.



Lipid nanoparticles formulation process

- Low-energy and reproducible process
- Faster and cheaper than liposome formulation
- Lipid nanoparticles are more stable than liposomes

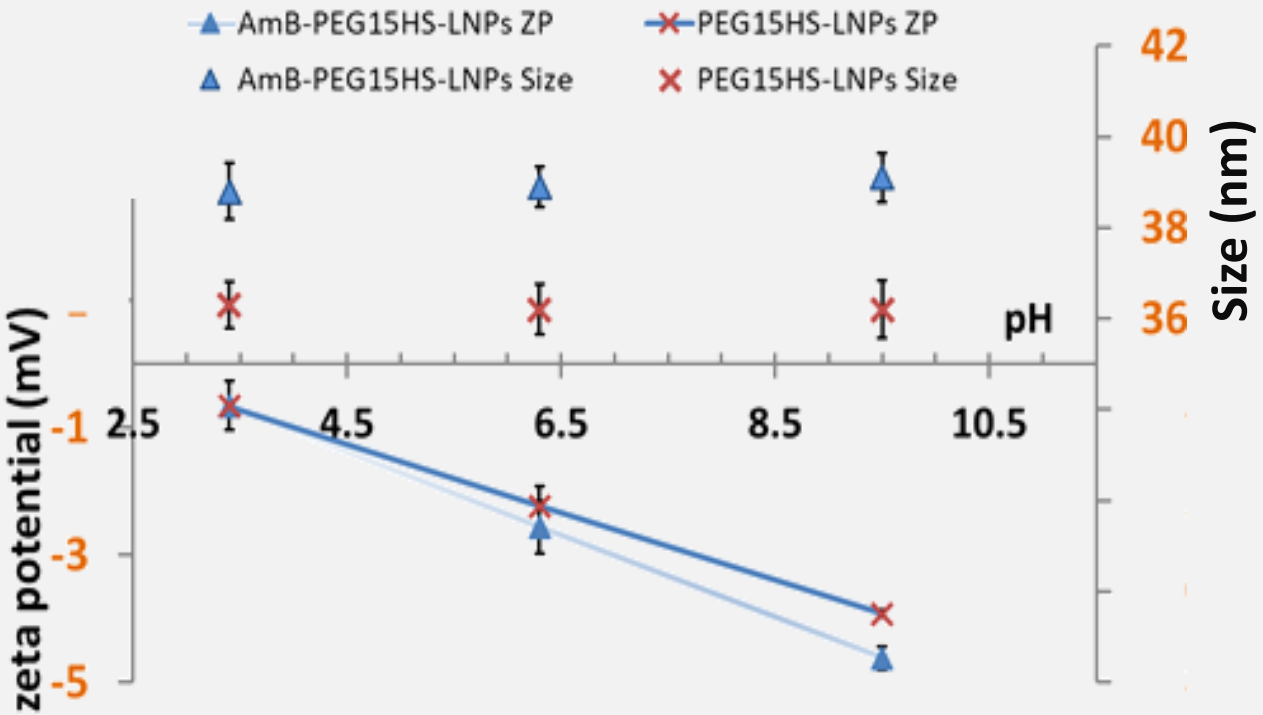


Nanoparticles size is controlled by the surfactant/oil weight ratio (SOR)

T.F. Vandamme and N. Anton, International Journal of Nanomedicine 2010:5 867–873

Lipid nanoparticles characterisation

Particles size and zeta potentional



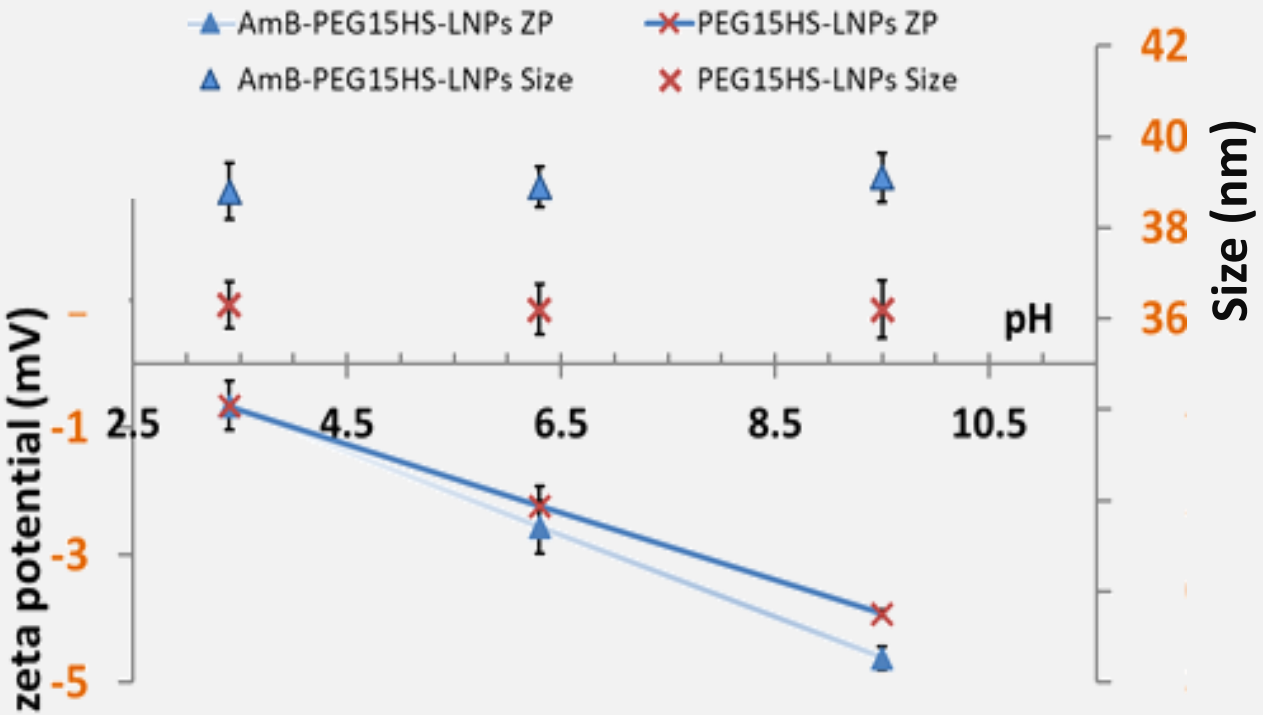
Size (DLS) and zeta potential of AmB-loaded LNPs and PEG15HS-LNPs at different pH

Zeta potential and median size of nanoparticles versus pH measured by DLS

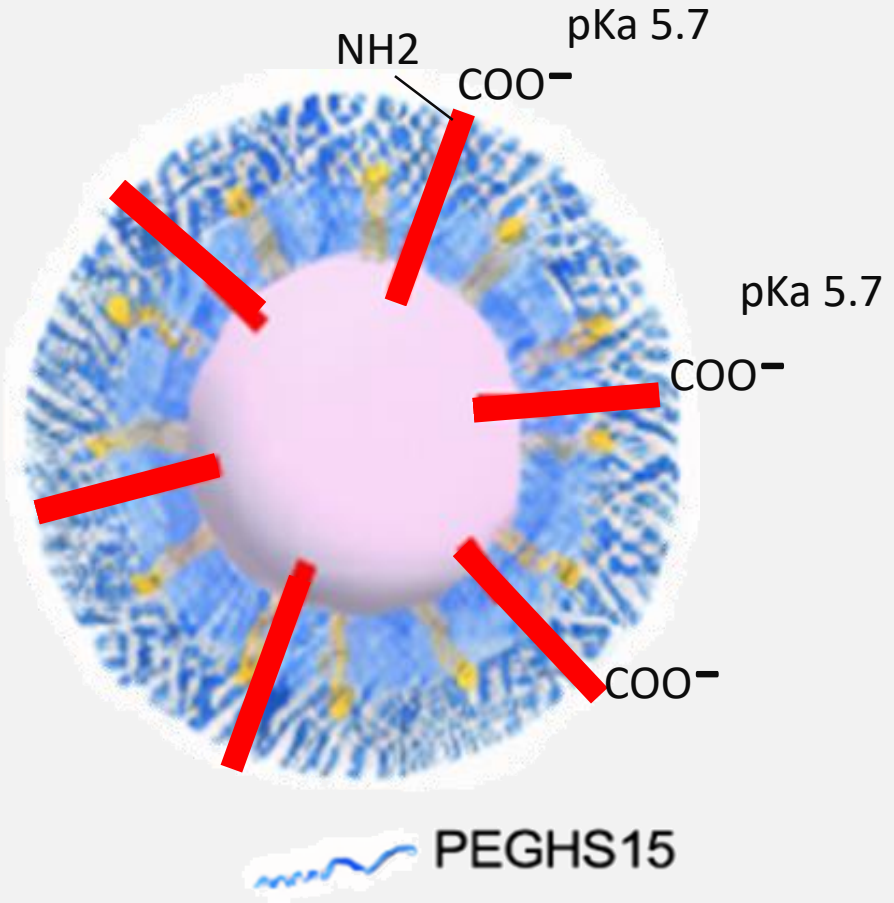
SOR of 1.5
[AMB] = 50 mg/L

Lipid nanoparticles Characterisation

Particles size and zeta potentional

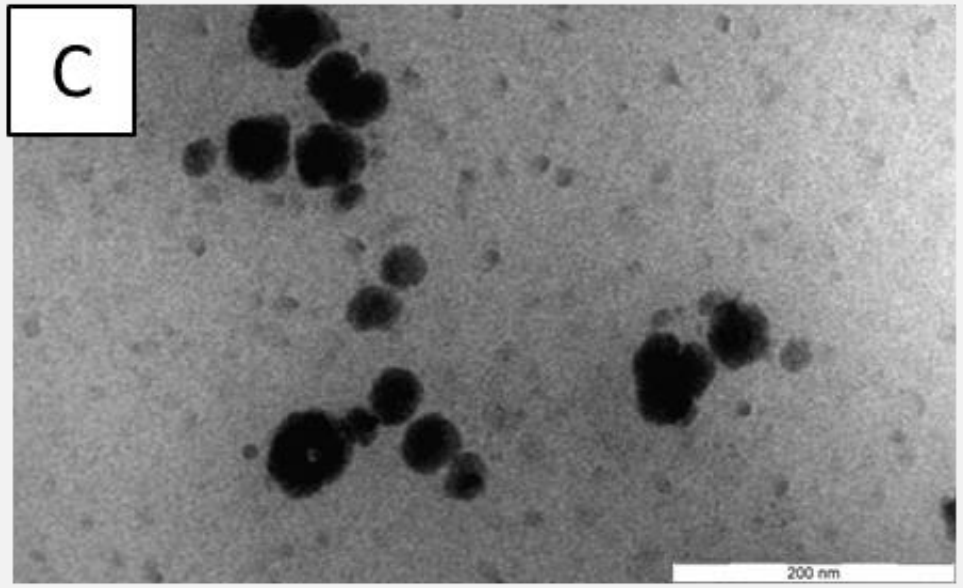


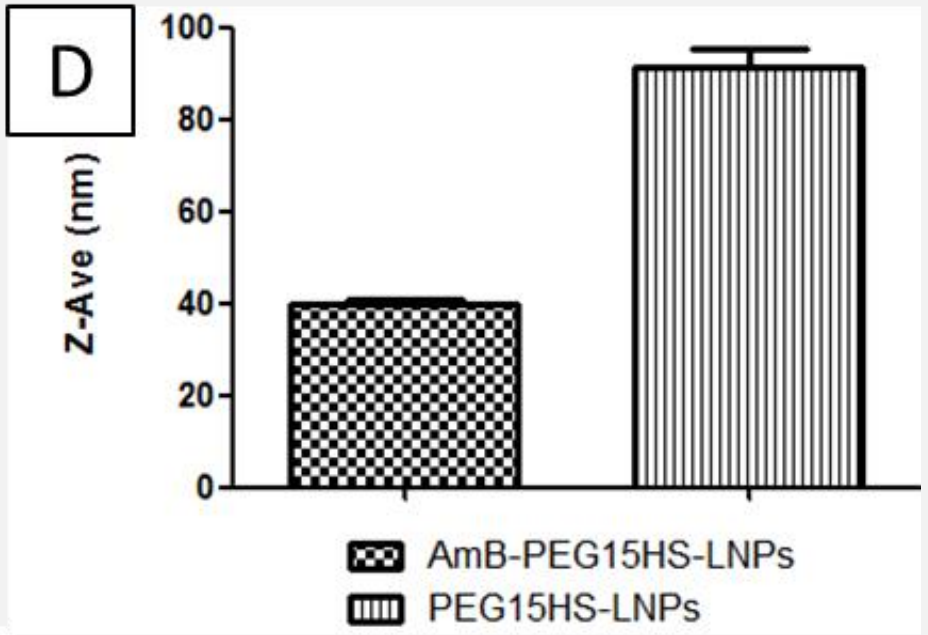
Size (DLS) and zeta potential of AmB-loaded LNPs and PEG15HS-LNPs at different pH



Lipid nanoparticled Characterizaztion

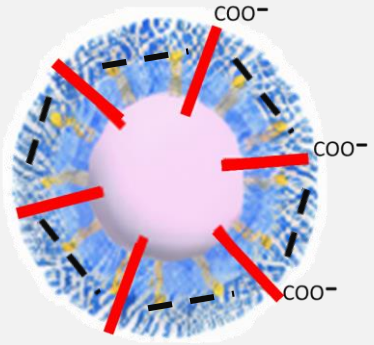
Transmission electron microscopy (TEM)





- (C) AmB-loaded LNPs observed by TEM at 80000X
- (D) Size of AmB-loaded and blank lipid nanoparticles measured by TEM

While the blank particles appear larger and more spread out in TEM compared to the values measured in DLS, the same sizes were obtained in TEM and DLS for AmB-loaded nanoparticles.



Efficacy - Minimal inhibitory concentration

Clinical Strains	MIC (mg/L)		Diminution of AmB MIC
	AmB	LNP-AmB	LNP-AmB
C1	1	0.03	32
C2	0.5	0.016	32
C3	1	0.03	32
C4	1	0.03	32
C5	1	0.06	16
C6	1	0.06	16
C7	1	0.03	32
C8	1	0.06	16

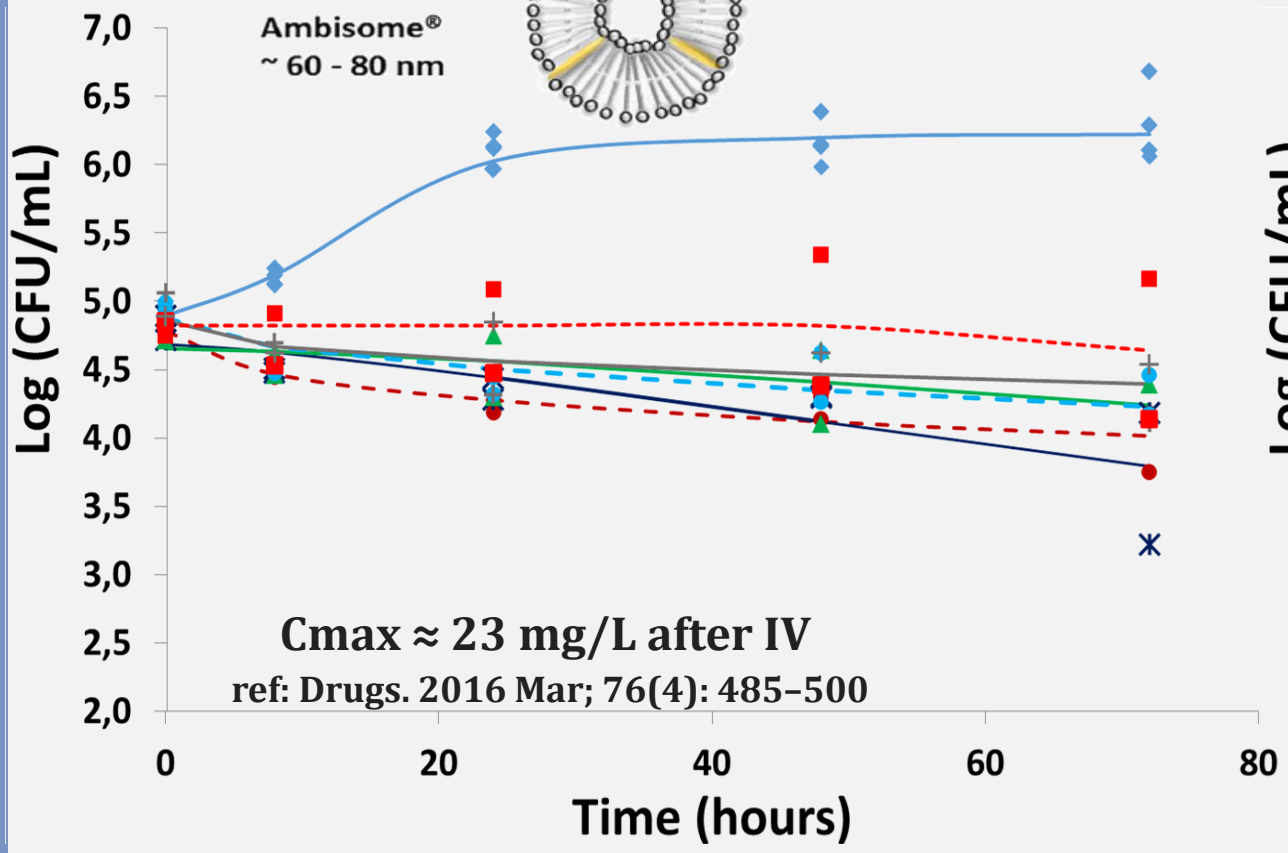
MICs were evaluated according to EUCAST guidelines.
LNP-AmB: AmB-loaded lipid nanoparticles

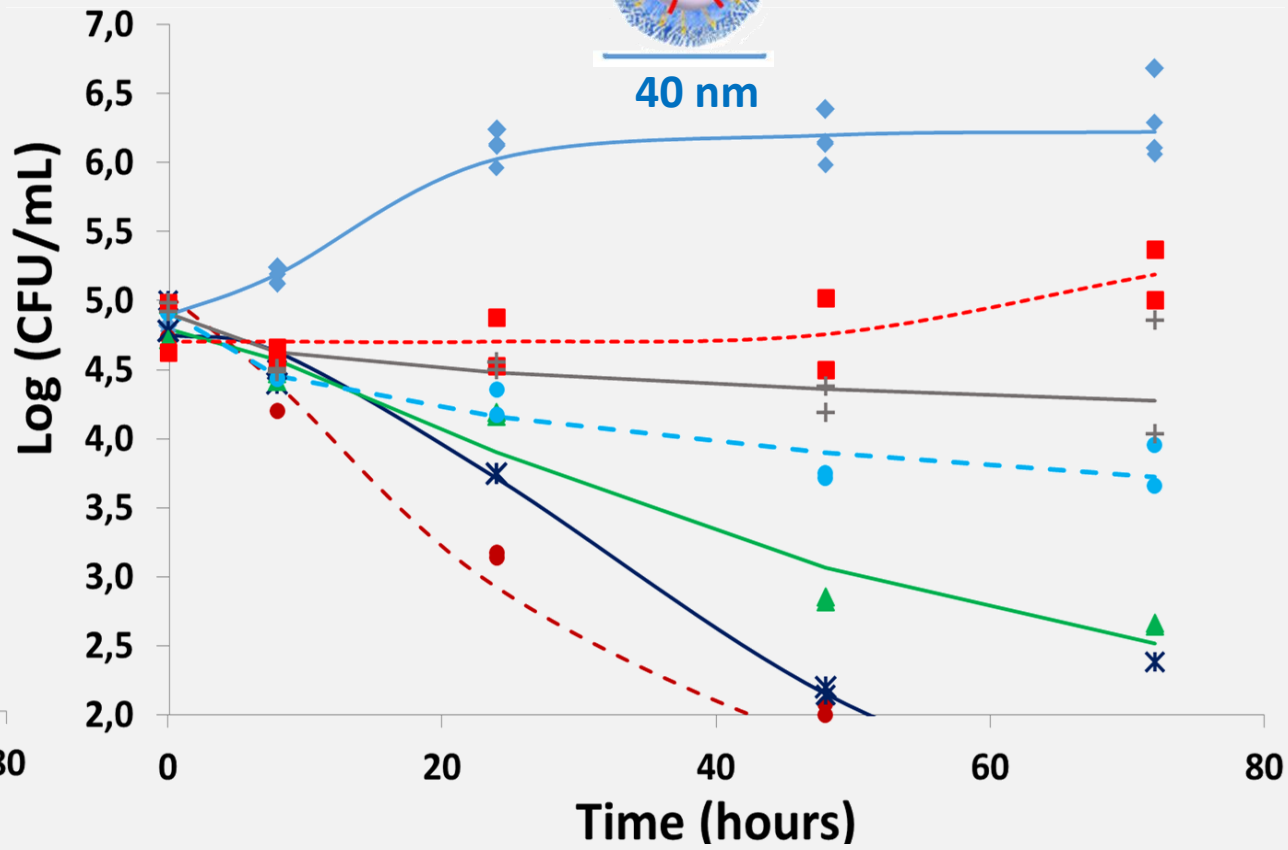
- AmB-loaded lipid nanoparticles have lower MICs (better efficacy) than AmB in solution against several *Cryptococcus neoformans* clinical isolates

- But, MICs of polyenes against *C. neoformans* do not always correlate with efficacy.

Evaluation of the in vitro activity of amphotericin B by time–kill curve methodology against large and small capsulate C. neoformans isolates. **Diagnostic Microbiology and Infectious Disease 71 (2011) 260 – 262**

Efficacy- Time kill experiments





No treatment — 8 mg/L — 4 mg/L — 2 mg/L — 1 mg/L — 0.5 mg/L — 0.125 mg/L

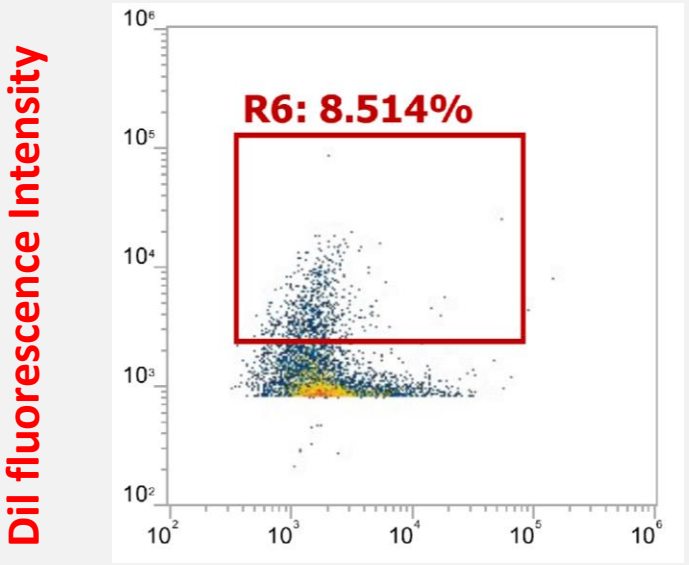
AmB-loaded nanoparticles are more effective than Ambisome® to kill *Cryptococcus neoformans*

Interaction between nanoparticles and *Cryptococcus neoformans*

Flow cytometry

40 nm

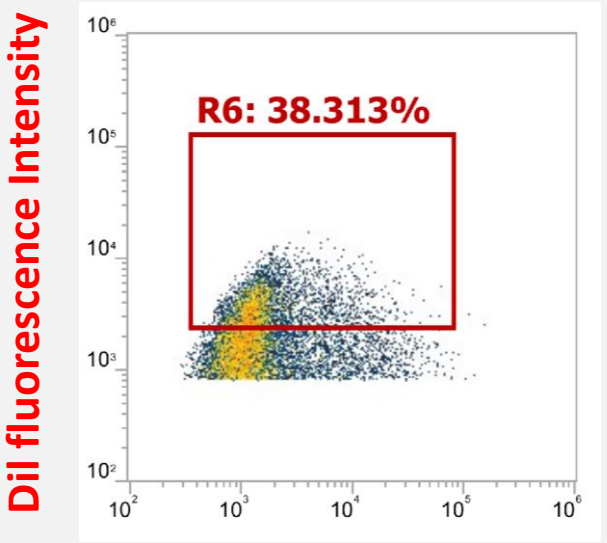
Blank nanoparticles stained with DiL



40 nm

AmB-loaded nanoparticles stained with DiL

[AmB] = 0.5 mg/L



- ***Cryptococcus*** **cells were stained with CMFDA (green)**
- **Nanoparticles were stained with lipophilic dye DiL (Red)**
- **18 hours of incubation**
- **10 000 cells were analysed**

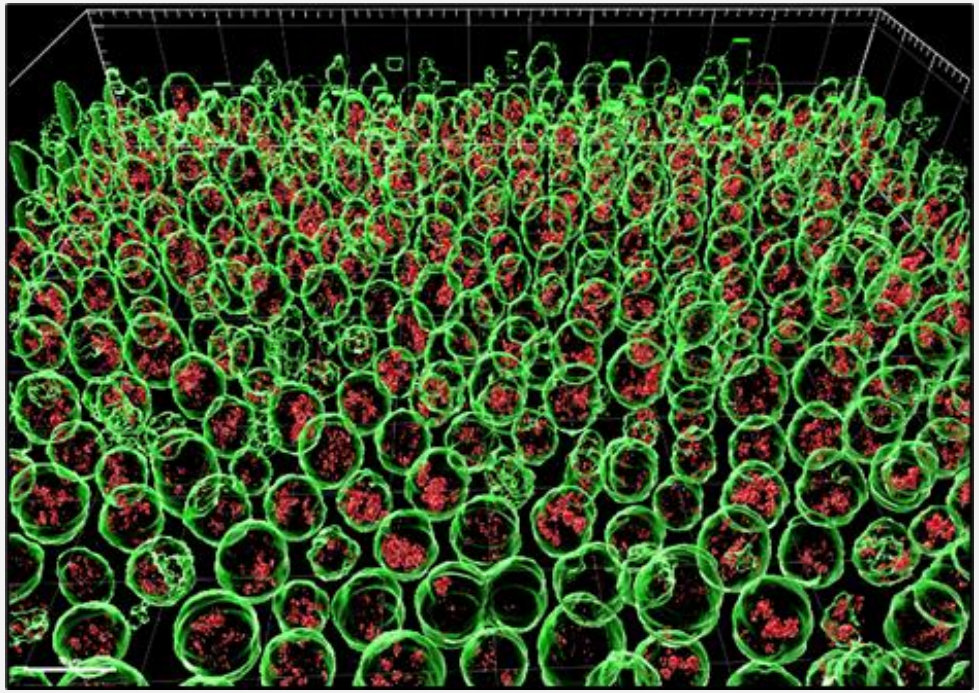
The presence of AmB in the nanoparticles increases their interaction with *Cryptococcus neoformans* cells.

Are the AmB-loaded nanoparticles inside or on the surface of *Cryptococcus neoformans* cells?

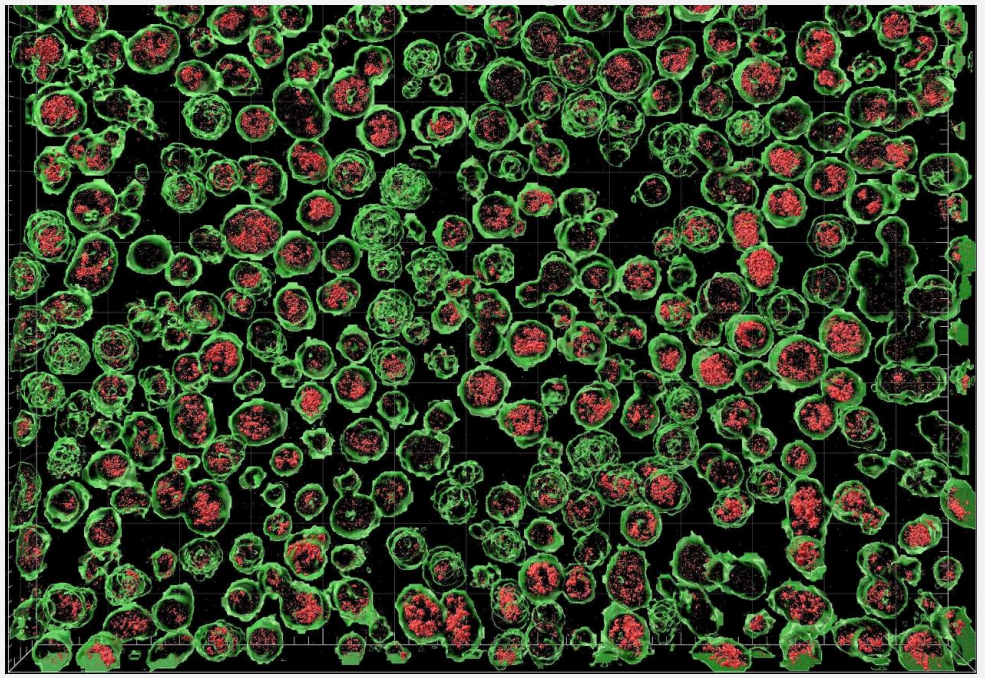
Interaction between nanoparticles and *Cryptococcus neoformans*

Confocal microscopy

Cryptococcus cells were stained with CMFDA (green)
Nanoparticles were stained with lipophilic dye DiL (Red)



40 nm (SOR of 1.5)
AmB-loaded nanoparticles stained with DiL

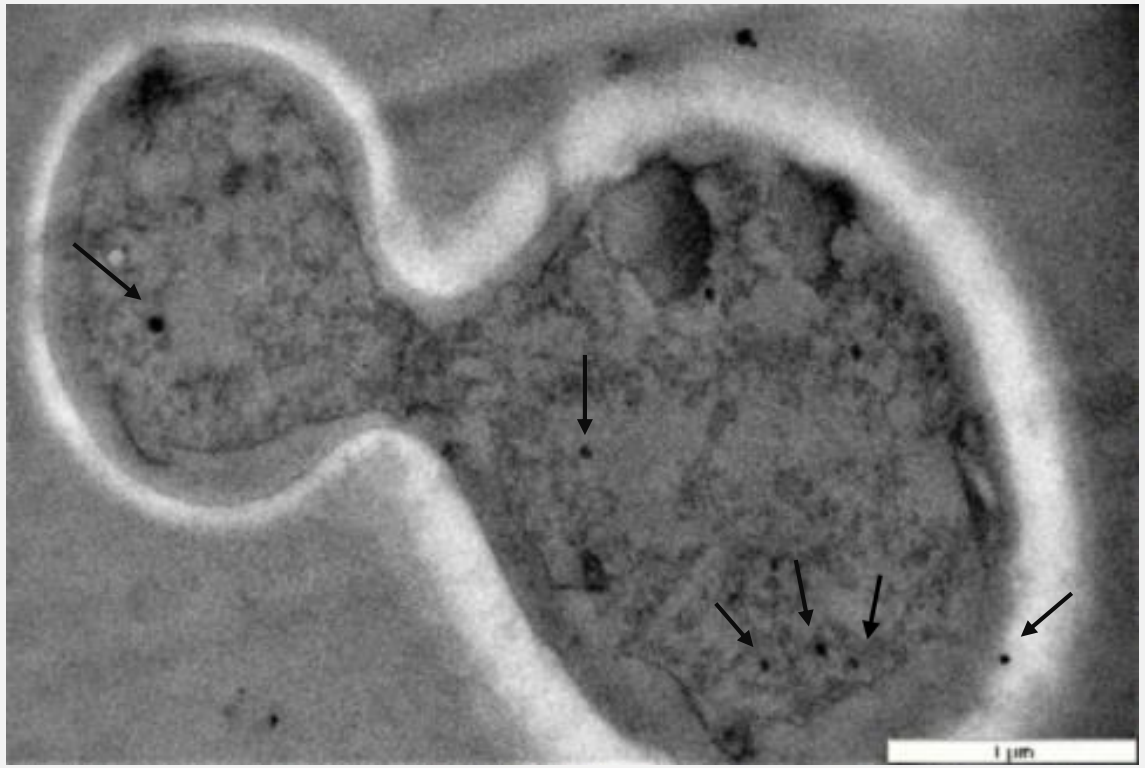


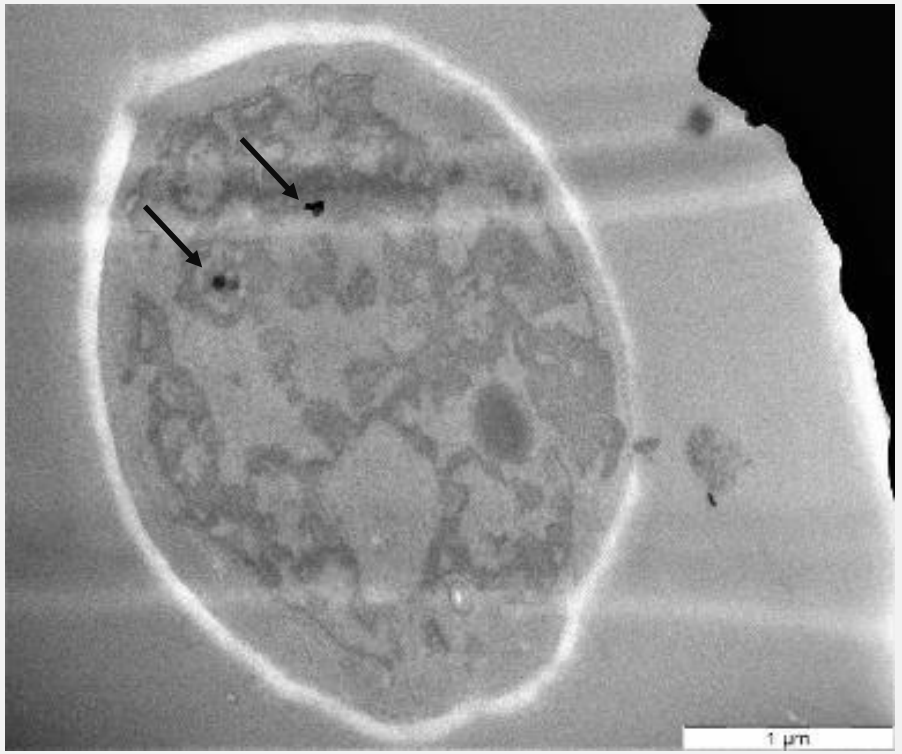
120 nm (SOR of 0.67)
AmB-loaded nanoparticles stained with DiL

Nanoparticles with size up to 120 nm accumulated inside *Cryptococcus* cells after 18h of incubation

Interaction between nanoparticles and *Cryptococcus neoformans*
Transmission electron microscopy

TEM micrographs of non-contrasting *Cryptoccocus neoformans* incubated 18 hours with 40 nm AmB-loaded nanoparticles labeled with iodinated oil. [AmB] = 0.5 mg/L





The nanoparticles were present everywhere in *Cryptococcus* neoformans cells after 18 hours of incubation.

- **The presence of amphotericin B at the surface of nanoparticles having a diameter up to 120 nm seems allows a significant improvement of the nanoparticles penetration inside the *Cryptococcus neoformans***
- However, fungal cell wall is a structure that represents a permeability barrier with pore size of around 6 nm which is theoretically too small to allow these 120 nm lipid nanoparticles to cross through.

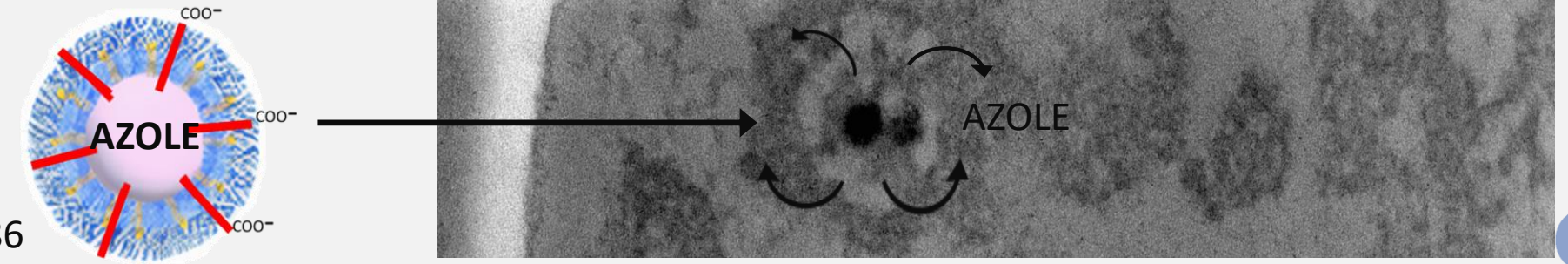
Walker L, *et al*. **2018**. **The viscoelastic properties of the fungal cell wall allow traffic of AmBisome as intact liposome vesicles**. mBio 9:e02383-17.

60- to 80-nm liposome of Ambisomes remained intact during transit through the cell wall of *Candida albicans* and *Cryptococcus neoformans*

The absence of amphotericin B in the liposomes were also associated with a significant reduction in liposome penetration

Conclusion

- We developped AmB-loaded nanoparticles that are more effective to kill *Cryptococcus neoformans* and cheaper than Ambisome®
- AmB seems to be at the interface of the nanoparticles and improve their accumulation inside *Cryptococcus neoformans* cells
- Preliminary data have shown that it is possible to load a large amount of lipophilic azole antifungals into these nanoparticles, which should allow them to accumulate the azoles in fungal cells and bypass resistance mechanisms (efflux or target overexpression).



Patent : PCT/EP2023/050486

Thank you for listening

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