Experimental report

19/10/2016

Proposal: 8-02-773 **Council:** 4/2016

Title: Quantification of sterol removal by amphotericin B and impact of sterol content on its interaction with natural yeast membranes

Research area: Biology

This proposal is a continuation of 8-02-746

Main proposer: Robin DELHOM

Experimental team: Hanna WACKLIN
Robin DELHOM

Local contacts: Giovanna FRAGNETO

Samples: Silicon blocks
Natural Pichia pastoris lipids (Hydrogenated)
Natural Pichia pastoris lipids (Deuterated)
Ergosterol (hydrogenated)
Ergosterol (deuterated)

Instrument	Requested days	Allocated days	From	To
FIGARO	4	3	30/09/2016	04/10/2016
D17	4	0		

Abstract:

The activity of Amphotericin B against systemic fungal infections is widely accepted to result from its specific interaction with ergosterol. We have recently carried out a detailed characterization of the lipid composition and structure of native fungal membrane extracts from Pichia pastoris, as well as the consequences of AmB action. The structure of yeast membranes differs considerably from typical model lipid bilayers composed of synthetic lipids and depends on the degree of lipid polyunsaturation. AmB inserts in yeast membranes both in the absence and presence of ergosterol, and our results confirmed directly that AmB forms a thick extramembraneous aggregate, proposed recently to act as an ergosterol-extracting sponge. We propose to complete our investigation of P. pastoris membranes by quantifying the effect of the different lipid fractions (polar/apolar lipids, sterols) on the ergosterol and cholesterol extraction and AmB insertion by using membranes where the lipid fractions and sterol are selectively labeled.

Experimental Report
FIGARO 8-02-773

Abstract:

The activity of Amphotericin B against systemic fungal infections is widely accepted to result from its specific interaction with ergosterol. We have recently carried out a detailed characterization of the lipid composition and structure of native fungal membrane extracts from Pichia pastoris, as well as the consequences of AmB action. The structure of yeast membranes differs considerably from typical model lipid bilayers composed of synthetic lipids and depends on the degree of lipid polyunsaturation. AmB inserts in yeast membranes both in the absence and presence of ergosterol, and our results confirmed directly that AmB forms a thick extramembranous aggregate, proposed recently to act as an ergosterol-extracting sponge. We propose to complete our investigation of P. pastoris membranes by quantifying the effect of the different lipid fractions (polar/apolar lipids, sterols) on the ergosterol and cholesterol extraction and AmB insertion by using membranes where the lipid fractions and sterol are selectively labelled.

Background:

Physiologically, natural yeast membranes are functional at the growth temperature (in our case 30°C) and are composed of around 15-20mol% of ergosterol. The viability of the cells depends on its membrane composition, and the strong antifungal agent amphotericin B (AmB) is known to interact with the ergosterol and induce fungal or protozoal cell death. Moreover, due to its preference to ergosterol, mammalian cells containing cholesterol as main sterol, are less impacted, explaining the use of AmB as antifungal treatment. In our experiments and studies, we perform deposition of bilayers of yeast lipids by vesicle fusion, allowing us to use different compositions in order to investigate the influence of sterol content in the mechanism of AmB. Most of the studies are performed with simple model membranes, composed of one phospholipid molecular species and sterol content, when we use a natural mixture of phospholipids, hydrogenated and deuterated, to which we add sterol content. This experiment aimed at investigating the extraction of ergosterol by AmB and at the same time, the mechanism inducing toxic side effect, by interaction with cholesterol.

Results:

We measured 6 different samples during the four days allocated on Figaro. The mixtures were sonicated in the corresponding water solution (D_2O or H_2O) containing 100mM of NaCl and 20mM of $CaCl_2$, before being injected in the sample cells at a controlled temperature of 65°C. The nature of the water, light or heavy, was selected in order to match the overall density of the lipid mixture. Thus, a fully deuterated sample was prepared in a D_2O solution, when a partially hydrogenous sample (ex h-Polar d-Erg*) was prepared in H_2O.

Samples (30mol% sterol)		Reason
h-Polar h-Ergosterol	✔	change in full structure/AmB insertion
d- Polar d-Ergosterol	✔ 1.	AmB insertion/H2O penetration
h-Polar d-Ergosterol	✔	ergosterol removal by AmB
h-Polar h-Cholesterol	✔	change in full structure
d-Polar h-Cholesterol	✔	cholesterol removal by AmB
d-Polar h-Ergosterol	✔ 2.	ergosterol removal by AmB
d-total	✔ 3.	change in full structure/AmB insertion
h-total	✔ 4.	change in full structure/AmB insertion
h-total - Ergosterol	✔ 5.	effect of other apolar lipids
d-total - Ergosterol	✔ 6.	effect of other apolar lipids

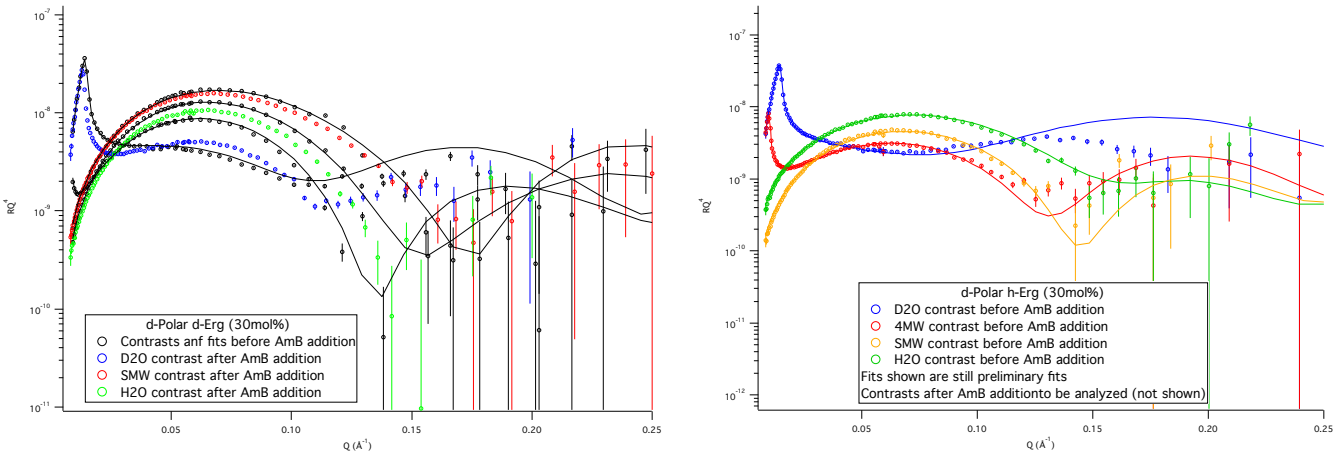
*Polar stands for Polar fraction (phospholipids), Erg for ergosterol and Chol for cholesterol. h & d are the indication of hydrogen or deuterium-labelled molecules.

Samples to perform in order to complete our study;

Samples (30mol% sterol)		Reason
h-Polar d-Cholesterol	A.	Is Cholesterol removed by AmB or not?
d-Polar d-Cholesterol	B.	AmB insertion/H_2O penetration
h-Polar + d-apolar lipids (no sterol)	C.	Effect of other apolar lipids (more contrast)
d-Polar + h-apolar lipids (no sterol)	D.	Effect of other apolar lipids (more contrast)

Due to the observations made during the Test experiment 2599 (see experimental report), we settled the deposition process for such complex mixtures and only one deposition did not give 100% coverage (90% indeed) during this experiment. The data look really good and effects can be seen upon AmB addition. The complexity of the system and of the contrasts performed by the preparation of samples does not allow us to give conclusions before going through the data analysis process, but we do have all the data and contrasts needed.

Once all samples will be fitted, the results will be compared in order to determine the difference(s) in the mechanism of AmB between mammalian sterol, cholesterol and fungal sterol, ergosterol. Obviously, the availability of the fully deuterated cholesterol would give us two crucial additional samples (see above and proposal 76702, *Deuterated cholesterol and final contrasts for completeness of the investigation of AmB mechanism within natural yeast biomembranes*)



Samples 1. fully detailed; black curves for contrasts before AmB addition (fully characterized) and colour ones for contrasts after AmB addition. Sample 2. Bilayer detailed with preliminary fits.

References:

1. Production and analysis of perdeuterated lipids from Pichia pastoris cells, de Ghellinck A., Schaller H., Laux V., Haertlein M., Sferrazza M., Marechal E., Wacklin H., Jouhet J., Fragneto G., PloS One, (2014), 9, e92999-1-e92999-9

2. Lipid polyunsaturation determines the extent of membrane structural changes induced by Amphotericin B in *Pichia pastoris* yeast, de Ghellinck A, Fragneto G, Laux V, Haertlein M, Jouhet J, Sferrazza M, Wacklin H. Biochim Biophys Acta. 9, 2317-2325 (2015)

3. Anderson, T.M. et al. Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol 10, 400-406 (2014).