Experimental report

09/02/2016

Proposal: 8-02-717 **Council:** 10/2014

Title: The new anti-fungal mechanism of Amphotericin B in natural yeast membranes: quantifying the extraction of ergosterol

Research area: Biology

This proposal is a continuation of 8-02-593

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Samples: lipids
single crystal silicon substrates

Instrument	Requested days	Allocated days	From	To
FIGARO	4	4	18/06/2015	22/06/2015
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 extra-membraneous aggregate, proposed recently to act as an ergosterol-extracting sponge responsible for the anti fungal activity instead of the classic model based on AmB pore formation. We propose to continue the previous investigation by quantifying ergosterol extraction by using membranes where the lipids and ergosterol are selectively labelled in turn. We will also investigate the ability of AmB to extract cholesterol found in mammalian membranes, to elucidate the mechanism of AmB's toxic side effects.

Experimental Report
FIGARO 8-02-717

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[1], as well as the consequences of AmB action[2]. 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 responsible for the anti fungal activity instead of the classic model based on AmB pore formation[3]. We proposed to continue the previous investigation by quantifying ergosterol extraction by using membranes where the lipids and ergosterol are selectively labelled in turn. We also investigated the ability of AmB to extract cholesterol found in mammalian membranes, to elucidate the mechanism of AmB's toxic side effects.

Background :

Physiologically, natural yeast membranes are functional at the growth temperature (in our case 30°C) and are composed of around 15-30mol% of ergosterol. The viability of the cells depends on it's 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 it's 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 D_2O containing 100mM of NaCl and 20mM of $CaCl_2$, before being injected in the sample cells at a controlled 52°C temperature. Only one deposition went wrong, due to formation of stacks instead of a unique bilayer over the silicon block. To investigate the impact of the temperature on the physical properties of the membrane, we measured the 3 first samples, before injection of AmB, both at 52°C and at the physiological temperature of 30°C. In those 3 samples, only small changes were observed due to the temperature shift, because there is no observable lipid phase transition in this temperature range, we only observed small changes of thickness of the layers, instead of larger effects.

- Sample 1 : **hPolhErg (30mol%)**, 4 contrasts before AmB injection and 4 contrasts after.
- Sample 2 : **dPoldErg (30mol%)**, 4 contrasts before AmB injection and 3 contrasts after.
- Sample 3 : **hPoldErg (30mol%)**, 4 contrasts before AmB injection and 4 contrasts after.
- Sample 4 : **hPolChol (30mol%)**, 4 contrasts before AmB injection and 3 contrasts after.
- Sample 5 : **dPolChol (30mol%)**, 4 contrasts before AmB injection and 4 contrasts after.

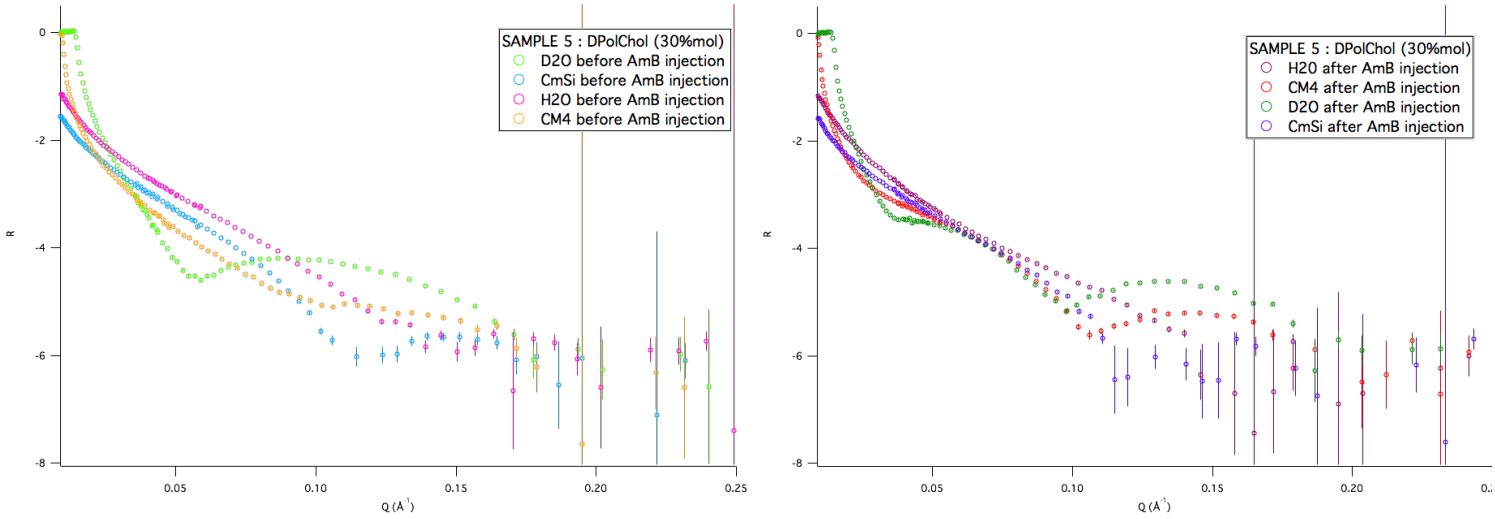
- Sample 6 : **dPolhErg (30mol%)**, 4 contrasts before AmB injection and 3 contrasts after

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

The data look really good and effects can be seen upon AmB addition. In opposition to the small changes observed by the change in temperature, the addition of AmB has a significant effect on all samples. And as expected and already seen previsously, those effects are much larger than the ones observed in simple model membranes, reinforcing our beliefs in the validity of our bio-mimetic models.

Moreover, the effects are much more important in the ergosterol-containing membranes as for cholesterol containing ones. Fitting is nearly completed and few samples showed poor bilayer deposition, and need to be done again. Nevertheless, the datas look good and are in accordance with our hypotheses..

Once all samples measured (all contrasts and combinations of hydrogenated/deuterated components), those result will be compared and this will allow us to determine the difference in the mechanism of AmB between mammalian sterol, cholesterol and fungal sterol, ergosterol.



Sample 5 : **dPolChol (30mol%)** : Changes before/after AmB injection.

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).