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Determination of the lipid membrane composition of J774 macrophages cells surexpressing MRP proteins (resistant to Ciprofloxacin)

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Abstract

Ciprofloxacin (CIP) is a fluoroquinolone antibiotic with an activity towards both extracellular and intracellular bacteria (Seral *et al.*, 2005). Diffusion and efflux processes modulate accumulation of this drug within eukaryotic cells. When J774 macrophages were grown in presence of ciprofloxacin, the antibiotic is subject to constitutive efflux through the activity of an MRP-related transporter (Michot *et al.*, 2004).
In view of the critical role of lipids for both drug uptake and activity of MRP proteins (Hinrichs *et al.*, 2004), together with the ability of fluoroquinolones to interact with lipids (Bensikaddour *et al.*, 2008 (a,b)), we investigated the composition of lipids in resistant and sensitive J 774 macrophages to ciprofloxacin.
Firstly, we characterized by thin layer chromatography the phospholipids composition of J774 macrophages cells sensitive (WT) and resistant to ciprofloxacin (CIP). Results showed that sphingomyelin (SM) decreased 2 times whereas phosphatidylinositol increased 1.5 fold in resistant cells. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol didn't show any significant change. Secondly, we studied membrane fluidity of liposomes mimicking WT and CIP resistant J774 cells, by fluorescence polarization spectroscopy. In this respect, we observed a decrease in the melting temperature in vesicles mimicking the membrane composition of CIP resistant cells, indicating that changes in the fluidity of these membranes may be due to the decrease of SM. Studies are currently performed to investigate potential changes in other components of rafts like glucosylceramides. These data might have important relevance to relate the interaction of fluoroquinolones with lipids and change in the processes involved in cellular accumulation of fluoroqinolones.

Materials and methods

a) Materials

Standards lipids (SM, Chol, PC, PE, PI and PS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Plates of thin layer chromatography (concentrating zone) were purchased from VWR international (Leuven, BE) and Amplex Red cholesterol Assay Kit was purchased from Invitrogen, Molecular probes ® (Leuven, BE)

b) Methods

Lipid extraction and separation of phospholipids by TLC

J774 mouse macrophages wild type and Ciprofloxacin resistant cells were routinely grown in RPMI 1640 medium containing 10% fetal bovine serum and incubated at 37°C in a humidified incubator (95% air and 5% CO_2). They were plated in 145 cm^2 cell culture dishes. Extraction of lipids was performed as described in Bligh and Dyer (1959) and Dennison et al., 2006. The phospholipids subclasses present in the wild type and Ciprofloxacin resistant cells were identified by TLC using concentrating zone plates silica gel 60 F_{254} with a mobile phase of chloroform- methanol-acetic acid-water (65:50:4:1, v/v). The plates were then exposed to Copper II reagent and the phospholipid bands were identified by comparing with known standards run in parallel. The spots were scraped and analyzed with phosphorus assay (Bartlett, 1959).

Cholesterol extraction and quantification

Cells were cultured in the same condition as described above. Cells were washed with Tris 10mM pH7.4 buffer and centrifuged at 1000g for 15min at 4°C. The pellet was then resuspended in water miliQ and washed with chloroform: methanol (2:1; v/v), one time with NaCl 0.05M and two times with $CaCl_2$, $2H_2O$ (0.36M: Methanol; 1:1;v/v). The organic phase was then collected with syringe, added to Triton X-100 (1:20, v/v) and evaporated under air efflux. The lipidic film was then resuspended in miliQ water. Cholesterol amount was determined using Amplex Red cholesterol Assay Kit and proteins were quantified using a Lowry assay (Lowry *et al.*, 1951).

Fluorescence polarization studies

Florescence polarization studies were performed on LUVs liposomes prepared in Tris 10 mM pH7.4 buffer at a final concentration of 0.3mM. The proportion of phospholipids used in liposomes was identical to that identified by TLC method and liposomes mimicking wild type membranes J774 macrophages cells and Ciprofloxacin resistant ones were prepared. To investigated the role of cholesterol, it was added in both type of liposomes with a percentage of 5% and 20 %
Incorporation of diphenylhexatriene (DPH) at a molar ration of 1:250 was obtained by preincubation at 37°C during 1 h of liposomes with the probe. The measurement was performed on a LS-55 Perkin Elmer fluorimeter (Perkin-Elmer, Beacons-field, UK). Temperature was raised from 8°C to 40°C with equilibration time of 5 minutes. Excitation and emission wavelengths were 381 and 426 nm respectively.

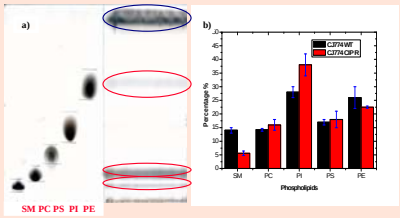

Figure 1. Composition of different phospholipids in J 774 macrophages Cells (WT and CIP Resistants) as revelled by TLC

In (a) phospholipids bands corresponding to SM, PC, PE, PI and PS as revelled by Copper II reagent. In (b), the individual phospholipids were quantified by phosphorus assay for WT J774 cells (dark bars) and CIP resistant cells (red bars).

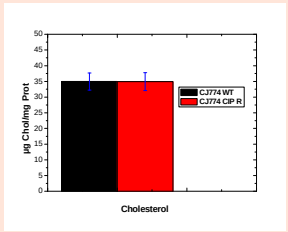

Figure 2. Cholesterol quantification in macrophages cells J774

WT J774 cells (dark bars); CIP resistant cells (red bars)

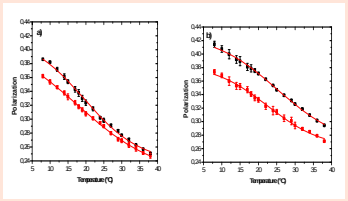

Figure 3. Fluorescence polarization thermotropic curves of liposomes

The liposomes mimicking wild type (dark square ■) and ciprofloxacin resistant (red square ■) membranes J774 macrophages cells. Cholesterol was added at percentage of five in (a) and twenty in (b). *Liposomes (0.3 mM) were prepared at pH7.4 and DPH probe was incorporated with a molar ration of (1:250).*

Results

1- Phospholipids contained in macrophages cells J774 (Wild type and Ciprofloxacin resistant cells)

Firstly, we checked if the macrophage cells J774 wild type and those surexpressing MRP protein (CIP resistant) present a modification in phospholipids composition. Using thin layer chromatography, we detected phospholipids bands corresponding to SM, PC, PE, PI and PS. Indeed, the following composition was quantified by phosphorus assay: 14%, 14.2%, 28%, 17% and 26 % for SM, PC, PI, PS and PE respectively for WT J744 cells and 5.6%, 16%, 38%, 18% and 22.5% for CIP resistant J774 cells. Nevertheless, a large band in front of the migration of the plate was observed and is under investigation. This band may be related to glycosylceramide lipids (Fig.1 a). As shown in Fig. 1b no significant changes were observed for all phospholipids examined, except sphingomyelin (SM) and phosphatidylinositol (PI). In fact, the SM decreased two fold whereas the PI increased 1.5 fold in resistant cells.

2- Cholesterol quantification in WT and CIP resistant J774 cells

From TLC results, the decrease of SM in CIP resistant J774 cells should be related to a change in free cholesterol (FC). Indeed, SM and free cholesterol are the most components of rafts domains that are involved in activity of MRP proteins (Hinrichs *et al*., 2004). We quantified 35 ±3µg FC/mg proteins for both extract of J774 cells (WT and CIP Resistant) (Fig. 2). These data suggest that free cholesterol is not involved in modification of lipids environment of J774 CIP resistant cells

3- Membrane fluidity of liposomes mimicking WT and CIP resistant membranes J774 cells

Since Ciprofloxacin have previously been shown to interact with negatively charged and zwitterionic vesicles (Bensikaddour *et al*., 2008 (b)), we tested whether the changes of lipids compositions in resistant J774 cells membranes could be responsible of changes in fluidity of these membranes. To this end, two types LUVs liposomes were used mimicking the lipid composition of WT and CIP resistant cells. The thermotropic fluorescence polarization profiles of the DPH-labelled vesicles are shown in Figure 3.
To further investigate the role of cholesterol, it was added at percentage of five (Fig. 3a) (as find from our calculation), and of twenty (Fig. 3b). A significant change in the thermotropic curves was observed, for all cases. In fact, a significant decrease of polarization values was observed in function of increasing in the temperature. Vesicles mimicking CIP resistant cells showed lower values of polarization suggesting a higher fluidity. This difference was more marked when vesicles contained 20 % of cholesterol.
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Summary and Conclusion

In the present study, we characterized the lipids composition of J774 membranes of wild type and resistant cells to ciprofloxacin. We also determined fluidity of vesicles mimicking the two types of membranes. Our results shows:
- No significant changes in PC, PS, PE and cholesterol between two types of cells. A decrease of SM by two folds and increase of PI by 1.5 fold in CIP resistant J774 cells.
- An increase of the fluidity of vesicles mimicking membrane composition of CIP resistant cells as compared to those mimicking WT macrophages cells. This could be related to modification of lipids composition such sphingomyelin.

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