APPENDIX A

Proton NMR Visible Mobile Lipid Signals in Sensitive and Multidrug Resistant K562 cells are Modulated by Rafts.

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Proton NMR visible mobile lipid signals in sensitive and multidrug resistant K562 cells are modulated by rafts.

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Abstract

Background: Most cancer cells are characterized by mobile lipids visible on proton NMR (1 H-NMR),these being comprised mainly of methyl and methylene signals from lipid acyl chains. Erythroleukemia K562 cells show narrow signals at 1.3 and 0.9 ppm, corresponding to mobile lipids (methylene and methyl, respectively),which are reduced when K562 cells are multidrug resistant (MDR).While the significance of the mobile lipids is unknown,their subcellular localization is still a matter of debate and may lie in the membrane or the cytoplasm. In this study, we investigate the role of cholesterol in the generation of mobile lipid signals.
Results: The proportion of esterified cholesterol was found to be higher in K562–sensitive cells than in resistant cells,while the total cholesterol content was identical in both cell lines. Cholesterol extraction in the K562 wild type (K562wt) cell line and its MDR counterpart (K562adr),using methyl–β–cyclodextrin,was accompanied by a rise of mobile lipids in K562wt cells only.The absence of caveolae was checked by searching for the caveolin–1 protein in K562wt and K562adr cells. However,cholesterol was enriched in another membrane microdomain designated as "detergent–insoluble glycosphingomyelin complexes"or rafts.These microdomains were studied after extraction with triton X-100, a mild non-ionic detergent,revealing mobile lipid signals preserved only in the K562wt spectra.Moreover,following perturbation/disruption of these microdomains using sphingomyelinase,mobile lipids increased only in K562wt cells.
Conclusion: These results suggest that cholesterol and sphingomyelin are involved in mobile lipid generation via microdomains of detergent-insoluble glycosphingomyelin complexes such as rafts. Increasing our knowledge of membrane microdomains in sensitive and resistant cell lines may open up new possibilities in resistance reversion.
Keys Words : Multi-drug resistance, NMR spectroscopy, cholesterol, raft.

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1. INTRODUCTION

When studied by NMR proton spectroscopy, most cancer cells are characterized by increased narrow signals at 0.9 and 1.3 ppm corresponding, respectively, to methyl and methylene resonances that belong to lipid acyl chains moving isotropically. This so–called "mobile lipid signal" (or ML signal) has been studied for several decades (for review see Hakumaki *et al.*, 2000). However, research has failed to elucidate the molecular origin of mobile lipids, their subcellular localization or their physiological significance.

Regarding their molecular origin, acyl chains can form part of triglycerides or esterified cholesterol. Phospholipidic acyl chains may also be involved if they are not embedded in membrane lipid bilayers. As far as localization is concerned, two types of subcellular origin are still debated. As early as 1988 (Mountford *et al.*, 1988), Mountford *et al.* proposed an origin in lipoprotein-like microdomains within the plasma membrane. More recently, some authors (Iorio *et al.*, 2003) have suggested the occurrence of cytosolic droplets, which appear concomitantly with the ML in stressed cells (Barba *et al.*, 1999) or which are associated with necrosis (Zoula *et al.*, 2003) and apoptosis. However, it has been shown that ML intensity is not always correlated with the number of cytoplasmic droplets (Le Moyec *et al.*, 1997). These studies (op cit.) tend to show that cytosolic lipid droplets generate mobile lipid signals in NMR spectra, without excluding the possibility that such signals could arise independently of the presence of cytosolic lipid bodies.

^{1}H–NMR spectroscopy shows that ML are decreased in erythroleukemia K562 cells when these become resistant (K562adr) (Le Moyec *et al.*, 1996), whereas the compositions of lipids extracted from K562wt (drug sensitive) and K562adr cells are the same, except for sphingomyelin content which is increased in resistant cells (Le Moyec *et al.*, 2000). Thus, although we may explain this by a structural difference in lipid "organization", the significance of such a behaviour is not yet elucidated. In a previous study (Mannechez *et al.*, 2001), we showed that the ML signal is not linked to the externalization of phosphatidylserine (a membrane phospholipid), this being a phenomena that precedes apoptosis.

In model membranes, free cholesterol interacts with phospholipids and sphingolipids to influence membrane fluidity (Finegold *et al.*, 1991). *In vivo*, cellular–free cholesterol is located in the plasma membrane (Lange *et al.*, 1993), which exhibits increasing structural order as demonstrated in erythrocytes (Rooney *et al.*, 1984), LM and CHO cells (Rintoul *et al.*, 1979; Sinensky, 1978). Moreover, cholesterol in model membranes is able to promote microdomains towards an intermediate state called the liquid–ordered phase (Lo), with less fluidity than the gel phase state and more fluidity than the surrounding membrane in the liquid crystalline state (Sankaram *et al.*, 1990).

The aim of this study was to investigate a possible contribution from the cholesterol-containing membrane domain to the ML detected in the NMR proton spectra of wildtype K562 and K562 adriamycin-resistant cells.

In a first set of experiments, cell membrane cholesterol was extracted by methyl–β–cyclodextrin (MCD). MCD

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is an oligosaccharid able to pump cholesterol out of the cell (Kilsdonk *et al.*, 1995). Moreover, cholesterol extracted by MCD originates primarily from the plasma membrane (Ilangumaran *et al.*, 1998). Cell incubation with MCD was expected to change the membrane microdomains via cholesterol extraction, and consequently produce variations in ML. In a second set of experiments, we used triton–X100 to separate membrane microdomains and then investigated their role in ML generation. In this study, we use the term "Detergent InsolubleGlycosphingolipid Complexes "(DIGCs) or "rafts" to refer to the membrane microdomains isolated by treatment with this mild non-ionic detergent (for review see Brown *et al.*, 2000). Finally, as these raft microdomains are also enriched in sphingolipids, we used sphingo-myelinase to modify their "organization", with the aim of inducing changes in ML signal intensity.

2. MATERIAL AND METHODS

2.1 *Chemicals*

Methyl-β-cyclodextrin, sphingo-myelinase, cholesterol, chloroform, isopropanol and paraformaldehyde, as well as deuterium oxide (D_2O) and all chemicals for western-blot analysis were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Triton X–100 was supplied by Merck (Schuchardt, Darmstadt, Germany), and methanol by Acros Organics (Geel, Belgium).

2.2 Cell culture

Human erythroleukemia K562 cells were grown in the culture medium RPMI 1640 (Bio-Whittaker Europe, Verviers, Belgium) supplemented with foetal calf serum (FCS) (10%) and glutamine (2 mM) (R10 medium) at 37°C and 5 % CO_2 . The K562 resistant variant cells (K562adr) were a gift from F. Calvo, Université Paris 7, and were grown in the same medium with 0.5 µg/ml adriamycin.

2.3 Cholesterol quantification

Lipids were extracted as previously described (Bligh *et al.*, 1959) and then desiccated and resuspended in isopropanol. Total cholesterol and free cholesterol were assayed using a kit (Cholesterol RTU, Biomérieux Craponne and Boeringher, France) based on cholesterol esterase. This was followed by cholesterol oxidase reaction and colour development measured on an absorption spectrometer at 500 nm. Concentration was determined using a standard solution of 0.5 mg/ml cholesterol. We first checked that the solvent (isopropanol or chloroform) did not have any effect on colour development.

2.4 Cholesterol extraction using methyl-β-cyclodextrin

Cells were incubated in RPMI, glutamin 2 mM, containing methyl–β–cyclodextrin 5 mM for 2 hours at 37°C, 5% CO_2. Controls were incubated in RPMI, glutamin 2 mM, with or without FCS 10% for 2 hours.

2.5 Proliferation

After incubation with methyl–β–cyclodextrin, with RPMI+ FCS or with RPMI (the last being used as a control for RPMI+FCS, considering that methyl–β–cyclodextrin is not used in the medium with FCS), cells were collected, washed and resuspended at 150,000 cells/ml in RPMI, glutamin 2 mM, FCS 10%. Cells were seeded in triplicate on 96-well microplates and

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incubated at 37°C and 5% CO_2. Proliferation was assessed by counting cells with a cell coulter (Beckman Coulter, ZI model) at 24, 48 and 72 hours after seeding.

2.6 Caveolin-1 detection: western-blot

Cells were collected in late log-phase and washed twice in PBS 150 mM and were resuspended in loading buffer (urea 4 M, lauryl sulphate 3.8%, glycerol 20%, tris base 75 mM pH 6; β -mercaptoethanol 5%; bromophenol blue 5%) for 5 mn at 95°C. They were centrifuged at 12500 g, 4°C for 10 minutes, and 50 µg of extracted protein were deposited per well on an 8% acrylamide gel. Migration was performed in an electrophoresis buffer (TRIS base 125 mM; glycine 250 mM; laurylsulphate 0.1%, pH 8.1) for 1 hour at 125 V with a size marker (Kaleidoscope prestained standards Bio-rad). Protein was transferred on a nitrocellulose membrane for 2 hours at 80 V and 4°C in a transfer buffer (tris-base 48 mM; glycine 39 mM, SDS 0.037%, methanol 20%). Non-specific sites were blocked with 1% milk (Regilait, skimmed milk, Saint-Martin-Belle- Roche, France) in TBS 1X (tris-base 20 mM, NaCl 500 mM, and pH 7.5). The nitrocellulose membrane was incubated for 1 hour at room temperature with 1 µg/ml anti-caveolin 1 rabbit antibody (Tebu, France) and washed three times for 10 minutes with TBS before incubation for 1 hour at room temperature with goat anti-rabbit and conjugate secondary antibody (1:3000). After three washes in TBS for 10 minutes, staining was developed in a phosphatase alkaline buffer ($NaHCO_3$ 0.1 M; $MgCl_2$, $6H_2O$ 1 mM) with tetrazolium nitro-blue 3% in N,N-dimethylformamide 70% and bromo-4-chloro-3-indolylphosphate p-toluidine 1% in N,N-dimethylformamide 70% for 30 minutes at room temperature in the dark.

2.7 Triton treatment

Cells were collected at the end of the log-phase, 4 days after seeding, when ML were at a maximum, and washed twice with 1 ml PBS 150 mM at 4°C. They were resuspended in paraformaldehyde (PFA) 3% in PBS 150 mM for 30 minutes at 4°C and washed twice with 1 ml PBS 150 mM at 4°C to be resuspended in triton X-100 1% in PBS 150 mM at 4°C for 30 minutes. Cells or triton-insoluble fractions were collected and washed for NMR experiments at 25°C.

2.8 Sphingomyelinase treatment

After triton treatment, cells were collected, washed with PBS 150 mM and treated for 35 mn at 37°C with 0.5 units of sphingomyelinase. Cells were washed as described for NMR experiments at 25°C.

2.9 NMR analysis

Cells were washed twice in 1 ml PBS 150 mM, twice in PBS/D 2 O, centrifuged at 250 g and counted. Then, 10^7 cells were resuspended in 400 µl PBS/D 2 O before transfer to a 5-mm Shigemi NMR tube. Experiments were performed without rotation, and the analysed cell pellet was maintained in the coil volume in the Shigemi NMR tube. The NMR proton spectra of whole cells were obtained at 25°C on a Unity Inova spectrometer (Varian, France) working at 500 MHz. One-dimensional runs were per-formed by accumulating 128 transients of 90° pulse with 2 s relaxation time. The signal from the residual water was suppressed by the presaturation technique, by using 0.03 mW irradiation for 2 s. Acquisition time was 1.34 s on 16K data points, corresponding to a spectral width of 6

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kHz. The Fourier transform was applied without zero-filling using an exponential window multiplication function corresponding to 1 Hz line broadening. The resonances were integrated after automatic baseline correction. Two-dimensional COSY runs were performed with 2K data points in the F2 direction and 256 data points in the F1 direction. The two-dimensional Fourier transformation was applied after zero filling to 512 data points in the F1 direction with a sine-bell function in both directions. Each run consisted of a one-dimensional acquisition and a two-dimensional COSY spectrum. Peak assignments were based on data from the literature. The peak areas were measured by manual integration with the Bruker WINNMR software using a manual tangential baseline correction for each peak, and the assigned peak areas were normalized to the creatine peak area. The following resonances were integrated: methyl group (CH_3 at 0.9 ppm), methylene group (CH_2 at 1.3 ppm), choline N–trimethyl group ($N^+(CH_3)_3$ at 3.2 ppm) and creatine (CH_3 at 3.05 ppm). The values obtained for the different treatments of the two cells lines were compared by ANOVA analysis followed by a Student–Neumann–Keuls test for group–to–group comparison. $P < 0.05$ was considered as a significant value.

NMR proton spectra of PFA or triton– or SMase–treated cells were acquired at 500 MHz and 25°C on a Bruker Advance DMX500 spectrometer (Bruker, Wissembourg, France). The runs consisted of a 1D acquisition with similar parameters as the previous 1D spectra, and the spectrum analysis was performed as previously with Bruker WINNMR software.

The possible contribution of lactate to the 1.3 ppm signal was eliminated by analysing 2D Cosy spectra in whole cells, which resolved the resonances of lactate from fatty acid chains. In fixed cells, we calculated the ratio of the double-bond signal (CH=CH at 5.4 ppm) to CH_2 peak area. As both groups belong to fatty acyl chains, this ratio remained constant since lactate did not significantly contribute to the increase of the CH_2 signal (Hakumaki *et al.*, 2000).

3.RESULTS

3.1 Cholesterol quantification

K562wt and K562adr cells do not differ in their total cholesterol contents. The proportion of cholesterol-ester were found to be lower in the resistant cells than in the wild type cells (Table 1). This implies that K562wt cells contain more free cholesterol than K562adr cells. Total cholesterol content after MCD treatment (Table 1) shows a 45% decrease in both K562wt and K562adr cells with respect to the control.

Table 1: Quantification of cholesterol in K562 cells. Results are expressed as means ± SD.

	K562 wt	**K562adr**
Total cholesterol (μg/10^6 cells)	3.6 ± 0.5 n=5	3.5 ±0.3 n=4
Cholesterol ester (percentage of total cholesterol)	26.5 ±1.3 n=6	19.4 ±2.4 n=5
Cholesterol variation with MCD treatment (% of control)	57 ± 13 n=5	54 ± 15 n=4

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3.2 Cell proliferation after incubation with methyl-β-cyclodextrin :

The cell proliferation results for K562wt and K562adr are presented in Figs. 1 and 2. The two–hour incubation without FCS had no effect on proliferation in K562wt or K562adr when compared to cells incubated in RPMI+FCS. Treatment with MCD inhibited proliferation in K562wt. This was not the case with K562adr, which grew normally. When K562adr cells were resuspended in R10 containing daunorubicin (0.25 nM), they were able to proliferate at this daunorubicin concentration. However, after treatment with MCD, the K562adr proliferation rate was decreased as shown in figure 2. This shows that MCD treatment reversed the resistance phenotype in K562adr; despite having no effect on cell growth, MCD affected the K562adr cell membrane.

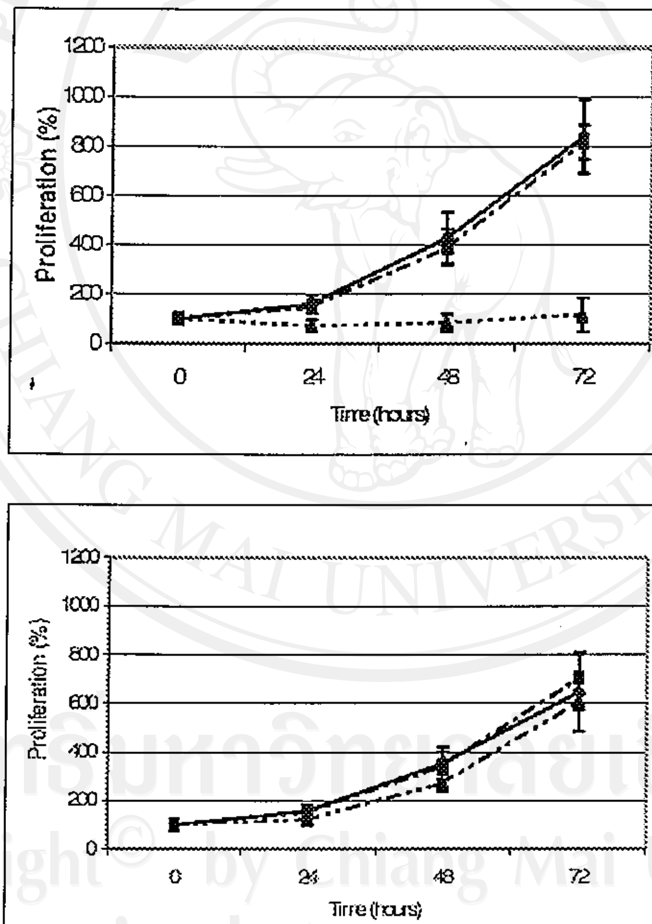

Figure 1 K562wt cells (up)and K562adr cells (down)were incubated for 2 hours in medium alone (WT RPMI; full line and square, n =6) supplemented in FCS 10%(dot line and lozenge, WT R10;n =6),in methyl-β-cyclodextrin 5 mM (dot line and triangle, WT MCD; n =6)and successively incubated in R10 for 72 hours. Data points are the percentages of the cellular concentration normalized to cellular concentration at T =0 hours expressed as means, with vertical bars representing standard deviation (SD).

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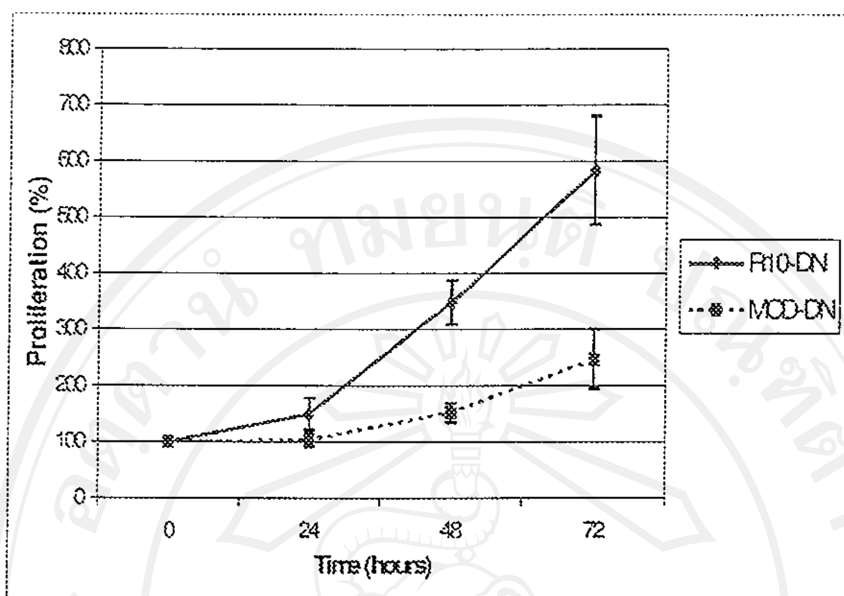

Figure 2. K562adr resistance modulation after MCD treatment. K562adr cells were incubated for 2 hours in medium supplemented in SVF 10% (R10-DN; n =4) or in methyl-β-cyclodextrin 5 mM (CD–DN; n =4) and seeded in R10 containing daunorubicin for 72 hours. Data points are the percentages of the cellular concentration normalized to cellular concentration at T = 0 hours expressed as means, with vertical bars representing standard deviation (SD).

3.3 Caveolin-1 expression : Western-Blot

Caveolin–1 expression was studied in K562 cells by SDS-page and western-blotting. Caveolin–1 is a protein of 21 kD (Kurzchalia *et al.*, 1992). As a positive control, we chose a protein extract of NIH-3T3 cells that are known to express caveolin-1. In this control, a band appeared between the 19 and 32 kD size markers. In the present study, however, neither K562wt nor K562adr expressed caveolin-1.

3.4 ^{1}H-NMR spectra

a) Incubation with methyl-β-cyclodextrin : Figure 3 shows spectra obtained on K562wt and K562adr cells, and resonance ratios are presented in Table 2. These results show that MCD increases the lipid signals only in K562wt cells. This increase can also be seen in Table 3, which reports a significant increase of CH_3 /Ct and CH_2 /Ct ratios in MCD–treated K562wt cells compared with non-treated cells.

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Table 2. NMR peak ratios measured on K562wt and K562adr spectra. Cells were incubated for 2 hours in RPMI, or in RPMI containing methyl–β–cyclodextrin 5 mM (MCD).Results are expressed as means of arbitrary units ±SD multiplied by ten. Statistical analysis: *P <0.05 when compared with RMPI incubated cells.

Group	K562wt		K562adr	
	RPMI (*n=6*)	MCD (*n=5*)	RPMI (*n=4*)	MCD (*n=4*)
CH_3/Ct	50 ± 5	101 ± 22*	34 ± 3	34 ± 2
CH_2/Ct	247 ± 35	537 ± 13*	142 ± 22	130 ± 18
$N^+(CH_3)_3$/Ct	23 ± 3	22 ± 7	17 ± 3	21 ± 16

Table 3. NMR peak ratios measured on K562wt and K562adr spectra. PBS: incubation in PBS; PFA: incubation in PFA; Triton: incubation in PFA and triton X; Smase: incubation in PFA, triton X–100 and 0.5 units sphingomyelinase. Results are expressed as means of arbitrary units ± SD multiplied by ten.

Group	K562wt				K562adr			
	PBS (*n=4*)	**PFA** (*n=7*)	**Triton** (*n=7*)	**Smase** (*n=4*)	**PBS** (*n=4*)	**PFA** (*n=7*)	**Triton** (*n=8*)	**Smase** (*n=5*)
CH_3/Ct	198 ± 21	231 ± 12	312 ± 42*	392 ± 105°	161 ± 19	192 ± 22	265 ± 62*	267 ± 42*
CH_2/Ct	541 ± 52	611 ± 78	1060 ± 164*	1696 ± 441°	308 ± 93	386 ± 56	695 ± 141*	565 ± 167*
$N(CH_3)_3$/Ct	132 ± 6	141 ± 18	53 ± 10*	79 ± 17°	114 ± 12	132 ± 36	60 ± 15*	94 ± 39*

Statistical analysis : * : P<0.05 when compared to PBS incubated cells,
° : P<0.05 when compared to Triton treated cells,

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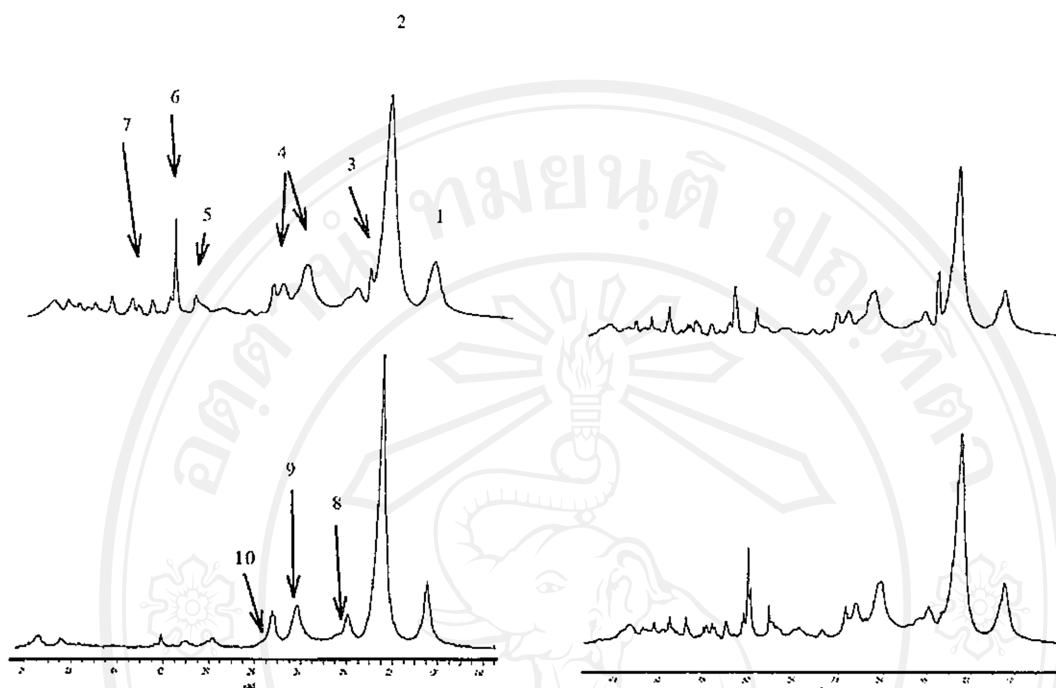

Figure 3. NMR proton spectra obtained at D3 on K562wt (left side) and K562adr (right side) on control cells (top spectra) or treated with 5 mM MCD during 2 hours (bottom spectra). Peak assignment 1: CH_3 from fatty acid chains; 2: CH_2 from fatty acid chains; 3: Alanine; 4: glutamine and glutamate; 5: creatine; 6: N-trimethyl from choline; 7: Inositol; 8: CH_2 in β position from ester function in fatty acid ester; 9: CH_2 in a position of a double bond in fatty acids; 10: CH_2 in α position from ester function in fatty acid ester.

b) Triton X-100 and sphingomyelinase treatments: Spectra obtained after treatments with PFA, triton and sphingomyelinase are presented in Figures 4 and 5 for K562wt and K562adr cells, respectively. The ratios of the peak intensities with respect to the creatine peak are reported in Table 3. When compared to the control, PFA treatment did not modify the cell spectra. After microdomain separation by triton treatment, the CH_2/Ct ratio was increased for K562wt cells and, to a lesser extent, for K562adr cells. N–trimethyl to creatine peak ratios were decreased in both cell lines. After treatment of these microdomains with sphingomyelinase, the spectra showed an increased CH_2/Ct ratio compared with the previous spectrum obtained on "DIGCs" in K562wt cells. However, the spectra of K562adr cells treated with Smase remained unchanged compared with spectra obtained on DIGCs from K562adr cells.

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4. DISCUSSION

We studied the modification of cell spectra using cholesterol and sphingomyelin because: i) cholesterol levels affect membrane fluidity; ii) membrane microdomains, rafts and caveolae are all enriched in these lipids. Western–blot did not detect the presence of caveolin-1 in either K562wt or K562adr. This confirms previous results showing the absence of caveolin–1 in K562wt (Hatanaka *et al.*, 1998). Even if membrane microdomains exist in this cellular type, this implies they represent rafts and not caveolae

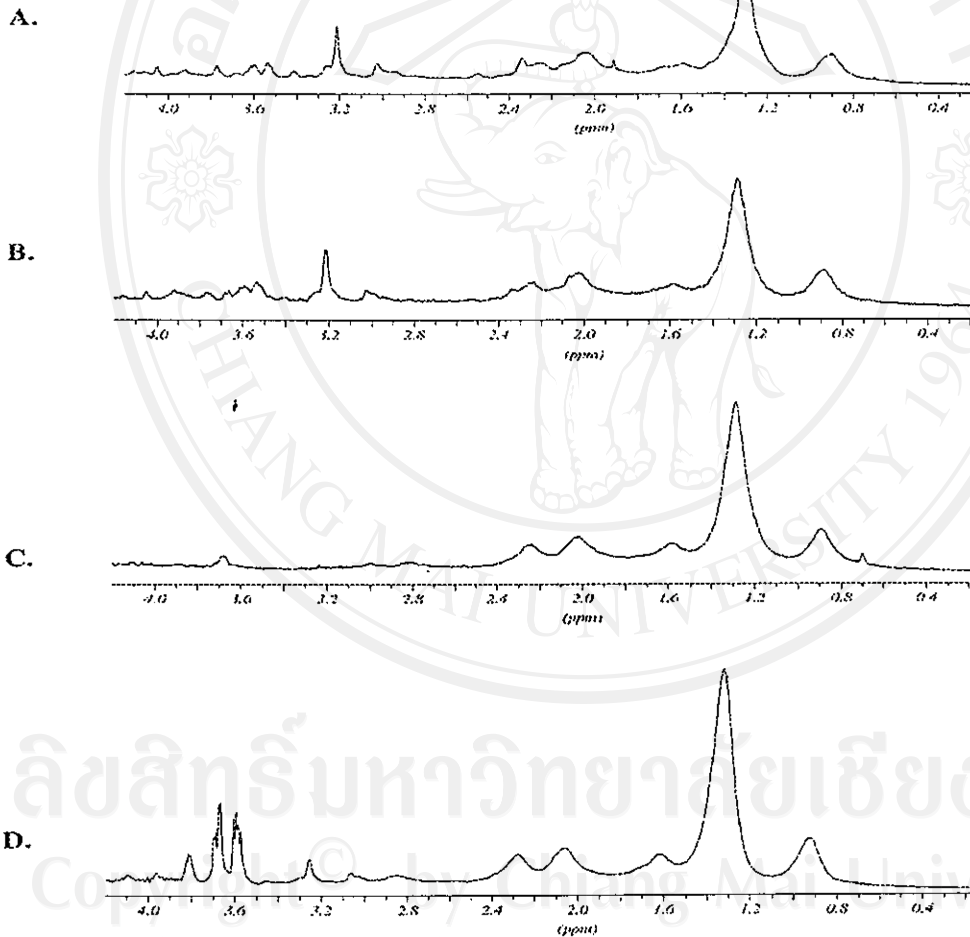

Figure 4. K562wt ^{1}H–NMR spectra : effects of Triton X–100 and sphingomyelinase treatments. A. non-treated cells; B. cells fixed with PFA; C. cells fixed with PFA 4% in triton-X100 1%; D: cells fixed with PFA 4% incubated in triton-X–100 1% and with 0.5 units sphingomyelinase. For peak assignment see figure 4. Peaks at 3.6ppm after SMase treatment arise from enzyme working buffer.

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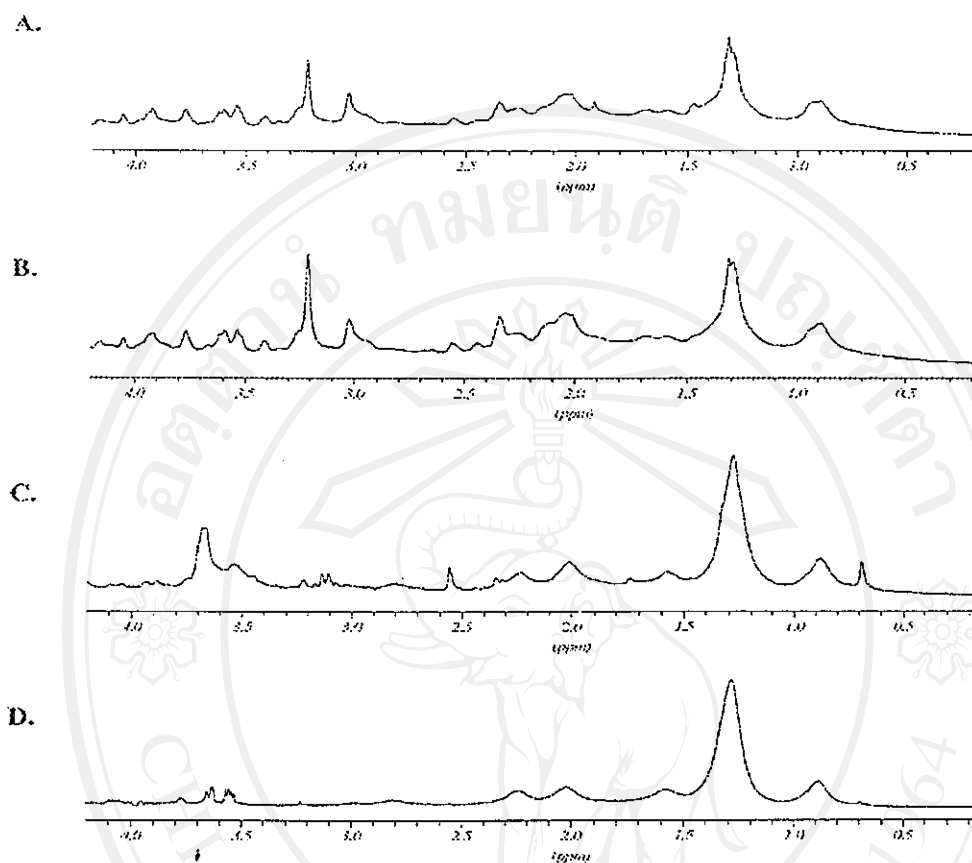

Figure 5. K562*adr* ^{1}H–NMR spectra: effects of Triton X–100 and sphingomyelinase treatments. A. non–treated cells; B. cells fixed with PFA; C. cells fixed with PFA 4% in triton–X100 1%; D: cells fixed with PFA 4% incubated in triton–X100 1% and with 0.5 units sphingomyelinase.H-NMR spectra: Triton X–100 and sphingomyelinase treatment. For peak assignment see figure 4. Peaks at 3.6ppm after SMase treatment arise from enzyme working buffer.

Rafts have been described as corresponding to membrane micro-domains enriched in cholesterol and sphingomyelin lipids (Brown *et al.*, 2000). In our first set of experiments, we disrupted the rafts using MCD to extract cholesterol from the cells. Before MCD treatment, K562wt and K562adr cells displayed the same cholesterol concentrations, which were found to be in agreement with previous results obtained for other cancer cells (Adam *et al.*, 1975; Mackinnon, *et al.*, 1992). In K562wt cells, this cholesterol was preferentially in the form of cholesterol-ester, implying that the absolute concentration of free cholesterol is lower in K562wt cells compared with K562adr cells. 90% of the free cholesterol is contained in plasma membrane (Lange *et al.*, 1993). On the other hand, cholesterol-ester synthesized in the endoplasmic reticulum is generally localized in the

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cytosol and is able to form cytoplasmic lipid bodies. This cholesterol is less available to MCD extraction. Thus, MCD treatment may affect the free–cholesterol plasma membrane pool (Ilangumaran *et al.*, 1998) and not the intracellular pool that mainly contains cholesterolester in K562wt cells. Consequently, in our study, a higher proportion of membrane cholesterol (corresponding to free cholesterol) was extracted in K562wt cells compared against K562adr cells. This may account for the fact that the proliferation rate for K562wt was more affected than for K562adr. Moreover, the removal of a greater proportion of membrane cholesterol modified the NMR proton spectra of the cells, since the mobile lipid signals only increased in the K562wt cells.

Nevertheless, we observe that MCD treatment affected the K562adr cell line. This is demonstrated by the reversion of daunorubicin resistance. In the plasma membrane, K562adr cells express the P–gp glycoprotein responsible for the extrusion of drugs. When K562adr cells were treated with MCD, the resistance to daunorubicin was decreased, showing that the P–gp is unable to extrude daunorubicin from the cells. P–gp has been widely reported to be sensitive to the lipid content of plasma membrane (Romsicki *et al.*, 1999). The present study shows that MDR is sensitive to the cellular cholesterol content. Indeed, it has been shown that drugs can accumulate in MDR cells grown in the presence of MCD (Grosse*et al.*, 1997).

This first set of experiments suggests that ML signals are generated during the disruption of membrane microdomains in K562wt cells, because MCD has been shown to extract cholesterol preferentially from membrane microdomains (Ilangumaran *et al.*, 1998). Overall, these results tend to show a link might exist between microdomains and the ML signal in K562 cells.

In a second set of experiments, we isolated rafts using triton X–100 at 4°C, a mild detergent in which they are insoluble (Parolini *et al.*, 1996). We applied this method to K562wt and K562adr cells. NMR spectra obtained on these isolated raft fractions show an increase of the ML signals for K562wt and K562adr cells. In this particular case, this implies that ML could be produced by raft domains. Although it has been shown that raft structures are tightly packed at 4°C, there is no report of their organization/structure at 25°C, which the temperature is chosen for the NMR experiments (London *et al.*, 2000). At this temperature, microdomain lipids could be in another physical state allowing them to move more isotropically and produce an NMR–visible signal: their solubility in triton at 25°C supports this hypothesis (Melkonian *et al.*, 1995).

In the final set of experiments, we incubated detergent insoluble fractions with sphingomyelinase, which was supposed to metabolise the sphingomyelin included in the rafts. Only the K562wt spectra exhibited an increase in ML signal compared with the raft spectra. Again, this suggests that microdomains are differently organized between K562wt and K562adr. Several hypotheses may be proposed to explain the difference between K562wt and K562adr: i) sphingomyelin concentrations are higher in K562adr; ii) lipid microdomains in K562adr may be organized in such a way that sphingomyelin is inaccessible to sphingomyelinase and iii) there are

more microdo-mains in K562adr, as suggested previously by some authors (Lavie *et al.*, 2001).

5. CONCLUSIONS

These results suggest that, while isolated rafts generate ML signals within the membrane, the presence of rafts seems to maintain a relatively tight organization. When this organized structure is disrupted by MCD or sphingomyelinase, the ML signal may be detected in K562wt cells and the P–gp function can then be modulated in K562adr cells. Taken together, the results suggest that ML signals may be sensitive to modifications in the lipidic organization of the cells and membrane, in which microdomains behave as rafts enriched in cholesterol and sphingo-myelin. A better knowledge of these microdomains, as well as the dif ferences in behaviour between sensitive and resistant cells, could open up new therapeutic perspectives for reversing the drug–resistance phenotype.

LIST OF ABBREVIATIONS:

NMR Nuclear Magnetic Resonance, MDR: multi-drug resistance, ML : mobile lipid, K562wt: K562 wild type, K562adr : K562 adriamycin-resistant, MCD : methyl-β-cyclodextrin, DIGC : dtergent insoluble glycosphingolipid complex, FCS : fœtal calf serum, R10: RPMI culture medium with 10% FC, PBS : Phosphate buffer saline, TRIS: Tris(hydroxymethyl)-1,3-propanediol, TBS : TRIS buffer Saline, PFA : paraformaldehyde, COSY : correlated-spectroscopy, CH_3: methyl, CH_2: methylene, $N(CH_3)_3$: N-trimethyl, Ct : creatine

PARTICIPATION OF AUTHORS:

all authors contributed equally to this work

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APPENDIX B

Decrease of P-Glycoprotein Activity in K562/ADR Cells by MβCD and Filipin and Lack of Effect Induced by Cholesterol Oxidase Indicate That This Transporter is Not Located in Rafts

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Decrease of P-glycoprotein Activity in K562/ADR Cells by MβCD and Filipin and Lack of Effect Induced by Cholesterol Oxidase Indicate That This Transporter is Not Located in Rafts

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ABSTRACT

The effect of low–density membrane domains on function of the plasma membrane transporter P–glycoprotein (P–gp), involved in multidrug resistance (MDR) phenotype, has been investigated in K562/ADR cells. To this end we reversibly altered the cholesterol content of K562/ADR cells by using methyl–β–cyclodextrin as a ,cholesterol chelator and conversely we repleted them through incubation with cholesterol in culture medium. We also used the cholesterol-binding fluorochrome filipin and cholesterol oxidase. Our data show that either cholesterol depletion or complex formation with filipin resulted in a strong decrease of P–gp activity. However, when cells were incubated with cholesterol oxidase that are known to disrupt rafts, no modification of the P–gp activity was observed. In addition, using a free–detergent methodology to separate by ultracentrifugation, "light," "heavy," and "extra heavy" fractions we show that no P–gp is found in the "light" fraction where rafts are usually detected. Altogether, our data strongly suggest that, in this cell line, P-gp is not localized in rafts.

Key words: Multidrug resistance; P–glycoprotein; transport; cholesterol; raft

Key to abbreviations: P–gp, P–glycoprotein; MDR, Multidrug Resistance; MCD, Methyl–β–cyclodextrin; Amplex Red, 10-acetyl-3,7-dihydroxyphenoxazine; HRP, Horseradish peroxidase; COase, cholesterol oxidase; FCS, fetal calf serum; PIRA, pirarubicin; DOX, doxorubicin; DNR, daunorubicin; IDA, idarubicin; CTB, cholera toxin B; TX, Triton X–100.

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1. INTRODUCTION

Cancer is a major worldwide health concern, with an estimated 6 millions new cases per year. Until now, the only possible methods of curing systemic cancers, such as leukemia, lymphoma, and unifocal tumors that have spread by metastasis, have involved systemic treatments such as chemotherapy and immunotherapy. Unfortunately, clinical resistance to anticancer drugs has been observed from the onset of chemotherapy. In addition to this intrinsic and primary resistance to first-line chemotherapy, clinicians are often confronted by the progressive resistance of tumors that were initially sensitive to treatment. Therefore, the appearance of cell populations resistant to multidrug–based chemotherapy constitutes the major obstacle to cure (Bordow *et al.*, 1994; Baldini *et al.*, 1995; Leith *et al.*, 1995).

Multidrug resistance (MDR) involves resistance to naturally derived anticancer agents, such as anthracyclines, vinca alkaloids, epipodophyllotoxins, and taxanes. This form of resistance is due to multifactorial mechanisms, such as decreased cellular topoisomerase II, increased cellular glutathione, and increased ATP–dependent efflux of the cytotoxic agent over the cellular plasma membrane due to the presence of drug efflux pumps (Alvarez *et al.*, 1998; Beck *et al.*, 1995; Brock *et al.*, 1995; Hasegawa *et al.*, 1995).

Biochemical research has uncovered three types of drug pumps that may play a role in MDR. The most intensively studied is the 170–kDa P–glycoprotein (P–gp) encoded by the MDR1 gene in humans. A variety of studies have shown that P–gp acts as a drug pump, localized in the plasma membrane of tumor cells, which can recognize an astonishing range of cytotoxic molecules and remove them from the cell. The effects of various lipid species on P–gp functions have been reported and overall it appears that optimal function of the protein may be supported by lipids such as cholesterol and the nonbilayer–forming phosphatidyl ethanolamine. MDR cells contain increased numbers of cholesterol– and sphingolipid-rich microdomains containing caveolins known as caveolae. Furthermore, localization of P–gp in these ordered microenvironments within the membrane has been reported (Rothnie *et al.*, 2001). Also, Luker *et al.*,(2000), have shown that in polarized cells, P–gp is localized in apical membranes, domains that are enriched in sphingolipids and cholesterol compared with basolateral surface. In general, changes in the cholesterol content of biological membranes are known to alter the lipid fluidity and thus membrane integrity (Yeagle, 1989; 1991).

Considering other transport proteins, it has been shown (i) that membrane cholesterol modulates serotonin transport activity (Scanlon *et al.*, 2001), (ii) that cholesterol has modulator effects on the function of two structurally related peptide receptors, the oxytocin receptor and the brain cholecystokinin receptor in plasma membranes as well as in intact cells (Gimpl *et al.*, 1997).

The effects of low density membrane domains on function of the protein remain almost unexplored in whole cells systems and in this study we have investigated whether membrane cholesterol also modulates

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the functional properties of the P–gp transporter expressed in K562/ADR cells with respect to substrate transport. To this end, in a first approach we reversibly altered the cholesterol content of K562/ADR cells by using methyl–β–cyclodextrin as a cholesterol chelator and conversely we repleted them through incubation with cholesterol in culture medium. We also used the cholesterol-binding fluorochrome filipin and cholesterol oxidase. Our data show that either cholesterol depletion or complex formation with filipin resulted in a strong decrease of P–gp activity. However, when cells were incubated with cholesterol oxidase that is known to disrupt rafts, no modification of the P–gp activity was observed. In addition, using a free-detergent methodology to separate by ultracentrifugation, "light", "heavy", and "extra heavy" fractions we show that no P–gp is found in the "light" fraction where rafts are usually detected. Altogether, our data strongly suggest that P–gp is not localized in rafts.

2. MATERIALS AND METHODS

2.1 Cell lines and culture

K562 leukemia cells and P-glycoprotein expressing K562/ADR cells (Lozzio *et al.*, 1975) were cultured in RPMI 1640 (Sigma, St Louis, MO) medium supplemented with 10% fetal calf serum (Biomedia, Boussens, France) at 37 °C in a humidified incubator with 5% CO_2. Every month, resistant K562/ADR cells were cultured for three days with 400 nM doxorubicin. The cell line was then used, 1 week later, during 3 weeks. Before each experiment the P–gp expression stability was checked by measuring the P–gp functionality, i.e., the rate of the P–gp–mediated efflux of pirarubicin (see later). Under these conditions, P–gp activity did not change. Cell cultures used for experiments were split 1:2 one day before use in order to assure logarithmic growth. Cells (10^6 /ml; 2 ml/cuvette) were energy–depleted via preincubation for 30 min in Hepes buffer with sodium azide but without glucose.

2.2 Drugs and chemicals.

Purified doxorubicin (DOX), daunorubicin (DNR), idarubicin (IDA) and pirarubicin (PIRA) were kindly provided by Laboratoire Pharmacia-Upjohn. Concentrations were determined by diluting stock solutions to approximately 10^{-5} M with ε_{480}= 11500 $M^{-1}cm^{-1}$. Stock solutions were prepared just before use. Methyl–β–cyclodextrin (MβCD) (mean degree substitution: 10.5–14.7), horse raddish peroxidase (HRP), cholesterol oxidase (COase), filipin and Triton X–100 (TX) were from Sigma and were dissolved in water. 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) was from Molecular Probes (Eugene, OR). C219 anti–P–gp antibody was from DAKO (Carpinteria, CA). Other chemicals were from the highest grade available. Before the experiments, the cells were counted, centrifuged and resuspended in Hepes buffer solutions containing 20 mM Hepes plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ at pH = 7.3, with or without 5 mM glucose. All other reagents were of the highest quality available. Deionized double-distilled water was used throughout the experiments.

2.3 Determination of the cholesterol content of K562 cells

Nonesterified cholesterol assay was adapted from a spectrofluorometric

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method used in the kit assay from Molecular Probe (Amundson *et al.*, 1999). Briefly, cells, 2.5×10^6 /ml, were washed once with PBS buffer and suspended in 1ml of reaction buffer. The reaction buffer at pH 7.4 contained 0.1 M PBS, 0.05 M NaCl, 0.1 % TX. Samples were incubated at 37 °C for 15 min, followed by 3 times 30 s sonication on ice and then one additional hour incubation at 37°C under continuous stirring. Unless otherwise indicated, 160 µl of this sample were added to the reaction buffer (total volume 1.6 ml) and the fluorescence signal at 560 nm (λ_{ex} = 585 nm) was monitored continuously when the following reactant were added: 50 µM Amplex Red, 0.5 U/ml HRP, 0.1 U/ml COase. The concentration of cholesterol in the solution was proportional to the difference of the fluorescence signal $\Delta F = F_{COase} - F_{HRP}$ (Fig. 1). Because the interaction of the cell suspension with HRP yielded a small change in the fluorescence signal (not shown), standard was systematically carried out in the presence of 160 µl of sonicated cells suspension to which 0 to 10 µM of cholesterol solution was added. The curve $\Delta F = F_{COase} - F_{HRP}$ against the exogenous cholesterol concentration was linear within the range 0 – 5 µM. Cholesterol titration was not affected by the presence of MβCD at concentrations used in this study.

2.4 Cellular anthracycline accumulation

The rationale and validation of our experimental setup for measuring the kinetics of the transport of anthracyclines in tumor cells has been extensively described and discussed before (Frézard *et al.*, 1991 a, b; Borrel *et al.*, 1994; Mankhetkorn *et al.*, 1996; Marbeuf–Gueye *et al.*, 1999). It is based on the continuous spectrofluorometric monitorring (Perkin Elmer LS 50B spectrofluorometer) of the decrease in the fluorescence signal of anthracycline at 590 nm (λ_{ex} = 480 nm) after incubation with the cells in a 1-cm quartz cuvette (Fig. 2). The decrease in fluorescence occurring during incubation with cells is due to the quenching of fluorescence after intercalation of anthracycline between the base pairs of DNA. We have previously shown that this methodology allows the accurate measurement of the free cytosolic concentration of anthracyclines under steady-state conditions, their initial rates of uptake, and kinetics of active efflux (Frézard *et al.*, 1991 a, b; Borrel *et al.*, 1994; Mankhetkorn *et al.*, 1996; Marbeuf–Gueye *et al.*, 1999). This methodology was used to check the effect of the treatment of cells with MβCD, filipin and COase on membrane permeability. For this purpose doxorubicin was used because the rate of uptake of doxorubicin by cells being very low (Kimmich *et al.*, 1975), the impact of membrane permeabilisation by any compound can be easily detected. Cells were thus treated with these different compounds, as explained later, and the decrease of the fluorescent signal was recorded as a function of time.

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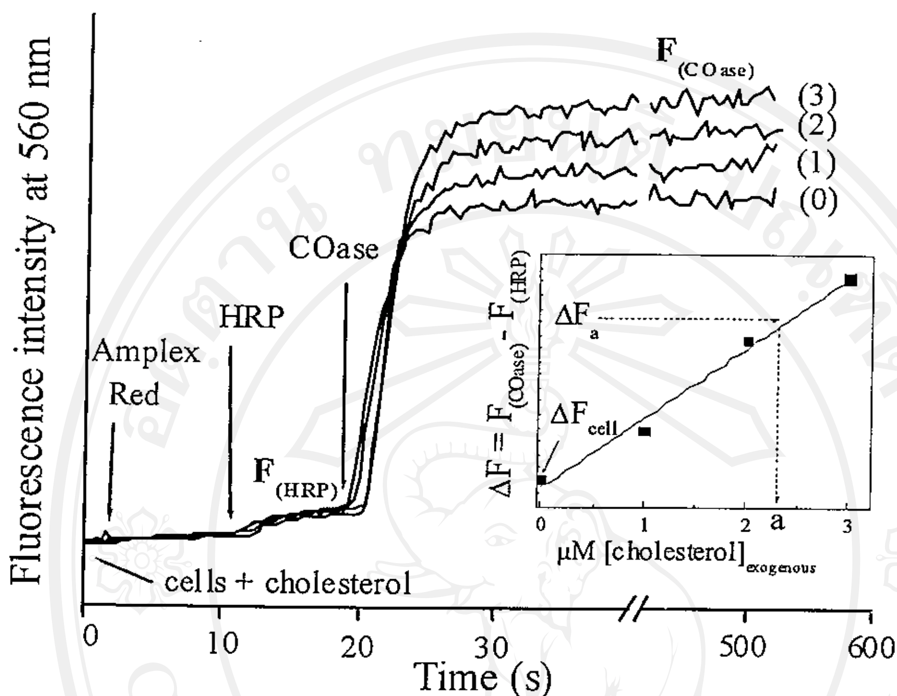

Figure 1. Spectrofluorometric method for the quantification of the cellular non-esterified cholesterol. The fluorescence intensity at 585 nm (λ_{ex} = 560 nm) was recorded when the following compounds were added to cells (10^6/ml) suspended in Hepes buffer: (1) a μM cholesterol with a = 0, 1, 2 or 3; (2) 50 μM Amplex Red; (3) 0.5 U/ml HRP; (4) 0.1 U/ml COase. $\Delta F = F_{(COase)} - F_{(HRP)}$ represents the increase of fluorescence intensity due after the addition of COase. Inset, $\Delta F = F_{(COase)} - F_{(HRP)}$ has been plotted as a function of the exogenous cholesterol concentration (a) added to the cells, the slope is $\rho = (\Delta F_a - \Delta F_{cell})/a$ where ΔF_{cell} and ΔF_a are the variation of fluorescence in the absence and in the presence of a μM exogenous cholesterol. The endogenous molar concentration of cholesterol in the assay is then $C = a \cdot 10^{-6} \cdot \Delta F_{cell} / (\Delta F_a - \Delta F_{cell})$ and the number of mole of cholesterol per cell is $4 \times 10^{9} \cdot C$ (see Material and Method)

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2.5 Determination of the P-gp-mediated efflux of anthracyclines

Cells (1× 0⁶/ml; 2 ml per cuvette) were preincubated for 30 min in HEPES buffer with 10 mM sodium azide, but without glucose (energy-deprived cells). Depletion of ATP in these cells was 90 %, as checked with the luciferin–luciferase test (Marbeuf–Gueye *et al.*, 1998). The cells remained viable throughout the experiment, as checked with Trypan blue and calcein vital stain (not shown). After addition of anthracycline, the decrease in the signal was monitored until steady state was reached. Since the pH of the buffer

was chosen to equal the intracellular pH, at steady state the extracellular free drug concentration (C_e) was equal to the cytosolic free drug concentration (C_i). At this point, $C_i = C_e$ was calculated from the non–quenched fluorescence: $C_e = C_T(F_0–F_n)/F_0$, where F_0 is the fluorescence of a C_T micromolar anthracycline solution and F_n, the fluorescence at steady state. Then glucose was added, which led to the restoration of control ATP levels within 2 min and to an increase in the fluorescence signal due to the efflux of anthracycline. Since under these conditions, at the moment of addition of glucose $C_i = C_e$, the passive influx and efflux were equal, the net initial efflux represents the P–gp–mediated active efflux only (Mankhetkorn *et al.*, 1996; Marbeuf–Gueye *et al.*, 1998), which was calculated $V_a = C_T/F_0(dF/dt)$, where dF/dt is the slope of the tangent to the curve (see Fig.3).

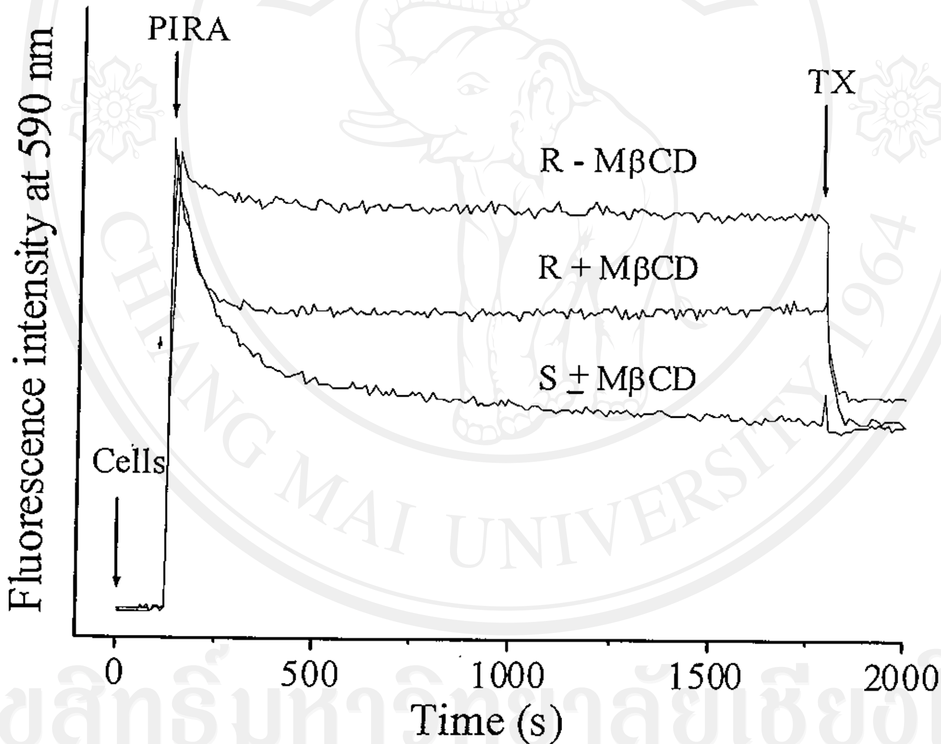

Figure 2. Time course of uptake of pirarubicin (PIRA) by K562 cells after incubation with MβCD. Cells either sensitive (S) or resistant (R), 10^6/ml, were incubated with 0 or 10 mM MβCD for 10 min. Cells were then centrifuged as was explained under Materials and Methods and incubated with 1 μM PIRA. The fluorescence intensity at 590 nm (λ_{ex} = 480 nm) was recorded as a function of the time of incubation. Arrow indicates the addition of 0.05% (w/v) triton X–100

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2.6 Treatment of cells with MβCD

The methylated derivatives of β–cylodextrin are known to trap preferentially membrane cholesterol compared to other cyclodextrins, which have also affinity to phospholipids and proteins (Ohvo *et al.*, 1996). We then used a 10.5 –14.7 times methylated β–cylodextrin (MβCD) to study the effect of cholesterol depletion on K562/ADR and K562 cells. Cells were grown as described. The standard culture medium was replaced with Hepes buffer to which 2 to 20 mM MβCD had been added. Unless otherwise stated, cells were then incubated for 30 min at 37°C. MβCD interacted with anthracycline and modified the fluorescence signal, therefore cells were washed with Hepes buffer and then transport activity was measured as described above.

2.7 Treatment of cells with filipin

To study the effect of filipin on P–gp functionality, transport activity was measured as described above with the difference that 1 to 10 μM filipin were added 10 min prior to the addition of glucose. The effect of filipin on the passive diffusion of doxorubicin was also determined.

2.8 Incorporation of cholesterol into cholesterol-depleted cells

Cells were treated with MβCD as described above to remove endogenous cholesterol. Cells were then incubated with 100 μM cholesterol in standard culture medium with gentle agitation for 30 min to 2 hours at 37°C. The cells were then washed and resuspended in Hepes buffer and transport activity was measured as described above

2.9 Treatment of cells with cholesterol oxidase.

K562/ADR cells overexpressing P–gp were pretreated at 37°C for 30 min with 0.5 U/ml COase in Hepes buffer.

2.10 Isolation of "light" and "heavy" membrane fractions

Purification of membrane domains was performed using a detergent free procedure."Light" and "heavy" fractions were isolated according to the method of Monneron and d'Alayer (1978 a, b) with the following modifications. K562/ADR cells, 10–15 × 10^6 cells, were washed 2 times with PBS buffer and suspended in this buffer, 2 ×10^6 cells/ml. They were then incubated for 30 min in the absence or presence of 15 mM MβCD at 37° under stirring. Cells were then again washed 2 times with PBS buffer. The pellet was suspended in 500 μL of sodium carbonate buffer at pH 9–10, and sonicated in a cold bath 3 times 30s (50 Hz, 117 v, and 80 W) (Vibra cell, Sonics& materials inc., Danbury, Connecticut, CT). The lysate was mixed with 80% sucrose to yield 2 ml of 40% sucrose solution. This mixture was transferred to the bottom of the ultracentifugation tube (Beckman Instruments, Palo Alto, CA) and was overlaid with 3 ml 35% sucrose, 3 ml 22% sucrose, and 3 ml 10% sucrose. The weight of the ultracentrifugation tubes was equilibrated with 10% sucrose and the gradients were inserted into a SW41 rotor. The ultracentrifugation was performed at 160 000 g for 14 h at 4°C. Light–scattering bands confined at the 10-22% sucrose and 22-35% sucrose interfaces respectively were observed. Eleven 1–ml fractions

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were collected by suction with a syringe from the top to the bottom. The velocity of aspiration was kept low in order to avoid disturbance of the sucrose layers. The first fraction was called F1 and the last fraction was called F11. The total protein concentration was measured in each fraction using the Bradford reagent (BC assay kit, Interchim France).

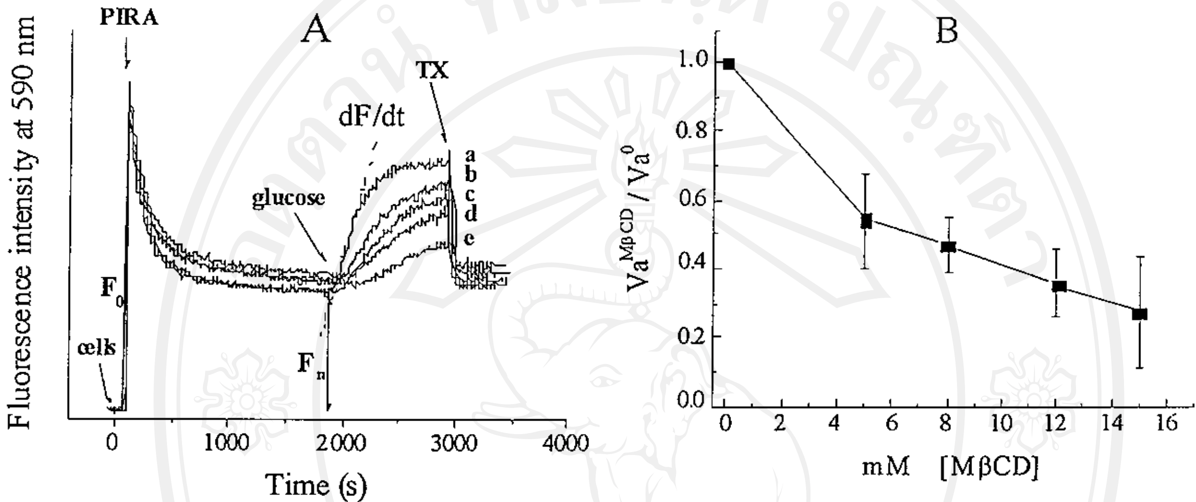

Figure 3. Incorporation of pirarubicin in energy–depleted cells K562/ADR cells and determination of the active efflux rate (V_a). Cells, 10^6/ml, were incubated for 10 min with MβCD at concentrations equal to 0 mM (a), 5 mM (b), 8 mM (c), 12 mM (d) and15 mM (e). Cells were then centrifuged as explained under Materials and Methods and incubated with 1 μM PIRA. (A) The fluorescence intensity at 590 nm (λ_{ex} = 480 nm) was recorded as a function of the time of incubation of cells with PIRA. The active efflux rate (V_a) was determined from dF/dt after the addition of glucose. (B) The ratio $V_a^{\beta MCD}/V_a^0$, has been plotted as a function of the MβCD concentrations. $V_a^{\beta MCD}$and V_a^0 are the rate of P–gp–mediated efflux of daunorubicin in the presence and absence of MβCD respectively. The points are the mean ± SD of three independent experiments.

2.11 Western blotting measurement of P-glycoprotein expression

Equal volume (12 μl) of sucrose density gradient fractions were mixed with concentrated SDS reducing buffer (final concentrations are 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, bromophenol blue). Protein samples were separated on 7.5% SDS-PAGE then transferred onto PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The membrane was blocked with 5% non–fat dry milk in 0.1% Tween–PBS and treated with 0.5 μg/ml C219 anti–P–glycoprotein antibody (DAKO). Detection was carried out by enhanced chemiluminescence (ECL plus[R] kit with mouse IgG, HRP linked whole antibody from Amersham Pharmacia Biotech). The P–gp expression has been evaluated after densitometric scanning of the films and analysis with Image J 1.30 software.

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2.12 Localization of ganglioside GM1.

GM1 is commonly found in high concentrations in rafts (Harder *et al.*, 1998), and it can be labeled using fluorescent FITC–conjugated cholera toxin B. Cholera toxin B (CTB) (Dietrich *et al.*, 2001) binds to the glycosphingolipid GM1 and we used it as marker for raft localization in "light" and "heavy" plasma membrane fractions. We employed a CTB–horseradish peroxidase (CTB–HRP) conjugate for fast simple detection of ganglioside GM1. Briefly, after activation of a PVDF membrane with methanol and washes with water and 0.1% Tween–PBS (PBS–T), 4 µl of each gradients were dotted onto the wet membrane. The air–dried membrane was reactivated and blocked with 5% non–fat dry milk in PBS–T. After washes with PBS–buffer, the membrane was incubated with HRP conjugated cholera (dilution 1:5000, Sigma) in PBS–T for 90 min, rinsed several times with PBS-bufer and then detected by enhance chemiluminescence (Amersham Pharmacia Biotech) and analysed with Image J 1.30 software.

3. RESULTS

3.1 Non-esterified membrane cholesterol in sensitive and resistant cell lines

Cholesterol content in K562 and P–gp overexpressing cell lines K562/ADR was similar and equal to 2.7 ± 0.4 x10^{-14}mol/cell. However, the rate of cholesterol depletion by MβCD was slightly different. This can be seen in Table I in which the number of moles of cholesterol per cell has been indicated after various times of incubation with different concentrations of MβCD.

Table 1.Percentage of cellular cholesterol content after treatment with different MβCD concentrations for various times.

MβCD, mM	% mole chol/cell after Δt = 10 min K562	% mole chol/cell after Δt = 10 min K562/ADR	% mole chol/cell after Δt = 15 min K562	%mole chol/cell after Δt = 15 min K562/ADR
0	100 [a]	100	100	100
5	52	65	37	42
10	31	48	12	30
15	25	36	10	30

[a] corresponds to 2.7 ±0.3 × 10^{-14} mole/cell

3.2 Effect of MβCD treatment on P-gp-mediated anthracycline transport

The incubation of cells, either sensitive or resistant, with MβCD yielded an increase of the cell membrane permeability to the drugs. In other words, the rate of passive uptake of the drug in presence of MβCD,

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$V_+^{M\beta CD}$, is higher than the rate, V_+, in its absence. The slow diffusing anthracycline, DOX, was used to clearly check this point: the number of mole of DOX accumulated per sensitive cell after one hour was $1.1\pm 0.1 \times 10^{-15}$ mole/cell in the absence of MβCD and $3.9 \pm 0.3 \times 10^{-15}$ mole/cell after incubation for 1 hour with 15 mM MβCD.

The incorporation of PIRA, IDA or DNR in resistant and sensitive cells was then measured in the presence of various MβCD concentrations, after different times of incubation. Fig. 2 shows the data obtained when cells were incubated with 1 μM PIRA after incubation, for 10 min, with 0 to 10 mM MβCD. As can be seen, the amount of PIRA accumulated inside the resistant cells, at steady state, increased in a dose–dependent manner. No modification was observed for sensitive cells. As, in resistant cell, this parameter depends on both the rate of passive diffusion of the drug and on the rate of its P–gp–mediated efflux, it was difficult to determine an eventual impact of MβCD on the rate of P–gp–mediated efflux of DNR. For this reason, experiments were performed with energy-depleted resistant cells, which were incubated with MβCD as previously described. At steady state the incorporation of PIRA was the same as in sensitive cells (Frézard *et al.*, 1991a, b; Borrel *et al.*, 1994; Mankhetkorn *et al.*, 1996; Marbeuf–Gueye *et al.*, 1999). At this stage, the addition of 5mM glucose yielded an increase of the fluorescent signal due to the P–gp–mediated efflux of the drug only, and as there was no gradient of concentration across the plasma membrane the effect of MβCD on passive PIRA diffusion had not to be taken into account. Fig. 3A shows the records of such experiments performed after cells incubation with different MβCD concentrations. One observed a dose–dependent effect of MβCD on the rate of P–gp–mediated efflux of PIRA (Fig. 3B). Similar experiments were performed with other anthracyclines, IDA and DNR, and analogous data were obtained. These experiments clearly show that the pump functionality is highly sensitive to cholesterol removal.

To determine whether V_M and/or K_m were modified by cholesterol depletion, we have measured the rate of P–gp–mediated efflux of pirarubicin, V_a, as a function of intracellular pirarubicin concentration, (i) in intact cells, (ii) in cells that had been incubated with 15 mM MβCD for 10 min and which, according to the above data, contained 50 ± 5 % of cholesterol versus untreated cells. The variation of V_a as a function of C_i is shown in Fig. 4. The relation between V_a and C_i is

$$V_a = V_M \cdot C_i^{\,h} / (K_m^{\,h} + C_i^{\,h}) \quad (1)$$

A curve fitting of the data of Fig. 4 with equation (1) yielded $V_M = (5.7 \pm 0.4) \times 10^{-18}$ mole/cell/s, $K_m = 0.93 \pm 0.13$ μM, $h = 1.6 \pm 0.4$ in untreated cells and $V_M = (3.1 \pm 0.5) \times 10^{-18}$ mole/cell/s, $K_m = 1.3 \pm 0.4$ μM, $h = 1.7 \pm 0.4$ in MβCD treated cells. The two V_M values are significantly different whereas the two K_m values are similar within the limit of incertitude.

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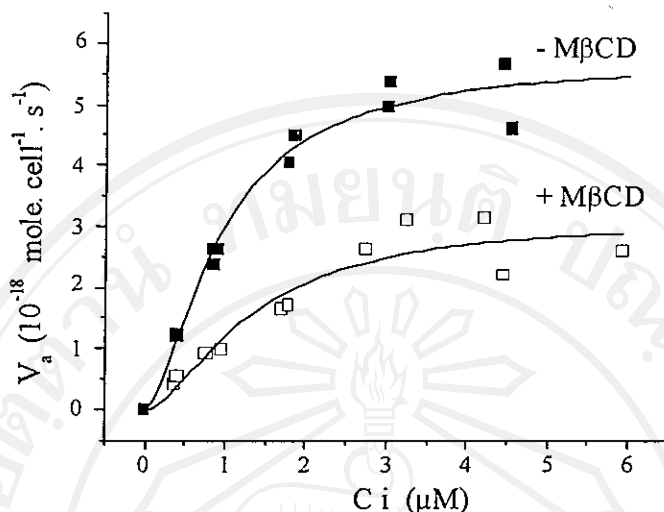

Figure 4. Rate of the P–gp–mediated efflux of pirarubicin plotted as a function of the intracellular free pirarubicin concentration. The cells were incubated without (full square) or with (empty square) 15 mM MβCD for 10 min. Data points are from 3--5 independent experiments performed on different days. V_a and C_i were determined as described under materials and methods. The data, from a typical experiment, were fitted using equation (1).

To determine if this decrease of the P-gp functionality was reversible, we studied the effect of cholesterol incubation after treatment of cells with MβCD. We observed that when cells, cholesterol–depleted through incubation with MβCD, were incubated with cholesterol in culture medium the P–gp–mediated efflux of PIRA was almost fully restored. The cholesterol content of the cells was also determined after such treatment. Figure 5 shows the plot of the rate of P–gp–mediated efflux of PIRA as a function of the cholesterol content of the cells (i) during cholesterol depletion of the cells through MβCD action and (ii) during cholesterol repletion. A very interesting data is that the rate of the P–gp–mediated efflux of pirarubicin is closely correlated to the cholesterol content of the cells.

3.3 Effect of cholesterol oxidation on P-gp activity

In a second approach, membrane cholesterol was chemically modified using cholesterol oxidase. The rate of P–gp–mediated efflux of PIRA was measured on cells without any treatment (t=0) and after incubation with 0.5 U/mL COase during 10 and 30 min respectively. The amount of non-oxidized cellular cholesterol was also measured under the same experimental conditions. Fig. 6 shows that the incubation of cells with COase gave rise to the oxidation of about 90% of cholesterol without yielding modification of V_a. Therefore, we can infer that the P–gp activity does not depend on the oxidation state of cholesterol.

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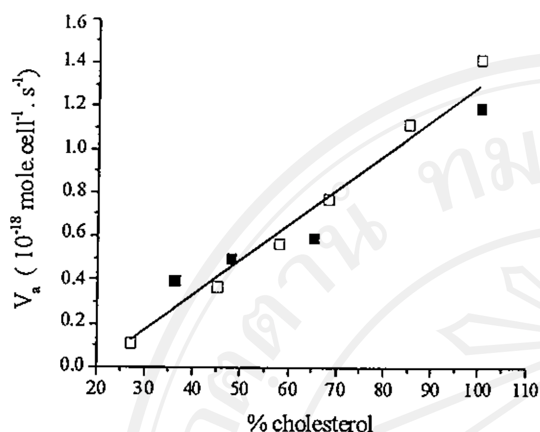

Figure 5. Rate (*V*a) of the P–gp–mediated efflux of PIRA as a function of the percentage of cholesterol in the cells. Cholesterol depletionwas obtained through incubation of cells for 10 min with variousM*β*CD ranging from 5 to 10 mM (full square). Cholesterol repletion was performed through incubation of the cells in culture medium in the presence of exogenous cholesterol (open square).

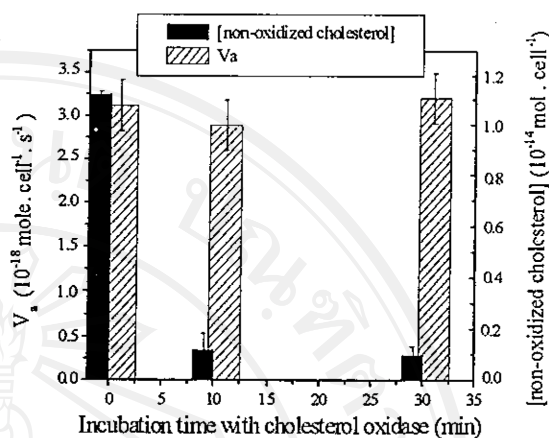

Figure 6. Effect of cholesterol oxidase on the rate of P–gp–mediated efflux of pirarubicin and on the cellular cholesterol content. Cells, 10^6/ml, were incubated for 0, 10 and 30 min with 0.1 U/ml cholesterol oxidase.

3.4 Effect of filipin on P-gp functionality

In another approach, filipin, a cholesterol binding fluorochrome, was used to assess the dependence of P–gp functionality on cholesterol. Within the membrane, filipin forms complex with cholesterol therefore altering the cholesterol repartition. The effect of a 10 min-filipin treatment on both the passive diffusion of DOX and the P–gp–mediated efflux of PIRA was measured. Fig. 7 shows that the efflux rate of PIRA was totally inhibited at 2 μM without increase of the membrane permeability to DOX. At higher concentrations, filipin drastically increased the permeability of the cells likely because of a detergent or pore forming effect.

3.5 Assignment of the source of membrane fractions

Digitalization of the images allowed the determination of GM1 and P–gp present in the different fractions (Fig. 8). To determine the effect of MβCD on the integrity of rafts, K562/ADR cells were treated for 30 min in the absence or in the presence of 15 mM MβCD. Low density membrane fractions were then isolated, and the distribution of GM1 and P–gp was assessed by Western and dot blotting of the analytical density gradients. Fraction 1 represents the top of the gradient, and fraction 11 is the bottom of the gradient. Fractions 3–4 and fractions 6–7 contain the 10/22% and 22/35% sucrose interface respectively. Fractions 3–4 were found to have low concentrations of proteins, about nine times less than in fractions 6–7. In untreated cells, about 25% of the GM1was found in fractions 3–4, 34% in

fractions 6–7 and 41% in fraction 8–11 corresponding to the cytoplasm and pellet. After mild treatment with MβCD (15 mM, 30 min) the amount of GM1 present in fractions 3–4 decreased to ~16% whereas in fractions 6–7 it increased up to ~43%. Following more drastic treatment, (30 mM, 1 h) GM1 was lost from the low density region of the gradient and recovered instead in the high density region of the gradient. We therefore identified fraction 3–4 as the raft fractions. No significant amount of P-gp was detectable in fraction 3–4, 20% was recovered in fractions 6–7 and the rest in fractions corresponding to cytoplasm and pellet. Treatment with MβCD did not yield modification of this distribution.

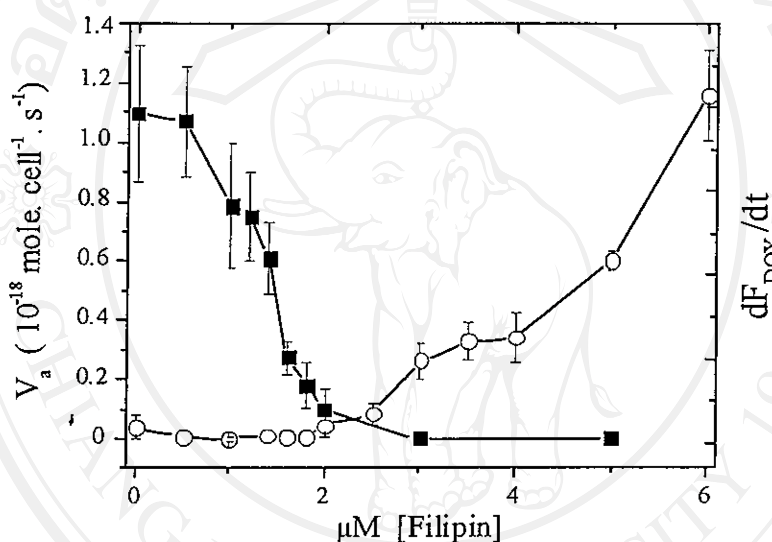

Figure 7. Effect of filipin on the rate of P–gp–mediated efflux of pirarubicin and on the membrane permeability to doxorubicin. Energy-deprived resistant cells, 10^6/ml, were incubated with 1 μM pirarubicin. After 20 min, filipin, at concentration ranging from 0 to 6 μM, was added, i.e., 10 min before the addition of glucose, and the rate of P–gp–mediated efflux of PIRA was measured (full square). Resistant cells, 10^6/ml, were incubated with 1 μM doxorubicin. After 20 min filipin, at concentration ranging from 0 to 5 μM was added, and after 10 min the slope *dF/dt* of the decrease of the DOX fluorescence was measured (open circle)

4. DISCUSSION

Domains rich in cholesterol and sphingomyelin have been subject of great interest recently in cell biology because some important integral membrane proteins may be preferentially located within them. Such domains that form around the protein caveolin, referred to as caveolae, have been unambiguously shown to exist (Anderson, 1998). When caveolin is not present, the domains are known as rafts. Raft association of proteins can be

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assayed by manipulating the lipid composition of rafts. If cholesterol or sphingomyelin are depleted from membrane, lipid rafts are dissociated and previously associated proteins are no longer in rafts (Simons *et al.*, 2000).

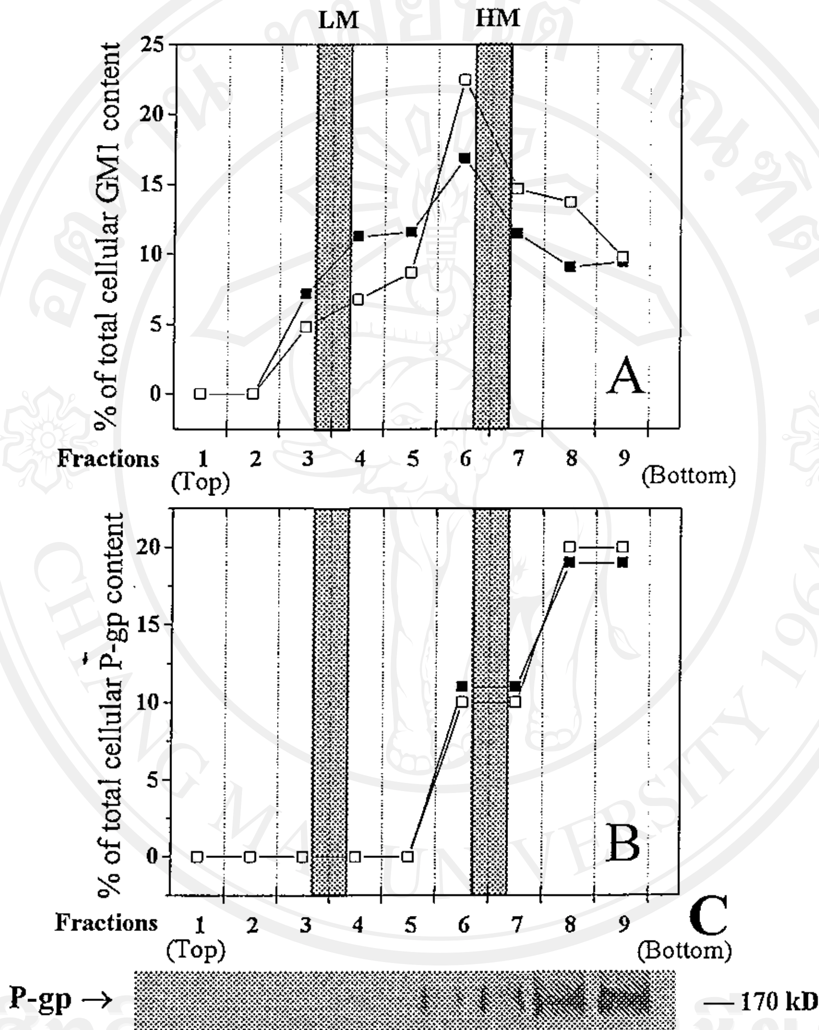

Figure 8. Detection of GM1 and P–gp in K562/ADR cell lysates. K562/ADR cells were lysed by sonication, before (full square) or after (empty square) treatment with MβCD. The lysate was separated by density centrifugation and collected from the top in 1 ml fractions. Fraction 1 is from the top of the gradient. (A) GM1 content and (B) P–gp expression have been analysed with Image J v1.30 software. (C) Immunodetection of P–gp in the absence of treatment with MβCD. Fractions 3–4 (LM, dashed) and fractions 6–7 (HM, dashed) contain the 10%/22% and 22%/35% sucrose interface respectively.

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In terms of their cholesterol metabolism and transport, most oncogenically transformed and cancer cells have been classified as type A cells, i.e., cells having many cell surface lipoprotein receptors but few, if any, caveolae and in which regulation of cholesterol homeostasis occurs at the level of the influx (Liscovitch *et al.*, 2000). However, recent results have shown that MDR cancer cells express very high caveolin levels and exhibit a high surface density of caveolae (Lavie *et al.*, 1998). One immediate implication of the up–regulation of caveolin and caveolae in MDR cells is that during acquisition of the MDR phenotype the cells revert from being cholesterol homeostasis type A cells to cholesterol homeostasis type B cells (i.e., cells having few cell surface lipoprotein receptors but many caveolae and in which regulation of cholesterol homeostasis occurs at the level of the efflux). Being type B cells, MDR cancer cells must have an active cholesterol active pathway and therefore one question is: does P–gp play a role in cholesterol transport and /or metabolism? Actually, a recent paper has proposed a coupling between the basal ATPase activity of P–gp and its intramembrane cholesterol redistribution function consistent with the possibility that P–gp may actively translocate cholesterol in the membrane (Garrigues *et al.*, 2002). This P–gp–mediated cholesterol redistribution in the cell membrane makes it likely that P–gp contributes in stabilizing the cholesterol-rich microdomains, rafts and caveolae, and that it is involved in the regulation of cholesterol trafficking in cells.

Given that the functioning of P–gp is intimately connected with the membrane, we might expect the transport properties of P–gp to be strongly influenced by the physical properties (composition) of the bilayer. The K562/ADR cells used in this work do not express caveolin and therefore no caveolae are expected (Mannechez *et al.*, in press). However the possibility that P–gp could be localized within rafts still remains and in the present work, our goal was to check this possibility and to explore the effect of lipid environment on the rate of ATP–driven drug transport by P–gp in intact cells. For this purpose we have used different approaches to affect the putative microdomains at the cholesterol level. In a first set of experiments, in order to determine whether cholesterol affects P–gp function, we used MβCD to extract cholesterol from the lipid phase of intact living cells. MβCD is a highly hydrophilic cyclic oligosaccharide that specifically binds sterol, rather than other membrane lipids, to form water-soluble complexes (Ohvo *et al.*, 1996), without causing further membrane perturbation by insertion (Gimpl *et al.*, 1997). This treatment gave rise to a strong decrease of the P–gp–mediated efflux of anthracycline. This was reversible and repletion of cells with cholesterol restored the P–gp functionality. Interestingly, the P–gp functionality, which was quantified by the V_a measurement, was quasi linearly linked with the amount of non–esterified cholesterol present in the cells down to about 30 % (see Fig. 5). It must be remind that about 90% of non–esterified cholesterol is localized in the plasma membrane (Lange *et al.*, 1993). On the other hand, when cells cholesterol content was decreased to about 50% of the initial value, the decrease of the rate of P–gp–mediated efflux of pirarubicin was due to a decrease of the V_M value whereas the K_m value does not change significantly.

This suggest that the site of binding of pirarubicin to P–gp is not strongly affected by 50% cholesterol removal

In a second set of experiments, filipin which is known to complex cholesterol within the membrane and therefore to destroy the rafts was added to the cells. Here also the P–gp–mediated efflux of anthracycline was strongly affected, the rate being close to zero at 2 μM filipin. However, in this case, the inhibition of the P–gp–mediated efflux of pirarubicin due to a specific filipin–transporter interaction cannot be excluded.

Treating cells with MβCD to deplete them with cholesterol is the common method to eliminate biological rafts, but it is a harsh treatment. The use of COase appears to be a gentler procedure (Samsonov *et al.*, 2001): COase converts cholesterol to cholestenone and because cholestenone does not possess a 3β–hydroxyl group, it does not interact with sphingomyelin. Recently, it has been shown that application of COase eliminated rafts in bilayer membrane (Samsonov *et al.*, 2001). In addition, the whole cholesterol present in the plasma membrane is rapidly oxidized by the action of extracellular COase, the transbilayer diffusion (flip–flop) of cholesterol proceeding with a half time of < 1s at 37°C (Steck *et al.*, 2002). It must also be emphasized that the oxidation of cholesterol to cholestenone does not modify the membrane fluidity (Gimpl *et al.*, 1997). Therefore, in a third set of experiments cholesterol was oxidized by action of COase. No modification of the efflux was observed.

These last sets of experiments strongly suggest that P–gp is not localized in rafts and it is likely that the decrease of the P–gp–functionality observed with cholesterol depletion is due to an increase in membrane fluidity. The promotion of ordered lipid phases within bilayers is a characteristic effect of cholesterol incorporation and such domains may modulate the function of P–gp. Actually, many membrane transporters show very low, or negligible, rates of transport when the host lipid bilayer is in the rigid gel phase, i.e. below T_m. In contrast, it has been shown by Lu *et al.* (2001) that the rate of tetramethylrosamine, a Rhodamine derivative, transport by P–gp in PamMyrGroPCho bilayers is actually at its highest in the gel phase, below 28°C. These authors have also reported that P–gp catalyzed ATP hydrolysis quite well in the gel phase, and the energy activation value is substantially lower than that measured in liquid crystalline bilayers (Romsicki *et al.*, 1998). Overall, P–gp–mediated transport of tetramethylrosamine displayed highly unusual temperature–dependence characteristics. Thus, the uptake of [^{3}H] colchicine into reconstituted proteoliposomes of $Myr_2GroPCho$ is more than twofold higher at the T_m of 24°C than in the fluid liquid crystalline phase at 32°C (Sharom, 1997). On the other hand, it has been shown that the addition of chemical fluidizing agents led to a decrease in P–gp–mediated transport of vinblastine and DNR in canalicular membrane vesicles (Sinicrope *et al.*, 1992) of calcein–AM and pirarubicin in MDR cells (Regev *et al.*, 1999; Borrel *et al.*, 1995).

Concerning cholesterol and P-gp functionality other authors have reported data that are at variance with ours. Luker *et al.* (2000) have studied the effects of cholesterol on P–gp localization and function in low-density membrane domains in different cell lines. They have observed that depletion

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of cholesterol with MβCD shows cell-type- and substrate-dependent effects on the transport activity of P–gp. Thus, depletion of cholesterol affects both function and inhibition of class I P–gp differently in CHO cells than in any of the cells expressing human MDR1 P–gp. It seems that such observation is not a general rule since, in the present work, we have used a human cell line and observed that depletion of cholesterol strongly affects P–gp functionality. On the other hand, Garrigues *et al.* (2002) have observed that in cholesterol-depleted MDR vesicles, P–gp exhibits an ATPase activity in the presence of various P–gp substrates, such as verapamil or progesterone, which stimulate P–gp ATPase activity in native membranes. They have concluded that therefore, P–gp is fully functional in cholesterol-depleted MDR vesicles and is able to interact with its substrates.

To support our data, we have also performed experiments to localize the transporter in relation to rafts. It is widely believed that rafts can be isolated from biological membranes by detergents such as TX that solubilizes the lipids and proteins of the fluid regions and leaves rafts as large, detergent–resistant membrane fragments (Yu *et al.*, 1973; Brown *et al.*, 1992; London & Brown, 2000). However, Heerklotz *et al.* (2003) have recently performed a thermodynamic description of the interactions governing the formation or disintegration of domains and shown that unfavorable interactions between TX and sphingomyelin in a mixed membrane drive the separation of these molecules into separate domains above a critical TX content. This result is in excellent agreement with the phenomenon of TX–induced raft formation reported by Heerklotz (2002). On the other hand, Luria *et al.* (2002) have shown that light membrane fractions isolated from *Xenopus* egg plasma membrane in a detergent-free environment have properties similar to those of detergent–resistant membranes. For these reasons we chose to isolate membrane fractions without detergent extraction. It should be noticed that we have found comparable amount of GM1 in “light” and “heavy” membranes. However, only the one present in light fraction was modified by MβCD treatment. On the other hand, we have found a high percentage of P–gp in the cytoplasm fraction of the cells. This was not unexpected as similar observation has been made by Labroille *et al.* (1998).

In conclusion, from our experiments we can infer that in K562/ADR cells P–gp is not localized in raft. However, cholesterol depletion strongly affects the rate of P–gp–mediated efflux of anthracycline that can be assigned to an increase in membrane fluidity.

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4. **Reungpatthanaphong, P.**, Marbeuf-Gueye, C., Le Moyec, L., Salerno, M., Garnier-Suillerot, A. (2004). Decrease of P-glycoprotein activity in K562/ADR cells by MβCD and filipin and lack of effect induced by cholesterol oxidase indicate that this transporter is not located in rafts. *Journal of Bioenergetic Biomembrane.* 36(6), 533–543.
5. **Reungpatthanaphong, P.,** Dechsupa, S., Meesungnoen, J., Loetchutinat, C. and Mankhetkorn, S. (2003). Rhodamine B as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential in drug–sensitive and –resistant cells. *J. Biochem. Biophys. Methods.*, 1663, 1–16.
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Oral Presentation

1. **Reungpatthanaphong, P.**, Dechsupa, S., Meesungnoen, J., Loetchutinat, C. and Mankhetkorn, S. Rhodamine B as mitochondrial probe for measurement

and monitoring cellular responses to toxins and xenobiotics. 2nd International Scientific Conference on Occupational and Environmental Health at Melia Hotel, Hanoi, Vietnam 16-18 November 2005.

2. Hauet, N., Deschsupa, S., Kothan, S., Martineau, A., Ratana, P., **Reungpatthanaphong, P.,** Weinmann, P., Beranger, S., Moretti, JL., Mankhetkorn, S. Evaluation de l'induction apoptotique par la quercetine chez la souris nude tumoree: Etudes par scintigraphie (Annexine V-99mTc) et histochimie. 42ème Nucléaire de Langue Française Pointe-au-Pic -Québec, 30 Septembre au 7 Octobre 2004.

3. **Reungpatthanaphong, P**., Dechsupa, S., Meesungnoen, J., Loetchutinat, C. and Mankhetkorn, S. Rhodamine B as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential in drug-sensitive and -resistant cells. RGJ seminar series XXVI: Molecular biology of cancer, Khon Kaen University, Khon Kaen , Thailand. 8 February 2004. P.12

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6. **Reungpatthanaphong, P.**, Dechsupa, S., Meesungnoen, J. and Mankhetkorn, S. Modulation of mitochondrial membrane potential ($\Delta\Psi_m$) in situ by MTT and artemisinin derivatives in multidrug resistance cancer. 26th congress on science and technology of Thailand, Bangkok, Thailand.18-20 October 2000, ISBN 974-346-611-8.

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