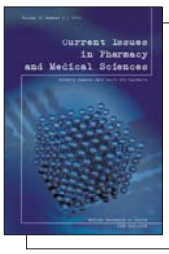

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Influence of fluphenazine dihydrochloride on model dipalmitoylphosphatidylcholine membranes and human genotoxically damaged lymphocyte cultures

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ABSTRACT

The membrane interactions can play a vital role in chemosensitizing activity of fluphenazine dihydrochloride, an inhibitor of P-glycoprotein transport function. In *in vitro* testings of the influence of fluphenazine dihydrochloride on model dipalmitoylphosphatidylcholine membranes and on human lymphocyte cultures, genotoxically damaged by benzo[*a*]pyrene, differential scanning calorimetry and rhodamine 123 retention test were applied. The results of both testings shown that the membrane interactions of fluphenazine dihydrochloride in model membrane structure and the chemosensitizing effect of this compound in examined human lymphocyte cultures, depend on its concentration in a sample.

Keywords:chemosensitizing agents, differential scanning calorimetry, fluphenazine dihydrochloride, membrane interactions, P-glycoprotein, rhodamine 123

INTRODUCTION

The P-glycoprotein (P_{gp}) is the major player responsible for multidrug resistance (MDR) in tumors and/or genotoxically damaged cells. This protein acts as a cell membrane efflux pump for cytostatic drugs, is dependent on the biophysical properties of the lipid membrane [14].

It was proved that the inhibitors of P_{gp} transport function, including phenothiazine derivatives (Pht), can destabilize the well-ordered cell membrane structure [2]. The interaction of Pht with membrane lipid molecules can indirectly modulate the activity of P_{gp} and be a vital mechanism of their chemopreventive activity [13]. Pht possess the ability to reverse MDR dependent on P_{gp} transport function; they increase an intracellular accumulation of cytostatics and are included to the group of chemosensitizing compounds with potential application in late cancer chemoprevention for enhancement of the effects of cytostatic drugs on cancer cells [10].

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The main goal of presented studies was to examine the effect of piperazine Pht – fluphenazine dihydrochloride (FPh-2HCl) exerted on model lipid bilayer and on the P_{gp} transport function in human genotoxically damaged lymphocytes using differential scanning calorimetry (DSC) and the rhodamine 123 (Rod-123) accumulation test, respectively.

MATERIALS AND METHODS

Chemicals. Chloroform, methanol and Rod-123 were obtained from Sigma-Aldrich (Germany, HPLC grade; St. Louis, MO, USA). Benzo[*a*]pyrene (B[*a*]P), lymphflot and phytohaemagglutinin were purchased from Fluka (Buchs, Switzerland), BioRad Medical Diagnostics (Dreieich, Germany) and GIBCO (Gaithesburg, MD, USA), respectively. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) with a purity 99.8% was supplied by Avanti Polar Lipids (Alabaster, Al, USA) and used as received. FPh-2HCl was purchased from Jelfa (Jelenia Góra, Poland). Water used for liposome preparation was obtained from a Millipore filtration device (Millipore, Milli Q).

The chemical structure of FPh-2HCl was presented on Figure 1.

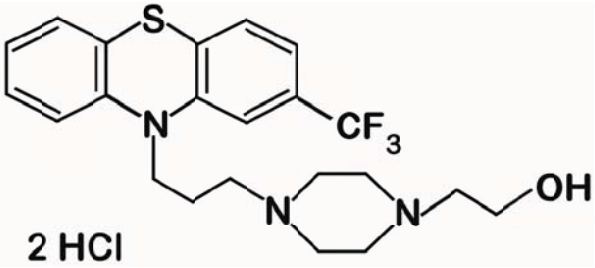

Fig. 1. Chemical structure of FPh-2HCl

Liposome preparation and DSC measurements. Samples were prepared using method described by Hendrich et al. [6] with some modifications. Stock solution of FPh-2HCl (10 mM) using in experiments was prepared by dissolving FPh-2HCl in chloroform/methanol (1:1, v/v). To obtain required FPh-2HCl/DPPC (0-50 mol%) molar ratio, 2 mg of DPPC was diluted in appropriate volume of FPh-2HCl stock solution in eppendorf tube (1 ml). Then the organic solvent was evaporated under a stream of nitrogen and simultaneously heated at 50˚C. The sample was placed in a desiccator for 3 h to remove any traces of solvent and to receive perfectly FPh-2HCl/DPPC dry film. The lipid vesicles were prepared by addition of 15 ml water and by vigorous vortexing for 5 min at 50˚C to obtain homogenous dispersion. The sample was enclosed in an aluminum pan and measured against a reference sample using Rigaku calorimeter. For each sample, four scans were carried out in the heating directions between 25-50˚C at a rate 1.25˚C/min. The temperature at the peak maximum was defined as the transition temperature.

Lymphocyte cultures and measurements of the Rod-123 retention. The research was approved by the Ethics Committee of Wroclaw Medical University. All experiments were repeated 10 times (n = 10). Lymphocytes were isolated from venous blood (20 ml) of healthy, non-smoking men aged below 26 years, by a gradient method using solution of the Lymphoflot, as it was described in detail in the literature [1].

Lymphocytes were used in own research as they are considered as model cells to test the P_{gp} activity [12]. The lymphocyte cultures containing a mitogen – phytohaemagglutinin [2% v/v], were seeded on the plastic 96-well culture plates; 4 x 10^4 cells were placed in each sample with a capacity of 200µl. Then the B[*a*]P [7,5µM] was added to the cultures for 48-hours to increase the expression of P_{gp} and to induce the genotoxic damage in the lymphocytes [8]. At the end of culture time lymphocytes were incubated with FPh-2HCl [concentration range: 0.125-100µM] for 30 min or 120 min and afterwards the fluorochrome Rod-123 (5µM, 60 min) was added to the cultures. Genotoxically damaged lymphocytes not incubated with FPh-2HCl, were considered the control culture. The P_{gp} activity in the cultures was assessed by measurements of the Rod-123 fluorescence (fluorescence arbitrary units, FAU) in cell homogenates at 488-530 nm using Victor2 reader.

Statistical analyses. The statistical estimation of data obtained in the Rod-123 retention test was carried out using a computer program *STATISTICA* 10.1 PL and t-test and ANOVA tests were used to assess the differences in the intensity of the Rod-123 accumulation in the lymphocyte cultures, assuming confidence interval as $p < 0.05$.

RESULTS AND DISCUSSION

Differential Scanning Calorimetry. DSC is useful technique to study modification in model biomembranes induced by incorporation of different compounds. To characterize the interaction between FPh-2HCl and model dipalmitoylphosphatidylcholine (DPPC) lipid bilayer, we applied DSC method. The studies using DSC technique carried out for DPPC model lipid multilayers containing increasing amount of piperazine Pht – FPh-2HCl, have shown significant impact of FPh-2HCl on structure and physicochemical properties of lipid bilayer. Data suggests that this effect is strictly correlated with FPh-2HCl/DPPC molar ratio (0-50 mol%). Even at the lowest FPh-2HCl/DPPC molar ratio, the gel-liquid crystalline phase transition (T_m) occurred at the lower temperature than in a pure DPPC dispersion. This effect indicates that FPh-2HCl strongly interacts with DPPC model membranes and destabilizes its hydrophobic part. Increasing the content of FPh-2HCl we observed a gradual reduction in the T_m values, which is accompanied by a decreasing of cooperation of the main phase transition from a well ordered gel to more disordered liquid-crystalline phase. It indicates that FPh-2HCl significantly intercalates into DPPC model membranes rising the membrane fluidity and permeability in the whole range of the studied FPh-2HCl /DPPC molar ratios [3]. In accordance with Figure 2, the dependence of T_m values on FPh-2HCl concentration exhibits biphasic run [4], what shows different manner of FPh-2HCl-DPPC interactions at low and higher molar ratios [5]. Moreover, an addition of 15 mol% of FPh-2HCl is a critical concentration, at which the maximum increase in the fluidity of lipid bilayers was found. At molar ratios up to 15 mol%, FPh-2HCl decreased almost linearly the temperature of main phase transition from 41.5 ± 0.1˚C for pure DPPC to 32.3 ±0.4˚C for 15 mol% FPh-2HCl/DPPC system, while for higher molar ratios FPh-2HCl caused only slight changes in T_m values. For molar ratios equal 15 mol% and higher, a low susceptibility of lipid systems to increasing FPh-2HCl concentrations, indicated the formation of the different type of lipid packing. This new lipid phase is characterized by less

lipid packing and increased lipid chain fluidity. This allows the amphiphilic FPh-2HCl molecules to aggregate. Therefore, in the range 15-50 mol% FPh-2HCl exerted similar effect of these analyzed lipid systems. The identical concentration dependence for decrease of T_m values was observed also for another phenothiazine derivative by other authors i.e. for chlorpro- mazine (CPZ)/DPPC systems [4]. For the FPh-2HCl/DPPC molar ratios lying in the range 15-50 mol% structural defects in DPPC model membrane caused by the incorporation of FPh-2HCl are so significant and the structure of lipid membrane is such disturbed, that we did not observe any additional effects.

The Rod-123 Retention Test. The spectroflurometric

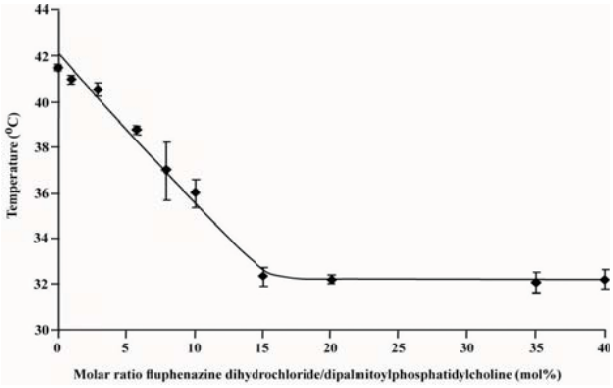

Fig. 2. The influence of FPh-2HCl (0-50 mol%) on the temperature of the main phase for DPPC lipid bilayer. The DSC studies

in vitro test of Rod-123 retention is a method recommended in the literature to assess at the same time the P_{gp} activity and a chemosensitizing effect of transport function inhibitors of this protein. Rod-123 is a fluorochrome, non-toxic for cells, which mimics *in vitro* an intracellular fate of the most of cytostatic drugs, bonded and transported by P_{gp} [7]. The results of Rod-123 measured in fluorescence arbitrary units (FAU) in human lymphocyte cultures, genotoxically damaged with B[*a*]P (7,5μM, 48h) and incubated with FPh-2HCl [0,125-100μM] (E) were compared with the results obtained in control culture (E_c) and were shown in Table 1 as E/E_c.

The results proved that FPh-2HCl influenced inversely on the intensity of the Rod-123 accumulation in lymphocytes. Together with the increased concentration of FPh-2HCl in culture medium, the ability of genotoxically damaged lymphocytes to accumulate Rod-123 decreased. Intracellular retention of Rod-123 in the presence of FPh-2HCl in the highest used doses [10μM], was equal or below the level of control culture. We did not establish the significant difference (p>0.05; ANOVA) of the Rod-123 accumulation in lymphocytes between cultures incubated for 30 min and for 120 min with FPh-2HCl (Tab. 1; Fig. 3).

Table 1. The Rod-123 accumulation in human genotoxically damaged lymphocyte cultures in comparison to control culture (E/E_c) and statistical analyses of the results obtained in Rod-123 retention test. Significant results are given in bold

Rod-123 retention test	Preincubation 30 min	*t*-test	Preincubation 120 min	*t*-test

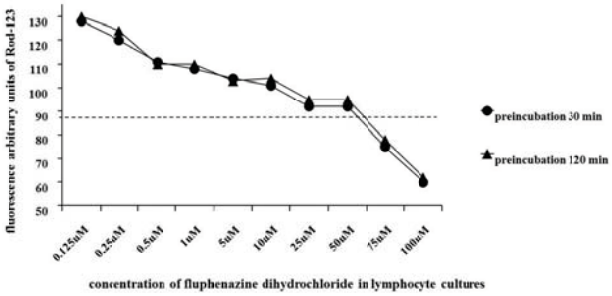

Fig. 3. Accumulation of Rod-123 in lymphocyte cultures in the presence of FPh-2HCl [0.125-100μM] depending on the preincubation time of cells with the tested compound. The level of control culture was marked with a dashed line in the figure. Spectroflurometric *in vitro* test of Rod-123 retention

Concentration of fluphenazine dihydrochloride	0.125 μM	1.47	**p<0.05**	1.49	**p<0.05**
	0.25 μM	1.38		1.43	
	0.5 μM	1.28		1.26	
	1 μM	1.24		1.26	
	5 μM	1.20		1.18	
	10 μM	1.16		1.20	
	25 μM	1.06	p>0.05	1.09	p>0.05
	50 μM	1.06		1.09	
	75 μM	0.86	**p<0.05**	0.90	**p<0.05**
	100 μM	0.69		0.71	
ANOVA		Preincubation 30 min		Preincubation 120 min	
		F = 0.0869; df = 1; p = 0.9799			

The literature data indicates that chemosensitizing compounds with characteristics of P_{gp} transport function inhibitors can decrease the activity of this protein by three independent mechanisms: 1. directly combine with transmembrane protein fragments, 2. impede ATP hydrolysis or 3. disorganize the structure of cell membrane which in effect, regardless of the mechanism, can lead to P_{gp} dysfunction [8].

We previously showed that the FPh-2HCl blocks the transport function by all three mechanisms, in a way dependent on the concentration [2,8,9]. It was suggested that FPh-2HCl in low concentration [10μM] shows the affinity to P_{gp} places (domains) which bond ligands and/or impairs the phosphorylation of this protein. While in higher doses [≥ 10μM], its interactions with cell membrane lipids predominate, probably lead to a significant destruction of cell membrane in higher concentration

[9,11]. The results presented in this paper confirm our preliminary findings that the higher FPh-2HCl concentration exerts the weaker chemosensitizing activity in damaged human lymphocyte cultures [11].

CONCLUSIONS

The DSC results clearly show that FPh-2HCl incorporates into hydrophobic part of lipid bilayer and alters its biophysical properties. We found that membrane interactions of FPh-2HCl in model membrane system and FPh-2HCl chemosensitizing activity in human lymphocyte cultures depend on its concentration in tests. Considering the destabilization (substantial fluidization) of DPPC model membranes in the presence of FPh-2HCl over 10mole%, as it was stated in DSC, it can be assumed that the observed in the Rod-123 retention test lack of chemosensitizing effect for FPh-2HCl in the concentration above 10 μM, could be caused by breaking (destroying) the organized structure of lymphocyte cell membrane in this range of concentration. Albeit, the FPh-2HCl in the concetrations below 10 μM significantly and inversely influenced on the Rod-123 accumulation in lymphocytes; higher concentration of the FPh-2HCl caused a weaker chemosensitizing activity in genotoxically damaged human lymphocyte cultures. Further research of FPh-2HCl chemopreventive effect in human lymphocytes cultures is necessary to establish precisely the range of concentration in which FPh-2HCl will have the optimal chemosensitizing effect conditioned on its potential membrane interaction.

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ABBREVIATIONS

B[*a*]P – benzo[*a*]pyrene; DPPC – dipalmitoylphosphatidylcholine; DSC – differential scanning calorimetry; FAU – fluorescence arbitrary units; FPh-2HCl – fluphenazine dihydrochloride; MDR – multidrug resistance; P_{gp} – P-glycoprotein; Pht – phenotiazine derivatives; Rod-123 – rhodamine 123.

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