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Short Communication

**THIORIDAZINE INDUCES ERYTHROCYTE STOMATOCYTOSIS
DUE TO INTERACTIONS WITH NEGATIVELY CHARGED LIPIDS**

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Abstract: Despite the fact that thioridazine is used clinically as a neuroleptic drug, little is known about the molecular mechanisms underlying its biological effects, in particular about its interactions with membranes. In the present work we investigate the influence of thioridazine on model and cell membranes, using calorimetry, DPH fluorescence polarization measurements, studies of haemolysis and scanning electron microscopy. The experiments show that thioridazine interacts with lipid bilayers and intercalates into bilayer structure. We found that erythrocyte stomatocytosis induced by the drug might be related to preferential interaction of thioridazine with charged lipids.

Key Words: Phenothiazine Derivatives, Erythrocyte Shape, Calorimetry, DPH Fluorescence Polarization

INTRODUCTION

Thioridazine (TDZ) is a phenothiazine derivative used as a tranquilliser and antidepressant in the management of certain psychiatric disorders. Despite the fact that thioridazine is used clinically, little is known about the molecular mechanisms underlying its biological effects. In particular, the literature dealing with TDZ-membrane interactions is scarcely available. In cultured glial cells TDZ was found to increase the fluidity of membranes and to alter the fatty acid composition of membrane lipids [1]. The antioxidant activity of thioridazine towards the mitochondrial membranes was proposed to be involved in processes with potential implications in apoptosis [2]. Due to the structural similarity to chlorpromazine (and other phenothiazine derivatives) one can expect that TDZ should intercalate into the lipid bilayer and change its biophysical properties. In

the present work we investigate the influence of TDZ on model and cell membranes.

MATERIALS AND METHODS

Thioridazine and chlorpromazine (CPZ) hydrochlorides were purchased from ICN Biomedicals (Costa Mesa, CA, USA). The chemical structure of TDZ is presented in Fig. 1. 1,2-dimyristoyl-sn-glycero-3-phosphatidylglycerol (DMPG) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 1,6-diphenyl-1,3,5-hexatriene (DPH), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phosphatidylethanolamine (DMPE) were from Sigma (St. Louis, MO, USA). Lipids were used without further purification. All other chemicals were of analytical grade.

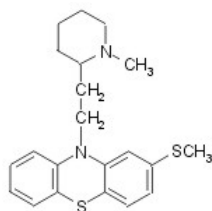


Fig. 1. Chemical structure of thioridazine.

Samples for calorimetry were prepared as described in [3], calorimetric measurements were performed using a Rigaku microcalorimeter with the measuring thermostat rebuilt in our laboratory.

Fluorescence polarization measurements were performed according to the methods described in [4], except that TDZ stock solutions were prepared in Tris-HCl buffer instead of DMSO.

Erythrocyte preparation and determination of haemolytic activity

Blood was drawn from healthy volunteers by vein puncture into tubes with sodium versenate according to the procedure described in [5]. After washing with PBS containing 0.9% NaCl and following centrifugation, erythrocytes were purified on an α -cellulose column. After next three centrifugations, erythrocyte suspension in buffer (138 mM NaCl, 5mM KCl, 6.1 mM Na₂HPO₄ x 12 H₂O, 1.4 mM NaH₂PO₄ x H₂O, 5.6 mM glucose, 1mM MgCl₂, pH=7.4) was obtained (hematocrit 30%).

Haemolysis was measured in samples containing 50 μ l of the above erythrocyte suspension and 950 μ l of thioridazine solutions of different concentrations. The final hematocrit of samples was 1.5%. After incubation with a phenothiazine derivative (for 60 min. at 37⁰C), erythrocytes were once more centrifuged for 3

minutes at 13000 rpm. The absorbance of supernatant at 545 was a measure of haemolysis.

Scanning electron microscopy (SEM)

Scanning electron micrographs were obtained with a LEO 435 electron microscope (Karl Zeiss, Jena, Germany) operating at 20 kV. The samples were prepared using methods described in [6]. Erythrocytes were suspension-fixed in 4% glutaraldehyde in phosphate buffer and postfixed in OsO_4 . After dehydration in a graded series of acetone/water, the samples were gold-sputtered and then examined.

RESULTS AND DISCUSSION

Both calorimetric and fluorescence polarization measurements revealed that TDZ interacts with lipid bilayers in a concentration-dependent manner. The phase transition parameters: temperature (Fig. 2A) and enthalpy (Fig. 2B) as well as DPH fluorescence polarization (Fig. 3) were the most profoundly altered in the case of TDZ/DMPG mixtures. Effects exerted by TFP on the systems composed of zwitterionic lipids (DPPC, DMPC or DMPE) are smaller than those observed for negatively charged DMPG. This highest capability of TDZ to interact with negatively charged lipids correlates with the similar properties of CPZ as described by Nerdal *et al.* [7]. Transition temperature decrease is usually interpreted as a result of perturbation induced in the polar head-group region, while enthalpy decrease as an effect of loosening of hydrocarbon chains packing. Therefore we conclude that TDZ presumably intercalates into the lipid bilayer and interacts with its polar/apolar interface. A similar location of thioridazine molecules was proposed by Moncelli and Becucci [8] in the study on TDZ/dioleoyl-phosphatidylcholine monolayers. Other phenothiazines, like CPZ [7] or trifluoperazine [3], are also supposed to locate in this region of lipid bilayer. TDZ molecules present in the bilayer reduce the mobility of lipid molecules and thus decrease membrane fluidity (as detected by elevation of DPH fluorescence polarization).

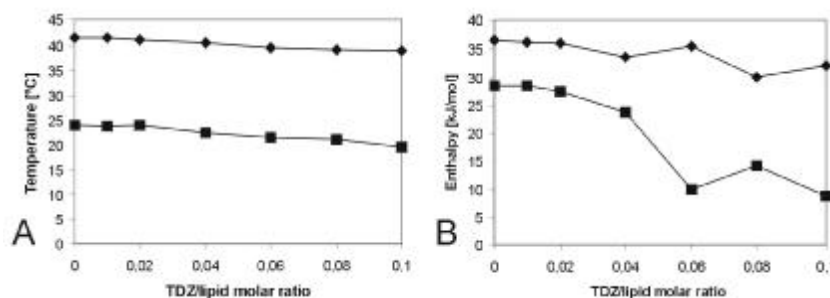


Fig 2. Influence of TDZ on lipid phase transition parameters: temperature (A) and enthalpy (B). Lipids used in experiments: ◆ – DPPC, ■ – DMPG.

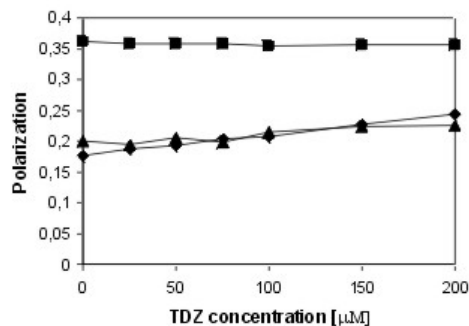


Fig. 3. Dependence of DPH fluorescence polarization on TDZ concentration in liposome suspension. Liposomes were prepared from: ♦ - DMPG, ▲ - DMPC and ■ - DMPE. Each point represents average of at least ten measurements, maximal SD = 0.005.

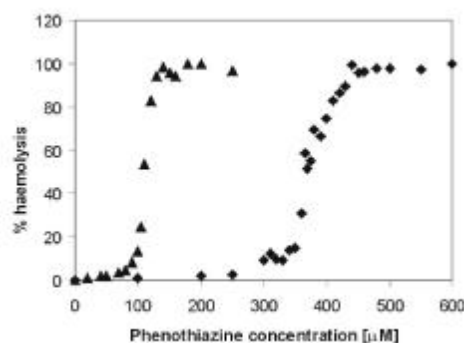


Fig. 4. Erythrocyte haemolysis induced by thioridazine (▲) and chlorpromazine (♦).

TDZ also interacts with cell membranes, as it was revealed in experiments on erythrocytes. We compared the ability of TDZ and CPZ to induce erythrocyte haemolysis (Fig. 4). It was found that TDZ is a much more effective haemolytic agent than CPZ, since haemolysis onset (c_0), 50% (c_{50}) and completion (c_{100}) concentrations ($c_0 = 100 \mu\text{M}$, $c_{50} = 110 \mu\text{M}$, $c_{100} = 180 \mu\text{M}$) were much lower for thioridazine than the corresponding values for chlorpromazine ($c_0 = 300 \mu\text{M}$, $c_{50} = 370 \mu\text{M}$, $c_{100} = 440 \mu\text{M}$).

The electron scanning microscopy investigation showed that thioridazine (100 μM) causes stomatocytosis of erythrocytes (Fig. 5, right panel). Induction of erythrocyte shape changes has been well known for many years and could be caused, among others, by anionic and cationic amphiphiles [9,10], as well as by phenothiazines [5,11]. According to the bilayer couple hypothesis [12], the stomatocytic effect points to the favourable interaction of TDZ with the inner layer of the erythrocyte's membrane. Such a conclusion seems to be in agreement with the results of our calorimetric and fluorescence polarization studies. In erythrocytes the inner layer of the membrane is rich in phosphatidylserine – a lipid bearing net negative charge at neutral pH like

DMPG studied in our work. Negatively charged lipids are absent in the outer layer. On the other hand, thioridazine at neutral pH occurs mostly in protonated form and bears positive charge ($pK_{aTDZ} = 9.50$, according to [13]). Due to the electrostatic attraction, phenothiazine molecules can interact with negatively charged lipids stronger than with zwitterionic ones. Thus the higher affinity of TDZ to lipids like phosphatidylserine might be responsible for phenothiazine accumulation in the inner membrane layer and erythrocyte stomatocytosis. Preferential binding of TDZ with the inner leaflet of the membrane can also be caused by its interaction with negatively charged proteins of the membrane skeleton, as it was proved for lipids (reviewed in [14]) and chlorpromazine [15]. These interactions are supposed to be involved in the modulation of membrane fluidity, as well as in the regulation of membrane-related processes (e.g. signalling or shape control).

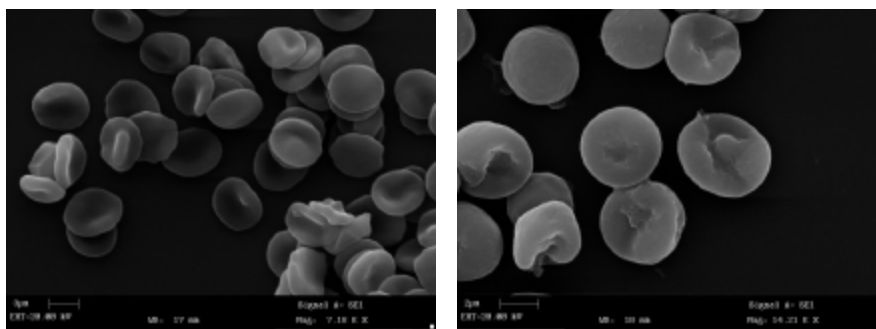


Fig. 5. SEM micrographs showing erythrocyte shape transformations. Normal erythrocytes are shown in the left panel. TDZ (100 μ M) induces erythrocyte stomatocytosis – right panel.

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