Colloids and Surfaces B: Biointerfaces 150 (2017) 106–113

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Membrane effects of dihydropyrimidine analogues with larvicidal activity

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ARTICLE INFO

Article history:
Received 15 June 2016
Received in revised form
18 November 2016
Accepted 22 November 2016
Available online 24 November 2016

Keywords:
Dihydropyrimidine
Larvicidal
Membrane interaction
Monolayers
Membrane fluidity
Molecular dynamic simulations

ABSTRACT

Two recently synthesized dihydropyrimidines (DHPMs) analogues have demonstrated larvicide and repellent activity against *Anopheles arabiensis*. DHPMs high lipophilicity suggests that these compounds may interact directly with the membrane and modify their biophysical properties. The purpose of the present study was to characterize the interaction of both compounds with artificial membranes. Changes on the properties of DPPC films were studied using Langmuir monolayers. The presence of DHPMs in the subphase modified the interfacial characteristics of DPPC compression isotherms, causing the expansion of the monolayer, inducing the disappearance of DPPC phase transition and increasing the molecular packing of the film. Moreover, both compounds showed ability to penetrate into the lipid monolayers at molecular pressures comparable to those in biological membranes. The effects of both DHPMs on the molecular organization of DPPC liposomes were measured by fluorescence anisotropy. The results indicate that their presence between lipid molecules would induce an increasing intermolecular interaction, diminishing the bilayer fluidity mainly at the polar region. Finally, we performed free diffusion MD simulations and obtained spatially resolved free energy profiles of DHPMs partition into a DPPC bilayer through Potential of Mean Force (PMF) calculations. In agreement with the experimental assays, PMF profiles and MD simulations showed that DHPMs are able to partition into DPPC bilayers, penetrating into the membrane and stablishing hydrogen bonds with the carbonyl moiety. Our results suggest that DHPMs bioactivity could involve their interaction with the lipid molecules that modulate the supramolecular organization of the biological membranes and consequently the membrane proteins functionality.

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1. Introduction

The pyrimidine system is an important pharmacophore with abundant occurrence in nature. Natural and synthetic pyrimidine derivatives have a wide range of actions with biological activities such as anticancer, by altering the mitotic spindle and arresting mitosis [1], as well as antiviral [2,3], antihypertensive [4], calcium channel blocking [5], antitubercular [6], antimicrobial [7–9], anti-inflammatory [10,11], larvicidal and insecticide actions [12,13]. Antimalarial drugs include halogenated dihydropyrimidine analogues [14] and the commercially available pyrimethamine, a folic acid antagonist used for treatment and prevention of malaria or, with a sulfonamide, to treat toxoplasmosis [15].

Dihydropyrimidines (DHPMs) are useful scaffolds for the design of pharmacological agents. Despite the fact that the exact mechanisms responsible for their biological activity remain unknown, disturbance of the cell membrane has been suggested as the mode of actions of several larvicide compounds [16]. The wide range of effects produced by DHPMs and their high lipophilicity suggest that these compounds may interact directly with the membrane and thus activate different signals.

Previous studies have shown that the interaction of lipophilic compounds with the membrane can be explained by an equilibrium model of partition [17] and that their partition into the membrane

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http://dx.doi.org/10.1016/j.colsurfb.2016.11.028
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modifies its biophysical properties. This behavior has been confirmed for lipophilic compounds such as benzodiazepines (BZD), whose location in the polar region of the membrane explains their effects, decreasing the size of DPPC vesicles, inducing lipid bilayer redistribution and inhibiting inverted phases formation, which are crucial intermediaries in fundamental biological phenomena such as membrane fusion [18–20]. The possibility that BZDs affect transduction mechanisms at several levels of receptor binding has also been shown for other lipophilic substances [21,22].

Many experimental evidences show that changes in the cell membrane microenvironment, where intrinsic proteins as specific receptors and channels are inserted, can modify their function [23–27]. Such is the case described by our group, where several gabaergic compounds (i.e. monoterpenes and phenols) that modulate the $GABA_A$ receptor allosteric behavior, also are capable of modifying membrane properties [28–32].

In the present work, we investigated the ability of two recently synthesized DHPMs with insecticidal activity (Fig. 1) [13], to interact with the membrane by analyzing their effects on the biophysical properties of two artificial model membranes: i) Langmuir monolayers at the air-water interface, where compression and penetration isotherms were analyzed, and ii) phospholipid liposomes yielded to fluorescence anisotropy analysis. Furthermore, we combined these experimental techniques with molecular dynamics (MD) simulations of DHPMs partition into the bilayer.

2. Materials and methods

2.1. Materials

2-(3-Bromophenylamino)-6-(4-chlorophenyl)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chloride (DHPM1) and 2-(4-bromophenylamino)-6-(4-chlorophenyl)-5-(methoxycarbonyl)-4-methyl-3,6-dihydropyrimidin-1-ium chloride (DHPM6) were synthesized according to Venugopala et al., 2013 [13]. DPH and TMA-DPH (*N,N,N*-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium*p*-toluenesulfonate) were obtained from Sigma-Aldrich Chem (St. Louis, MO; USA) and l-2-dipalmitoyl-sn-glycero-3-phospocholine (DPPC) lipid were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other reagents were of the highest analytical grade. Solutions were prepared with double-deionized water.

2.2. Langmuir monolayers

2.2.1. Surface pressure (π) – molecular area (A) isotherms

Compression isotherms (π/A isotherms) were performed by the compression of monolayers containing DPPC using a Minitrough II (KSV, Finland). Phospholipidic monolayers on the air–water interface were prepared by spreading pure DPPC dissolved in chloroform on the aqueous surface of a Teflon™ trough filled with bidistilled-deionised water containing each DHPM at a final concentration of 100 μM as subphase. After 5 min for solvent evaporation, the film was compressed isometrically at a constant rate of 5 ± 1 mm/min until reaching the target pressure. Lateral surface pressure (π) was measured with a platinum plate by the Wilhelmy plate method [33] at different molecular areas (A) of the phospholipid. Isotherms were averaged at least from duplicates. All assays were performed at 25 ± 1 °C.

2.2.2. Compressibility analysis

The onset of phase transition points was identified from a minimum and π_c from a maximum in the variation of the *compressibility modulus* (*K*) vs. molecular area plot. For this, *K* values were calculated from π-A isotherms data applying Eq. (1):

$$K = -(A_\pi)\left(\frac{\delta_\pi}{\delta_A}\right)\pi \quad (1)$$

where $A\pi$ is the molecular area at the indicated surface pressure. Control isotherms obtained in the presence of DMSO 0.25% (v/v) (used as DHPMs dissolvent) were not different from those at 0% DMSO (data not shown).

2.2.3. Penetration isotherms: partition into lipid interface

To study the penetration of the DHPMs into DPPC lipid monolayers, π *vs.* time plots in a Langmuir trough at constant total surface area were performed, using double deionized water as subphase.

Penetration experiments were carried out in circular homemade Teflon through by injections of DHPMs, from stock solutions in DMSO, into the subphase (100 μM final concentrations) under continuous stirring (150–250 rpm) at different initial π (π_i), in order to measure the increment in π induced by the penetration into a preformed DPPC monolayer as a function of time. The injection of each DHPM in the sub-phase was done after the stabilization of the π_i of the film (between 5 and 10 min) at 25 ± 1 °C. Initially, π vs. time plots were measured until an equilibrium surface pressure was reached (changes in pressure less than 1 mN m^{-1} per hour). Finally, plots of $\Delta\pi$ vs. π_i were graphed in order to determine the "*cut off*" point for both DHPMs.

2.3. Fluorescence anisotropy

2.3.1. Preparation of large unilamellar vesicles

Multilamellar large vesicles (MLVs) were prepared as described elsewhere [34]. Briefly, the appropriate amount of DPPC dissolved in chloroform was placed in a glass tube and evaporated under a stream of nitrogen with constant rotation to facilitate the formation of a thin lipid film; traces of solvent were removed under vacuum. The dried lipid was suspended in water at a final concentration of 0.03 mg/mL [35] by repeating seven consecutive cycles of heating at 65 °C for 1 min plus vortexing for 30 s, and MLVs were formed. Large unilamellar vesicles (LUVs) of homogeneous size were obtained by extruding 19 times the MLVs suspension trough 100-nm pore size Whatman polycarbonate filters using a miniextruder Liposofast (Avestin, Canada).

2.3.2. Steady-State fluorescence

The fluorescent probes DPH (4 μM) and TMA-DPH (6 μM) were added to the DPPC-LUV suspension (prepared as described above) and incubated for 1 h at room temperature [36]. The effect of DHPMs (100 μM) on DPH and TMA-DPH steady-state fluorescence anisotropy was studied. Anisotropy values were calculated from the emission fluorescence intensities at λ_{em} = 430 nm (λ_{ex} = 356 nm) measured with the excitation and the sample polarizer filters oriented parallel and perpendicularly one with respect to the other, in a L-format FluoroMax-3 spectrofluorometer (JovinYvon, Horiba, Japan). Slits width and integration time were set at 2 nm and 1 s, respectively. Control samples containing DMSO used as vehicle were tested to rule out the effect of this solvent.

Steady-state fluorescence anisotropy (FA) was calculated using Eq. (2):

$$FA = \frac{I_{VV} - I_{VH}.G}{I_{VV} + 2I_{VH}.G}, G = \frac{I_{HV}}{I_{HH}} \quad (2)$$

where I_{VV}, I_{HH}, I_{VH}, and I_{HV} are the values of the different measurements of fluorescence intensity taken with both polarizers in vertical (VV) and horizontal (HH) orientations or with the excitation polarizer vertical and the emission polarizer horizontal (VH) or

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