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Edelfosine disturbs the sphingomyelin–cholesterol model membrane system in a cholesterol-dependent way – The Langmuir monolayer study

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ABSTRACT

Synthetic alkyl-lysophospholipids, represented by edelfosine (ED), reveal strong anticancer activity and therefore are promising drugs used in anticancer therapy. Primary target for edelfosine is cellular membrane, which is in contrast to traditional cytostatics affecting DNA. The mechanism of antitumor activity of edelfosine was hypothesized to be related to its accumulation in membrane rafts. Inspired by these findings, we have performed the Langmuir monolayer studies on the influence of edelfosine on systems composed of sphingomyelin (SM) and cholesterol (Chol), being the principal components of membrane rafts. Sphingomyelin–cholesterol proportion in monolayers was varied to reflect the composition of solely membrane rafts (SM/Chol = 2:1) and contain excess of cholesterol (SM/Chol = 1:1 and 1:2). Into these systems, edelfosine was added in various concentrations. The analysis of surface pressure–area isotherms, complemented with films visualization with Brewster angle microscopy (BAM) allowed us to compare the effect of edelfosine on condensation and ordering of SM/Chol monolayers. The results evidenced that the influence of ED on the interactions in model membranes and its fluidizing effect is highly cholesterol-dependent. The strongest decrease of monolayer ordering was observed for model raft system, while the excess of cholesterol present in the remaining mixtures was found to weaken the fluidizing effect of the drug.

1. Introduction

Intensive investigations on the organization of biological membranes led to the concept that within cellular bilayer, heterogeneous domains enriched in cholesterol and sphingolipids, acting as platforms for specific proteins, are formed [1,2]. These assemblies, called membrane rafts, form in model membranes a liquid ordered phase, which exist in more fluid liquid disordered state [3–6]. Membrane rafts participate in various vitally important cellular processes (e.g. cell signaling and membrane trafficking), however, they are also used by various pathogens (e.g. bacterias or viruses) to invade a cell; also they facilitate a fusion of human immunodeficiency virus 1 (HIV-1) with a host cells and additionally accumulate proteins involved in Alzheimer's disease [1,2,7]. On the other hand, many compounds of therapeutic properties are known, for which a raft-based mechanism of action has been evidenced [8–10]. For example, edelfosine (ED), a new generation anticancer drug, has been suggested to act at the membrane level via accumulation in rafts [11–13]. Edelfosine (1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine, *abbr.* Et-18-OCH_3) belongs to the group of synthetic antitumor lipids (ATLs) of phospholipid-like structure, which introduction in clinical treatment was a breakthrough in anticancer therapy. Synthesis of these compounds was inspired by cytolytic properties observed for natural lysophosphatidylcholines (LPCs), being intermediate products of phospholipids metabolism [14]. Chemical modifications in LPCs structure, directed towards increasing of their metabolic stability, led to the creation of alkyl-phospholipids (ALPs), which were found to exert cytostatic and cytotoxic activity on certain tumor cells [15], edelfosine being the most effective drug of the ALPs series [16].

Although a plethora of studies on cell lines and artificial systems have been done so far, the mechanism of action of ATLs, in general, has not been clarified yet and several hypotheses have been put forward [12,16,17]. However, for anticancer activity of a drug, its incorporation into membrane and subsequent penetration throughout bilayer in therapeutic amount is required [16]. So far little is known on this issue. For example, it is still unclear, which membrane component(s) target the drug molecule to the neoplastic cell membrane, sparing the normal cells, and why some tumor cells are resistant while the others are sensitive to this drug (see for example Refs. [18–20]).

To get a deeper insight into these questions, series of experiments were performed on artificial membrane systems, which in a controllable manner enable to study a particular aspect of a given phenomenon, contrary to highly variable and quite complicated natural systems (living cells or isolated natural membranes)

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[21,22]. One of the methods useful to examine the behavior of a drug molecule in membrane environment is the Langmuir monolayer technique, which has been successfully applied to study the interactions between edelfosine and various membrane lipids [23–26]. To summarize the obtained results, it was found that due to strong affinity of edelfosine to cholesterol and gangliosides [23,26], these lipids may be of particular importance when the selectivity of the drug is concerned. Gangliosides, which are overexpressed in tumor progression (e.g. [27]), seem to be of high importance in attracting edelfosine selectively to neoplastic cells. On the other hand, cholesterol content in the membrane was found to "control" the penetration of the drug through the membrane by having the ability to bind edelfosine [23]. Moreover, it can be postulated that sterol, as a regulator of membrane fluidity, is responsible for the selectivity of this drug – namely, membranes of higher sterol concentrations were not penetrated by ED in contrast to the membranes of lowered cholesterol level [20]. Also, cholesterol is important in the mechanism of action of ED in the sense of being a basic component of lipid rafts, which were hypothesized to be a site of activity of edelfosine in biomembrane [11–13]. Recent studies on model membranes performed on POPC/SM/cholesterol (1:1:1) bilayers evidenced that the incorporation of edelfosine into the foregoing mixture modifies membrane organization due to associations of ED with cholesterol [28].

To investigate the effect of edelfosine on rafts at molecular level, in this work we have analyzed the influence of ED on model raft system mimicked by SM/Chol = 2:1 monolayers. Additionally, to find a correlation between the effect of ED and sterol concentration, we furthered our studies, performing similar experiments on model sphingomyelin/cholesterol systems containing excess of cholesterol–SM/Chol = 1:1 and 1:2 (~25% and ~50% excess cholesterol, respectively). The obtained results evidenced for significant differences induced by edelfosine on raft-mimicking membrane *versus* the remaining sphingomyelin/cholesterol systems.

2. Experimental

2.1. Materials

The lipids used in our experiments: edelfosine (1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine, ED), egg sphingomyelin (SM) as well as cholesterol (Chol) were synthetic products of high purity (≥99.1% for ED and ≥99% for SM and Chol) purchased from Biaffin GmbH&Co KG, Germany (edelfosine), Avanti Polar lipids (sphingomyelin) and Sigma (cholesterol). Spreading solutions of the edelfosine and sphingomyelin were prepared in chloroform/methanol 9:1 (v/v) mixture, while cholesterol was dissolved in chloroform (both chloroform and methanol were purchased from Aldrich, HPLC grade, ≥99.9%). Mixed solutions of desirable compositions were prepared from the respective stock solutions and deposited onto water subphase with the Hamilton micro syringe, precise to 1.0 μL. The monolayers were left after spreading for 10 min before the compression was initiated with the barrier speed of 20 cm^2/min.

2.2. Methods

The experiments were performed with the NIMA (UK) Langmuir trough (total area = 300 cm^2) placed on an anti-vibration table. Surface pressure was measured with the accuracy of ±0.1 mN/m using Wilhelmy plate made of filter paper (ashless Whatman Chr1) connected to an electrobalance. The subphase temperature (20 °C) was controlled thermostatically to within 0.1 °C by a circulating water system. UltrapureMilli-Q water used as the subphase in the monolayer experiments, at 20 °C ± 0.1° C, has surface tension of 72.6 mN/m, pH 6.5 and resistivity of 18 MΩ cm.

Brewster angle microscopy experiments were performed with UltraBAM instrument (Accurion GmbH, Goettingen, Germany) equipped with a 50 mW laser emitting p-polarized light at a wavelength of 658 nm, a 10× magnification objective, polarizer, analyzer and a CCD camera. The spatial resolution of the BAM was 2 μm.

2.3. Composition of monolayers

The experiments were performed for binary SM/Chol monolayers and ternary SM/Chol/ED films. The investigated mixtures differed in the proportion of sphingomyelin to cholesterol, namely: SM/Chol = 2:1 (system imitating raft composition) and two systems of higher content of cholesterol, namely SM/Chol = 1:1 and 1:2. The SM/Chol ratio in the respective ternary monolayers was always kept constant, while the concentration of ED, expressed as mole fraction of edelfosine, varied (X_{ED} = 0; 0.025; 0.05; 0.1; 0.2; 0.3 and 0.5).

3. Results and discussion

Fig. 1 presents the isotherms recorded for sphingomyelin/cholesterol monolayers of various proportion of lipid components (namely SM/Chol = 2:1; 1:1 and 1:2), pure edelfosine (ED) film as well as the curves for the mixtures of SM/Chol/ED of constant SM/Chol ratio and various concentrations of the drug. As can be observed in Fig. 1A, the progressive addition of edelfosine into 2:1 monolayer causes its gradual shift towards larger areas. On the other hand, when edelfosine is incorporated into SM/Chol films of higher content of cholesterol (namely: SM/Chol = 1:1 and 1:2) the curves for ternary mixtures containing lower concentration of ED (X_{ED} = 0.025–0.1) are nearly identical with those recorded for the respective binary SM/Chol films (see Fig. 1B and C). For all the investigated systems, the effect of edelfosine on the characteristics of the isotherm seems to be the strongest at 20–50% of ED content in the monolayer. At the foregoing concentrations of the drug in the model systems, the alterations in the position of the isotherms (i.e. their shift to larger areas) are most pronounced; the curves shift towards the isotherm for pure edelfosine and in their course two collapses appear at high surface pressure region.

Taking into account that edelfosine is a biologically active molecule, which incorporates into cellular membranes, the analysis of the obtained results was performed at a higher pressure region (30 mN/m), where the monolayer properties can be linked to a bilayer system [29]. In the first step, the area per lipid values (A_{123}) were estimated from the isotherms and compared with those resulting from the additivity rule [30] (Eq. (1)).

$$A_{123}^{id} = A_{12}(X_1 + X_2) + A_3X_3 \quad (1)$$

where A_{12} is the mean area per molecule in SM/Chol films, A_3 is the molecular area of edelfosine in its pure film at a given surface pressure and X_1, X_2 and X_3 are the mole fractions of the three components in the mixed film. The obtained results are presented in Fig. 2A. The deviations from the additivity rule, observed in A_{123} *versus* composition plots (Fig. 2A), suggest a non ideal behavior and miscibility of the monolayer's components at a given surface pressure. It is evident that the results obtained for mixtures of the lowest content of cholesterol (SM/Chol = 2:1) differ from those collected for the other investigated systems. Namely, the area per lipid values obtained for SM/Chol/ED (SM/Chol = 2:1) ternary films of X_{ED} = 0.025–0.1 are slightly lower than those resulting from the additivity rule. At the same region of edelfosine concentration for the remaining SM/Chol/ED mixtures, the deviations are slightly positive. When edelfosine comprises 20, 30 or 50% of monolayer's components, the deviations from additivity rule observed for the