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Effect of anti-tumor ether lipids on ordered domains in model membranes

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Abstract 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (OMPC, edelfosine) and 1-hexadecylphosphocholine (HePC, miltefosine) represent two groups of synthetic ether lipid analogues with anti-tumor activity. Because of their hydrophobic nature, they may become incorporated into plasma membranes of cells, and it has been argued that they may act via association with lipid rafts. With the quenching of steady-state fluorescence of probes preferentially partitioning into sterol-rich ordered domains (cholestatrienol and *trans*-parinaric acid), we showed that OMPC and HePC by themselves did not form sterol-rich domains in fluid model membranes, in contrast to the two chain ether lipid 1,2-*O*-dihexadecyl-*sn*-glycero-3-phosphocholine. Nevertheless, all three ether lipids significantly stabilized palmitoyl–sphingomyelin/cholesterol-rich domains against temperature induced melting. In conclusion, this study shows that anti-tumor ether lipids are likely to affect the properties of cholesterol–sphingomyelin domains (i.e., lipid rafts) when incorporated into cell membranes.

Keywords: Quenching; Cholestatrienol; *trans*-Parinaric acid; Hexadecylphosphocholine; Octadecylmethylphosphatidylcholine; Dihexadecylphosphatidylcholine

1. Introduction

Synthetic ether analogues of phosphatidylcholine and lysophosphatidylcholine constitute a new class of anti-tumor agents which, in contrast to most of the currently used chemotherapeutic drugs, do not target DNA but act at the cell membrane. Antineoplastic ether-lipids comprise two classes – alkyllysophospholipid derivates with the prototypical 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (OMPC, Et-18-OCH3, edelfosine), and alkylphosphocholines with the prototypical hexadecylphosphocholine (HePC, miltefosine). In addition to its antineoplastic effect, miltefosine is useful for the treatment of human visceral leishmaniasis [1,2] and is also toxic in vitro to other protozoan parasites [3–5].

The mechanism of cytotoxic and cytostatic action of ether lipids is so far poorly understood [6]. The presence of ether links protects these lipids from fast turn-over by phospholipases and they tend to accumulate in the cell membranes [7,8]. Both alkyllysophospholipids and alkylphosphocholines have been shown to inhibit de novo phosphatidylcholine (PC) synthesis at the rate-limiting CTP:phosphocholine cytidylyltransferase step [9–11], possibly by inducing a translocation of the active membrane-bound form of the enzyme to the cytosol [12]. Synthesis of PC is metabolically coupled to sphingomyelin (SM) synthesis, and indeed treatment of cells with HePC decreased the transfer of PC-derived phosphorylcholine moiety thereby increasing the mass of SM precursor ceramide [13]. Except for their impact on lipid metabolism, anti-tumor ether lipids also affect a variety of cell surface receptors and signal transduction pathways including EGF receptors, GM-CSF receptors, Fas, PI-PLC, PtdIns 3-kinase, PKC, the MAPK pathway, and the SAPK/JNK pathway [14–19].

Lipid rafts are considered to be dynamic plasma membrane domains enriched in saturated sphingolipids and cholesterol together with specific proteins. Recent studies with cultured cells suggested that alkyllysophospholipids are internalized by lipid raft-dependent endocytosis [20,21]. Anti-tumor lipids could also act on cellular signaling by affecting the protein composition of rafts. Treatment of human leukemic cells with edelfosine resulted in translocation of Fas into membrane rafts and this apparently trigged apoptosis [16]. In yeast cells edelfosine caused a redistribution of sterol from the plasma membrane into the cell and also a mislocalization of essential proton pump ATPase Pma1p out of rafts [22].

In this study, we employed domain selective fluorescent probes to look on the behavior of alkyllysophosphocholine edelfosine (OMPC) and alkylphosphocholine miltefosine (HePC) in model membrane systems containing unsaturated phosphatidylcholine, saturated sphingomyelin and cholesterol. We were particularly interested in the effect of OMPC and HePC on the stability of sphingomyelin/cholesterol-rich domains in bilayer membranes. For comparison we included in our study also 1,2-*O*-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC), a double chain ether-lipid with no reported anti-tumor activity.

2. Materials and methods

2.1. Material

D-*Erythro*-*N*-palmitoyl–sphingomyelin (PSM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids, Alabaster, AL, USA) as described in [23]. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and

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Abbreviations: 7SLPC, 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine; CTL, cholesta-5,7,9(11)-trien-3-beta-ol; DHPC, 1,2-*O*-dihexadecyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; HePC, 1-hexadecylphosphocholine; DSC, differential scanning calorimetry; OMPC, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; PSM, D-*erythro*-*N*-palmitoyl–sphingomyelin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; tPar, *trans*-parinaric acid; tPar-SM, D-*erythro*-*N*-*trans*-parinoyl–sphingomyelin

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DHPC were obtained from Avanti Polar Lipids. HePC and OMPC were obtained from A.G. Scientific (San Diego, CA, USA). Cholesterol was from Sigma Chemicals (St. Louis, MO, USA). (7-Doxyl)-stearic acid was obtained from TCI (TCI Europe N.V., Belgium) and was used for the synthesis of 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) [24]. All organic solvents were obtained from Merck (Darmstadt, Germany). Stock solutions of lipids were prepared in hexane/2-propanol (3/2, v/v), stored in the dark at −20 °C, and warmed to ambient temperature before use. Millipore UF Plus produced water (resistivity 18.2 MΩ cm) was used.

trans-Parinaric acid (tPar) was obtained from Molecular Probes (Eugene, OR, USA). D-Erythro-*N*-*trans*-parinoyl–sphingomyelin (tParSM) was synthesized from *trans*-parinaric acid and D-*erythro*-sphingosylphosphorylcholine (Matreya LLC, Pleasant Gap, PA, USA) according to [25]. Cholesta-5,7,9(11)-trien-3-beta-ol (cholestatrienol, CTL) was synthesized and purified using the method published in [26]. Probes were stored dry under argon in the dark at −87 °C until solubilized in argon-purged ethanol (CTL) or methanol (tPar, tParSM). Solvents were saturated with argon before use to minimize the risk of oxidation. Stock solutions of fluorescent lipids were stored in the dark at −20 °C and used within a week.

2.2. Quenching of steady-state fluorescence

In fluorescence quenching studies, *F* samples consisted of POPC:7SLPC:variable lipid:cholesterol (30:30:30:10 molar ratio) or POPC:7SLPC:PSM:variable lipid:cholesterol (30:30:15:15:10) or POPC:7SLPC:PSM:cholesterol (45:30:15:10). In F_0 samples 7SLPC was replaced by POPC. The variable lipids used were PSM, DPPC, DHPC, OMPC and HePC. The samples were studied with CTL, tPar or tParSM as the fluorescent probe, which replaced 1 mol% of cholesterol, POPC or PSM, respectively. CTL is fluorescent cholesterol analog which mimics well cholesterol behavior [27] and in lipid mixtures containing coexisting liquid ordered (L_o) and disordered phases preferentially associates with cholesterol-rich L_o domains [23]. Due to its molecular shape, fluorescent tPar also partitions preferentially into ordered phases where its quantum yield is higher then in less tightly packed phases [28,29]. tParSM, as a fluorescent analog of sphingomyelin, was used to probe sphingomyelin-rich domains.

The fluorescence quenching method is described in more detail in [23]. Briefly, in lipid vesicles in which ordered and disordered domains coexist, POPC together with the nitroxide-label quencher 7SLPC form the disordered phase [31]. Fluorescent probes residing in sphingomyelin/cholesterol-rich ordered domains are shielded from quenching by 7SLPC. The melting of ordered domains is seen as a decrease of fluorescence intensity of probe when that comes to closer contact with quencher due to more homogenous bilayer. In our measurements, the fluorescence intensity in the *F* samples is compared to the fluorescence intensity in F_0 samples giving the fraction of quenched fluorescence.

Vesicles used in steady-state fluorescence measurements were prepared to yield a final lipid concentration of 50 μM. Sample preparation and fluorescence measurement details have been given recently and were used here exactly as described [30]. Excitation of tPar was carried out at 305 nm and the emission was recorded at 410 nm.

2.3. Differential scanning calorimetry

Miscibility of lipids with PSM was determined by differential scanning calorimetry (DSC) using Nano II high-sensitivity scanning calorimeter (Calorimeter Science Corporation, Provo, UT, USA). Samples containing pure PSM, or mixture of lipid and PSM (50:50 mol%) were dried under nitrogen and excess solvent was removed by vacuum drying at room temperature for at least 2 h. The dry lipids were resuspended in water, heated above the phase transition temperature of particular lipids, shortly vortexed and sonicated for 4 min at 60 °C in a Bransonic 2510 bath sonicator (Branson Ultrasonics Corporation, CT, USA). The final concentration of lipids in the solution was 1 mM. Two consecutive heating and cooling scans from 0 to 100 °C at a scan rate of 0.5 °C/min were performed.

3. Results

3.1. Formation of ordered domains by ether lipids

In ternary systems containing cholesterol together with low and high temperature melting lipid, cholesterol promotes phase separation into liquid disordered (L_d) cholesterol-poor and liquid ordered (L_o) cholesterol-rich domains [31–33]. In this study as a control system we have used bilayer membranes consisting of POPC as a low T_m lipid, PSM as a high T_m lipid and cholesterol in the ratio POPC:PSM:cholesterol 60:30:10 or 75:15:10 mol%. According to the phase diagram published by de Almeida and coworkers [32], at 23 °C both these mixtures are composed only of a L_d phase enriched in POPC and a L_o phase enriched in PSM and cholesterol.

The formation of sterol-rich ordered domains by anti-tumor ether lipids and by PSM was studied by quenching of fluorescence intensity of CTL (Fig. 1A). In agreement with published results [23,34], the melting of PSM/sterol-rich domains in bilayers containing 30 mol% PSM was clearly detected over a temperature interval of 15–40 °C. Importantly, by comparing melting temperature of ordered domains as reported by domain selective probes and by scanning calorimetry for corresponding bilayer compositions, Alanko and coworkers [34] confirmed that the presence of probe and quencher does not disturb the temperature behavior of domains significantly.

As apparent from Fig. 1A there are significant differences in formation of sterol-rich domains in bilayers containing ether lipids. No melting of sterol-rich domains demonstrated as decrease of F/F_0 ratio could be observed in bilayers containing OMPC (30 mol%) or HePC (30 mol%). DHPC at 30 mol% on the other hand was able to form sterol-rich domains which had an intermediate melting stability compared to DPPC/sterol and PSM/sterol-rich domains. The formation of cholesterol-rich condensed domains in cholesterol-DHPC mixed monolayers has previously been demonstrated by fluorescence microscopy [35].

To look at the possible formation of ordered domains other than sterol-rich ones, we probed the same systems with tPar (Fig. 1B). In bilayers containing 30 mol% HePC and OMPC we observed no ordered domains into which tPar could partition. DPPC, DHPC or PSM at 30 mol% formed ordered domains into which tPar could partition, and the melting of these domains as reported by tPar was similar to the melting reported by CTL.

3.2. Effect of ether lipids on sphingomyelin/sterol-rich domains

To study the influence of ether lipids on sphingomyelin/sterol-rich domains we included OMPC, HePC, or DHPC in the bilayer membranes together with PSM and cholesterol and looked at the quenching susceptibility of CTL (Fig. 2A). The temperature stability of PSM/sterol-rich domains was clearly dependent on the amount of sphingomyelin with different melting temperatures when 15 or 30 mol% of PSM was present in bilayers. The temperature interval of melting of PSM (15 mol%)/sterol-rich domains is in good agreement with previous studies [30,34,36]. Including 15 mol% of ether lipid to the bilayer membranes together with 15 mol% of PSM and 10 mol% cholesterol clearly stabilized the sterol-rich domains against temperature induced melting. Still, the ether lipid induced stability of PSM/sterol-rich domains had not reached the stability of PSM (30 mol%)/sterol-rich domains. The incorporation of ether lipids into bilayers containing ordered domains also enlarged the amplitude of F/F_0 ratio decrease suggesting increased partitioning of sterol (CTL) into PSM/sterol-rich domains in presence of ether lipids. When the ordered domains were probed with tPar (Fig. 2B) and tPaSM