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Perifosine induced release of contents of trans cell-barrier transport efficient liposomes

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ABSTRACT

Perifosine (OPP) containing liposomal formulation was previously found to deliver almost half of liposome encapsulated content through a tight cellular barrier in vitro. In order to understand the role of different liposome components, especially perifosine, in transendothelial transport the physical characteristics of liposome membranes composed of phosphatidylcholine, and cholesterol, as a main lipid constituents, and variable amount of helper lipids: dioleoyl phosphatidylethanolamine (DOPE), and alkylphospholipid perifosine.

For this purpose, electron paramagnetic resonance (EPR) with computer aided EPR spectra simulation and fluorescence polarization spectroscopy were used to investigate how different membrane components influence membrane characteristics and the release of liposome entrapped substances. Beside methylester of palmitic acid with nitroxide group at different position on acyl chain usually used for such studies, the spin labeled and fluorescent labeled analog of perifosine were introduced.

OPP increases membrane fluidity of liposomes as well as the release of liposome encapsulated content. The release of neutral molecules increases with OPP concentration, while the release of charged molecules is about an order of magnitude slower. Optimal OPP concentration, for release of charged molecules, is about 15 mol%.

These results are one step further toward the conclusion that the lysolipid-containing liposomes could be promising trans endothelial delivery system, since lysolipids, such as OPP, open tight cellular barriers, as was published before, and in the same time induce the release of liposome encapsulated content at physiological temperature, as shown here. Since many drug delivery systems are being developed, which mainly exploit the transcellular route of delivery through barrier-forming cells, we hope that the uniqueness of lysolipid-containing liposomes, exploiting the paracellular route, and thus avoiding efflux transporters, will foster further research in formulating other lysolipid-containing liposomes as drug delivery systems.

1. Introduction

Delivery of therapeutics across cell barriers (trans cell barrier transport), especially to the disease-affected brain tissue, in a controlled and non-invasive manner remains one of the key goals of drug development (Karkan et al., 2008). Since the blood–brain barrier (BBB), a dynamic interface between the blood and the brain formed by endothelial cells of the brain capillaries, efficiently prevents the uptake of most therapeutically active compounds, many diseases of central nervous system (CNS), such as Alzheimer's disease, are undertreated. Various drug delivery systems were tested for delivery of drugs to tumors of the CNS with different efficiencies (Tiwari and Amiji, 2006; Orthmann et al., 2010; Zeisig et al., 2007; Patel et al., 2009). Liposomes seem to be a promising delivery system, which enables high cellular uptake and efficient transport across cell barriers, since their composition can be easily adjusted according to the properties of targeted cells and tissues (Cornford and Cornford, 2002). The effect of liposome bilayer properties on cellular uptake and trans cell barrier transport of the encapsulated hydrophilic marker calcein through a barrier formed by epithelial Madin–Darby canine kidney (MDCK) cells, which are often used as an in vitro BBB model system (Garberg et al., 2005; Veronesi, 1996; Alavijeh et al., 2005), was investigated recently (Orthmann et al., 2010). A correlation between trans cell barrier transport and fluidity of liposome membrane was found, however it was unclear

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which liposome component affected the transport most, since the most efficient liposomal formulation in trans cell-barrier delivery of liposome encapsulated content contained high amount of perifosine as well dioleoyl phosphatidylethanolamine. Since perifosine as well as other synthetic analogs of lysophosphatidylcholine (LPC) reversibly open epithelial tight junctions, probably through their incorporation into the plasma membrane lipid bilayer (Leroy et al., 2003), I hypothesized previously (Koklic and Štrancar, 2012) that perifosine might facilitate efficient trans cell-barrier drug delivery provided that the following two assumptions are true:

1) perifosine is transferred in sufficient amount from liposomes to barrier forming cells, where it can increase permeability of the barrier and
2) perifosine containing liposomes release enough of its content, which can then pass through the compromised cellular barrier.

In this work I have therefore concentrated my attention to perifosine, which has been intensively investigated in our laboratories as its liposomal formulations seem promising for breast cancer therapy (Zeisig et al., 1991, 1998). Perifosine belongs to the group of alkylphospholipids (APL), a new class of anticancer agents, targeting directly cell membrane and not DNA. They show a selective apoptotic response in tumor cells, sparing normal cells (van der Luit et al., 2007; Gills and Dennis, 2009; van Blitterswijk and Verheij, 2008). APLs have shown promising results in several clinical studies (Mollinedo, 2007) and among them perifosine and miltefosine seem to be the most promising candidates for breast cancer therapy (Fichtner et al., 1994). Due to the unwanted side effects, reflected in gastrointestinal toxicity and hemolytic activity, which limits the application of higher doses of APLs different liposomal formulations with APLs have been tested and showed diminished hemolytic activity (Zeisig et al., 1991, 1998).

To test the two above stated assumptions we synthesized fluorescent and spin labeled analog of perifosine and incorporated them into the liposomes of different composition. Spin labeled APLs were synthesized previously (Mravljak et al., 2005), whereas fluorescent perifosine analog was first used by Testen et al. only recently (unpublished data). Due to possible artifacts introduced by the reporter probe it is generally advisable to use several probes, since it is unlikely that different probes would give the same artifactual results. Complementary character of EPR and fluorescence makes the two techniques a suitable combination. High sensitivity of fluorescence offers one to use a low concentration of the probe and can be used to locate the distribution of probes within cells using a microscope. However, the steric perturbations caused by the large size of fluorescent probes should be considered carefully. Spin-labels on the other hand are more representative of naturally occurring lipids, since nitroxide rings are smaller than most fluorescent groups. Thus the combination of both techniques is advantageous in order to draw conclusions about endogenous lipids. Synthetic lipids with a nitroxide or a fluorescent probe proved to be useful in determining influence of different compounds on membrane fluidity, giving comparable results (Jurkiewicz et al., 2012; Abram et al., 2013). Results obtained by fluorescence or EPR are also comparable to those obtained by ^{2}H NMR data, where the membrane is not perturbed by insertion of the probes (de Paula et al., 2008). In this study we used a spin labeled analog of perifosine as well as palmitic acid and its methyl ester with nitroxide group at different locations in combination with DPH and TMA–DPH which are frequently used for membrane fluidity measurements (Litman and Barenholz, 1982; Prendergast et al., 1981; Kaiser and London, 1998).

In agreement with the first assumption, we have recently shown that perifosine is rapidly transferred from liposomal formulation to cell plasma membrane in sufficient amount to increase permeability of a tight cell barrier (unpublished data). In this work we test the second assumption and show how perifosine affects liposome membrane properties, and subsequently induces release of liposome encapsulated hydrophilic substances.

2. Materials and methods

Egg yolk phosphatidylcholine (PC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were products of Sigma–Aldrich (Steinheim, Germany). Perifosine ((1,1-dimethylpiperidin-1-ium-4-yl) octadecyl phosphate, also referred as OPP) was a generous gift from Dr. Hilgard (ASTA Medica, Frankfurt, Germany). Cholesterol (CH), and dicetylphosphate (DCP) were purchased from Serva (Heidelberg, Germany). Solvents were purchased from Merck (Darmstadt, Germany). The spin probes 5- and 12-doxylpalmithoyl-methyl ester (MeFASL(10,3), MeFASL(2,11)), 5-doxayl palmitic acid (HFASL(10,3)), spin labeled perifosine containing the nitroxide group at the 5th C atom (P5) (counting from the polar head group), fluorescently labeled perifosine, where 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) is attached to perifosine at the 7th C atom (MTV-24) (counting from the polar head group), 4-(N,N-dimethyl-N-(2-hydroxyethyl))ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide, (TEMPO-choline, ASL) and 1-oxyl-2,2,6,6-tetramethyl-4-(2′,3′,4′,5′,6′-pentahydroxyhexanoyl-1′-amino)-piperidine, (the spin-labeled glucose GluSL) were synthesized by Slavko Pečar and Janez Mravljak (Faculty of Pharmacy, University of Ljubljana, Slovenia). TMA–DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate) and DPH (1,6-diphenyl-1,3,5-hexatriene) were products of Sigma–Aldrich (Steinheim, Germany).

2.1. Synthesis of fluorescently and spin-labeled perifosine

Detailed description of different spin labeled analogs of perifosine including the synthesis of P5 was published previously (Mravljak et al., 2005). The synthesis of fluorescently labeled perifosine MTV-24 was accomplished similarly to the synthesis of spin labeled perifosine analogs except that nitroxide moiety was replaced by the NBD fluorophore. Detailed description of the MTV-24 synthesis was described by Testen et al. (unpublished data – manuscript sent for publication). All of the chemicals used for the synthesis were obtained from commercial sources (Acros, Aldrich, Fluka, Merck, Avanti Polar Lipids) and used without further purification. Methyl 7-oxooctadecanoate and ASL were synthesized according to literature data (Samuni and Barenholz, 1997; Hünig et al., 1958).

2.1.1. Spin-labeled perifosine (P5)

2.1.1.1. 4-{[{[13-(2-Butyl-3-oxyl-4,4-dimethyl-1,3-oxazolidine-2-yl)tridecyl]oxy}(oxy)phosphoryl]oxy}-1,1-dimethylpiperidinium Inner Salt (P5). Reactions were monitored using analytical TLC plates (Merck, silica gel 60 F254) with rhodamine G6 staining. Mass spectra were obtained with a VG-Analytical Autospec Q mass spectrometer with EI or FAB ionization (MS Centre, Jožef Stefan Institute). IR spectra were recorded on a Perkin-Elmer FTIR 1600 spectrometer. EPR spectra of nitroxide solutions were measured at room temperature in a glass capillary (1 mm inner diameter) using a BRUKER X-band CW-ESR spectrometer ESP 300 (EPR Centre, Jožef Stefan Institute) at 10 mW microwave power. Elemental analyses were performed by the Department of Organic Chemistry, Faculty of Chemistry and Chemical Technology, Ljubljana, on a Perkin-Elmer elemental analyzer 240C.

The procedure described in ref. (Mravljak et al., 2005), afforded compound P5 (0.40 g, 79%) as yellow solid. R_f ($CHCl_3$: MeOH: 25% NH_3, 78:30:4.7) = 0.12; IR (NaCl) 3375, 2925, 2860,