

How perifosine affects liposome-encapsulated drug delivery across a cell barrier

Background: The development of efficient drug delivery systems to transport therapeutics across barrier-forming cells remains a challenge. Recently it was shown that liposomes containing perifosine, a synthetic analog of lysophosphatidylcholine, efficiently deliver liposome encapsulated content across barrier-forming cells. **Methods:** To elucidate the mechanism of the delivery, fluorescent and spin labeled analog of perifosine were synthesized and their transport from liposomes to the barrier-forming MDKC cells was measured. **Results & Conclusion:** Perifosine analogs are rapidly transported from liposomes into cell membranes. The total amount of perifosine accumulated in plasma membranes seems to be the most important factor in efficient transepithelial transport of liposome-encapsulated substances. Lysolipid-containing liposomal formulations seem to be promising candidates as drug delivery systems in general.

The transport of drugs across epithelial or endothelial cells, which form barriers in vascular systems, glandular tissues, intestines or brain, is of significant importance for drug efficacy. Designing efficient delivery system, capable to overcome these barriers and deliver therapeutics to pathologically affected tissues, in a controlled and noninvasive manner remains one of the key goals of drug development [1,2]. Depending on the mode of administration drugs should cross different physiological barriers. For intravenous delivery the endothelial barrier, the interface between blood and tissue, represents important biomedical goal. Besides, nowadays the noninvasive, needle free, routes of administration are in progress where the epithelial barrier of nose, mouth, eye, skin of GI tract greatly limit the *in vivo* accessibility of drugs, especially the hydrophilic drugs or large biopharmaceuticals, to their intended target sites of action.

The important factor for successful delivery of drugs or drug loaded nanocarriers is their ability to either cross the biological barrier or allow the encapsulated drug to cross the barrier and to achieve the sites of its action. Unfortunately, drugs have no affinity to endothelial or epithelial barriers, therefore limiting specificity,

safety and effectiveness of therapeutic intervention. In order to achieve targeted delivery of drugs to or across the endothelial barrier, delivery systems were developed, which enable their adhesion to the endothelial membranes. For this purpose drug carriers were conjugated with affinity ligand of endothelial surface determinants such as cell adhesion molecules, endothelial surface receptors or enzymes, transport proteins and specific domains in cell membrane [3]. The drugs or drug carrier systems are usually transferred into the endothelial cells by the endocytotic pathways, which generally deliver material via endosomes to lysosomes. Only clathrin-mediated or caveolin-related endocytosis can also traverse cytosol and transport the material across endothelium (**transcellular route**), but are suitable only for delivery systems with size less than 100 nm [4]. Therefore the intracellular delivery is more convenient for transport of drugs into the endothelial cells, but is less effective for **trans cell barrier transport**. The other possibility is the delivery systems that loosen the tight cell junction between endothelial or epithelial cells and allow the paracellular drug transport (**paracellular route**). In the last 50 years, several methods and molecules have

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Key terms

Transcellular route: Transport of molecules and ions through the cells.

Trans cell barrier transport: Epithelial or endothelial cell barriers are tightly connected by tight junctions, which regulate the passage of molecules across the barrier.

Paracellular route: Transport between the cells – through tight junctions.

Barrier-forming cells: *In vitro* model of epithelial or endothelial cell layers in multicellular organisms. These cell layers form selectively permeable barriers between compartments.

Spin-labeled analog: Molecule with attached nitroxide group – a radical (free – unpaired electron), resembling the molecule of interest. Such molecule can be detected using electron paramagnetic resonance spectroscopy, which gives information about the environment in which the analog is located.

been investigated for the safe and reversible opening of these junctions to enhance drug absorption and penetration. Recently nanostructured thin films were developed which in contact with epithelial barrier increase the epithelial permeability for the transport of high-molecular-weight proteins [5]. Another promising candidates to improve drug delivery are tight junction modulators (specific peptides, enzymes or lipids) which interact directly with tight junction proteins or the associated membrane micro domains [6]. Various drug delivery systems, such as liposomes, surfactant coated polymeric nanoparticles, solid-lipid nanoparticles, microspheres, nanogels, dendrimers and bionanocapsules, have been tested for the delivery of drugs to tumors of the CNS [7–9], to retina [10], lung [11,12], kidney [13,14], intestine [15,16], skin [17–19] and oral mucosa [20]. They were found to enhance the permeability of the corresponding epithelial barriers and show some promise in therapy of tumors and other diseases. However, due to nonspecific mode of action and high toxicity their application in pharmaceutical therapy is strongly limited. Therefore development of new, efficient and safe delivery systems is still an exponentially growing field of biomedical research [1,21–22].

Among drug delivery systems, liposomes were one of the first, introduced already in the 1970s [23]. Due to their biocompatible lipid matrix, they become promising tool in medicine. Their application especially as anticancer drug carriers has experienced fast development in last few decades. They can be engineered in different sizes with different permeability properties and can be loaded with various drugs or particles. In addition, their composition can be easily adjusted according to the properties of targeted cells and tissues [24]. Recently Orthmann *et al.* [8] showed that liposomes can be formulated as an efficient drug delivery system for delivering

liposome content across **barrier-forming cells**. Liposomal formulations composed of phosphatidyl choline (PC) and cholesterol (CH) as basic components of the liposomal lipid matrix, with dioleoyl phosphoethanolamine (DOPE) and an alkylphospholipid, a synthetic analog of lysophosphatidylcholine (LPC), perifosine as helper lipids, efficiently delivered liposome-encapsulated hydrophilic calcein across a tight monolayer of MDCK (Madin–Darby canine kidney) cells. A liposomal formulation with equimolar DOPE and perifosine was shown to be especially efficient in transepithelial transport, delivering approximately one third of encapsulated liposome content across the cell monolayer in a trans-well system. These liposomes were shown to have a more fluid membrane, as well as a reduced solute retention capacity in serum-containing buffer, leaking approximately one quarter of their contents within 8 h of incubation with serum proteins. It was proposed that greater efficiency in transepithelial drug delivery is based on higher membrane fluidity and leakage of these liposomal formulations [8]. In the recent investigations of larger series of liposomes with different amount of perifosine it was shown that perifosine increases membrane fluidity of liposomes as well as the release of liposome encapsulated content [25]. On the basis of these results, we proposed a hypothesis explaining a probable transport mechanism of liposome-encapsulated molecules across cell barriers [26]. The hypothesis is based on the published results, which show that perifosine as well as other synthetic analogs of LPC reversibly open epithelial tight junctions, probably through their incorporation into the cell membrane lipid bilayer [21]. The main assumptions of our hypothesis were that liposomal formulations efficient in transepithelial drug delivery should contain an alkylphospholipid, which is present not only in the liposome membrane, but also in the form of micelles and as free, single molecules. The free alkylphospholipid molecules could compromise tight cell layer permeability, allowing passage of liposome content across the barrier. However, the correlation between perifosine concentration and their relation to transepithelial transport still remains to be proven.

Therefore the main questions explored in this work are:

- Is perifosine transferred from liposomes into barrier-forming cells? If so, how much of it is transferred from liposomes to cells and does the transport depend on the liposome membrane composition?
- Is the release of the liposome-encapsulated content and its transepithelial transport accompanied with the depletion of perifosine from liposomes?

To answer these questions, we have synthesized fluorescent and **spin-labeled analog** of perifosine

ine and incorporated them into the liposomes of different composition. Their transport from liposomal formulation to MDKC epithelial cells was investigated by fluorescence microscopy (FM) and electron paramagnetic resonance (EPR), respectively. Fluorescently labeled perifosine was synthesized and used in this study for the first time (Figure 1).

Synthetic lipids with a nitroxide or a fluorescent probe proved to be useful in determining the transmembrane diffusion of phospholipids in lipid membranes [27]. For example the active transport of aminophospholipids in red blood cells characterized by spin-labeled lipid [28,29] gave similar results as fluorescent probes [30], as well as radio-labeled lipids [31] or addition of exogenous unlabeled lipids [32]. Similarly was shown for calcium triggered phospholipid scrambling where results obtained with unlabeled lipids [33] were in agreement with results obtained using spin-labeled or fluorescent lipids [34–36]. 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 suitable combination to gather valuable information about the interaction and transport of substances from liposomes to cells. 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 size of the probe should be considered carefully. The large size of the fluorescent moiety can modify the affinity towards proteins. Spin-labels on the other hand are more unstable due to oxy-redox reactions in biological systems [37], but are more representative of naturally occurring lipids, since nitroxide rings are smaller than most fluorescent groups.

Thus the combination of both techniques is needed in order to draw conclusions about endogenous lipids.

Materials & methods

Materials

Egg yolk phosphatidylcholine (PC), 1,2-dioleoyl-*s,n*-glycero-3-phosphoethanolamine (DOPE) and dimethyldioctadecylammonium bromide (DDAB) were products of Sigma-Aldrich (Steinheim, Germany). (1,1-dimethylpiperidin-1-ium-4-yl) octadecyl phosphate (OPP, also referred to as perifosine in Figure 2) was a generous gift of P Hilgard (ASTA Medica, Frankfurt, Germany). CH, dicetyl phosphate (DCP) and calcein were purchased from Serva (Heidelberg, Germany) and *N*-methyl-(polyethyleneglycol)₂₀₀₀-1,2-distearyl-*s,n*-glycero-3-phosphatidylethanolamine (PEG₂₀₀₀-DSPE) was obtained from Lipomed (Arlesheim, Switzerland). Solvents were purchased from Merck (Darmstadt, Germany). Lipid fluorophore 1-palmitoyl-2-(6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl)-*sn*-glycero-3-phosphocholine (NBD-PC) was purchased from Avanti polar lipids (Alabaster, AL, USA) and the spin probes: 4-(*N,N*-dimethyl-*N*-(2-hydroxyethyl)) ammonium-2,2,6,6-tetramethyl piperidine-1-oxyl iodide (ASL), spin-labeled perifosine (P5) [38] and fluorescently labeled perifosine (MTV) were synthesized at The Faculty of Pharmacy, University of Ljubljana, Slovenia. Structural formulas of all labeled substances and perifosine are shown in Figure 2.

Methods

Synthesis of fluorescently & spin-labeled perifosine

Synthesis and characteristics of spin-labeled perifosine (P5) was described previously by

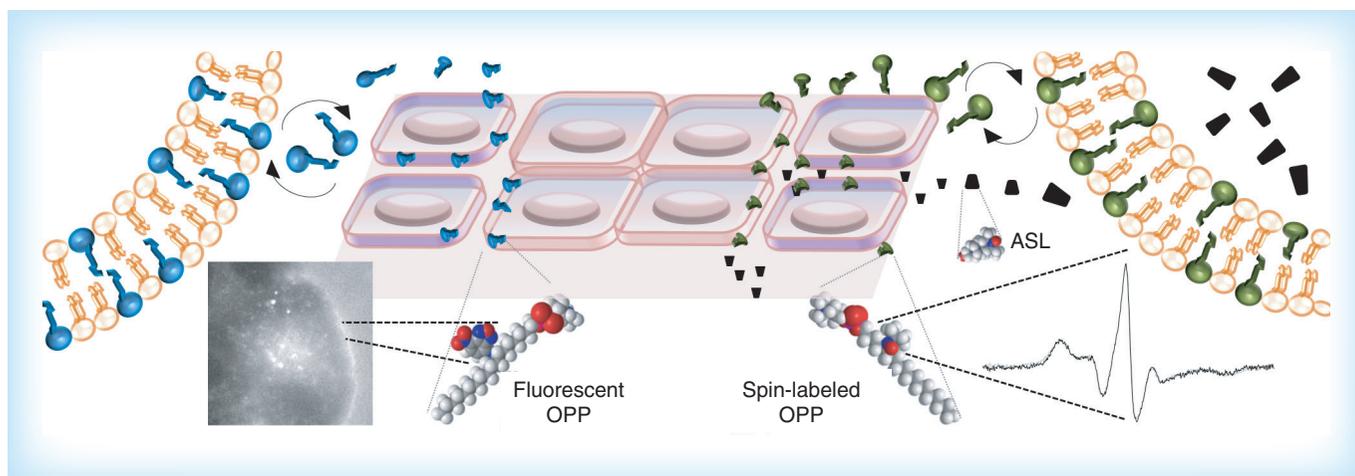


Figure 1. Perifosine-induced opening of the tight cell barrier (pink) and the subsequent transfer of liposome-encapsulated content (ASL - black) across the cell barrier.

ASL: 4-(*N,N*-dimethyl-*N*-(2-hydroxyethyl)) ammonium-2,2,6,6-tetramethyl piperidine-1-oxyl iodide; OPP: Perifosine. For color images please see online www.future-science.com/doi/full/10.4155/TDE.14.127

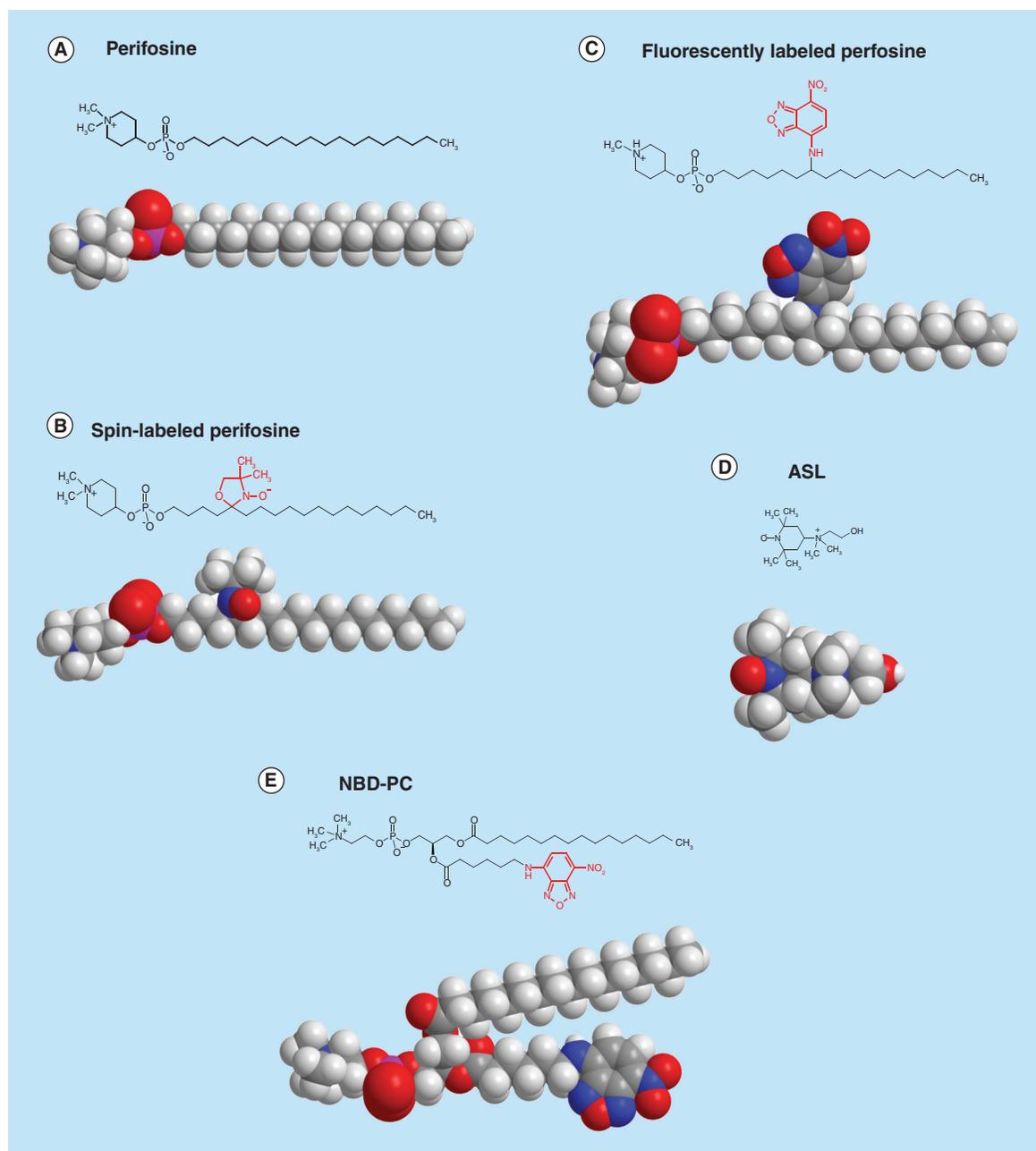


Figure 2. Structural formulas. (A) Perifosine, (B) spin-labeled perifosine (P5), (C) fluorescently labeled perifosine (MTV), (D) water-soluble spin probe ASL, (E) NBD-PC. Space filling models of the molecular probes are minimal structures in vacuum calculated by ChemBioDraw Ultra 12.0 (PerkinElmer Informatics Inc).

ASL: 4-(*N,N*-dimethyl-*N*-(2-hydroxyethyl)ammonium-2,2,6,6-tetramethyl piperidine-1-oxyl) iodide;

NBD-PC: 1-palmitoyl-2-(6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl)-sn-glycero-3-phosphocholine.

Mravljak *et al.* [38]. The complete synthesis of fluorescently labeled perifosine is provided in Appendix A: [Supplementary material](#).

Preparation of liposomes

Liposomes composed of different concentrations of PC, CH, DOPE and perifosine (OPP) were prepared with lipid film hydration technology as described before [9,39] from appropriate mixtures of stock solu-

tions of the components in chloroform:methanol (7:3, v/v). The exact composition of liposomes is given in [Table 1](#). In some cases, NBD-PC was added to the organic phase to prepare fluorescent membrane-labeled liposomes. The molar ratio of the fluorescent probe with respect to other components was 0.5 mol%. Lipid films were hydrated with PBS (pH 7.4) or, to obtain liposomes loaded with a hydrophilic probe, with 10 mmol/l ASL solution in PBS for EPR measurements

Table 1. Characteristics of different liposomal formulations: their composition, average particle size, calcein release and delivery of calcein across the Madin–Darby canine kidney tight cell barrier.

Code	Molar ratio				Size(nm) [†]	PI [‡]	Calcein content (mmol/ol TL)	Calcein Leakage (%) [§]	Trans-epithelial delivery (%)	OPP (mol%)
	PC	CH	DCP	DOPE/OPP						
L1	70	30	10	0/0	110 ± 9	0.54 ± 0.19	34.5	21 ± 1	4 ± 2	0
L3	40	30	10	30/0	114 ± 8	0.26 ± 0.09	44.3	17 ± 2	2 ± 1	0
L4	50	20	10	20/20	129 ± 9	0.19 ± 0.07	34.9	36 ± 17	8 ± 4	17
E2–2	50	20	10	10/30	151 ± 1	0.10 ± 0.06	22.5	62 ± 1	24 ± 13	25
E2–3	50	20	10	30/10	141 ± 2	0.15 ± 0.04	24.8	13 ± 1	3.3 ± 0.2	8
E2–5	40	20	10	40/20	130 ± 1	0.18 ± 0.04	22.8	14 ± 1	4 ± 1	15
E2–6	40	20	10	50/20	106 ± 3	0.17 ± 0.02	20.0	8.7 ± 0.4	6 ± 3	14
E2–7	50	20	10	50/0	134 ± 2	0.07 ± 0.02	34.3	7.3 ± 0.3	3 ± 1	0
E3	70	20	10	0/20	128 ± 1	0.16 ± 0.01	23.4	55 ± 2	3.7 ± 0.3	17
E4	0	50	10	0/50	140 ± 1	0.14 ± 0.06	29.0	44 ± 1	21 ± 4	45

The composition of selected liposomal formulations (molar ratios), vesicle size, calcein content per total lipid concentration, liposome stability (as determined by calcein leakage), transepithelial delivery of liposome-encapsulated calcein across the Madin–Darby canine kidney cell barrier (based on calcein measurements in the basal media), relative content of alkylphospholipid OPP and the ratio of OPP versus cholesterol are shown for selected liposomes. Details are described in section 'Materials & methods'.

[†]Unimodal diameter: mean ± SD for three measurements.

[‡]Polydispersity index.

[§]Determined after 7 h of storage in fetal calf serum containing PBS (1:1 V/V) at 25°C.

CH: Cholesterol; DCP: Dicapryl phosphate, negative charge; DOP: Dioleoyl phosphoethanolamine; DOPE: Dioleoyl phosphoethanolamine; OPP: Perifosine; PC: Phosphatidyl choline.

and with 50 mM calcein solution (pH 7.5) for fluorescence and transepithelial transport measurements. Large unilamellar vesicles (LUV) were made from multilamellar vesicles (MLV) with the extrusion technique specified by MacDonald *et al.* [40] with repeated extrusion through polycarbonate filters (diameter of pores 100 nm) using a LipoFast Basic system (Avestin, Ottawa, ON, Canada).

Calcein-loaded vesicles were separated from non-encapsulated fluorescence marker by gel filtration using a G50 sepharose column, while ASL-loaded vesicles were separated by overnight dialysis at 4°C. The encapsulated ASL was determined as the ratio of EPR spectra intensity after dialysis (I_{dialysis}) and before dialysis (I_{total}). EPR spectra intensity was measured with double integration of the EPR spectra as well as with the peak-to-peak height of the middle line.

Vesicle size and polydispersity index (PI) were determined by dynamic light scattering with a N5 Coulter submicrone particle analyzer (Beckman Coulter Electronic, FL, USA) as described in more details elsewhere [8] and are presented in Table 1. For some liposomes these characteristics were already determined and published before (L1 to L4) [8], and they are again summarized in Table 1. All liposomes were prepared in a concentration range of 10–20 mM total lipid.

Characterization of liposomes

Liposome stability (calcein leakage) was determined by measuring the release of calcein, encapsulated at a

self-quenching concentration in liposomes, by fluorescence spectroscopy. Dequenching of calcein fluorescence was measured as follows: liposomes were diluted with fetal calf serum containing PBS (1:1 V/V) to obtain a final concentration of 5 μM (total lipid concentration) and were kept at 25°C for 48 h with gentle stirring. At different time intervals, two aliquots were taken and fluorescence was determined with a Fluostar fluorescence spectrometer at wavelengths of F_{ex} 485 nm and F_{em} 538 nm in microtiter plates (FluoroNunc™ MaxiSorp Surface). Calcein leakage was calculated according to:

$$\text{leakage [\%]} = \frac{m_{\text{calcein}}}{m_{\text{calcein total}}} \times 100$$

where m_{calcein} is the amount of calcein released and $m_{\text{calcein total}}$ is the total amount of calcein in the liposomal formulation determined after vesicle destruction with the addition of 10 μl of 10% Triton solution to 100 μl of the sample. The experiment was performed twice and results are expressed as mean% ± SD.

Cell culture

Dog epithelial MDCK cells were used as a model of endothelial cell barrier. MDCK cells were cultured in DMEM medium (Gibco), supplemented with 2 μmol/ml l-glutamine (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml) and heat-inactivated fetal calf serum (10% FCS, Gibco) in a humidified 5% CO₂ atmosphere.

Key term

Transepithelial electric resistance: Permeability of a cell barrier is correlated with electric resistance across the cell barrier.

Transepithelial transport

Transepithelial transport of liposome content was determined as described recently [8]: aliquots of 10^5 MDCK cells were seeded on a collagen-coated permeable support insert (Millipore; 0.2 μm pore size diameter, 0.6 cm^2 area, 0.2% collagen) in a 24-well microtiter plate. A tight monolayer was obtained under these conditions within 3 to 4 days. The tightness of each cell monolayer was checked before and after treatment with **transepithelial electrical resistance** (TEER) measurements directly in culture media using a Millicell[®] ERS voltohmmeter (Millipore, Germany). Cell monolayers with TEER values less than $120 \Omega\text{cm}^2$ after background correction were excluded from the experiment.

After obtaining a tight cell barrier, MDCK cells were incubated with 400 μl nutrient deficient DMEM containing liposomes (200 nmol total lipid/ml) in the apical chamber of the transwell system at 37°C . The calcein concentrations in the basal media and in cells after lysis were determined after 24 h with fluorescence measurements in microtiter plates (FluoroNunc[™] MaxiSorp Surface) with a Fluostar fluorescence spectrometer at wavelengths of F_{ex} 485 nm and F_{em} 538 nm. Two experiments were performed, each done in triplicate.

Interaction of liposomes with cells

Liposome content tracking

To track liposome-encapsulated content upon the interaction of liposomes with cells, MDCK cells were plated in 25 cm^2 tissue culture flasks (TPP, Techno Plastic Products AG, Trasadingen, Switzerland) and allowed to grow for a day. The cell medium was then removed and cells were rinsed with fresh PBS before 3 ml of fresh PBS supplemented with 80 μl of L3 or L4 liposomal formulation with encapsulated ASL was added to cells attached to the bottom of the culture flask. At different time points after incubation, cell supernatant was removed and the intensity of EPR spectra measured. As long as the liposomes remain in the supernatant (above the cells attached to the bottom of the culture flask), EPR spectra intensity should remain unchanged. EPR spectra intensity in cell supernatant is therefore a measure of ASL in liposomes that do not interact with cells and ASL that is released from liposomes, but remains in solution.

To distinguish between the amount of ASL encapsulated in liposomes and ASL released from liposomes

after incubation with cells, aliquots of supernatant were taken from the culture flask at different time points and mixed with 0.16 M solution of Na ascorbate. Ascorbate is a reducing agent which reduces nitroxides to the corresponding hydroxylamines, which are not visible with EPR. Due to their charges, neither ascorbate nor ASL penetrates the liposome membrane easily [41,42]. As long as the liposomes are intact, EPR spectra intensity remains constant. When ASL is released from liposomes into the solution, it is reduced to the corresponding hydroxylamine. EPR spectra intensity in the presence of ascorbate is therefore a measure of the concentration of intact liposomes above the cells.

EPR experiments were performed on a Bruker ELEXSYS E500 EPR X-band spectrometer at 37°C , magnetic field 0.332 T, microwave frequency 9.59 GHz, incident microwave power 20 mW, modulation frequency 100 kHz and amplitude 0.1 mT. EPR spectra intensity was measured by measuring the second integrals of EPR spectra.

Lipid mixing

The mixing of liposome membranes lipids with cell membrane lipids (lipid mixing) was evaluated with FM, which measured the transport of fluorophore incorporated in the liposome membrane to MDCK cells. For this purpose, the cells were seeded onto a Nunc[®] Lab-Tek[®] chambered coverglass (Thermo Scientific, Denmark) and allowed to grow for a day. The cell medium was then removed and cells were rinsed with fresh PBS before 200 μl of PBS with 10 μl of an appropriately diluted liposomal formulation containing the fluorescently labeled lipid probe NBD-PC was added to the cells. The intensity of fluorophore signal in MDCK cells with time after incubation with liposomal formulation was recorded with an inverted microscope Nikon Eclipse TE 2000-E, using a water immersion 60 \times objective lens [43].

Transport of perifosine from liposomes to MDCK cells

In order to get information about the transport of perifosine from liposomes to MDCK cells, the spin-labeled analog of perifosine (P5) as well as the fluorescently labeled analog of perifosine (MTV) were used and their transport was evaluated by EPR or fluorescence spectroscopy, respectively. Liposomes were labeled with the lipophilic probes with a molar ratio 1:200 of the probe to total liposome lipids, as described elsewhere [44].

In order to follow the transfer of P5 into cells, the liposomal formulations (80 μl , 10 mM total lipid) were spin-labeled with P5 (40 μl , 0.1 mM). Cells were treated in the same way as described for the liposome content tracking experiment (Liposome content track-

ing paragraph in ‘Materials & methods’), except that the cells were incubated with P5-labeled liposomal formulations. At different time points after incubation, aliquots of cell supernatant with P5-labeled liposomes were removed from the flasks and EPR spectra intensity was measured. In this way, the rate of transfer of P5 from liposomal formulations to cells was determined. After 10 min of incubation, the cells were removed from the flasks by trypsinization and the cell suspension was centrifuged at $120 \times g$. The cell pellet (10^7 cells, cca $10 \mu\text{l}$) was put into a glass capillary and EPR spectra measured. The EPR spectra intensity of cells in glass capillary was followed with time in order to get the information about the amount of P5 transferred from liposomal formulations to cell membranes and about the transfer of P5 to cell interior, where the nitroxide group of P5 was reduced to EPR non-visible hydroxyl amine by oxy-redox systems in cells [37]. EPR spectra intensity was determined by measuring the amplitude of the middle line, as the line shape of the spectra did not change with time.

In order to follow the transfer of MTV from liposomes to cells, the cells were treated in the same way as described above, except that the cells were incubated with MTV-labeled liposomal formulations. At different time points of incubation, aliquots of the supernatant containing the MTV-labeled liposomes were taken and the fluorescence intensity was measured in order to determine the amount of fluorophore transferred to cells (with TECAN Infinite M1000, Tecan Trading AG, Switzerland). Increasing incubation time would increase the fluorescence signal intensity of cell membranes, and also decrease the fluorescence signal of the supernatant. After 15 min of incubation, fluorescence intensity of MTV in cells was also measured by lysing the cells with SDS (Sigma-Aldrich, Steinheim, Germany).

Results

To elucidate the mechanism of transepithelial transport of substances applied to barrier-forming cells in liposomal formulation containing perifosine, we investigated the role of perifosine in these formulations. Therefore in the first phase of our study we wanted to see how is perifosine transferred from liposomes to cells. Therefore we selected liposomal formulation L4 with high amount of perifosine, which exhibit low liposome uptake by cells and high transepithelial transport [8]. The formulation was labeled by perifosine analogs (P5 or MTV) and their transport to cell membrane was followed by EPR or fluorescence spectroscopy, respectively. To get information about the role of liposome composition, especially the amount of perifosine in the formulation and liposome

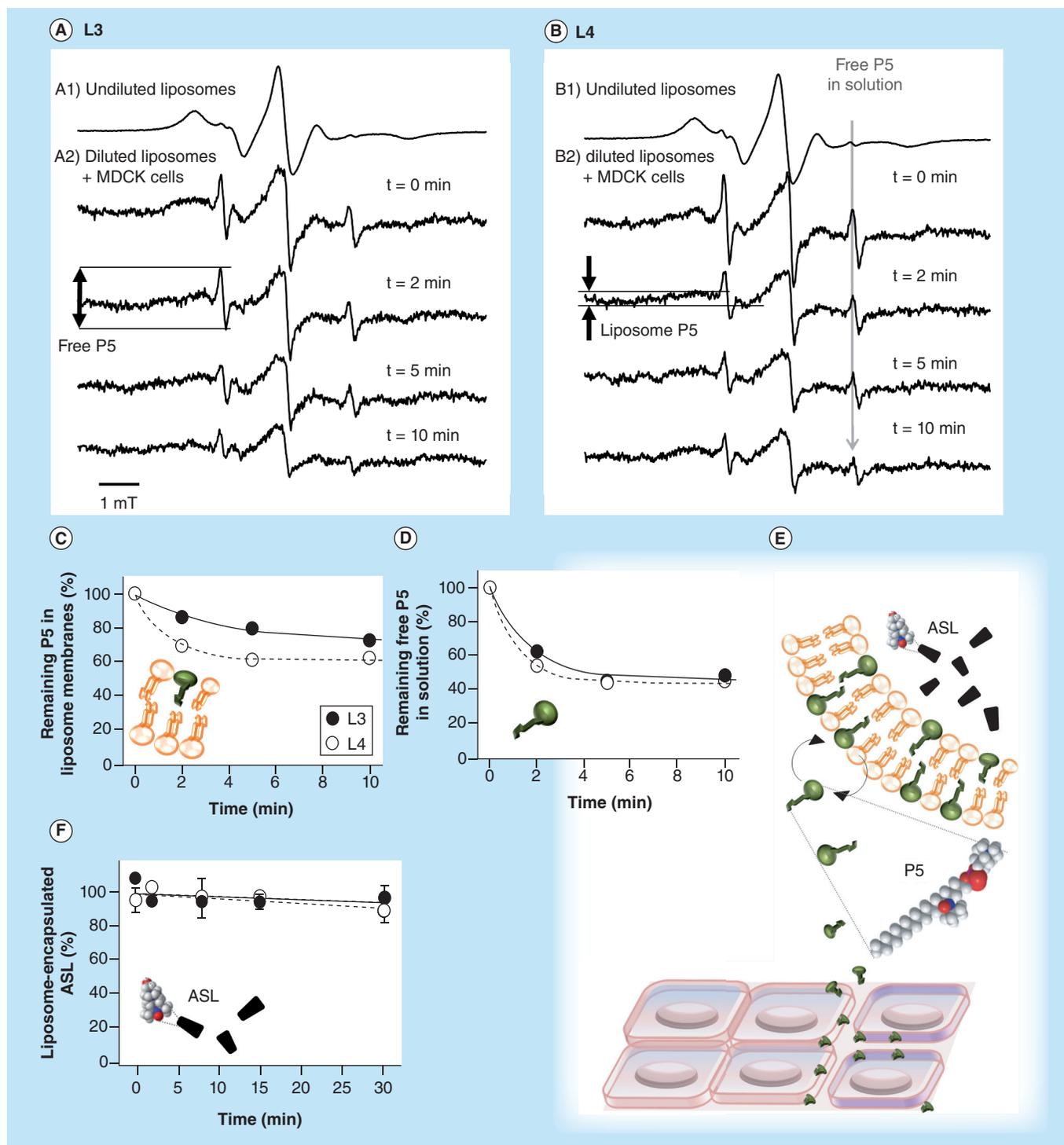
characteristics the same experiments were performed on L3 liposomal formulation without perifosine, which, according to our previously published results differ the most from L4 [8] and showed high liposome uptake by cells and low transepithelial transport. L4 liposomes were also shown to have more fluid membranes and a higher leakage of liposome-encapsulated contents than L3 liposomes [8]. After labeling with perifosine analog L3 formulation can be treated as liposomal formulation with a very low amount of perifosine (0.5 mol%).

Transport of perifosine from liposomes into barrier-forming cells

To answer the first question, whether perifosine is transferred from liposomes into barrier-forming cells and whether this depends on the liposomal formulation, we labeled the two liposomal formulations with either fluorescently labeled perifosine – MTV (Figure 2C) or with spin-labeled perifosine – P5 (Figure 2B). Both probes are chemical analogs of perifosine (OPP) (Figure 2A) and differ only by the presence of different labels shown in red in Figure 2. The advantage of using two probes to determine the action of perifosine is in the combination of the two techniques: EPR and fluorescence. P5 has a smaller nitroxide label, which should change the structure of the molecule less than a fluorophore on MTV. Both methods are expected to yield similar results, supposing that the labels do not significantly affect the measured properties of perifosine. The probes reflect the amount of labeled perifosine in cell membranes. However, MTV can be measured also if located inside the cells using FM, while the possible location of P5 inside the cells cannot be determined, since the nitroxide group of P5 is reduced to EPR nonvisible hydroxyl amine by redox reactions in cells [37].

Transfer of spin-labeled analog of perifosine (p5) into MDCK cells

Transfer of P5 from liposomes to cells was followed by measuring the EPR spectra intensity of cell supernatant with P5-labeled liposomes taken from the flask with MDCK cells at different time points after incubation at 37°C . In this way, only liposomes remaining in the supernatant, which did not interact with cells, contributed to measured signal as the intensity of EPR spectra is proportional to the amount of spin probes in supernatant. EPR spectra line shape (Figure 3A&B) is very sensitive to the environment of the spin probe. From the line shape of the EPR spectra of the liposomal formulations (Figure 3A&B), one can see that the spectra are composed of the components typical for probes incorporated into a



membrane (a broad spectral component) and probes, which are free in solution with hyperfine splitting constant $a = 1.55$ mT, typical for spin probes in polar environments (narrow spectral component, indicated by the arrow in Figure 3B). Intensity of each component was estimated by measuring the amplitude of the left line of the corresponding EPR spectrum as indicated in Figure 3A. We could therefore follow the

concentration of P5 in liposomes separately from the concentration of free P5 remaining in the supernatant. Figure 3A&B demonstrate that the intensity of both EPR spectra components decreases with incubation time, proving that P5 is transferred out of the supernatant. From the EPR spectra intensity decrease (Figure 3C&D) one can see that the concentration of P5 in L4 liposomes (open circles) containing

Figure 3. Kinetics of transport of spin-labeled perifosine analog (P5) from liposomes into cells (see facing page). (A & B) electron paramagnetic resonance (EPR) spectra of P5 in cell supernatant after incubation of barrier-forming MDCK cells with L3 and L4 liposomal formulations, respectively. Undiluted liposomal formulation (A1 & B1). Liposomal formulation diluted in PBS in the culture flask with MDCK cells at different time points of incubation (A2 & B2). (amplitude of the EPR signal corresponding to free P5 is denoted with black vertical double arrow on the spectra A) $t = 2$ min (free P5), while the amplitude of the signal of membrane component is denoted on the spectrum B $t = 2$ min (liposome P5)); (C & D) amount of P5 transferred from liposomal formulations to MDCK cells. EPR spectra intensity of the membrane component (C) and the isotropic component (free molecule in solution) (D) of P5 in liposomal formulations L3 (closed circles) and L4 (open circles) at different time points after the addition of the liposomal formulations to MDCK cells. Lines are best fits with a two parameter single exponential function; (E) Scheme of the experiment: Transfer of free P5 (green) from liposome membrane (phospholipids – orange, P5 – green, ASL in liposome interior – black) to tight cell monolayer of live cells (pink) growing on the bottom of a culture flask in incubator at 37°C. Opening of tight cell junction at the site where P5 interacts with the membrane is shown. The space filling model of P5 and ASL is also given. (F) EPR spectra intensity of hydrophilic spin probe ASL encapsulated within L3 (closed circles) and L4 liposomes (open circles) at different time points after incubation with cells in presence of the reducing agent ascorbate. All measurements were performed at 37°C. ASL: 4-(*N,N*-dimethyl-*N*-(2-hydroxyethyl))ammonium-2,2,6,6-tetramethyl piperidine-1-oxyl iodide; MDSK: Madin–Darby canine kidney; P5: Spin labeled perifosine.

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17 mol% perifosine decreases similarly as the concentration in L3 liposomes (closed circles) without perifosine, indicating that presence of perifosine in liposomal formulation does not influence the rate of P5 transfer from liposomes to cells significantly. From the rapid drop of the EPR spectra intensity of both spectral components, it can be concluded that P5 is rapidly redistributed between liposome membranes and cells. After approximately 5 min equilibrium between P5 in the supernatant and in cell membranes was reached. Kinetics of P5 transfer from liposomes and from supernatant was adequately described with a single exponential decay (lines through the symbols in Figure 3C&D). The rate of transfer is similar for free P5 in solution and P5 in liposome membranes, as it is expected for two species in thermodynamic equilibrium. Approximately 40% of perifosine is transferred from a liposome formulation into cells.

Using computer program for simulation of EPR spectra line-shape, EPRSIM [45], we estimated relative proportions of the free P5 component in the EPR spectra of the diluted L3 liposomal formulation in cell flasks (Figure 3A2) to be $4 \pm 0.4\%$, which corresponds to a concentration of $0.16 \pm 0.01 \mu\text{M}$ P5 in supernatant as a free molecule. Whereas in the diluted liposomal formulation L4 (Figure 3B2), the relative proportion of free P5 was estimated to be $6.1 \pm 0.3\%$, which corresponds to a concentration of $0.25 \pm 0.01 \mu\text{M}$. Concentrations of free P5 from both liposomal formulations are well below the critical micelle concentration (CMC) of P5 ($10 \mu\text{M}$ [46]), indicating that perifosine is transported into the cells as a free molecule.

Transfer of fluorescent analog of perifosine (MTV) into MDCK cells

Fluorescence intensity of the MTV-labeled L3 or L4 liposomes at different time points after incubation with cells (Figure 4E) also show fast transfer of MTV from liposomes to cells, similar to that observed with

P5. After 5 min, equilibrium was reached and approximately 40% of the probe was transferred from liposomes into cells, independent of the amount of perifosine in liposomes (Figure 4E). The results indicate that both analogs of perifosine are transferred to cell membranes at a fast rate of well below 5 min.

Liposome content tracking during incubation with cells

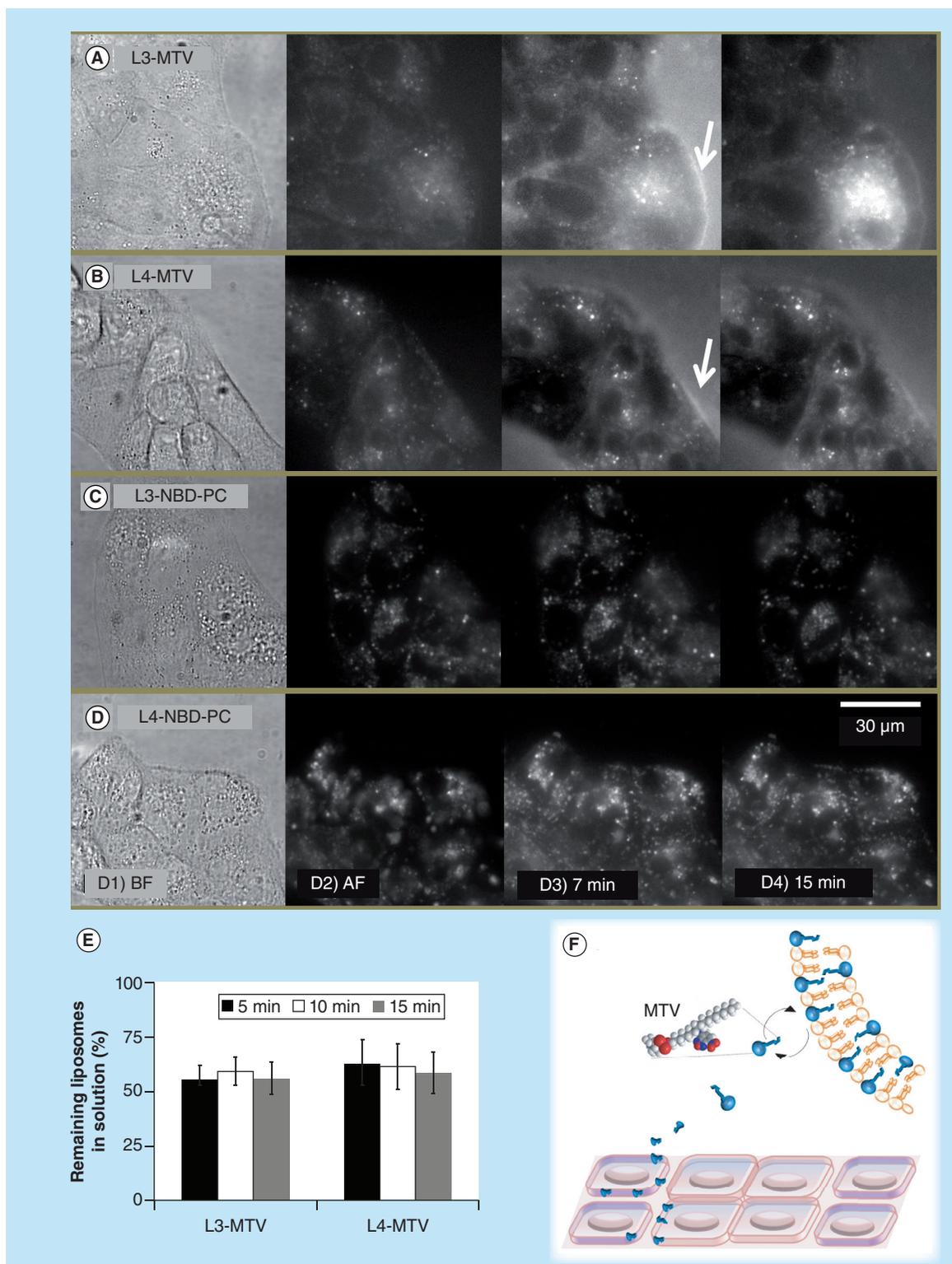
In addition to the observed transfer of perifosine to cells (Figures 3C&D & Figure 4E), there are several other possible ways of transporting perifosine from liposomes into cells. These include fusion of liposomes with cells, which would deliver perifosine directly into the cell membrane, endocytosis of liposomes, which would deliver perifosine into the cells, or the adsorption of liposomes to cells. In all these cases, the amount of liposomes in the cell supernatant should decrease with time. However, if there is no interaction of liposomes with cells, the concentration of liposomes in the cell supernatant should remain constant.

In order to test whether any change of liposome concentration in the supernatant occurs, we encapsulated a hydrophilic spin probe ASL inside the liposomes and followed the intensity of EPR spectra in the supernatant at different time points after the addition of the liposomal formulation to the cells in presence of reducing agent sodium ascorbate (Figure 3F). EPR spectra intensity should be proportional to the amount of encapsulated ASL and is therefore a measure of the amount of liposomes in the cell environment and their stability. Any ASL released from liposomes would be reduced to EPR nonvisible hydroxyl amine. It was found that the amount of ASL within both L3 (Figure 3F, closed circles) or L4 liposomes (Figure 3F, open circles) did not significantly change with incubation time. This indicates that intact liposomes remain in the cell environment and are not taken up by any of the ways described above in both formulation, irrespective of the presence of perifosine.

These experiments with membrane-incorporated probes P5 or MTV indicate that perifosine is rapidly equilibrated among the available membranes, while liposomes remain intact in solution above the barrier-forming cells, as shown with ASL-containing liposomes.

Accumulation of perifosine in barrier-forming MDCK cells

In order to prove that perifosine molecules, which are depleted from liposomes, incorporate into cell membranes, liposomes labeled with MTV were introduced



to MDCK cells plated on the bottom of a cover glass chamber at room temperature under a microscope. The FM images were taken at different time points after liposome addition to the cells. **Figure 4A1–D1** show bright field images of cells, while **Figure 4A2–D2** show auto fluorescence images of the cells. Auto fluorescence signals originated from the cell cytoplasm, whereas no auto fluorescence signals could be observed from cell membranes. As suggested by the EPR results shown in **Figure 3**, MTV was observed in cell membranes after 7 min of incubation (white arrows in **Figure 4A3&B3**). No significant difference could be observed between the two liposomal formulations proving again that the concentration of perifosine in liposomal formulations is not important for the rate of transfer. The fluorescence signal from cell membranes decreased with time (**Figure 4A4&B4**) for both formulations. It should be taken into account that the amount of MTV in liposomal formulation should be about ten-times smaller than the number of lipids in cell membranes, therefore the source of MTV molecules is finite and after certain time the transport from liposomes to cells reaches an equilibrium. The fluorescence intensity decrease in cell membrane can be explained by the slow transport of MTV into the cell interior which is supported by the observed increase of fluorescence intensity inside the cells at later times.

In order to confirm our EPR experiments, which indicated that perifosine is transported as a free molecule, we labeled both liposomal formulations with the fluorescent probe NBD-PC. This lipid has a very slow lipid exchange rate, since it has two alkyl chains, and consequently a low CMC. The transfer of this probe into MDCK cells with time was measured using FM (**Figure 4C&D**). The fluorescence in cells could only occur if liposomes were transferred to cells or if lipid mixing occurred. No fluorescence of NBD-PC was observed in the cell membranes during the incubation with L3 or L4 liposomes, indicating that there was no probe transfer from the liposomes to the cell membrane. This suggests that the liposomes did not interact with the cells, and confirms the EPR experimental results of ASL-loaded liposomes (**Figure 3F**).

The transfer of perifosine into the MDCK cells, as suggested by FM of the MTV probe, was supported by spin labeled perifosine (P5). After incubation of cells with P5-labeled liposomes a broad EPR signal, typical for a signal from a plasma membrane, was detected in cells (**Figure 5A**), proving the incorporation of P5 into the cell membrane. EPR spectra of P5 from the cells were identical for both liposomal formulations used. This is expected since the target membrane was the same, assuming that both liposomal formulations do not interact with the cells, and therefore do not change the lipid composition of the cell membrane. The EPR spectra intensities from cells incubated with either of the liposomal formulations were within the range of experimental error (**Figure 5C**), indicating that the transfer of P5 into the cells is similar for both liposomal formulations. The same was observed after the incubation of cells with MTV-labeled liposomes (**Figure 5D**).

The EPR spectra intensity decrease of P5 in cells, due to the reduction of the nitroxide group of P5 by redox reactions in cells, is slow (**Figure 5B**), proving a slow transport of P5 into the cell interior observed previously with fluorescence measurements. This is not surprising considering the positive charge of the polar head of the perifosine and its analogs (**Figure 1**), which prevents fast transport of P5 from the outer cell layer into the cell, as already observed before [46].

Influence of perifosine & leakage of liposome-encapsulated content on transepithelial transport

Since around 50% of labeled perifosine is rapidly transported from liposomal formulation to cells irrespective of its composition and presence of perifosine, it can be expected that the amount of perifosine in a liposomal formulation is a decisive factor in formulating an efficient transepithelial delivery system. It can be concluded that the liposomes with perifosine represent a source of active free perifosine. However, calcein transported across the MDCK cell barrier was found to be released from liposomes [8], indicating that whole, intact liposomes, cannot be transported across the cell barrier. Therefore, we proposed a hypothesis that leakage of liposome-encapsulated content is as

Figure 4. Transport of fluorescently labeled perifosine analog from liposomes into cells (see facing page). Madin–Darby canine kidney cells were incubated either with the liposomal formulation L3 labeled with **(A)** MTV or **(C)** NBD-PC, or with the liposomal formulation L4 labeled with **(B)** MTV or **(D)** NBD-PC. Each row displays 1) a bright field image, 2) autofluorescence, 3) a fluorescence image 7 min and 4) 15 min after the addition of liposomal formulations. **(E)** Amount of MTV remaining in cell supernatant. Fluorescence intensity of MTV in liposomal formulations L3 and L4 at different time points after the addition of the liposomal formulations to Madin–Darby canine kidney cells attached on the bottom of a culture flask. **(G)** The transfer of perifosine analog MTV (blue) from liposome membrane (phospholipids – orange, MTV – blue) to cells (pink) is shown. AF: Autofluorescence image; BF: Bright field image; MTV: Fluorescently labeled perifosine; NBD-PC: Fluorescently labeled phosphatidylcholine.

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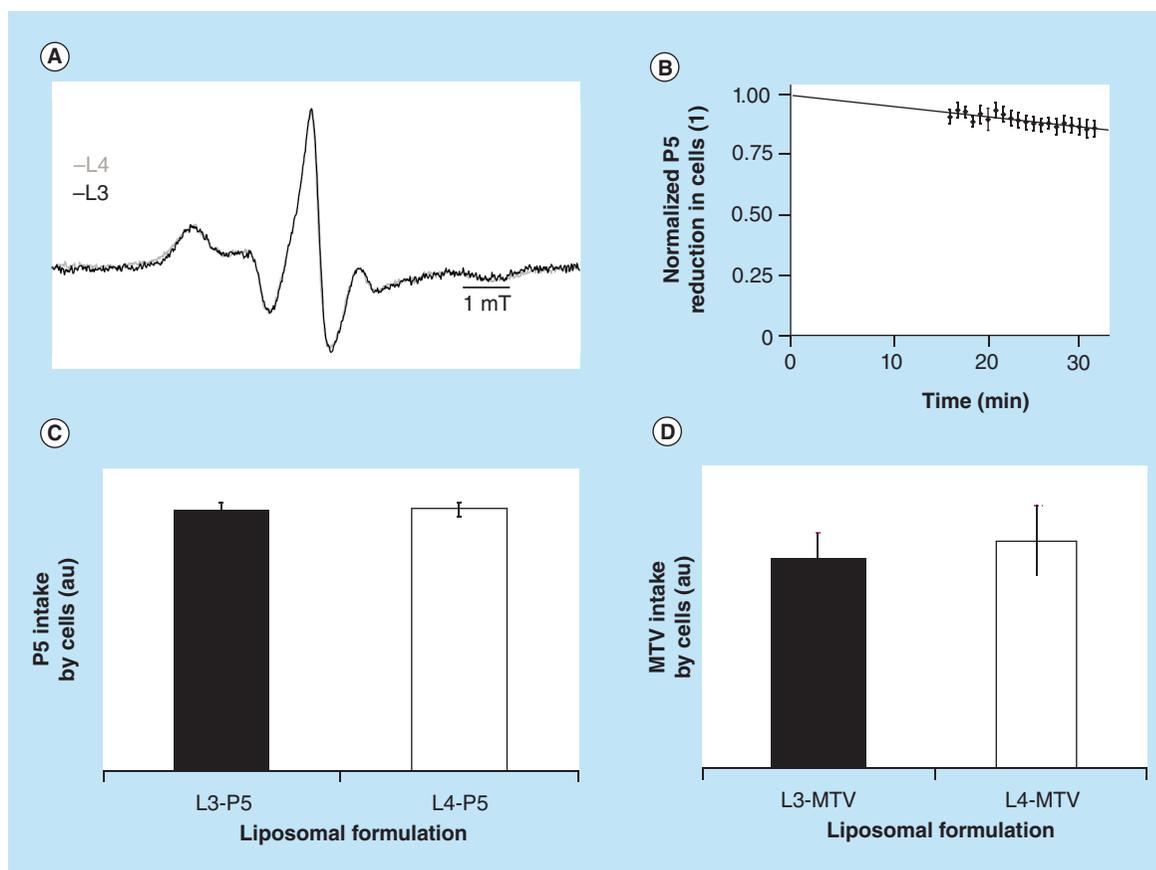


Figure 5. Transport of perifosine into cells. (A) Electron paramagnetic resonance spectra of spin-labeled perifosine (P5) delivered into Madin–Darby canine kidney (MDCK) cells either with liposomal formulation L3 (black line) or L4 (gray line); (B) EPR spectra intensity decrease of P5 in MDCK cells after incubation with liposomes, average intensity of all five experiments with SD is shown; (C) Intake of P5 by cells as measured by electron paramagnetic resonance intensity of P5 in MDCK cells delivered with L3 liposomes (black) or L4 liposomes (white); (D) Intake of MTV by cells as measured by fluorescence intensity of MTV in MDCK cells delivered with L3 liposomes (black) or L4 liposomes (white).

MTV: Fluorescently labeled perifosine.

important as the amount of perifosine in the liposomal formulation [26]. To test this hypothesis, we formulated different liposomal formulations with different lipid compositions, as presented in Table 1, and measured transepithelial transport of liposome-encapsulated calcein. Since perifosine opens endothelial barrier transiently for more than 4 h [21], we measured release of liposome contents after 8 h of incubation with cell medium containing serum (Table 1). The transepithelial transport varied from only 2% of the total liposome-encapsulated calcein for liposomes L3 and E2–7, which do not contain perifosine, to almost 25% for liposomes E2–2 and E4, which contain 25 and 45 mol% of perifosine, respectively, confirming the correlation between the relative proportion of perifosine in the liposomal formulations and transepithelial transport (Figure 6A) observed before [8]. Other lipid components of liposomes, or even their ratios, did not show any significant correlation (data not shown).

Transepithelial delivery of more than 5 mol% calcein was obtained when the amount of perifosine in liposomal formulations was at least 17 mol% and when liposomes concurrently released more than 30% of their content in a period of several hours (Figure 6B). Taking into account that about half of all perifosine in a liposomal formulation is transferred to cells and that total lipid concentration added to cells was 40 μ M, one can easily see that cells are exposed to more than about 4 μ g/ml of perifosine when high transepithelial transport is achieved. This is in accordance with previously published results showing that transepithelial electrical resistance in enteric T84 cell layers drops to 80% in presence of 5 μ g/ml of perifosine [21]. This therefore shows that a threshold concentration of perifosine has to be exceeded, which in our case corresponds to about 20 mol% of perifosine in a liposomal formulation. Since epithelial barrier is open for several hours, liposomes have to release most of their content in this

time period. High liposome leakage alone is therefore not enough for efficient transepithelial transport, as it can be seen in [Figure 6B](#). Only liposomal formulations which exhibit high leakage and contain threshold amount of perifosine simultaneously are suitable for transepithelial transport (red peak in [Figure 6C](#)).

In order to confirm that calcein found in the basal medium did not result from passive diffusion of free calcein across an intact cell monolayer, we quantified transepithelial transport of the free calcein. Free calcein (4.3 nmol/well) was incubated in serum containing medium in the apical chamber of the transwell system. After 24 h at least two 100 μ l samples were taken from basal chamber and the amount of calcein was quantified spectrophotometrically. We found that only 1.5% of free calcein was transported across a tight MDCK barrier, whereas almost 38% was transferred in absence of the MDCK cell barrier. Taking into account that the highest leakage of liposomes used in this study was at most 60% ([Table 1](#)), we can assume that less than 1% of transepithelial transport can be attributed to the transport of free calcein, independent of transepithelial transport of liposome-encapsulated calcein.

Discussion

In our previous work, we have shown that the presence of perifosine in liposomal formulations significantly increases the transport of calcein across a tight cell barrier [8]. On the basis of these findings, we proposed the hypothesis that perifosine-containing liposomes could be efficient delivery systems for the transport of drugs across barrier-forming cells. We assumed that perifosine-containing liposomal formulations represent a source of perifosine, which might be transported into barrier-forming endo- or epithelial cells and induce changes in their cell shape. As a result, gaps are formed between cells which enable the passage of liposomes through the barrier [26].

In this study, we tested the proposed hypothesis on a larger series of liposomal formulations than previously [8], containing different concentrations of perifosine and other lipid components ([Table 1](#)), aiming to contribute to the understanding of transepithelial transport mechanisms. For this purpose, we investigated whether perifosine is transferred from liposomes into barrier-forming cells and whether the release of liposome-encapsulated content and its transepithelial transport is accompanied with the depletion of perifosine from liposomes.

In the first part of our investigation, we focused on two liposomal formulations, one with high transepithelial transport and high leakage of liposome content (L4 liposomes) and the other with low transepithelial transport and low leakage (L3 liposomes). Both liposomal formulations contained spin-labeled (P5) or fluorescently

labeled (MTV) perifosine. The summary of the experimental setup is schematically shown in [Figure 7](#). Biological activity of P5 was tested before and was found to have similar hemolytic activity to perifosine, but to be slightly less cytotoxic [38]. The CMC of P5 is 4.22 μ M and is comparable to the CMC of perifosine [19]. According to our results, MTV interacts with cells in a similar way as P5, which supports our assumption that both perifosine derivatives reflect properties of perifosine. Both perifosine derivatives show fast transport from liposomes to cell membranes ([Figure 3](#) & [Figure 4](#)). We have shown that P5 is not only present in the liposome membrane, but also as a free molecule, as is evident from its EPR spectra, where the membrane and isotropic part of P5 are well resolved ([Figure 3A](#) & [Figure 4B](#)). During incubation with cells, both fractions tend to be in equilibrium, as their intensity decreases in a similar manner. As was observed before, alkylphospholipids in concentrations below CMC insert progressively into lipid monolayers as monomers from the aqueous medium. However, above CMC also groups of monomers (micelles) are transferred into lipid monolayers [47]. A high affinity between alkylphospholipids and CH has been observed in lipid monolayers, and maximum condensation is reached at around a 1:1 ratio of alkylphospholipid:cholesterol (mol:mol) [47]. This is generally known as the condensing effect of CH toward phospholipids [48,49]. We have previously hypothesized that perifosine might interact with barrier-forming cells if the liposome membrane contains more perifosine than CH [26]. In the present work, we demonstrated that this is not the case. Both perifosine analogs, were rapidly transported from both liposomal formulations L4 (fluid liposomes) and L3 (rigid liposomes which contains only labelled perifosine, i.e., a much lower amount than CH) ([Figure 3C&D](#) & [Figure 4A,B&E](#)). It should be mentioned that P5 does not detect and fluidity increase with increasing concentration of perifosine, which was observed with other probes, as it monitors membrane characteristics close to water-lipid interface [25]. Therefore the spectra of P5 in both liposomal formulations are the same.

Our results also show that perifosine incorporates into the cell membrane, but is not transferred into the cell interior very quickly, as its reduction by redox reactions in cells is very slow ([Figure 5B](#)), as well as is the clearance of fluorescently labeled perifosine from the cell membrane ([Figure 4A4&B4](#)). Therefore it can induce significant changes in the cell membrane, which, according to published data [21], results in the formation of gaps between cells.

Using a series of liposomal formulations with different lipid compositions, we confirmed that the higher the amount of perifosine in liposomal formulations is, the greater is the transport of the liposome-encapsulated content across barrier-forming cells. However, for

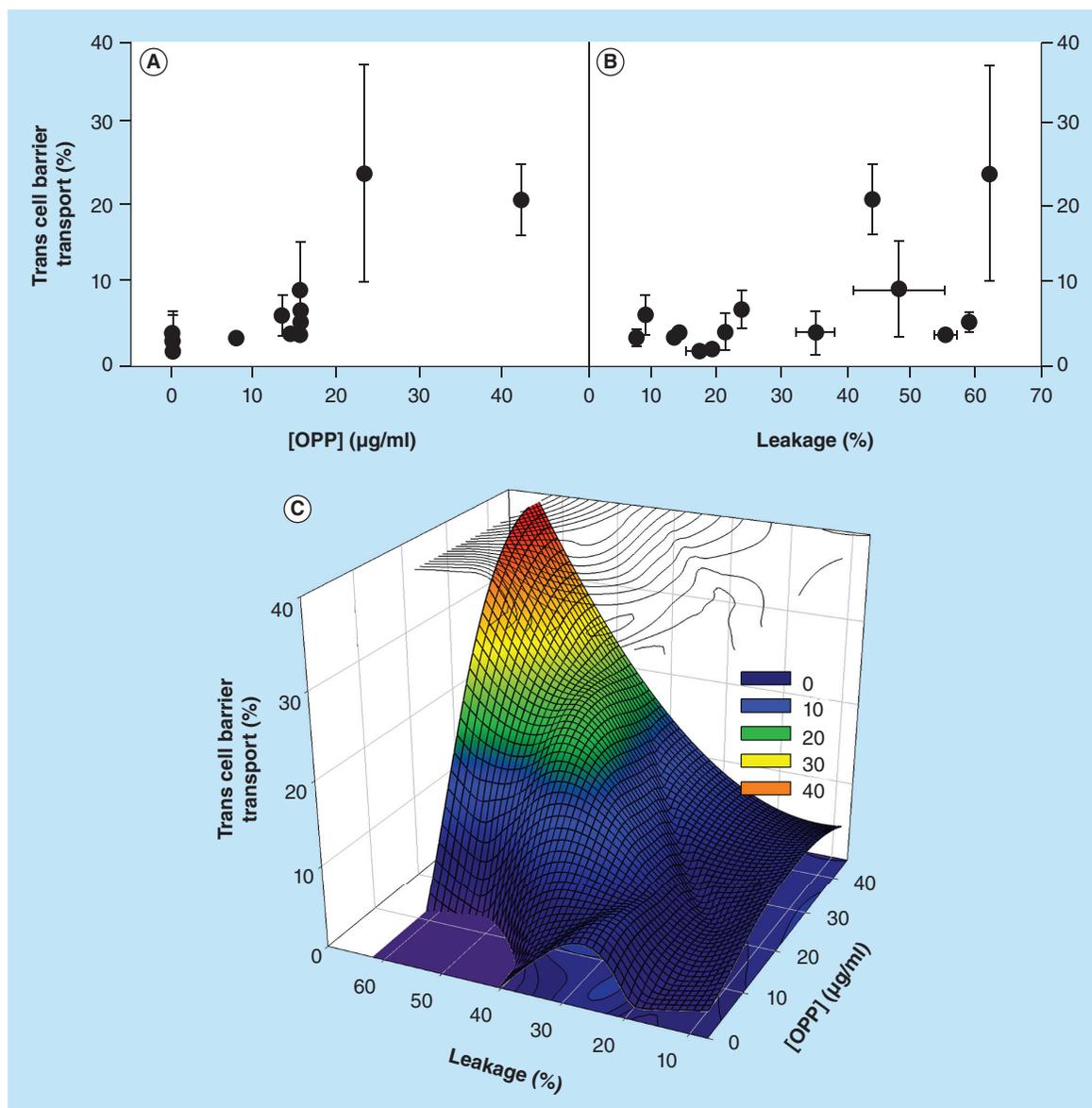


Figure 6. Trans cell barrier transport of liposome-encapsulated calcein across the Madin–Darby canine kidney tight cell barrier. The amount of calcein in the basal media was determined by fluorescence measurements after incubating liposomes with Madin–Darby canine kidney cells for 24 h at 37°C. Trans cell barrier transport is shown as a function of: **(A)** the amount of OPP in liposomal formulations; **(B)** liposome leakage (calcein release); and **(C)** leakage and perifosine concentration. OPP: Perifosine.

efficient transport of liposome-encapsulated calcein, a high liposome leakage (>20 mol% calcein) is also necessary (Figure 6 & Table 1). It was shown before that perifosine at concentrations of 10–15 µg/ml (corresponding to approximately 20–30 µM) reversibly opens the epithelial tight junction [21], which is in the same concentration range as was found in our experiment to be the limiting concentration for inducing transepithelial transport. It was also shown that the leakage of liposomes increases with increasing concentration of perifosine [25]. This supports our hypothesis about the role of perifosine, or other LPC derivatives, in transepithelial transport. Recently

it was shown that LPC transiently increases endothelial permeability [50] by disrupting endothelial barrier function, resulting in inflammatory responses in the blood vessel wall. Proinflammatory mediators increase endothelial permeability by activating signaling cues that induce endothelial cell contraction, resulting in the formation of gaps between endothelial cells [51,52].

To summarize (Figure 7), our results prove that liposomal formulations can provide a source of free perifosine, which is rapidly transferred into cell membranes. As is already known, perifosine increases endothelial permeability [21] similarly to other LPC

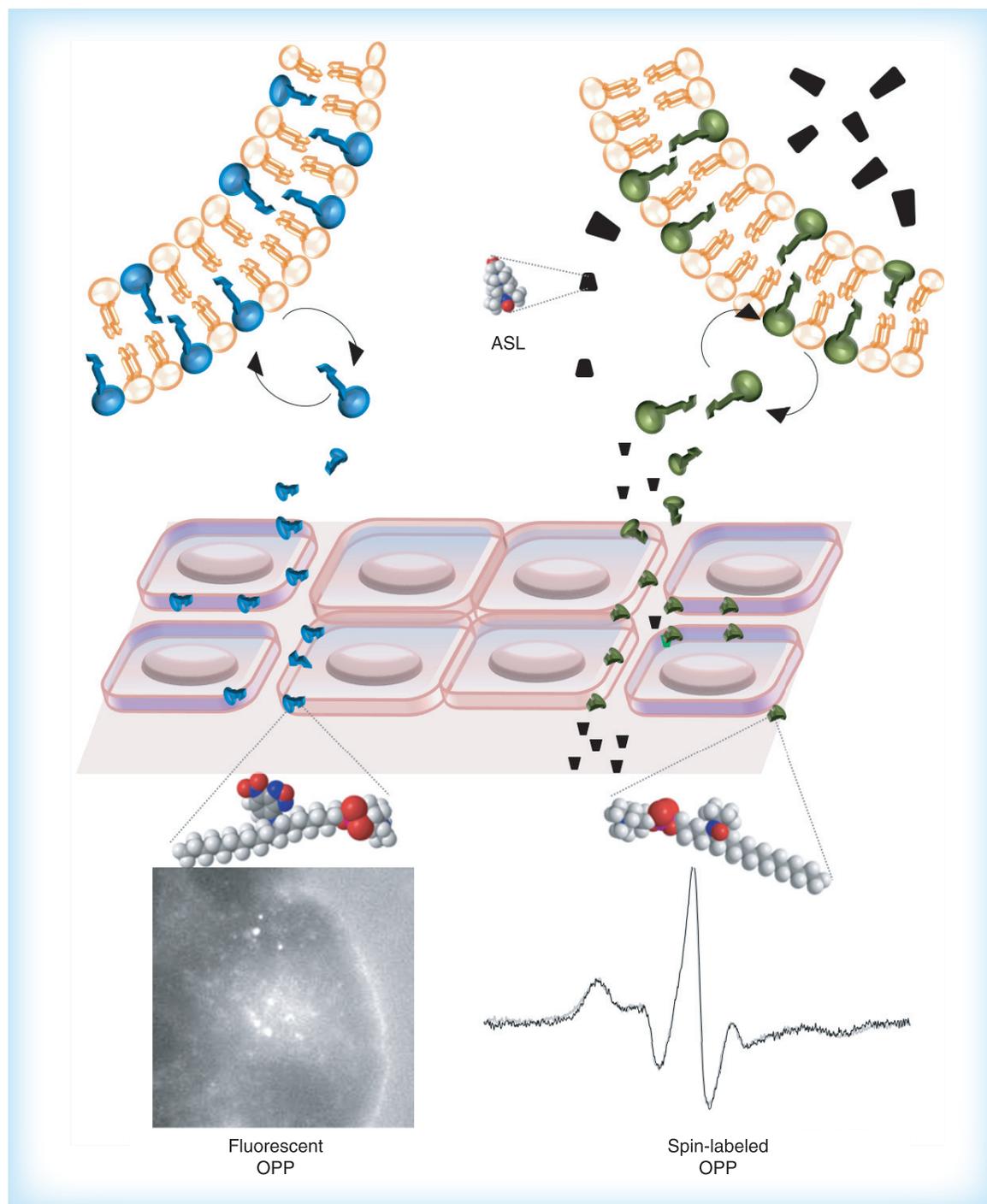


Figure 7. Perifosine-induced opening of the tight cell barrier (pink) and the subsequent transfer of liposome-encapsulated content (ASL - black) across the cell barrier. The experiments presented in this work involving liposomes with fluorescently labeled perifosine analog MTV (blue) and spin-labeled analog P5 (green) are summarized. ASL: 4-(*N,N*-dimethyl-*N*-(2-hydroxyethyl))ammonium-2,2,6,6-tetramethyl piperidine-1-oxyl iodide; OPP: Perifosine. For color images please see online www.future-science.com/doi/full/10.4155/TDE.14.127

derivatives, by activating cell contraction, resulting in the formation of gaps between endothelial cells [51,52] and thus enabling the transport of substances to the basal side. Therefore it is necessary that liposomes release their content when the cell barrier is com-

promised. For efficient transepithelial transport of liposome-encapsulated hydrophilic compounds, two conditions should be fulfilled. First, a sufficient amount of perifosine should be present in the liposomal formulation (more than 17 mol%, resulting in

approximately 15 µg/ml of final concentration, when added to cells) to open the cell barrier and second, liposome leakage must be sufficient to enable the release of encapsulated drugs.

Conclusion

In this work, we used a combination of two techniques to follow the transport of perifosine from liposomes into barrier-forming cells (Figure 7). For this purpose, spin-

Executive summary

Background

- Development of drug delivery systems across barrier-forming cells remains a challenge.
- Liposomes containing perifosine efficiently deliver drugs across barrier-forming cells.

Aim of the study

- Is perifosine transferred from liposomes into barrier-forming cells? If so, how much of it is transferred from liposomes to cells and does the transport depend on the liposome membrane composition?
- Is the release of the liposome-encapsulated content and its transepithelial transport accompanied with the depletion of perifosine from liposomes?

Materials & methods

- Spin-labeled and fluorescently labeled perifosine analogs were synthesized and inserted in liposomes of different composition.
- Transport of labeled analogs from liposomes to the barrier-forming Madin–Darby canine kidney (MDCK) cell was measured by electron paramagnetic resonance (EPR) and fluorescence spectroscopy.

Results

- Transport of perifosine from liposomes to barrier-forming cells
 - Approximately 50% of perifosine analogs are transferred from liposomes to cell, within 5 minutes after addition of liposomes to cells.
 - Rate of transfer does not depend significantly on liposome characteristics and composition
- Liposome content tracking during incubation with cells
 - During incubation with cells liposomes remain intact in solution above MDCK cells.
- Accumulation of perifosine in barrier-forming cells
 - Perifosine analogs P5 and MTV were detected in MDCK cell membranes within 5 min after incubation of liposomes with cells proving again fast transfer of perifosine analogs from liposomes to cell membrane.
 - There was no transfer of phospholipid fluorescence analog NBD-PC from liposomes to cells, since it has very slow lipid exchange rate. This proves that liposomes are not transferred into the cells, but only the free perifosine analogs are transferred to cell membrane, as was already shown in section 'Liposome content tracking during incubation with cells'.
 - As shown by fluorescence intensity measurements as well with EPR spectra intensity decrease in cells after incubation with liposomes the transfer of perifosine analogs from cell membrane to cell interior is a slow process.
- Influence of perifosine & leakage of liposome-encapsulated content on transepithelial transport
 - A transepithelial transport and release of calcein from liposomes after 8 h was measured for a series of ten liposomal formulations with different amount of perifosine.
 - The higher the amount of perifosine in liposomal formulation is, the greater is the transport of the liposome encapsulated content across barrier-forming cells.
 - For efficient transport high liposome leakage is necessary.
 - Only liposomal formulations which exhibit high leakage and contain more than 20 mol% perifosine are suitable for transepithelial transport.

Discussion

- Biological activity of P5 was tested before and was found to have similar biological activity as perifosine. According to our results MTV interact with cells in similar way as P5. Therefore we supposed that the used analogs mimic well the properties of perifosine. Therefore, we suppose that fast transport of perifosine analogs from liposome to cell membrane measure is valid also for perifosine.
- Taking this into account we suppose that perifosine is transferred from liposomes to cell as a single molecule and is not transferred into the cell interior very fast. Therefore, it can induce significant changes in the cell membrane, which according to published data results in the formation of gaps between cells. The liposome content which is released from liposomes can then be transferred through the barrier.

Conclusion

- An efficient transport of liposome-encapsulated hydrophilic drugs across cell barriers is possible with liposomal formulations that contain sufficient amounts of a lysolipid and release sufficient amounts of their content, while the cell barrier is compromised.
- This opens a new possibility of using lysolipid-containing liposomal formulations as a drug delivery system across cell barriers.
- To confirm this assumption, further investigations with a series of liposomal formulations with different lysolipids are necessary.
- For *in vivo* application it would be necessary to develop the liposomal formulations which would enable targeted delivery to the endothelium and to ensure that the liposomes will remain in the desired region long enough to loosen the tight junction and deliver the content through the barrier.

labeled and fluorescently labeled perifosine analogs were synthesized. Electron paramagnetic resonance and FM yielded comparable results, which indicate that approximately one half of perifosine is rapidly transferred from liposomes into cells, while liposomes remain intact. As perifosine is a derivative of LPC, which was shown to induce the formation of gaps between endothelial cells [51,52], we believe that an efficient transport of liposome-encapsulated hydrophilic drugs across cell barriers is possible with liposomal formulations that contain sufficient amounts of a lysolipid and release sufficient amounts of their content while the cell barrier is compromised. This opens a new possibility of using liposome formulations as a drug delivery system across cell barriers. To confirm this assumption, further investigations with a series of liposomal formulations with different lysolipids are necessary. Additionally, localization of liposomes to the target site remains a challenge that still needs to be addressed.

Future perspective

The drugs or drug carrier systems are usually transferred into the endothelial cells by the endocytotic pathways. Only clathrin-mediated or caveoli-related endocytosis can also traverse cytosol and transport the material across endothelium, but are suitable only for delivery systems with size less than 100 nm [53]. Therefore the intracellular delivery is more convenient for transport of drugs into the endothelial cells, but is less effective for trans cell barrier transport. We speculate that future research will focus on the delivery systems which loosen the tight cell junction between endothelial or epithelial cells and allow the paracellular drug transport.

Lysolipid containing liposomes are promising candidates for this since according to the results presented, they are transferred fast from liposomes to cell membrane where they can open the tight junction and enable trans cell barrier transport of the entrapped molecules as was shown by other authors [21]. In future work it would be necessary to test our hypothesis with other lysolipids and to investigate the possible side effects of lysolipid containing liposomal formulations for *in vivo* application.

Besides, for *in vivo* application it would be necessary to develop the liposomal formulations, which would

enable targeted delivery to the endothelium and to ensure that the liposomes will remain in the desired region long enough to loosen the tight junction and deliver the content through the barrier. This could be achieved by incorporation of targeting ligands into the liposome membrane that would enable binding to tight junction proteins or the associated membranes [6]. Another challenging approach are superparamagnetic iron oxide nanoparticles (SPIONs), which can be entrapped into the liposomes and are recently extensively investigated for this purpose. SPIONs can be concentrated at a particular point of the body using external magnetic field [54].

A proper combination of lysolipids and other lipids resulting in a temperature dependent, burst-like release of majority of liposome contents at the specific site in the body therefore holds challenging prospects for application in drug delivery across cell barriers.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/TDE.14.127

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