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Interaction of Alkylphospholipid Formulations with Breast Cancer Cells in the Context of Anticancer Drug Development

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1. Introduction

Alkylphospholipids have shown promising results in several clinical studies (Mollinedo 2007) and among them Perifosine (octadecyl(1,1-di-methyl-4-piperidinium-4-yl)phosphate, OPP), and miltefosine (hexadecylphosphatidylcholine (HPC)) seems to be most promising for breast cancer therapy (Fichtner, Zeisig et al. 1994). For this type of tumor, an antitumor effect was found only for hormone receptor negative tumors *in vivo*, while no effect was found for receptor positive tumors. The reason for this difference is not yet understood and requires further studies. The exact mechanism of action of alkylphospholipids on the molecular level is still not well known in detail. It is clear that they do not target DNA, but they insert into the plasma membrane and subsequently induce a broad range of biological effects, ultimately leading to cell death.

Unfortunately, administration of free (micellar) alkylphospholipids results in unwanted side effects, reflected in gastrointestinal toxicity and hemolytic activity, which limits the application of higher doses of alkylphospholipids. To achieve better therapeutic effects of alkylphospholipids *in vivo* with less side effects, different liposomal formulations of alkylphospholipids have been tested and showed diminished hemolytic activity. On the other hand, in most cases, cytotoxic activity of liposomes was also lower as compared to free alkylphospholipids (Zeisig et al., 1998).

For efficient application of liposomes as nanocarriers in breast cancer therapy it is not only necessary to investigate the properties of the nanocarrier, which has to transport the drug to the (target) cell, but also the properties of the target cell. The main difference between Perifosine (OPP) resistant MCF7 cells and OPP sensitive MT-3 cells is in the uptake of OPP liposomes by cells and the transport of OPP across plasma membrane. At physiological temperatures the rate of transfer of OPP across plasma membrane increases to greater extent in OPP resistant MCF7 cells, while the uptake of liposomal OPP formulations is lower for

OPP resistant MCF7 cells as compared to OPP sensitive MT3 breast cancer cells. On the other hand the properties of an efficient OPP formulation are mainly determined by cholesterol concentration, which should be below 50 mol%.

2. Alkylphospholipids in clinical trials

In the late 1970's and early 1980's systemic investigations of structure – activity relationship were performed to screen lysophospholipids, alkylphospholipids and etherlipids to identify new candidates for cancer treatment. Among them, especially Miltefosine (Fig. 1), basically a simple phosphorus acid diester, displayed high inhibitory activity against chemically induced mammary carcinomas in rats (Eibl & Unger, 1990). It became the first drug based on a phospholipid structure demonstrating the high potential of this simple structured molecule class. The main advantage of this class of drugs is the target. In contrast to most anti-cancer drugs, which interfere at the DNA level with cell proliferation, alkylphospholipids act at the cell membrane, where they disturb the PI3K/Akt/mTOR signal transduction pathway (Fig. 2). Initial preclinical tests were promising, indicating a good anti-cancer activity against several human tumour xenograft models in the mouse (Arndt et al., 1997; Fichtner et al., 1994), including different breast cancer cell lines like: MT-3 (Zeisig et al., 1998), MDA-MB 435 and MDA-MB 231 (Sobottka & Berger, 1992), MaTu (Arndt et al., 1999), MT-1 (Naundorf et al., 1992), C3H, Ca 755 (Zeisig et al., 1991) and also syngeneic models like murine P388 leukemia, and B 16 melanoma (Zeisig et al., 1991). Preclinical experiments further demonstrated that alkylphospholipids, if used in liposomal form, are able to abolish multi drug resistance in human breast cancer xenografts (Zeisig et al., 2004) and inhibit metastasis if combined with an aggregation inhibitor inside liposomes in murine syngene (Wenzel et al., 2010) and human xenograft breast cancer models (Wenzel et al., 2009). Perifosine in combination with dioleoylphosphoethanolamine, as a component of the liposome bilayer, also enhances transport of drugs across the blood brain barrier and in this way improves the treatment of intracerebral tumours and metastases (Orthmann et al., 2010). Miltefosine was also tested as an alternative approach for the treatment of patients with progressive cutaneous lesions from breast cancer in Phase I and II studies, which indicated that Miltefosine (either used alone or in conjunction with other therapies for distant metastases) is an effective and tolerable local treatment for cutaneous breast cancer (Clive et al., 1999; Unger & Eibl, 1991).

Only small changes in the molecular structure (slightly longer alkyl chain, a modified head group) while maintaining molecular size and shape resulted in Perifosine (OPP). Gills et al. (Gills & Dennis, 2009) summarised the clinical trials with Perifosine as single agent until 2009. Seven Phase 1 single agent studies of Perifosine have been completed. The trials demonstrated that Perifosine can be safely given to humans with a manageable toxicity profile. The dose limiting toxicity in the Phase I studies was, similar to Miltefosine, gastrointestinal: nausea, vomiting and diarrhea. Perifosine as single agent has been further evaluated in Phase II studies for the treatment of most common cancers, including breast, prostate, head and neck, pancreatic cancers, melanoma, renal cell carcinoma, advanced brain tumours, soft-tissue sarcomas, hepatocellular carcinoma, as well as in haematological malignancies including multiple myeloma and Waldenstrom's macroglobulinemia (WM). Potent activity with Perifosine, given as single-agent, has been observed so far in sarcoma and WM patients.

Fig. 1. Structural formula of pharmaceutically tested alkylphospholipids.

Name	Abbreviation(s) CAS-number	IUPAC-name	Formula, molecular weight (g/mol), reference
Miltefosine	HPC, HePC, 58066-85-6	Hexadecyl-2-(trimethylazaniumyl) ethylphosphat	C ₂₁ H ₄₆ NO ₄ P, 407.57, (Eibl & Unger, 1990; Unger et al., 1988)
Perifosine	OPP, D21266 57716-52-4	(1,1-dimethylpiperidin-1-ium-4-yl) octadecyl phosphate	C ₂₅ H ₅₂ NO ₄ P, 461.66, (Hilgard et al., 1997)
Erucyl phosphocholine	EuPC; C22:1-PC 143317-74-2	[(Z)-docos-13-enyl] 2-(trimethylazaniumyl) ethyl phosphate	C ₂₇ H ₅₆ NO ₄ P, 489.71, (Erdlenbruch et al., 1998)
Edelfosine	ET-18-OCH3 77286-66-9	(2-methoxy-3-octadecyloxypropyl) 2-(trimethylazaniumyl) ethyl phosphate	C ₂₇ H ₅₈ NO ₆ P, 523.73, (Heesbeen et al., 1991)

Table 1. Names, abbreviation, IUPAC names, formula, molecular weights and references of most common alkylphospholipids

Erucylphosphocholine is an alkylphospholipids derivative with a 22 carbon atom chain and a cis-13,14 double bond. Although it differs from miltefosine only in alkyl chain length and the presence of a double bond (Fig. 1), significant differences were found in pharmacological properties. This structural modification increases hydrophobicity resulting in the formation of lamellar supramolecular structures, which abolished hemolytic side effects and allows Erucylphosphocholine to be administrated intravenously (Erdlenbruch et al., 1999; Kaufmann-Kolle et al., 1996; van Blitterswijk & Verheij, 2008). It is a potent inducer of apoptosis (Jendrossek et al., 2003) that exerts more potent antineoplastic effects *in vitro* and *in vivo* than Miltefosine.

3. Mode of action of APL

Anti-cancer mechanisms of alkylphospholipids have been described and extensively discussed in some recent reviews (Danker et al., 2010; Gajate & Mollinedo, 2002; Gills & Dennis, 2009; van Blitterswijk & Verheij, 2008). Early interest focussed on immune stimulating activity of alkylphospholipids. It could be demonstrated that Miltefosine and other lipids of this class are able to activate T-cells and macrophages to express and release chemokines like GM-CSF (Vehmeier et al., 1992), IF γ (Hochhuth et al., 1992) and/or nitric oxide (NO) (Zeisig et al., 1995). This effect could be improved if the alkylphospholipids were used in liposomal form. Because of their amphiphilic structure, alkylphospholipids are able to form lamellar bilayers, if combined with lipids of opposite molecular shape. Liposomes were taken up by macrophages much better than the free, micellar lipids and induced, after cellular uptake, the release of IF γ and NO (Eue et al., 1995). But their potency as immune stimulator was limited and not sufficient enough amount of chemokines was released for a complete inhibition of tumor cell proliferation.

3.1 Uptake and absorption of alkylphospholipids

Due to their amphiphilic nature alkylphospholipids are easily incorporated into cell membranes in substantial amounts and then spread among intracellular membrane compartments, where they accumulate and interfere with a wide variety of key enzymes (Unger et al., 1992; van Blitterswijk et al., 1987). At lower, clinically relevant concentrations alkylphospholipids interfere with phospholipid turnover and lipid-based signal transduction pathways. In mouse S49 lymphoma cells alkylphospholipids accumulate in detergent-resistant, sphingolipid- and cholesterol-enriched lipid raft domains and are rapidly internalized by clathrin-independent, raft-mediated endocytosis (van der Luit et al., 2007). Alkylphospholipid uptake in KB carcinoma cells, however, appears to be raft-independent and mediated by a yet unidentified ATP-dependent lipid transporter (Vink et al., 2007). In leukemic cells treatment with alkylphospholipids induces the formation of membrane raft aggregates containing Fas/CD95 death receptor and the adaptor molecule Fas-associated death domain-containing protein (FADD), which are critical in the triggering of apoptosis (Gajate et al., 2009). Miltefosine and other alkylphospholipids also alter intracellular cholesterol traffic and metabolism leading to an increased uptake, synthesis and accumulation of cholesterol in the cell (Carrasco et al., 2008; Jimenez-Lopez et al., 2006; Marco et al., 2009). As cholesterol and sphingomyelin content are critical for the integrity and functionality of membrane lipid rafts, the disturbance of the cholesterol/sphingomyelin ratio could alter signaling pathways associated with these membrane domains.

3.2 Inhibition of phosphatidylcholine biosynthesis

Inhibition of phosphatidylcholine (PC) biosynthesis is a major alkylphospholipid target (Fig. 2). Inhibition of the biosynthesis of PC causes stress on cells sufficient to trigger apoptosis. In the endoplasmic reticulum, alkylphospholipids inhibit CTP (phosphocholine cytidyltransferase, CT), which catalyses the rate-limiting step of the *de novo* PC synthesis. Alkylphospholipids inhibit CT in all exponentially growing tumor and normal cells, including leukemic and endothelial cells (Zerp et al., 2008). Synthesis of PC is essential for cell proliferation and is upregulated in tumor cells. PC is not only the most abundant membrane lipid and crucial for new membrane formation, but also the precursor for the second messengers diacylglycerol (DAG) and phosphatidic acid (PA) and for

sphingomyelin (SM) in membrane lipid rafts. Inhibition of PC biosynthesis blocks the downstream sphingomyelin synthase (SMS) that catalyzes synthesis of sphingomyelin and diacylglycerol in the trans-Golgi (van Blitterswijk et al., 2003). Possible consequence is the accumulation of the ceramide, which is a second SMS substrate and can trigger apoptosis (Wieder et al., 1998). Another consequence of the PC shortage is the oxidative stress with reactive oxygen species (ROS) formation (Smets et al., 1999; Vrablic et al., 2001; Wagner et al., 1993).

Fig. 2. Alkylphospholipid targets in lipid metabolism and signalling pathways summarized after van Blitterswijk et al. (van Blitterswijk & Verheij, 2008).

3.3 Influence of alkylphospholipids on major signaling pathways

Alkylphospholipids interfere with phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB)/Akt survival pathway, which is important for proliferation, differentiation, survival and intracellular trafficking (Fig. 2). They inhibit phosphorylation and recruitment of PKB/Akt to the membrane, which is essential for its activation (Elrod et al., 2007; Kondapaka et al., 2003; Rahmani et al., 2005; Tazzari et al., 2008) probably by decreased production of PIP₃ (Gills & Dennis, 2009; van Blitterswijk & Verheij, 2008).

Alkylphospholipids inhibit PC hydrolysis to phosphatidic acid (PA) by phospholipase D and further to diacylglycerol (DAG) (Kiss & Crilly, 1997; Lucas et al., 2001). PA and DAG are second messengers, essential for the mitogen-activated protein kinase (MAPK) pathways, which regulate mitosis, metabolism, survival, apoptosis and differentiation (Chen et al., 2001; Kyriakis & Avruch, 2001; Pearson et al., 2001). PA is also involved in the activation of protein kinase C- ζ (Limatola et al., 1994), mTOR (Fang et al., 2001) and c-Raf (Rizzo et al., 2000). DAG activates proteins with the C1 domain, such as protein kinases C and D, Ras guanine-releasing protein (RasGRP) and indirectly MAPK/ERK pathway (Carrasco &

Merida, 2007; van Dijk et al., 1997; Yang & Kazanietz, 2003). Alkylphospholipids also inhibit DAG formation by phospholipase C (Maly et al., 1995; Ruiter et al., 2001; Strassheim et al., 2000). Alkylphospholipids also activate the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) pathway responsible for apoptosis in tumor cells (Gajate et al., 1998; Nieto-Miguel et al., 2007; Nieto-Miguel et al., 2008; Nieto-Miguel et al., 2006; Ruiter et al., 2001; Ruiter et al., 1999). Apoptosis can be triggered by imbalance between apoptotic and survival signals (Ruiter et al., 2001; Ruiter et al., 1999), which can be influenced by alkylphospholipids, since they have an influence on cross-talk between several membrane dependent signaling pathways.

4. Interaction of free Perifosine (OPP) with breast cancer cells

In this chapter the investigation of the interaction of Perifosine (octadecyl(1,1-di-methyl-4-piperidinium-4-yl)phosphate – OPP), with ER positive (ER+) and ER negative (ER-) breast cancer cell lines is emphasized. Perifosine was chosen since it is one of the most cancerostatically active lipids, with strong antitumor effect on xenotransplanted human breast cancer. We summarize results obtained mostly by the electron paramagnetic resonance (EPR) method in order to measure the influence of Perifosine on cell membrane fluidity and to measure transport of free and liposome incorporated Perifosine into breast cancer cells.

4.1 Influence of Perifosine (OPP) on cell membrane fluidity as studied by EPR

To get the information about the influence of a biologically active substance on cell membrane by EPR it is necessary to introduce a lipophilic paramagnetic probe into the membrane bilayer. This so called spin probe serves as a marker, which reflects the motion of the alkyl chains in the vicinity of the nitroxide group of the spin probe and in this way gives information about its surrounding. Motional characteristics that determine membrane fluidity are reflected in the EPR spectra line-shape. Main parameters obtained directly from the line-shape of the EPR spectra are order parameter (S) and correlation time (τ_c). Order parameter describes the orientational order of the phospholipids alkyl chains with $S = 1$ for perfectly ordered chains and $S = 0$ for isotropic alignment, and rotational correlation time (τ_c) describes the dynamics of the spin probe motion; more fluid membranes are characterized by a small τ_c . The changes in the EPR spectra line-shape give direct information about the external influences (temperature, interactions, damages) on cell membrane fluidity. More exact information about the membrane alterations can be obtained by computer simulation of the EPR spectra taking into account that the membrane is heterogeneous, composed of several coexisting domains with different fluidity characteristics. Therefore the EPR spectrum is composed of several spectral components reflecting different modes of restricted rotational motion of the spin probe molecules in different membrane environments (Pabst et al., 2007; Stopar et al., 2006; Strancar et al., 2003; Strancar et al., 2005).

In order to see how Perifosine (OPP) influences the plasma membrane fluidity of ER+ MCF7 and ER- MT-3 breast cancer cells, the cells were labeled with the spin probe 5P. This is a spin labeled OPP (5P), containing the nitroxide group at the 5th C atom (counting from the polar head group), (Mravljak et al., 2005). Structural formula of 5P is shown in Fig. 3.

It enters the plasma membrane easily but due to its charge, it only slowly crosses from outer to inner side of cell membrane, therefore it is suitable spin probe for detecting changes in the properties of the outer layer of plasma membrane. For spin labeling of cell membranes, MCF7 and MT-3 cells were mixed with 5P (2 μM) as a spin probe and with different amounts of Perifosine (OPP) to achieve final concentrations of OPP in extracellular medium: 0 μM , 25 μM , 50 μM or 150 μM .

Fig. 3. Structural formulas of spin probes. MeFASL(10,3) – 5-doxylpalmitoyl methylester; HFASL(10,3) – 5-doxyl-palmitic acid, ASL – spin labeled tempocholine; 5P – spin labeled OPP, containing the doxyl group at the 5th C atom (counting from the polar head group).

From the spectra the order parameter was calculated. In the absence of OPP, $S = 0.66$ for MT-3 and $S = 0.68$ for MCF7 cells were obtained. After addition of 150 μM OPP, the order parameter decreased to $S = 0.57$ for MT-3 and to 0.60 for MCF7 cells. At lower concentrations of Perifosine no significant differences in order parameter were observed. This result indicates that OPP increases membrane fluidity of both cell lines at concentrations higher than 50 μM . The influence of OPP is less pronounced for MCF7 as for MT-3 cells. This indicates that OPP either doesn't incorporate into the alkylphospholipid resistant, ER⁺ MCF7 cell membranes as well as into alkylphospholipid sensitive, ER⁻ MT-3 cells, or it doesn't concentrate in plasma membrane of MCF7 cells at such high concentrations as it does in MT-3 cells.

4.2 Transport of Perifosine (OPP) into the cell

To get information about the transport of Perifosine (OPP) into breast cancer cells by EPR, several spin labeled OPPs were synthesized in our group (Mravljak et al., 2005), which have an EPR detectable nitroxide group at various positions along the alkyl chain. We have chosen spin labeled OPP (5P) (structural formula in Fig. 3) with the lowest critical micellar concentration (CMC) of all synthesized spin labeled OPPs (Mravljak et al., 2005). Its CMC is around 10 μM , while 14P (spin labeled OPP, containing the nitroxide group at the 14th C atom) exhibited the CMC of around 200 μM (Mravljak et al., 2005). Edelfosine and OPP disperse in water in the form of micelles, due to their inverted-cone shape (Busto et al., 2007), displaying a CMC at 2.5 - 3 μM (Rakotomanga et al., 2004). It appears that addition of the doxyl group to OPP distorts the inverted-cone shape of molecule to greater extent when it is placed further away from the polar head group, which results in increased CMC. Since the CMC of 5P is similar to CMC of other similar alkylphospholipids one can assume that

the disturbance caused by attaching doxyl group to OPP is small. Therefore we assumed that from all of synthesized spin labeled OPPs, 5P is the best candidate as a model molecule for studying behavior of Perifosine (OPP).

When 5P is transported into the cell, the EPR spectra intensity decreases due to the reduction of the nitroxide group to the corresponding EPR non-visible hydroxylamine by oxy-redox systems inside the cells (Chen et al., 1988; Swartz et al., 1986; Ueda et al., 2003) and can be detected by measuring the amplitude of the middle line of EPR spectra with time. From the kinetics of EPR spectra intensity decrease, information about the transport and/or interaction of spin probe with cells can be obtained. Reduction kinetics of 5P was found to be much slower as for spin probes usually used in EPR investigations of cell membranes MeFASL(10,3) and HFASL(10,3) (Chen et al., 1988; Yonar et al., 2010) indicating that its transport into the cell cytoplasm and organelles is slower as for the other probes. This is not surprising due to the charge at the head group of 5P (Fig. 3), which prevents passive transport of OPP across the membrane. For human KB carcinoma cells it has been demonstrated that OPP is internalized by an ATP-dependent translocase activity across the plasma membrane (Munoz-Martinez et al., 2008).

In order to investigate whether there is a difference in the uptake of OPP by alkylphospholipid (APL) resistant MCF7 cells (estrogen receptor positive, ER+) and APL sensitive MT-3 cells (estrogen receptor negative, ER-), both cell lines were incubated with 5P and EPR spectra intensity decrease was measured with time after incubation (Fig. 4).

Fig. 4. EPR spectra intensity decrease with time after incubation of MCF7 cells (●), and MT-3 (○) cells with 5P at A) room temperature and B) 37 °C MCF7 (estrogen receptor positive) and MT-3 (estrogen receptor negative) breast cancer cells ($5-7 \times 10^6$ MCF7 cells and $12-20 \times 10^6$ MT-3 cells) were incubated with 5P (2-3 μM concentration, depending on the estimated number of total cell membrane bilayer lipids), which was adsorbed to the wall of a glass tube in order to achieve gradual accumulation of OPP in cells during 10 min incubation at room temperature and EPR spectra intensity decrease was measured with time after incubation.

From the kinetics of EPR spectra intensity decrease (Fig. 4) the rate of transfer of spin labeled OPP (5P) across the cell membrane was calculated using a similar model as

described previously (Koklic et al., 2008). The rate of nitroxide reduction inside the cells does not differ significantly between the two cell lines and is faster at physiological temperature as at room temperature, while by increasing the temperature from room to physiological temperature the rate of transfer remains in the range of error for MT-3 cells (estrogen receptor negative), but increases significantly for estrogen receptor positive MCF7 cells (Podlipec et al., manuscript in preparation).

5. Interaction of liposomal Perifosine (OPP) with breast cancer cells

5.1 Effect of supramolecular organization of liposomal OPP formulations on their interaction with breast cancer cells

Alkylphospholipids are amphiphilic molecules and usually form micelles under physiological conditions. Unfortunately, administration of free (micellar) alkylphospholipids results in unwanted side effects, reflected in gastrointestinal toxicity and hemolytic activity, which limits the application of higher doses of alkylphospholipids. To achieve better therapeutic effects of alkylphospholipids *in vivo* with less side effects, different liposomal formulations of alkylphospholipids were prepared. This is possible only in the presence of lipids or other amphiphiles with a complimentary molecular shape. Usually cholesterol fulfills this role and enables the preparation of stable liposomal formulations from alkylphospholipids and lipids of different chain length and head groups. Among different alkylphospholipids, most investigations with liposomal formulations were performed with Perifosine (OPP). *In vivo* data show that the hemolytic effect of OPP is significantly diminished in liposomal formulations, but unfortunately in most cases, cytotoxic activity of OPP liposomes was also lower than of free OPP (Zeisig et al., 1998).

In an early study (Zeisig et al., 2001) we investigated the influence of cholesterol in liposomes consisting of Perifosine (OPP), dicetylphosphate and cholesterol (CH) on liposome stability and *in vitro* cytotoxicity. It was found that the ratio between the alkylphospholipid and cholesterol affects the cytotoxicity of the liposomes (Table 2). An increase in the OPP/CH ratio correlated directly with an increase in cytotoxicity against breast cancer cells. In the same time it was shown that a portion of 10 – 30% of OPP was present as micelles in liposomal formulations with OPP/CH ratio between 10:10 and 10:5, while the remaining OPP was stabilised by CH and forms liposomes. This was concluded, using ¹H-NMR spectroscopy, by the analysis of lipid composition after centrifugation of liposomal formulations, where micelles remain in supernatant in comparison to the initial sample. This micellar part of OPP molecules can easily be exchanged with the external environment and is able to become incorporated into other (bi)layers, as monolayer incorporation experiments demonstrated. It was assumed that this part of OPP is also mainly responsible for the cytotoxicity against tumor cells, which are not able to internalize the vesicles very well (Zeisig et al., 2001). A similar composition dependent effect was found *in vivo*, when the hemolytic effect of differently composed liposomes was followed. Again, liposomes with higher OPP/CH ratio, and thus containing a higher proportion of micellar OPP, were more hemolytically active than liposomal OPP formulations with a lower CH content (Zeisig et al., 1998).

Recently we developed a new method achieving more accurate estimates of the relative proportion of micelles, in comparison to the previously used methods (Koklic et al., 2010). The method is based on the spectral decomposition of EPR spectra. We confirmed findings of previous studies, which showed that the amount of micelles in liposomal OPP formulations increases with decreasing amount of cholesterol (Table 2).

According to the results presented in Table 2 we concluded that the amount of micelles in OPP liposome formulations is too small to be the main reason for better efficiency of liposomes with low amount of cholesterol in experimental breast cancer therapy (Koklic et al., 2010). Therefore we proposed that better efficiency of liposomes with lower amount of cholesterol depends also on the physical and chemical characteristics of liposome membranes and their interaction with cells.

Code	Molar ratio OPP:CH:X:PEG	OPP*	CH*	X*	Micelles** (%)	Hemolysis increase# (%)	Cytotoxicity IC ₅₀ MT-3#
N5 PEG	10:5:2:1	55.6	27.8	11.1	18 ± 7	179 ± 23	23 ± 1
N5	10:5:2:0	58.8	29.4	11.8	20 ± 9	126 ± 30	18 ± 3
N7.5	10:7.5:2:0	51.3	38.5	10.2	11 ± 4	147 ± 32	18 ± 3
N10	10:10:2:0	45.5	45.5	9	5 ± 2	127 ± 29	28 ± 5
P10	10:10:2:0	45.5	45.5	9	0.5 ± 0.6	nd	19 ± 2
N15	10:15:2:0	37.0	55.6	7.4	0 ± 1	nd	n.d.
Micelles	100:0:0:0	100	0	0	100	248 ± 11	17 ± 9

N and P denote charge of the formulation (- or +, respectively)
X is a charged compound (DCP (dicetylphosphate) for N formulations and DDAB (dimethyloctadecylammonium bromide) for P formulation)
PEG are stearily stabilized liposomes with PEG₂₀₀₀DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[cyanur(polyethylene glycol)-2000])
* mol% of total lipids
** reference (Koklic et al., 2010)
IC₅₀ concentration (μM) required for 50% inhibition of cell growth for MT-3 breast cancer cells, (Zeisig et al., 1998)

Table 2. Composition of OPP liposomes, relative portion of micelles obtained by EPR and their hemolytic and cytotoxic activity.

5.2 Membrane domain structure of liposomal OPP formulations

For a better understanding of the interaction between liposomal OPP formulations and tumor cells, a deeper understanding of liposomal bilayer organization is necessary. Therefore, EPR with spin labels was used to study the influence of cholesterol, charge and sterical stabilization by PEG₂₀₀₀ DSPE on physical and chemical characteristics of liposomal OPP formulations. For this purpose liposomes with the composition presented in Table 2 were spin labeled with 5-doxylpalmitoyl methyl ester (MeFASL(10,3), Fig. 3) and EPR spectra were measured. By computer simulation of the EPR spectra line-shape, information about membrane fluidity and membrane domain structure was obtained, taking into account that the membrane is heterogeneous, composed of regions with different fluidity characteristics (Koklic et al., 2002; Koklic et al., 2008). Typical spectra are presented in Fig. 5A.

It was found that in general the experimental spectra are composed of at least three spectral components. Each spectral component corresponds to a mode of motion of a portion of spin probes partitioned in different parts of the membrane with the same physical properties and characterizes a certain type of lateral membrane domains with different fluidity characteristics. EPR parameters (order parameter S, rotational correlation time τ_c , polarity correction factor p_A), which describe the motional modes of nitroxide in a certain domain

Fig. 5. A) EPR spectra of lipophilic spin-probe methyl ester of 5-doxyipalmitate (MeFASL(10,3)) in the membrane of OPP liposomes with different concentrations of cholesterol (the amount of cholesterol is indicated in mol%) in PBS buffer at 39 °C. The arrow points to a peak, which vanishes at around 50 mol% of cholesterol in the liposome membrane. B) - E) Dependence of EPR spectral parameters of spectral components on cholesterol concentration ([cholesterol]). Spectral parameters of EPR spectra of spin-probe MeFASL(10,3) in membranes of liposomal OPP formulations were derived by fitting of the calculated to the experimental spectra. B) Relative proportions of spectral components with: the lowest order parameter – domain type 1 (●); middle order parameter – domain type 2 (◇); and the highest order parameter – domain type 3 (□). Solid black line is a linear fit to the relative proportion of domain type 1, C) Order parameter, D) rotational correlation time, and E) polarity correction factor of the less ordered domain type (domain type 1). (republished with permission from (Koklic et al., 2008)).

type and reflect the fluidity characteristics of the domains as well as the proportion of spin probes in each domain type were determined. They were found to depend mainly on the amount of cholesterol, and only to a minor part on charge and sterical stabilization (Koklic

et al., 2002). Dependence of EPR parameters, reflecting the properties of the least ordered domain type on cholesterol concentration is presented in Fig. 5 B, C, and D. A sudden increase in order parameter and rotational correlation time was observed when cholesterol concentration increases from 45 mol% to 50 mol%, while at the same time the polarity correction factor decreased, indicating that the spin probes in the domain type with lowest order parameter are less accessible to water. Relative proportion of this domain type (Fig. 5B) decreases at higher cholesterol concentrations, whereas the relative proportion of the domain type with the highest order parameter increases. It seems that above 50 mol% cholesterol the least ordered domains are transformed into a new type of domains with higher order parameter ($S = 0.15$) and with proportion of 15 %.

5.3 Release of liposome encapsulated material during the interaction of Perifosine (OPP) liposomal formulations with breast cancer cells

In order to better understand the factors that determine the therapeutic activity of liposomal OPP formulations, the interaction of liposomal OPP formulations at different cholesterol/Perifosine (CH/OPP) ratios with MT-3 and MCF7 breast cancer cells was measured and correlated with the membrane domain structure of liposomal OPP formulations (Koklic et al., 2008). For this purpose, spin labeled tempocholine (ASL) (Fig. 3), which cannot penetrate an intact liposome membrane easily, was entrapped into the liposomes. Labeled liposomal formulations were mixed with the cells and the kinetics of ASL reduction in the presence of human breast cancer cells was measured by EPR. ASL gets reduced to EPR non-visible hydroxylamine when it is released from liposomes and exposed to the oxy-redox systems inside the cells (Chen et al., 1988; Swartz et al., 1986; Ueda et al., 2003), which is reflected in an EPR spectra intensity decrease. Therefore, from the kinetics of EPR spectra intensity decrease information about the interaction of liposomes with cells can be obtained. Results are presented in Fig. 6.

Fig. 6. EPR spectra intensity decrease after mixing of MCF7 cells (closed signs) or MT-3 cells (open signs) with Perifosine (OPP) liposomal formulations with two concentrations of cholesterol: 29 mol% (N5) (circles) and 56 mol% (N15) (squares) at A) room temperature and B) 37 °C. Symbols represent mean values of two to three measurements with error bars representing standard deviations.

For liposomal OPP formulation with low cholesterol content N5 (circles) a fast decrease of the EPR signal was observed in first 10 minutes after mixing liposomes with cells (Fig. 6), indicating that about 30% of spin-probes were released fast from the liposome interior into the cell cytoplasm. On the other hand, for liposomal OPP formulation with high cholesterol content N15 (Fig. 6 squares), only a very small amount of liposome entrapped ASL was released into the cells, since the intensity decrease was less than 10% at room and physiological temperature. This indicates that the liposomes remained intact either in the extracellular space or entered the cells by endocytosis, but remained intact at least for the time of measurement. It is important to note that at room temperature both cell lines behave similarly, while at physiological temperature significantly higher amount of liposomes with low CH (N5) interact with alkylphospholipid sensitive, estrogen receptor negative, MT-3 cells (open circles in Fig. 6B) than with alkylphospholipid resistant, estrogen receptor positive, MCF7 cells (Podlipec et al., manuscript in preparation). These results, obtained on trypsinated cells, which are presented here, agree well with the results published by Koklic et al. (Koklic et al., 2008), which were obtained on scraped MT-3 cells, although small differences could originate from different procedures of removal of cells from the culture flasks (Batista et al., 2010).

In order to investigate interaction of OPP liposomes with breast cancer cells in more detail, we have added 0.5 mol% of a phospholipid fluorescent probe C6-NBD-PC, where 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) is attached to the phosphatidylcholine phospholipid (16:0-06:0 NBD PC, Avanti polar lipids, Alabaster, AL, USA) to OPP liposomes as described previously (Arsov et al., 2011). Results with the lipophilic fluorescence probe C6-NBD-PC (Fig. 7) confirm the EPR measurements, indicating that liposomes with high amount of

Fig. 7. Interaction of fluorescently labeled Perifosine (OPP) liposomal formulations with MCF7 breast cancer cells. Fluorescence microscopy was performed to localize C6-NBD-PC labeled liposomal formulation immediately after addition to cells, at room temperature. A) Negatively charged liposomal OPP formulation (N5) with 29 mol % of cholesterol (1 mM final total lipid concentration) were added to MCF7 cells attached to the bottom of a well and the distribution of the lipophilic fluorescent probe C6-NBD-PC was followed with time as indicated at the bottom of each image. B) Negatively charged liposomal OPP formulation (N15) with 56 mol % cholesterol were added to MCF7 cells and measured under same conditions as in experiment A.

cholesterol do not interact with cells. Fluorescence microscopy clearly shows that OPP liposomes with high amount of CH (N15) remain outside the cells, while low cholesterol – containing, N5 liposomes interact with cell membranes because the fluorescent probe distributes in the cell interior. In addition for liposomes with low amount of cholesterol (N5), for which C6-NBD-PC distributes inside the cells, maximum of fluorescence emission spectrum shifts for a few nanometers, indicating that either micelles or liposomes interacted with cells and delivered C6-NBD-PC into lipophilic compartments of MCF7 cells, where the environment of the fluorescent probe changed (Arsov et al., 2011).

Comparing liposome membrane characteristics, derived from EPR spectra (Fig. 5) and summarized in Table 2, with liposome cell interaction experiments (Fig. 6 and Fig. 7) we can see that the propensity of Perifosine (OPP) liposomal formulations for interaction with tumor cells and for delivery of OPP into cells coincides with the existence of disordered domains as well as with the existence of micelles. We have shown that by increasing the concentration of cholesterol above 50 mol% the domains with the lowest order parameter (between 0.06 and 0.03, Fig. 5) are transformed into a new type of domains with higher order parameter ($S = 0.15$). This suggests that disordered motion of lipid alkyl chains within the liquid-disordered domains, which coexist with liquid-ordered domains, is necessary for fast delivery of liposome encapsulated probe into the cells. On the other hand, the presence of micelles in liposome formulations with concentration of cholesterol below approximately 50 mol% suggests that micelles are necessary for efficient delivery of liposome encapsulated probe into cells.

5.4 Transport of spin labeled Perifosine (OPP) from liposomes to cell membrane does not depend on the liposome cell interaction

In order to get information about the rate of transfer of OPP from liposomes containing two different concentrations of cholesterol to cells, a portion of OPP molecules in liposomal OPP formulations was replaced with spin labeled OPP (5P), so that the final concentration of 5P in OPP liposomes was 17 mol%. Because of such high amount of P5 in the liposomal formulations, the EPR lines are highly broadened (Fig. 8A) due to the spin exchange interaction between paramagnetic probes.

EPR spectra in Fig. 8B are similar as obtained for MCF7 cells labeled with 5P, indicating that high amount of 5P from liposomal OPP formulation was transferred to cell membranes in a time shorter than 2 minutes. It was not possible to resolve any difference in the rate of transfer from N5 or N15 liposomes after 2 minutes of mixing liposomes with cells. Very fast transfer was also observed when giant liposomes (composition: POPC:POPE:POPS:CH molar ratios 40:20:10:40), which represent a model for cell membrane, were incubated with N5 or N15 liposomes (Mravljak et al., 2010), proving that spin labeled OPP molecules are transferred from one type of membrane to other membranes within several minutes, and the rate of transport does not depend significantly on the membrane composition. We can conclude from the above experiment that analkylphospholipid-like molecule can easily exchange between membranes and can accumulate in cells when they are in contact with liposomal OPP formulations. This is in agreement with lipid monolayer experiments, which showed that alkylphospholipids, below the critical micellar concentration (CMC), insert progressively into lipid monolayers as monomers from the aqueous medium, while above CMC, not only monomers but also groups of monomers (micelles) are transferred into the monolayers (Rakotomanga et al., 2004). It was also shown that, while the alkylphospholipid HePC is miscible with POPC, there is high affinity between HePC and sterols (ergosterol, and cholesterol) and that maximum

condensation is reached at a ratio of HePC/sterol around 50:50 (mol/mol) (Rakotomanga et al., 2004). This kind of behavior is generally known as the condensing effect of cholesterol towards phospholipids (Chapman et al., 1969; Ghosh & Tinoco, 1972). Micelles constituted a reservoir of monomers both for monomer insertion between condensed phospholipids and for insertion of groups of monomers between fluid phospholipids. Since biological membranes are composed of dynamically condensed domains surrounded by fluid domains, it has been suggested that, above the CMC, alkylphospholipids can insert into both kinds of phases: as monomers into the condensed phase and as a group of monomers into the fluid phase. (Rakotomanga et al., 2005). The presence of albumin in the medium has the effect of increasing the CMC value by binding molecules of lipids and, hence, reducing the concentration of free monomers in the medium (Kim et al., 2007). Like albumin acts as an alkylphospholipid reservoir - it binds reversibly to the cell surface and may release the drug gradually - the role of liposomes seems to be similar.

Fig. 8. EPR spectra of lipophilic spin-probe - spin labeled OPP (5P)

A) in the membrane of liposomal OPP formulation with different concentrations of cholesterol (the amount of cholesterol is indicated in mol%) in PBS buffer at room temperature. The arrows point to peaks corresponding to free 5P, which is neither incorporated in liposomes, neither in micelles. A broad spectrum corresponds to supramolecular structures of OPP (liposomes and micelles);

B) in the membrane of MCF7 cells. Spectra were recorded 2 minutes after mixing of spin labeled OPP liposomes (1.5 μ L) with pellet of MCF7 cells (1.5 μ L with 5 -10 $\times 10^6$ cells).

At first glance these results are in stark contrast with the experiments with fluorescently labeled liposomal OPP formulations (Fig. 7) and with OPP liposomes with the entrapped hydrophilic probe (Fig. 6), since those experiments suggest that OPP liposomes with high amount of cholesterol almost do not interact with breast cancer cells. Fast transfer of OPP from liposomes to other membranes would lead to destabilization of liposomes, which was not the case for N15 liposomes with high amount of CH. It seems that OPP differs from spin labeled OPP (5P), which is not surprising with respect to the doxyl group attached to the alkyl chain, which probably prevents the condensing effect of cholesterol.

6. Summary of differences between MT-3 and MCF7 breast cancer cell lines

Main differences between OPP sensitive MT-3 and OPP resistant MCF7 cells are:

1. Plasma membrane fluidity is slightly larger for estrogen receptor negative (ER-) MT-3 as for estrogen receptor positive (ER+) MCF7.

2. OPP increases plasma membrane fluidity of both cell lines at concentrations higher than 50 μM . The influence of Perifosine (OPP) is less pronounced for MCF7 as for MT-3 cells. This indicates that OPP either doesn't incorporate into alkylphospholipid resistant, ER+ MCF7 cells as well as it incorporates into alkylphospholipid sensitive, ER- MT-3 cells, or it doesn't concentrate in plasma membrane of MCF7 cells at such high concentrations as it does in MT-3 cells.
3. Transport of alkylphospholipids across plasma membrane and subsequent reduction in breast cancer cells (Fig. 4) showed that the transport and the reduction of spin labeled OPP is faster for MT-3 than for MCF7 cells at room temperature, whereas it is just the opposite at physiological temperature. The main difference between MCF7 and MT-3 cells is the transport of OPP across the plasma membrane, which increases significantly for MCF7 cells at physiologic temperature, but remains almost unchanged for MT-3 cells. Because of this we suspect that OPP uptake by OPP resistant MCF7 cells might be mediated, similarly as in the case of KB carcinoma cells (Vink et al., 2007), by a lipid transporter. This observation could explain lower influence of OPP on cell membrane fluidity of MCF7 cells and support the hypothesis that OPP doesn't concentrate in the plasma membrane of MCF7 cells.
4. Liposomal OPP formulations with low CH concentration (N5) quickly release a portion of their content when mixed with breast cancer cells. At room temperature the release is comparable for MT-3 and MCF7 cells (Fig. 6). However, at physiological temperature the amount of released content increases for OPP sensitive MT-3 cells, but remain in the same range for MCF7 cells.

7. Conclusions with respect to experimental breast cancer therapy with alkylphospholipids

For efficient application of liposomes as nanocarriers in breast cancer therapy it is not only necessary to investigate in detail the physical properties of the nanocarrier, which has to transport the drug to the (target) cell, but also the properties of the target cell. In the case of application of alkylphospholipids, where plasma membrane is the specific target, one has to know the properties of the plasma membrane and differences among membranes of different breast cancer cell lines. We have shown that plasma membrane of MT-3 cells is more fluid (lower order parameter) as the membrane of MCF7 cells and was influenced more by OPP. Besides, it should be taken into account also that the properties of plasma membrane depend on external factors. For example, it has been shown that confluent MT-3 breast cancer cells have significantly higher membrane fluidity and higher relative proportion of disordered membrane domains as compared to cells harvested during exponential growth (Koklic et al., 2005). The fluidity of plasma membrane of MT-3 breast cancer cells also has an important role in metastasis development. The increase in membrane fluidity of MT-3 breast cancer cells was correlated with 2-fold increase in sialyl Lewis X and/or A ligand-mediated adhesion of these cells and a higher motility of ligands in the membrane of confluent cells, together with an accumulation of these ligands in distinct areas on a cell membrane (Zeisig et al., 2007).

In order to better understand the interaction of OPP micelles or liposomes with breast cancer cells, one has to take into account the following main characteristics of alkylphospholipids and of liposomal formulations:

1. In mouse S49 lymphoma cells, alkylphospholipids accumulate in detergent-resistant, sphingolipid- and cholesterol-enriched lipid raft domains and are rapidly internalized by clathrin-independent, raft-mediated endocytosis (van der Luit et al., 2007).
2. Alkylphospholipid uptake in KB carcinoma cells appears to be raft-independent and mediated by a yet unidentified ATP-dependent lipid transporter (Vink et al., 2007).
3. Lipid monolayer experiments showed that alkylphospholipids, below the critical micellar concentration (CMC), insert progressively into lipid monolayers as monomers from the aqueous medium, while above CMC, not only monomers but also groups of monomers (micelles) are transferred into the monolayers (Rakotomanga et al., 2004).
4. While alkylphospholipid HePC is miscible with POPC, there is high affinity between HePC and sterols (ergosterol, and cholesterol) and that maximum condensation is reached at a ratio of HePC/sterol around 50:50 (mol/mol) (Rakotomanga et al., 2004). This kind of behavior is generally known as the condensing effect of cholesterol towards phospholipids (Chapman et al., 1969; Ghosh & Tinoco, 1972).
5. Alkylphospholipid OPP increases membrane fluidity of both MCF7 and MT-3 cell line at concentrations higher than 50 μ M, indicating that OPP incorporates into the membrane of breast cancer cells and is slowly transferred into the cell interior as it was detected by reduction kinetics of spin labeled OPP (Fig. 4). OPP transfer increases at physiologic temperature for OPP resistant MCF7 cells, but remains almost the same for OPP sensitive MT-3 cells. We believe that OPP uptake by OPP resistant MCF7 cells might be mediated, similarly as in the case of KB carcinoma cells (Vink et al., 2007), by a lipid transporter.
6. Liposomal OPP formulations efficient in experimental breast cancer therapy should have cholesterol concentration below 50 mol%.
7. *In vivo* data show that hemolytic effects of liposomal OPP formulations is diminished as compared to free OPP, but cytotoxic activity of liposomal formulations is also lower (Zeisig et al., 1998).
8. Liposomal formulations with lower cholesterol/OPP ratio containing higher proportion of micellar OPP, are hemolytically more active than liposomal formulations with lower cholesterol concentration (Zeisig et al., 1998). At approximately 55 mol% cholesterol liposomal formulations do not contain any OPP micelles (Koklic et al., 2010). While there is almost no release of content from liposomal OPP formulations with 56 mol% cholesterol for both cell lines, liposomal formulations with low cholesterol concentration quickly release a portion of their content when mixed with breast cancer cells (Fig. 6). Similarly lipid phase of liposomal OPP formulations with low cholesterol concentration quickly enters and crosses plasma membrane of OPP resistant MCF7 cells, but remains outside of cells in the case of liposomal formulations with 56 mol% cholesterol, as measured by C6-NBD-PC labeled liposomal formulations.
9. Fluidity characteristics of liposomal OPP formulations depend mainly on the amount of cholesterol, and only to a minor part on charge and sterical stabilization (Koklic et al., 2002). A sudden increase in order parameter of most disordered domain type occurs at cholesterol concentration around 50 mol%, while at the same time the polarity correction factor decreases, indicating that the spin probes are located in an environment less accessible to water.
10. Surface activity of an alkylphospholipid Edelfosine is decreased by other lipids, especially sterols, which indicates that Edelfosine is slowly released from the lipid mixture to the aqueous environment (Busto et al., 2008).

Based on all of the above mentioned properties, there are two competing hypothesis of OPP liposome formulation – breast cancer cell interaction, which still need further experimental validation. Either OPP liposome formulations with low cholesterol concentration are able to deliver OPP into breast cancer cells by fusing with plasma membrane of breast cancer cells, due to liposome membrane properties, or alkylphospholipids whether in free or micellar form insert with high affinity into cholesterol containing target membranes (cells) as long as liposomal carriers contain low amounts of cholesterol. Once the remaining liposomal carriers have cholesterol concentration above around 50 mol% the remaining alkylphospholipids are stabilized in liposomes and do not interact with cells anymore. In this way liposomes serve as a reservoir capable of releasing alkylphospholipids and preventing side effect associated with high alkylphospholipid concentrations.

8. References

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