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Interference of phosphatidylcholines with in-vitro cell proliferation — no flock without black sheep

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ABSTRACT

According to early experiments with natural extracts, phosphatidylcholines (PCs) are widely considered essentially non-toxic. In addition to these physiological mixed-chain PCs, many different synthetic diacyl-PCs are currently available, but they have never been systematically evaluated for any interference with cell proliferation. We thus investigated the cell proliferation of several cell lines in the presence of various liposomes consisting of a single PC component and cholesterol.

Most of the PCs investigated did not interfere with cell proliferation, supporting the notion that most PCs are safe excipients. Significant IC_{50} values below 0.5 mM were detected for PC(12:0/12:0), PC(14:1/14:1)trans and all diacyl-PCs containing two polyunsaturated fatty acids (PUFAs). The ω-3 PC(22:6/22:6) was the most toxic PC assessed, revealing IC_{50} values below 100 μM, but no rule concerning ω-3/6 configuration or acyl chain length could be observed. Physiological mixed-chain PCs containing PUFAs were much less toxic than respective non-physiological diacyl-PCs. All trans fatty acids in diacyl-PCs interfered more with proliferation than their respective cis-configured counterparts. Depending on the concentration, those diacyl-PCs not only inhibited proliferation but also induced cell death.

Unlike the non-toxic PCs usually used for liposomal drug delivery, the elucidated diacyl-PCs may be worthy of further examination to eventually construct a toxic shell for toxic drugs, thereby enhancing anticancer drug delivery via lipid particles.

1. Introduction

Phosphatidylcholines (PCs) are omnipresent building blocks of almost all living organisms. As a key feature of this amphipathic substance class, PCs spontaneously form lipid bilayer structures in aqueous surroundings, and are major constituents in animal and human cell membranes [1]. They are chemically characterized by a glycerol core linked via an sn-3-phosphodiester to a choline head group, and two acyl chains are esterified in sn-1- and sn-2-positions. While the choline head group is the defining moiety, the fatty-acid (FA) acyl chains vary for different PCs. With respect to biological functionality, the sn-1 and sn-2 positions differ widely. Most natural PCs are mixed-chain PCs, having different FAs in their sn-1 and sn-2 positions. Diacyl-PCs, showing the same FAs in sn-1 and sn-2 positions, are common in neither yeast [2] nor mammalian cells [3], except for PC(16:0/16:0), PC(16:1/16:1) and PC(18:1/18:1). With regard to their spontaneous membrane-building properties, PCs are often referred to as a homogeneous class of substances, although it is well known that many biophysical and biochemical properties of various PCs depend on their FA acyl chain components. Even minor chemical changes in acyl chain structure, such as adding a double bond or two hydrocarbons, trigger major differences in membrane characteristics such as phase transition or miscibility with other membrane components. Poor miscibility of membrane lipids leads to microdomain formation in cellular membranes. These microdomains are crucial for cellular function, as the functionality of several receptors and membrane enzymes relies on a specific lipid environment [4].

PCs were initially characterized from natural extracts such as egg PC or soy PC. Not surprisingly, these PC mixtures were shown to be non-toxic in vitro [5] and in vivo. IC_{50}-values of several grams of egg PC per kg body weight were determined in mice [6,7]. A growing number of synthetic and non-natural PCs became available over the years, mostly diacyl-PC. Interestingly, PCs are generally regarded as non-toxic even though these pure components have never undergone systematic evaluation with respect to interference with cell proliferation.

Abbreviations: FA, fatty acid; FFA, free fatty acid; PUFA, polyunsaturated fatty acid; HSPC, hydrogenated soy PC; LB, liposome buffer; lyso-PC, 1-acyl-2-hydroxy-phosphatidylcholine; PC, 1-acyl-2-acyl-phosphatidylcholine; diacyl-PC, 1,2-acyl-phosphatidylcholine; NMT, N-myristoyltransferase; CI, confidence interval

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As a naturally occurring substance group that forms self-organized vesicles in water, PCs have been intensively investigated for drug-delivery purposes. The most successful vesicular approach to drug delivery are small unilamellar liposomes in a size range of about 100 nm, already in clinical practice for many years [8]. Major constituents of these liposomes are PCs and cholesterol besides minor components like PEG-linked lipids used for functional surface modifications. While many different drugs have been encapsulated and their impact intensively characterized in vitro and in vivo, possible interaction between a specific liposomal PC and cell proliferation has been generally neglected.

PCs may be hydrolyzed to free fatty acids (FFAs) and lysolipids, a reaction catalyzed by omnipresent phospholipases A. Saturated lysolipids have been shown to inhibit the metastatic spread of cancer cells in a metastatic mouse model [9]. In contrast to PCs, FFAs are generally considered to possess some toxic potential by uncoupling the electron chain in mitochondria [10] or acting as a detergent to permeabilize membranes [11]. FFA toxicities vary with the FA acyl chain's chemical structure. FFAs are utilized as a natural defense against bacteria in several species including humans [12], and polyunsaturated fatty acids (PUFAs) have attracted some interest as anticancer agents in vitro (e.g.[13,14,15] and in vivo [16,17]).

In mammalians, lipids are transported by lipoproteins, which are covered by a phospholipid monolayer. As major functional components of lipoproteins, PCs are related to severe human threats like cardiovascular diseases and diabetes [18]. Several epidemiologic studies have demonstrated FAs' key impact on human health concerning the nutritional intake of ω-3 and ω-6 FAs or cis/trans-configured unsaturated FAs [19,20].

Unfortunately, the systematic evaluation of FFA toxic effects is made difficult because of differences in solubility, binding characteristics to cells, materials and transporting proteins like albumin. Further, various FFAs possess different membrane-transport properties, hindering systematic comparison. The use of liposomes as delivery vehicles for diacyl-PCs of different FA compositions may overcome this drawback. The aim of this examination was to systematically evaluate various diacyl- and some mixed chain-PCs with respect to possible interference with cell proliferation. Furthermore, we attempted to identify possible candidates to construct a “toxic shell for toxic drugs”, such as chemotherapeutic agents, to potentially enhance the impact of liposomal drugs in anticancer therapy.

2. Material and methods

2.1. Materials

PCs were purchased from Avanti Polar Lipids (Alabaster, AL, USA), cholesterol as well as $CHCL_3$ essential purity 99.8% stabilized with amylenes was derived from Sigma-Aldrich® (St. Louis, MO, USA). [1,2-^{3}H]Cholesterylhexadecylether (1.9 TBq/mMol) was obtained from American Radiolabeled Chemicals (Saint Louis, MO, USA) and l-α-dipalmitoyl-1-^{14}C-phosphatidylcholine was obtained from PerkinElmer Life Science Products (Waltham, MA, USA). All other chemicals were of the highest quality available from commercial sources.

2.2. Chemistry of PCs

The 1-acyl-2-acyl-PCs used were noted as PC(1-acyl/2-acyl), respective FAs as FA (number of carbon atoms:double bonds) as is standard. The exact position and stereochemistry of double bonds are not stated in the text for reasons of enhanced legibility. The following chemical names and CAS numbers apply to the PCs used:

PC(10:0/10:0): 1,2-didecanoyl-*sn*-glycero-3-phosphocholine, [3436-44-0]; PC(12:0/12:0): 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine [18194-25-7]; PC(14:0/14:0): 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine [18194-24-6]; PC(16:0/16:0): 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine [63-89-8]; PC(20:0/20:0): 1,2-dieicosanoyl-*sn*-glycero-3-phosphocholine [61596-53-0]; PC(14:1/14:1)cis: 1,2-di-(9Z-tetradecenoyl)-*sn*-glycero-3-phosphocholine [56750-90-4]; PC(14:1/14:1)trans: 1,2-di-(9E-tetradecenoyl)-*sn*-glycero-3-phosphocholine [76733-52-3]; PC(16:1/16:1)cis: 1,2-di-(9Z-hexadecenoyl)-*sn*-glycero-3-phosphocholine [4724-96-3]; PC(16:1/16:1)trans: 1,2-di-(9E-hexadecenoyl)-*sn*-glycero-3-phosphocholine [56816-00-3]; PC(18:1/18:1)cis: 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine [4235-95-4]; PC(18:1/18:1)trans: 1,2-di-(9E-octadecenoyl)-*sn*-glycero-3-phosphocholine [56782-46-8]; PC(18:2/18:2)allcis: 1,2-di-(9Z,12Z-octadecadienoyl)-*sn*-glycero-3-phosphocholine [998-06-1]; PC(18:3/18:3)allcis: 1,2-di-(9Z,12Z,15Z-octadecatrienoyl)-*sn*-glycero-3-phosphocholine [2701-19-1]; PC(20:4/20:4)allcis: 1,2-di-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine [17688-29-8]; PC(22:6/22:6)allcis: 1,2-di-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-*sn*-glycero-3-phosphocholine [99296-81-8]; PC(18:0/14:0): 1-octadecanoyl-2-tetradecanoyl-*sn*-glycero-3-phosphocholine [20664-02-2]; PC(16:0/18:1cis): 1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine [26853-31-6]; PC(18:1cis/16:0): 1-(9Z-octadecenoyl)-2-hexadecanoyl-*sn*-glycero-3-phosphocholine [59491-62-2]; PC(16:0/20:4allcis): 1-hexadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine [35418-58-7]; PC(16:0/22:6allcis): 1-hexadecanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-*sn*-glycero-3-phosphocholine [59403-54-2].

2.3. Cell lines and cultivation

Chinese hamster ovarian wild type (CHO-K1) cells (ATCC® CCL61™, American Type Culture Collection, Manassas, USA) were cultured in HAM's F-12 medium (Promo Cell®, Heidelberg, Germany), supplemented with 10% (v/v) FCS (PAA, Pasching, Austria). *Rattus norvegicus*, mammary gland adenocarcinoma (MAT-B-III) cells (ATCC® CRL1666™) and MES-SA cells (ATCC® CRL1976™) were cultured in McCoy's 5A medium (Sigma-Aldrich®), supplemented with 10% (v/v) FCS and 1.5 mM L-glutamine. HL-60 cells (ATCC® CCL240™) were cultivated using RPMI 1640 (Sigma-Aldrich®) supplemented with 2 mM L-glutamine and 10% FCS. Mouse embryonic fibroblast (MEF) cells (ATCC® SCRC1008™) were cultured in DMEM – high glucose – medium containing 4.5 g/l glucose (Biochrom GmbH, Berlin, Germany) supplemented with 10% (v/v) FCS and 1 μl/ml EtSH. Pooled human umbilical vein endothelial cells (HUVEC, cat. no. 1223) were purchased from PromoCell GmbH (Heidelberg, Germany) and cultivated using Endothelial Cell Growth Medium supplemented with Supplement Mix (containing 2% FCS) purchased from PromoCell as well.

Usually 75 ml cell culture flasks (Cellstar®, Greiner Bio-One, Kremsmünster, Austria) were used to harbor the cells. All cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C, and MEF cells were protected by penicillin/streptomycin antibiotics.

2.4. Preparation and characterization of liposomes

Liposomes were prepared by extrusion technique [21]. Briefly, PCs were dissolved in $CHCl_3$ (10 mg/ml), mixed to a total amount of ~40 mg with 70 mol% PC and 30 mol% cholesterol. For radioactive measurements 0.6 μCi/μmol lipid [1,2-^{3}H]cholesterylhexadecylether (cholesterylhexadecylether) and/or l-α-dipalmitoyl-1-^{14}C-phosphatidylcholine were added to the lipids during preparation.

A dry film was prepared by evaporating the solvent in a vacuum. Lipids were then suspended in 1 ml of liposome buffer (LB) containing 118 mM NaCl, 4.74 mM KCl, 1.2 mM $MgCl_2$, 0.59 mM KH_2PO_4, 0.59 mM Na_2HPO_4, 10 mM HEPES adjusted to pH 7.4. The extrusion device LiposoFast™ (Milsch-Equipment, Laudenbach, Germany) used was equipped with polycarbonate filters (Milsch-Equipment). Routinely, the samples were first extruded 21 times through one filter with a pore diameter of 200 nm and subsequently 21 times through two stacked filters having a pore diameter of 100 nm. The prepared liposomes were stored at 4 °C for a maximum of 7 days.

Particle size distribution of manufactured liposomes was determined by photon correlation spectroscopy on a Submicron Particle Sizer NICOMP™ 380 (Particle Sizing Systems, St. Barabara, California, USA). Liposomes were diluted with LB to yield a count rate of ~300 kHz. The measurement was done at 23 °C for 10 min. For viscosity and refractivity standard parameters were used. Data were processed by NICOMP-software according to "Gaussian intensity weighting". To use this calculation, the chi^2 had to be less than 3. Depending on the PC, the average size varied between 77 (±52) nm and 119 (±44) nm. The measured sizes fell within the range expected for liposomes prepared by extrusion.

PC concentrations were measured via the colorimetric method using ferrothiocyanate [22]. In 2-ml-vials 1 ml of $CHCL_3$ and 750 µl of a ferrothiocyanate solution containing 0.4 M ammonium thiocyanate and 0.1 M $FeCl_3$ in dest. water were prepared. Liposomal preparations were diluted 1:19 (v/v) with LB and 25 µl of this dilution were added to each vial. LB was used as blank. The vials were mixed vigorously. To separate phases the vials were centrifuged at 4000 *g* for 5 min. The aqueous supernatant was aspired; remaining traces of red ferrothiocyanate solution were removed with the help of cotton buds. The extinctions of the organic phases were measured in glass cuvettes at 485 nm. For each preparation 5 replicates were measured. Individual calibration curves for respective lipids had to be used since the different PCs showed variations in extinction.

2.5. Cell proliferation assay

Adherent cells were seeded at 0.5×10^6 cells/ml in 96-well tissue culture plates (Cellstar®, Greiner Bio-One, Kremsmünster, Austria). After 4 h of incubation, the cell culture medium was removed and media containing liposomes at different concentrations were added. HL-60 cells were seeded at 2×10^6 cells/ml and allowed to settle for 2 h until the medium was aspired carefully and liposomes containing media were added. Since liposomes were not prepared under strict sterile conditions, liposome preparations were first diluted to ~2.2 mM in respective cell culture media that were phenol-red free. To prepare sterile cell culture media, these liposome suspensions were subjected to filtration under sterile conditions by a filter with 0.45 µm pore size (Millex®-HV, Merck Millipore, Merck KGaA Darmstadt, Germany). Loss of PC due to filtration was about 10% on average. PC contents were measured and respective volumes added to the cell culture wells containing media to yield a total volume of 100 µl/well. To investigate possible interference of PCs with cell proliferation, liposomes were added to yield final concentrations of 0.01; 0.05; 0.1; 0.25; 0.5 and 1 mM liposomal PCs and proliferation was measured after 96 h. With respective mixed-chain PCs 2 mM liposomal PCs were investigated as well. To investigate the time course of proliferation in presence of PC(12:0/12:0), PC(14:1/14:1)trans or PC(18:3/18:3)allcis respective liposomes were added in concentrations of 0.05, 0.25 or 1 mM liposomal PC and proliferation was compared to untreated controls after 4, 8, 24, 48, 72 or 96 h.

Proliferation was measured using the MTT-assay (Roche, Basel, Switzerland) [23]. Media containing liposomes were removed and cells were washed twice with LB. Cell culture wells were then filled with 100 µl of respective cell culture media without FCS and without phenol red and 10 µl of MTT solution containing 5 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide were added to each well. After 4 h of incubation, cells were killed by adding 100 µl of 10% (w/v) SDS in 0.01 M HCl. Culture plates were kept for 24 h under cultivating conditions to solubilize possible tetrazolium crystals, then adsorption was measured at 500 nm wavelength. All measurements were taken in triplicate.

Each culture plate contained untreated cells, and proliferation was estimated as percent of these untreated controls. Interference with cell proliferation was investigated between 0.01 and 1 mM liposomal PC. When the course of proliferation was measured, proliferation was estimated as percent of untreated cells at t = 0.

2.6. Binding and uptake of liposomes into cells

Cells were cultivated as described and seeded into a 24-well cell culture plate. Isotopic labeled liposomes were added to the cell culture wells to yield a final PC-concentration of 1 mM. Association between liposomes and cells was measured by adding labeled cholesterylhexadecylether, possible PC hydrolysis was investigated by adding radioactive labeled cholesterylhexadecylether as marker for whole particle uptake and I-α-dipalmitoyl-1-^{14}C-phosphatidylcholine as marker for uptake of isolated PC or hydrolysis products thereof. After different time points, the media containing liposomes were aspired and cells washed 3 times with 0.5 ml LB. Then cells were lysed by adding 0.5 ml of 0.5% (w/v) SDS and 0.1% (v/v) Triton-X-100 in 200 mM NaCl.

Radioactivity was determined via a liquid scintillation counter Wallac 1411 (Berthold, Wildbad, Germany) using internal quench correction. The samples (200 µl) were mixed with 3.5 ml of liquid scintillation cocktail (Ultima Gold®, Canberra Packard GmbH, Frankfurt, Germany) and allowed to rest for at least 6 h in the dark before we started to count liquid scintillation.

Protein content of cultured hepatocytes was determined by a modified Lowry procedure [24], using the DC protein determination assay (BioRad Life Science Group, Germany). Bovine serum albumin, dissolved in the solution used for dissolving the cells, was used as a standard.

2.7. Thin layer chromatography of lipids

Cells were cultivated in FCS free medium containing 0.5 mM liposomal PC, and cell culture supernatant was collected after different cultivation periods. Lipids were extracted according to Bligh & Dyer [25]. PC, lyso-PC and free fatty acids separated by TLC on silica coated plates 60F254 (E. Merck, Darmstadt, Germany) with $CHCl_3$/methanol/H_2O (65/25/4) (v/v/v). Phospholipids were visualized by spraying with 1.3% molybdenum blue spray (Sigma-Aldrich) [26]. Oleic acid (Sigma-Aldrich®), 1-palmitoyl-2-hydroxy-*sn*-glycero-phosphocholine (Avanti Polar Lipids) and PC(14:1/14:1)cis were used as references. Oleic acid was determined using 0.1 M $KMnO_4$. Trace amounts of possible hydrolysis products were investigated by adding I-α-dipalmitoyl-1-^{14}C-phosphatidylcholine labeled liposomes to the cells. Spots were scraped off and radioactivity measured by liquid scintillation counting (Wallac 1411, Berthold, Wildbad, Germany).

2.8. Mathematical methods

To compute and visualize data, Slide Write Plus 7.01 (Advanced Graphics Software Inc., Ranco Santa Fe, CA, USA) was used. Mean and s.d. were calculated according to standard equations. IC_{50} values were calculated by fitting a 4 parameter logistic dose response curve (Eq. 1) to the experimental data using the least square method.

$$y = a0 + a1/\left(1 + (x/a2)^3\right) \quad (1)$$

with ai being the coefficients to fit and a2 the parameter corresponding to the IC_{50}.

The confidence intervals (CI) of fitted parameter were calculated according to Eq. (2):

$$CI_i = a_i \pm t(1\text{-}\alpha/2,\ n\text{-}p) * SE_{ai} \quad (2)$$

$$SE_{ai} = \mathrm{sqrt}(MSE * c_i) \quad (3)$$

The respective diagonal c is given by:

$$c = X^T X - \mathbf{1_n} \tag{4}$$

wherein X is the design matrix, X^T the transposed matrix X and $\mathbf{1_n}$ the identity matrix.

MSE is the mean square error given by the error sum of squares (SSE) according to:

$$MSE = SSE/(n-p) \tag{5}$$

with n the number of sample data and p the number of coefficients.

In general 95%-CI is given.

3. Results

3.1. PC interference with cell proliferation

To detect possible PC interference with cell proliferation, the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide was used (MTT-Assay system) [23]. This reaction is directly related to cells' metabolic activity, most likely to the activity of mitochondrial enzymes [27]. Proliferation in the presence of liposomal PC is given as a percentage of proliferation in the absence of liposomal PC. Generally, non-treated cells were used as proliferation control on each assay plate. Since adding liposomes suspended in LB dilutes the cell culture medium, the cell culture media of control experiments were diluted with the same LB volumes (sky-blue diamonds in Fig. 1).

The experiments were conducted with different cell lines from human or mammalian sources. The full panel of investigated lipids was used for CHO and MAT-BIII cells, while only lipids displaying a certain effect on both of those cell lines were investigated further with HUVEC, HL-60, MEF and MES-SA cells (see Table 1). In general, all the cell lines we investigated revealed a rather similar overall tendency with some differences in IC_{50} values for certain PCs. Exemplary, detailed data on the CHO cells' dose response are illustrated in Fig. 1.

The proliferation of CHO cells in the presence of various saturated diacyl-PCs is shown in Fig. 1A. Short chain PC(12:0/12:0) showed elevated toxicity along a broad range of concentrations with an estimated IC_{50} of approximately 190 (90–280) μM. Interestingly, short chain PC(10:0/10:0) interfered significantly less with cell proliferation (IC_{50} of 680 (410–930) μM) in CHO cells. We observed the same on MAT-

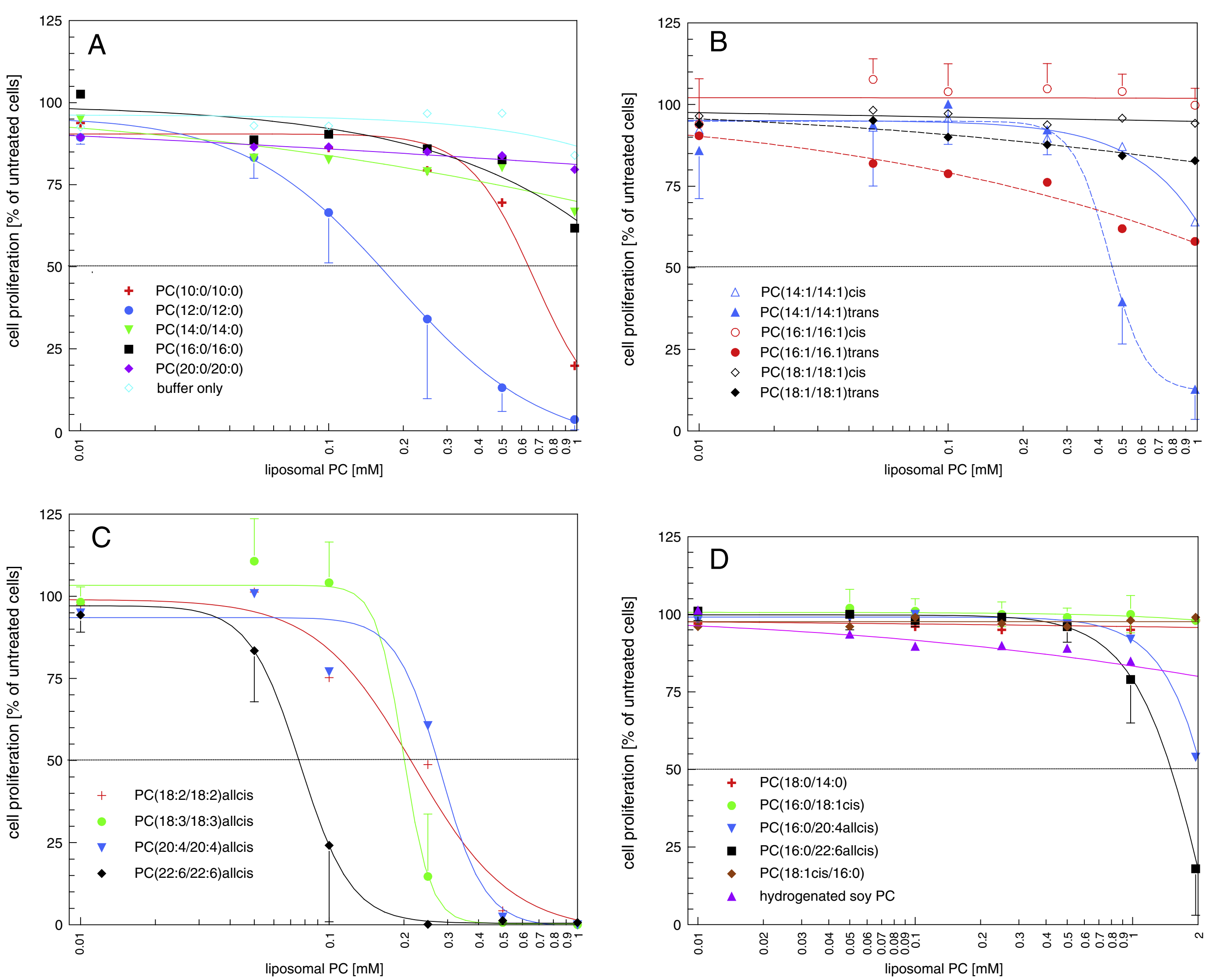

Fig. 1. Interference of PCs with cell proliferation. CHO-K1 cells were grown in the presence of liposomes (70 mol% PC/30 mol% cholesterol) for 96 h at various concentrations. Cell proliferation was measured via MTT-assay and proliferation is given as percent of untreated control (means of 3–6 individual measurements, s.d. is given exemplarily). Where appropriate, a 4-parameter logistic dose–response function was fitted to the data (respective correlation coefficients and 95%-confidence intervals are given in Table 1). FA acyl chains are given in carbon reference systematic. A) Saturated diacyl-PCs, B) monounsaturated diacyl-PCs in Δ9-cis or -trans configuration. C) Polyunsaturated diacyl-PCs (all cis), D) mixed chain PCs (1-acyl/2-acyl).

Table 1
Inhibition of cell proliferation by different diacyl PC. Cells of various cell lines were grown in the presence of liposomes (70 mol% PC/30 mol% cholesterol) for 96 h. Cell proliferation was measured via MTT-assay and proliferation provided relative to untreated controls. Where appropriate, an IC_{50}-concentration was calculated using a 4-parameter logistic dose–response function and respective correlation coefficients are given as r while CI gives the 95%-confidence intervals. When no growth inhibition >50% up to a concentration of 1 mM was detected, respective IC_{50}-value is stated as >1, undetermined values are left empty. All PCs tested were 1,2-diacyl-PC and respective FAs are given in carbon reference systematic.

		PC saturated					PC monounsaturated						PC polyunsaturated			
							cis			trans			all cis			
Cell line		**10:0**	**12:0**	**14:0**	**16:0**	**20:0**	**14:1**	**16:1**	**18:1**	**14:1**	**16:1**	**18:1**	**18:2**	**18:3**	**20:4**	**22:6**
CHO-K1	IC50 [mM]	**0.68**	**0.19**	>1	>1	>1	>1	>1	>1	**0.44**	>1	>1	**0.22**	**0.20**	**0.28**	**0.08**
	CI	0.41–0.93	0.09–0.28							0.30–0.58			0.16–0.28	0.17–0.23	0.21–0.34	0.07–0.09
	r	0.97	0.99							0.98			0.99	0.99	0.98	0.99
MAT-B-III	IC50 [mM]	**0.51**	**0.36**	>1	>1	>1	>1	>1	>1	**0.42**	**0.58**	>1	**0.39**	**0.06**	**0.18**	**0.02**
	CI	0.36–0.67	0.14–0.49							0.19–0.65	0.31–0.85		0.07–0.71	0.04–0.07	0.05–0.32	0.01–0.03
	r	0.99	0.98							0.96	0.99		0.91	0.99	0.99	0.99
HL-60	IC50 [mM]	**0.16**	**0.35**	>1	**0.85**		>1			**0.17**				**0.09**	**0.07**	**0.07**
	CI	0.11–0.21	0.13–0.57		0.40–1.3					0.13–0.22				0.07–0.12	0.06–0.08	0.04–0.11
	r	0.99	0.99		0.99					0.99				0.86	0.99	0.99
MES-SA	IC50 [mM]		**0.26**	>1	>1					**0.55**				**0.10**		
	CI		0.25–0.28							0.46–0.64				0.09–0.11		
	r		0.99							0.98				0.99		
MEF-WT	IC50 [mM]	**0.65**	**1.00**	>1	>1					>1				**0.26**	**0.28**	**0.09**
	CI	0.47–0.83	0.83–1.2											0.23–0.29	0.23–0.33	0.05–0.12
	r	0.99	0.98											0.99	0.99	0.97
HUVEC	IC50 [mM]		**0.18**	>1	>1					**0.13**				**0.08**		
	CI		0.09–0.28							0.05–0.27				0.02–0.13		
	r		0.99							0.98				0.97		

The bold entries are highlighted to identify measured IC50 values.

BIII cells, while the toxicity was roughly identical on HL-60 cells, which appear slightly more susceptible to PC-mediated toxicity in general. Saturated PCs with longer side chains than PC(12:0/12:0) displayed no or minimal interference with cell proliferation. Interestingly, the natural PC(16:0/16:0) always revealed slight inhibition of cell proliferation in all cell lines. Again, HL-60 cells were more susceptible, and we observed an IC_{50} of ~0.9 mM. Stable PC(18:0/18:0)-liposomes were not preparable by the simple extrusion technique used, so we used hydrogenated soy PC (HSPC) as a surrogate for PC(18:0/18:0). According to the manufacturer, HSPC contains ~89% FA(18:0), thus ≥78% of HSPC has to be PC(18:0/18:0). Like all extracts from natural sources investigated in earlier work, HSPC did not demonstrate increased inhibition of cell proliferation. Taken together, there is no clear-cut correlation between toxicity and chain length, but short chain diacyl PCs do reveal elevated toxicity, and PC(16:0/16:0) can interfere with cell proliferation at high concentrations.

All the monounsaturated PCs in cis-configuration we investigated showed little or no interference with cell proliferation, with only PC(14:1/14:1)cis showing a slight inhibition of cell proliferation at 1 mM PC (Fig. 1B). Interestingly, all trans PCs displayed greater interference with proliferation than their respective cis configured counterparts, with toxicity increasing with shorter chain length. This effect was more pronounced with MAT-B-III cells than with CHO cells (Table 1). Interestingly, PC(14:1/14:1)trans showed elevated toxicities at higher concentrations in all the cell lines we investigated (IC_{50} ranging from 130–550 μM).

In contrast to monounsaturated PCs, all diacyl-PCs containing fatty acids with two or more double bonds in sn-1 and sn-2 positions displayed significant cell-proliferation inhibition (Fig. 1C). The long chain ω-3 PC(22:6/22:6) was the most toxic PC investigated, with IC_{50} values well below 100 μM. Again, we cannot claim that there is a general tendency toward increasing toxicity in conjunction with increasing chain length or the number of double bonds. Interestingly, the ω-3 PCs PC(18:3/18:3) and PC(22:6/22:6) revealed greater toxicity than the respective ω-6 PC(20:4/20:4).

Except for PC(16:0/16:0) and PC(18:1/18:1), diacyl-PCs with similar FAs are not common in mammalian cells [3]. To reveal whether the fatty acid position plays a key role in the toxicities observed, we investigated different mixed chain PCs also (Fig. 1D). In these experiments, the maximum concentration was 2 mM liposomal PC in order to have the same amount of a potentially toxic FA as with 1 mM diacyl-PC. These as well as further investigations were done on CHO cells only, since we had observed no general differences between the investigated cell lines as stated above. As expected, PCs with PUFAs in the sn-2 position showed a certain degree of toxicity, but the IC_{50} values were all >1 mM. Most strikingly, interference of PC(16:0/22:6) and PC(16:0/20:4) was not seen at concentrations <1 mM, while the respective diacyl-PCs showed much lower IC_{50} values (~80 μM for PC(22:6/22:6) and ~280 μM for PC(20:4/20:4)). Thus non-natural diacyl-PCs containing PUFAs in the (common) sn-2 position *and* in the sn-1 position show significantly higher toxicity than their natural mixed-chain counterparts that is unrelated to the total amount of PUFAs delivered to the cell. Within natural mixed-chain PCs, the unsaturated PC is usually found in the sn-2 position, but the non-natural additional PUFAs in the sn-1 position seemed to add significantly to the toxicities we observed.

Absolute IC_{50} values depend on cell type and many boundary conditions such as the media being used, cultivation period, cell density, etc. The estimated values are very useful to compare the effects of different PCs within the described set of conditions, but should be considered cautiously when applied to other conditions like in vivo situations. Although absolute IC_{50} values vary, the overall trend is robust and reproducible for all the cell lines we have investigated so far.

3.2. Proliferation time course

Several diacyl-PCs displayed significant cell-proliferation inhibition compared with untreated controls. A more detailed analysis of the time course of cell proliferation was done for PC(12:0/12:0), PC(14:1/14:1)trans and PC(18:3/18:3) with different concentrations (Fig. 2). For these experiments, we regarded the vitality of cells present at $t = 0$ as being 100% vital. The untreated control showed normal growth behavior; the cells grew 2–2.5 folds within the first 24–48 h with growth slowing down afterwards. As expected, all PCs in low concentrations (0.05 mM) demonstrated no or minimal effect on cell proliferation. PCs present at 0.25 mM inhibited cell proliferation significantly, depending on the IC_{50} of the given lipid. When PCs were present in concentrations above their respective IC_{50} (1 mM), all three lipids showed a decline in initial cell vitality, indicating the ability of these PCs to kill a certain amount of cells in the initial population. PC(18:3/18:3) killed the entire cell population within 24 h when present at 1 mM.

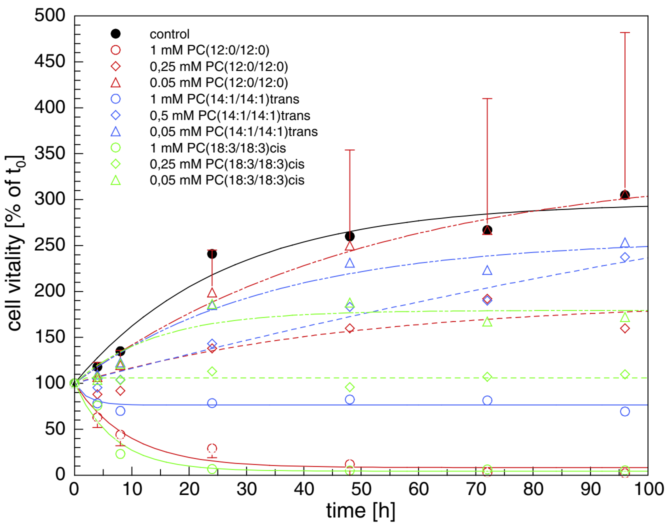

Fig. 2. Cell proliferation in presence of potentially toxic PCs. CHO-K1 cells were grown in the presence of liposomes (70 mol% PC/30 mol% cholesterol). After certain intervals, cell proliferation was measured via MTT-assay. The MTT signal of untreated cells at the beginning of the experiment ($t = 0$) was defined as 100% and proliferation/cell vitality is given as respective percent. Untreated cells are shown in black (control). PC(12:0/12:0) is shown in red, PC(14:1/14:1)trans in blue and PC(18:3/18:3)allcis in green ($n = 3$).

Interestingly, cells seem to be able to compensate for PC effects, since cell vitality drops within the first 10 h after adding PC(14:1/14:1)trans liposomes, remaining constant thereafter. Whether the constant cell viability we observed is a result of growth arrest or a steady state between growing and dying cells cannot be determined by the methods used.

3.3. Uptake

To rule out that the impact of certain PCs on cell proliferation we observed is related to various amounts of liposomal PCs reaching the cells, liposomes were labeled by [^{3}H]cholesterylhexadecylether as a nonexchangeable membrane marker. The amount of cellular liposomal lipid was estimated for PC(12:0/12:0), PC(14:1/14:1)trans and PC(18:3/18:3) as lipids showing elevated toxicity, and PC(14:1/14:1) cis and PC(16:0/16:0) as lipids revealing no or minimal interference with cell proliferation (Fig. 3). To compare lipids in a relevant concentration range, uptake was measured at a concentration of 0.5 mM over a 12 h period. While 0.5 mM is well beyond the IC_{50} of respective toxic lipids in long-term experiments, we observed cell killing only within the first hours at higher concentrations (see Fig. 2). As the significant toxicity of the lipids we investigated appeared within the first hours (see Fig. 2), experiments were not continued for longer than 12 h.

Cellular uptake followed a typical endocytosis pattern revealing linear initial uptake and saturation over time. As Fig. 4 illustrates, we observed no general differences in cellular uptake between these PCs. Uptake of the more toxic PCs PC(12:0/12:0) and PC(18:3/18:3) occurred slightly more slowly than uptake of the less toxic PCs. The varying toxicities we observed are thus not a result of the toxic liposomes' higher rates of uptake.

Brügger et al. reported a total amount of ~21 pmol PC per 1000 CHO cells. In our hands 1000 CHO cells had a protein content of ~0.37 µg. Thus, a total uptake of about 10 pmol PC/µg cellular protein after 24 h (see Fig. 3) converts to 3.7 pmol PC/1000 cells. According to this, the liposomal PC taken up by the cells corresponds to about 18% of the total cellular PC, as a rough estimate.

3.4. Contribution of lysolipids

Degradation of PCs yields lyso-PC and FFA. Both are well known to be toxic to cells. The elevated toxicities of certain PCs might be related to lysolipid toxicity, while the PCs themselves exhibit no elevated interference with cell proliferation. To rule out this hypothesis, we subjected cell culture supernatant to TLC (Fig. 4A). No lysolipid was seen for PC(18:3/18:3), PC(14:1/14:1)trans and PC(16:0/16:0) after up to 36 h of observation. To detect trace amounts of lysolipid, we added di-[^{14}C] palmitoyl-phosphatidylcholine to some liposomes. Again, no lysolipid was detected in the supernatant (Fig. 5B). Thus there is no accumulation of lysolipid (and FFA) in the cell culture supernatant, leading to interference with cell proliferation. Lysolipids and FFAs are readily taken up by cells [9]. Lysolipid products might not accumulate in cell culture supernatant, but they may be taken up by the cells in significant amounts to reveal toxicity. To rule out this mechanism, liposomes carrying di-[^{14}C]palmitoyl-phosphatidylcholine and [^{3}H]cholesterylhexadecylether were added to cells and the amounts of cell-associated radioactivity measured (Fig. 3B). In case of

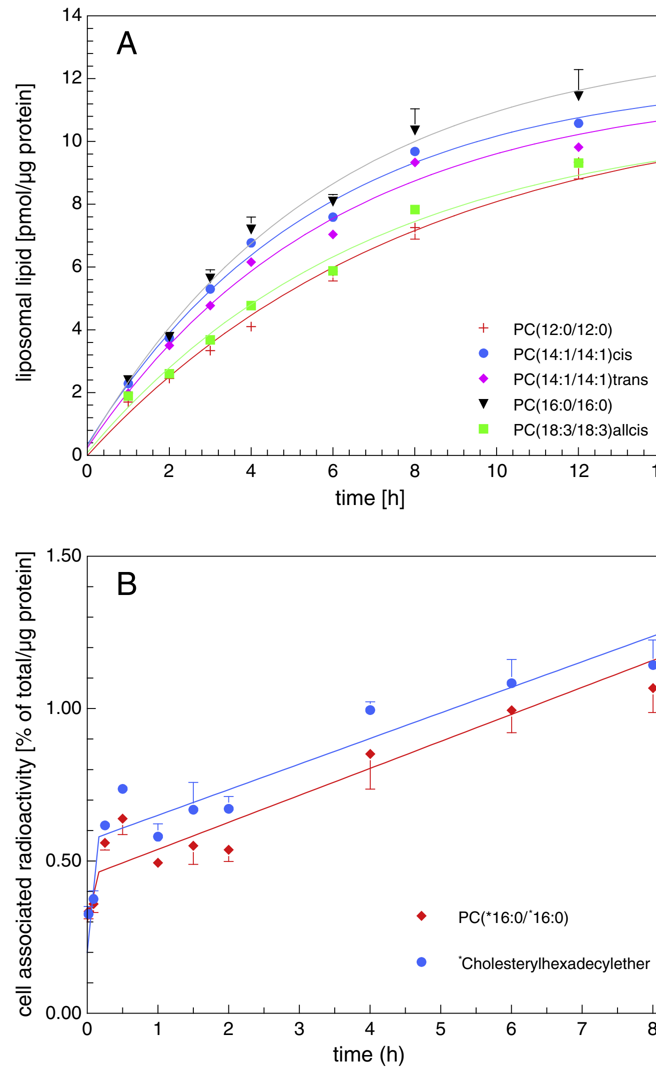

Fig. 3. Uptake of liposomal lipid. CHO-K1 cells were grown in the presence of radioactively labeled liposomes (70 mol% PC/30 mol% cholesterol). A) Liposomes were marked with [1,2-^{3}H]cholesterylhexadecylether and specific radioactivities of liposomal suspensions (counts/µmol PC) were determined. Cell associated liposomal lipid was calculated based on the amount of radioactivity associated with the cells (mean and exemplary s.d. of $n = 3$). B) Liposomes were marked with l-α-dipalmitoyl-1-^{14}C-phosphatidylcholine PC(16:0*/16:0*) as a marker that can be hydrolyzed and taken up as FFA and lyso-PC as well as [1,2-^{3}H]cholesterylhexadecylether as an uncleavable liposome marker. Uptake of both marker lipids is given as percentage of respective total radioactivity present in the cell culture supernatant. PC(18:3/18:3)allcis is given as example for potentially toxic PCs ($n = 3$, mean and s.d.).

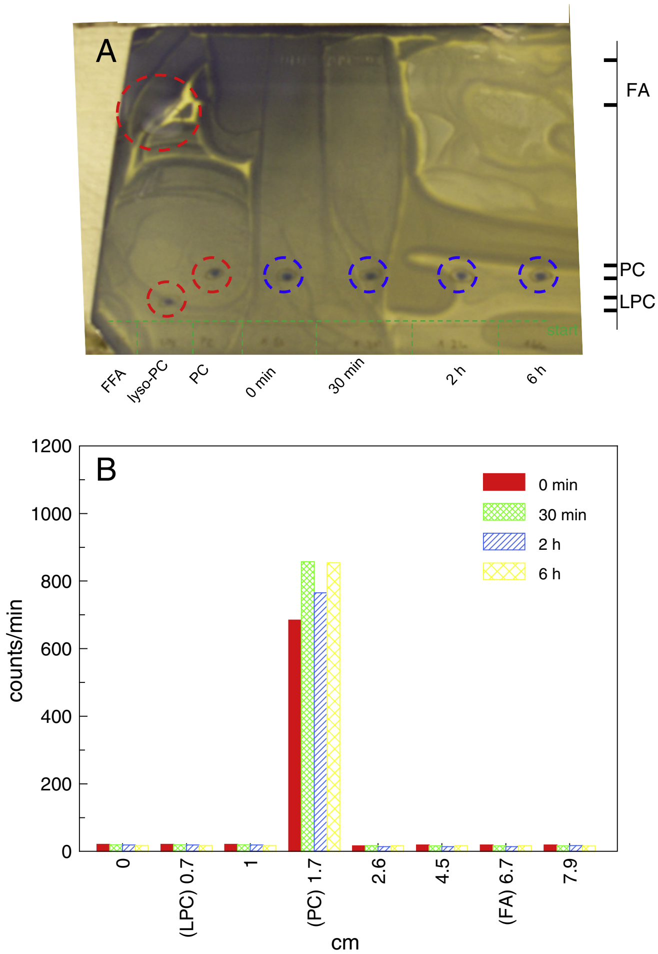

Fig. 4. Liposomal lipids in cell culture supernatant. CHO-K1 cells were grown in the presence of liposomes (70 mol% PC/30 mol% cholesterol). After certain times supernatant was aspired and analyzed for PC, lyso-PC and FFA. A) TLC of supernatant, spots visualized by molybdenum blue spray. References: PC(14:1/14:1)cis, LPC(16:0) and FA(18:1)cis. B) TLC of liposomes radioactive labeled by PC($16:0^*/16:0^*$). After drying, respective areas were scraped off and subjected to liquid scintillation counting.

accelerated lysolipid/FFA production from added liposomes and the cells' uptake of these degradation products, the amount of radioactivity related to the –[^{14}C]acyl chains should be much higher than that of cell-associated radioactivity related to the liposome membrane marker [^{3}H]cholesterylhexadecylether. As shown in Fig. 3B, we observed no significant differences in markers associated with the cells, indicating no significant uptake of PC-degradation products. Thus, the differences observed in cell proliferation and toxicity are not mediated by the degradation of respective PCs into lysolipids and FFA.

4. Discussion

PCs are a group of substances chemically characterized by their choline headgroup linked via a phosphodiester to glycerol. Two varying acyl chains are esterified in sn-1- and 2-positions of the glycerol moiety, creating many different species. PCs were initially characterized from natural extracts like egg or soy PC. Not surprisingly, these PC mixtures proved to be non-toxic in vitro [5] and in vivo [28,7]. Over the years, more and more synthetic PCs became available. Interestingly, PCs still are generally regarded as non-toxic even though these synthetic components have never undergone systematic assessment. We used neutral liposomes with a fixed content of cholesterol and a single PC species. These liposomes were taken up by the cells in nearly equal amounts, irrespective of the differences in FA acyl-chain composition (Fig. 3). In contrast to FFAs revealing varying bioavailability due to different binding and uptake characteristics, the liposomal approach enabled us to directly compare the effects of PCs with different FAs on cell proliferation. Except for the (non-toxic) PC(16:0/16:0), PC(16:1/16:1) and PC(18:1/18:1), diacyl-PCs are not common in nature, neither in yeast [2] nor mammalian cells [3]. Natural extracts like soy or egg PC are already known to be non-toxic, and the mixed-chain PCs investigated in this project showed no significant inhibition of cell proliferation as well, thereby supporting the notion that PCs from natural extracts are non-toxic in general. These efforts thus focused on synthetic diacyl-PCs to elucidate the effects of a single species of FA component and possibly identify a certain subset of toxic PCs to possibly create a toxic carrier for toxic drugs.

A small set of diacyl-PCs displayed significant toxicity to different cell lines, while most other PCs did not interfere with cell proliferation at concentrations up to 2 mM. This work investigated interference with cell proliferation with different mammalian and human cell lines. All the PCs revealing proliferation-inhibiting potential displayed the same overall effect on all the cell lines we investigated, with slight variations in IC_{50} values (Table 1). Interestingly, we observed few general rules concerning structure–toxicity relationships. For saturated PCs, short chain diacyl-PCs PC(12:0/12:0) and PC(10:0/10:0) interfered significantly with cell proliferation, while PC(14:0/14:0) had no effect. Interestingly, the natural PC(16:0/16:0) always demonstrated slight cell-proliferation inhibition at concentrations ≥0.5 mM, and we estimated an IC_{50} of ~0.9 mM for HL-60 cells. Probably, natural PC(16:0/16:0) is readily incorporated into cellular membranes, thereby decreasing fluidity, while larger saturated PCs tend to be metabolized. Fatty acids function as a natural defense against bacteria in several species including humans [12], and among saturated FAs, FA(12:0) showed remarkably high antibacterial activity [29]. Membrane leakage, disrupted protein–membrane interaction and disrupted oxidative phosphorylation are currently being considered as antibacterial mechanisms [12].

Monounsaturated FAs in PCs from natural sources are usually cis-configured. During our experiments, only PC(14:1/14:1)cis displayed a slight inhibition of proliferation at higher concentrations. Not surprisingly, the natural cellular diacyl-PCs PC(16:1/16:1)cis and PC(18:1/18:1)cis revealed no toxicity. In contrast, monounsaturated diacyl-PCs in trans-configuration were all significantly more toxic than their respective cis-diastereomers. Again, there is a tendency to higher toxicity in conjunction with shorter chain length. Trans-FAs have been characterized as a nutritional health hazard for years [20], but unfortunately there have been no systematic investigations on the cellular level. Zapolska-Dovnar et al. demonstrated apoptosis induced by FA(18:1)trans and FA(18:2)trans, but the percentage of cells in apoptosis was low even when 5 mM were used [30]. Trans-FAs possess membrane properties that are more similar to their unsaturated parent than to their cis-configured sister [31]. Since PC(14:0) revealed no interference with cell proliferation, perturbations in membrane properties by PC(14:1/14:1)trans are unlikely to explain the toxicity we observed. N-myristoyltransferase (NMT) is an essential enzyme for cellular proliferation, and NMT inhibition has been discussed as an anticancer-therapy option [32]. FA(14:1)trans showed similar K_M and V_{max} as FA(14:0) itself, while FA(14:1)cis had higher K_M and significantly lower V_{max} than PC(14:1/14:1)trans. Calculating relative specificity constants K_{Srel} ($=V_{max}/K_M$) from this data, K_{Srel} for FA(14:1)trans is about 72% of K_{Srel} for FA(14:0), while K_{Srel} for FA(14:1)cis is only ~9% of K_{Srel} of FA(14:0). The resulting peptides of the monounsaturated FAs were less hydrophobic than the peptides properly coupled to FA(14:0) [33]. A disturbance of myristylation patterns and thereby protein sorting and function may underlie the PC interferences we observed for PC(14:1/14:1). Interestingly, FA(12:0) and FA(10:0) are also potential substrates of NMT, with FA(12:0) showing a K_{Srel} of 120%, even higher than the “proper” substrate FA(14:0) [33]. Interference with NMT may also contribute to the PC(12:0/12:0) toxicities we noted. High concentrations of trans-FA-PCs reduce proliferation compared to the cis-configured counterparts, at least regarding the diacyl-PCs investigated in this project. Whether the same holds true for trans-FFAs or trans-FAs in nutritional triglycerides cannot be concluded by our findings, since metabolism and uptake are different.

All the polyunsaturated diacyl-PCs we investigated showed elevated interference with cell proliferation, and polyunsaturated diacyl-PCs were the most toxic PCs observed. Interestingly, PC(18:2)cis already revealed markedly increased toxicity, while PC(18:1/18:1)cis demonstrated no interference at all. However, FFA(18:2)trans displayed only moderate toxicity on HUVEC cells [30], probably because the trans isomer leads to similar membrane structures as in FA(18:0), while the divenyl-methane structures of FA(18:2)cis led to the membrane perturbations common for PUFAs. Despite PC(22:6/22:6) being the most toxic PC we investigated, we detected no general rule concerning chain length or number of double bonds. As ω-3 FAs show anti-inflammatory effects, they are often considered beneficial [34], while pro-inflammatory ω-6 FAs are often regarded as unhealthy, although debate continues [19]. On the cellular level regarding PCs, we observed no differences between the toxicities of ω-6 and ω-3 configured diacyl-PCs. More likely, any observed differences between these species arise on a systemic level, where ω-6 and ω-3 FAs oppose each other's pro- and anti-inflammatory effects.

PUFAs have been investigated as anticancer agents, and IC_{50} values between 1 and 10 μM have been observed on Mia-Pa-Ca-2 cells and HL-60 cells [13], with a slight increase in toxicity with increasing numbers of double bonds. On MCF-7 cells, 50% inhibition of cell growth was not attained at 30 μM for all the PUFAs we investigated except for FA(22:6). FA(18:2) demonstrated no inhibition whatsoever. During these experiments, media were supplemented with fetal bovine serum, albumin and FA(18:1), thereby possibly counteracting certain toxicities [14]. In this investigation, PC-liposomes were used as well, and the PCs showed effects similar to those of the corresponding FFAs. On Jurkat cells, 50% growth inhibition by PUFAs was observed around approx. 50 μM or less while FA(18:1) showed no inhibition [15]. Taken together, PUFAs and their respective non-physiological diacyl-PCs reveal similar toxic potential, although they may differ in uptake routes and the mechanisms leading to cell death.

PUFAs are much more susceptible to oxidation than other FAs, and oxidized FAs are closely associated with oxidative stress [35]. PUFAs have been reported to cause apoptotic cell death presumably via oxidative stress [13], and enhanced oxidative stress may likely contribute to the toxicities of respective PCs we observed. Mixed chain PCs containing PUFAs showed no or minimal interference with cell proliferation (Fig. 1D), even when the same amounts of FAs were present. Natural mixtures usually contain mixed-chain PCs for the most part, and these findings are in line with an earlier examination [5] in which these mixtures' compatibility with cell proliferation was already proven. Since these mixed-chain PCs are much less toxic than their synthetic diacyl-PC counterparts even when the same amounts of PUFAs are delivered to the cells, oxidative stress caused by delivered PUFAs cannot be the sole cause of the toxicities we observed. Natural mixed-chain PCs contain only a single polyunsaturated FA usually in sn-2 position with a saturated FA in sn-1 position [36], and PUFAs in non-natural sn-1-position seem to be a source of elevated toxicity. Possibly, natural mechanisms (such as defense mechanisms against PUFAs' oxidative modification) are less well-equipped to deal with oxidized PUFAs in the sn-1 position. Substantial differences in biophysical membrane properties were recorded between ω-6 PC(16:0/20:4) and ω-3 PC(16:0/22:6) [37], but the similar toxicities we observed cannot be attributed to these differences. In this study, a model was proposed displaying the tip of the bulky PUFA in sn-2 position extending into the intermembrane space beyond the end of the FA(16:0) in sn1-position, thereby containing a PC's overall cylindrical shape. With regard to a second PUFA in sn-1 position, such a configuration is no longer possible, probably leading to a more conical shape for the diacyl-PC's and thereby to alterations in membrane integrity.

PCs might be cloven into FFAs and lysolipid, and both components are known to be toxic. Within our study's time span, we detected no cleavage of liposomal PCs outside the cells. Thus the effects on proliferation we observed are mediated by the uptake of given liposomal PCs, but once within the cells, the PCs might be cloven into respective FFAs and lysolipids. There is evidence that LPC(18:0) inhibits growth on different cell lines with IC_{50}-values between 120 and 180 μM [38]. Reported LPC(18:0) toxicity contrasts with that of long-chain saturated PCs, which did not display elevated growth inhibition in our experimental setting. Furthermore, LPC(17:0) is known to be effective against metastasis by altering the surface structure of cancer cells in vivo [9]. No toxicity was reported in vitro for LPC(17:0) and LPC(16:0) on cancer cells even though these LPC were administered repeatedly over several days at a concentration up to 450 μM. As with FFA, experimental conditions such as albumin concentration may affect observed toxicities.

Liposomes are readily taken up by cells primarily via endocytosis, while the cellular uptake of phospholipids is more likely obtained via specific lipid transfer under physiological conditions [18]. Since endocytosis does not seem to distinguish between different liposomal PCs, this entry route probably circumvents cellular regulation of PC uptake. As a rough estimate, about 18% of our cellular PC was taken up by the cells within 24 h. With regard to just one non-natural diacyl-PC species, this is a fairly high amount. Interestingly, a similar amount of incorporated FFA into MCF-7 cells has been measured for FFA [14]. Unspecified endocytosis of liposomal PC may be a sort of “back-door” entry for PC that may lead to lipid overload which can be managed with natural mixed-chain PCs and most diacyl-PCs, but certain non-physiological diacyl-PCs pose a toxic threat to the cells.

Once taken up, PCs are used either as membrane building blocks without modification or they are metabolized. Headgroup exchange is a common route of metabolizing PCs, generating phosphatidylserine (PS) and phosphatidylethanolamine (PE). Both components play a crucial role in membrane asymmetry [39]. A disturbance of these compounds' function may contribute to the observed effects as well. Interestingly, remarkable differences between PC, PS and PE occur regarding FA acyl chain composition, leading to the assumption that different routes of lipid homeostasis must exist for PC, PS and PE [36]. With a fairly high amount of a single PC species entering the cells, PS and PE homeostasis might be disturbed as well.

Since there is no obvious rule concerning toxicity, different mechanisms might be behind the interferences with cell proliferation that we observed in conjunction with some PCs. So far, these underlying mechanisms are a matter of speculation, and more detailed investigation will be the focus of further work.

The hypothesis of using FAs against cancer was developed decades ago [40], and several studies showed PUFAs' beneficial effects against cancer [13,14]. The use of hybrid liposomes consisting of essential non-toxic PC(14:0) and PUFAs is a means of combining liposomal delivery technique with FAs' antitumor efficiency [16]. Another way to combine these techniques is to employ certain toxic diacyl-PCs as suggested by the data presented in this work. Essential non-toxic PCs like HSPC have been used in liposomal anticancer drug delivery for years. The toxic diacyl-PCs we have identified could perhaps be used to construct a toxic shell, enhancing the impact of a transported cytostatic drug. Targeted drug delivery and anticancer properties would thereby be combined within a single vehicle by choosing the appropriate PC.

Chemotherapeutic agents show IC_{50} concentrations in the μM range, e.g. doxorubicin has an IC_{50} value around 1 μM, vincristine around 4.6 μM [41]. IC_{50} values for the most “toxic” diacyl-PCs PC(22:6/22:6), PC(20:4/20:4) and PC(18:3/18:3) were well below 100 μM, revealing some toxic potential. On the other hand, the same toxicity pattern has been observed on all cell lines investigated so far, and generally high toxicity may create a narrow therapeutic window for a given “toxic” carrier. A moderately-toxic lipid like PC(12:0/12:0) or PC(14:1/14:1) would probably be more appropriate for constructing a toxic shell for chemotherapeutic agents. In terms of pegylated liposomal doxorubicin, the lipid concentration in tumor interstitium might be as high as 1 mM (for a rough estimation, see supplement), and adding a second constraint on cell proliferation via toxic PCs might present a realistic option. Several other aspects of drug delivery beyond synergistic toxicities, such as systemic toxicity, drug entrapment, shelf life, etc. must be taken into account to enable successful in vivo drug delivery. We maintain that it might prove worthwhile to assess some of the PCs that potentially interfere with cell proliferation for improved drug delivery.

5. Conclusion

Most of the diacyl PCs we investigated revealed no elevated interference with cell proliferation at concentrations up to 1 mM. The same was true regarding all the mixed-chain PCs we investigated. Our data thus support the notion that most PCs are safe excipients. Certain non-natural diacyl-PCs, however, do display marked interference with cell proliferation, namely all non-physiological diacyl-PCs containing PUFAs and short-chain saturated PCs, as well as trans-configured mono-unsaturated PCs. Since there are no evident comprehensive rules concerning FA structure, these PCs might perform via different mechanisms. These “black sheep” deserve further investigation to explore the potential of constructing a “toxic carrier for toxic drugs”.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.04.010.

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