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Effect of nucleoside analogues and oligonucleotides on hydrolysis of liposomal phospholipids

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Abstract

The hydrolysis of the bilayer forming phospholipids resulting first of all in lysophospholipids and fatty acids is one limiting factor determining the shelf-life of liposomes. In several studies the influence of pH, buffer, lipid composition and other parameters on the hydrolysis of phospholipids have been demonstrated, but the influence of drugs has not yet been investigated systematically. In this study the influence of nucleoside analogues, especially 2′,2′-difluoro 2′-deoxycytidine (gemcitabine, dFdC) on the degradation of phospholipids was elucidated in more detail. It could be demonstrated that the interaction of dFdC with phospholipid bilayers promotes the hydrolysis of phospholipids in a concentration-dependent manner. Obviously two parts of the molecule, the amino group bound to the pyrimidine moiety and the 2′,2′-difluoro-2′-deoxyribose, seem to be responsible for the forced phospholipid hydrolysis. The dFdC-induced hydrolysis of phospholipids was influenced by pH, buffer, lipid composition and different anions. Optimization of the above parameters resulted in prolonged shelf-life of dFdC liposome dispersions, which is an important prerequisite for clinical practice.

Keywords: Hydrolysis; Lysophospholipid; Liposome; Nucleoside analogue; Gemcitabine

1. Introduction

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdC), 1-β-D-arabinofuranosylcytosine (Ara-C) and 5-fluorouridinedeoxyribose (FUdR) are cytotoxic nucleoside analogues used for anticancer therapy of solid tumors and leukemia (Schwendener et al., 1987; Heinemann et al., 1988, 1992; Hertel et al., 1990; Abbruzzese et al., 1991; Chu et al., 1993; Lund et al., 1993; Zhang et al., 1993; Kaye, 1994). Due to their short plasma half-lives,

Abbreviations: Ara-C, 1-β-D-arabinofuranosylcytosine; CHOL, cholesterol; dFdC, gemcitabine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSPC, distearoyl-phosphatidylcholine; EPC-3, hydrogenated egg phosphatidylcholine; FudR, 5-fluorouridine-deoxyribose; PLA_2, phospholipase A_2.

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the drugs have to be applied in high doses to guarantee sufficient plasma levels for cytotoxic efficacy over a sufficient time period. In order to protect the nucleoside analogues from rapid metabolic degradation and to enhance the efficiency of nucleoside analogues in anticancer therapy, there are efforts to entrap these drugs into liposomes as drug delivery systems for sustained release (Schwendener et al., 1987; Allen et al., 1992; van Borssum Waalkes et al., 1993; Moog et al., 1998a,b).

A long shelf-life of a liposomal formulation is a prerequisite for a broad use in clinical practice and is reduced by lipid hydrolysis. The products of lipid hydrolysis during the storage of liposomes are lysophospholipids, fatty acids and glycerophosphate. They may lead to an increased leakage of entrapped drugs (Inoue and Kitagawa, 1974; Grit and Crommelin, 1992), to an increase in particle size due to aggregation (Petersen and Chan, 1978) or a decrease (Zuidam et al., 1995) and to a change of the tolerability of these formulations. It has been demonstrated that a high concentration of lysophospholipids enhances RES depression and increases the acute toxicity of liposomes in a dose dependent manner (Lutz et al., 1995). In several studies the influence of buffer and lipid composition, charge, particle size, pH and ionic strength on the stability of liposomes in terms of hydrolytic degradation of the phospholipids has been demonstrated (Piraube et al., 1988; Grit and Crommelin, 1992, 1993; Grit et al., 1993a,b; Zuidam et al., 1995). Drugs might also increase the hydrolysis rate of phospholipids. Their influence, however, has not yet been investigated systematically to our knowledge.

To develop liposomal formulations of cytotoxic nucleoside analogues for anticancer therapy and of oligonucleotides for gene therapy, which should show long-term stability, we investigated the influence of dFdC, Ara-C, FUdR and an oligonucleotide on the stability of different lipids and lipid compositions in extruded, small unilamellar vesicle (SUV) and multilamellar vesicle (MLV) dispersions. The hydrolysis of lipids in different buffer systems, at different pH and under the influence of different drugs was assessed in stress tests by aging the formulations artificially. At a defined temperature above the phase transition temperatures of all tested lipids (60°C) lipid hydrolysis was accelerated for several hours. On the basis of these investigations optimal conditions for a liposomal dFdC formulation could be established.

2. Material and methods

2.1. Materials

Distearoylphosphatidylcholine (DSPC) and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Sygena, Liestal (Switzerland). Hydrogenated egg phosphatidylcholine (EPC-3) was a kind gift of Lipoid, Ludwigshafen (Germany). Lysophosphatidylcholine was prepared in our group by hydrolysis of DPPC by Phospholipase A_2 (PLA_2) from Naja mozambique mozambique, which was purchased from Sigma, Deisenhofen (Germany). Cholesterol (CHOL) and 5-fluorouridinedeoxyribose (FUdR) were obtained from Fluka, Neu-Ulm (Germany). The hydrochloric salt of gemcitabine (dFdC) was a kind gift of Eli Lilly, Indianapolis, (USA). The hydrochloric salt of 1-β-D-arabinofuranosylcytosine (Ara-C) and cytosine were purchased from Sigma, Deisenhofen (Germany). The oligonucleotide GTACGTCACTCAGTCACG was synthesized by Birsner & Grob Biotech GmbH, Denzlingen (Germany). All other reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of the liposome dispersions

The lipid composition consisted of EPC-3, EPC-3/CHOL (60/40 molar ratio) or DSPC/DPPG/CHOL (55/10/35 molar ratio). Total lipid concentration was 80 mM. Liposomes were prepared by the thin film method of Szoka and Papahadjopoulos (1980). The lipids of each liposome composition were dissolved in ethanol at 60–70°C in a round bottom flask. The solvent was removed under vacuum by rotary evaporation. The lipid film obtained was hydrated by adding the respective buffer at 60°C. The lipo-

somes were formed either by hand-shaking for 10 min or by hand-shaking and subsequent extrusion through polycarbonate membranes of different pore size (first 0.4 μm, followed by 0.1 μm, 11 cycles each) using Avestin LiposoFast, Ottawa (Canada). Although no investigations of liposome size and lamellarity have been performed, the resulting liposomes are called multilamellar vesicles (MLV) and small unilamellar vesicles (SUV), respectively. To investigate the influence of cytosine, dFdC, Ara-C and FUdR on the hydrolysis of phospholipids each substance was added in raising concentrations from 20 to 80 mM, the oligonucleotide in raising concentrations from 3.3 to 13.3 mM and was compared with the hydrolysis in no drug containing dispersions. The pH value of the dispersions was measured and adjusted, if necessary. For calorimetrical studies liposomes made of DSPC (1 mg/ml) in 10 mM HEPES buffer containing 150 mM NaCl, pH 7.4 were prepared by extrusion as described above.

2.2.2. Accelerated hydrolysis by aging

In our study the liposome dispersions were aged artificially by incubation at 60°C in a constant temperature water bath for 66 h in order to accelerate phospholipid hydrolysis. For investigation of the pH with best phospholipid stability in the presence of dFdC, the incubation time at 60°C was 15 h.

2.2.3. Analytical methods

2.2.3.1. HPTLC. HPTLC plates consisted of silicagel 60, 100 × 200 mm, obtained from Merck, Darmstadt (Germany). For chromatography, the HPTLC plates were prerun with the mobile phase $CHCl_3$/MeOH/triethylamine/water (30:35:34:8, v/v) (Kötting et al., 1992). After incubation the liposome dispersions were diluted with 2-propanol to a final phospholipid concentration of 1 mM. In order to remove the buffer salts, which were not soluble in 2-propanol, the vials were centrifuged for 5 min (centrifuge JH2, Beckman, Munich, Germany) at 4000 rpm and 4°C. Calibration was performed by applying standard solutions of lysophosphatidylcholine in 2-propanol in the range of 0.025–0.5 nmol/μl (corresponding to 0.375–7.5 nmol lyso-PC). Fifteen microlitres of samples and calibration solutions, respectively, were streaked automatically to the silicagel plates with a Linomat IV (Camag, Berlin, Germany). The samples were applied as lines of 5 mm length at 10 mm from the lower edge of the plate. The distance to the next sample was 5 mm. After evaporation of the solvent the plates were developed in glass tanks with the mobile phase over a distance of 8 cm.

After development, the plates were dried at 180°C on a heating plate (Thermoplate S, Desaga, Heidelberg, Germany) for 10 min. In order to visualize the lipids by staining the HPTLC plate was dipped for 15 s in a dye solution consisting of cupric sulfate (10%, w/v) in phosphoric acid (8% w/v) (Kötting et al., 1992) and dried on the heating plate at 110°C for 2 min followed by 10 min at 180°C.

The amount of lysophosphatidylcholine formed was quantified densitometrically at 660 nm (Personal Densitometer, Molecular Dynamics). The analyses were carried out three times each. Limit of detection was 0.225 nmol per sample, limit of quantitation was 0.375 nmol as determined by the method of Shah et al. (1992).

2.2.3.2. Differential scanning calorimetry. Calorimetric data were obtained using MicroCal scanninig calorimeter Type MC-2D (MicroCal Inc., USA-MA-Northampton). Cell volume was 1.12 ml (Skalko et al., 1996). The reference consisted of HEPES buffer (10 mM isotonated with 150 mM NaCl), pH 7.4. Samples were scanned in the temperature range 10–95°C at a heating rate of 1 K/min. At least five consecutive runs were performed for each sample. The data were evaluated using Origin Vers. 1.16 software (MicroCal Inc., USA-MA-Northampton).

3. Results and discussion

In the course of developing a liposomal dFdC formulation we observed an unexpected fast hydrolysis of phosphatidylcholine (PC) in the presence of dFdC (data not shown). This

phenomenon caused a dramatic shortening of the shelf-life of the liposomes and needed to be investigated in more detail.

The hydrolysis of the first of the two fatty acid ester bonds of phosphatidylcholine (PC) yield in lyso-phosphatidylcholine (lyso-PC) and fatty acid. For this study, it was important to be able to quantify PC-hydrolysis at minimal extent. Because the expected decrease in PC content due to hydrolysis is too small, we decided not to quantify intact PC but the first hydrolysis product lyso-PC. This study design bears the risk to underestimate hydrolysis rates because lyso-PC itself is an intermediate which further degrades into glycerophosphocholine and a second fatty acid. This second hydrolysis step, however, is, at least in the beginning of the hydrolytic degradation of PC, of minor importance only, thus resulting in an accumulation of the intermediate degradation product lyso-PC. As it was not intended to follow the hydrolysis beyond its initial phase, and to use it merely for comparison purposes, the quantitation of lyso-PC appeared satisfactory for the aims of this study.

The hydrolysis rate of PC is dependent on the temperature. An artificial aging of liposomes at elevated temperatures leads to an accelerated degradation of the phospholipids. This fact can be used to make a prediction on the long-term stability of PC by incubation of the respective formulation at increased temperatures for a few hours (Grit et al., 1993a).

3.1. Influence of dFdC on PC-hydrolysis

Extruded EPC-3/CHOL liposomes containing dFdC in phosphate buffered solution (150 mM of Na_2HPO_4/KH_2PO_4) at pH 7.3 were aged artificially at 60°C and lysoPC was determined. We found that the formation of lysoPC was accelerated by dFdC concentrations of 40 mM and above and increased nearly linearly with rising dFdC concentrations (Fig. 1). It was therefore concluded that dFdC acts as a concentration-dependent promoter for PC hydrolysis in this case at least above dFdC concentrations of 40 mM.

3.2. Influence of liposome characteristics on dFdC promoted hydrolysis of phospholipids

3.2.1. Influence of cholesterol

In EPC-3 liposomes without cholesterol lysoPC reached the same concentration as was determined for EPC-3/CHOL liposomes (Fig. 1) at all corresponding dFdC concentrations. Thus, the dFdC-promoted hydrolysis of PC was not influenced by cholesterol. This result correlates with studies from other groups, that demonstrated that cholesterol as a part of the liposome bilayer has no effect on the hydrolysis rate of phospholipids (Zuidam et al., 1995) of empty liposomes.

3.2.2. Influence of the type of liposomes

Hitherto our experiments were performed using extruded liposomes with a diameter of approximately 100 nm. To assess whether the accelerated degradation of PC was influenced by the size and lamellarity of the liposome dispersion, the experiments, which were performed for investigating the influence of dFdC, were repeated with hand-shaken EPC-3/CHOL-liposomes (MLVs) in phosphate buffer. Again the hydrolysis rate was dependent on the dFdC concentration and was of similar extent as compared to the extruded vesicles (Fig. 2). Thus, in order to simplify the following experiments, it was possible to use

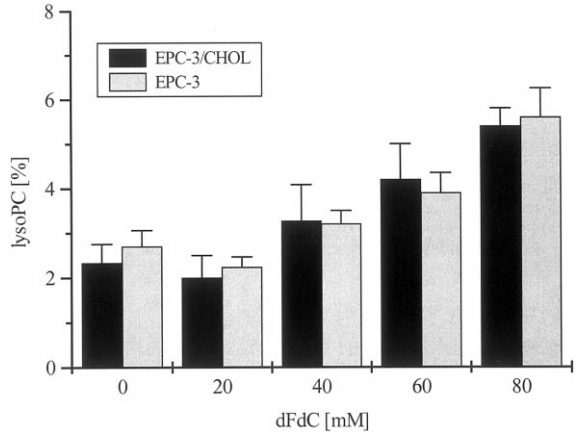

Fig. 1. Influence of dFdC on the hydrolysis of PC at 60°C in EPC liposomes with and without cholesterol in phosphate buffer at pH 7.3. Values represent mean ± SD ($n = 3$).

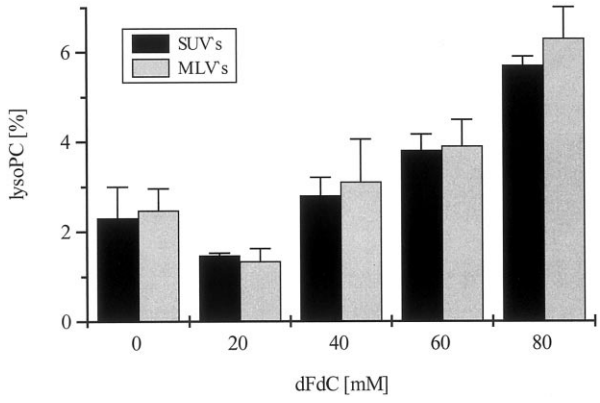

Fig. 2. Comparison of the hydrolysis of PC at 60°C in extruded EPC-3/CHOL-SUVs and non-extruded EPC-3/CHOL-MLVs with rising dFdC concentrations in phosphate buffer pH 7.3. Values represent mean ± SD ($n = 3$).

non-extruded liposomes for investigating the influence of pH and buffer species.

3.2.3. Influence of surface charge of liposomes

The different lipid compositions EPC-3/CHOL or DSPC/DPPG/CHOL were used for preparing liposome dispersions containing dFdC at increasing concentrations. We could confirm the results of Grit and Crommelin (1993), who demonstrated that, in general, the hydrolysis rate of PG was higher than the hydrolysis rate of PC under the same conditions. Furthermore, in 150 mM phosphate buffer (pH 7.3) the hydrolysis rate of DSPC/DPPG/CHOL slightly exceeded that of EPC-3/CHOL, especially at high dFdC concentrations (Table 1). This might be a fact of negative surface charge on the DSPC/DPPG/CHOL liposomes, which on the one hand influences the stability of the phospholipids as described by Grit and Crommelin (1993) and on the other hand might lead to a higher association of dFdC with the surface of the liposomes (see below).

3.3. Influence of the medium on dFdC promoted hydrolysis of phospholipids

3.3.1. Influence of pH value

As described in the literature, the hydrolysis of phospholipids is increased by hydrogen as well as hydroxyl ions (Grit et al., 1993a). In order to find

Table 1
dFdC promoted hydrolysis of PC in EPC-3/CHOL or DSPC/DPPG/CHOL liposomes, respectively, in phosphate buffered dispersion: compared to EPC-3 the degradation of DSPC was enhanced in all dispersions[a]

dFdC (nM)	lysoPC (%) in EPC-3/CHOL liposomes	lysoPC (%) in DSPC/DPPG/CHOL liposomes
0	2.3 ± 0.7	2.3 ± 0.2
20	2.0 ± 0.4	3.0 ± 0.8
40	3.3 ± 0.6	4.5 ± 0.4
60	4.2 ± 0.5	5.3 ± 0.5
80	5.4 ± 0.3	6.9 ± 0.6

[a] Again, the degradation of PC increased with increasing dFdC concentrations. Values represent mean ± SD ($n = 3$).

the pH yielding the highest phospholipid stability in the presence of dFdC, we investigated MLV dispersions of EPC-3/CHOL (80 mM total lipid) containing 40 mM dFdC and various pH values. The buffer consisted of 150 mM phosphate in the pH range between 6.1 and 7.8. In the phosphate buffered dispersion a pH dependency could be clearly demonstrated as it is shown in Fig. 3. The lowest amount of lysoPC was determined at pH of 7.3. Higher and lower pH values caused an increase of dFdC promoted hydrolysis of PC to lysoPC. In contrast, Grit et al. reported a pH

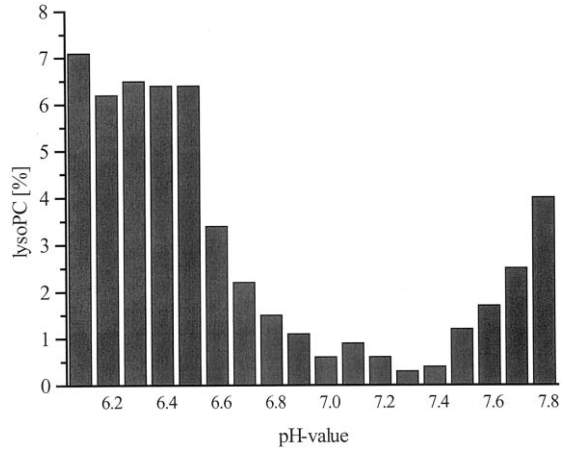

Fig. 3. pH-dependent lyso-transformation of EPC-3 liposomes in phosphate buffered solution: the optimum pH is shifted towards higher pH-values after addition of 40 mM dFdC. Values represent mean ± SD ($n = 3$).

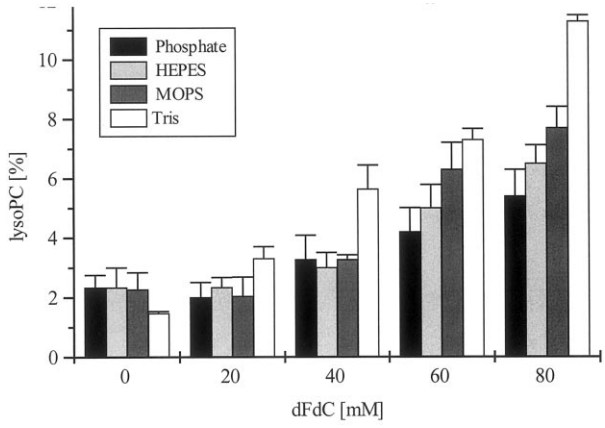

Fig. 4. The hydrolysis of PC at 60°C in EPC-3/CHOL liposomes is dependent on the buffer species (150 mM each). Values represent mean ± SD ($n = 3$).

optimum of 6.5 for empty liposome dispersions (containing no dFdC) (Grit et al., 1993a). They demonstrated, that the hydrolysis of phospholipids is not dependent on the bulk pH, but on the pH at the membrane surface (Grit and Crommelin, 1993). A possible explanation for the difference of hydrolysis rates between empty and dFdC containing liposomes is that an interaction of dFdC with the membrane surface might change the surface pH to more acidic values. It can be discussed that dFdC, which shows a pK_a value of 3.6 in water, works as a local surface associated buffer.

3.3.2. Influence of buffer composition

A linear relationship could be demonstrated between the hydrolysis of PC and the buffer concentration Grit et al. (1993a). In order to investigate whether dFdC-promoted hydrolysis rate varies with varying buffers at fixed pH of 7.3, dFdC-containing liposomes were aged artificially in 150 mM HEPES, MOPS and Tris buffer, respectively. Compared to the phosphate buffered dispersion, the stability of PC against hydrolysis was reduced in all of the investigated buffers (Fig. 4). This effect was most obvious for Tris. No clear influence of buffers could be observed up to a dFdC concentration of 40 mM. At dFdC concentrations between 60 and 80 mM the stability ranking was phosphate > HEPES > MOPS > Tris. In all cases the hydrolysis in liposome dispersions, containing in addition 50 mM NaCl, exceeded the hydrolysis rate in non-NaCl containing formulations (Table 2).

3.3.3. Influence of anions

In order to adjust iso-osmolarity in solutions for parenteral applications, mainly NaCl is used. However, the hydrolysis rate of PC in all studied buffers was much lower than in buffers supplemented with 50 mM NaCl (Table 2). The effect of ionic strength by addition of NaCl in non-drug containing liposome dispersions has recently been investigated. In these studies no clear correlation between ionic strength and hydrolysis rate could be observed Grit et al. (1993a). Thus, the fact that NaCl supplementation could influence the phospholipid hydrolysis in the presence of dFdC was unexpected. In order to examine the influence of NaCl in more detail, the effects of other halogen anions and sulfate anion were investigated and compared with chloride. Fig. 5 shows the hydrolysis of EPC-3, which was strongly dependent on the kind of anion supplemented at least at dFdC concentrations of 40 mM and above. In the absence of dFdC the different anions did not show any significant influence of EPC-3 hydrolysis. With increasing dFdC concentrations the different

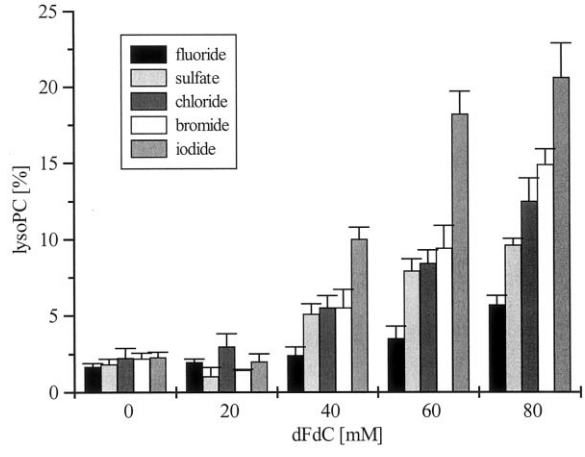

Fig. 5. Hydrolysis of PC in EPC-3/CHOL liposomes, phosphate buffered solution, containing different anions (50mM each in 100 mM phosphate buffer). Values represent mean ± SD ($n = 3$).

Table 2
Comparison of the hydrolysis of PC in different buffers with or without NaCl supplemented: buffer species influenced the degradation of EPC-3 in the presence of dFdC (see Fig. 4)[a]

DfdC (mM)	lysoPC (%) in phosphate buffer		lysoPC (%) n HEPES buffer		lysoPC (%) in MS buffer		lysoPC (%) in Tris buffer	
	100 mM with NaCl (50 mM)	150 mM	100 mM with NaCl (50 mM)	150 mM	100 mM with NaCl (50 mM)	150 mM	100 mM with NaCl (50 mM)	150 mM
0	2.2 ± 0.2	2.3 ± 0.4	3.0 ± 0.4	2.3 ± 0.7	3.5 ± 0.5	2.3 ± 0.6	2.1 ± 0.6	1.5 ± 0.1
20	3.0 ± 0.9	2.0 ± 0.5	3.6 ± 0.6	2.3 ± 0.3	2.7 ± 0.4	2.0 ± 0.6	7.3 ± 1.0	3.3 ± 0.4
40	5.5 ± 0.8	3.3 ± 0.8	4.8 ± 0.6	3.0 ± 0.5	9.7 ± 0.5	3.3 ± 0.2	10.9 ± 1.4	5.6 ± 0.8
60	8.4 ± 0.7	4.2 ± 0.8	11.6 ± 1.3	5.0 ± 0.8	14.9 ± 1.4	6.3 ± 0.9	13.9 ± 1.3	7.3 ± 0.4
80	12.5 ± 1.0	5.4 ± 0.9	15.4 ± 1.4	6.5 ± 0.6	17.3 ± 0.8	7.7 ± 0.7	18.8 ± 1.2	11.3 ± 0.2

[a] Generally the degradation of EPC-3 in 50 mM NaCl containing buffers exceeded that in non-NaCl containing buffers. Values represent mean ± SD ($n = 3$).

influences of the various anions became more and more apparent. A rising hydrolysis rate could be observed from the very small and 'hard' fluoride to the very big and 'soft' iodide. The influence of sulfate was in between that of fluoride and chloride. These results indicate that the degradation of PC correlates with the 'lipophilicity' of the supplemented anions. The softer or more 'lipophilic' an anion was, the more the hydrolysis was enhanced.

3.3.4. Calorimetrical studies

In order to investigate the interaction of dFdC and the phospholipids more detailed, DSC measurements were performed. Differential scanning calorimetry (DSC) is a sensitive and accurate method for determining the interaction between lipid bilayers and drug molecules (Skalko et al., 1996) The influence of dFdC on the liposome membrane was studied by comparing the chain-melting phase transition of empty DSPC liposomes with 40 mM dFdC-containing DSPC liposome dispersion in terms of transition temperature, enthalpy and half width of the peak

The main transition peak of pure DSPC was detected at 54.49 ± 0.08°C and the phase transition enthalpy being 44.7 ± 0.69 KJ/mol. The addition of 40 mM dFdC did not cause any change in phase transition temperature (54.42 ± 0.07°C) and the phase transition enthalpy slightly decreased to 40.9 ± 1.93 KJ/mol. However, dFdC enlarged the half width of the peak evidently from 0.73 ± 0.003 K (pure DSPC) to 1.40 ± 0.029 K. As the half width of the peak is a measure for the cooperativity of transition the widening of the peak clearly indicates an interaction between the lipid bilayer and the drug molecule.

3.4. Mechanism of deacylation in the presence of dFdC

3.4.1. Systematic elimination of dFdC molecular sites

Although interaction between dFdC and the phospholipids could be demonstrated by DSC, the detailed mechanism of the promotion of phospholipid hydrolysis by dFdC could not be explained so far. We suggest that three sites in the molecular structure of dFdC might be involved in the hydrolysis process: (1) the ribose moiety; (2) substitution of 2′-position of ribose moiety with two fluorine atoms; and (3) the basic amino-group at the pyrimidine moiety. We investigated the influence of each of these sites by its systematic elimination (Fig. 6). For this, the influence of cytosine, Ara-C and FUdR, which are molecules of similar pyrimidine structure to dFdC, but missing either at least one of the three sites, were investigated in terms of their PC hydrolysis promotion. Cytosine is lacking the ribose moiety, FUdR is lacking the amino moiety at C-4 of the pyrimidine ring, and Ara-C is a cytidine analogue like dFdC, but the 2′,2′-difluoro2′-deoxyribose is replaced by arabinose. Phosphate buffered EPC-3/CHOL liposome dispersions at pH 7.3, containing these three drugs in rising concentrations were aged and the formation of lysoPC was compared with the formation of lysoPC in dFdC containing formulations.

The extent of the hydrolysis of PC was dependent on the structure of the present nucleoside analogue. dFdC as well as Ara-C induced the deacylation of phospholipids in a dose-dependent manner above concentrations of 40 mM (Fig. 7). For both drugs, the concentration of lysoPC increased with increasing drug concentration, respectively. The formation of lysoPC was faster with dFdC than with Ara-C. The other analogue FUdR and cytosine showed no effect on the hydrolysis of PC.

These results confirmed our hypothesis that all three moieties of the dFdC molecule are necessary for the promoted PC hydrolysis. As FUdR did not enhance the hydrolysis of PC, an amino-moiety at C4 of the cytosine group seemed to be necessary. However, this structure did not much influence the hydrolysis of PC alone. Secondly, a ribose moiety carrying thirdly strongly electron-withdrawing substituents was of absolute need. The two electron-withdrawing fluorine atoms at 2′-position are responsible for the low pK_a value of dFdC, which is 3.6. The pK_a of Ara-C is higher ($pK_a = 4.5$), because the hydroxyl group at 2′-position is less electron-withdrawing compared to the fluorine atoms of dFdC. Thus, the lower pK_a value leads to a higher polarity of dFdC compared to the polarity of Ara-C. This might be the

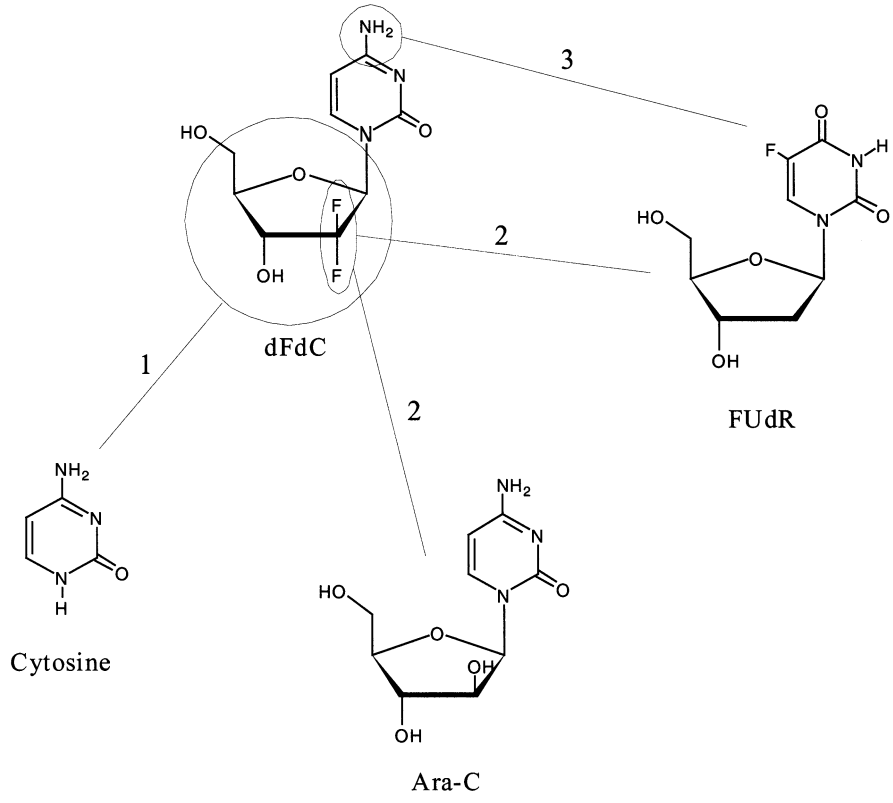

Fig. 6. Three sites of the molecular structure of dFdC might be responsible for the forced lipid hydrolysis. Their systematic elimination leads to structures of Cytosine, Ara-C and FUdR, which have been investigated in terms of their influence on phospholipid hydrolysis.

reason, why dFdC induced the hydrolysis of PC to a higher extent than Ara-C. Again the hydrolysis of phosphatidylcholine in phosphate buffer without NaCl was lower than in phosphate buffered dispersion (data not shown).

3.4.2. Influence of oligonucleotides

Not only single nucleoside analogues are of interest for liposomal encapsulation. For gene therapy purposes, oligonucleotides, which also contain cytidine moieties, are entrapped in liposomes. To this end, we investigated the influence of oligonucleotides on the hydrolysis of PC.

As shown in Fig. 7, the degradation of EPC-3 was not enhanced in the presence of the oligonucleotide GTACGTCACTCAGTCACG. Possibly the arrangement of the oligonucleotide molecules prevent the interaction of the cytidine moiety with the membrane surface.

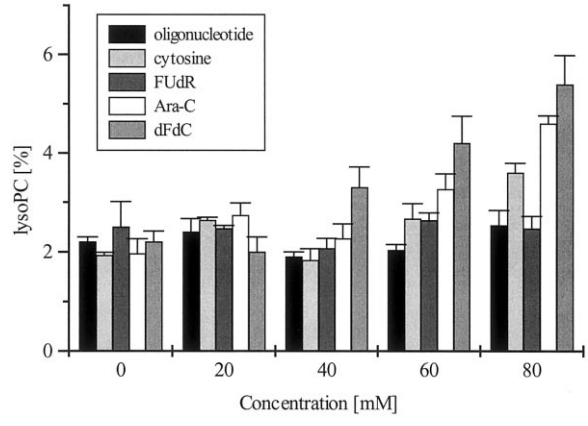

Fig. 7. Dependency of PC hydrolysis (in EPC-3/CHOL) liposomes at 60°C on the molecular structure of the added cytosine, nucleoside analogue or oligonucleotide, repectively, at pH 7.3. Values represent mean ± SD ($n = 3$).

For transfection in gene therapy, often a different lipid composition, namely positively charged liposomes are used. As this experiment was performed with neutral liposomes, further investigations on the interactions between oligonucleotides or plasmids and cationic lipids are necessary to assess whether the hydrolysis of phospholipids might be a determining factor for the shelf-life of positively charged liposomal DNA formulations.

4. Conclusion

The hydrolysis of phospholipids is one of the most determining factors for the long term stability of liposome dispersions. We could demonstrate that nucleoside analogues, depending on their molecule structure, obviously are able to interact with the phospholipid bilayer, and that they can promote the hydrolysis of phospholipids. We could further show that the dFdC-promoted hydrolysis depends on pH, buffer composition and presence of salts. By careful choice of these parameters we were able to minimize the hydrolysis of the phospholipids to an extent that development of nucleoside analogue containing liposomes for pharmaceutical purposes appears feasible.

References

Abbruzzese, J.L., Grunewald, R., Weeks, E.A., Gravel, D., Adams, T., Nowak, B., Mineishi, S., Tarasoff, P., Satterlee, W., Raber, M., Plunkett, W., 1991. A phase I clinical, plasma and cellular pharmacology study of gemcitabin. J. Clin. Oncol. 9, 491–498.

Allen, T.M., Mehra, T., Hansen, C., Chin, Y.C., 1992. Stealth liposomes: an improved sustained release system for 1-β-D-arabinofuranosylcytosine. Cancer Res. 52, 2431–2439.

van Borssum Waalkes, M., van Galen, M., Morselt, H., Sternberg, B., Scherphof, G.L., 1993. In-vitro stability and cytostatic activity of liposomal formulations of 5-fluoro-2′-deoxyuridine and ist diacylated derivatives. Biochim. Biophys. Acta 1148, 161–172.

Chu, E., Johnston, P.G., Takimoto, C.H., Politi, P.M., Grem, J.L., Chabner, B.A., Allegra, C.J., 1993. Anitmetabolites. Cancer Chem. Biol. Response Mod. 14, 1–25.

Grit, M., Crommelin, D.J.A., 1992. The effect of aging on the physical stability of liposome dispersions. Chem. Phys. Lip. 62, 113–122.

Grit, M., Crommelin, D.J.A., 1993. The effect of surface charge on the hydrolysis kinetics of partially hydrogenated egg phosphatidylcholine and egg phosphatidylglycerol in aqueous liposome dispersions. Biochim. Biophys. Acta 1167, 49–55.

Grit, M., Zuidam, N.J., Underberg, W.J.M., Crommelin, D.J.A., 1993a. Hydrolysis of partially saturated egg phosphatidylcholine in aqueous liposome dispersions and the effect of cholesterol incorporation on hydrolysis kinetics. J. Pharm. Pharmacol. 45, 490–495.

Grit, M., Underberg, W.J.M., Crommelin, D.J.A., 1993b. Hydrolysis of saturated soybean phosphatidylcholine in aqueous liposome dispersions. J. Pharm. Sci. 82, 362–366.

Heinemann, V., Hertel, L.W., Grindey, G.B., Plunkett, W., 1988. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. Cancer Res. 48, 4024–4031.

Heinemann, V., Xu, Y.Z., Chubb, S., Sen, A., Hertel, W., Grindey, G.B., Plunkett, W., 1992. Cellular elimination of 2′,2′-difluorodeoxycytidine 5′-triphosphate: a mechanism of self-potentation. Cancer Res. 52, 533–539.

Hertel, L.W., Boder, G.B., Stan Kroin, J., Rinzel, S.M., Poore, G.A., Todd, G.C., Grindey, G.B., 1990. Evaluation of the antitumor activity of gemcitabin (2′,2′-difluoro-2′-deoxycytidine). Cancer Res. 50, 4417–4422.

Inoue, K., Kitagawa, T., 1974. Effect of exogenous lysolecithin on liposomal membranes: its relation to the membrane fluidity. Biochim. Biophys. Acta 363, 361–372.

Kaye, S.B., 1994. Gemcitabin: current status of phase I and II trials. J. Clin. Oncol. 12, 1527–1531.

Kötting, J., Marschner, N., Unger, C., Eibl, H., 1992. Determination of alkylphosphocholines in biological fluids and tissues. In: Eibl, H., Hilgard, P., Unger, C. (Eds.), Alkylphosphocholines: New Drugs in Cancer Therapy. Karger, Basel, pp. 6–11.

Lund, B., Kristjansen, P.E.G., Hansen, H.H., 1993. Clinical and preclinical activity of 2′,2′-difluorodeoxycytidine (gemcitabine). Cancer Treatment Reviews 19, 45–55.

Lutz, J., Augustin, A.J., Jäger, L.J., Bachmann, D., Brandl, M., 1995. Acute toxicity and depresson of phagocytosis in vivo by liposomes: influence of lysophosphatidylcholine. Life Sciences 56, 99–106.

Moog, R., Häring, A.B., Brandl, M., Schubert, R., Unger, C., Fiebig, H.H., Massing, U., 1998a. Entrapment of gemcitabine into vesicular phospholipid gels for anticancer treatment: in vivo studies on human bladder xenograft s.c. growing in athymic nude mice. J. Liposome Res. 8 (1), 87–88.

Moog, R., Häring, A.B., Brandl, M., Schubert, R., Unger, C., Burger, A.M., Fiebig, H.H., Massing, U., 1998b. Increased antitumoral activity of gemcitabine upon entrapment into vesicular phospholipid gels. J. Cancer Res. Clin. Oncol. 124(suppl.), R163.

Petersen, N.O., Chan, S.I., 1978. The effects of the thermal prephase transition and salts on the coagulation and flocculation of phosphatidylcholine bilayer vesicles. Biochim. Biophys. Acta 509, 111–128.

Piraube, C., Postaire, E., Lize, J.M., Prognon, P., Pradeau, D., 1988. Evidence of chemical instability of phospholipids in liposomes. Chem. Pharm. Bull. 36, 4600–4602.

Schwendener, R.A., Schott, H., Hartmann, H.R., Supersaxo, A., Rubas, W., Hengartner, H., 1987. Liposomen als Träger von lipophilen Cytosinarabinosid- und Fluorodeoxyuridin-Derivaten. Ihre zytostatische Wirkung und Möglichkeiten zur tumorzellspezifischen Therapie. Onkologie 10, 232–239.

Shah, V.P., Midha, K.K., Dighe, S., McGliveray, I.J., Skelly, J.P., Yacobi, A., Layloff, T., Viswanathan, C.T., Cook, C.E., McDowell, R.D., Pittman, K.A., Spector, S., 1992. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Pharm. Res. 9, 588–592.

Skalko, N., Brandl, M., Becirevic-Lacan, M., Filipovic-Grcic, J., Jalsenjak, I., 1996. Liposomes with nifedipine and nifedipine-cyclodextrin complex: calorimetrical and plasma stability comparison. Eur. J. Pharm. Sci. 4, 359–366.

Szoka, F., Papahadjopoulos, D., 1980. Comparative properties and method of lipid vesicles (liposomes). Annu. Rev. Biophys. Bioengng. 9, 467–508.

Zhang, R., Lu, Z., Liu, T., Soong, S., Diasio, R., 1993. Relationship between circadian-dependent toxicity of 5-fluorodeoxyuridine and circadian rhythms of pyrimidine enzymes: possible relevance to fluoropyrimidine chemotherapy. Cancer Res. 53, 2816–2822.

Zuidam, N.J., Gouw, H.K.M, Barenholtz, Y., Crommelin, D.J.A., 1995. Physical (in)stability of liposomes upon chemical hydrolysis: the role of lysophospholipids and fatty acids. Biochim. Biophys. Acta 1240, 101–110.