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In-vitro stability and cytostatic activity of liposomal formulations of 5-fluoro-2′-deoxyuridine and its diacylated derivatives

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The water-soluble antineoplastic agent 5-fluoro-2′-deoxyuridine (FUdR) was encapsulated in the water phase of liposomes of different lipid compositions. The retention of this drug upon storage and during contact with plasma was assessed. It was found that, upon refrigeration, diffusion of FUdR across the liposome bilayer was considerably faster when the drug was encapsulated in fluid-type liposomes (egg PC/PS/CHOL) than in solid-type liposomes (DSPC/DPPG/CHOL). With either composition, leakage of the drug from the liposomes was accelerated upon contact with plasma. To achieve improved liposomal retention of the drug, FUdR was converted to a lipophilic prodrug by esterifying the free hydroxyl groups in the deoxyribose moiety with fatty acids of different chain lengths. Thus FUdR-dipalmitate (C-16) and FUdR-dioctanoate (C-8) were synthesized and incorporated in liposomes. The dipalmitoyl derivative could be incorporated upto 13 mol% in solid-type liposomes but to only 2 mol% in fluid-type liposomes. Freeze-fracture electron microscopy revealed no major differences between control liposomes and those containing the prodrug. FUdR-dipalmitate was found to be firmly associated with the liposomal bilayer in both liposome-types: no exchange of the pro-drug with blood constituents or hydrolysis by serum esterases could be registered when the liposomes were incubated with serum. On the other hand, liposome-incorporated FUdR-dioctanoate was found to be readily extracted from the liposomes by serum components (predominantly albumin) and was found to be degraded rapidly by serum esterase activity. The antitumor activity of FUdR-prodrugs was determined using C26 colon adenocarcinoma cells. This cell line was found to be highly sensitive to FUdR. Liposomal FUdR-dioctanoate inhibited cell growth in the same concentration range as unesterified FUdR. FUdR-dipalmitate, however, was more than two orders of magnitude less potent in inhibiting cell proliferation. Its antiproliferative activity was dependent on the liposome-type used: when incorporated in fluid-type liposomes, antiproliferative activity of FUdR-dipalmitate was several-fold higher than in solid-type liposomes. The difference in antitumor activity between FUdR-dipalmitate and FUdR-dioctanoate and between FUdR-dipalmitate in the fluid- and solid-type liposomes could be explained by differences in the rate of hydrolysis of the prodrugs to FUdR by esterase activity in the tumor cells or in the growth medium.

Introduction

One of the reasons for using liposomes as a drug-carrier system is their potential to protect drugs against rapid intravascular or intracellular degradation (as reviewed in Ref. 1). This is especially relevant for antineoplastic drugs that have a short plasma half-life but require a long retention in vivo to obtain the desired antitumor activity. Examples of such drugs are the antineoplastic agents 1-β-D-arabinofuranosyl-cytosine (cytarabine, Ara-C) and 5-fluoro-2′-deoxyuridine (FUdR) [2]. Although there are several groups that have investigated the efficacy of liposomal Ara-C (for reviews, see Refs. 1,3–5), the experience with liposome-encapsulated FUdR is limited.

FUdR, although quite water-soluble, is not very well retained within liposomes because it can diffuse readily across lipid bilayers [6] thus counteracting an increase in its retention time in vivo. Therefore, we explored the possibility to prepare a liposomal formulation in which FUdR is firmly anchored. An attractive concept was reported by Schwendener and co-workers who used a

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Abbreviations: FUdR, 5-fluoro-2′-deoxyuridine; FUdR-dipalmitate, 3′-5′-*O*-dipalmitoyl-5-fluoro-2′-deoxyuridine; FUdR-dipalmitate; DSPC, distearoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; eggPC, egg-yolk phosphatidylcholine; PS, phosphatidylserine; CHOL, cholesterol; MLV, multilamellar vesicles; SUV, small unilamellar vesicles.

lipophilic derivative of FUdR incorporated in the liposomal lipid-bilayer [7–9].

The free hydroxyl groups of the deoxyribose moiety of FUdR can easily be esterified with fatty acids to obtain a lipophilic derivative. Because FUdR is only active as an antiproliferative agent after phosphorylation of its 5′-hydroxyl group [10,11], the fatty-acid chains have to be removed in order for FUdR to exert antitumor activity. In vivo, removal of the lipophilic residues proceeds via hydrolysis by aspecific esterases. These are spread throughout the body with high activity in liver, intestine and kidney and low activity in plasma, spleen, and several tumor cell lines [12]. The rate of hydrolysis depends on the chain length of the fatty acid residues: upon incubation of different FUdR prodrugs with porcine liver esterases it was found that the reaction rate increases when the chain is lengthened upto 8–10 carbon atoms and decreases again when fatty acids are used with longer chain lengths [13,14]. Thus, the nature of the lipophilic moiety of the prodrug is pertinent to a maximal retention of the drug in the body.

When liposomal FUdR-prodrugs are used the efficacy in vivo is thought to depend not only on the prodrug itself, but also on the characteristics and the stability of the FUdR-prodrug-liposome complex. It is known, for example, that liposome size, charge or lipid composition will considerably influence the behavior of liposomes in vivo. This is reflected as changes in (intrahepatic)-distribution, interaction with cells and intracellular degradation [15–17].

In this paper we report on in-vitro characteristics of liposome-incorporated FUdR-prodrugs: prodrug incorporation efficiency, particle size, charge and morphology of these liposomes and stability of the drug-liposome complexes towards serum. In addition, the antitumor activity of these liposome formulations towards C26 adenocarcinoma tumor cells was determined in vitro. Because of the importance of the nature of the prodrug, in this study we used two diacylated FUdR-derivatives which are known to be deacylated at different rates, i.e., FUdR-dipalmitate and FUdR-dioctanoate [14]. To assess the effect of liposome bilayer rigidity, we used liposomes composed of egg PC/PS/CHOL (fluid-type) and of DSPC/DPPG/CHOL (solid-type).

Materials and Methods

Chemicals. 5-Fluoro-2′-deoxyuridine (Floxuridine, FUdR) was generously supplied by Hoffmann-La Roche (Basel, Switzerland). Distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylglycerol (DPPG), egg-yolk phosphatidylcholine (egg PC) and phosphatidylserine (Bovine brain, PS) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). Cholesterol was obtained from Sigma (St. Louis, MO, USA). 5-Fluoro,6[^{3}H]-2′-deoxyuridine was from New England Nuclear (Boston, MA, USA); [^{14}C]sucrose and cholesteryl-[1-^{14}C]oleate were from Amersham (UK).

Synthesis of 3′-5′-O-dipalmitoyl-FUdR (FUdR-dP). FUdR-dipalmitate was synthesized as described by Schwendener and Supersaxo [7–9]. Briefly, FUdR (0.4 mmol) was dissolved in 1 ml of dimethylacetamide to which 0.8 mmol of freshly-distilled palmitoyl chloride was added. The mixture was incubated overnight under constant shaking at 40°C. To the gel-like solution formed, 10 ml of distilled water was added. The resulting white precipitate was applied to a glass filter and washed with distilled water. The FUdR-dipalmitate thus obtained was recrystallized three times from methanol. Purity of the compound was checked by TLC on silicagel F254 plates (Merck) with chloroform/methanol (95:5) as an eluent. The Rf values of the diester, the 3′-*O*-palmitoyl- and 5′-*O*-palmitoyl-monoester were 0.70, 0.28 and 0.17, respectively, in accordance with Ref. 7.

Synthesis of 3′-O-palmitoyl- and 5′-O-palmitoyl-FUdR (FUdR-mP). The monoesters were synthesized according to the method described above, except for the addition of 0.4 mmol palmitoyl chloride to the incubation mixture instead of 0.8 mmol. The monoesters were separated from each other and from the diester on preparative silicagel TLC plates. The material corresponding to the mono-esters was scraped off and the esters were extracted with chloroform/methanol (80:20).

Synthesis of 3′-5′-O-dioctanoyl-FUdR (FUdR-dioctanoate). To 0.4 mmol of FUdR in dimethylacetamide 0.8 mmol freshly-distilled octanoyl chloride was added. After incubation overnight at 40°C, 10 ml distilled water was added and the mixture was extracted with chloroform. The chloroform was evaporated and the residual oily material was applied to preparative silicagel TLC plates and eluted with chloroform/methanol (95:5). The spot corresponding to the diester was scraped off and was extracted with chloroform/methanol (95:5). The yields of all synthetic products were on average similar to those reported in Ref. 7.

Synthesis of [^{3}H]FUdR prodrugs. The ^{3}H-labeled prodrugs were prepared essentially in the same way as described for the synthesis of unlabeled esters. First, [^{3}H]FUdR, dissolved in water/ethanol (3:7), was diluted with unlabeled FUdR to the desired specific activity. Then the mixture was dried under a gentle stream of nitrogen. Since this procedure was not adequate to remove all residual water, the sample was freeze-dried from a small volume of distilled water. To the lyophilized material dimethylacetamide and palmitoyl- or octanoyl chloride was added. After incubation at 37°C for maximally 72 h, distilled water was added and the radioactive product was extracted with chloro-

form. The product was further purified on TLC as described above.

Preparation of liposomes. Phospholipids, cholesterol and FUdR-prodrugs were kept in chloroform/methanol (4:1) at −20°C under nitrogen. For the preparation of liposomes, aliquots of stock solution of lipids and prodrug were mixed and dried under nitrogen. The dried lipids were dissolved in cyclohexane, frozen and lyophilized. The lyophilized lipids were hydrated in HN buffer (10 mM Hepes, 135 mM NaCl (pH 7.4)) and thoroughly vortexed. The resulting preparation of multilamellar vesicles (MLV) was extruded twice through 0.4-μm polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) using an extruder (Lipex Biomembranes, Vancouver, Canada). The liposome preparations were stored under nitrogen at 4°C until they were used. As a rule, the liposomes were used within two weeks of preparation.

To obtain small unilamellar vesicles (SUV), the unextruded MLV preparation was sonicated using a probe type sonifier (Branson B12, output 40 W) until an optically clear dispersion was obtained. The resulting dispersion was centrifuged (15 min at 12000 × g) to remove probe-derived titanium particles. All liposome types containing DSPC were vortexed, sonicated and extruded at 60°C (above the gel-to-liquid-crystalline phase transition temperature of DSPC). Liposomes with egg PC were prepared at room temperature.

Liposomes containing cholesteryl-[^{14}C]oleate or ^{3}H-labeled FUdR-prodrugs were prepared as described above, except that the radioactive label was added to the lipid mixture in chloroform/methanol before freeze-drying.

When liposomes containing [^{14}C]sucrose and/or [^{3}H]FUdR were prepared, the label (diluted with carrier compound to a final concentration of 1 mM sucrose and 40 mM FUdR) was dissolved in HN buffer. Isotonicity of the buffer was assured by replacing part of the buffer with distilled water (iso-osmotic concentration of FUdR is 8.47%, [18]). This final mixture was added to the lyophilized lipids. After vortexing, the lipids were allowed to hydrate overnight at 4°C. Then, the preparation was extruded and non-encapsulated drug was separated from liposome-associated drug by gel-filtration on Sephadex G-75.

Characterization of liposomes. Phospholipid phosphorus was determined after destruction of the phospholipids with perchloric acid [19]. Particle size and polydispersity index (pd) were measured by dynamic light scattering using a Malvern 4700 system, equipped with a 25 mW He-Ne laser (Malvern, Malvern, UK). The polydispersity index is a measure for liposome

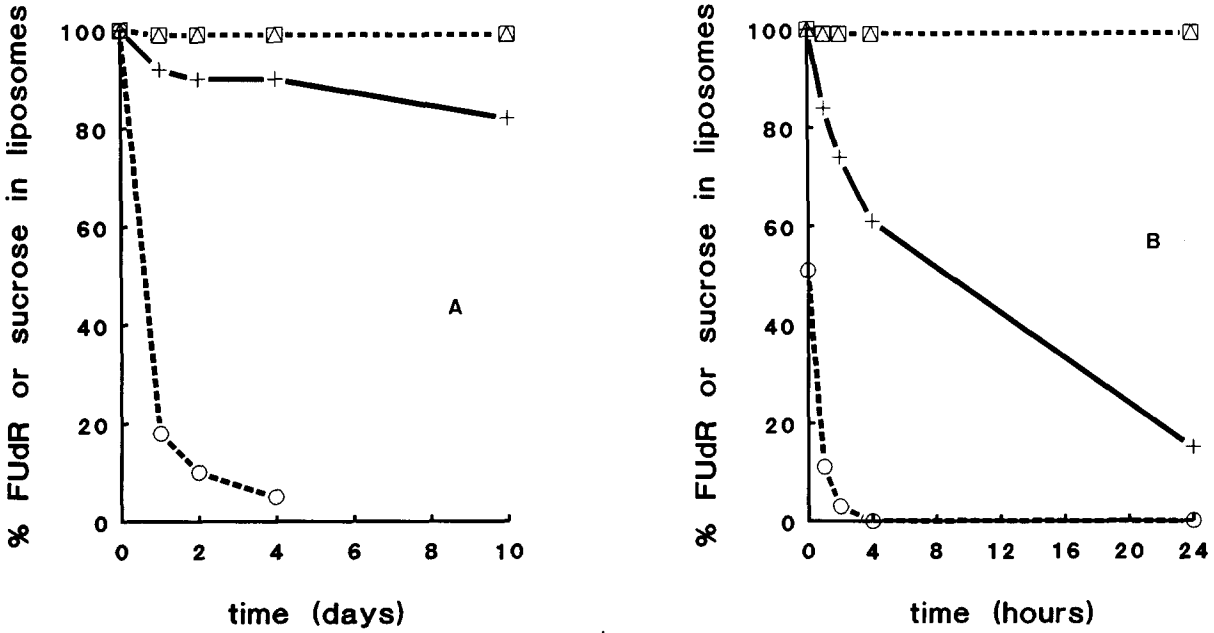

Fig. 1. Retention of liposome-encapsulated FUdR and sucrose upon storage and incubation in plasma. Extruded MLV, 0.4 μm, consisting of DSPC/DPPG/CHOL or PC/PS/CHOL and containing [^{3}H]FUdR and [^{14}C]sucrose were stored at 4°C under nitrogen (A). At the times indicated, samples of the liposome preparation were passed over a Sephadex G-75 column. After elution, the amounts of liposome-associated and free FUdR and sucrose were determined. The percentages of compounds retained within the liposomes were calculated. Liposomes were incubated with freshly obtained rat plasma for 1, 2, 4 and 24 h at 37°C (B). The percentage of encapsulated compound was calculated after separating the liposome-associated and free compound on a Sephadex G-75 column. [^{3}H]FUdR (+) and [^{14}C]sucrose (□) in DSPC/DPPG/CHOL liposomes [^{3}H]FUdR (○) and [^{14}C]sucrose (△) in egg PC/PS/CHOL liposomes.

homogeneity and ranges from 0.0 for a perfectly homogeneous to 1.0 for a completely inhomogeneous dispersion.

The zeta-potential of liposomes was estimated using a Mark II microelectrophoresis apparatus (Rank Bros., Cambridge, UK). Zeta-potentials were calculated from average mobilities of approx. 20 particles.

Freeze-fracture electron microscopy. Morphology and average particle size of SUV and MLV of the rigid as well as of the fluid type were investigated by freeze-fracture electron microscopy at two different prodrug concentrations. The samples were studied after quenching rapidly (10^4 KS^{-1}) from room or body temperature (22°C or 37°C, respectively) according to the sandwich technique and shadowed in a Balzers BAF 400D freeze-fracture device at −120°C. The cleaved replicas were examined in a Jeol JEM 100B electron-microscope [20].

Incorporation efficiency of FUdR-dipalmitate in liposomes. Liposomes of different lipid composition and increasing amounts of FUdR-dipalmitate were prepared as described above. Before and after extrusion, drug and lipid concentrations were measured and the amount of drug that could be incorporated was calculated. When the amount of FUdR-dipalmitate exceeded the maximal amount of drug that could be incorporated, aggregates were formed which remained on the extrusion filter.

Retention of liposomal FUdR upon storage and in plasma. MLV consisting of DSPC/DPPG/CHOL (10:1:10) or egg PC/PS/CH (4:1:5) labeled with [^{3}H]FUdR and [^{14}C]sucrose were prepared as described above. To measure the encapsulation efficiency, concentrations of sucrose, FUdR and phospholipid were assessed before and after gel-filtration, by measuring radioactivity and phospholipid phosphorus. In order to determine FUdR and sucrose leakage accross the liposomal bilayer upon storage at 4°C, part of the liposome suspension was passed over a Sephadex G-75 column 1, 2, 4, and 10 days after preparation. The amounts of liposome-associated and released radioactive FUdR and sucrose were measured and the percentage of residual liposome-associated label was calculated.

The stability of the liposomal formulations in plasma was determined by incubating the liposomes at 37°C with fresh rat plasma. At times indicated in Fig. 1B, free and liposome-associated drug were separated by passing a sample of the incubation mixture over a Sephadex G-75 column.

Deacylation of liposomal FUdR-dipalmitate and FUdR-dioctanoate in serum. Liposomal [^{3}H]FUdR-dipalmitate or [^{3}H]FUdR-dioctanoate preparations were incubated with fresh rat serum at 37°C (50 μl liposome-dispersion containing 0.6 μmol total lipid/ml serum). At 0, 1, 2, and 4 h after the start of the incubation a sample was drawn and extracted with chloroform/methanol according to the method of Bligh and Dyer [21]. Radioactivity contents of the aqueous and organic layers were measured. The percentage of label associated with the water phase, representing water soluble degradation products, was calculated.

Degradation of liposomal [^{3}H]FUdR-dioctanoate by aspecific esterase. Liposomal [^{3}H]FUdR-dioctanoate was incubated with 0.05 units of aspecific porcine liver esterase (carboxy-ester hydrolase, EC 3.1.1.1. Type 1) in 1 ml Hepes buffer at 37°C. At 0, 1, 2, and 4 h samples were drawn from the reaction mixture and analyzed as described for the determination of FUdR-prodrug degradation in serum.

Exchange of FUdR-prodrug from liposomes to serum components. Liposomes containing cholesteryl-[^{14}C]-oleate and [^{3}H]FUdR-dipalmitate or [^{3}H]FUdR-dioctanoate were incubated for 1 h at 37°C with freshly obtained serum from fasted rats (50 μl liposome dispersion containing 0.6 μmol total lipid/ml serum). After the incubation period, 0.8 ml of the mixture was applied to a Sepharose 6B column and eluted with HN buffer at 4°C. 1-ml fractions were collected, and fractions were assayed for radioactivity and absorbance at 280 nm.

Exchange of FUdR-prodrug between liposomal bilayers. Unextruded MLV consisting of PC/PS/CHOL (4:1:5) labeled with cholesteryl-[^{14}C]oleate were centrifuged for 10 min at 12000 × g. The pellet was dispersed in HN-buffer and the concentration of total lipid assessed as described above. A sample of this liposome preparation corresponding to 1 μmol of total lipid, was incubated at 37°C with 0.1 μmol of SUV-lipid containing [^{3}H]-labeled FUdR-esters (volume adjusted to 1 ml with HN-buffer). After 0, 1, 2, and 4 h the liposome mixture was centrifuged for 15 min at 12000 × g. The supernatant and pellet were separated and the pellet was washed with HN buffer. The washing buffer was added to the first supernatant after centrifugation. The pellet was dispersed in HN buffer. Radioactivity and phospholipid phosphorus were determined in the pooled supernatants and resuspended pellets. From this, the percentage of exchanged FUdR-prodrug was calculated.

In-vitro cytotoxicity assay. C26 colon adenocarcinoma cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco, Paisley, UK), glutamine (2 mM, Flow Labs, Irvine, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (both from Gist Brocades, Delft, The Netherlands). The cells were harvested by short trypsinization and transferred to 96-wells culture plates with 5000 cells per well in 100 μl medium. After overnight incubation at 37°C under 5% CO_2/ 95% air atmosphere, 100 μl medium aliquots with different concentrations of test compounds were added to the cells.

The cells were incubated with the test compounds for 72 h and cell growth inhibition was determined by a [^{3}H]thymidine incorporation assay: 20 h before the end of the experiment 0.02 μCi [^{3}H]thymidine was added to each well. At the termination of the experiment the cells were harvested and cell-associated radioactivity was measured. Cell growth was calculated as the total amount of radioactivity in the treated cells divided by the total amount of radioactivity in the control cells multiplied by 100.

Degradation of FUdR-dipalmitate and FUdR-dioctanoate by tumor cells. Tumor cells were plated in 96-well culture plates (5000 cells per well) in RPMI containing 10% FCS, glutamine and antibiotics as described above. Liposomal [^{3}H]FUdR-dipalmitate or [^{3}H]FUdR-dioctanoate was added to the tumor cells 24 h after plating. The cells and liposomes were incubated for another 24 h at 37°C under a 5% CO_2/95% air atmosphere. Then, the medium was pipetted off and extracted with chloroform/methanol according to the method of Bligh and Dyer [21]. Radioactivity in the organic and in the water phase was measured and the percentage of degradation was calculated.

Results

Encapsulation efficiency and retention of FUdR in liposomes

[^{3}H]FUdR and [^{14}C]sucrose were encapsulated in liposomes (0.4 μm extrusion MLV) composed of egg PC/PS/CHOL (4:1:5) or DSPC/DPPG/CHOL (10:1:10). After removing the liposome-associated drug from the free drug by gel-filtration chromatography on Sephadex G-75, the encapsulated aqueous volume was calculated. Using sucrose as a non-permeating polar compound, the encapsulated volume was calculated to be 2.29 μl/μmol lipid in the former and 3.29 μl/μmol lipid in the latter type of liposome. Based upon FUdR as the encapsulated solute, these numbers were 1.76 and 3.30 μl/μmol lipid, respectively. Thus, the encapsulation efficiency of sucrose and FUdR were equal for solid-type liposomes but differed considerably when fluid-type liposomes were used. Apparently, liposomes consisting of egg PC/PS/CHOL are not able to fully retain FUdR, not even during the process of liposome preparation (gel filtration).

The difference in stability between these two liposome types is also demonstrated in Fig. 1a and b. They show the encapsulation stability of these liposome types upon storage at 4°C and upon contact with plasma at 37°C. Sucrose is retained in both liposome-types during storage, as well as upon contact with plasma. FUdR, on the other hand, leaks out very rapidly when encapsulated in the fluid-type liposomes, especially when brought into contact with plasma. Just mixing the liposomes with plasma led to an immediate release of about 50% of the encapsulated material. FUdR encapsulated in the solid liposome type leaks out considerably more slowly during storage, but also in this case incubation with plasma at 37°C caused substantial additional leakage.

Incorporation efficiency of FUdR-dipalmitate in liposomes; determination of particle size and charge

FUdR-dipalmitate could be incorporated to a maximal amount of 13 mol% in liposomes consisting of DSPC/DPPG/CHOL and to no more than 2 mol% in liposomes consisting of PC/PS/CHOL (Table I). When attempting to incorporate larger amounts of FUdR-dipalmitate in the liposomes, the surplus formed aggregates which remained on the filter during extrusion.

TABLE I

Characterization of FUdR-dipalmitate-containing liposomes (Vesicle size and zeta potential)

Liposomes were prepared by mixing 10 μmol total lipid with various quantities of FUdR-dipalmitate. The lipid-to-drug ratio was assessed before and after extrusion through 0.4-μm membrane filters. From this, the amount of drug that was incorporated into the liposomes was calculated. Particle size and charge were measured as described in Materials and Methods. pd, polydispersity index; n.d., not determined.

Lipid composition liposome	Initial molar fraction FUdR-dipalmitate	% Incorporation FUdR-dipalmitate	Vesicle size diameter (nm)	pd	Zeta potential (mV)
DSPC/DPPG/CHOL	0	–	416	0.248	−13.0
(10:1:10)	2	100	416	0.237	−11.5
MLV, 0.4 μm	10	97	366	0.190	−15.9
	12.5	91	n.d.		n.d.
	15	89	n.d.		n.d.
PC/PS/CHOL	1	100	n.d.		n.d.
(4:1:5)	2	98	397	0.221	−16.4
MLV, 0.4 μm	3	61	n.d.		n.d.
DSPC/DPPG/CHOL (10:1:10), SUV	10	100	n.d.		n.d.

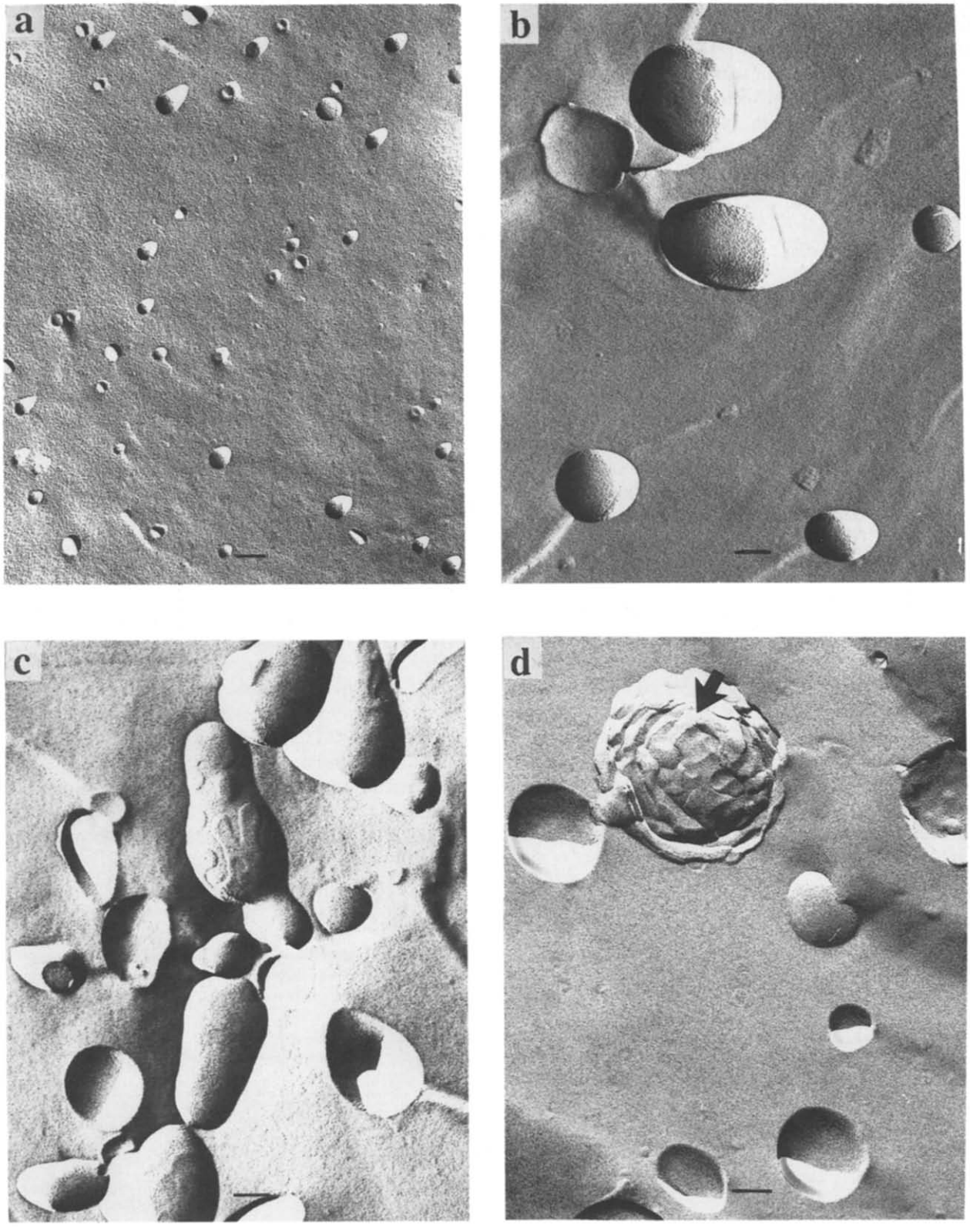

Fig. 2. Freeze-fracture electron micrographs of different liposome preparations, all quenched from room temperature (22°C): (a) SUV made of DSPC/DPPG/CHOL (solid type) and with 10% FUdR-dP incorporated; (b) MLV of the same lipid composition and prodrug concentration as in (a) showing a circular shape; (c) MLV of the same lipid composition as in (a), however with a prodrug concentration of only 2% showing elongated sizes; (d) MLV made of egg-PC/PS/CHOL (fluid type) and with 2% prodrug incorporated showing two vesicle populations, one example of the larger one is marked by an arrow. Bars represent 100 nm. Shadowing direction is from bottom to top of the micrographs.

Therefore, in subsequent experiments, DSPC liposomes were prepared with 10 mol% and PC-liposomes with 2 mol% incorporated FUdR-dipalmitate. FUdR-dipalmitate could also be incorporated upto 10% in SUV consisting of DSPC/DPPG/CHOL. In these experiments, the sonicated SUV were extruded through 0.1-μm filters. The drug to lipid ratio was determined before and after extrusion and was found not to change, implying that 100% of the initially added prodrug was incorporated.

The mean particle diameters and zeta potentials for some liposome preparations are given in Table I.

Liposome morphology and size studied by freeze-fracture electron microscopy

As is shown in Fig. 2 SUV were produced with diameters well below 100 nm (Fig. 2a), while MLV were below (Fig. 2b), around (Fig. 2c), and above 300 nm (Fig. 2d).

The fracture faces of all liposomes investigated are smooth and no appreciable differences were observed in the appearance of solid (Figs. 2a–c) and fluid (Fig. 2d) type liposomes, either quenched from room or body temperature (not shown), and independent of prodrug content (10% FUdR-dP, Fig. 2a and b; 2% FUdR-dP, Fig. 2c and d). MLV of the solid type with 2% of the prodrug incorporated, however, appear somewhat elongated (Fig. 2c) with long-axis diameters of approx. 500 nm and short-axis diameters of approx. 250 nm. Two size populations are found for MLV of the fluid type with 2% prodrug incorporated (Fig. 2), either showing diameters around 500 nm (one of them marked by an arrow) or around 250 nm.

Sizes of spherical MLV of the solid type and with 10% FUdR-dP incorporated (Fig. 2b), as determined by dynamic light scattering (366 nm; Table I), are only slightly larger than those determined by freeze-fracture electron microscopy (200–300 nm). MLV preparations

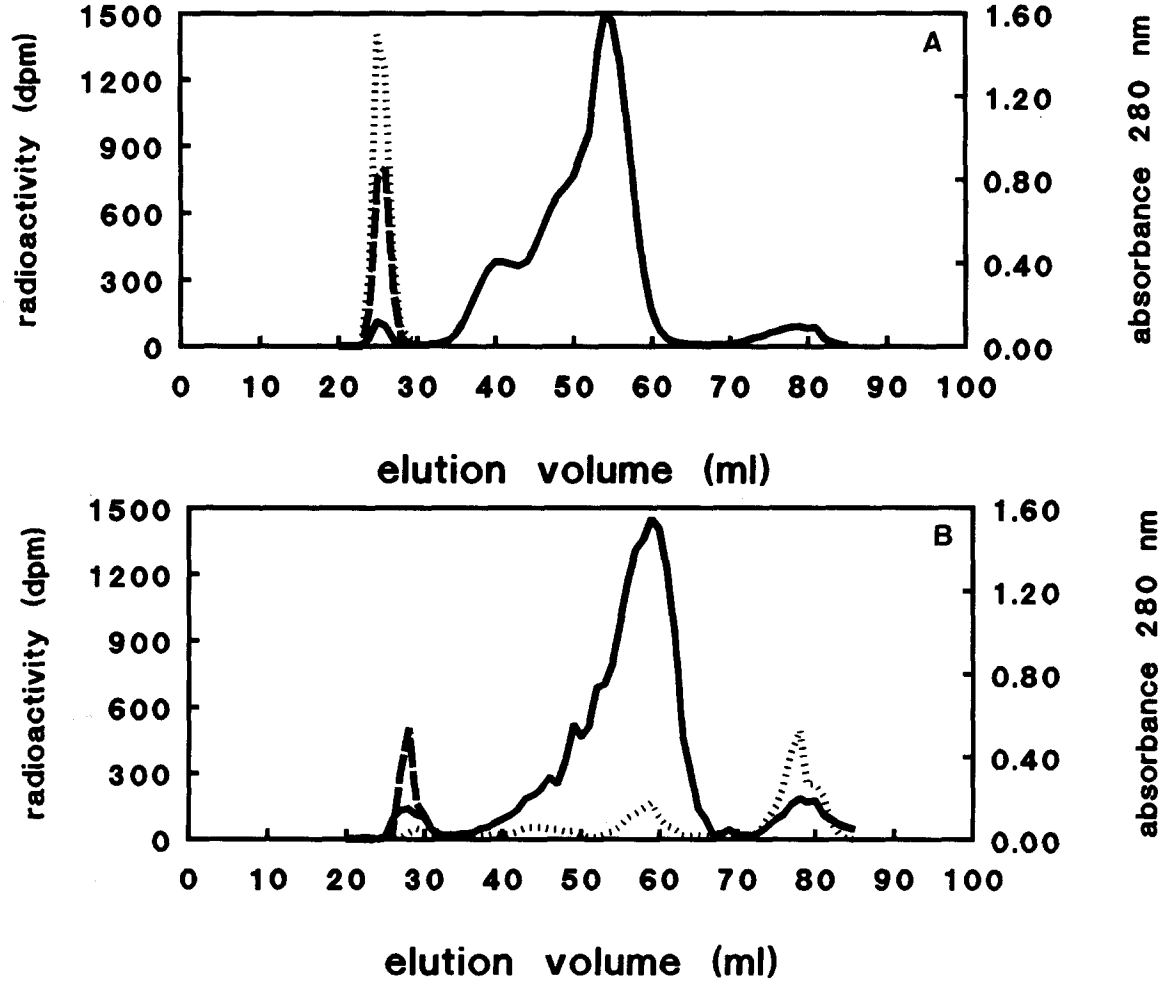

Fig. 3. Exchange of FUdR-dipalmitate and FUdR-dioctanoate between liposomes and serum components. Liposomes consisting of DSPC/DPPG/CHOL and containing cholesteryl-[^{14}C]oleate (– – –) and [^{3}H]FUdR-dipalmitate (– – –) (| | | | | |) (A) or [^{3}H]FUdR-dioctanoate (| | | | | |) (B) as radioactive labels were incubated with freshly obtained rat serum for 1 h at 37°C (50 μl liposome dispersion containing 0.6 μmol total lipid/ml serum). After the incubation, the mixture was passed over a Sepharose 6B column and eluted with HN buffer at 4°C. 90 ml elution fluid was collected in 1-ml fractions. Of each fraction absorbance at 280 nm (———) and radioactivity were measured.

containing ellipsoid vesicles (Fig. 2c) have an average diameter of 416 nm, as determined by dynamic light scattering, i.e., in between the length of the long (500 nm) and short (250 nm) axes of the ellipsoids as determined by electron microscopy. A similar observation is done for the MLV preparations consisting of two size populations (Fig. 2d; 500 and 250 nm); the average diameter obtained by light scattering measurement is 397 nm, in this case FUdR-dipalmitate (Table I). Thus, the morphological and the light-scattering data are quite compatible for all preparations examined.

The membranes of the MLV of the fluid type (Fig. 2d) are clearly more flexible than those of the solid type (Fig. 2c) in view of the occurrence, in the former type, of vesicles with diameters substantially larger than the 400-nm pore size of the extrusion filter. The lower flexibility of the membranes of the solid type may be the cause of some degree of deformation/elongation of such vesicles under shearing stress.

Exchange of liposome-incorporated FUdR-prodrugs with serum components

When liposomes, upon intravenous injection, come into contact with blood, destabilization or even disintegration of the liposomes may result, because liposomal phospholipids can exchange with or transfer to plasma (lipo)proteins, in particular HDL [22]. Because FUdR-prodrugs are lipophilic, it is conceivable that they also are subject to exchange with serum components. To examine this, liposomal [^{3}H]FUdR-dipalmitate and [^{3}H]FUdR-dioctanoate were incubated for 1 h with serum. After incubation, the radioactivity and UV-absorbance profile of the incubation mixture upon gel-filtration (Sepharose 6B) was determined (Fig. 3a and b). This revealed that, in contrast to FUdR-dipalmitate, FUdR-dioctanoate in DSPC/DPPG/CHOL liposomes does exchange with serum constituents: FUdR-dipalmitate elutes with the liposomes (labeled with [^{14}C]cholesteryl-oleate) in the void volume of the column, whereas the tritium label of FUdR-dioctanoate was found for the greater part in three peaks in the included volume. Extraction of one of these peaks (75–80 ml) with chloroform/methanol showed that this compound is water-soluble, implying that it is a degradation product. The remainder of the label was partly associated with lipoproteins (elution volume 40–50 ml) and partly with albumin (55–65 ml).

Just like FUdR-dipalmitate in liposomes consisting of DSPC/DPPG/CHOL, FUdR-dipalmitate incorporated in egg PC/PS/CHOL liposomes did not exchange with serum components (not shown).

Exchange of FUdR-dipalmitate and FUdR-dioctanoate between liposomal bilayers

The far superior association of FUdR-dipalmitate with liposomes as compared to liposomal FUdR-dioctanoate, was also demonstrated in the following experiment. SUV consisting of DSPC/DPPG/CHOL and containing [^{3}H]FUdR-dipalmitate or [^{3}H]FUdR-dioctanoate were incubated with egg PC/PS/CHOL liposomes (unextruded MLV) labeled with cholesteryl-[^{14}C]oleate. After incubation at 37°C and separation of MLV from SUV by centrifugation, FUdR-dipalmitate remained associated with SUV (Fig. 4). Even 4 h after initiation of the experiment, insignificant amounts of FUdR-dipalmitate were associated with MLV. In contrast to this, liposomal FUdR-dioctanoate was transferred almost instantaneously from SUV to MLV. Already at the first time point (upon mixing of the liposomes) 80% of the ^{3}H-label was recovered in the pellet (MLV).

Also the two monopalmitoyl-esters, 3′-*O*-palmitoyl- and 5′-*O*-palmitoyl-FUdR, were readily transferred from SUV to MLV, but at a slower rate than FUdR-dioctanoate. On the basis of these results it was decided not to continue experiments with the mono-acylated derivatives.

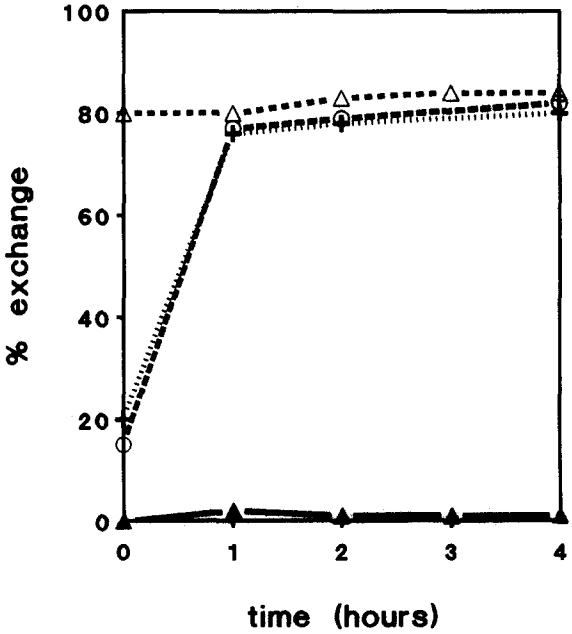

Fig. 4. Exchange of mono- and diacyl FUdR derivatives between liposomal bilayers. SUV consisting of DSPC/DPPG/CHOL and containing [^{3}H]FUdR-dipalmitate (▲), 3′-*O*-palmitoyl-[^{3}H]FUdR (+), 5′-*O*-palmitoyl-[^{3}H]FUdR (○) or [^{3}H]FUdR-dioctanoate (△) were incubated at 37°C in HN buffer with unextruded cholesteryl-[^{14}C]oleate-labeled egg PC/PS/CHOL MLV. After the incubation period (0, 1, 2 and 4 h) SUV and MLV were separated by centrifugation and the ratio of [^{3}H] or [^{14}C] to lipid was determined in supernatant and pellet. From these values the percentage of exchanged ^{3}H-label and thus the amount of exchanged FUdR-prodrug, was calculated.

Enzymatic deacylation of liposomal FUdR-dipalmitate and FUdR-dioctanoate in serum and by aspecific porcine liver esterase

We compared the susceptibility of the two diacyl derivatives of FUdR to hydrolysis by lipolytic activity in serum and by a commercial hepatic esterase preparation. When FUdR-dipalmitate, incorporated in egg PC/PS/CHOL liposomes was incubated with serum, only 2% was hydrolyzed during a 4-h incubation (not shown). This percentage was even below 0.5% when this prodrug was incorporated in solid-type liposomes (DSCP/DPPG/CHOL). When, on the other hand, liposomal FUdR-dioctanoate was incubated with serum, the prodrug was rapidly hydrolyzed (about 60% in 1 h; Fig. 5). Because FUdR-dioctanoate exchanges readily with serum components, as described above, we examined whether hydrolysis can take place at the liposome surface or rather has to be preceded by transfer of the substrate to plasma components. Therefore, we incubated liposomal FUdR-dioctanoate with isolated porcine liver esterase in an aqueous environment without serum. Fig. 6 demonstrates that also in this case FUdR-dioctanoate is rapidly hydrolyzed.

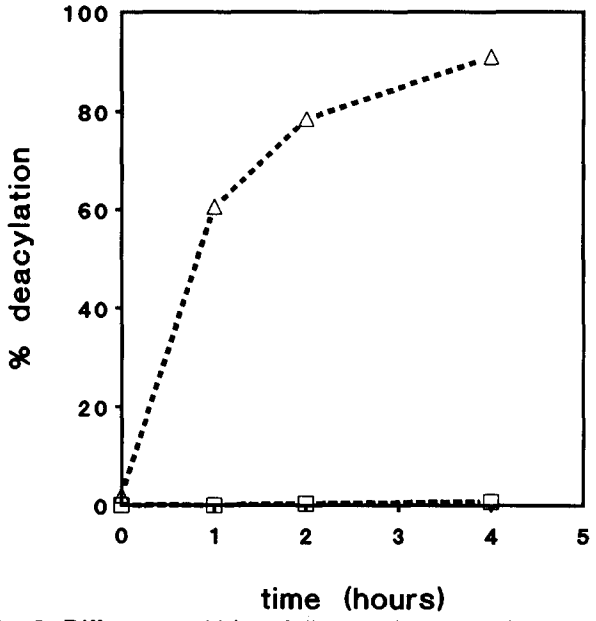

Fig. 5. Different sensitivity of liposome-incorporated FUdR-dipalmitate and FUdR-dioctanoate to deacylation in rat serum. [^{3}H]FUdR-dipalmitate (□) or [^{3}H]FUdR-dioctanoate (△) incorporated in DSPC/DPPG/CHOL liposomes was incubated with rat serum (50 μl liposome-dispersion containing 0.6 μmol total liposomal lipid/ml serum). At 0, 1, 2 and 4 h samples of the incubation mixture were extracted with chloroform/methanol. Radioactivity of the organic and of the water phase was measured and the percentage of deacylation was calculated.

Cell-growth inhibition of C26 adenocarcinoma tumor cells in vitro by FUdR and liposomal FUdR-diesters

The effects of liposome encapsulation and acyl derivatization of FUdR on its cytostatic potency towards a colon tumor cell line were investigated. The C26 adenocarcinoma cell line appears to be highly sensitive to the antiproliferative effect of free FUdR (Table II). When encapsulated in liposomes (DSPC/DPPG/CHOL), FUdR induced cell-growth inhibition equal to that of free FUdR. Also, the inhibitory potency of liposomal FUdR-dioctanoate was not significantly different from that of free FUdR. However, the dipalmitoyl ester of FUdR, incorporated in liposomes, induced cell-growth inhibition only at concentrations 2 orders of magnitude higher than any of the other forms of FUdR. Furthermore, the cell-growth inhibition caused by FUdR-dipalmitate was dependent on the liposome type: the concentration to reach 50% inhibition was between 10^{-6} and 10^{-7} M when the compound was incorporated in egg PC/PS/CHOL liposomes and was even as high as 10^{-4} M when incorporated in DSPC/DPPG/CHOL liposomes. In that concentration range liposomes not containing any drug were also found to cause cell-growth inhibition.

Deacylation of FUdR-dipalmitate and FUdR-dioctanoate by tumor cells and cell culture medium

In order to explain the results on the antiproliferative potency of the various liposomal FUdR formulations described in the previous paragraph, we deter-

TABLE II

Antiproliferative action of FUdR and diacylated FUdR on C26 adenocarcinoma tumor cells

Tumor cells were incubated for 72 h at 37°C in a 5% CO_2 air atmosphere with various concentrations of 5FU, FUdR or liposomal FUdR-derivatives. 20 h before the end of the experiment [^{3}H]thymidine was added. At the end of the experiment, the cells were harvested and the cell-associated radioactivity was measured. Data represent ^{3}H incorporation (cell proliferation) as percent of control.

Liposome composition	(pro-)Drug	Drug concentration (M)					
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
	FUdR			2±1	3± 1	66±15	95± 9
DSPC/DPPG/CHOL	FUdR			16±8	27±11	88±24	90±10
DSPC/DPPG/CHOL	FUdR				2± 1	54±10	89± 4
PC/PS/CHOL	FUdR-dipalmitate		9±5	14±4	100± 3		
DSPC/DPPG/CHOL	FUdR-dipalmitate	52±2	86±8	99±9	93± 3		

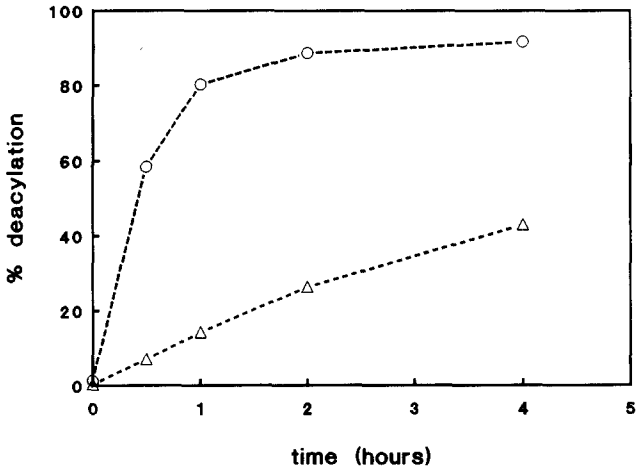

Fig. 6. Deacylation of liposome-incorporated FUdR-dioctanoate by aspecific liver esterases. [^{3}H]FUdR-dioctanoate in DSPC/DPPG/CHOL liposomes (0.16 (○) or 0.7 (△) μmol/ml total liposomal lipid) was incubated with 0.05 U of aspecific liver esterase in 1 ml HN buffer at 37°C. At 0, 1, 2 and 4 h samples of the incubation mixture were extracted with chloroform/methanol; radioactivity in organic and water phase was measured and the percentage of deacylation was calculated.

mined the susceptibility of the diacyl FUdR derivatives incorporated in liposomes towards deacylating activity in tumor cells and culture medium. FUdR-dioctanoate was far more susceptible to hydrolysis than FUdR-dipalmitate as is apparent from the results presented in Table III. Almost all added liposomal FUdR-dioctanoate is degraded to water-soluble compounds within 24 h of incubation. In contrast, only approx. 1% of FUdR-dipalmitate is hydrolyzed when the compound is incorporated in PC/PS/CHOL liposomes and even less than 0.1% when incorporated in DSPC/DPPG/CHOL liposomes. Thus, the rate of hydrolysis of FUdR-dipalmitate is strongly dependent on the liposomal lipid composition. Hydrolysis of FUdR-dioctanoate in the presence of tumor cells is more pronounced than degradation in cell culture medium under the conditions applied. Degradation of the FUdR-prodrugs in the medium is likely to be due to esterases present in the fetal calf serum supplement.

Discussion

The results presented in this paper show that FUdR, although it is water-soluble, has sufficiently lipophilic properties to allow appreciable diffusion through liposomal bilayers (at physiological pH, FUdR is virtually uncharged ($pK_a = 7.6$) [23]). The diffusion rate was shown to depend on liposome bilayer rigidity and on the presence of plasma, in agreement with generally accepted views concerning liposome permeability to solutes [24]. These findings are also compatible with results obtained by Simmons and Kramer who compared the stability of FUdR liposomes on storage in a fluid (egg PC/CHOL/dicetylphosphate) and solid (sphingomyelin/CHOL/dicetylphosphate) liposome-type [6].

To circumvent the leakage of the drug across the liposomal bilayer, we synthesized lipophilic derivatives of FUdR which, because of their incorporation in the bilayer, were expected to remain much more tightly associated with the liposome. Two lipophilic diesters were synthesized: (1) FUdR-dioctanoate, because it was reported to display good antitumor activity in vivo when administered in the lymphographic agent Lipiodol® [25–27] and (2) FUdR-dipalmitate, which already has been subject to investigation in a liposomal formulation [7–9].

The amount of FUdR-dipalmitate that can be incorporated in liposomes depends on the liposome composition. The diester can be accomodated to higher proportions in the bilayers of liposomes consisting of DSPC than in those containing egg PC as the bulk phospholipid: FUdR-dipalmitate could be incorporated upto 13 mol% in the solid-type liposome and to only 2 mol% in the fluid-type. Attempts to incorporate more prodrug resulted in the formation of prodrug aggregates remaining on the extrusion filter. Therefore, the liposomes used in our experiments contained 10 mol% in DSPC/DPPG/CH liposomes and 2 mol% in PC/PS/CH liposomes.

FUdR-dipalmitate is firmly anchored in liposomes consisting of either DSPC/DPPG/CH or egg PC/

TABLE III

Deacylation of liposomal FUdR-dipalmitate and FUdR-dioctanoate by tumor cells and medium

Samples of liposomal FUdR-dipalmitate or FUdR-dioctanoate were incubated for 24 h in medium with or without tumor cells. Medium contained 10 mol% FCS. At the end of the incubation period the medium was extracted with chloroform/methanol and radioactivity in the water (hydrolyzed FUdR-prodrug) and in the organic layer was measured. From these data, the percentage of degradation was calculated. TL, total liposomal lipid.

Liposome composition	Prodrug	Concentration TL/prodrug	% Degradation by tumor cells + medium	% Degradation by medium alone
PC/PS/CHOL	FUdR-dipalmitate	1.0/0.02 mM	0.90	0.90
DSPC/DPPG/CHOL	FUdR-dipalmitate	1.0/0.10 mM	< 0.1	< 0.01
DSPC/DPPG/CHOL	FUdR-dioctanoate	1.0/0.10 mM	91.5	
		2.5/0.25 mM	69.0	25.7

PS/CH. This is evident from the retention of this prodrug with the liposomes upon incubation with serum and from the lack of exchange between liposomal bilayers. In contrast, FUdR-dioctanoate is readily transferred to serum components (mainly albumin) and exchanges rapidly between liposomal bilayers. The 3′- and 5′-palmitoyl monoesters of FUdR also exchanged between liposomal bilayers, but far more slowly than FUdR-dioctanoate.

It was shown earlier that the hydrolysis of FUdR-prodrugs by (serum) esterases depends on the chain length of the fatty-acid residue: FUdR-dioctanoate is degraded more readily than FUdR-dipalmitate [12–14]. Our results show that this difference in hydrolysis rate is preserved when the compounds are incorporated in liposomes. Insertion in liposomal bilayers could not protect FUdR-dioctanoate from being rapidly degraded in serum (60% in 1 h), probably because of weak drug-bilayer interaction. The presence of serum components, although probably facilitating extraction of the prodrug from the bilayer, was not required for enzymatic hydrolysis of FUdR-dioctanoate to proceed because aspecific porcine liver esterases in absence of serum also induced considerable degradation of the liposomal prodrug.

The antiproliferative activity of the two FUdR-prodrugs was measured by a [^{3}H]thymidine incorporation assay. This method may result in an underestimation of cell-growth inhibition because FUdR can stimulate DNA [^{3}H]thymidine incorporation by blocking thymidylate synthase. Thymidylate synthase converts deoxyuridinemonophosphate to thymidine-5′-monophosphate and inhibition of this enzyme causes the cells to take up thymidine more avidly [29]. However, at long incubation times such as used in our experiments, cell damage is severe and stimulation of [^{3}H]thymidine incorporation is negligible. To exclude incorrect results we compared this assay with an alternative assay [30,31], which is not hampered by the problem mentioned above. In this assay total cell-associated protein is measured after precipitation with trichloroacetic acid and staining with sulfo-rhodamine B. With this assay the antiproliferative activity was consistently found slightly higher for each compound tested than with the [^{3}H]thymidine incorporation assay. Because this was found for each compound the relative antiproliferative activity between the different compounds did not change. Therefore, we conclude that the [^{3}H]thymidine incorporation assay is quite reliable under the conditions applied.

The antiproliferative activity of liposomal FUdR-dioctanoate was equal to that of free FUdR, when the drug was allowed to interact for 72 h with C26 adenocarcinoma tumor cells. This implies that FUdR-dioctanoate is either hydrolyzed outside the cells by serum esterases (the growth medium contained 10% FCS) or is internalized as such and converted to free FUdR within the cells. We have shown that both events can occur: 26% of the prodrug was degraded in the medium without tumor cells in 24 h (at 0.25 mM drug concentration) while in the presence of tumor cells 69% was hydrolyzed. We consider it more likely that FUdR-dioctanoate is degraded after transfer from the liposomes to the cells than after whole liposome uptake, in view of the observed rapid equilibration of FUdR-dioctanoate between liposomal lipid bilayers. In addition, most tumor cell lines do not actively phagocytose liposomes [3].

Liposomal FUdR-dipalmitate exhibited over 100-fold lower antiproliferative activity than either underivatized FUdR or FUdR-dioctanoate, reflecting the superior stability of this liposome-prodrug complex. The observed difference in activity between the egg PC/PS/CH and DSPC/DPPG/CH liposomal FUdR-dipalmitate formulations is noteworthy. It is probably caused by the difference in deacylation rate of FUdR-dipalmitate, which was higher when the compound was incorporated in the fluid than in the solid liposome type. In the latter, FUdR-dipalmitate is almost completely protected from esterase activity.

We conclude that liposomal preparations of FUdR are useful for in-vivo purposes, provided the liposomal composition is chosen so as to ensure retention of FUdR within the liposomes. Fluid-type liposomes with egg PC as the bulk lipid are definitely not suitable for this purpose even if fortified with cholesterol. Solid-type liposomes were found unsuitable carriers for the medium-chain derivative FUdR-dioctanoate, which is readily released from the liposomal bilayer. In contrast, the lipophilic prodrug FUdR-dipalmitate remains tightly anchored in both fluid and solid liposome types and could be incorporated upto 2 mol% and 13 mol%, respectively. The greatly limited antiproliferative effect of liposomal FUdR-dipalmitate in vitro, suggests that this formulation is not likely to be effective in a direct therapeutic approach. Nonetheless, liposomal FUdR-dipalmitate may provide an adequate drug formulation for the treatment of tumors, especially in the liver, as we showed in a recently published study [32]. Due to the avid uptake of liposomes by liver macrophages, a single injection of liposomal FUdR-dipalmitate will create a large depot of this prodrug in these cells, in close proximity of the tumor cells. Subsequent sustained release of active drug from the cells following intralysosomal deacylation will provide effective concentrations of the drug over an extended period of time [33].

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