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Controlled release of a protein kinase inhibitor UCN-01 from liposomes influenced by the particle size

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Abstract

A protein kinase inhibitor UCN-01 binds with high affinity to human α_1-acid glycoprotein (hAGP) which may compromise the drugs therapeutic effectiveness. Liposomal formulations of UCN-01 have been evaluated as a means of reducing the impact of binding to hAGP. However, in an initial study, UCN-01 was released rapidly from liposomes added to rat plasma containing hAGP. The purpose of this study was to develop a liposomal formulation of UCN-01 that only slowly released drug. Liposomes composed of lipids with a high phase transition temperature and having an average particle size of 120 nm and above reduced leaking of UCN-01 when the formulations were evaluated by adding to rat plasma containing hAGP. Furthermore, formulations composed of larger liposomes were also more effective in vivo; in tests in which liposomal preparations were injected together with hAGP into rats, more UCN-01 was retained in liposomes for 24 h after administration of 155 nm liposomes as compared to 112 nm liposomes.

Keywords: α_1-Acid glycoprotein; Release; Liposomes; Pharmacokinetics; Lamellarity

1. Introduction

UCN-01 (7-hydroxystaurosporine, (3*R**,8*S**,9*R**,10*R**,12*R**)-2,3,9,10,11,12-hexahydro-3-hydroxy-9-methoxy-8-methyl-10-(methylamino)-8,12-epoxy-1*H*,8*H*-2,7*b*,12*a*-triazadibenzo[*a*,*g*]-cyclonona[*cde*]triden-1-one, $C_{28}H_{26}N_4O_4$, Fig. 1) is a new type of anticancer drug that acts via inhibiting protein kinases (Kawakami et al., 1996; Akiyama et al., 1997). Originally UCN-01 was selected as an inhibitor of protein kinase C (PKC) but it is now known that it affects additional kinases. UCN-01 preferentially induces G1 phase accumulation in several human tumor cells and this effect is associated with dephosphorylation of the retinoblastoma protein caused by inhibition of cyclin-dependent kinase 2 (CDK2). During clinical evaluation, the plasma concentrations of UCN-01 were much higher than predicted from non-clinical studies in experimental animals (Fuse et al., 1998). The unexpectedly high plasma concentrations of UCN-01 in humans were explained by specific, high affinity binding of the compound to human α_1-acid glycoprotein (hAGP) (Fuse et al., 1999, 2000; Hedaya and Daoud, 2001). In general, only unbound drug can traverse biological membranes and produce a pharmacological effect and, therefore, the therapeutic activities of drugs usually correlate with the concentration of unbound drug in blood (Yoo et al., 1996; Chiang and Oie, 1990). Thus, high affinity binding to hAGP may prevent UCN-01 being delivered to the cytoplasm of the tumor and interacting with the target molecules, PKC and CDK2. Consequently it would be advantageous to prevent or reduce binding of UCN-01 to hAGP in order that the drug is delivered efficiently to tumor cells resulting in improved antitumor activity in patients.

Liposomes are widely used as carriers for many anticancer drugs such as doxorubicin (James et al., 1994; Gabizon et al., 1994), daunorubicin (Bellott et al., 2001; Offidani et al., 2003) and cisplatin (Newman et al., 1999). Doxil, which is a formulation of doxorubicin in stealth liposomes, has been approved for use in AIDS-related Kaposi's sarcoma and refractory ovarian cancer (James et al., 1994; Gabizon et al., 1994). The surfaces

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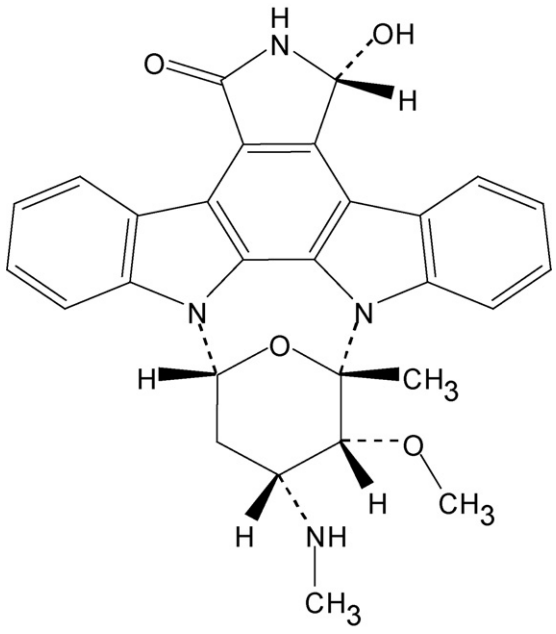

Fig. 1. Chemical structure of UCN-01.

of these stealth liposomes are covered with polyethylene glycol (PEG) and such liposomes have a long circulation half-life because the steric barrier formed by the PEG coating decreases the uptake of liposomes by the cells of the reticuloendothelial system (Allen et al., 1991). In general, encapsulation into PEG liposomes increases the efficacy and decreases the side effects of anticancer drugs because the drugs are delivered preferentially to tumor tissue due to the enhanced permeability and retention effect (Tsukioka et al., 2002). Drugs that are weak bases or acids can be concentrated efficiently in liposomes in response to a pH gradient (Mayer et al., 1990) or an ammonium sulfate gradient (Haran et al., 1993). However, the release of the drug from liposomes depends on the drug (Maurer-Spurej et al., 1999; Maurer et al., 1998). For example, doxorubicin is retained effectively in liposomes, whereas both ciprofloxacin and vincristine leak very rapidly. Liposomes that do not retain drugs cannot deliver them to the target sites efficiently; liposomes that release drug slowly have a better therapeutic activity than liposomes that release drug rapidly (Charrois and Allen, 2004). Drug released from liposomes can distribute into normal tissues and may cause side effects. Thus, in order that liposomal formulations are effective, the drug should be released slowly thereby increasing efficacy and decreasing side effects.

In our previous study, encapsulation of UCN-01 in liposomes was proposed as a possible method of avoiding the therapeutic impact of the high affinity binding of UCN-01 to hAGP (Yamauchi et al., 2005). Thus, after injection of PEG liposomes encapsulating UCN-01, the liposomes would be expected to accumulate in tumors and gradually release UCN-01; the local levels of UCN-01 would be higher than those of hAGP so that it may be possible to deliver therapeutically effective concentrations of UCN-01 to the cytoplasm of the tumor. However, in the initial study, UCN-01 was released from liposomes relatively rapidly and was then bound to hAGP if present.

In the present study, we prepared various liposomal formulations and compared the processes involved in the release of drug by monitoring the concentration of UCN-01 in different fractions after adding liposomes containing drug to rat plasma with added hAGP. Blood levels of hAGP bound drug and drug retained in liposomes were also measured after intravenous administration to rats of mixtures of UCN-01 liposomes and hAGP in order to gauge the feasibility of using liposomal preparations of UCN-01 to deliver therapeutic doses of the drug safely and effectively.

2. Materials and methods

2.1. Materials

UCN-01 was produced by a fermentation technique in our laboratories as described previously (Kawakami et al., 1996). Hydrogenated soy phosphatidylcholine (HSPC), distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC) and eggyolk phophatidylcholine (EPC) were purchased from Nippon oil and fat (Tokyo, Japan). Cholesterol (Chol) and hAGP were obtained from Sigma Chemical (St. Louis, MO). Methoxypolyethyleneglycol (Mw 2000)-distearoylphosphatidylethanolamine (PEG-DSPE) was acquired from Avanti Polar Lipids (Alabaster, AL). Sepharose CL-6B was purchased from Amersham-Pharnacia Biotech (Uppsala, Sweden). All other chemicals and solvents were of analytical grade.

2.2. Animals

Male SD strain rats, 7–8 weeks of age (Nihon SLC, Hamamatsu, Japan, 240–300 g at the start of the experiments), were housed for about 1 week under controlled conditions with free access to food and water. The Welfare Committee for Experimental Animals in our institute approved all the experiments performed in the study.

2.3. Preparation of UCN-01 liposomes

Each liposomal preparation was formed by hydrating the respective lipid with a 100 mmol/L citric acid solution (pH 4.0). When cholesterol was used, lipids containing cholesterol were dissolved in diethylether, then the solvent was evaporated to produce a thin layer of lipid that was subsequently hydrated with 100 mmol/L citric solution. The resulting liposomes were sequentially extruded through two stacked Nuclepore polycarbonate membranes of well-defined pore sizes of 0.4, 0.2, 0.1 and 0.08 μm in an extruder (Lipex Biomembranes Inc., Canada). These operations were performed at a temperature above the gel to liquid crystalline phase transition temperature of each lipid; at 25 °C to generate EPC and HSPC/Chol/PEG-DSPE (=56.5/38.2/5.3, mol/mol) liposomes, at 40 °C to produce DMPC liposomes, at 50 °C for DPPC liposomes, and 70 °C for HSPC and DSPC liposomes. Liposomes were sized after every critical extrusion to check the resulting particle size and the

extrusion was stopped when the liposomes reached a defined particle size. The liposomes were diluted with 100 mmol/L citric acid solution (pH 4.0) to give a lipid concentration of approximately 62.5 mg/mL.

UCN-01 was added to the liposome preparations to give a UCN-01-to-lipid weight ratio of 0.02 (Yamauchi et al., 2005). The exterior pH of the liposomal vesicles was then titrated to approximately 8.0 with 1.0 mol/L NaOH, thus creating a pH gradient (acidic inside) across the vesicles. Water was added to give a UCN-01 concentration of 1 mg/mL and then to encapsulate UCN-01 into liposomes, the mixture was heated for 5 min at the same temperature as used when sizing.

PEG-DSPE/ethanol solution was added to the HSPC liposomes to give a ratio of PEG-DSPE 6.7 mol%, when present, and ethanol 4 vol.% and heated for 2 min at the same temperature as used when sizing. The pH of the resultant liposome suspensions was about 7.

The concentration of UCN-01 was measured by high-performance liquid chromatography (HPLC) (Kurata et al., 1998). The concentrations of lipids were determined using the phosphorus assay (Wako, Tokyo, Japan). The mean size of the liposome vesicles was measured by dynamic light scattering (ELS-800, Otsuka electronics, Osaka, Japan). Encapsulation efficiency was determined using ultracentrifugation (Optima™ XL-A, Beckman, Fullerton, CA); liposomes were centrifuged for 1 h at approximately 110,000 × *g*, the supernatant containing unencapsulated drug was removed for determination of UCN-01 and these data, together with knowledge of the total concentration of drug in the liposomal preparations, were used to calculate the encapsulation efficiency.

Liposomes for use in PK studies were diluted with four volumes of water and centrifuged for 1 h at 110,000 × *g*. The supernatant containing unencapsulated UCN-01 and excess citric acid was removed and the liposomal pellet was resuspended in 20 mmol/L citric acid solution (pH 7) to provide a preparation containing 1 mg/mL UCN-01.

2.4. Microscopy observation

A drop of UCN-01 liposomal preparations (HSPC/PEG-DSPE, 93.3/6.7, mol/mol, 98 or 153 nm) was placed on a specimen carrier and then rapidly frozen by immersion in slush liquid nitrogen. The specimen carrier was placed on a cooled sample holder, and transferred to a Freeze Fracture and Freeze Etching Equipment, JFD-9010 (JEOL, Tokyo, Japan). Immediately following fracture at −120 °C, the sample was coated using a platinum/carbon electrode at a 60° and 90° angle. Replicas were removed by floating in chloroform/methanol, cleaned by rinsing with chloroform/methanol, placed on a 300 or 400-mesh copper grid, and then examined under a JEM-1010 electron microscope (JEOL, Tokyo, Japan) at 100 kV (Kumar et al., 1988).

2.5. ^{31}P NMR analysis

^{31}P NMR spectroscopy of HSPC liposomes (85 and 140 nm) was performed in the presence and absence of aqueous praseodymium chloride ($PrCl_3$) in order to determine the number of membranes in each liposomal preparation (Fröhlich et al., 2001). In general, Pr^{3+} ions cannot permeate lipid membranes so adding $PrCl_3$ results in a shift of the spectra downfield, thereby distinguishing the different groups of phospholipid. The final concentration of both HSPC and $PrCl_3$ in 40% D_2O was 39 mmol/L. Spectra were recorded using a Bruker DRX500 spectrometer (Karlsruhe, Germany) with a ^{31}P-probe, at 202.46 MHz using the following parameters: acquisition time, 0.49 s; spectrum width, 33,333 Hz; data size, 32 K complex; 90° pulse width, 10 μs; relaxation delay, 2 s; number of acquisitions, 737; chemical shift reference, external H_3PO_4; temperature, 60 °C. ^{31}P NMR spectroscopy of HSPC liposomes revealed a peak that was shifted downfield, which was attributed to HSPC-headgroups accessible to $PrCl_3$, i.e. the outer leaflet of the outermost bilayer. In contrast, the peak remaining at about 2 ppm were HSPC-headgroups inaccessible to $PrCl_3$, i.e. the inner leaflet of the outermost bilayer and both the inner and outer leaflet of the other bilayer. The in/out ratio was estimated from the ratio of intensities of shifted versus non-shifted ^{31}P-resonances when $PrCl_3$ was added.

2.6. In vitro release experiments

UCN-01 liposomes (100 μg/mL UCN-01) were diluted with 99 volumes of rat plasma supplemented with hAGP and incubated at 37 °C. The final concentrations of UCN-01 and hAGP in the mixtures were 1 μg/mL and 0.5 mg/mL, respectively (i.e. the approximate UCN-01:hAGP molar ratio was 1:6; Mw of UCN-01 and AGP are 482.5 and 42,000, respectively); a concentration of 0.5 mg/mL hAGP was selected as this is similar to the concentration of the protein in normal human plasma (Kremer et al., 1988). Aliquots were removed at different times for determination of liposomal and protein-bound UCN-01; liposomal UCN-01 was separated from hAGP-bound UCN-01 and free UCN-01 by Sepharose CL-6B gel chromatography and the concentration of drug in each fraction was measured by HPLC (Kurata et al., 1998).

2.7. Rat PK

Aliquots of UCN-01 liposomal preparations (HSPC/PEG-DSPE, 93.3/6.7, mol/mol, 112 or 155 nm) were diluted with physiological saline to provide solutions containing 0.175 mg/mL UCN-01 as described previously (Yamauchi et al., 2005). Similarly, aliquots of UCN-01 liposomes were mixed with a solution of hAGP to give mixtures containing 0.175 mg/mL UCN-01 and 76.5 mg/mL hAGP. Each of these four solutions was administered rapidly to rats via the tail vein at a volume of 2 mL/kg. The doses of UCN-01 and hAGP were 0.35 and 153 mg/kg (0.725 and 3.625 μmol/kg), respectively. For the analysis of the blood concentrations of UCN-01, approximately 0.3 mL blood was withdrawn from the jugular vein using heparinized syringes at 0.083, 1, 3, 6 and 24 h after dosing. The concentration of total UCN-01 in each blood sample was measured by HPLC (Kurata et al., 1998) and the percentage of the injected dose present in the blood compartment was calcu-

lated assuming that the blood volume of a 250 g rat is 13.5 mL (Dvies and Morris, 1993). Also, a portion of each blood sample was immediately applied to a column of Sepharose CL-6B, as described for the in vitro release experiments. The concentrations of UCN-01 in the hAGP-bound, liposomal and free fractions were measured by HPLC.

2.8. Data analysis

All data are presented as the mean ± S.D. of three animals or experiments. The pharmacokinetic parameters in rats were calculated from the individual blood concentration–time curves after intravenous injection by a non-compartment model using the pharmacokinetics software package WinNonlin^{TM} Professional Ver. 4.0.1 (Pharsight Co., Mountain View, CA). The areas under the blood concentration–time curves ($AUC_{0-\infty}$) were calculated using the trapezoidal rule and by extrapolating the time to infinity using the elimination rate constant (k_{el}) values. The elimination half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$. The total body clearance (CL_{tot}) was calculated from dose/$AUC_{0-\infty}$. The mean residence time (MRT) was calculated as AUMC/AUC (AUMC is the area under the first moment curve). The volume of distribution at steady-state (V_{dss}) was calculated as CL_{tot}·MRT. Statistical significance was estimated by using the Student's *t*-test with equal variance recognized by the *F* test or the Aspin–Welch test with unequal variance by the *F* test. *p* value of less than 0.05 were considered statistically significant.

3. Results

3.1. Preparation of UCN-01 liposomes

Formulations of UCN-01 in liposomes were prepared successfully using the procedure described in Section 2. Neither the lipid used to prepare the liposomes nor the average size of the liposomes affected the uptake of the drug. The encapsulation efficiencies were greater than 95%.

3.2. Microscopy observation

UCN-01 liposomes composed of HSPC/PEG-DSPE, which were 98 and 153 nm as determined by dynamic light scattering, were examined using freeze-fracture electron microscopy. The electron micrographs revealed the presence of spherical liposomes (Fig. 2), the size and polydispersity of which were in reasonable agreement with the dynamic light scattering data. The smaller (98 nm) liposomes were comprised of unilamellar vesicles (Fig. 2a), whereas the larger (153 nm) liposomes exhibited a freeze-fracture behaviour that is characteristic of oligolamellar lipid structures (Fig. 2b).

3.3. ^{31}P NMR analysis

Attempts to determine the number of membranes in a preparation of HSPC/PEG-DSPE liposomes (HSPC/PEG-DSPE, 93.3/6.7, mol/mol) with encapsulated UCN-01 using ^{31}P NMR failed because transmembrane permeation of Pr^{3+} occurred during the time taken to acquire spectra thereby compromising interpretation. Therefore, the ^{31}P NMR spectra of HSPC liposomes (85 and 140 nm) were acquired (Fig. 3) instead of using HSPC/PEG-DSPE liposomes with encapsulated UCN-01. It was assumed that the presence of UCN-01 and PEG caused the change of membrane permeability to Pr^{3+} but that the presence of UCN-01 and PEG would not change the number of membranes because the particle size of the liposomes was not significantly different between HSPC liposomes and HSPC/PEG-DSPE liposomes with encapsulated UCN-01. Therefore, it is considered that the data generated using HSPC

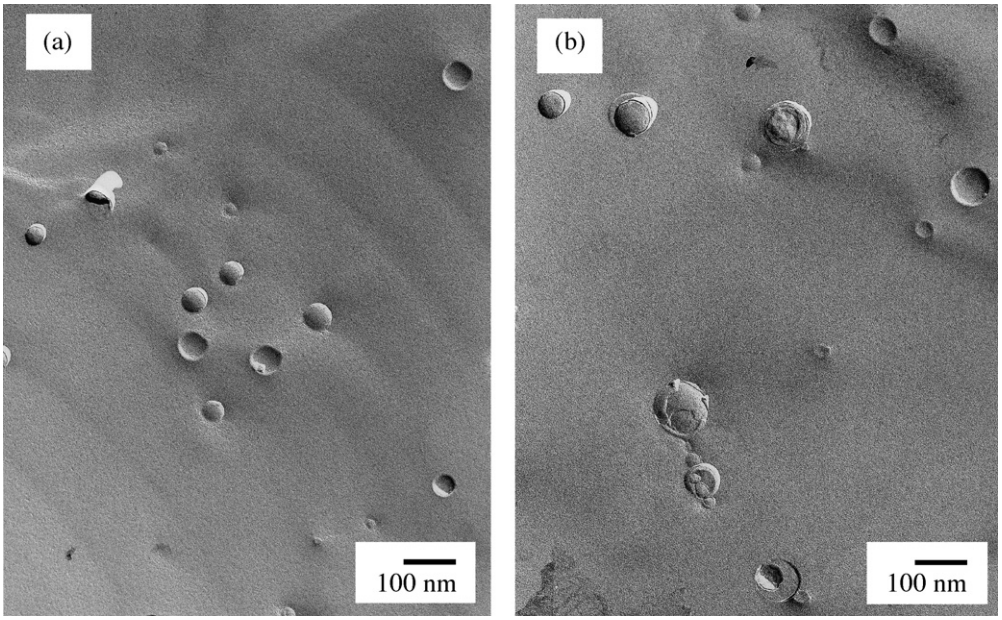

Fig. 2. Freeze-fracture electron micrographs of UCN-01 liposomes (HSPC/PEG-DSPE, 93.3/6.7, mol/mol) (a) 98 nm and (b) 153 nm. The mean size of the liposome vesicles was measured by dynamic light scattering.

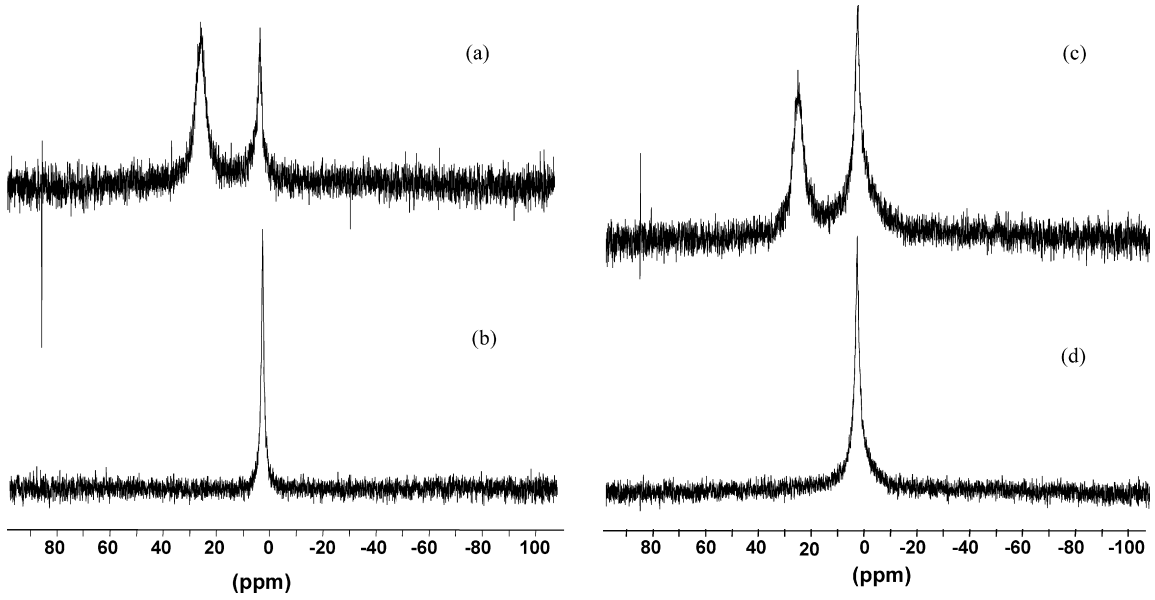

Fig. 3. ^{31}P NMR spectra of HSPC liposomes after (a and c) and before (b and d) addition of $PrCl_3$. 'In' and 'out' correspond to phospholipid-headgroups inaccessible or accessible, respectively, to the reagent. (a and b) 85 nm liposomes and (c and d) 140 nm liposomes.

only liposomes has relevance to HSPC/PEG-DSPE liposomes with encapsulated UCN-01. Addition of $PrCl_3$ resulted in a shift of the spectra downfield, which was attributed to differences in the signals for the outer and inner compartments of the vesicles. The signal that was shifted downfield was ascribed to phospholipid (PL)-headgroups accessible to $PrCl_3$, i.e. the 'out'-side of the vesicles. In contrast, the signal remaining at about 2 ppm was due to PL-headgroups that were inaccessible to $PrCl_3$, i.e. 'in'-side of the vesicles. The in/out ratio of PLs was estimated from the intensities of ^{31}P-resonances following addition of $PrCl_3$, and these ratios for the 85 and 140 nm liposomes were 0.7 and 1.3, respectively. Assuming that all of the vesicles were unilamellar, the thickness of the outer monolayer was 2.1 nm ($=t_o$), the thickness of the inner monolayer was 1.6 nm ($=t_i$), the occupancy volume (v) per phospholipid molecule was the same for the inner and outer monolayers (Huang and Mason, 1978) and did not depend on the vesicle size (r) and lipid composition, then the in/out ratio for liposomes was calculated by:

$$\text{in/out} = \frac{[(4/3)\pi((r-2t_o)/2)^3 - (4/3)\pi((r-2(t_o+t_i))/2)^3]/v}{[(4/3)\pi(r/2)^3 - (4/3)\pi((r-2t_o)/2)^3]/v}$$

The in/out ratio for the 85 and 140 nm liposomes was calculated to be 0.695 and 0.722, respectively. The in/out ratio for the 85 nm liposomes was similar to the calculated figure indicating that these are unilamellar. However, there was a significant discrepancy between the measured and calculated ratios for the larger liposomes (140 nm) implying that these were oligolamellar. Assuming that all of the vesicles had two lipid bilayers and the thickness of the first aqueous layer was 2 nm, the in/out ratio for 140 nm liposomes was calculated to be 2.2. Therefore, 140 nm liposomes were mixture of unilamellar and oligolamellar. This conclusion is consistent with the data generated using microscopy.

3.4. In vitro release experiments

Rapid release of UCN-01 from liposomes was observed when 112 nm liposomes were incubated in rat plasma containing hAGP (Fig. 4). A slower release of UCN-01 from 155 nm liposomes was observed (Fig. 4); after 3, 6 and 24 h incubation, the retention of UCN-01 was 26.5%, 12.3% and 2.2%, and 51.2%, 28.0%, and 12.8% in 112 and 155 nm liposomes, respectively. Thus, the release of UCN-01 from liposomes is influenced markedly by the particle size.

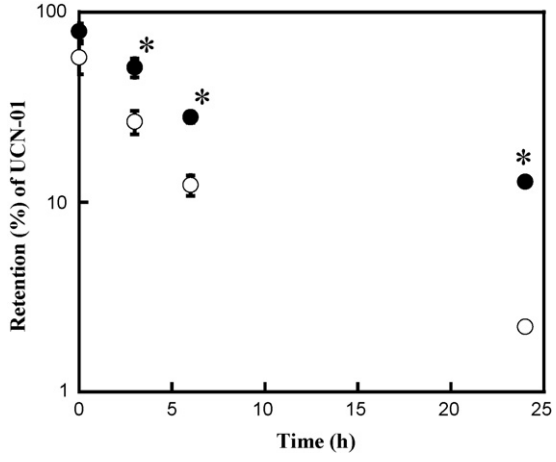

Fig. 4. In vitro retention of UCN-01 in liposomes (HSPC/PEG-DSPE, 93.3/6.7, mol/mol) of size 112 nm (◯) and 155 nm (●). Retention was measured after incubation in rat plasma supplemented with hAGP at 37 °C. UCN-01:hAGP = 1:6 (mol ratio), [UCN-01] = 1 μg/mL, [Lipids] = 50 μg/mL. Data represent the mean ± S.D. from three experiments. *$p < 0.05$, significantly different from 112 nm liposomes.

3.5. Effect of particle size on release of UCN-01 from liposomes

The retention of UCN-01 obtained 0 and 3 h after adding liposomes to hAGP-enriched rat plasma revealed that UCN-01 in the liposomal fraction increased with increasing particle size. The retention of UCN-01 improved significantly when UCN-01 was encapsulated in HSPC, DSPC and HSPC/PEG-DSPE liposomes with an average particle size of more than 120 nm. In the case of the liposomes with two or more membranes, the release of the drug was suppressed relative to unilamellar liposomes, presumably because UCN-01 in the first aqueous layer is released rapidly and UCN-01 in deeper aqueous layers retained. However, no improvement in the retention was observed in EPC, DMPC, DPPC and HSPC/Chol/PEG-DSPE liposomes even when UCN-01 was encapsulated in larger liposomes (Fig. 5).

3.6. Rat PK

After intravenous administration of UCN-01 liposomes, the blood level of total UCN-01 (i.e. the sum of the concentrations of drug in liposomes and bound to protein, as well as free drug) was much higher than that after administration of UCN-01 solution as described before. After intravenous administration of 155 nm UCN-01 liposomes, the blood level of total UCN-01 was higher than that after administration of 112 nm UCN-01 liposomes. After bolus intravenous administration of 112 and 155 nm UCN-01 liposomes, the blood level of total UCN-01 was 66.2% and 74.8% of the injected dose at 5 min after injection and 2.4% and 6.2% at 24 h (Fig. 6). The $AUC_{0-\infty}$ of total UCN-01 after injection of 155 nm UCN-01 liposomes was 1.4 times that after injection of 112 nm UCN-01 liposomes. The $t_{1/2}$ and MRT of total UCN-01 were longer and the $AUC_{0-\infty}$ was larger after administration of the 155 nm liposomal formulation of the drug relative to the respective parameters after administration of 112 nm UCN-01 liposomes (Table 1). The CL_{tot} of total UCN-01 was lower after administration of 155 nm liposomal formulation of the drug than after administration of 112 nm UCN-01 liposomes. A previous study demonstrated that the plasma PK of liposomes does not depend on the particle size of the liposomes when the size is within the 82–154 nm range (Charrois and Allen, 2003). Therefore, the differences between the pharmacokinetic parameters observed after administration of drug encapsulated

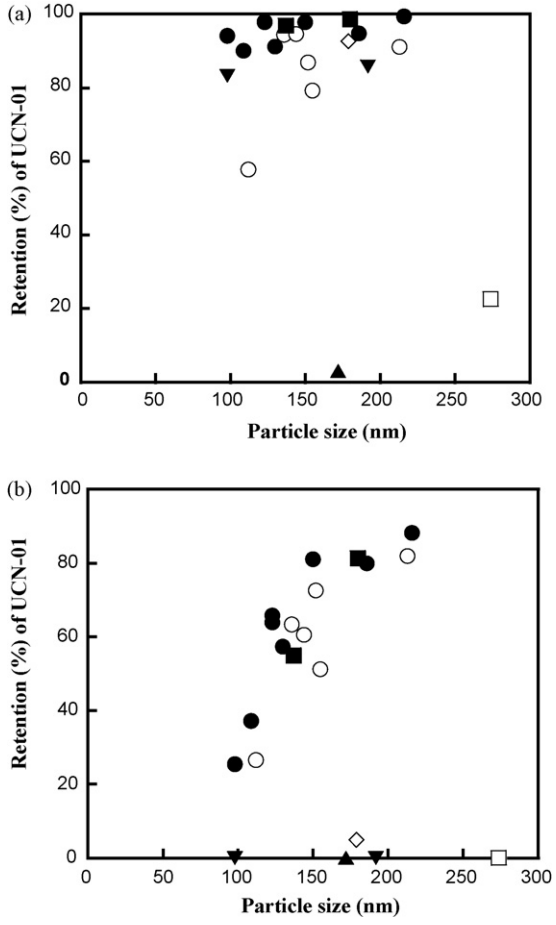

Fig. 5. Retention of UCN-01 in liposomes composed of HSPC (●), HSPC/PEG-DSPE (◯), DSPC (■), EPC (□), DMPC (▲), DPPC (◊) and HSPC/Chol/PEG-DSPE (▼). Retention was measured after incubation at 37 °C in rat plasma supplemented with hAGP for (a) 0 h and (b) 3 h. UCN-01:hAGP = 1:6 (mol ratio), [UCN-01] = 1 μg/mL, [Lipids] = 50 μg/mL.

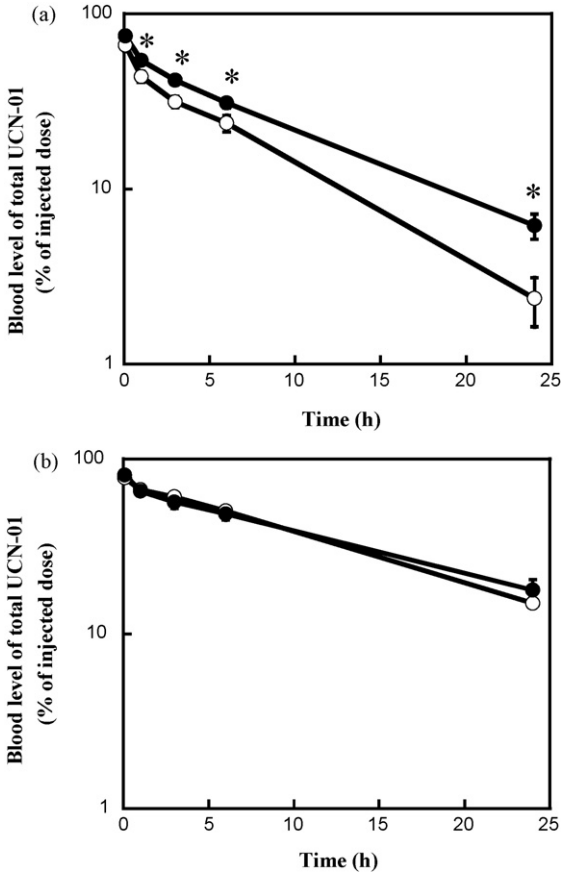

Fig. 6. Blood level of total UCN-01-time profiles after intravenous administration to rats of UCN-01 liposomes (HSPC/PEG-DSPE, 93.3/6.7, mol/mol) of 112 nm (◯) and 155 nm (●) (UCN-01; 0.35 mg/kg) (a) and together with hAGP (UCN-01; 0.35 mg/kg, hAGP; 153 mg/kg, UCN-01:hAGP = 1:5 (mol ratio)) (b). Data represent the mean ± S.D. from three animals. *$p < 0.05$, significantly different from 112 nm liposomes.

Table 1
Pharmacokinetic parameters of UCN-01 liposomes (HSPC/PEG-DSPE, 93.3/6.7, mol/mol) of (a) 112 nm and (b) 155 nm

	$t_{1/2}$ (h)	C_{max} (μg/mL)	$AUC_{0-\infty}$ (μg h/mL)	MRT (h)	CL_{tot} (L/h/kg)	V_{dss} (L/kg)
Without hAGP						
UCN-01 liposomes 112 nm	5.53 ± 0.54	4.29 ± 0.33	30.4 ± 3.4	6.23 ± 0.60	0.012 ± 0.001	0.072 ± 0.005
UCN-01 liposomes 155 nm	7.68* ± 0.84	4.85 ± 0.17	43.4* ± 1.6	9.06* ± 1.17	0.008* ± 0.0003	0.073 ± 0.008
With hAGP						
UCN-01 liposomes 112 nm	10.4 ± 0.6	5.08 ± 0.11	76.7 ± 1.6	13.3 ± 0.9	0.005 ± 0.00009	0.061 ± 0.003
UCN-01 liposomes 155 nm	12.5 ± 1.2	5.26 ± 0.10	82.7 ± 9.6	16.4 ± 1.7	0.004 ± 0.00046	0.070 ± 0.005

* Shown are: $p < 0.05$, compared with UCN-01liposomes 112 nm without hAGP.

in 112 and 155 nm liposomes can be attributed to variations in the retention of the drug in the liposomes.

As described previously (Yamauchi et al., 2005), coadministration of UCN-01 liposomes with hAGP did alter the PK parameters compared to the parameters obtained in the absence of human protein (Table 1). However, the PK parameters obtained after administration of 112 and 155 nm liposomes together with hAGP were not significantly different (Table 1). As shown in Fig. 6, coadministration of UCN-01 liposomes with hAGP to rats slightly, but not significantly, increased the total UCN-01 blood level. The $AUC_{0-\infty}$ of UCN-01 after injection of 155 nm UCN-01 liposomes with hAGP was 1.1 times that after injection of 112 nm UCN-01 liposomes with hAGP.

3.7. In vivo release experiments

As described above, the blood levels of total UCN-01 were similar after intravenous administration to rats of 112 nm UCN-01 liposomes with hAGP and 155 nm UCN-01 liposomes with hAGP (Fig. 6). However, measurement of total UCN-01 did not reveal whether UCN-01 was retained in liposomes and how much drug was bound to hAGP. In order to clarify the distribution of the drug in blood, aliquots of blood samples were subjected to Sepharose CL-6B gel chromatography as described in Section 2. When UCN-01 in 112 nm liposomes was administered with hAGP, the proportion of UCN-01 in liposomes gradually decreased with time; 5 min, 6 and 24 h after injection of UCN-01 liposomes plus hAGP to rats, 81.4%, 35.3% and 9.5%, respectively, of the UCN-01 in the blood was retained within liposomes (Fig. 7). More UCN-01 was retained in liposomes for longer after coadministration of UCN-01 in 155 nm liposomes with hAGP; 5 min, 6 and 24 h after injection of UCN-01 liposomes plus hAGP to rats, 88.0%, 53.6% and 24.6%, respectively, of the UCN-01 in the blood was retained within liposomes (Fig. 7).

4. Discussion

In our previous study, encapsulation of UCN-01 in liposomes was proposed as a possible method of avoiding the therapeutic impact of the high affinity binding of UCN-01 to hAGP (Yamauchi et al., 2005). Thus, after injection of PEG liposomes encapsulating UCN-01, the liposomes would be expected to accumulate in tumors and gradually release UCN-01; the local levels of UCN-01 would be higher than those of hAGP so that it may be possible to deliver therapeutically effective concentrations of UCN-01 to the cytoplasm of the tumor. However, in the initial study, UCN-01 was released from liposomes relatively rapidly which might be expected to result in an inadequate therapeutic improvement. Therefore, the present study was undertaken to explore methods to prepare liposomal formulations of UCN-01 that would release the drug more slowly thereby potentially improving the clinical effectiveness of the drug.

Many methods for improving the retention of drugs in liposomes have been reported. For example, Maurer-Spurej et al. improved the retention of ciprofloxacin and vincristine by substitution of the anionic lipid distearoylphosphatidylglycerol for distearoylphosphatidylcholine in the large unilamellar vesicle bilayer (Haran et al., 1993). However, in general, the addition of anionic lipids causes the rapid elimination of the liposomes from blood (Chiu et al., 2001). Semple et al. reported that liposomes composed of sphingomyelin and cholesterol improved retention of vinorelbine and vincristine (Semple et al., 2005). Our attempts to develop a liposome formulation to improve retention of UCN-01 were restricted to the use of those lipids that are used in marketed formulations, such as EPC, DMPC and HSPC.

Presumably diffusion across the individual bilayers of multilamellar vesicles occurs at similar rates and hence the overall release rate would be sum of permeation across each bilayer.

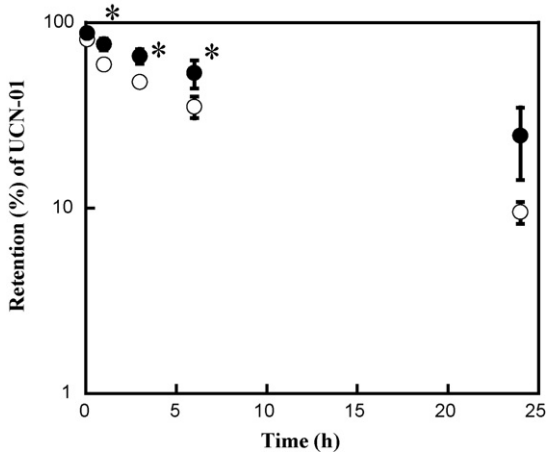

Fig. 7. In vivo retention of UCN-01 in liposomes (HSPC/PEG-DSPE, 93.3/6.7, mol/mol) of 112 nm (◯) and 155 nm (●). Retention was measured after intravenous administration to rats of UCN-01 liposomes with hAGP. UCN-01:hAGP = 1:5 (mol ratio), UCN-01; 0.35 mg/kg, hAGP; 153 mg/kg. Data represent the mean ± S.D. from three animals. *$p < 0.05$, significantly different from 112 nm liposomes.

Liposomes with multiple lipid membranes are thought to result in a more effective barrier thus increasing the retention of drugs and consequently in the present study, multilamellar as well as unilamellar liposomes were considered in the experimental approach to achieving a high-retention liposomal formulation of UCN-01. Extrusion of vesicles through a 0.1 μm filter results primarily in unilamellar vesicles whereas a high proportion of liposomes remain multilamellar when the vesicles are passed through a 0.2 μm polycarbonate filter (Hope et al., 1985). Our study confirmed that 0.1 μm-filtered liposomes are unilamellar and 0.2 μm-filtered liposomes are oligolamellar by both electron microscopic observation and ^{31}P NMR spectroscopic investigation. Retention of UCN-01 was improved by encapsulating the drug into 0.2 μm-filtered liposomes as compared to 0.1 μm-filtered liposomes (Fig. 4). A possible explanation for this observation is as follows: When UCN-01 is loaded into multilamellar liposomes by the pH gradient method, UCN-01 would initially enter the first aqueous layer and this would increase the pH of the aqueous layer thus producing a pH gradient between the first aqueous layer and the next layer. UCN-01 would then move to the next layer and the cycle would repeat itself until the drug reached the core of the liposome. As a result, UCN-01 would need to pass through multiple lipid membranes in order to be released from multilamellar liposomes. Therefore, it is logical that these liposomes would increase retention of UCN-01.

An investigation of the relationship between the retention property and the particle size of liposomes revealed that retention was improved as the particle size increased. Liposomes having an average particle size of 120 nm or more reduced leaking of UCN-01.

The release of drugs from liposomes in blood is generally caused by serum proteins such as high-density lipoproteins (HDL) (Allen and Cleland, 1980; Gregoriadis, 1995). HDLs remove phospholipid molecules from the vesicle bilayer thereby causing gaps to appear through which the drug can pass. The release of UCN-01 from liposomes is also likely to be influenced by the presence of the soluble protein, hAGP, as ideal sink conditions would exist on the outside of the liposome due to the high affinity binding of the drug to hAGP. Oligolamellar liposomes would be more effective in protecting the release of UCN-01 caused both by HDLs and hAGP than unilamellar liposomes. No improvement in the retention of UCN-01 was observed in oligolamellar liposomes whose phase transition temperatures were low (EPC, DMPC, DPPC liposomes) nor in liposomes which contained cholesterol. In general, factors that lead to decreased order in the lipid bilayer, such as acyl chain shortening, acyl chain unsaturation (Cullis et al., 1997) or the addition of cholesterol to liposomes with high phase transition temperatures (DSPC, HSPC liposomes) (Gier et al., 1969), result in more rapid release properties. The effect of these factors to increase the release of UCN-01 probably negates the decreased release of drug caused by increasing the particle size, although further research will be needed to clarify the relation between the transition temperature of each lipid and the increased lamellarity of liposomes caused by increasing the particle size.

As described before, the blood levels of total UCN-01 and PK parameters were similar after intravenous administration to rats of UCN-01 solution with hAGP and UCN-01 liposomes with hAGP. However, the distribution of UCN-01 in the blood was quite different. When UCN-01 solution was administered with hAGP, all the UCN-01 in blood bound to hAGP at all time points. On the other hand, when UCN-01 liposomes were administered with hAGP, part of the UCN-01 was retained in liposomes. In the present study, a similar phenomenon was observed. The blood levels of total UCN-01 and PK parameters were similar after intravenous administration to rats of UCN-01 liposomes 112 and 155 nm. However, the distribution of UCN-01 in the blood was quite different (Fig. 7). More UCN-01 was retained in liposomes for 24 h after administration of 155 nm liposomes as compared to 112 nm liposomes. Therefore, larger liposomes may enable delivery of more UCN-01 to the tumor in the presence of hAGP.

In future studies, we plan to investigate whether larger UCN-01 liposomes containing multiple lipid bilayers improve the antitumor activity of the drug when hAGP is present and to clarify whether variations in hAGP levels have any effect on the efficacy. We would like to study whether other drugs are also retained or not when they are encapsulated in larger liposomes.

In summary, the present study demonstrated that encapsulation of UCN-01 into liposomes was a viable method of formulating UCN-01 and that liposomes having an average particle size of at least 120 nm and with a high phase transition temperature are most effective. After a mixture of these liposomes and hAGP was injected into rats, UCN-01 was retained in liposomes for a long time. Therefore, it is feasible that more drug can be delivered to the site of action, tumors, by administering the drug in large liposomes. It is hoped that these liposomal formulations of UCN-01 will offer improved clinical effectiveness and safety profile relative to the intravenous administration of the simple solution that has been evaluated clinically thus far.

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