Optimum Formulation of Thermo-Sensitive Liposome for Targeted Tumor Drug Delivery

熱感受性リポソームを用いたＤＤＳの開発研究　～薬物の腫瘍へのターゲッティング～

Katsumi Iga

(1)

Optimum Formulation of Thermosensitive Liposome for Targeted Tumor Drug Delivery

熱感受性リポソームを用いたＤＤＳの開発研究　～薬物の腫瘍へのターゲッティング～

Katsumi Iga

伊　賀　勝　美

Optimum Formulation of Thermosensitive Liposome for Targeted Tumor Drug Delivery

A Thesis
Submitted to the Faculty
of
Pharmaceutical Sciences
of
Tokyo University

by

Katsumi Iga

1991

Acknowledgement

This study was conducted as part of the targeted liposome drug delivery system research in the Central Research Division of Takeda Chemical Industries, Ltd.

I gratefully acknowledge the following people at Takeda for their various contributions and support during the study: Dr. N. Hamaguchi, Dr.Y. Igari, Dr. Y. Ogawa, Mr. K. Gotoh, Dr. K. Ootsu, Dr. H. Toguchi, Dr. T. Shimamoto, and the many members of the project for their collaboration and helpful discussion; Mrs. K. Asano and Mrs. S. Yonezawa for their generous assistance; Mr. J. A. Hogan for smoothing out my English and Dr. K. Morita, Dr. Y. Sugino and Dr. M. Nishikawa for their constant support and encouragement.

I also wish to express my sincere gratitude to Professor Dr. T. Iga of Tokyo University for reviewing the thesis and for his valuable suggestions and guidance.

September 1991
Katsumi Iga

Contents

Chapter 3 Clearance Kinetics and Tissue Distribution in Rats

Chapter 4 Increased Cisplatin Levels in Heated Tumors in Mice

Chapter 5 Heat-Induced Drug Release Rate and Maximal Targeting Index

Chapter 6 Enhanced Antitumor Activity in Mice

Chapter 7 General Conclusion

Abbreviations

AAS	Atomic Absorption Spectrometry.
AUC	Area Under Curve.
BUN	Blood Urea Nitrogen.
CDDP	Cisplatin.
6-CF	*6*-Carboxyfluorescein.
DDS	Drug Delivery System.
DDTC	Sodium Diethyldithiocarbamate.
DPPC	Dipalmitoylphosphatidylcholine.
DPPG	Dipalmitoylphoshatidylglycerol.
DSPC	Distearoylphosphatidylcholine.
HT	Hyperthermia.
LUV	Large Unilamellar Vesicles.
MLV	Multilamellar Vesicles.
Pt	Platinum.
PTC	Positive Temperature Coefficient Thermister.
RES	Reticuloendothelial System.
REV	Reverse Phase Evaporation Vesicles.
RT	Room Temperature.
SDP	Size-Distribution Processor.
SMT	Sodium Stearoylmethyltaurate.
SUV	Small Unilamellar Vesicles.
TDD	Targeted Drug Delivery.
TDDS	Targeted Drug Delivery Systems.
TI	Targeting Index.

Chapter 1 Introduction

1.1 Targeted Drug Delivery Systems

The concept of targeted drug delivery systems (TDDS) [20,23,51,89,92] originates from the desire to increase the therapeutic index of a drug whose *in vivo* efficacy is small as compared to its side effects. TDDS employ special drug carriers to direct the drug to its site of action so as to obtain an optimal pharmacological effect at a minimum dose.

The carriers currently being studied include soluble macromolecular carriers such as monoclonal antibodies and paticulate carriers such as liposomes.[92] However, it has not been long since these carriers were first proposed for TDDS, and none has been put to practical use in therapy. Once a great deal of attention has been focused on the antibody TDDS as a magic bullet.[92] However, the carrier molecule which is too large to extravasate does not guarantee that this TDDS will result in high *in vivo* affinity of the drug for the targeted site. The same is true for TDDS using particulate carriers.[92]

1.2 DDS Using Liposomes

Liposomes are vesicles composed of lipid bilayers that enclose an aqueous volume. Since they were used by Bangham,[40] liposomes have been extensively studied as carriers of therapeutic agents. Techniques of drug encapsulation in liposomes have progressed greatly.[58,87,88] However, with some exceptions (macropharge targeting [4,7,72,82]), liposomal TDDS [81] have not been successfull, mainly because the liposomes do not have the ability to direct the drug to the targeted site.[12,16,17,22,73,101,109] The encapsulation of a toxicic drug in liposomes can mask the toxicity of the drug. In fact, the encapsulation of certain antitumor agents [46,60,78,83,91,] and antibiotics [42,48,62] in liposomes have been proposed as a way of reducing the toxic effects of free drugs on normal organs. The encapsulation of the drug in liposomes can also prolong the time the drug circulates in the blood. However, we cannot expect such liposomes to exhibit a high TDD effect.
[24,25,43,70,71,79,81,103]

Some workers have tried to endow liposomes with targeting ability by conjugating small unilamellar vesicles (SUV) with an antibody.[57] However, this trial has proven unsuccessful partly because the liposomes cannot easily extravasate and therefore cannot to direct the drug to the targeted site.

1.3 Combination of Thermosensitive Liposomes and Hyperthermia

The concept of target-stimulated local drug release has been proposed as a method to create more usefull liposomal TDDS.[15,47,86,93,98] In this system, local drug release is designed to occur in response to small physiological changes at the target or in the blood adjacent to the target, and therefore extravasation of the administered liposomes is not necessary. Thermosensitive liposomes [95-100,104-107] and *p*H sensitive liposomes[15] are typical examples.

Thermosensitive liposomes are designed to release the drug in response to local hyperthermia (HT: tumor heating at temperatures of 41°C to 45°C).[18,56] Generally, the lamellar structure of the liposomal membrane at the gel-to-liquid-crystalline transition phase is somewhat loose and porous. Thus, when a liposome is heated to the phase-transition temperature, it should release its content (*Fig. 1.1*).[39] The use of thermosensitive liposomes facilitates this.[95-100,104-107] In order to achieve a high TDD effect, the liposomes should release the drug in the short time that they travel concurrently with the blood through the targeted tumor (*Fig. 1.2*). However, the early thermosensitive liposomes prepared using SUV, did not show a good release rate.[105] One reason may be that SUV have too large a membrane curvature to get an accute phase transition. In this respect, the appropriate choice of liposomal type is a key factor.

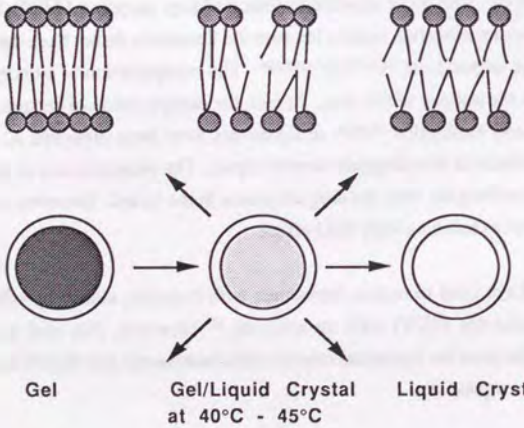

Fig. 1.1 Illustration of the phase-transition induced drug release from liposome.

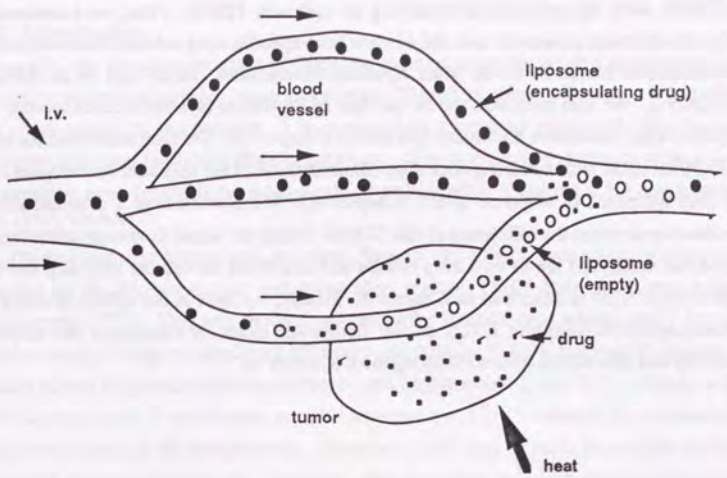

Fig. 1.2 Illustration of thermosensitive liposome and hyperthermia mediated tumor drug targeting.

1.4 Pharmacokinetic Consideration of TDD

A common problem in almost all TDDS currently being studied is the lack of pharmacokinetic parameter analysis in relation to the TDD effect.[6,12,13,27,28,85] However, this analysis is very important if we are to learn the uses and limitations of TDDS. For example, the TDD efficiency of thermosensitive liposomes will be determined not only by the local drug release rate but also by the systemic clearance of the free drug. If the clearance of the free drug is very small, the drug concentration at the targeted site after liposome administration will not be different from that after the administration of a conventional dosage form. The TDD efficiency also depends on the systemic clearance of the liposomes, as the amount of drug released at the targeted site is a function of the release rate and the liposome concentration in the blood. Thus, the pharmacokinetic parameter analysis affords us a criterion for the systemic clearance of the drug and the liposomes.

1.5 Aim of the Present Study

In the present study,[5,31-38,66] we focussed on thermosensitive LUV encapsulating cisplatin (CDDP), with the intention of obtaining an optimum TDDS. First, we examined the physicochemical properties and the *in vitro* heat-specific drug release characteristics of liposomes in search of an optimum liposome formulation, which will be described in *Chapter 2*. We also examined the *in vivo* fate of liposomes (systemic clearance and RES uptake) after intraveous administration to rats (*Chapter 3*). We then examined the tumor CDDP levels in tumor-bearing mice after administration of the optimum formulation to see if they increased in response to HT (*Chapter 4*). We also derived a pharmacokinetic method to evaluate the efficiency of this TDDS. Using the actual *in vivo* pharmacokinetic data, we annalyzed the *in vivo* drug release and examined the factors affecting the TDD efficiency. This is described in *Chapter 5*. Finally, we look at the effects of using this thermosensitive liposome TDDS on the therapeutic index by examining the antitumor activity and side effects in tumor-bearing mice (*Chapter 6*).

Chapter 2 *In Vitro* Heat-Specific Drug Release of Large Unilamellar Vesicle [33]

2.1 Introduction

Generally, the lamellar structure of liposome membrane at the gel-to-liquid-crystalline transition phase is somewhat loose and porous. Therefore, when a liposome is heated at the phase-transition temperature, it is supposed to release its content.[39] The thermosensitive liposome facilitates this characteristic. The liposome is prepared with a lipid composition so as to exhibit the gel-to-liquid-crystalline phase transition at HT temperatures (40-45°C).[95-100,104-107]

The heat-specific drug release, however, depends not only on such a lipid composition but also on the liposomal type and preparation method. SUV does not release a drug satisfactorily.[50,90,105] This may be disadvantageous for the local drug release. SUV has also disadvantages in drug-encapsulating efficiency and stability.[88] LUV is generally thought to exhibit higher drug-encapsulating efficiency and higher stability than SUV. Magin et al.,[52-55] Maynard et al.,[61] and Bassett et al.[9,10] reported that LUV's released an encapsulated drug more rapidly at HT temperatures. Therefore, LUV may be more favourable for drug release in response to local HT. However, there has been little information on the drug release mechanism.[67,68]

This chapter described the heat-specific drug release characteristics and other physichochemical properties of a thermosensitive LUV which encapsulates carboxyfluorescein (6-CF) or CDDP. The purpose of the study is to find an optimum formulation and to elucidate the mechanism of the heat-specific drug release.

2.2 Materials and Methods

2.2.1 Liposome Preparation

Dipalmitoylphosphatidylcholine (DPPC, Nippon Fine Chemical), distearoylphosphatidylcoline (DSPC, Nippon Fine Chemical), 6-CF (Sigma), and CDDP (Aldrich) were used for SUV and LUV preparations. LUV's were prepared by reverse-phase evaporation (REV) method.[58,87] To get a lipid composition with the phase transition temperature near HT temperatures, we preferably used the mixture of DPPC and DSPC. A CDDP solution of 1000μg/ml (sodium chloride solution) and a 6-CF solution of 50μmole/ml (phosphate buffer, *p*H7) were prepared for the aqueous phase. The osmotic pressure of the aqueous phase was ajusted so that the osmotic pressure of the internal aqueous fluid in the obtained liposome was 1.5 or more times higher than the physiological osmotic pressure. Six

hundred mg of the lipid mixture was dissolved in 200 ml of isopropylether-chloroform mixture [1:1 (v/v)]. The lipid solution was mixed with 30 ml of the 6-CF solution or the CDDP solution in a 500 ml round-bottom glass flask by a mixer (Polytron, Kinematica). The obtained w/o emulsion was homoginized with a sonicator (Ohtake). Then, the emulsion was transferred to a 1000 ml round-bottom glass flask, and the organic solvent in the emulsion was evaporated gradually by a rotary evaporator at 60°C to form a LUV suspension. The free CDDP or the free 6-CF in the LUV suspension was removed by dialysis against saline at room temperature (20-25°C) for 2 days, using a dialyzing tube (Spectrapor, melecular weight 8000 cut-off, Spectrum Medical). The medium (2000 ml) was changed at least 5 times during the dialysis. A SUV liposome containing CDDP was prepared according to a reported method.[88] Five ml of the aqueous drug solution was added to 400 mg of a dry lipid film which was prepared in a round flask, and mixed with a vortex mixer (Thermolyne, Sybron). The consequent multilamellar vesicle (MLV) was sonicated to form a SUV suspension. The free CDDP in the suspension was removed by the same technique as above.

2.2.2 Liposomal 6-CF and CDDP Content

The liposomally-entrapped amount of 6-CF or CDDP was determined by measuring the free and the total amount of 6-CF or CDDP in the liposomal suspension. The free 6-CF or CDDP was separated from the liposomal suspension by a filter (Centrisart, molecular weight cut-off 20000, Sartorius). The 6-CF concentration was assayed by fluorescene spectrometry (fluorescene spectrometer, F-3000, Hitachi). The excitation and emission wave-lengths were 494nm and 515nm, respectively. The CDDP concentration was assayed by atomic absorption spectrophotometry (flameless, F7000, Hitachi)[41] or HPLC.[8] In the HPLC assay, the sample was mixed with an equal volume of 10 % sodium diethyl-dithiocarbamate (DDTC, Wako Pure Chemical) and stood at room temperature for 30 min to form platinum DDTC adduct. The adduct was extracted with *n*-hexane and applied for HPLC (column, Zorbax CN; eluent, heptane/isopropylalchol = 8/1(v/v); flow rate, 1ml /min; detector, UV 254nm).

2.2.3 Differential Scanning Calorimetry

Phase transition temperatures of hydrated lipid mixtures and liposomal suspensions were determined by differential scanning calorimetry (SSC 5000, Seiko). The hydrated lipid mixtures were prepared by sonicating the mixtures of DPPC and DSPC in saline. The liposomal suspensions were prepared by suspending the LUV (DPPC/DSPC=9/1, w/w) in sodium chrolide solutions of different concentrations. The sample volume for the assay was 15μl. The heating rate was 2°C/min.

2.2.4 Mean-Size and Size-Distribution of Liposome

The mean size or size distribution of liposomes was determined by three different methods; electron micrography (negative stain and freeze fracturing), light scattering and filter extrusion. For the negative stain electron micrography, a SUV (DPPC/DSPC= 9/1, w/w) and a LUV (DPPC/DSPC= 9/1, w/w), both of which did not contain the drug, were diluted 40 fold with saline. Carbon- and collosion-coated grid (400 mesh) was floated onto the surface of a drop of each liposome. After removing excess fluid, a drop of 2% ammonium molybdate (*p*H7.4) was placed on the grid for negative staining. The grid was allowed to air dry, and then observed in a JEM-1200EX (JEOL) at 120 kV. For freeze fracturing electron micrography,[94] a small amount of a LUV (DPPC/DSPC=9/1, w/w) containing CDDP was placed in a holder and rapidly freezed in liquid nitrogen (-180°C). The freezed specimen was fractured under high vacuum. The resulting fractured specimen was replicated by shadowing with platinum/carbon at angles of about 45°, followed by carbon shadowing to improve the mechanical stability of the replica for subsequent electron microscopy (JEM-1200EX, JEOL). For the light scattering, a submicron analyzer (N4 submicron particle analyzer, Coulter Electronics) was used. The liposomes were diluted with saline 1:100 for the assay. The normalized size distribution was obtained by size-distribution processor (SDP) analysis. For the filter extrusion, filters of different pore sizes (Acrodiscs, Gelman; 0.45μm, 1.2μm and 5.0μm, respectively) were used. The liposomes were diluted with saline 1: 20 and the liposomal suspensions (2 ml) were extruded from the filter. The percentage of the liposome passing through the filter without disruption was evaluated by measuring the concentration of the liposomally-entrapped drug in the extruded fluid.

2.2.5 Osmotic Pressure of Internal Aqueous Fluid in Liposome

The osmotic pressure of the internal aqueous fluid in a liposome was measured by directly applying 3 ml of the liposome obtained before dialysis (pre-dialysis liposome) to an osmometer (Osmette A, Amuco Corp.). A preliminary study indicated that the osmotic pressure of such a sample was equal to that of the external aqueous fluid. Also, the osmotic pressure of the internal aqueous fluid did not change after dialysis for removing the free drug. Therefore, the osmotic pressure of the internal aqueous fluid in the liposome was assumed to be the osmotic pressure of such a liposomal sample before the dialysis.

2.2.6 *In Vitro* Drug Release

The rate of release of 6-CF from a thermosensitive LUV as a function of heating time was examined by passing the liposome through a heated thin polyethylene tube (PE50, Intramedic). The tube consisted of two parts. The first part (about 20 cm) was immersed in a water bath maintained at 37°C for pre-heating the liposome, and the second part was immersed in another water bath maintained at various temperatures for heating the liposome. The length of the second part was varied from 1 cm to 5 cm in order to change the sample resident time in the heated tube. The liposome was diluted 10-fold with saline and was passed through the tube at 0.42 ml/min. It was ascertained that the time required for the sample to reach the water bath temperature in the heated tube was less than 1 s. This was achieved by measuring the temperature of the sample flowing out of the heated tube. The sample flowing out was pooled in a plastic tube. The release rate of 6-CF was determined as a function of the time required for the sample to pass through the heated tube.

Temperature release-rate profiles of 6-CF and CDDP liposomes were determined by incubating the liposomes at various temperatures as follows. The liposomes were diluted 10-fold with saline. Two ml of each sample was placed in a Centrisart tube and incubated for 15 min in a water bath (BT-21, Yamato) maintained at constant temperatures (variation was less than 0.1°C). The released 6-CF or CDDP was separated from the liposomal suspension and assayed by the same method as in the liposomal drug-content assay.

2.2.7 Liposomal Membrane Permeability

Membrane permeabilities of a 6-CF thermosensitive LUV at the gel phase, the gel-to-liquid-crystalline transition phase and the liquid-crystalline phase were evaluated by examining the release of 6-CF from the liposome at temperatures of the three phases. Preliminary temperature release-rate test suggested that when the liposome was heated above its phase transition temperature, it released 6-CF before reaching the test temperature. To avoid this, the pre-dialysis liposome was used for the test sample. It was ascertained that the liposome did not release 6-CF during the heating process, unless the liposome was placed in the test medium since the concentrations of 6-CF in the internal aqueous space and in the liposome suspending fluid were in equilibrium. Three ml of the pre-dialysis liposome heated to the test temperature was placed in a dialysis tube and was dialyzed for 3 hr or 16 hr in 1000 ml of sodium chloride solutions of various concentrations (0.6%, 0.9% and 1.35%, respectively) at constant temperatures (25°C, 37°C, 41°C, 47°C and 57°C, respectively). The release rate was determined by measuring the remaining unreleased 6-CF during dialysis. It was ascertained that the volume of the dialyzing fluid (release test medium) was large enough and the diffusion of free 6-CF out of the dialysis tube was quick enough to assume that 6-CF release from the liposome occured under sink condition.

2.3 Results and Discussion

2.3.1 Phase-Transition Temperature of DPPC/DSPC Mixtures

Thermosensitive liposomes can be prepared from membrane-lipid compositions so as to exhibit the phase transition temperature at HT (41-45°C). It has been shown that appropriate combination of DPPC with DSPC or dipalmitoylphoshatidylglcerol (DPPG) exhibited a phase transition temperature near HT temperatures. Yatvin et al.[104] and Weinstein et al.[95] reported the use of the combination of DPPC and DSPC in the ratio from 7/1 to 7/3 (molar ratio) for thermosensitive SUV. Magin et al.[54] and Bassett et al.[9] reported the use of the combination of DPPC and DPPG (4/1, w/w) for thermosensitive LUV.

In the present study, we used the combinations of DPPC and DSPC. In order to obtain an optimum combination, we examined the DSC's of mixtures of these lipids (*Fig. 2.1*). The phase diagram is similar to the previously reported one in using a mixture of DMPC and DSPC.[49] As the portion of DSPC in the mixture was increased, the peak phase transition temperature shifted to the values intermediate between those of the pure components. The peak phase-transition temperatures for DPPC/DSPC=9/1 (w/w) and DPPC/DSPC=7/3 (w/w) were found to be 41.5 °C and 42.5 °C, respectively, showing close correspondence to the reported values.[49] For achieving larger chemotherapeutic effect of a HT-mediated liposome delivery with minimum side effect, it is favourable that HT temperature at which the drug release occurs is as low as possible. Therefore, we chose DPPC/DSPC=9/1 (w/w) for the lipid composition so as to get the phase transition temperature near the lower limit of HT temperature.

2.3.2 Mean-Size and Size-Distribution of Liposome

The negative stain electron micrograph of the SUV and the LUV are shown in *Fig. 2.2.*, and the freeze fracturing electron micrograph of the LUV is shown in *Fig. 2.3*. Both of the liposomes appeared unilamellar.[19] The mean particle diameters of the SUV and the LUV were approximately 0.08μm and 0.2μm, respectively. The size distribution of the CDDP encapsulated LUV obtained by the filter extrusion is shown in *Table 2.1*. Almost 70 % of the liposome was distributed in the size smaller than 0.45 μm but the remaining percent was distributed beween 0.45 μm and 5 μm. The mean size of the LUV estimated by this method appeared larger than the size estimated by the electron micrograph. The size distribution of the same LUV determined by the light scattering is shown in *Fig. 2.4*. The

profile showed a bi-modal distribution. The one peak located near 0.2μm and the other located near 2μm. The size of the LUV as a single particle obtained by the electron micrograph and the bi-modal size distribution obtained by the light scattering suggest that the liposome may exist in the fluid as multidispersion.

Table 2.1 Size distribution of a CDDP-encapsulated LUV liposome (DPPC/DSPC=9/1, w/w) obtained by extrusion through Acrodiscs filters of different pore sizes.

Pore size of filter [a]	Percent filtered
5μm pass	97.4 [b]
1.2μm pass	84.9
0.45μm pass	69.6

[a] Acrodisc. [b] Percentage of the liposome passing through the filter without disruption was evaluated by measuring the concentration of the liposomally-entrapped drug in the filtered fluid.

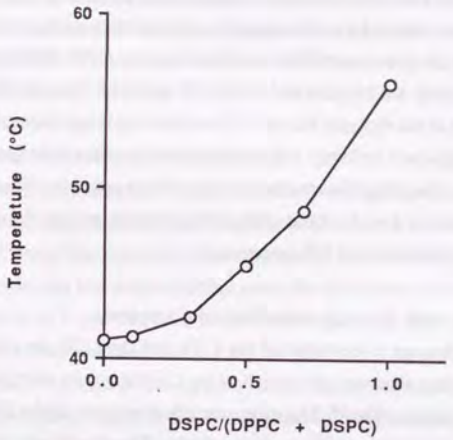

Fig.2.1 Peak phase-transition temperatures of DPPC/DSPC mixtures measured by differential scanning calorimetry.

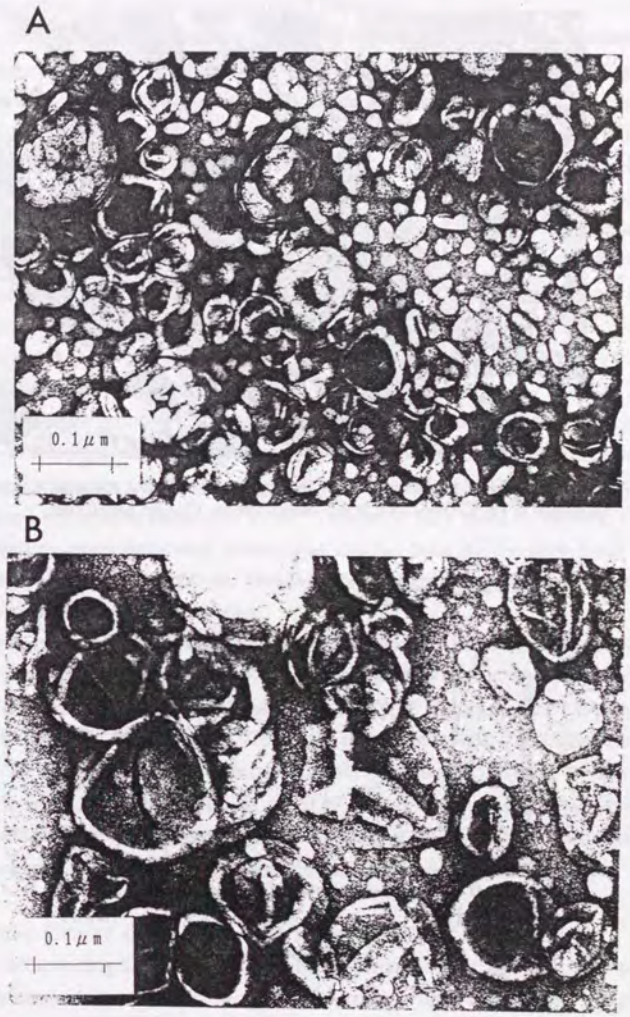

Fig. 2.2 Negative-stain electron micrographs of a SUV liposome (*A*) a LUV liposome (*B*) both of which were composed of DPPC/DSPC (9/1, w/w). Magnification, 150,000; bar =0.1μm.

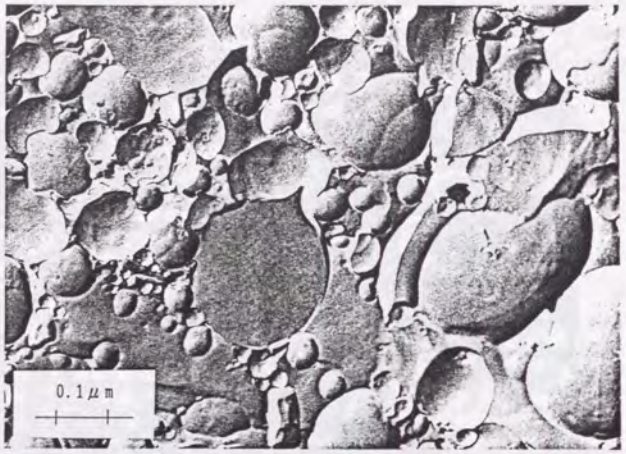

Fig. 2.3 Freeze fracturing electron micrographs of a LUV liposome which was composed of DPPC/DSPC (9/1, w/w). Magnification, 150,000; bar =0.1μm.

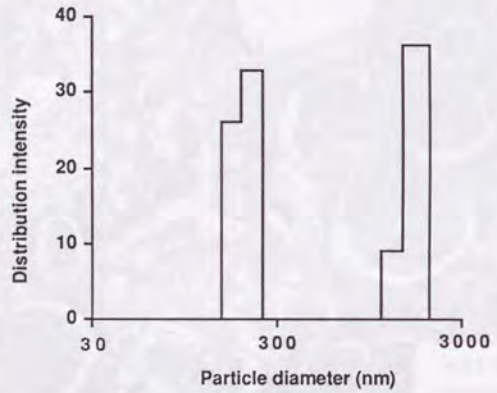

Fig. 2.4 The normalized particle-size distribution plotted against particle diameter for CDDP-encapsulated LUV liposome composed of DPPC/DSPC (9/1, w/w). The normalized size-distribution was obtained by size-distribution processor analysis in Coulter N4 submicron analyzer.

2.3.3 Liposome Drug-Encapsulating Efficiency

Generally, higher drug-encapsulating efficiency of liposome is thought to be preferable for its clinical use. When an encapsulated drug is soluble in an aqueous fluid, the liposome drug-encapsulating efficiency is obviously determined by the volume of the internal aqueous fluid. Because of its larger aqueous fluid volume, LUV is thought to exhibit higher encapsulating efficiency than SUV. In the present study, the LUV encapsulated about 25% CDDP (250μg CDDP and 20mg lipid/ml). On the other hand, the SUV encapsulated about 15 % of the drug (150μg CDDP and 80mg lipid/ml). The entrapment per the lipid in the SUV was about one sixth of that in the LUV. This indicates that the injection of CDDP as LUV can save the lipid, thus offering a greater advantage for the therapeutic use of the liposome.

2.3.4 Long-Term Storage Stability

Generally, liposome stability is thought to be affected by lipid composition and type of liposome. It was reported that a liposome prepared with saturated phospholipid is more stable than that prepared with unsaturated phospholipid such as *Egg* PC.[88] It was also reported that LUV is more stable than SUV.[88] Generally, SUV tends to coalesce. Coalescence caused by aggregation may induce drug leakage from the liposome even if it is prepared with saturated phospholipid. However, the long-term storage stability of thermosensitive liposome has not been reported.

In the present study, the stabilities of a CDDP-encapsulated thermosensitive SUV and a CDDP-encapsulated thermosensitive LUV were examined by storing the liposomes at 4°C and room temperature (RT) for 6 months (*Table 2.2*). The latencies of the LUV stored at 4°C and RT were both more than 97% even after 6 months. No coalescence was observed. On the other hand, the latencies of the SUV stored at 4°C and RT for one month were 91.8% and 9.2%, respectively. In the SUV, remarkable coalescence was observed one week after storage at RT or one month after storage at 4°C. These results indicate that the LUV prepared with DPPC/DSPC is stable even upon long-term storage at RT.

2.3.5 Time Course of Heat-Specific Drug Release

In order to achieve drug targeting by HT-mediated thermosensitive liposome, the liposome should release the highest possible amount of the drug within a short time. Magin and Niesman[54] demonstrated that drug release from a thermosensitive LUV occurred very rapidly (in a few seconds) after the liposome was heated in a small glass capillary tube. To evaluate time-dependent drug release from a thermosensitive LUV, we heated the liposome

in a flow system where the liposome passed through heated thin tubes with different lengthes.

Fig. 2.5 shows time-dependant 6-CF release from the LUV when it passed through a heated tube. At 41 and 42°C, the liposome released about 70% of entrapped 6-CF within 2 sec. Thereafter, the release profile reached a plateau level. At 40°C, the liposome released about 25% of the entrapped 6-CF within 15 sec the release profile also reaching a plateau level after the initial release. However, at 38°C, the liposome did not release 6-CF even after 1 min.

The data correspond to the previous report,[54] the drug release occurred explosively within a short time (a few seconds) in response to heating, and the rate depended on the incubation temperature rather than the incubation time. By considering the data of HT-mediated release and the increase in the tumor CDDP level (*Chapter 4*), the heating time of a few seconds is probably short enough for the *in vivo* drug release.

Table 2.2 Stabilities of a CDDP-encapsulated SUV and a CDDP-encapsulated LUV liposome (DPPC/DSPC=9/1, w/w) when stored at 4°C and room temperature (RT).

Liposome	Month	4°C	RT
SUV	0	97.5 a)	—
	1	91.8 b)	9.2 b)
LUV	0	98.2	—
	1.5	98.2	95.1
	3	98.2	99.9
	6	97.1	96.3

a) The latencies (%) of the liposomes were used as a measure for liposomal stability.
b) Remarkable coalescence was observed.

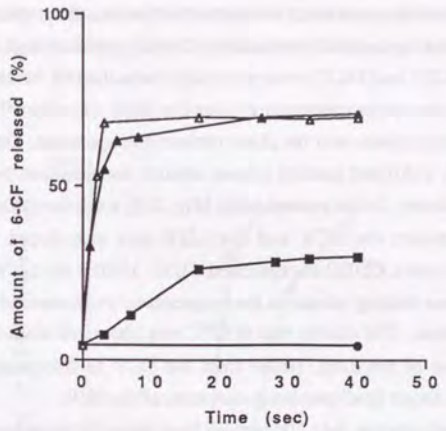

Fig. 2.5 Time-dependent 6-CF release from a 6-CF encapsulated thermosensitive LUV liposome (DPPC/DSPC=9/1, w/w) when the liposome passed through a tube heated at different temperatures. The release rate was plotted against time for the liposome to pass through the heated tube. (●), 38°C; (■), 40°C; (▲), 41°C; (△), 42°C.

2.3.6 Effect of Lipid Composition on Drug Release

Heat-specific drug release is thought to be primarily determined by the lipid composition to exhibit a phase transition at HT temperatures. Temperature-dependent release of CDDP from LUV's prepared with different ratios of DPPC and DSPC is shown in *Fig. 2.6*. A LUV composed of DPPC/DSPC=9/1 released the drug at and above 41°C, but did not release at 38°C. The increase in release-rate was very sharp between 40°C and 41°C. The lowest temperature at which the drug release occurred, appeared slightly below the peak phase transition temperature as observed above. The amount of the drug released at 42°C was more than 80%.

As far as LUV's composed of DPPC/DSPC=7/3 and DPPC/DSPC=5/5, the drug-release temperature was shifted higher by approximately 1°C for DPPC/DSPC=7/3, and by approximately 3°C for DPPC/DSPC=5/5. The LUV composed of DPPC/DSPC=5/5 did not show sharp drug-release increase at its phase transition temperature. The LUV composed of DSPC alone, did not show drug release at the temperature range of HT.

These results indicate that the lipid composition of DPPC/DSPC= 9/1 is optimum for HT-sensitive drug release.

2.3.7 Effect of Liposome Type on Drug Release

Heat-specific drug release depends on the liposomal type and the preparation method, but is primarily determined by the lipid composition. The drug release from the three classes of liposomes (SUV, LUV and MLV) were previously examined by measuring the release of Ara-*C* from the liposomes at temperatures near the phase transition.[61] The LUV showed sharp increase in drug-release near the phase transition temperature. On the other hand, the SUV and the MLV exhibited gradual release around the transition temperature, and the release rates were lower. In the present study (*Fig. 2.6*), a similar difference of the release characteristics between the SUV and the LUV was also found between a CDDP encapsulated SUV and a CDDP encapsulated LUV. Unlike the LUV, the SUV showed only a slight increase in drug release as the temperature was increased through the phase-transition temperature. The release rate at 42°C was about one sixth of that found in the LUV. Smaller rate of the drug release from the SUV as compared with the LUV is probably due to the larger lipid-membrane curvature of the SUV.

These results indicate that the LUV is more favourable for thermosensitive drug release than the SUV.

2.3.8 Effect of Osmotic Pressure on Drug Release

The difference of the osmotic pressures between the internal aqueous fluid in the liposome and the release test medium (external fluid of the liposome) is also a factor influencing thermosensitive drug release. There have been few reports on the effect of the differential osmotic pressure, and the osmotic permeability of a liposome have been reported.[11,64,67,68,108] *Fig. 2.7* shows the temperature-dependent release of CDDP from the thermosensitive LUV in media with different osmotic pressures (sodium chloride solution). The heat-specific drug release from the LUV depended on the osmotic pressure of the medium. Higher osmotic pressure of the medium as compared with physiological osmotic pressure remarkably decreased the heat-specific drug release.

The internal-fluid osmotic pressure for the CDDP-encapsulated LUV estimated by the present method was about 1.7 times the physiological osmotic pressure. *Fig. 2.8* showed the dependency of the release rate on the ratio of the internal fluid osmotic pressure to the external fluid osmotic pressure. There seemed to be a critical point at a ratio near 1.5. On the other hand, DSC profiles of the liposome suspensions showed that the phase transition was influenced by the ratio of the osmotic pressure of the internal aqueous fluid to the osmotic pressure of the external fluids in the liposome (liposomal suspension fluid). The higher osmotic pressure of the internal fluid shifted the transition temperature to lower value as compared to the transition temperature of the hydrated lipid mixture of the same

composition (data not shown). One reason may be that the membrane of the liposome with higher inside osmotic pressure is flexing outward by the internal fluid and the outer lipid layer is tensioned by this force. This may contribute to the mechanism for heat-specific drug release (discussed below).

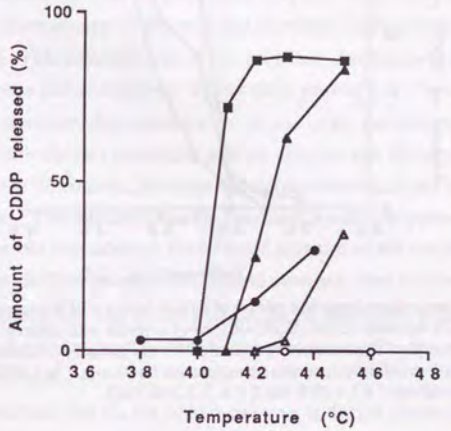

Fig. 2.6 Temperature-dependent release of CDDP from a CDDP encapsulated SUV liposome composed of DPPC/DSPC (9/1, w/w) and CDDP encapsulated LUV liposomes composed of DPPC/DSPC (9/1, 7/3, 5/5 and 0/10, w/w). The liposomes were diluted with saline by 10 times and incubated in a water bath maintained at constant temperatures for 15 min. (●), SUV, DPPC/DSPC=9/1; (■), LUV, DPPC/DSPC=9/1; (▲), LUV, DPPC/DSPC=7/3; (△), LUV, DPPC/DSPC=5/5; (○), LUV, DSPC alone.

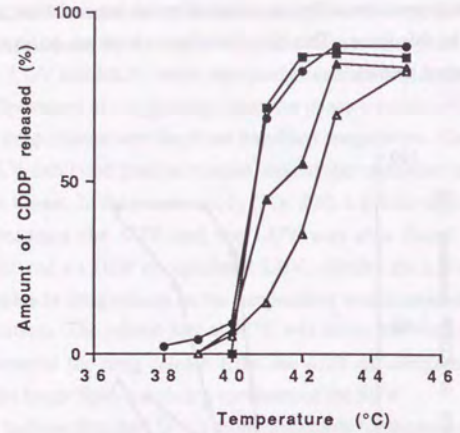

Fig. 2.7 Temperature-dependent release of CDDP from a CDDP encapsulated thermosensitive LUV liposome (DPPC/DSPC=9/1, w/w) in media with different concentrations of sodium chloride. The liposome was diluted with the media by 10 times and incubated in a water bath maintained at constant temperatures for 15 min. (●), 0.72% NaCl; (■), 0.9% NaCl (saline); (▲), 1.08% NaCl; (△), 1.35% NaCl.

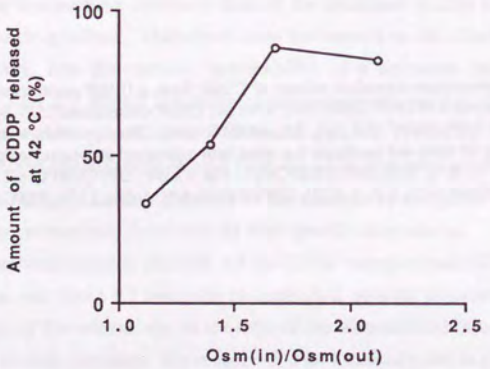

Fig. 2.8 The plots of the rates of CDDP release from a CDDP-encapsulated thermosensitive LUV liposome (DPPC/DSPC=9/1, w/w) at 42°C *versus* the ratios of the internal aqueous space osmotic pressure to the external-fluid osmotic pressure. The release rate data were the same as presented in *Fig. 2.7*, and the internal-fluid osmotic pressure was assumed to be 1.7 times as the saline osmotic pressure.

2.3.9 Liposomal-Membrane Permeability

Fig. 2.9 shows the membrane permeability of the *6*-CF encapsulated thermosensitive LUV at the gel phase, the transition phase and the liquid-crystalline phase. This was evaluated by measuring *6*-CF release from the pre-dialysis liposome and after dialysis at various temperatures using media with different osmotic pressures. At room temperature and 37°C (gel phase), the amounts of *6*-CF released were very low even after 16 hr-dialysis in any medum. However some erroneous data exsisted in estimating the released amount from the unreleased amount. This indicates that, at the gel phase, the liposome membrane was not permeable and it was not changed by the osmotic pressure of the medium. At 41°C (transition phase), however, the released amount was large, particularly in lower osmotic pressure media. Upon dialysis with 0.6% sodium chloride and saline the release amounts were more than 90%. In contrast, the released amount when dialized with 1.35% sodium chloride was smaller. This indicated that the liposomal membrane permeability was high at the transition phase but depended on the osmotic pressure of the test medium. At 47°C and 57°C (liquid-crystalline phase), the released amounts were smaller than those at the phase transition temperature. The results indicate that at this phase, *6*-CF release depended on the time of incubation rather than on the temperature or the osmotic pressure of the medium.

These results indicate that (*i*), the lipid membrane at the gel phase is impermeable and osmotically insensitive rendering the stability to the liposome; (*ii*), the lipid membrane at the transition phase is permeable and osmotically sensitive so that the liposome releases *6*-CF very rapidly under lower osmotic pressure of the suspension medium; (*iii*), the lipid membrane at the liquid crystalline phase is somewhat permeable but not much osmotically sensitive, and the liposome is not much stable.

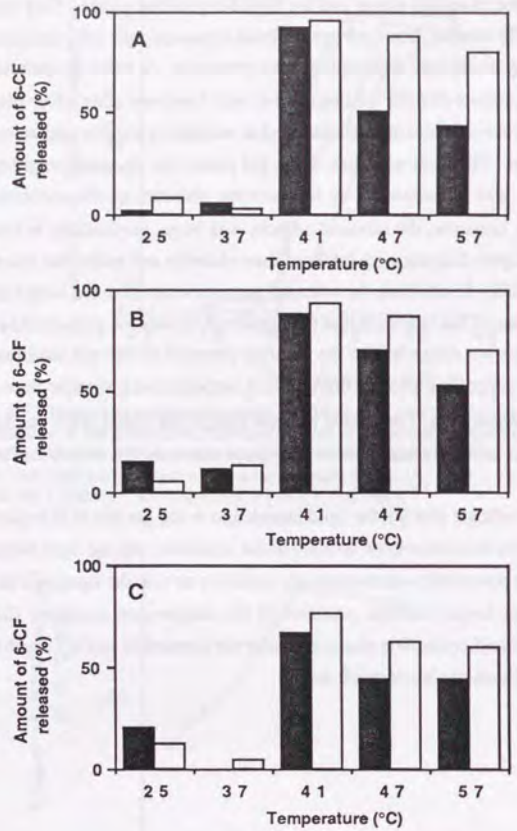

Fig. 2.9 The liposomal-membrane permeabilities of a *6*-CF encapsulated thermosensitive LUV liposome at the gel phase, the transition phase and the liquid-crystalline phase as evaluated by *6*-CF-release from the pre-dialysis LUV liposome when it was dialyzed at different temperatures using aqueous sodium chloride solutions with different concentrations. Panels *A*, *B* and *C* show the percentage of released *6*-CF when dialized with media of 0.6% , 0.9% and 1.35% sodium chloride, respectively. Solid columns and open columns show the results in 3 hr and 16 hr dialysis, respectively.

2.3.10 Postulated Mechanism of Thermosensitive Drug Release

The findings in the above experiments suggest that higher inside osmotic pressure contributes to the mechanism of thermosensitive drug-release and is a major driving force of the release.[74] It is possible that at the phase-transition temperature, the high inside osmotic pressure enlarges *aqueous pores* existing at the lipid bilayer, and thereby causes the internal aqueous fluid to diffuse out through the pores explosively in a short period. The drug release occurs concurrently with this fluid flow. After diffusion of the inside fluid, the pores may shrink or close and the explosive drug release will not occur any more. This mechanism can explain the plateau level of the release rate as observed in the temperature release-rate profile.

2.4 Conclusion

LUV is the most favourable liposomal type for TDDS using thermosensitive liposome. The optimum lipid composition for HT-specific drug release is DPPC/DSPC=9/1 (w/w). The higher internal osmotic pressure is essential for the rapid drug release and the optimum osmotic-pressure ratio as compared to the liposomal suspension fluid is 1.7. In this optimum formulation, the liposome is stable in long-term storage and shows sharp release-rate increase between 40°C and 41°C. The release occurrs explosively in a few seconds.

2.5 Summary

The heat-specific *6*-CF or CDDP release characteristics and liposomal properties of thermosensitive LUV's which were prepared with DPPC and DSPC have been demonstrated in comparison to a thermosensitive SUV. The entrapped amount of *6*-CF or CDDP per lipid in the LUV was about 6 times as high as that in the SUV. The LUV was stable in long-term storage (more than 97% latency at room temperature after 6 months). Unlike the LUV, the SUV was unstable. The LUV showed very sharp release-rate increase between 40°C and 41°C. The amount released at 42°C was about 80%. The release occurred explosively in a short time (a few seconds). Unlike the LUV, the SUV showed only a small release rate increase. The optimum lipid composition of the LUV for HT-mediated drug release was found to be DPPC/DSPC= 9/1 (w/w). Heat-specific drug release from the LUV and the drug permeability of the LUV at the phase transition temperature depended on the ratio of the osmotic pressure of the internal aqueous fluid to the osmotic pressure of the liposomal suspension fluid (release test media).

These results indicate that the LUV is more favourable than the SUV for thermosensitive delivery with respect to drug encapsulation capacity, liposome stability and

drug release and that the osmotic pressure of the internal aqueous space should be 1.5 or more times as high as the physiological osmotic pressure for heat-specific drug release.

Chapter 3 Clearance Kinetics and RES Distribution of Liposome in Rats [38]

3.1 Introduction

Before a liposome can be used therapeutically in humans, more must be known about their pharmacokinetics.[3,4,26,44,77] The encapsulation of a drug in liposomes can alter the pharamcokinetics of the drug. It can prolong the systemic circulation of the drug. However, the encapsulated drug tends to be distributed to the RES, which may cause undesirable side effects.

In this chapter, we describe the pharmacokinetics of CDDP-encapsulated liposomes with different heat-sensitive lipid compositions (*Chapter 2*) after intravenous administration in rats. The systemic liposome degradation rate and the RES-uptake rate are determined both theoretically and experimentally.

3.2 Theory

3.2.1 Pharmacokinetics after Administration of Liposome

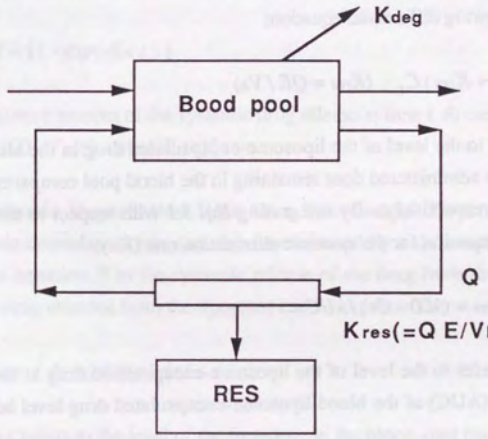

Fig. 3.1 Anatomical tissue-perfusion model describing the pharmacokinetics of liposomes.

The pharmacokinetics of liposome-encapsulated drug after intravenous administration can be described using an anatomical tissue-perfusion model (*Fig. 3.1*).[12,85] Liposomes when administered, first enter the systemic circulation (blood pool compartment) and are mixed. In the repeated circulation, a portion of the liposomes will be degraded by the action of serum components in the systemic blood and the remainder will be taken up by the RES of which the liver and the spleen are major components (RES compartment). Liposomes do not easily distribute to normal tissues or organs except the RES because they do not easily extravasate.[26] The systemic degradation of a liposome results in the drug's being released from the liposome. Similarly, the RES uptake of a liposome results in the distribution of the encapsulated drug to the RES. Therefore, the systemic degradation of liposomes can be characterized as the release of the drug from the liposomes in the blood pool compartment (K_{deg}, degradation rate). The RES uptake of liposomes can be characterized as the extraction of the liposome-encapsulated drug by the RES when the liposomes pass through the RES compartment concurrently with the blood (E, extraction ratio; Q, blood flow rate; K_{res}, RES uptake rate).

3.2.2 Systemic Clearance of Liposomes

The levels of the liposome-encapsulated drug in the blood after administration can be described by the following differential equation:

$$dC/dt = -(K_{deg} + K_{res})\,C, \quad (K_{res} = QE / V_b) \tag{3.1}$$

where C and V_b refer to the level of the liposome-encapsulated drug in the blood which is expressed as % of the administered dose remaining in the blood pool compartment and the blood pool volume, respectively. By integrating *Eq. 3.1* with respect to time t, we can obtain the following equation for the systemic elimination rate (K_{el}):

$$K_{el} = K_{deg} + K_{res} = (100 - C_t)\,/AUC_t \tag{3.2}$$

where C_t and AUC_t refer to the level of the liposome-encapsulated drug at time t and the area under the curve (AUC) of the blood liposome-encapsulated drug level between time zero and time t.

3.2.3 RES-Uptake Rate and Systemic Degradation Rate

Assuming that the liposomes taken up by the RES accumulate in the RES without degradation, the level of the liposome-encapsulated drug in the RES can be described by the following differential equation:

$$dT/dt = K_{res}\,C \tag{3.3}$$

where T refers to the level of the liposome-encapsulated drug in the RES which is expressed as % of the administered dose.

By integrating *Eq. 3.3* with respect to time t, we can obtain the following equation for K_{res}:

$$K_{res} = T_t\,/AUC_t \tag{3.4}$$

where T_t refers to the level of the liposome-encapsulated drug in the RES at time t (% of the administered dose existing in the RES). Assuming $t = \infty$ in *Eq. 3.2* and *3.4*, we can also obtain the following equation for the level of the liposome-encapsulated drug in the RES at infinitive time:

$$T_\infty = 100\,K_{res}\,/K_{el} \tag{3.5}$$

Then,

$$T_t = T_\infty\,[1 - exp(-K_{el}\,t)] \tag{3.6}$$

The cummulative amount of the systemic drug release at time t, R_t can be expressed as

$$R_t = (100 - T_\infty)[1 - exp(-K_{el}\,t)] \tag{3.7}$$

3.2.4 Systemic Degradation Rate as a Function of Time

The systemic degradation rate can also be evaluated by a different way. By applying a convolution equation [30] to the systemic release of the drug from the liposome, the blood level of the drug released from the liposome can be expressed as

$$C_{free,\,t} = \int I_n(x)\,G(t\text{-}x)\,dx \tag{3.8}$$

where $C_{free,t}$ refers to the level of the free-drug in the blood after liposome administration, $G(t)$ refers to a multi-exponential function describing the level of the free drug in the blood after CDDP solution administration and $I_n(x)$ refers to an input function and equal to the release rate (dRx/dt). Then, using the same deconvolution method as that reported pre-

viously,[30] we can estimiate the cumulative rate of the systemic drug release and therefore the rate of the systemic degradation of the liposomes.

3.3 Materials and Methods

3.3.1 Liposome Preparation

Three types of liposome encapsulating CDDP were prepared and each had different heat-sensitive lipid compositions [DPPC/DSPC=9/1, 7/3 and 5/5 (w/w)]. The preparation method was the same as described in *Chapter* 2. Each liposome contained 200-250 μg of CDDP and 20-30 mg of lipid per ml. The average particle size was almost the same for the 3 liposomes (negative-stain electron micrograph, about 0.2 μm).

3.3.2 Measurement of Pt Concentration

Pt was assayed by AAS or assayed by HPLC after Pt diethyldithiocarbamate adduct formation as described in *Chapter* 2. The HPLC method was preferable for the determination of the low concentrations of Pt.

3.3.3 Measurement of Liposomal Lipid Concentration

The concentrations of DPPC and DSPC were determined by HPLC (column, μBondasphere, 5μ C4-100A, 3.9mm *id.*, 15cm, Waters; eluent, methyl alchol/0.1M KH_2PO_4 =9/1(v/v); flow rate, 1ml/min; detector, refraction index analyzer, Showadenko; retention times: 8 min for DPPC, 14 min for DSPC). The liposomal lipid concentration was considered to be the sum of the concentrations of DPPC and DSPC.

3.3.4 Blood and RES Liposome Distribution Experiments in Rats

SD-JCL rats (male, 8 weeks old and weighing about 300g) were used for the experiment. The liposomes and a CDDP solution were administered intravenously *via* the femoral vein. In the blood liposome distribution experiment, the blood samples (n=3) were taken from the tail vein and collected in a heparinized tube periodically after administration. In the RES liposome distribution experiment, rats (n=3) were sacrificed at appropriate time intervals by exsanguination from the abdominal aorta, and the liver and the spleen were removed. Each was homogenized with 10 volumes of water with a tissue homogenizer (Polytron Kinematica).[29] The distribution of liposome was then determined by measuring the concentration of Pt or lipids in the blood samples or the tissue homogenates.

3.3.5 Blood and RES Total-Pt Level

The total Pt (encapsulated Pt + protein-bound Pt + free Pt) levels in the blood or in the RES were determined by measuring the Pt in the solubilized blood or tissue samples by the AAS. The blood or the tissue homogenate was solubilized with methybenzethonium hydroxide (Sigma) at 60°C. In determination of the RES Pt levels, the amount of Pt in the blood contained by the RES was subtracted by assuming the RES contained 7 % of the whole blood.[3] The blood levels or the RES levels were expressed as % of the administered dose. The volume of the whole blood was assumed to be 8 % of the body weight.[33]

3.3.6 Blood Free-Pt Level

The blood sample was diluted with 10 volumes of 5% glucose and centrifuged. The free Pt in the supernatant was separated from the liposome-encapsulated Pt and the plasma-protein-bound Pt using a filter (Centrisart) as described in *Chapter 2* and assayed by the HPLC method.

3.3.7 Blood and RES Liposomal-Lipid Level

The blood or the tissue homogenate was diluted with 10 volumes of saline and centrifuged. Two milliliters of the supernatant was mixed with 1 ml of 10 % trichloroacetic acid and 4 ml of chloroform. The liposomal lipid extracted with the chloroform was assayed by the HPLC method.

3.4 Results and Discussion

3.4.1 Systemic Clearance of Pt after Solution Administration

The blood total-Pt (protein-bound Pt + free Pt) levels and the blood free-Pt levels after administering a CDDP solution to rats are shown in *Fig. 3.2*. The Pt in the blood, particularly the free Pt in the blood, was eliminated very rapidly.[41] The elimination half life and the total clearance of the free Pt were about 10 min and about 600 ml/h, respectively. The free Pt in the blood was gradually changed to the protein-bound Pt whose elimination from the blood was a little slower than that of free Pt.[41] However, the elimination of the total Pt was much more rapid than that of the total Pt levels after liposome administration (see below).

3.4.2 Systemic Clearance of Pt after Liposome Administration

The blood total-Pt (liposome-encapsulated Pt + protein-bound Pt + free Pt) levels and the blood free-Pt levels after administering CDDP liposomes to rats are shown in *Fig. 3.3*.

Following the administration of any of the 3 types of liposomes, the total Pt levels were much higher than the free Pt levels. Thus, the blood total Pt-levels represent the blood liposome-encapsulated-Pt levels and the blood liposome levels. Six minutes after liposome administration, almost all of the administered dose remained in the blood. Unlike solution administration, at this time, the liposomes were distributed only in the blood. Thereafter, the liposomes were eliminated at a first order rate. The K_{el} values at various times for each of the 3 liposomes calculated using *Eq. 3.2* were almost the same (*Table 3.1*). The average elimination rate was faster when the content of DSPC was smaller (the phase-transition temperature, lower). Certain liposomes have been reported to accumulate rapidly in the RES and then reenter the circulatory system.[80] Similar results were obtained with liposomes composed of DSPC alone in our study (data not shown). However, this characteristic was not obtained with the present DPPC/DSPC liposomes.

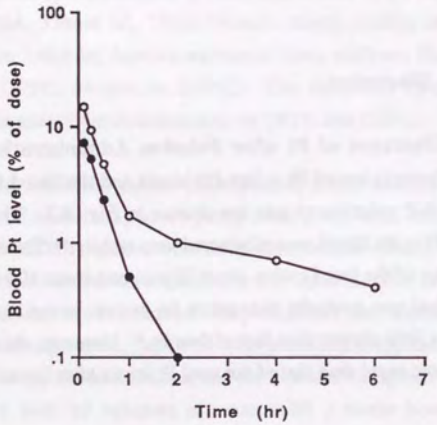

Fig. 3.2 The levels of the total-Pt (protein-bound Pt + free Pt, ○) and the free-Pt (●) in the blood after administering a CDDP solution to rats (dose: 1.5 mg of CDDP/rat).

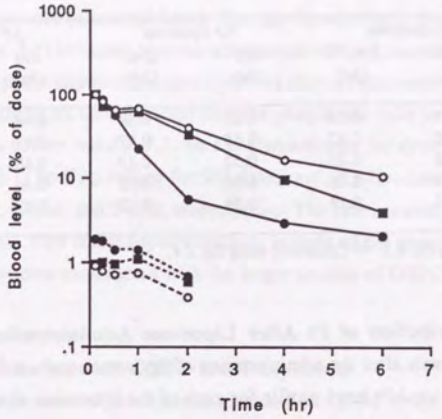

Fig. 3.3 The levels of the total-Pt (liposome-encapsulated Pt + protein-bound Pt + free Pt, solid line) and the free Pt (broken line) in the blood after administering CDDP liposomes composed of DPPC/DSPC (9/1, 7/3, and 5/5, w/w) to rats (dose: 3 ml of liposomal suspension/rat). (●), 9/1; (■), 7/3; (○), 5/5

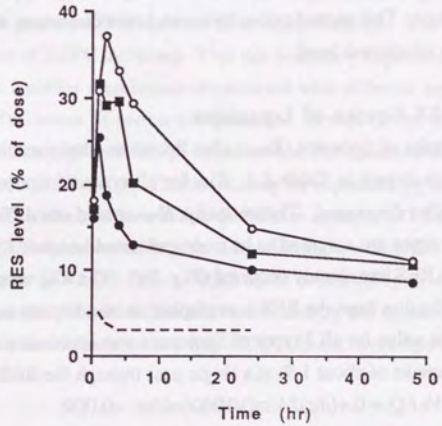

Fig. 3.4 The levels of the total Pt in the RES after administering CDDP liposomes composed of DPPC/DSPC (9/1, ● ; 7/3, ■ ; 5/5, ○)(solid line) and a CDDP solution (broken line) to rats (dose: 3 ml of liposomal suspension/rat).

Table 3.1 The systemic elimination rate (*Kel*) and the RES uptake rate (*Kres*) of liposome-encapsulated CDDP after administering CDDP liposomes composed of DPPC/DSPC (9/1, 7/3, and 5/5 w/w) to rats.

time (hr)	*9/1-liposome*		*7/3-liposome*		*5/5 -liposome*	
	Kel a) (/hr)	*Kres* b) (/hr)	*Kel* (/hr)	*Kres* (/hr)	*Kel* (/hr)	*Kres* (/hr)
0.5	1.31	0.43	0.65	0.39	0.48	0.40
1	1.30	0.42	0.68	0.47	0.47	0.42
2	1.28	0.25	0.62	0.27	0.48	0.29
4	1.18	0.18	0.60	0.19	0.46	0.18
6	1.12	0.14	0.59	0.12	0.42	0.14

a) Calculated using *Eq. 3.2*. b) Calculated using *Eq. 3.4*.

3.4.3 RES Distribution of Pt After Liposome Administration

The RES total-Pt levels after the administration of liposomes and a solution are shown in *Fig. 3.4*. The RES total-Pt level profile for each of the liposomes showed biexponential decay with a maximal level (25% at 1 hr for *9/1-liposome*, 32 % at 1 hr for *7/3-liposome*, 37% at 2 hr for *5/5-liposome*). The maximal level was larger with larger amount of DSPC. The result indicates that the distribution of the liposomes to the RES took place relatively early and that the liposomes distributed in the RES were gradually eliminated from the RES at time-dependent rates. This second point, however, is not consistent with the assumption in the present theory (discussed later).

3.4.4 Rate of RES Uptake of Liposomes

The rates of RES uptake of liposome (*Kres*) after liposome administration were calculated using *Eq. 3.4* and are shown in *Table 3.1*. *Kres* for all types of liposomes was constant upto 1 hr and thereafter decreased. Theoretically, *Kres* should not differ with times. The *Kres* values for later times are assumed to be underestimated because significant liposome elimination from the RES has already occurred (*Fig. 3.4*). The *Kres* value at 30 min or 1 hr when liposome elimination from the RES is negligible, is an adequate representation of the RES uptake rate. The value for all 3 types of liposomes was approximately equal to 0.4/hr, which accounts for uptake of about 1 % at a single pass through the RES :

$$E = Kres\ Vb / Q = 0.4(/hr)24(ml)/1060(ml/hr) = 0.009.$$

This suggests that the difference in the maximal RES level of the liposome-encapsulated drug among these liposomes resulted from the difference in their systemic degradation rate (K_{deg}). The hypothetical RES level of the liposome-encapsulated drug at infinitive time (T_∞) for each of the 3 liposomes can be estimated using *Eq. 3.5* and the *Kres* value at 1 hr.

The resulting T_∞ values (about 30 % for *9/1-liposome*, about 60 % for *7/3-liposome* and 80 % for *5/5-liposome*) apprear realistic when we consider the difference in the systemic stability of the liposomes (discussed later). The gap between such T_∞ values and the actual RES Pt levels (*Fig. 3.4*) indicates biphasic elimination of the liposome from the RES. The relatively fast rate in the elimination phase upto 4 hr after administration reflects partial lysis of the liposome during its incorporation into the phagocytic cells in the RES. Using the above *Kel* and *Kres* values and *Eq. 3.2*, we can also estimate the systemic liposome degradation rate (K_{deg}). The K_{deg} values for *9/1-liposome*, *7/3-liposome*, and *5/5-liposome* were about 0.9/hr, 0.3/hr, and 0.1/hr, respectively. The rate became larger as the content of DSPC increased. This result appears realistic in light of the general consideration that the lamellar membranes constructed with the larger amount of DSPC were more stable in the blood.[3]

3.4.5 Systemic Release of CDDP from Liposome

The time-dependent rates of the systemic release of CDDP from the liposomes were calculated using the deconvolution method described in *Theory*. $G(t)$ was assumed to be $13.2\ exp(-3.44\ t)$ which was obtained from the curve fitting in the blood-free-Pt levels after the solution administration (*Fig. 3.2*). The time-dependent rate of systemic CDDP release from the liposomes after administration is shown in *Fig. 3.5*. All 3 types of liposomes showed gradual CDDP release after administration. The release rate became faster as the content of DSPC increased. This can be mainly explained by the difference in the stability of the lamellar membranes constructed with different amounts of DPPC and DSPC under the influence of serum components. However, the time-dependent drug release amounts obtained as such for *7/3-liposome* and *5/5-lipoaome* were somewhat larger than those obtained from the K_{deg}. This result may be explained by the release of the free CDDP from the RES to the systemic blood: the release amounts of CDDP from the RES for *7/3-liposome* and *5/5-liposome* 2 hr after administration which are estimated using the above rate constants [$T_\infty\ exp(-2K_{el})$ - RES level at 2hr](*Fig. 3.4*) are 13% and 9%, respectively. However, the release amounts estimated by the deconvolution method may contains some data error because the free CDDP levels were too low to estimate the accurate release amounts.

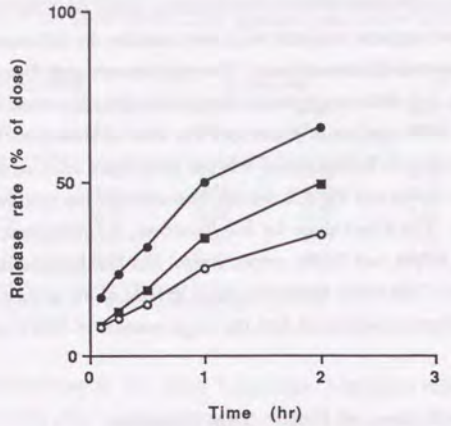

Fig. 3.5 Time-dependent rate of systemic CDDP release from the liposomes after administering CDDP liposomes composed of DPPC/DSPC (9/1, 7/3 and 5/5, w/w) to rats (dose: 3 ml of liposomal suspension/rat). (●), 9/1; (■), 7/3; (○), 5/5

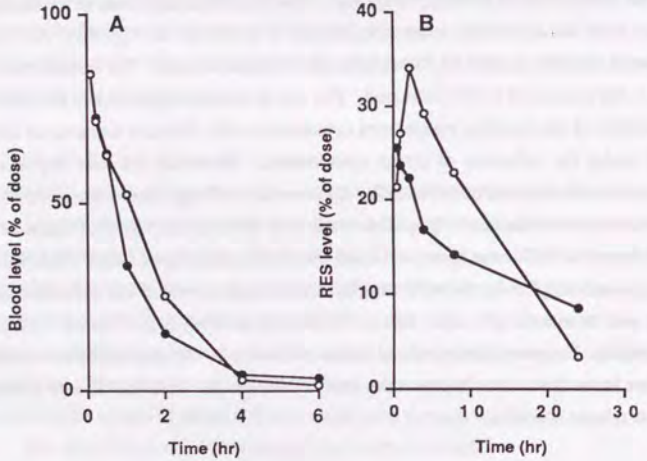

Fig. 3.6 The levels of the total-Pt (●) and the liposomal-lipid (○) in the blood (Panel *A*) and in the RES (Panel *B*) after administering CDDP liposomes composed of DPPC/DSPC (9/1, w/w) to rats (dose: 1 ml of liposomal suspension/rat).

3.4.6 Fate of Liposomal Lipid

The total Pt levels and the liposomal-lipid levels in the blood and in the RES after the administration of *9/1-liposome* to rats (the dose in this case, 1ml of liposomal suspension /rat) are shown in *Fig. 3.6*. At each time, the lipid levels were almost the same as the total Pt levels. In our preliminary study, the lipids when administered in a solubilized form showed the rapid elimination from the blood (data not shown). The above results indicate that the elimination of the liposomes either from the circulatory system or from the RES was due largely to the breakedown of the liposomes in the body. The relative constancy of the RES total-Pt level profiles for the two doses [1 ml/rat (*Fig. 3.6*) and 3 ml/rat (*Fig. 3.5*)] suggests that the RES was not saturated at these doses.

3.5 Conclusions

Measuring the level of the liposome-encapsulated drug in the blood and the RES and applying first order rate kinetics to this data, we have demonstrated important pharmacokinetic parameters (systemic liposome degradation and RES liposome uptake) for CDDP encapsulated heat-sensitive liposomes. The apparent good correlation between the experimental data and the theoretically calculated values suggests that these parameters adequately describe the pharmacokinetics of the present liposomes. The described pharmacokinetics will offer useful information when investigating factors that control the efficacy of heat-sensitive-liposome-based CDDP delivery.

3.6 Summary

The clearance kinetics and RES distribution of cisplatin (CDDP) encapsulated in liposomes composed of DPPC and DSPC (DPPC/DSPC=9/1, 7/3 and 5/5, w/w) after intravenous administration were examined in rats. All 3 types of liposomes were eliminated from the systemic circulation at a first order rate, and the smaller the DSPC content the faster the elimination rate (K_{el}): K_{el} values for *9/1-liposome*, *7/3-liposome* and *5/5-liposome* were approximately 1.3/hr, 0.7/hr and 0.5/hr, respectively.

The RES distribution of all 3 types of liposomes occurred relatively early, within 4 hr after administration, and the liposomes were then gradually eliminated from the RES. The amount of the RES distribution appeared to be dependent on both the rates of the systemic release of CDDP from the liposomes (liposome degradation) and the uptake of the liposomes by the RES. A liposome composed of a smaller amount of DSPC released CDDP in the systemic blood at a faster rate and therefore distributed in the RES to a smaller extent.

The rate kinetics based on an anatomical tissue-perfusion model indicated that the rate of liposome uptake by the RES, K_{res} was almost the same (0.4/hr) for all 3 types of liposomes, while the systemic degradation rate, K_{deg} (K_{el} - K_{res}) became smaller as the content of DSPC decreased. The K_{deg} value for each type of liposome corresponded with the systemic CDDP release rate.

Chapter 4 Increased Tumor Cisplatin Levels in Heated Tumors in Mice [35]

4.1 Introduction

The concept behind the present TDD system is HT-dependent phase transition of liposome and phase-transition-induced drug release from the liposome.[96,98] The site of the drug release is in the local blood at or adjacent to the heated tumor.[96,98] The TDD efficiency will, therefore, depend largely on the heat sensitivity of the liposome and its concentration in the local or systemic blood.[12]

Chapter 2 described that a LUV released CDDP at HT temperatures at a higher rate than a SUV.[105] However, the TDD efficiency of this type of liposome is not yet known. Therefore, the purpose of the present study is to examine whether or not the tumor CDDP levels in mice after the administration of CDDP in thermosensitive LUV and thermosensitive SUV are increased in response to HT, and also to investigate the factors governing their TDD efficiency, in comparison with CDDP solution (*Sol*) and non-thermosensitive liposomes.

4.2 Theory

4.2.1 Pharmacokinetics after Administration of Thermosensitive Liposomes

The pharmacokinetics of encapsulated drug in liposome and free drug after administration can be described using an anatomical tissue-perfusion model (*Fig. 4.1*).[12] Following the administration of liposome without HT, although a certain amount of the drug encapsulated in the liposome is taken up by the RES, the residual amount will be released gradually in the systemic blood (*Chapter 3*). As a result, the free drug will be distributed to the tumor or to the normal tissue (organ). Liposome itself, however, is assumed not to be distributed to the tumor or the normal tissues.[26] If liposome administration is combined with HT, rapid drug release will occur in the blood at or adjacent to the heated tumor when the liposome travels concurrently with the blood through the tumor. The released drug will be distributed in the tumor, resulting in high tumor drug level. Therefore, the TDD efficiency of the present delivery system will depend largely on the concentration of the liposome in the local or systemic blood as well as the heat sensitivity of the liposome.

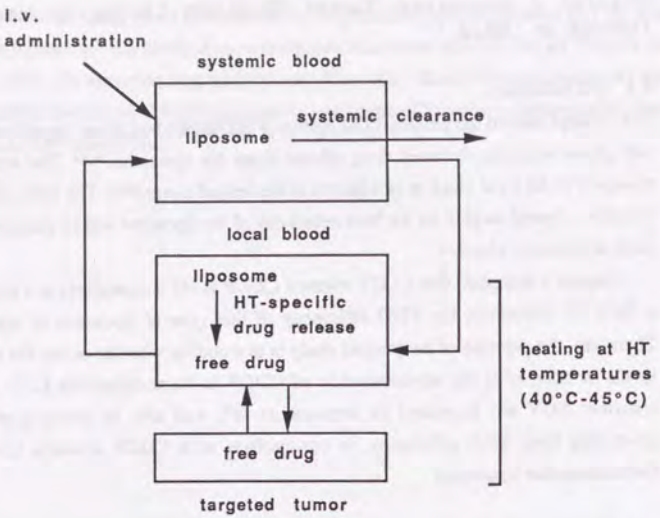

Fig. 4.1 Schematic description of pharmacokinetics after administration of thermosensitive liposome.

4.3 Materials and Methods

4.3.1 Liposome Encapsulating CDDP

Two types of thermosensitive LUV which release the encapsulated CDDP at the HT temperatures at a faster rate (*LUV-1* and *LUV-2*) were prepared by REV method (*Chapter 2*). A thermosensitive SUV (*SUV-1*) was prepared with the same lipid composition as that for *LUV-1* according to the film method (*Chapter 2*). The phase transition temperature of each liposome was adjusted near the HT temperature using a mixture of DPPC and DSPC for *LUV-1* and *SUV-1* and a mixture of DPPC and sodium stearoylmethyltaurate (SMT, Nikko Chemical, Tokyo) for *LUV-2* (*Table 4.1*). A non-thermosensitive LUV which does not release CDDP at HT temperatures (*LUV-3*) was prepared using a mixture of DSPC and SMT with the phase transition temperature higher than HT temperatures (*Table 4.1*). The SMT in *LUV-2* and *LUV-3* was used as a modifier for liposome dispersion.

Table 4.1 Liposomal composition, phase transition temperature and CDDP content of the liposomes.

Liposome	Composition (w/w)	Phase transition temperature °C	CDDP content μg/ml [a]
LUV-1	DPPC/DSPC (9/1)	41	250
LUV-2	DPPC/SMT (10/1)	41	350
LUV-3	DSPC/SMT (10/1)	58	300
SUV-1	DPPC/DSPC (9/1)	41	150

a) The CDDP content was determined by separating the liposome-encapsulated CDDP from the free CDDP; each LUV contained about 20 mg of lipid per ml, and *SUV-1* contained 4 times as much lipid as the LUVs.

4.3.2 *In Vivo* Tumor Heating

Tumor-bearing mice were prepared by inoculating Meth A fibrosarcoma (*Meth A*) cell ($1x10^6$) into the left flank of BALB/*c* mice (female, 8 weeks old and weighing about 25 g).[69] The tumor was heated by placing a PTC heater (Positive Temperature Coefficient Thermistor, 10 mm in length and 10 mm in width; Tokyo Denki Kagaku, Tokyo) on the tumor surface on day 7 after the inoculation (*Fig. 4.2*). The mean of the length and the width of the tumor was about 10 mm, and the thickness of the tumor was about 2 mm. The heater was fixed to the tumor with surgical tape. The mouse was fixed on a sheet with adhesive tape while conscious until the end of the heating.

For the tumor CDDP distribution study, a heater temperature of 47°C was used. The heating was started at 15 min after drug administration and was continued for 30 min. This HT timing is usually used in HT therapy.[56]

4.3.3 CDDP Level in Blood and Tumor after Administration

The liposomes and the CDDP solution (*Sol*) were administered intravenously *via* the tail vein (dose, 40μg CDDP/mouse). At each time point, 5 mice from each treatment group were sacrificed by exsanguination from the heart with a heparinized syringe, and the blood and tumor samples were obtained. The tumor sample was homogenized with a tissue homogenizer (Polytoron, Kinematica). The concentration of CDDP in the blood and the tumor was determined by the same method as described in *Chapter 3*. The CDDP concentration in all cases except that following *SUV-1* administration was determined by HPLC (*Chapter 2*) and by AAS (*Chapter 2*). The CDDP concentration in the case of SUV

administration was determined only by HPLC since AAS was not available in this experiment.

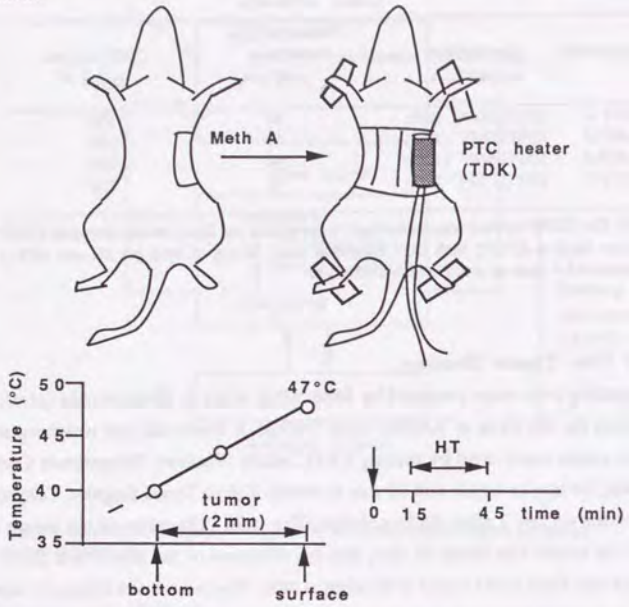

Fig. 4.2 Schematic illustration of tumor heating in a mouse.

4.4 Results and Discussion

4.4.1 Dispersion Characteristics of Liposomes

In the previous study (*Chapter 2*), the light-scattering analysis of thermosensitive LUV composed of DPPC and DSPC showed that it existed in fluid as a multidispersion with an apparent size larger than the single particle size (about 0.2μm).

Generally, small liposomes are taken up by the RES to a lesser extent and stay in the systemic circulation for a longer time than large liposomes.[2] In a preliminary study, we found that adding SMT to the lipid constituent improved the dispersion characteristics of the liposome. In the preparation of *LUV-2* and *LUV-3*, therefore, we used SMT in an effort to decrease their systemic clearance by improving their dispersion characteristics. However, we got the opposite effect in the systemic clearance after *LUV-2* administration (discussed below).

4.4.2 *In Vitro* Release Characteristics of Liposomes

The heat-specific drug release from a thermosensitive liposome has been demonstrated to occur explosively and completely in a few seconds, indicating that the release rate does not change much if the heating time is longer than 1 min (*Chapter 2*). Therefore, as a convenient temperature-dependent release test, we employed the method of incubating the liposomes at various HT temperatures for 15 min. *Fig. 4.3* shows the temperature-dependent release rate profiles for *LUV-1*, *LUV-2*, *SUV-1* and *LUV-3*. The *LUV-1* and *LUV-2* showed very sharp temperature-dependent release profiles. They released CDDP at HT temperatures almost completely, but did not release CDDP at 39°C. The critical release temperatures for *LUV-1* and *LUV-2* were slightly different, being 41°C and 42°C, respectively. It is suggested that the higher temperature obtained with *LUV-2* is due to the relatively high phase transition temperature of SMT. Although *SUV-1* released CDDP at HT temperatures, the rate was slower than those observed with the thermosensitive LUVs. The mean amounts of the drug released from *LUV-1*, *LUV-2* and *SUV-1* between 41°C and 47°C were 82, 76 and 32%, respectively. On the other hand, *LUV-3* did not release the drug at HT temperatures.

4.4.3 *In Vivo* Tumor Heating

Water bath heating is usually employed to heat mouse tumors.[105] However, this results in the elevation of the body temerature. To avoid body temperature elevation, we employed a PTC heater. In a preliminary heating experiment, the temperatures at different sites in the tumor were monitored by instilling a thermocouple into the tumor. A heater temperature of 43-49°C afforded the heating of the tumor to HT temperatures without raising the systemic temperature. The temperatures at the surface, center and bottom of the tumor reached steady state within 5 min, and a linear temperature gradient was observed inside the tumor. Using a heater temperature of 47°C, the temperatures at the surface, center and bottom of the tumor were about 47°C, 44°C and 41°C, respectively, indicating that a heater temperature of 47°C afforded the heating of about 80 % of the tumor to above 42°C.

4.4.4 Systemic Clearance of Liposomes

Fig. 4.4 shows the CDDP levels (% of the administered dose) in the blood after administration of the liposomes or the solution with or without HT. The levels upto 1 hr

after liposome administration with or without HT were 10-20 times as high as those after solution administration. Because of the much faster systemic elimination of the free CDDP, the CDDP levels after liposome administration are assumed to be nearly equal to the levels of the CDDP encapsulated in the liposome (*Chapter 3*). The CDDP levels after *LUV-2* administration were a little lower than those after administration of the other liposomes. In an additional study, the liver Pt levels at 4 hr after *LUV-2* administration were shown to be two-thirds that after *LUV-1* administration and one-third that after *LUV-3* administration (data not shown). The larger systemic clearance after *LUV-2* administration is, therefore, attributed to the faster degradation of the liposome in the systemic circulation rather than the RES uptake (*Fig. 4.1*).

Yatvin et al.[105] reported that the systemic clearance of the encapsulated drug was increased by HT, but in the present study, the clearance was decreased a little. It was possible that the heating of the tumor with a water bath raised the body temperature and thereby increased the systemic degradation of the liposome. In the present study, such body temperature elevation was avoided.

4.4.5 CDDP Distribution in the Tumor

The tumor CDDP levels were measured using an HPLC assay (*Fig. 4.5*) and an AAS assay (*Fig. 4.6*). The levels obtained with the AAS assay were about three times as high as those obtained with the HPLC assay, suggesting that about two-thirds of the Pt was associated with the protein or the other macromolecules in the tumors.

In both the assays, the tumor CDDP levels after the administration of the thermosensitive liposomes with HT were shown to be significantly higher than those after the administration of the liposomes alone (unpaired Student's t test: $p<0.01$ in the 1 hr tumor levels). However, such higher CDDP levels in response to HT were not observed after the administration of the non-thermosensitive liposome or solution. The tumor CDDP level increase with only the combination of the thermosensitive liposomes and HT indicates that CDDP is delivered to the tumor *via* the heat-specific CDDP release in these modalities. Moreover, the lack of the tumor CDDP level increase in *Sol + HT* or in *LUV-3 + HT* indicates that HT-specific CDDP uptake by the tumor or HT-specific liposome endocytosis does not occur in the present liposome + HT delivery system. The tumor CDDP levels after the administration of the liposomes, except *LUV-3*, without HT were similar to those after *Sol* administration. It is possible that these liposomes released the encapsulated CDDP gradually in the systemic circulation and the released CDDP was distributed to the tumor as following *Sol* administration. On the other hand, the levels after *LUV-3* administration without HT were lower than those after *Sol* administration. It is supposed

that this resulted from the relatively high distribution of the liposome into the RES (discussed above). Both results indicate that liposome administration without HT does not have any active means of directing the drug to the targeted tumor.

Upon administration of *LUV-1* or *SUV-1* with HT, high tumor CDDP levels were maintained for a long time. It is suggested that this was due to the slow elimination of CDDP from the tumor after distribution. Generally, platinum compounds tend to bind tightly within the tissues and tend not to be eliminated quickly after their distribution to the tissues.[84] The *LUV-2 + HT* exposure resulted in a rather rapid elimination of the drug from the tumor. However, the time to reach the peak levels was different in the two assays. There may be some error in the peak level determination in *LUV-2 + HT*.

4.4.6 TDD Efficiency (Targeting Index)

The efficiency of the present TDD system can be evaluated by calculating targeting index (*TI*) which is expressed as the ratio of the AUC of the tumor CDDP-levels after liposome administration with HT to that after *Sol* administration with HT.[23,28] *Table 4.2* shows the *TI*s after the administration of the thermosensitive liposomes with HT. The *TI* for *LUV-1* (about 5) is the largest among those for the present thermosensitive liposomes and is larger than that for a thermosensitive SUV reported earlier (about 3).[105]

4.4.7 *In Vivo* Heat Sensitivity

According to theory, the TDD efficiency is a function of the heat sensitivity of the liposome and the concentration of the liposome in the local or systemic blood. The ratio of the AUC of the blood levels of the encapsulated drug during HT after liposome administration to the AUC of the blood levels of the free CDDP after *Sol* administration can give a *TI* for complete drug release.[28] The value for *LUV-1* administration with HT is about 6, which is close to the actual *TI* (about 5), indicating that the HT-dependent drug release after *LUV-1* administration occurred almost completely. The heat sensitivity of *LUV-2* and *SUV-1* can be compared with that of *LUV-1* on a similar theoretical basis. The AUC of the blood levels of the encapsulated drug during HT after *SUV-1* administration was equivalent to that of *LUV-1*. Therefore, it could be suggested that the smaller *TI* for *SUV-1* was due only to its lower heat sensitivity. Similarly, the AUC of the blood levels of the encapsulated drug during HT after *LUV-2* administration was about one-third that after *LUV-1* administration. It is possible that the HT-dependent drug release after *LUV-2* administration occurred almost completely and that the smaller *TI* for *LUV-2* administration was due to larger systemic clearance of *LUV-2*.

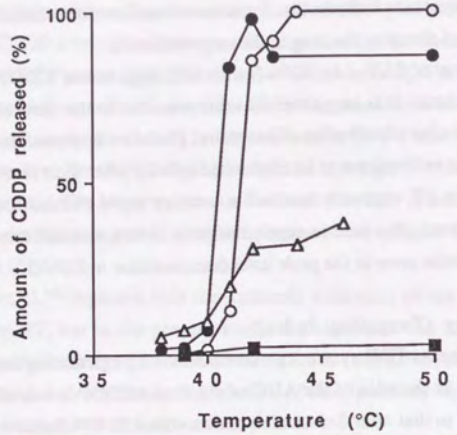

Fig. 4.3 The CDDP release-rate *versus* temperature profile after incubating *LUV-1* (●), *LUV-2* (○), *SUV*-1 (△) or *LUV*-3 (■) in saline for 15 min.

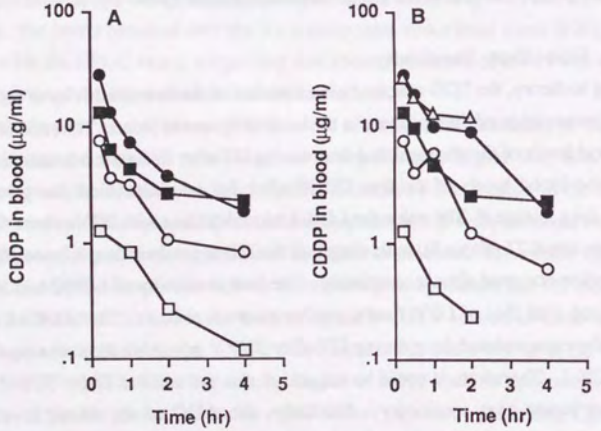

Fig. 4.4 The blood CDDP levels after administration of *LUV-1* (●), *LUV-2* (○), *SUV-1* (△), *LUV-3* (■) or *Sol* (□) to tumor-bearing mice with (Panel *B*) or without HT (Panel *A*). The CDDP levels after liposome administration represent the levels of the free + encapsulated CDDP. The CDDP levels after *SUV-1* administration were determined by the HPLC assay, while in all other cases they were determined by the AAS assay.

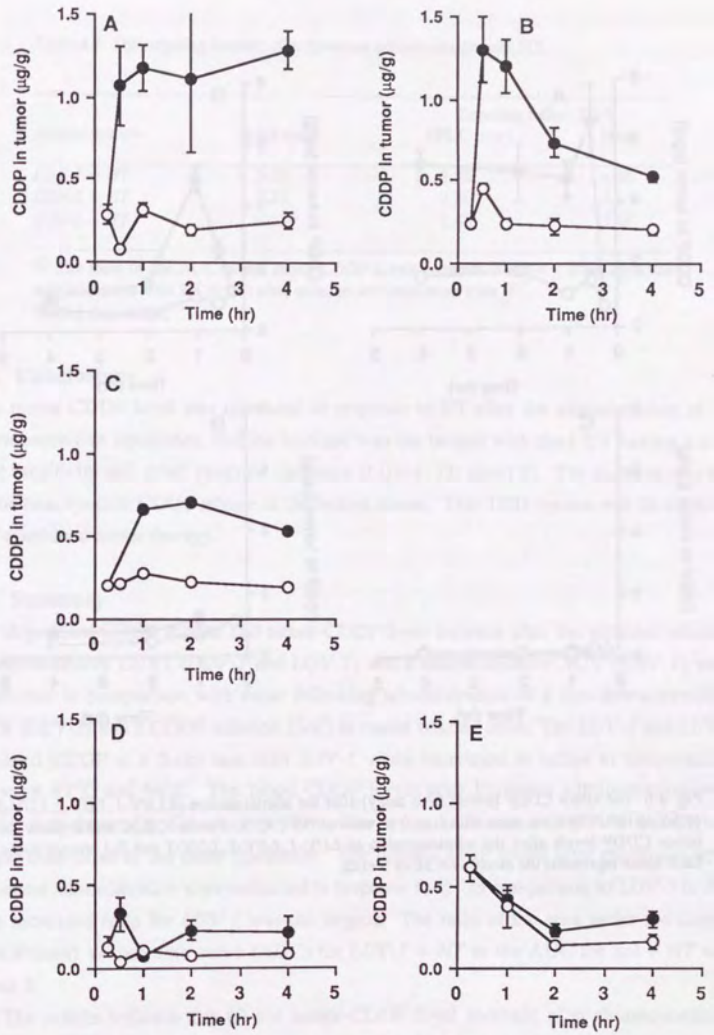

Fig. 4.5 The tumor CDDP levels (HPLC assay) after the administration of *LUV-1*, *LUV-2*, *SUV-1*, *LUV-3* or *Sol* to tumor-bearing mice with (●) or without HT (○). Panel *A*, *B*, *C*, *D* and *E* show the tumor CDDP levels after the administration of *LUV-1*, *LUV-2*, *SUV-1*, *LUV-3* and *Sol*, respectively. Each result represents the mean with SE of 5 mice.

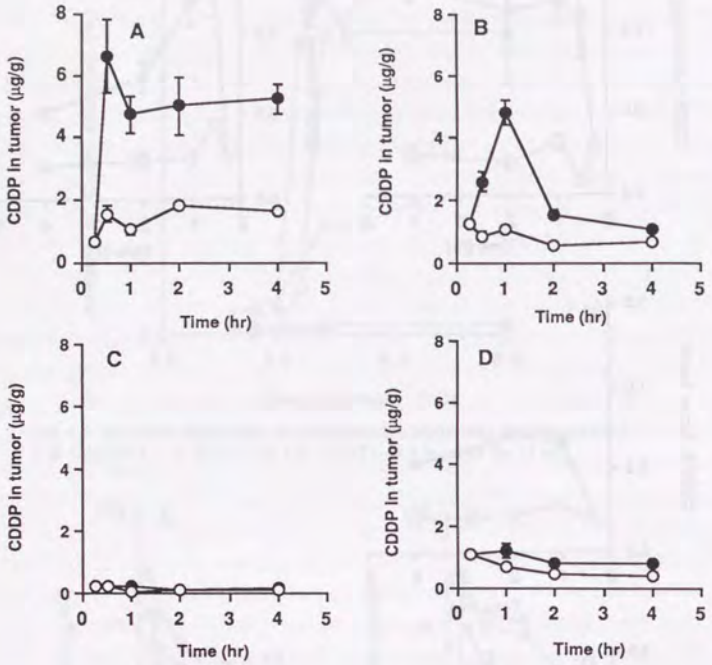

Fig. 4.6 The tumor CDDP levels (AAS assay) after the administration of *LUV-1*, *LUV-2*, *LUV-3* or *Sol* to tumor-bearing mice with (●) or without HT (○). Panels *A*, *B*, *C* and *D* show the tumor CDDP-levels after the administration of *LUV-1*, *LUV-2*, *LUV-3* and *Sol*, respectively. Each result represents the mean with SE of 5 mice.

Table 4.2 The targeting indexes after liposome administration with HT.

Administration	Targeting Index (*TI*) [a]		
	AAS assay	HPLC assay	Mean
LUV-1 + HT	5.29	3.98	4.63
LUV-2 + HT	2.31	2.80	2.55
SUV-1 + HT	ND [b]	2.65	2.65

[a] The ratio of the AUC of the tumor CDDP-levels between 0 and 4 h after liposome administration with HT to that after solution administration with HT.
[b] Not determined.

4.5 Conclusions

The tumor CDDP-level was increased in response to HT after the administration of the thermosensitive liposomes, and the increase was the largest with the LUV having a high heat sensitivity and small systemic clearance (*LUV-1*; *TI*: about 5). The increase was due to the heat-specific CDDP release at the heated tumor. This TDD system will be useful in HT-combined tumor therapy.

4.6 Summary

HT-dependent CDDP release and tumor-CDDP-level increase after the administration of thermosensitive LUVs (*LUV-1* and *LUV-2*) and a thermosenstive SUV (*SUV-1*) were examined in comparison with those following administration of a non-thermosensitive LUV (*LUV-3*) and a CDDP solution (*Sol*) in tumor bearing mice. The *LUV-1* and *LUV-2* released CDDP at a faster rate than *SUV-1* when incubated in saline at temperatures between 41°C and 44°C. The blood CDDP levels after liposome administration were higher than those after *Sol* administration. The systemic clearance of *LUV-2* was slightly larger than those of the other liposomes. The tumor CDDP levels after thermosensitive liposome administration were increased in response to HT in comparison to *LUV-3* or *Sol*. The increased ratio for *LUV-1* was the largest. The ratio of the area under the tumor-CDDP-level *versus* time curve (AUC) for *LUV-1 + HT* to the AUC for *Sol + HT* was about 5.

The results indicate that (*i*) the tumor-CDDP-level increase after thermosensitive liposome administration is due to CDDP release from the liposome in the blood at or adjacent to the heated tumor, (*ii*) the increase is highly dependent on the heat sensitivity and

systemic stability of the liposome, and (*iii*) LUV, such as *LUV-1*, exhibite higher heat sensitivity and larger targeted drug delivery efficiency than SUV.

Chapter 5 Heat-Induced Drug Release Rate and Maximal Targeting Index (*TI*) [34,37]

5.1 Introduction

Theoretical basis of drug targeting have been reported by several workers,[12,13,28] but none afford a quantitative evaluation of the site-specific drug release and the upper limit of the targeting efficiency. This chapter describes a theoretical and experimental method to estimate the fraction of drug released at the heated tumor (*F*) and the maximal drug targeting index (TI_{max}, *TI* determined assuming complete drug release) and the evaluation of the *F* and TI_{max} for the thermosensitive liposomes described in *Chapter 4*.

5.2 Theory

5.2.1 Pharmacokinetic Concept behind HT-Combined Thermosensitive Liposome Delivery

As described in *Chapters 3* and *4*, the pharmacokinetics of the liposome-encapsulated drug and the free drug after intravenous administration of a liposome can be described using an anatomical tissue-perfusion model. The administered liposome first enters the systemic circulation and is mixed. In the repeated circulation, it is degraded in the systemic blood or taken up by the RES (*Chapter 3*). However, little is taken up by the tumor and the normal tissue (organ).[26] Therefore, although a certain amount of the drug encapsulated in the liposome is taken up by the RES, the residual amount is eliminated from the systemic blood *via* drug release from the circulating liposomes. As a result, the free drug is distributed in the tumor or in the normal tissue (organ). The elimination kinetics are the same as those after the administration of the drug as a solution. If a liposome is thermosensitive and its administration is followed by tumor heating, drug release occurs from the liposomes at or adjacent to the tumor when the liposomes travel concurrently with the blood through the heated tumor, and the released drug is distributed in the tumor resulting in a high drug concentration in the tumor. This is the concept behind the HT-combined thermosensitive liposome delivery system.[96,98]

5.2.2 Drug Targeting Index (*TI*) as a Function of Blood Drug Concentration

Assuming rapid drug partition between the blood and the tumor,[6] the distribution of drug in the tumor after the administration of a solution can be expressed by the following differential equation:

$$V_T dT_{drug} / dt = Q(B_{in} - B_{out}) = Q(B_{drug} - T_{drug}/K) \qquad (5.1)$$

where V_T, T_{drug}, Q, B_{in}, B_{out} and K refer to the volume of the tumor, the concentration of drug in the tumor, the blood flow rate, the concentration of drug in the arterial blood which is equal to the concentration of drug in the systemic blood (B_{drug}), the concentration of drug in the venous outflow (effluent) blood from the tumor compartment, and the ratio of tumor concentration to the venous outflow-blood concentration, respectively (*Fig.5.1*).

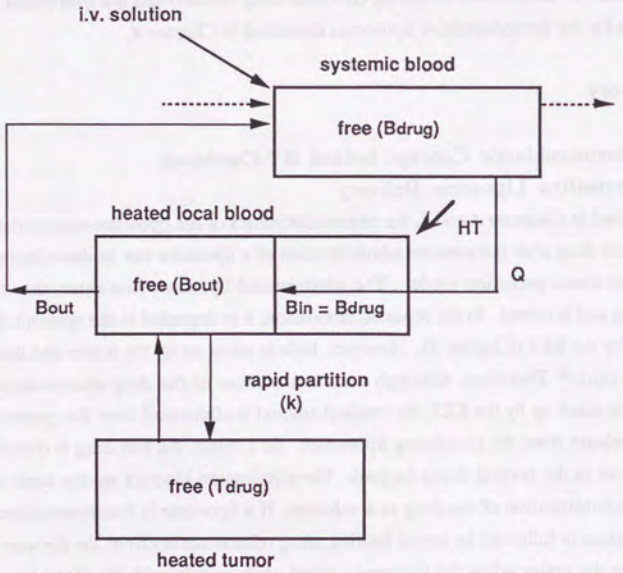

Fig. 5.1 Distribution of drug in the tumor after administration of a solution with hyperthermia.

The integration of *Eq. 5.1* with respect to time, *t*, from zero to infinity gives:

$$AUC(T_{drug}, sol) = K\, AUC(B_{drug}, sol) \qquad (5.2)$$

where $AUC(T_{drug}, sol)$ and $AUC(B_{drug}, sol)$ refer to the area under the curve (AUC) of the concentration of drug in the tumor after solution administration and the AUC of the concentration of drug in the systemic blood after solution administration, respectively.

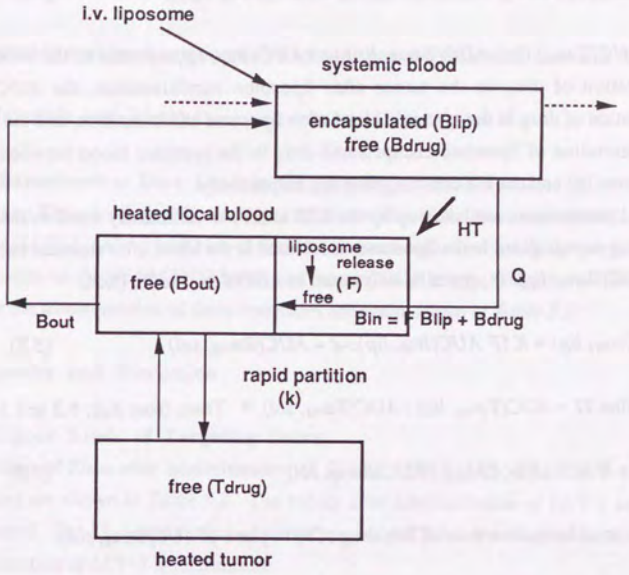

Fig. 5.2 Distribution of drug in the tumor after administration of a liposome with hyperthermia.

The distribution of drug in the tumor after administration of a thermosensitive liposome can also be expressed by a differential equation similar to *Eq. 5.1*. In this case, B_{in} is equal to the concentration of drug released from the liposomes in the blood at or adjacent to the heated tumor in a single pass plus the concentration of free drug which has already been released at the tumor or at the systemic circulation (the concentration very low), and the concentration of the drug released from the liposomes in response to heat is assumed to be the product of the concentration of liposome-encapsulated drug in the arterial blood which is equal to the concentration in the systemic blood (B_{lip}) and the fraction of the drug released from the liposomes in a single pass through the heated tumor (F) (*Fig. 5.2*).

$$B_{in} = F\, B_{lip} + B_{drug} \text{ (except HT time, } F = 0) \qquad (5.3)$$

The integration of the differential equation gives:

$$AUC(T_{drug}, lip) = K\,[F\,AUC(B_{lip}, lip)_{ts\text{-}tf} + AUC(B_{drug}, lip)] \qquad (5.4)$$

where $AUC(T_{drug}, lip)$, $AUC(B_{drug}, lip)$ and $AUC(B_{lip}, lip)_{ts\text{-}tf}$ refer to the AUC of the concentration of drug in the tumor after liposome administration, the AUC of the concentration of drug in the systemic blood after liposome administration, and the AUC of the concentration of liposome-encapsulated drug in the systemic blood between the HT starting time (ts) and the HT finishing time (tf), respectively.

If the liposomes are not taken up by the RES and V_T is sufficiently small so that almost all the drug encapsulated in the liposomes is released in the blood after repeated circulation, then, $AUC(B_{drug}, lip)$ is approximately equal to $AUC(B_{drug}, sol)$. Then,

$$AUC(T_{drug}, lip) = K\,[F\,AUC(B_{lip}, lip)_{ts\text{-}tf} + AUC(B_{drug}, sol)] \qquad (5.5)$$

If we define $TI = AUC(T_{drug}, lip) / AUC(T_{drug}, sol)$.[28] Then, from *Eqs. 5.2* and *5.5*,

$$TI = 1 + F\,AUC(B_{lip}, lip)_{ts\text{-}tf} / AUC(B_{drug}, sol) \qquad (5.6)$$

Using the total body clearance of free drug (Cl_t) in place of $AUC(B_{drug}, sol)$,

$$TI = 1 + F\,Cl_t\,AUC(B_{lip}, lip)_{ts\text{-}tf} / dose \qquad (5.7)$$

5.2.3 Upper Limit of *TI* as a Function of Blood Liposome Concentration

The aim of targeting delivery systems is to release the drug at the target site.[13] Therefore, the concentration of liposome-encapsulated drug in the blood at the targeted site is a primary factor limiting the targeting specificity. The *TI* value, assuming complete drug release ($F = 1$), gives an upper limit of *TI* (TI_{max}) which is determined just by the concentration of liposome in the blood during HT and the systemic clearance of the drug:

$$TI_{max} = 1 + Cl_t\,AUC(B_{lip}, lip)_{ts\text{-}tf} / dose \qquad (5.8)$$

Thus, the TI_{max} value can be an indicator how the targeting specificity of the present targeting delivery system is limited by the systemic clearance of the liposome or by the systemic clearance of the drug.

5.2.4 *F* as a Function of *TI* and TI_{max}

Combining *Eqs. 5.7* and *5.8* gives an equation for *F*:

$$F = 100\,(TI - 1) / (TI_{max} - 1) \qquad (5.9)$$

This equation enables us to estimate *F* when we know the values of TI_{max} and *TI*.

5.3 Materials and Methods

5.3.1 Experimental Data Used in Calculation of *F* and TI_{max}

The *F* and TI_{max} for four different types of thermosensitive liposomes containing CDDP (*LUV-1*, *LUV-2*, *LUV-3* and *SUV-1*; *Table 4.1*) were estimated using the present method and the data on the blood CDDP levels and tumor CDDP levels (*Chapter 4*). The values of *TI* after the administration of these liposomes are again shown in *Table 5.1*.

5.4 Results and Discussion

5.4.1 Upper Limit of Targeting Index

The values of TI_{max} after administration of *SUV-1*, *LUV-1*, *LUV-2* and *LUV-3* with HT treatment are shown in *Table 5.2*. The values after administration of *LUV-1* and *SUV-1* are about 6. The TI_{max} value after administration of *LUV-2* is less than half, and that after administration of *LUV-3* is intermidiate.

As described in the theory, TI_{max} can be an indicator of how the targeting specificity of the delivery system is limited by the systemic clearance of the liposomes. Assuming that the liposomes are not eliminated from the systemic circulation, the resulting TI_{max} (about 10) indicates the upper limit of *TI* for the present drug delivery system:

$TI_{max} = 1 + Cl_t\,(tf - ts) / blood\ volume = 10$

(Cl_t = 20ml /h; $tf - ts$ =1/2h; blood volume=1ml)

The values of TI_{max} for *LUV-1* and *SUV-1* are about 60% of this limit whereas the value of TI_{max} for *LUV-2* is only about 20%. The small TI_{max} obtained with *LUV-2* is due to the large systemic clearance of the liposomes and thus, a small *TI* in spite of high heat sensitivity for *LUV-2* is attributed to the large systemic clearance of the liposomes.

5.4.2 Rate of Heat-Specific Drug release

Table 5.3 shows the values of *F* after administration of *SUV-1*, *LUV-1*, *LUV-2* and *LUV-3* with HT treatment. The *F* values after administration of *LUV-1* and *LUV-2* with HT

treatment were 0.71 and 1.17, respectively. In contrast, the *F* values after administration of these liposomes without HT treatment were 0.08 and -0.12, respectively. The *F* value after the administration of *SUV-1* with HT treatment was 0.34. In spite of some error, these values obtained with the thermosensitive liposomes seem to reflect their *in vitro* heat-specific release characteristics (*Chapter 4*). The results suggest that unlike the small *TI* obtained with *LUV-2*, the small *TI* with *SUV-1* can be attributed to the small release rate. The *F* value after administration of *LUV-3* with or without HT was negative with a large absolute value (approximately -0.3). Theoretically *F* must not be negative. The value obtained with *LUV-3* may indicate error beyond that of normal data variation. In the theory, it was assumed that liposomes are not taken up by the RES, but actually about 20% of the dose after administration of *LUV-1* and and *LUV-2* and, about 50% of the dose after administration of *LUV-3* were taken up by the RES (*Chapter 3*). If we assume that the fraction of the dose taken up by the RES is α, then the fraction of the dose released in the systemic circulation is $1 - \alpha$, and *Eq. 5.6* can be rewriten as:

$$TI - (1 - \alpha) = F\, AUC(B_{lip}, lip)_{ts\text{-}tf} \,/\, AUC(B_{drug}, sol)$$

F can be recalculated using this equation. The *F* values for *LUV-1 + HT* and *LUV-1* alone ($\alpha = 0.2$) are 0.75 and 0.04, respectively, and the *F* value for *LUV-2 + HT* and *LUV-2* alone ($\alpha = 0.2$) are 1.32 and 0.03, respectively. These values are not much different from those above. The *F* values for *LUV-3 + HT* and *LUV-3* alone ($\alpha = 0.5$) are -0.1 and -0.13, respectively, and these are more realistic than those above in light of the *in vitro* release characteristics.

Table 5.1 The values of *TI* after administration of *SUV-1*, *LUV-1*, *LUV-2* or *LUV-3* with or without HT treatment.

liposome	*TI*
SUV-1	0.97
SUV-1 + HT	2.66
LUV-1	0.99
LUV-1 + HT	4.63
LUV-2	0.84
LUV-2 + HT	2.55
LUV-3	0.15
LUV-3 + HT	0.24

Table 5.2 The values of *TImax* after administration of *SUV-1*, *LUV-1*, *LUV-2* or *LUV-3* with HT treatment.

liposome	*TImax*
SUV-1 + HT	5.83
LUV-1 + HT	6.08
LUV-2 + HT	2.32
LUV-3 + HT	3.57

Table 5.3 The values of *F* after administration of *SUV-1*, *LUV-1*, *LUV-2* or *LUV-3* with or without HT treatment.

liposome	*F*
SUV-1	0.00
SUV-1 + HT	0.34
LUV-1	0.08
LUV-1 + HT	0.71
LUV-2	-0.12
LUV-2 + HT	1.17
LUV-3	-0.33
LUV-3 + HT	-0.29

5.4.3 Correlation between *F* and *In Vitro* Release Rate

In the theory, *F* is assumed to be constant during HT. However, the fraction released in the first pass is likely to be larger than the fraction released from the same liposomes in each additional pass. Our earlier study showed thermosensitive *LUV* liposomes release encapsulated carboxyfluorescein within a few sec (*Chapter 2*). Therefore, highly heat-sensitive liposomes are supposed to release almost all the drug in the first pass. This, however, does not contradict the present theory because, with highly heat-sensitive liposomes, B_{lip} in *Eq. 5.3* represents the concentration of only the liposomes which are going to experience the first pass through the tumor.

Temperature distribution in the tumor is another factor to be considered in estimating theoretically the extent of heat-specific drug release. If the temperature distribution in the tumor is not uniform, the fraction of released drug will be different at different sites in the tumor. Our study on tumor heating with a PTC heater (heater temperature of 47°C) revealed that the temperature shows a linear gradient in the direction of the depth (*Chapter 4*). The temperature at the surface of the tumor was almost the same as the heater temperature while the temperature at the bottom was about 40°C. Therefore, the *F* value

represents the average fraction of drug release. Thus, we can compare the F values with the mean of the *in vitro* drug release rates at the temperatures from 40°C to 47°C as shown in *Materials and Methods*, and these are in good agreement. This indicates not only that the present method is highly reliable but also that the phase transition of the liposome in response to the heat occurs quickly enough for complete drug release when the liposomes pass through the heated tumor (*Chapter 2*).

5.4.4 Parameter Analysis

The response of F, $AUC(Blip,lip)_{ts\text{-}tf}$ and Clt to TI can be seen in *Fig. 5.3*. A larger TI is achieved with a larger area A or a smaller area B, and accordingly by a larger drug release rate (arrow *a*), a smaller systemic clearance of the liposome (arrow *b*), an earlier (arrow *c*) or longer (arrow *d*) HT period, or a larger systemic clearance of the drug (arrow *e*). Therefore, it is important not only to prepare a highly heat-sensitive liposome with a small systemic clearance, but also to choose a drug with a large systemic clearance. In this respect, CDDP is a suitable drug.

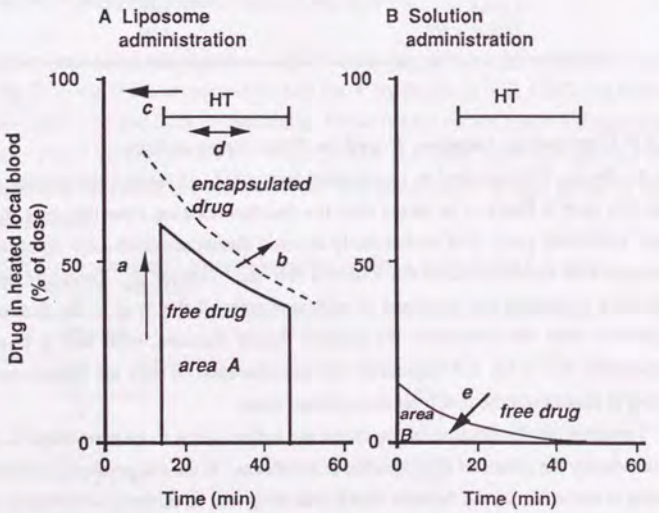

Fig. 5.3 Hypothetical drug concentration in the blood at or adjacent to the targeted tumor (*Panel A*, liposome administration and *Panel B*, solution administration) and the response of the heat-specific drug release rate (F), the AUC of the blood liposome level during HT [$AUC(Blip, lip)_{ts\text{-}tf}$] and the systemic clearance of a drug (Clt) to the drug targeting index (TI).

5.5 Conclusion

The present method using pharmacokinetic data obtained after administration of a thermosensitive liposome enabled us to evaluate F and TI_{max}. The value of TI_{max} after administration of *LUV-1* and *SUV-1* was around 6. The values of F (0.71 for *LUV-1* and 0.34 for *SUV-1*) are in good agreement with the *in-vitro* drug release rates. This confirms that the encapsulation of CDDP in a thermosensitive LUV liposome which is highly heat-sensitive and has a small systemic clearance is preferable for the present drug delivery system.

5.6 Summary

To evaluate the rate of drug release at heated tumor and maximal drug targeting after administration of thermosensitive liposomes with HT, a theoretical and experimental method was derived, assuming the fraction of drug released from liposomes in a single pass through the heated tumor, F, and drug targeting index when drug release occurs completely in response to heat (F=1), TI_{max}. The F and TI_{max} were evaluated for four different types of liposomes using the data on the blood liposome levels and the tumor drug levels described in *Chapter 4*. The TI_{max} values for *LUV-1* and *SUV-1* were approximately 6, while TI_{max} value for *LUV-2* with a relatively large systemic clearance was only 2.3. The F values for *LUV-1*, *LUV-2* and *SUV-1* with HT were about 0.71, 1.17 and 0.34, respectively, whereas the values for those liposomes without HT and for *LUV-3* with or without HT were nearly zero. These results confirm earlier findings that *LUV-1* and *LUV-2* release CDDP almost completely at the heated tumor and that the large TI value obtained in *LUV-1* (TI = 4.6) was due to its high heat sensitivity and its small systemic clearance.

Chapter 6 Enhanced Antitumor Activity[36]

6.1 Introduction

Targeted drug delivery (TDD) is one way to increase the therapeutic index of antitumor drugs.[24,25,43,70,71,79,81,103] A TDD system combining thermosensitive liposomes with HT should attain a large TDD effect, thereby increasing the therapeutic index.[95-100,104-107] This chapter describes the TDD effect and the enhanced antitumor activity in the tumor-bearing mice after the administration of CDDP-encapsulated thermosensitive LUV in combination with HT is presented. The purpose is to determine whether the therapeutic index of CDDP is increased by the TDD effect.

6.2 Materials and Methods

6.2.1 Liposome-Encapsulating CDDP

A thermosensitive LUV which releases the encapsulated CDDP at the HT temperatures (*LUV-1*) was prepared as *Chapter 2*. The temperature-dependent CDDP release characteristcs of the liposome were examined using 15-min incubation method (*release test-1*) and short-time heating method using a heated tuble (*release-test 2*)(*Chapter 2*). In *release-test 1*, the liposomes were diluted with saline 1:10. In *release-test 2*, the liposomes were diluted with the rat plasma 1:10 (*in-vivo* simulation).

6.2.2 Tumor-Bearing Mice and HT

Tumor bearing mice and HT were the same as described in *Chapter 4*. *Meth A* cells ($1x10^6$) were inoculated *s. c.* into the left flanks of 8-week-old mice weighing about 25 g on day 0. Drug administration and/or HT treatment was started on day 7 after the tumor inoculation. At this time, the mean of the longest diameter and the widest diameter of the tumor was about 10 mm and thickness of the tumor was about 2 mm. The heating (heater temperature, 47°C) was started at 15 min after drug administration and was continued for 30 min.

6.2.3 Pt Distribution in Blood, Tissue and Organ

The Pt levels in the blood, the tumor and the normal organs (liver, left kidney and spleen), after the i. v. administration of *LUV-1* and a CDDP solution (*Sol*), each at a single dose of 40μg CDDP/mouse, to mice with or without HT, was examined as described in *Chapter 4*. The concentration of Pt in the tissues was determined by the AAS.

6.2.4 Antitumor Activity and Side Effects

As a preliminary study, we examined the inhibitory activity of *Sol* against *Meth-A* using continuous treatment schedules. The daily administration for 4 days at 80μg/mouse showed not only moderate antitumor activity [the weight inhibition of the tumor calculated from 100 minus the percentage of the tumor weight of the untreated control (*T/C*) on day 21; 54%], but also severe toxicity (two of five mice died before assay). Therefore, we selected the schedule of two consecutive-day treatment (days 7 and 8) at the CDDP dose ranging from 10μg/mouse to 40μg/mouse. The antitumor activity for *LUV-1* with HT was examined as compared to treated controls (*HT alone*, *Sol alone*, *LUV-1 alone*, and *Sol + HT*) and an untreated control. Five mice were used for each treatment and the untreated control.

The antitumor activity after each treatment was evaluated on the basis of the tumor growth delay obtained in the periodical measurement of the tumor size (the mean of the longest diameter and the widest diameter of the tumor) and the weight inhibition of the tumor obtained from the measurement of the weight on day 21. The tumor growth delay was expressed as the lag time in the tumor growth as compared to the untreated control (day).

The side effects after drug administration with HT were examined by measuring the loss in the body weight (the tumor weight subtracted) on day 21 as compared with the untreated control and blood urea nitrogen level (BUN) on day 13 as an indicator of CDDP nephrotoxicity.[65,110] The BUN level was determined by taking blood from the ocular vein and measuring the concentration of the urea nitrogen in the plasma by the Urea NB-test (Wako Pure Chemical, Osaka).

6.3 Results

6.3.1 Temperature-Dependent Release Rate Profle

Temperature-dependent release rate profiles for *LUV-1* obtained from two release tests are shown in *Table 6.1*. In *release-test 1* (15-min heating in saline), the drug release occurred at and above 41°C and the mean released amount at these HT temperatures was about 80%. At temperatures lower than 41°C, the drug release did not occur. In *release-test 2* (short-time heating in plasma), the drug release occurred at the HT temperatures within a few seconds but the released amounts were somewhat low; the released amount during 3.4-s heating at 41°C was about half of that obtained in 15-min heating at the same temperature in *release-test 1*. The critical release temperature in *release-test 2* was lower by about 1 °C than that in *release test-1*.

6.3.2 TDD Effect

6.3.2.1 Pt Distribution in Tumor

The Pt distribution in the tumor after *LUV-1* administration with and without HT and *Sol* administration with HT is shown in *Table 6.2*. The tumor Pt levels after *LUV-1* administration with HT were about 3 times higher than those after *LUV-1* administration without HT and *Sol* administration with HT and the levels were sustained for at least 24 hr. The AUC of the tumor Pt levels in *LUV-1 + HT* (calculation using a trapezoidal rule) was about 3 times larger than that in *LUV-1 alone* or *Sol + HT*.

6.3.2.2 Pt-Distribution in Normal Organs

The blood Pt levels and the Pt levels in the liver, spleen and kidney after *LUV-1* administration with and without HT or *Sol* administration are shown in *Table 6.3*.

The blood Pt levels at early time after *LUV-1* administration with and without HT were higher as compared to *Sol* administration with HT. The AUC of the blood Pt levels during HT for *LUV-1 + HT* (calculation using a trapezoidal rule) is about 4 times larger than that for *Sol + HT*. The liver Pt levels at early times after *LUV-1* administration with and without HT were higher as compared to *Sol* administration with HT. The 1-hr-liver levels in *LUV-1* and *LUV-1 + HT* were 1.7-2.3 times higher than those in *Sol + HT*. However, the 24 hr levels were similar to those in *Sol + HT*. The Pt levels in the spleen at 1 hr and 24 hr after *LUV-1* administration with or without HT were higher as compared to *Sol* administration with HT. The 1-hr-spleen levels in *LUV-1* and *LUV-1 + HT* were about 20 times higher than those in *Sol + HT*, and the levels were maintained at 24 hr. The kidney Pt levels after *LUV-1* administration with HT were similar to those after *Sol* administration with HT. However, the levels after *LUV-1* administration without HT are about half of those in *LUV-1 + HT* and *Sol + HT*. The kidney Pt levels in all modalities were sustained for at least 24 hr.

Table 6.1 Temperature-dependent CDDP release rate profile.

			Release-Test 1 [a]			
Temperature	38°C	39°C	40°C	41°C	42°C	43°C
Heating Time						
15 min	4.6	3.2	6.9	81.4	80.2	80.2
			Release-Test 2 [b]			
Temperature	39°C	40°C	41°C	42°C	43°C	45°C
Heating Time [c]						
1.6 sec	1.6	5.8	32.2	50.9	68.9	73.0
3.4 sec	2.8	16.7	42.1	60.8	74.1	75.3
5.0 sec	3.1	22.0	58.4	66.1	73.0	79.0

a) Amount released (%) after incubation of saline-diluted *LUV-1* (1:10)
b) Amount released (%) when plasma-diluted *LUV-1* (1:10) passed through a heated tube
c) Equal to the sample resident time in the heated tube.

Table 6.2 Pt distribution in tumor after the administration of *LUV-1* and *Sol* each at 40 μg CDDP/mouse to mice with or without HT. The all data except AUC and *TI* are expressed as mean ± SE (n=5).

	Tumor Pt Level		
	LUV-1 + HT	*LUV-1*	*Sol + HT*
		μg/g tumor	
1 hr	3.40 ± 0.33 [a]	0.93 ± 0.08	1.19 ± 0.23
2 hr	2.86 ± 0.37 [a]	1.09 ± 0.04	0.80 ± 0.08
4 hr	2.15 ± 0.13 [a]	0.96 ± 0.11	0.78 ± 0.03
24 hr	2.90 ± 0.45 [a]	0.58 ± 0.02 [a]	1.17 ± 0.14
AUC [b]	60.3	19.0	22.6
TI [c]	2.7	0.8	

a) $P<0.01$ compared with *Sol + HT* at the same time point.
b) AUC of the tumor Pt level (hr μg/g tumor) between 0 and 24 hr which was calculated using a trapezoidal rule.
c) *TI*= AUC(*LUV-1 + HT* or *LUV-1 alone*)/AUC(*Sol + HT*).

6.3.3 Antitumor Activity

6.3.3.1 Tumor Growth Rate

The tumor growth rates after 2 days *LUV-1* administration with HT are shown as compared to the treated control and the untreated control in *Fig. 6.1*. The tumor growth in *LUV-1 + HT* was delayed in a longer time than that in the other treatments. The delay was

dependent on the CDDP dose. The larger CDDP dose resulted in longer delay. *Sol(40) + HT* exhibited a certain degree of tumor growth delay. However, neither *LUV-1 alone* nor *Sol alone* showed tumor growth delay.

6.3.3.2 Tumor Growth Delay and Tumor Weight Inhibition

The tumor growth delay and the tumor weight inhibition on day 21 after *LUV-1* administration with HT are summarized as compared to the treated or the untreated control in *Table 6.4*. The activity rank orders in tumor growth delay and tumor weight inhibition were:

LUV-1(40) + HT>LUV-1(20) + HT>Sol(40) + HT>LUV-1(10) + HT>HT alone. The activity of *LUV-1(40) alone* and *Sol(40) alone* was very small.

6.3.3.3 Dose Response of the Antitumor Activity

The dose-response of the tumor growth delay and the tumor weight inhibition for *LUV-1 + HT* in comparison to *Sol + HT* is shown in *Fig. 6.2*. Both tumor growth delay and tumor weight inhibition after *LUV-1 + HT* were positively correlated with the CDDP dose. The CDDP doses in *LUV-1 + HT*, giving tumor growth delay and tumor weight inhibition equivalent to those after *Sol(40) + HT*, were 10μg/mouse and 17.4μg/mouse, respectively.

6.3.4 Side Effect

The body weight change on day 21 and the BUN levels on day 13 after *LUV-1* administration with HT are shown as compared to the treated or the untreated controls in *Table 6.5*. All treatments were shown to cause a small decrease in body weight when the weights were compared to non tumor-bearing normal mice (1.6g - 3.1g). The BUN levels tended to rise significantly in *HT alone*, *Sol(40) + HT* and *LUV-1(40) + HT*. The highest levels were observed in *Sol(40) + HT*. The levels were, however, only 150 % of the normal levels. The side effects reflected in body weight loss and BUN level rise appear to be independent of the liposome administration.

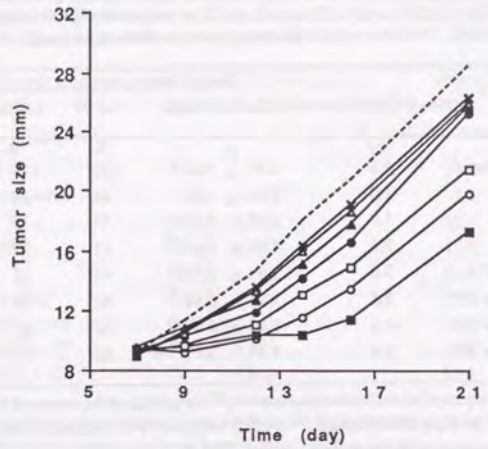

Fig. 6.1 Tumor growth rate after the two consecutive-day administration of *LUV-1* to tumor-bearing mice with HT as compared to the treated or the untreated control. Symbols: ●, *LUV-1(10)* [a] + *HT*; ○, *LUV-1(20) + HT*; ■, *LUV-1(40) + HT*; □, *Sol(40) + HT*; ▲, *HT* alone; △, *LUV-1(40)*; X, *Sol(40)*; ····· *Non-treatment*. [a] 10μg CDDP/mouse.

Table 6.3 Pt distribution in normal organs (liver, spleen and kidney) after the administration of *LUV-1* and *Sol* each at 40μg CDDP/mouse to mice with or without HT. All data are expressed as mean ± SE (*n*=5).

		Blood and Organ Pt level		
		LUV-1 + HT	*LUV-1*	*Sol + HT*
		μg/g organ		
Blood	0.25 hr	ND [a]	6.36 ± 0.90	1.27 ± 0.05
	0.5 hr	4.10 ± 0.39	3.39 ± 0.69	ND
	1 hr	1.61 ± 0.14 [b]	1.29 ± 0.12	0.66 ± 0.10
Liver	1 hr	6.42 ± 0.39	4.86 ± 0.52	2.77 ± 0.29
	24 hr	2.89 ± 0.12	1.97 ± 0.12	2.24 ± 0.32
Spleen	1 hr	13.46 ± 0.97 [b]	15.17 ± 1.05 [b]	0.73 ± 0.09
	24 hr	10.18 ± 0.84 [b]	10.02 ± 0.13 [b]	0.62 ± 0.09
Kidney	1 hr	4.77 ± 0.33	2.59 ± 0.28 [c]	5.64 ± 1.01
	24 hr	3.68 ± 0.35	2.59 ± 0.11 [c]	3.90 ± 0.73

[a] Not determined. [b] $P<0.01$ compared with *Sol + HT* at the same time point.
[c] $P<0.05$ compared with *Sol + HT* at the same time point.

Table 6.4 Tumor growth delay and tumor weight inhibition after the 2-consecutive-day administration of *LUV-1* with HT (days 7 and 8) as compared with the treated or the untreated control. The tumor weight data are expressed as mean ± SE (*n*=5).

	Tumor Growth Delay [a]	Tumor Weight Inhibition (Day 21) Tumor Weight	*T/C* [b]	Inhibition [c]
	day	*g*	%	%
Untreated control	0.0	6.34 ± 0.44	100	–
Sol(40 [d]*)*	1.0	5.45 ± 0.32	86	14
LUV-1(40)	1.0	5.37 ± 0.53	85	15
HT	2.1	4.61 ± 0.82 [e]	73	27
Sol(40) + HT	3.0	2.84 ± 0.71 [f]	45	55
LUV-1(10) + HT	2.5	3.96 ± 0.58 [f]	63	37
LUV-1(20) + HT	4.3	2.42 ± 0.39 [f]	38	62
LUV-1(40) + HT	5.6	1.63 ± 0.41 [f],[g]	26	74

[a] Lag time as compared to the untreated control. [b] Percentage of the untreated control.
[c] 100 - *T/C*. d) 40μg CDDP/mouse. [e] *P<0.05* compared with untreated control.
[f] *P<0.01* compared with the untreated control. [g] *P<0.2* compared with *Sol + HT*.

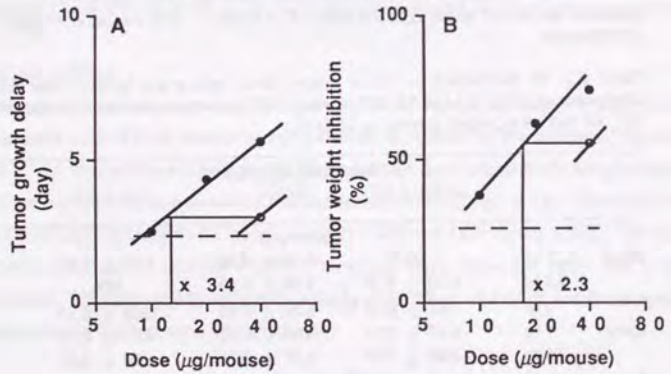

Fig. 6.2 Dose-response of tumor growth delay (*Panel A*) and tumor weight inhibition (*Panel B*) after *LUV-1* administration with HT as compared to that after Sol administration with HT. Symbols: ●, *LUV-1 + HT*; ○, *Sol + HT*; ---, *HT alone*.

Table 6.5 Body weight change and BUN after the 2-consecutive-day administration of *LUV-1* with HT (days 7 and 8) as compared with the treated or the untreated control . The BUN data are expressed as mean ± SE (*n*=5).

	Loss in Body Weight (Day 21) Net Body Weight [a]		BUN (Day 13)
	g		*g* *mg/dl*
Normal mice	24.1		17.8 ± 1.2
Untreated control	21.4	(-2.7 [b])	17.5 ± 1.3
Sol (40 [c]*)*	21.3	(-2.8)	18.0 ± 1.0
LUV-1(40)	21.4	(-2.7)	17.8 ± 1.0
HT	21.0	(-3.1)	25.4 ± 1.2 [d]
Sol (40) + HT	21.4	(-2.7)	27.0 ± 2.1 [d]
LUV-1(10) + HT	21.0	(-3.1)	18.1 ± 0.4
LUV-1(20) + HT	22.2	(-1.9)	18.9 ± 0.4
LUV-1(40) + HT	22.5	(-1.6)	23.4 ± 1.9 [e]

[a] Tumor weight was subtracted from the body weight in the tumor bearing mice.
[b] Difference as compared with the normal mice. [c] 40μg CDDP/mouse.
[d] *P<0.01* compared with the normal mice. [e] *P<0.05* compared with the normal mice.

6.4 Discussion

In order to achieve drug targeting by HT-mediated thermosensitive liposome, the liposomes should release the highest possible amount of the drug within a short time (a few sec). Temperature-dependent drug release was tested *in vitro* by two different methods (*Table 6.1*). In a 15-min heating in saline, the liposome showed a very sharp release-rate increase between 40°C and 41°C. The release at 41°C, 42°C and 43°C was almost complete. In short time heating in plasma, the liposome released about 60 % of the encapsulated CDDP within 5 sec at the HT temperatures (at and above 41°C), although the release rate increase was not sharp between 40°C and 41°C. The release rate obtained is thought to be fast enough for the *in vivo* HT-specific drug release at the heated tumor. The release starting temperature in *release-test 2* was somewhat low. This was due to the phase-transition temperature shift under the influence of the plasma. These release characteristics are consistent with those reported previously.[54]

The tumor Pt levels after *LUV-1* administration with HT were shown to be about 3 times higher than those after *LUV-1* administration without HT and after *Sol* administration with HT. The higher tumor-Pt levels are thought to be due to the HT-specific drug release at the tumor. The TDD efficiency of *LUV-1 + HT* can be evaluated by calculating the targeting index (*TI*) [28] expressed as the ratio of the AUC of the tumor Pt-level after *LUV-1* administration with HT to that after *Sol* administration with HT. The value (2.7) is a little

smaller than that for a thermosensitive LUV reported previously (*TI*=4.6)(*Chapter 5*). This might be due to relatively fast systemic clearance of *LUV-1* (the blood Pt level in *Table 6.3*). From the concept of the present TDD system, if the liposome releases the drug completely in the blood at the heated tumor, the targeting effect should be determined by the ratio of the AUC of the blood levels of the encapsulated drug during HT after liposome administration to the AUC of the blood levels of the free CDDP after *Sol* administration (*Chapter 5*). The ratio is close to the obtained targeting effect.

It is generally known that HT itself exerts a large antitumor effect and that it enhances the antitumor activity of CDDP in the combined treatment [18]. In the present study, a similar effect was observed. HT exerted a larger antitumor effect than the drug administration alone and the combination of *Sol* with HT, or *LUV-1* with HT, resulted in the synergistic antitumor activity enhancement. The antitumor activity of *LUV-1 + HT* was observed to be larger than that of *Sol + HT* at the same dose. The larger antitumor activity of *LUV-1 + HT* is thought to be due to the thermal enhancement of the CDDP activity and the activity enhancement by the TDD.

The TDD effect on the antitumor activity enhancement can be evaluated by calculating the ratio of the CDDP dose in *Sol + HT* to the dose in *LUV-1 + HT* to give equivalent antitumor activity on the logarithmic dose *versus* antitumor activity response curve (*Fig. 6.2*). The values from tumor growth delay and tumor weight inhibition are 3.4 and 2.7, respectively. The value obtained from tumor weight inhibition is somewhat small. The antitumor activity evaluated in tumor weight inhibition in the present study appears to be dependent on the assay period and not to be suitable for the assessment of the TDD enhancement ratio. However, these values are in fairly good agreement with the *TI* obtained above. The TDD enhancement ratio on the basis of the tumor growth delay is 1.6 times larger than that reported for the SUV[105], although the comparison is not on the same experimental basis.

In a preliminary study, we examined the antitumor activity of another type of liposome-encapsulating CDDP (DSPC alone was used for the membrane composition) without HT (data not shown). The antitumor activity was equivalent to those of *LUV-1* administration and *Sol* administration without HT, which indicates that the liposomes do not have a large ability to direct the drug to the targeted tumor.

The major side effect of free CDDP is the nephrotoxicity caused by the accumulation of the drug in the kidney during its excretion *via* that organ.[65,110] The encapsulation of CDDP in a liposome might be expected to reduce its nephrotoxicity.[83] In the present study however, the kidney CDDP levels 1 hr and 24 hr after *LUV-1 + HT* did not differ much from those after *Sol + HT* (*Table 6.3*). This result indicates that most of the encapsulated

CDDP is released in the systemic blood at a relatively early time and eliminated *via* the kidney, and that the liposome does not have a large ability to mask the nephrotoxicity of CDDP.

Severe nephrotoxicity is often reflected in a BUN-level rise occurring several days after the administration of CDDP and continuing for several days.[65,110] In the present study, the BUN levels after the administration of the higher CDDP dose with HT [(*LUV-1(40) + HT* and *Sol(40) + HT*] were obseved to be slightly high (*Table 6.5*). However, this effect appears not due to the nephrotoxic effect of CDDP because similar BUN level rise was observed in HT treatment alone. At the doses used in the present study, the nephrotoxic effect of CDDP both in liposome administration and solution administration might be very small.

In order to examine the toxicity of *LUV-1* on the reticuloendothelial system (RES),[75] we determined the RES Pt distribution after *LUV-1* administration, focusing on the liver and the spleen. The liver Pt levels at an earlier time after *LUV-1* administration with or without HT were observed to be significantly higher than those after *Sol* administration with HT (about 2 times higher than those for *Sol + HT*)(*Table 6.3*). However, the amount taken up by the liver is lower and the elimination rate is faster as compared to other CDDP liposomes reported.[83] The spleen Pt levels after *LUV-1* administration are higher for a long time period than those after *Sol* administration (about 20 times higher than those for *Sol + HT*). However, in the antitumor activity experiment, the change of splenic weight, which might be an indicator of the toxic effects of the liposome to that organ, was small (data not shown). The sum of the levels of Pt, including the liver and the spleen at 1 hr and 24 hr after *LUV-1* administration with HT, are only about 24% and 12% of the administered dose, respectively, and they are only 2.8 and 1.8 times those after *Sol* administration with HT (8.5% at 1 hr and 6.7% at 24 hr). These results suggest that the present thermosensitive liposome may be less toxic to the RES than other CDDP liposomes.

6.5 Conclusion

The combination of the thermosensitive liposome with HT resulted in the tumor CDDP level increase (*TI*, about 3) and enhancement of the antitumor activity (the TDD enhancement ratio, about 4) without severe side effects. This TDD system will be an effective way to decrease the CDDP dose, thereby increasing its therapeutic index.

6.6 Summary

The antitumor effect of cisplatin-(CDDP)-encapsulated thermosensitive large unilamellar liposome (*LUV-1*) administration with hyperthermia (HT) was examined in mice bearing

Meth A fibrosarcoma. The tumor Pt levels after *LUV-1* administration were increased in response to HT. The targeting index was approximately 3. The antitumor activity of *LUV-1 + HT*, as measured by tumor growth delay or tumor weight inhibition, was larger than that of *LUV-1* without HT or a solution (*Sol*) with or without HT. The CDDP dose in *LUV-1 + HT* to give equivalent tumor growth delay in *Sol(40μg/mouse) + HT* was about 10μg/mouse, and therefore the TDD enhancement ratio was about 4. The ratio correlates with the targeting index.

The blood urea nitrogen (BUN) level, as an indicator of CDDP nephrotoxicity, was increased 7 days after the administration of *LUV-1* (40μg CDDP/mouse) with HT. However, this BUN-level rise was independent of the activity enhancement by the liposome.

These findings suggest that the HT combined CDDP delivery system using thermosensitive large unilamellar liposome can decrease the effective CDDP dose, thereby increasing its therapeutic index.

Chapter 7 General Conclusion

7.1 Optimum Liposome Formulation

HT-specific drug release from liposome is highly dependent on the liposomal type, the phase transition temperature of the lipid constituent and the osmotic pressure of the internal aqueous fluid in the liposome. An LUV prepared using the lipid composition of DPPC/DSPC=9/1 and adjusting the osmotic pressure of the internal aqueous fluid (1.7 times as high as osmotic pressure) shows a very sharp release-rate increase between 40°C and 41°C. The amount released at 42°C is more than 80%. The release occurs explosively in a short time (a few seconds). In this optimum formulation, the liposomes are stable upon long-term storage.

7.2 Systemic Clearance and RES Uptake

The elimination rate and the RES uptake rate in rats after administration of the optimum thermosensitive formulation (LUV; DPPC/DSPC=9/1) were 1.3/hr and 0.4/hr, respectively. The obtained rate kinetics support the usefulness of the formulation in the present TDD system.

7.3 TDD Efficiency

The administration of thermosensitive liposomes with HT results in a tumor CDDP-level increase. The increase is the largest with LUV having a high heat-senstivity and small systemic clearance (*LUV-1*; DPPC/DSPC=9/1)(*TI*: about 4.6). The increase is due to the heat-specific CDDP release at the heated tumor.

7.4 Factors Affecting TDD Efficiency

A theoretical and experimental method to estimate the fraction of released drug (*F*) and maximal targeting index (*TImax*) for the present TDD system is derived. The *F* and *TImax* for various types of liposome formulations are determined using blood liposome levels and tumor drug levels.

The *TImax* value for *LUV-1* (the optimum thermosensitive formulation) is approximately 6, and the *F* value is approximately 0.7 which is in good agreement with the *in vitro* drug release rate. This confirms that heat-sensitive LUV having a small systemic clearance (*LUV-1*) is preferable for the present TDD system.

7.5 Improvement of Therapeutic Index

Combining the optimum themosensitive liposome formulation (*LUV-1*) with HT results in large enhancement of the antitumor activity without severe side effects (the activity enhancement ratio as compared with solution plus HT, about 4) . Thefore, this system can be used to decrease the necessary CDDP dose, thereby effectively increasing the therapeutic index.

Summary

In the introduction (*Chapter 1*), the possibility of TDD by combining thermosensitive liposomes with HT was described. Avoiding the problem of extravasation inability of drug carriers which is common in TDD sytems, this system is designed to release antitumor agents from liposomes in the blood at or adjacent to the heated tumor. Its targeting specificity is largely dependent on the concentration of liposomes in the local or systemic blood as well as the heat sensitivity of the liposomes. In search of an optimum CDDP-encapsulating-liposome formulation, the present study is foccused on examining (*i*) the heat-specific drug release characteristics of liposomes, (*ii*) the pharmokinetics, (*iii*) the tumor drug levels and (*iv*) the antitumor activity after administering to rats and mice.

In *Chapter 2*, the *in vitro* heat-specific drug release characteristics and liposomal properties of various types of thermosensitive liposomes were examined. An LUV prepared using the lipid composition of DPPC/DSPC=9/1 and adjusting the osmotic pressure of the internal aqueous fluid (1.7 times as high as osmotic pressure) showed a very sharp release-rate increase between 40°C and 41°C. The amount released at 42°C was more than 80%. The release occurred explosively in a short time (a few seconds). In this optimum formulation, the liposomes were stable upon long-term storage.

In *Chapter 3*, the clearance kinetics and RES-distribution in rats after administration of various types of LUVs encapsulating CDDP were examined. The elimination rate and the RES uptake rate for the optimum thermosensitive formulation (LUV; DPPC/DSPC=9/1) were 1.3/hr and 0.4/hr, respectively. The obtained rate kinetics supported the usefulness of this formulation in the present TDD system.

In *Chapter 4*, the tumor CDDP levels in *Meth-A* tumor-bearing mice after administration of various types of thermosensitive liposomes were examined. The administration of thermosensitive liposomes with HT resulted in a tumor CDDP-level increase. The increase was the largest with LUV having a high heat-senstivity and small systemic clearance (*LUV-1*). The *TI* calculated from the ratio of the area under the tumor-CDDP-level curve (AUC) for *LUV-1 + HT* to the AUC for *Sol + HT* was 4.6.

In *Chapter 5*, a theoretical and experimental method to estimate the fraction of released drug (*F*) and maximal targeting index (TI_{max}) for the present TDD was derived. The *F* and TI_{max} after administration of various types of thermosensitive liposomes were evaluated using the blood liposome levels and the tumor drug levels. The TI_{max} value for *LUV-1* was approximately 6, and the *F* value was approximately 0.7 which is in good agreement with the *in vitro* drug release rate. This confirms that the encapsulation of CDDP in a

highly heat-sensitive LUV having a small systemic clearance (*LUV-1*) is preferable for the present TDD system.

In *Chapter 6*, the antitumor activity in *Meth-A* tumor bearing mice after administration of the optimum thermosensitive LUV (*LUV-1*) with HT was examined. The activity as measured by the tumor growth delay was larger with *LUV-1 + HT* than with *LUV-1* without HT or *Sol* with or without HT. The antitumor activity enhancement ratio was about 4 while this treatment did not cause severe side effects.

In conclusion (*Chapter 7*), the optimum thermosensitive liposome formulation is achieved using an LUV preparation with a lipid composition of DPPC/DSPC = 9/1 and adjusting the osmotic pressure of the internal aqueous space to 1.7 times, or more, the physiological osmotic pressure. Combining administration of this liposome formulation with HT results in about 5 times tumor-CDDP-level increase (*TI* = 5) and about 4 times antitumor-activity enhancement, as compared with solution with HT and avoided severe side effects. Therefore, this TDD system is recommended as a way to decrease the necessary CDDP dose, thereby effectively increasing its therapeutic index.

References

1. Abraham, I., Goudalkar, A. and Mezei, M., Effect of liposomal surface charge on the pharmacokinetics of an encapsulated model compound. *Biopharm. Drug. Dispos.*, 1984, **5**, 387-398.

2. Allen, T. M., Everest, J. M. Effect of liposome size and drug release properties on pharmacokinetics of encapsulated drug in rats. *J. Pharmaco. Exp. Ther.*, 1983, **226**, 539-544.

3. Allen, T. M., Hansen, C. and Rutledge, J., Liposomes with prolonged circulation times: Factors affecting uptake by reticuloendothelial and other tissues. *Biochim. Biophys. Acta*, 1989, **987**, 27-35.

4. Alving, C. R., Macrophages as targets for delivery of liposome encapsulated antimicrobial agents. *Adv. Drug Deliv. Rev.*, 1988, **2**, 107-128.

5. Alving, C. R. and Hashimoto, Y., Drug delivery systems including liposomes as carriers. In Hadden, J. W., Spreafico, F., Yamamura, Y., Austen, K. F., Dukor, P. and Masek, K. (Eds.), *Advances in immunopharmacology 4* (Proceedings of the 4th international conference on immunopharmacology, Osaka, Japan, 16-19 May 1988), Pergamon, Oxford, 1989, pp. 317-319.

6. Awazu, S. Oguma, T., Iga, T. and Hanano, M., Generalized consideration of administration route dependence of drug disposition and use of urinary data for prediction of the dependence. *Chem. Pharm. Bull.*, 1977, **25**, 680-689.

7. Baldeschwieler, J. D., Phospholipid vesicle targeting using synthetic glycolipid and other determinants. In Tirrell, D. A., Donaruma, L. G. and Turek, A. B. (Eds.), *Macromolecules as drugs and carriers for biologically active materials*, The New York Academy of Sciences New York, New York, 1985, pp. 349-367.

8. Bannister, S. J., Sternson, L. A. and Repta, A. J., Urine analysis of platinum species derived from cis-dichlorodiammine platinum (II) by high-performance liquid chromatography following derivatization with sodium diethyldithiocarbamate. *J. Chromatogr.*, 1979, **173**, 333-342.

9. Bassett, J. B., Anderson, R. U. and Tacker, J. R., Use of temperature-sensitive liposomes in the selective delivery of methotrexate and cis-platinum analogues to murine bladder tumor. *J. Urol.*, 1986, **135**, 612-615.

10. Bassett, J. B., Tacker, J. R., Anderson, R. U. and Bostwick, D., Treatment of experimental bladder cancer with hyperthermia and phase transition liposomes containing methotrexate. *J. Urol.*, 1988, **139**, 634-636.

11. Blok, M. C., Deenen, L. L. M. Van, and Gier, J. De, Effect of the gel to liquid crystalline phase transition on the osmotic behaviour of phosphatidylcholine liposomes. *Biochim. Biophys. Acta*, 1976, **433**, 1-12.

12. Boddy, A., Aarons, L., Pharmacokinetic and pharmacodyanamic aspects of site-specific drug delivery. *Adv. Drug Deliv. Rev.*, 1989, **3**, 155-163.

13. Boddy, A., Aarons, L. and Petrak, K., Efficiency of targeting: Steady-state considerations using a three-compartmental model. *Pharm. Res.* 1989, **6**, 367-372.

14. Bradfield, J. W. B., The reticulo-endothelial system and blood clearance. In Davis, S. S., Illum, L., McVie, J. G. and Tomlinson, E. (Eds.), *Microspheres and drug therapy*. Elsevier, Amsterdam, 1984, pp. 25-38.

15. Connor, J., Yatvin, M. B. and Huang, L., *p*H-sensitive liposomes: Acid-induced liposome fusion. *Proc. Natl. Acad. Sci. U.S. A.*, 1984, **86**, 1715-1718.

16. Crearen, R. J., Brenner, D. E., Cowens, J. W., Dadey, B., Huben, R., Karakousis, C., Arbuck, S., Cushman, M. K., Han, T. and Andrejcio, K., Phase I study of MTP-PE encapsulated in liposomes. *J. Cell Biochem. (Suppl.)*, 1988, **12B**, 262.

17. Eichler, H. G., Senior, J., Stadler, A., Gasic, S., Pfunder, P. and Gregoriadis, G., Kinetics and disposition of fluorescein-labelled liposomes in healthy human subjects. *Eur. J. Clin. Pharmacol.*, 1988, **34**, 475-479.

18. Engelhardt, R., Hyperthermia and drugs. In Herfarth, C. and Senn, H. J. (Ser. Eds.) and Streffer, C. (Vol. Ed.), *Recent results in cancer research (hyperthermia and therapy of malignant tumors)*, Springer-Verlag, Berlin, 1987, pp. 166-167.

19. Franks, N. P. and Lieb, W. R., From physical structure to therapeutic applications. In Knight, C. G. (Ed.), *Liposomes*, Elsevir, North Holland Biomedical Press, Amsterdam, 1981, pp. 243-272.

20. Freeman, A. I. and Mayhew, E., Targeted drug delivery. *Cancer* 1986, **58** *(Suppl.)*, 573-583.

21. Freise, J. Mueller, W. H., Margerstedt, P. and Schmoll, H. J., Pharmacokinetics of liposome encapsulated cisplatin in rats. *Arch. Int. Pharmacodyn.*, 1982, **258**, 180-192.

22. Gabizon, A., Sulkes, A. and Ben-Baruch, N., Exploratory clinical phase of liposome-entrapped doxorubicin. *J. Cell Biochem. (Suppl.)*, 1988, **12B**, 251.

23. Gupta, P. K. and Hung, C. T., Quantitatve evaluation of targeted drug delivery systems. *Int. J. Pharm.*, 1989, **56**, 217-226.

24. Gregoriadis, G., Senior, J., Wolff, B. and Kirby, C., Fate of liposome *in vivo*: Control leading to targeting. In Gregoriadis, G., Poste, G., Senior, J. and Trouet, A. (Eds.), *Receptor-mediated targeting of drugs*. Plenum Press, New York, 1984, pp. 243-266.

25. Gregoriadis, G., Senior, J., Wolff, B. and Kirby, C., Targeting of liposomes to accessible cells *in vivo*. In Tirrell, D. A., Donaruma, L. G. and Turek, A. B. (Eds.), *Macromolecules as drugs and carriers for biologically active materials*, The New York Academy of Sciences New York, New York, 1985, pp. 319-340.

26. Gregoriadis, G., Fate of injected liposomes: Observations on entrapped solute retention, vesicle clearance and tissue distribution *in vivo*. In Gregoriadis, G. (Ed.), *Liposome as Drug carriers*, John Wiley and Sons Ltd., New York, 1988, pp. 3-18.

27. Hanano, M., Sawada, Y., Iga, T. and Sugiyama, Y., The integration of *in vitro* data with physiological data. In Breimer, D. D., and Speiser, P. (Eds.), *Topics in pharmaceutical sciences 1987*, Elsevier, Amsterdam, 1987, pp. 63-77.

28. Hunt, C. A., MacGregor, R. D. and Siegel, R. A., Engineering targeted *in vivo* drug delivery. I. The physiological and physicochemical principles governing opportunities and limitations. *Pharm. Res.*, 1986, **3**, 333-344.

29. **Iga, K.**, Hirai, S. and Yashiki, T., Assessment of local and systemic availability of tribenoside after rectal administration in rats. *Int. J. Pharm.*, 1980, **6**, 43-54.

30. **Iga, K.**, Ogawa, Y., Yashiki, T. and Shimamoto, T., Estimation of drug absorption rates using a deconvolution method with nonequal sampling times, *J. Pharmacokin. Biopharm.*, 1986, **14**, 213-225.

31. **Iga, K.**, Igari, Y., Hamaguchi, N., Ogawa, Y. and Toguchi, H., Cisplatin-entrapping thermosensitive liposomes for tumor targeting. In Koga, S. (Ed.), *Hyperthermic oncology in Japan '87* (Proceedings of the 4th annual meeting of the Japanese Society of Hyperthermic Oncology), Imai Printing and Publishing Co. Ltd., Yonago, Japan, 1987, pp. 275-280.

32. **Iga, K.** and Ogawa, Y., Drug targeting to tumor by thermosensitive liposome . In Iga, T. and Okumura, K. (Eds.), *Advances in biopharmaceutics,* Yakugyojiho-Sha (*Japanese*), Tokyo, 1989, pp. 416-425.

33. **Iga, K.**, Hamaguchi, N., Igari, Y., Ogawa, Y., Toguchi, H. and Shimamoto, T., Heat-specific drug-release of large unilamellar vesicle as hyperthermia-mediated targeting delivery. *Int. J. Pharm.*, 1989, **57**, 241-251.

34. **Iga, K.**, Hamaguchi, N., Igari, Y., Ogawa, Y. and Toguchi, H., Thermosensitive liposome-encapsulated cisplatin: *In vivo* drug release and targeting specificity. In Saito, M. (Ed.), *Hyperthermic oncology in Japan '89* (Proceedings of the 6th annual meeting of the Japanese Society of Hyperthermic Oncology), Institute of medical electronics, Faculty of medicine, University of Tokyo, Tokyo, Japan, 1989, pp. 40-43.

35. **Iga, K.**, Hamaguchi, N., Igari, Y., Ogawa, Y., Toguchi, H. and Shimamoto, T., Increased tumor cisplatin levels in heated tumor in mice after administration of thermosensitive large unilamellar vesicles encapsulating cisplatin. *J. Pharm. Sci.*, 1991, **80**, 522-525.

36. **Iga, K.**, Hamaguchi, N., Igari, Y., Ogawa, Y., Gotoh, K., Ootsu, K, Toguchi, H. and Shimamoto, T., Enhanced antitumor activity in mice after administration of thermosensitive liposome encapsulating cisplatin with hyperthermia. *J. Pharmaco. Exp. Ther.*, 1991, **257**, 1203-1207.

37. **Iga, K.**, Ogawa, Y. and Toguchi, H., Heat-induced release rate and maximal targeting index of thermosensitive liposome in tumor bearing mice. *Pharm. Res.*, in press.

38. **Iga, K.**, Ogawa, Y. and Toguchi, H., Clearance kinetics and RES distribution of liposome in rats. *J. Pharmacokin. Biopharm.*, in press.

39. Inoue, K., Permeability properties of liposomes prepared from dipalmitoyllecithin, dimyristoyllecithin, EGG lecithin, rat liver lecithin and beef brain sphingomyelin. *Biochim. Biophys. Acta*, 1974, **339**, 390-402.

40. Inoue, K. and Nojima, S., General concept of liposome. In Nojima, S., Sunamoto, J. and Inoue, K. (Eds.), *The liposomes*, Nanko-Do, Tokyo (*Japanese*), 1988, pp. 1-20.

41. Irie, T., Hashimoto, Y., Irie, A., Ishibashi, M., Miyazaki, H., Machida, H., Karasawa, Y. and Nakamori, K., Absorption, excretion and distribution of cisplatin in dogs and rats. *Iyakuhin Kenkyu* (*Japanese*), 1983, **14**, 384-410.

42. Juliano, R. L., Lopez-Berestein, G., Hopfer, R., Mehta, R. and Milis, K., Selective toxicity and enhanced therapeutic index of liposomal polyene antibiotics in systemic fungal

42. Juliano, R. L., Lopez-Berestein, G., Hopfer, R., Mehta, R. and Milis, K., Selective toxicity and enhanced therapeutic index of liposomal polyene antibiotics in systemic fungal infections. In Tirrell, D. A., Donaruma, L. G. and Turek, A. B. (Eds.), *Macromolecules as drugs and carriers for biologically active materials*, The New York Academy of Sciences New York, New York, 1985, pp. 390-402.

43. Juliano, R. L., Microparticulate drug carriers: Liposome, microsphere, and cells. In Robinson, J. R. and Lee, V. H. (Eds.), *Controlled drug delivery: Fundamentals and application.* Marcel Dekker, Inc., New york and Basel, 1987, pp. 555-572.

44. Juliano, R. L., Factors affecting the clearance kinetics and tissue distribution of liposomes, microspheres and emulsions. *Adv. Drug Deliv. Rev.*, 1988, **2**, 35-54.

45. Knight, G, Bard, D. R. and Thomas, D. P. P., Liposomes as Carriers of antiarthritic agents. In Tirrell, D. A., Donaruma, L. G. and Turek, A. B. (Eds.), *Macromolecules as drugs and carriers for biologically active materials*, The New York Academy of Sciences New York, New York, 1985, pp. 415-428.

46. Lautersztain, J., Perez-Soler, R., Khokhar, A. R., Newman, R. A. and Lopez-Berestein, G., Pharmacokinetics and tissue distribution of liposome-encapsulated cis-bis-N-decyl-iminodiacetato-1, 2,-diaminocyclohexane-platinum (II). *Cancer Chemother. Pharmacol.*, 1986, **18**, 93-97.

47. Liburdy, R. P., Tenforde, T. S. and Magin, R. L., Magnetic field-induced drug permeability in liposome vesicles. *Rad. Res.*,1986, **108**, 102-111.

48. Lopez-Berestein, G., Rosenblum, M. G., Mehta, R. T., Altered tissue distribution of amphotericin B by liposome encapsulation: Comparison of normal mice to mice infected with *Candida albicans. Caner Drug Delivery*, 1984, **1**, 199-205.

49. Mabrey, S., From physical structure to therapeutic applications. In Knight, C. G. (Ed.), *Liposomes*, Elsevir, North-Holland Biomedical Press, Amsterdam, 1981, pp. 105-138.

50. Maekawa, S., Ueo, H., Kai, H., Kitamura, M. and Sugimachi, K., Experimental study on treatment of metastatic lymph nodes with local hyperthermia and temperature-sensitive liposomes containing bleomycin. *Jpn. J. Cancer Chemother.* (*Japanese*), 1986, **13**, 271-274.

51. Magee, W. E. and Ristow, S. S., Strategies for targeting to lymphoid cells of the immune netwok. In Ihler, G. M.(Ed.), *Methods of drug delivery*, Pergamon Press, Oxford, 1986, pp. 233-268.

52. Magin, R. L. and Weinstein, J. N., Delivery of drugs in temperature-sensitive liposomes. In Gregoriadis, G., Senior, J. and Trouet, A (Eds.), *Targeting of drugs*, Plenum Press, New York, 1982, pp. 203-221.

53. Magin, R. L. and Morse, P. D., Rapid measurement of drug release from temperature-sensitive liposomes by electron paramagnetic resonance and radioisotope techniques. *Biochim. Biophys. Acta*, 1983, **760**, 357-362.

54. Magin, R. L. and Niesman, M. R., Temperature-dependant drug release from large unilamellar liposomes. *Cancer Drug Deliver.*, 1984, **1**, 109-117.

55. Magin, R. L., Hunter, J. M., Niesman, M. R. and Bark, G. A., Effect of vesicle size on the clearance, distribution, and tumor uptake of temperature-sensitive liposomes. *Cancer Drug Deliver*, 1986, **3**, 223-237.

56. Marmor, J. B., Interactions of hyperthermia and chemotherapy in animals. *Cancer Res.*, 1979, **39**, 2269-2276.

57. Martin, F. J. and Kung, V. T., Binding characteristics of antibody-bearing liposomes. In Tirrell, D. A., Donaruma, L. G. and Turek, A. B. (Eds.), *Macromolecules as drugs and carriers for biologically active materials*, The New York Academy of Sciences New York, New York, 1985, pp. 443-456.

58. Martin, F. J., Pharmaceutical manufacturing of liposomes. In Tyle, P. (Ed.), *Specialized drug delivery systems: Manufacturing and production technology*. Marcel Dekker, Inc., New York, 1990, pp. 267-316.

59. Mayhew, E. and Papahadjopoulos, D., Therapeutic applications of liposomes. In Ostro, M. J. (Ed.), *Liposomes*, Marcel Dekker, Inc., New York, 1983, pp. 289-341.

60. Mayhew, E., Goldresen, M., Vaage, J. and Rustum, Y., Liposomal adriamycin and survival of mice bearing liver metastases of colon carcinomas *26* or *38*. *Proc. Am. Assoc. Cancer Res.*, 1986, **27**, 402.

61. Maynard, V. M., Magin, R. L. and Dunn, F., Ultrasonic absorption and permeability for liposomes near phase transition. *Chem. Phys. Lipids*, 1985, *37*, 1-12.

62. Mehta, R. T., Hopfer, R. L., McQueen, T., Juliano, R. L. and Lopez-Berestein, G., Toxicity and therapeutic effects in mice of liposome-encapsulated nystatin for systemic fungal infection. *Antimicro. Agents Chemother.*, 1987, **31**, 1901-1903.

63. Nakagawa, Y., Utsumi, H., Inoue, K. and Nojima, S., Transfer of cholestane spin label between lipid bilayer membranes and its molecular motion in membranes. *J. Biochem.*, 1979, **86**, 783-787.

64. Neitchev, V. Z., Kostadinov, A. P. and Bideaud, F. A., Glycoprotein effect on the osmotic permeability of liposomes. *Int. J. Biochem.*, 1986, **18**, 459-464.

65. Nelson, J. A., Santos, G. and Herbert, B. H., Mechanisms for the renal secretion of cisplatin. *Cancer Treat. Rep.*, 1984, **68**, 849-853.

66. Nishimura, Y., Ono, K., Hiraoka, M., Masunaga, S., Jo, S., Shibamoto, Y., Sasaki, K., Abe, M., **Iga, K.** and Ogawa, Y. Treatment of murine SCC VII tumors with localized hyperthermia and temperature-sensitive liposome containing cisplatin. *Rad. Res.* 1990, **122**, 161-167.

67. Oku, N., Nojima, S. and Inoue, K., Selective release of non-electrolytes from liposomes upon perturbation of bilayers by temperature change or polyene antibiotics. *Biochim. Biophys. Acta*, 1980, **595**, 277-290.

68. Oku, N., Biomembrane Model. In Nojima, S., Sunamoto, J. and Inoue, K. (Eds.), *The liposomes*, Nanko-Do, Tokyo (*Japanese*), 1988, pp. 152-164.

69. Ootsu, K., Gotoh, K., and Houkan, T., Therapeutic efficacy of human recombinant interleukin-2 (TGP-3) alone or in combination with cyclophosphamide and immuno-competent cells in allogeneic, semi-syngeneic, and syngeneic murine tumors. *Cancer Immunol. Immunother.*, 1989, **30**, 71-80.

70. Papahadjopoulos, D., Heath, T., Bragman, K. and Matthay, K., New methodology for liposome targeting to specific cells. In Tirrell, D. A., Donaruma, L. G. and Turek, A. B. (Eds.), *Macromolecules as drugs and carriers for biologically active materials*, The New York Academy of Sciences New York, New York, 1985, pp. 341-348.

71. Papahadojopoulos, D., A personal view of liposomes: From membrane modeling to drug delivery. In Yagi, K. (Ed.), *Medical application of liposomes*. Japan Scientific Societies Press, Tokyo, 1986, pp. 1-5.

72. Peil, A., Targeting of the reticuloendothelial system by liposomes: Therapeutic aims and requirements for their realization. In Buri, P. and Gumma, A. (Eds.), *Drug targeting*, Elsevier, Amsterdam, 1985, pp. 119-134.

73. Perez-Soler, R., Liposomes as carriers of antitumor agents: Toward a clinical reality. *Cancer Treatment Reviews*, 1989, **16**, 67-82.

74. Prescott, L. F., and Nimmo, W. S., *Drug absorption: Novel drug delivery systems.* ADIS Press, Sydney, 1981, pp. 157-176.

75. Poste, G., Bucana, C., Raz, A., Bugelski, P., Kirsh, R. and Fidler, I. J., Analysis of the fate of systemically adminstered liposomes and implications for their use in drug delivery. *Cancer Res.*, 1982, **42**, 1412-1422.

76. Poste, G., Liposome targeting *in vivo*: Problems and opportunities. *Biol. Cell*, 1983, **47**, 19-38.

77. Poste, G., Kirsh, R. and Bugelski, P., Liposomes as a drug delivery system in cancer therapy. In Sunkara, P. S. (Ed.), *Novel approaches to cancer therapy*, Academic Press, New York, 1984, pp. 165-230.

78. Rahman, A., Carmichael, D., Harris, M. and Roh, J. K., Comparative pharmacokinetics of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes. *Cancer Res.*, 1986, **46**, 2295-2299.

79. Ryman, B. E. and Barratt, G. M. Liposomes-further considerations of their possible roles as carriers of therapeutic agents. In Gregoriadis, G., Senior, J. and Trouet, A. (Eds.), *Targeting of drugs*, Prenum Press, New York, 1982, pp. 235-260.

80. Sato, Y., Kiwada, H. and Kato, Y., Effects of dose and vesicle size of the pharmacokinetics of liposome. *Chem. Pharm. Bull.*, 1986, **34**, 4244-4252.

81. Scheider, M., Liposomes as drug carriers: 10 years of reserach. In Buri, P. and Gumma, A., *Drug targeting*, Elsevier, Amsterdam, 1985, pp. 119-134.

82. Scherphof, G. L., Dukstra, J., Spanjer, H. H., Derksen, J. T. P. and Roerdink, F. H., Uptake and intracellular processing of targeted and nontargeted liposomes by rat kupffer cells *in vivo* and *in vitro*. In Tirrell, D. A., Donaruma, L. G. and Turek, A. B. (Eds.), *Macromolecules as drugs and carriers for biologically active materials*, The New York Academy of Sciences New York, New York, 1985, pp. 368-384.

83. Steerenberg, P. A., Storm, G., Groot, G. De, Bergers, J. J., Claessen, A., and De Jong, W. H., Liposomes as drug carrier system for cis-diamminedichloroplatinum (II): I. Binding capacity, stability, tumor cell growth inhibition *in vitro*. *Int. J. Pharm.*, 1987, **40**, 51-62.

84. Steerenberg, P. A., Storm, G., Groot, G. De, Claessen, A., Bergers, J. J., Franken, M. A. M., Hoesel, Q. G. C. M. Van, Wubs, K. L. and Jong, W. H. De, Liposomes as drug carrier system for diamminedichloroplatinum (II): II. Antitumor activity *in vivo*, induction of drug resistance, nephrotoxicity and Pt distribution. *Cancer Chemother. Pharmacol.*, 1988, **21**, 299-307.

85. Sugiyama, Y., Reconstruction of drug dispositions *in vivo* from *in vitro* studies based on physiological pharmacokinetic modeling: From low-molecular weight drugs to poly-peptide compounds. *Yakugaku-Zasshi (Japanese)*, 1989, **109**, 199-231.

86. Sullivan, S. M. and Huang, L., Preparation and characterization of heat-sensitive immunoliposomes. *Biochim. Biophys. Acta*, 1985, **812**, 116-126.

87. Szoka, F. and Papahadjopoulos, D., Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. U. S. A.*, 1978, **75**, 4194-4198.

88. Szoka, F. and Papahadjopoulos, D., Comparative properties and methods of preparation of lipid vesicles (liposomes). *Ann. Rev. Biophys. Bioeng.*, 1980, **9**, 467-508.

89. Tomlinson, E., (Phatho)physiology and the temporal and spatial aspects of drug delivery. In Tomlinson, E. and Davis, S. S. (Eds.), *Site-specific drug delivery*, John Wiley and Sons, New York, 1986, pp. 1-26.

90. Tomita, T., Watanabe, M., Takahashi, T., Kumai, K., Tadakuma, T. and Yasuda, T., Temperature-sensitive release of adriamycin, an amphiphilic antitumor agents, from dipalmitoylphoshatidylcholine-cholesterol liposomes. *Biochim. Biophys. Acta*, 1989, **978**, 185-190.

91. Treat, J. and Rahman, A., Liposomal encapsulated doxorubicin (LED): Phase II study in brest cancer. *J. Cell Biochem. (Suppl.)*, 1988, **12B**, 252

92. Tyle, P. and Ram, B. P., Monclonal antibodies, immunoconjugates and liposomes as targeted therapeutic systems. In Tyle, P. and Ram, B. P. (Eds.), *Targeted therapeutic systems*. Marcel Dekker, Inc. New York, 1990, pp. 3-24.

93. Umemura, S., Yumita, N., Nishigaki, R. and Umemura, K., Mechanism of cell damage by ultrasound in combination with hematoporphyrin. *Jpn. J. Cancer Res.*, 1990, **81**, 962-966.

94. Verkleij, A. J. and Gier, J. De, Freeze fracture studies on aqueous dispersions of membrane lipids. In Knight, C. G. (Ed.), *Liposomes*, Elsevir, North Holland Biomedical Press, Amsterdam, 1981, pp. 243-272.

95. Weinstein, J. N., Magin, R. L., Yatvin, M. B. and Zaharko, D. S., Liposomes and local hyperthermia: Selective delivery of methotrexate to heated tumors. *Science*, 1979, **204**, 188-191.

96. Weinstein, J. N., Magin, R. L., Cysyk, R. L. and Zaharko, D. S., Treatment of solid *L1210* murine tumors with local hyperthermia and temperature-sensitive liposomes containing methotrexate. *Cancer Res.*, 1980, **40**, 1388-1395.

97. Weinstein, J. N., Klausner, R. D., Innerarity, T., Ralston, E. and Blumenthal, R., Phase transition release, a new approach to the interaction of proteins with lipid vesicles. *Biochim. Biophys. Acta*, 1981, **647**, 270-284.

98. Weinstein, J. N., Liposomes as drug carriers in cancer therapy. *Cancer Treat. Rep.*, 1984, **68**, 127-135.

99. Weinstein, J. N. and Leserman, L. D., Liposomes as drug carriers in cancer chemotherapy. *Pharmac. Ther.* 1984, **24**, 207-233.

100. Weinstein, J. N., Liposomes in the diagnosis and treatment of cancer. In Ostro, M. J. (Ed.), *Liposomes*, Marcel Dekker, New York, 1987, pp. 277-338.

101. Weissmann, G., Liposomes as vectors for calcium ionophores, In Tirrell, D. A., Donaruma, L. G. and Turek, A. B. (Eds.), *Macromolecules as drugs and carriers for biologically active materials*, The New York Academy of Sciences New York, New York, 1985, pp. 385-389.

102. Wondergem, J., Bulger, R. E., Strebel, F. R., Newman, R. A., Travis, E. L., Stephens, L. C. and Bull, J. M. C., Effect of cis-diamminedichloroplatinum (II) combined with whole body hyperthermia on renal injury. *Cancer Res.*, 1988, **48**, 440-446.

103. Yagi, K. and Naoi, M., Glycolipid insertion into liposomes for their targeting to specific organs. In Yagi, K. (Ed.), *Medical application of liposomes*, Japan Scientific Society Press, Tokyo, 1986, pp. 91-97.

104. Yatvin, M. B., Weinstein, J. N., Dennis, W. H. and Blumenthal, R., Design of liposomes for enhanced local release of drugs by hyperthermia. *Science*, 1978, **202**, 1290-1293.

105. Yatvin, M. B., Muhlensiepen, H., Porshen, W., Weinstein, J. N. and Feinendegen, L. E., Selective delivery of liposome-associated cis-dichlorodiammineplatinum (II) by heat and its influence on tumor drug uptake and growth. *Cancer Res.*, 1981, **41**, 1602-1607.

106. Yatvin, M., B., Cree, T. C. and Gipp, J. J., Hyperthermia-mediated targeting of liposome-associated anti-neoplastic drugs. In Gregoriadis, G., Senior, J. and Trouet, A. (Eds.), *Targeting of drugs*, Plenum Press, New York, 1982, pp. 223-234.

107. Yatvin, M. B., Tegmo-Larsson, I.-M., Dennis, W. H., Temperature- and pH-sensitive liposomes for drug targeting. *Methods Enzymol.* 1987, **149**, 77-99.

108. Yoshikawa, W., Akutsu, H., Kyogoku, Y. and Akamatsu, Y., An essential role of phosphatidylglycerol in the formation of the osmotically stable liposomes of *Escherichia coli* phospholipids. *Biochim. Biophys. Acta*, 1985, **821**, 277-285.

109. Zonnereld, G. M. and Crommelin, D. J. A., Liposomes: Parenteral administration to man. In Gregoriadis, G. (Ed.), *Liposomes as drug carriers*. John Wiley and Sons Ltd., New York, 1988, pp. 795-817.

110. Zwelling, L. A., Cisplatin and new platinum analogs. *Cancer Chemotherapy*, 1985, **7**, 105-122.