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RESEARCH PAPER

Formulation Design and Evaluation of Liposomal Sepantronium Bromide (YM155), a Small-Molecule Survivin Suppressant, Based on Pharmacokinetic Modeling and Simulation

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ABSTRACT

Purpose Sepantronium bromide (YM155) is administered by 168-hour continuous infusions in clinical studies due to its time-dependent pharmacological efficacy and rapid elimination from plasma. To enable more convenient administration, i.e., bolus injections with low frequency, we prepared liposomal formulations of YM155 and evaluated their antitumor activities.

Methods A kinetic simulation model of liposomal YM155 to predict the free drug concentration in both tumor and plasma was developed. A liposomal formulation with the target drug release rate was prepared based on the simulation. Antitumor activities of the formulation were examined in various tumor xenograft mouse models. In addition, antitumor activities of liposomal formulations with different drug release rates were compared in order to confirm the validity of the simulation-based prediction.

Results Liposomal YM155 with the release half-life of 48 h was prepared as a promising formulation. This formulation showed significantly potent antitumor activities in tumor xenograft models by weekly bolus injections. Further studies demonstrated that this release rate was optimal for YM155 in terms of both efficacy and safety.

Conclusions We successfully developed a liposomal formulation of YM155 that could substitute for long-term continuous infusion of the drug solution in clinical settings by being given as weekly bolus injections.

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KEY WORDS Drug release · Liposome · Modeling · Simulation · Sepantronium bromide (YM155)

ABBREVIATIONS

AS	Ammonium sulfate
CA	Cholesteryl anthracene-9-carboxylate
CR	Complete regression
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
HPLC	High-performance liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MPEG-DSPE	*N*-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt
PB	Phosphate buffer
PEG	Polyethylene glycol
RES	Reticuloendothelial system
$t_{1/2}$	Half-life
UV	Ultraviolet

INTRODUCTION

Sepantronium bromide (YM155) is a survivin suppressant that shows potent antitumor activities against a wide range of cancer cells [1]. The antitumor activity of YM155 is time dependent, and elimination from plasma is very rapid [2–4]. Owing to these characteristics, high *in vivo* antitumor efficacy of YM155 in tumor xenograft models is obtained by continuous infusions, and the potency diminishes when the drug is administered by bolus injections [2]. Based on the preclinical data, YM155 is administered by 168-hour continuous

infusions in clinical studies to expose tumor cells to the drug for a long duration [5, 6]. However, this administration method is apparently cumbersome for patients, and so a more convenient method, such as bolus injections, is desired. In the case of bolus injections, the dosing frequency should be as low as possible to reduce the burden of injection on the patients. In order to enable the efficacious bolus injections instead of continuous infusion, the development of an adequte delivery system is necessary. Given the insufficient antitumor activity of YM155 solution after bolus injections even with a daily administration schedule, the delivery system needs to attain not only enhancement of the efficacy but also a reduction in the dosing frequency.

Liposomes are artificial vesicles composed of lipid bilayers [7, 8]. A variety of drugs can be encapsulated in liposomes and released in a sustained manner [9, 10]. Furthermore, nanosized liposomes, especially those modified with polyethylene glycol (PEG), exhibit long circulation times in the bloodstream and accumulate in tumor tissue after systemic administration via the "enhanced permeability and retention effect" [11, 12]. A number of studies have demonstrated enhanced efficacy of anticancer drugs delivered as liposomal formulations [13–15]. Although the efficacies of conventional and liposomal drugs were compared at the same dosing schedules in most cases, the encapsulation of YM155 in liposomes would appear to have the potential to obtain potent antitumor activity by low-frequent bolus injections if the formulation is suitably designed.

In the formulation design of liposomes, the drug release rate is one of the most important factors to be examined [9]. The release rate affects the concentration profiles of free drug at the target site, and the optimal release rate varies among drugs depending on their pharmacological, pharmacokinetic, and toxicological characteristics [16–18]. The previous studies, however, evaluated the drug release rate and efficacy/safety of different formulations without thoroughly associating the release rate with the free drug concentration profiles. In the case of liposomal YM155, the free drug concentration profile after a single bolus injection needs to be comparable to that after continuous infusion of a drug solution. Accordingly, the efficacy range of the drug release rate is supposed to be narrow. If the relationship between the release rate and the free drug concentration profile are deliberated in advance, the optimal formulation can be designed efficiently.

In this study, at first we developed a kinetic simulation model of liposomal YM155 to predict the free drug concentration profile in tumors and plasma. The target drug release rate was set based on the simulation. A liposomal formulation of YM155 having this target release rate was then prepared, and its antitumor activities were examined in various tumor xenograft mouse models. Additionally, antitumor activities of liposomes with different drug release rates were compared in order to confirm the validity of the simulation-based formulation design.

MATERIALS AND METHODS

Pharmacokinetic Modeling and Simulation

A kinetic model of liposomal YM155 in mice was developed to predict the free drug concentrations in tumors and plasma [19, 20]. The overall structure of this model is shown in Fig. 1a. YM155 encapsulated in liposomes was assumed to be released with a first-order rate constant (k_{rel}) at both central (plasma and rapidly equilibrating tissues) and tumor compartments, and then be distributed and eliminated in the same manner as conventional YM155. The kinetic parameters for liposomes and YM155 were estimated separately by nonlinear least squares fitting, based on corresponding differential equations and measured concentration time course data in tumors and plasma as described later. All the fittings and simulations were conducted by using WinNonlin® software (Version 5.2; Pharsight, Mountain View, CA).

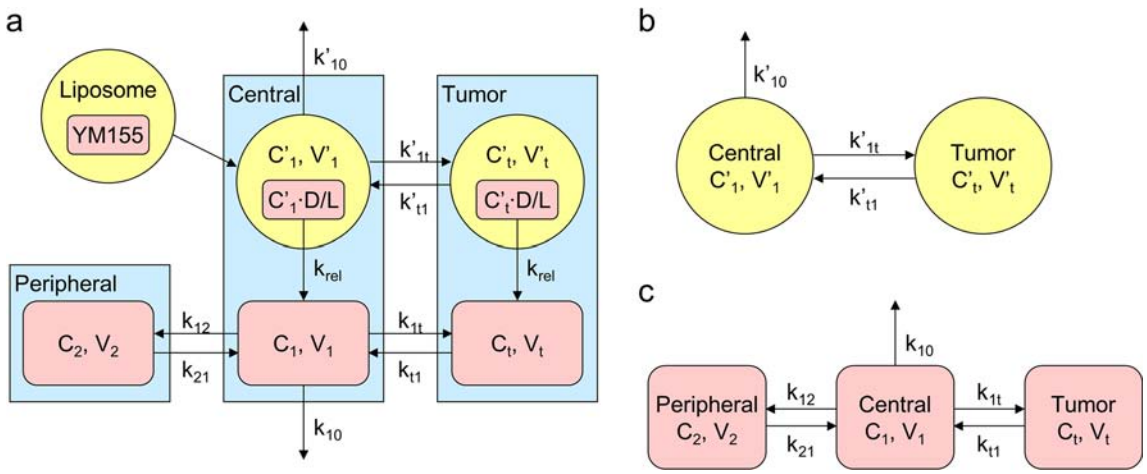

Fig. 1 Kinetic simulation models of liposomal YM155. (**a**) Overall structure and individual compartment models for (**b**) liposomes (**c**) YM155.

Pharmacokinetic Modeling of Liposomes

A kinetic model of liposomes composed of central and tumor compartments was developed as described in Fig. 1b. The differential equations for this model were given by using distribution rate constants k'_{1t} and k'_{t1} and elimination rate constant k'_{10} as follows (Eqs. 1 and 2):

$$V'_1 \cdot dC'_1 \Big/ dt = -(k'_{10} + k'_{1t}) \cdot C'_1 \cdot V'_1 + k'_{t1} \cdot C'_t \cdot V'_t \quad (1)$$

$$V'_t \cdot dC'_t \Big/ dt = -k'_{t1} \cdot C'_t \cdot V'_t + k'_{1t} \cdot C'_1 \cdot V'_1 \quad (2)$$

where V'_1 and V'_t represent the volume of the central and tumor compartment for liposomes, respectively. C'_1 and C'_t represent the concentration of liposomes in the central and tumor compartment, respectively. Each parameter was estimated by nonlinear least squares curve fitting using measured tumor and plasma concentration time course data of liposomal lipid in tumor-bearing mice, obtained as described later.

Pharmacokinetic Modeling of YM155

A kinetic model of YM155 composed of central, peripheral (slowly equilibrating tissues other than tumor), and tumor compartments was developed as described in Fig. 1c. The differential equations for this model were given by using distribution rate constants k_{12}, k_{21}, k_{1t}, and k_{t1} and elimination rate constant k_{10} as follows (Eqs. 3–5):

$$V_1 \cdot dC_1 \Big/ dt = -(k_{10} + k_{12} + k_{1t}) \cdot C_1 \cdot V_1 + k_{21} \cdot C_2 \cdot V_2 + k_{t1} \cdot C_t \cdot V_t \quad (3)$$

$$V_2 \cdot dC_2 \Big/ dt = -k_{21} \cdot C_2 \cdot V_2 + k_{12} \cdot C_1 \cdot V_1 \quad (4)$$

$$V_t \cdot dC_t \Big/ dt = -k_{t1} \cdot C_t \cdot V_t + k_{1t} \cdot C_1 \cdot V_1 \quad (5)$$

where V_1, V_2, and V_t represent the volume of the central, peripheral, and tumor compartments for YM155, respectively. C_1, C_2, and C_t represent the concentration of YM155 in the central, peripheral, and tumor compartments, respectively. Each parameter was estimated by nonlinear least squares curve fitting using measured tumor and plasma concentration time course data of YM155 after continuous infusion in tumor-bearing mice, reported previously [2].

Simulations

The kinetic model of liposomal YM155 was developed by linking the above models with a release rate constant (k_{rel}), as described by the following differential equations (Eqs. 6–11):

$$V'_1 \cdot dC'_1 \Big/ dt = -(k'_{10} + k'_{1t}) \cdot C'_1 \cdot V'_1 + k'_{t1} \cdot C'_t \cdot V'_t \quad (6)$$

$$V'_t \cdot dC'_t \Big/ dt = -k'_{t1} \cdot C'_t \cdot V'_t + k'_{1t} \cdot C'_1 \cdot V'_1 \quad (7)$$

$$d\left(D \Big/ L\right) \Big/ dt = -k_{rel} \cdot D \Big/ L \quad (8)$$

$$V_1 \cdot dC_1/dt = k_{rel} \cdot C'_1 \cdot V'_1 \cdot D/L - (k_{10} + k_{12} + k_{1t}) \cdot C_1 \cdot V_1 + k_{21} \cdot C_2 \cdot V_2 + k_{t1} \cdot C_t \cdot V_t \quad (9)$$

$$V_2 \cdot dC_2 \Big/ dt = -k_{21} \cdot C_2 \cdot V_2 + k_{12} \cdot C_1 \cdot V_1 \quad (10)$$

$$V_t \cdot dC_t \Big/ dt = k_{rel} \cdot C'_t \cdot V'_t \cdot D \Big/ L - k_{t1} \cdot C_t \cdot V_t + k_{1t} \cdot C_1 \cdot V_1 \quad (11)$$

where D/L represents the ratio of encapsulated drug to lipid within liposomes. Simulations were conducted to predict free YM155 concentration profiles in the tumor (C_t) and plasma (C_1) at various release rates.

Reagents

YM155 monobromide was synthesized at Astellas Pharma Inc (Tokyo, Japan). 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), *N*-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt (MPEG-DSPE), and cholesterol were purchased from NOF (Tokyo, Japan). Cholesteryl anthracene-9-carboxylate (CA) came from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were obtained from Kanto Chemical (Tokyo, Japan).

Cell Culture

Human hormone-refractory prostate cancer cell line PC-3, human non-small-cell lung cancer cell line Calu-6, and human malignant melanoma cell line A375 were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA) in a humidified incubator with 5% CO_2 at 37°C.

Preparation of Liposomes

Liposomes containing YM155 were prepared by the conventional thin film hydration and extrusion method [21]. Briefly, phospholipid, cholesterol, and MPEG-DSPE at predetermined molar ratios (62:33:5) were dissolved in chloroform. For some pharmacokinetic and biodistribution studies, 0.2 mol% of CA was also added as a lipid marker. Chloroform was evaporated under a stream of nitrogen gas, and the lipid was further dried under vacuum. The resulting lipid film was then hydrated in 20 mg/mL YM155 solution to form multilamellar vesicles. Subsequently, the vesicles were extruded sequentially through polycarbonate membrane filters having 400, 200, and 100 nm pores (Nuclepore, Pleasanton, CA) by using an extrusion device (Northern Lipids, Burnaby, BC). Unencapsulated YM155 was removed by dialysis against 10 mM sodium phosphate buffer (pH 6.0) containing 8.8 mg/mL of NaCl by using a dialysis cassette (Slide-A-Lyser 10 K MWCO, Thermo Fisher Scientific, Rockford, IL).

The particle size of the liposomes was measured with Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK), and the mean diameter was checked to be between 100 and 150 nm. The YM155 concentration in the liposome preparation was determined by a high-performance liquid chromatography-ultraviolet (HPLC-UV) method after dissolution of the liposomes in methanol. For this assay, a TSKgel ODS-80Ts column (5 μm, φ4.6 mm × 150 mm; Tosoh, Tokyo, Japan) was used, and the column temperature was set at 40°C. The mobile phase was 10 mM ammonium acetate buffer (pH 5.0)/acetonitrile (4:1, *v*/*v*), and the flow rate was maintained at 1.0 mL/min. The detection wavelength was 252 nm.

In Vitro Release Studies

Equal amounts of liposome suspension and mouse serum were mixed and incubated at 37°C. Samples were collected at predetermined periods, and the concentration of released YM155 was measured as described above after removal of liposomes by ultrafiltration using a 30-kDa molecular weight cut-off membrane (Microcon YM-30, Millipore, Billerica, MA). The drug release rate was calculated by fitting the time course of drug amount retained in the liposomes to first-order kinetics.

Table I Estimated Kinetic Parameters for Liposomes and YM155 in Mice[a]

Kinetic parameters for liposomes[b]		Kinetic parameters for YM155	
V'_1 (mL)	1.03	V_1 (mL)	71.8
k'_{10} (h^{-1})	0.0330	k_{10} (h^{-1})	1.38
V'_t (mL)	0.321	k_{12} (h^{-1})	0.0719
k'_{1t} (h^{-1})	0.000932	k_{21} (h^{-1})	0.395
k'_{t1} (h^{-1})	0.00963	k_{1t} (h^{-1})	0.0183
		k_{t1} (h^{-1})	0.131
		V_2 (mL)	20.0
		V_t (mL)	0.694

[a] Body weight of mice was assumed to be 20 g

[b] Compositions of liposomal lipid and inner phase were DPPC/cholesterol/MPEG-DSPE/CA (62:33:5:0.2, molar ratio) and 8.8 mg/mL NaCl/10 mM sodium phosphate buffer (pH 6.0), respectively

Animal Studies

All animal studies were conducted in accordance with institutional guidelines and approval of the internal committee at Astellas Pharma Inc. Five-week-old male BALB/c normal and nude (BALB/c nu/nu) mice were purchased from Japan SLC (Shizuoka, Japan) and Charles River Laboratories Japan (Kanagawa, Japan), respectively, and maintained on a standard diet and water under pathogen-free conditions throughout all experiments. In studies using tumor xenograft models, tumor cells (3×10^6) were mixed with BD Matrigel matrix (Becton, Dickinson and Company, Franklin Lakes, NJ), subcutaneously implanted into the flanks of nude mice, and allowed to reach more than 100 mm^3 in tumor volume (length × width^2 × 0.5).

Pharmacokinetic and Biodistribution Studies

The pharmacokinetic and tissue distribution characteristics of liposomal YM155 were examined in a PC-3 xenograft model. In another pharmacokinetic study to evaluate *in vivo* drug release rate of liposomes, normal mice were employed. Liposomes were administered to the mice via a tail vein, and samples (plasma and tumor) were collected at predetermined periods.

The YM155 concentration in each sample was determined by using liquid chromatography-tandem mass spectrometry (LC-MS/MS), as reported previously [22]. The concentration of CA, a marker of liposomal lipid [23], in each sample was measured as follows: Briefly, plasma or a tissue homogenate was added to an appropriate amount of methanol/chloroform (1:1, *v*/*v*) followed by sonication and vigorous shaking. Samples were then centrifuged, and CA concentrations in the supernatants were measured by using HPLC equipped with a fluorescence detector. For this assay, a TSKgel ODS-80Ts column (5 μm, φ4.6 × 250 mm; Tosoh, Tokyo, Japan) was used, and the column temperature was set at 40°C. The mobile phase was 2-propanol/acetonitrile (1:2, *v*/*v*), and the flow rate was maintained at 1.0 mL/min. The excitation and emission wavelengths for detection were 250 and 465 nm, respectively.

In Vivo Antitumor Activities against Tumor Xenograft Model

Tumor-bearing mice were randomized into groups ($n = 6$–8). The mice were treated with liposomal YM155 or physiological saline weekly by bolus injections via a tail vein.

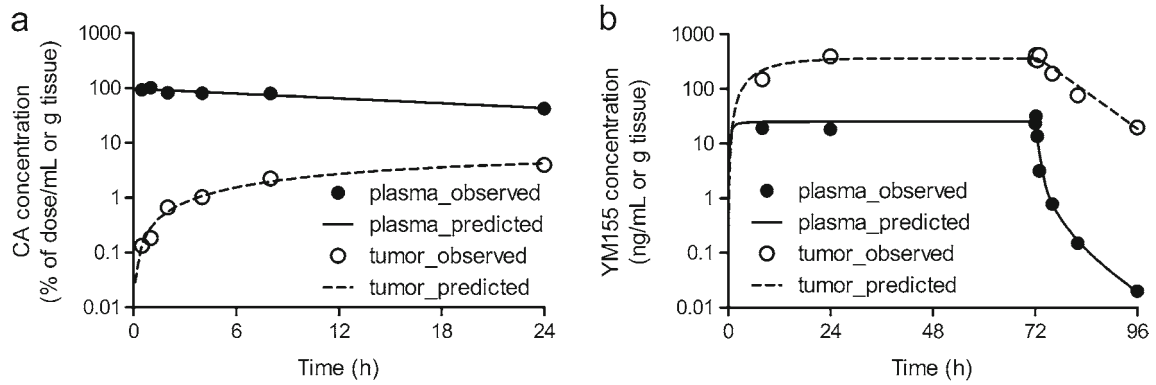

Fig. 2 Comparison of fitting curves to measured values. (**a**) Concentration time courses of CA-labeled liposomes in tumor and plasma after intravenous administration to PC-3 tumor-bearing mice. Compositions of liposomal lipid and inner phase were DPPC/cholesterol/MPEG-DSPE/CA (62:33:5:0.2, molar ratio) and 8.8 mg/mL NaCl/10 mM sodium phosphate buffer (pH 6.0), respectively. (**b**) Concentration time courses of YM155 in tumor and plasma after 3-day continuous infusion of drug solution to PC-3 tumor-bearing mice at 3 mg/kg/day [2].

Alternatively, mice were treated with YM155 solution in physiological saline by 7-day continuous infusion using an implanted osmotic pump (ALZET Osmotic Pump model 1007D, Durect, Cupertino, CA) [2]. Tumor volumes and body weights were measured periodically. Complete regression (CR) was defined as tumor regression to below the limit of palpation.

Statistical Analysis

Differences between groups were analyzed by performing Student's *t*-test or Dunnett's test. All data analyses were done with SAS software (SAS Institute, Cary, NC), and a *P* value of less than 0.05 was considered statistically significant.

RESULTS

Pharmacokinetic Modeling

Table I presents kinetic parameters for liposomes estimated by simultaneous curve fitting using tumor and plasma concentration time course data. Approximation of fitting curves to measured values was confirmed as shown in Fig. 2a. Kinetic parameters for YM155 were also estimated, and approximation was confirmed in the same way (Table I and Fig. 2b).

Simulations

YM155 solution administered by 3-day continuous infusions completely inhibits tumor growth at a dose of 9 mg/kg/week (3 mg/kg/day × 3 days/week) in a PC-3 xenograft mouse model, whereas the antitumor activity is not sufficient by bolus injections at 10 mg/kg/week (2 mg/kg/day × 5 days/week; ref. 2). Less frequent administration, such as once per week, is apparently to be preferred in terms of the burden of injections on patients. Accordingly, simulation of liposomal YM155 with various release rates was conducted assuming once-a-week administration at 10 mg/kg.

Fig. 3 shows the predicted free drug concentration profiles in the tumor and plasma. The simulation indicated that a faster drug release rate led to a higher maximum free drug concentration in the tumor. However, the high concentration

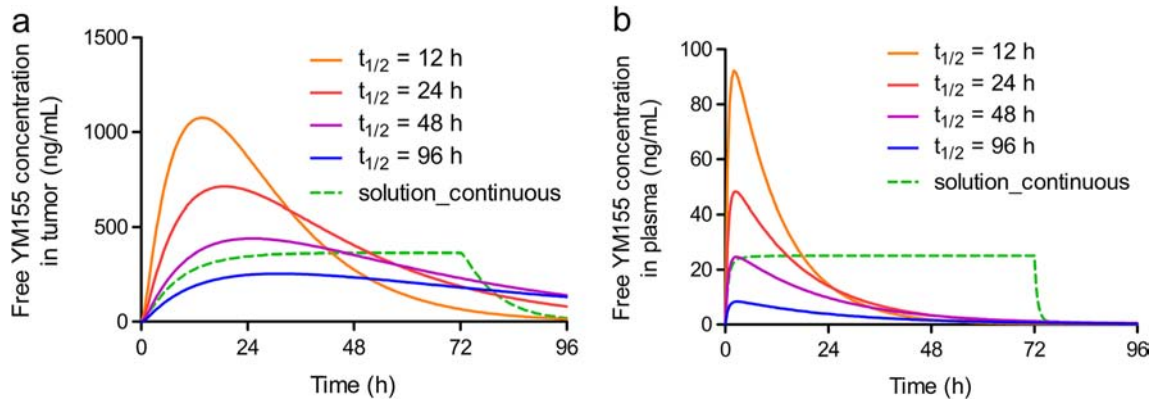

Fig. 3 Predicted free drug concentrations in (**a**) tumor and (**b**) plasma after intravenous administration of liposomal YM155 with various drug release rates at 10 mg/kg. For comparison, predicted drug concentrations after 3-day continuous infusion of YM155 solution at 3 mg/kg/day are also presented.

Table II Composition of Liposomal Formulations Studied

Sample name	Lipid composition (molar ratio)[a]	Solvent of inner phase[b]
DMPC/PB	DMPC/cholesterol/MPEG-DSPE = 62:33:5	osmotic phosphate buffer[c]
DPPC/PB	DPPC/cholesterol/MPEG-DSPE = 62:33:5	osmotic phosphate buffer[c]
DSPC/PB	DSPC/cholesterol/MPEG-DSPE = 62:33:5	osmotic phosphate buffer[c]
DSPC/AS	DSPC/cholesterol/MPEG-DSPE = 62:33:5	60 mM ammonium sulfate

[a] For pharmacokinetic studies, 0.2 mol% of CA was also added

[b] YM155 was dissolved in each solvent at 20 mg/mL for hydration

[c] 8.8 mg/mL NaCl/10 mM sodium phosphate buffer (pH 6.0)

cannot last for a long period, suggesting the possibility of insufficient efficacy due to the time-dependent antitumor activity of YM155. In addition, the free drug concentration in the plasma was predicted to become higher at faster release rates. Since a previous report indicated lower tolerability of YM155 after bolus injections than after continuous infusion [2], the high drug concentration in the plasma would likely cause toxicity. At slower release rates, the free drug concentration in the tumor would not reach a sufficient level. A release half-life ($t_{1/2}$) of 48 h showed free drug concentration profiles in the tumor and plasma comparable to those of continuously administered drug solution, and thus was considered to be optimal for YM155 from the viewpoint of both efficacy and safety.

Preparation of Liposomal YM155

Liposomal formulations containing YM155 prepared in this study are described in Table II. As shown in Fig. 4, the *in vitro* drug release rate of liposomal YM155 could be controlled by changing the acyl chain length of phospholipids, with the shorter chain giving faster release. Further, similarly to that for other drugs [24–26], the release of YM155 was retarded by adding ammonium sulfate to the inner phase.

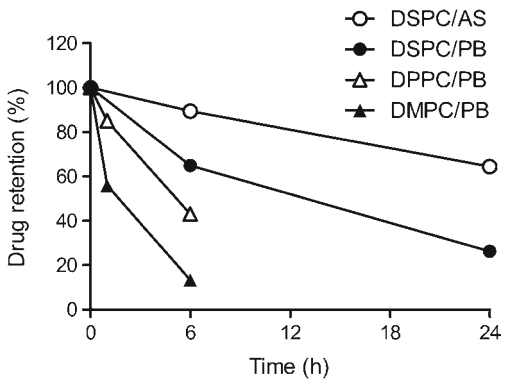

Fig. 4 *In vitro* drug release of liposomal YM155. Liposomes were incubated in 50% mouse serum at 37°C. Results are expressed as the mean ± SD ($n = 3$).

Determination of *In Vivo* Drug Release Rate

The *in vivo* drug release rate of liposomal YM155 was examined in a pharmacokinetic study where plasma concentrations of both liposomal lipid (CA) and drug were measured. The *in vivo* release rate constant was calculated by fitting the time course of the YM155/CA ratio to first-order kinetics [13, 18]. Fig. 5 shows the results of the study for DPPC/PB and DSPC/PB, with the data indicating an *in vivo* release $t_{1/2}$ of 23.4 and 48.2 h, respectively.

By using the obtained *in vivo* release rate, the validity of the pharmacokinetic modeling was further confirmed. The measured tumor and plasma concentration values of total (sum of liposomal and free) YM155 in DPPC/PB fitted well with the simulated curves for liposomal YM155 with a release $t_{1/2}$ of 24 h, as shown in Fig. 6.

Antitumor Activity of DSPC/PB

Based on the results of the simulation and pharmacokinetic study, antitumor activities of DSPC/PB, having an *in vivo* release $t_{1/2}$ of approx. 48 h, was examined as the most promising formulation. PC-3, Calu-6, and A375 xenograft models were employed in the pharmacological study, where liposomal YM155 was administered by weekly bolus injections at 10 mg/kg as YM155. In advance of the study, inter-model differences in tumor accumulation of liposomal YM155 and YM155 solution were assessed, and both accumulation levels were similar among the models (data not shown).

As shown in Fig. 7, liposomal YM155 exhibited potent antitumor activities after weekly bolus injections in all xenograft models. The therapeutic efficacy of liposomal YM155 was comparable to that of the YM155 solution administered by continuous infusion (Fig. 7c).

Effect of Drug Release Rate on Antitumor Activity

In order to investigate whether the simulation-based prediction was adequate, antitumor activities of liposomal YM155 with different drug release rates were compared in the Calu-6 xenograft model. The dose of liposomal YM155 in weekly

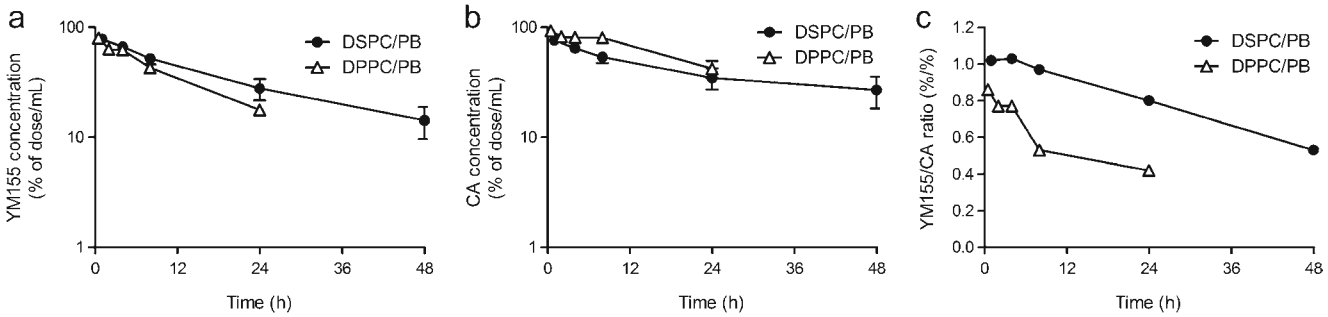

Fig. 5 Pharmacokinetics of liposomal YM155 in mice. (**a**) YM155 and (**b**) CA concentrations in plasma after intravenous administration of CA-labeled liposomal YM155 to normal BALB/c mice at 3 mg/kg as YM155. (**c**) Values of YM155/CA ratio. Results are expressed as the mean ± SD ($n = 3$).

bolus injections was set at 7 mg/kg (14 mg/kg in total), based on a previous study showing potent antitumor activity of YM155 solution obtained by 7-day continuous infusion at 2 mg/kg/day [27].

As shown in Fig. 8, DSPC/PB and DSPC/AS strongly inhibited tumor growth after weekly bolus injections. In addition, DSPC/PB caused CR of the tumor in 50% of the treated mice, whereas no CR was observed in the other groups including DSPC/AS. Relatively attenuated antitumor activity and great weight loss were observed for DPPC/PB. Furthermore, 2 out of 6 mice in the DPPC/PB group and all mice in the DMPC/PB group died within 4 days after the first injection (data not shown). Taken together, DSPC/PB demonstrated the most potent antitumor activity among the formulations tested.

DISCUSSION

In the formulation design of drug-containing liposomes, adjustment of release rate based on characteristics of the drug is important to obtain optimal performance in terms of both efficacy and safety [16–18]. Since the antitumor activity of YM155 solution is insufficient after daily bolus injections

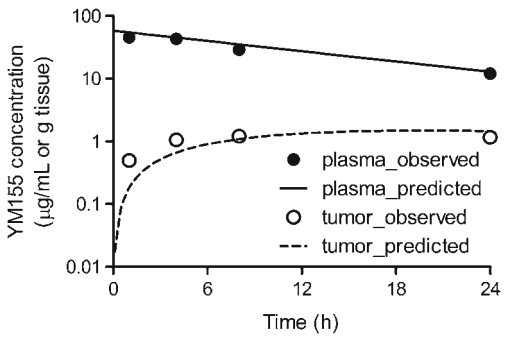

Fig. 6 Concentration time courses of total (sum of liposomal and free) YM155 in tumor and plasma after intravenous administration of DPPC/PB to PC-3 tumor-bearing mice at 3 mg/kg as YM155. The simulated *curves* represent the total drug concentrations for liposomal YM155 with a release $t_{1/2}$ of 24 h.

[2–4], the liposomal formulation for once-a-week bolus injections needs to be ingeniously designed to maintain an adequate free drug concentration in the tumor for a sufficient duration. Comprehension of the pharmacokinetic properties of the carrier and drug is essential for the formulation design [28, 29].

In this study, a kinetic simulation model of liposomal YM155 was developed to predict the free drug concentration profiles in the tumor and plasma (Fig. 1a; ref. 20). Based on the modeling and simulation, the release $t_{1/2}$ of 48 h was considered to be optimal for YM155 (Fig. 3). When the release rate is faster, the free drug concentration in the tumor cannot be maintained for a sufficient duration and that in the plasma will be too high to be a cause of toxicity. In contrast, when the release rate is slower, the drug concentration in the tumor cannot reach the pharmacologically effective level.

To prepare liposomal YM155 having the target drug release rate, we tested several formulations (Table II). Their drug release rates were first evaluated *in vitro* by using mouse serum, and we confirmed controllability of the release rate depending on the phase transition temperature of the phospholipid (Fig. 4). Even though the *in vitro* drug release rates may be different from *in vivo* release rates, the relative order among formulations is believed to be constant between *in vivo* and *in vitro* rates [16, 18]. Based on the subsequent *in vivo* drug release study, DSPC/PB, having the release $t_{1/2}$ of 48 h, was selected as the most promising formulation (Fig. 5).

As shown in the result for DSPC/AS, the addition of ammonium sulfate to the inner phase of the liposomes led to a delay in the drug release. The mechanism for the increased liposomal retention of YM155 in DSPC/AS was likely to be similar to that in the case of doxorubicin, topotecan, and staurosporine [24–26, 30] although the drug loading procedure was different. Some preliminary data suggested the importance of ammonium ions for the drug retention (data not shown), and detailed investigations are underway to elucidate the mechanism.

As expected, DSPC/PB after weekly bolus injections showed potent antitumor activity comparable to that of YM155 solution administered by continuous infusions in

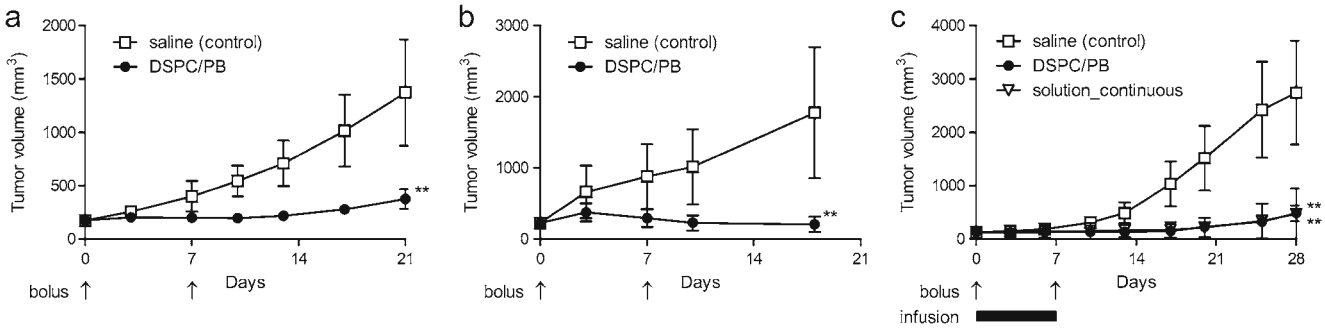

Fig. 7 Antitumor activities of liposomal YM155 (DSPC/PB) against (**a**) PC-3, (**b**) Calu-6, and (**c**) A375 xenograft tumors in mice. Liposomal YM155 was administered to the mice on days 0 and 7 at 10 mg/kg as YM155, and tumor volumes were measured periodically on the indicated days. YM155 solution was also administered to A375-bearing mice by 7-day continuous infusion starting on day 0 at 3 mg/kg/day (**c**). Results are expressed as the mean ± SD ($n = 6$–8). ** $P < 0.01$ *vs.* control by Student's *t*-test (**a, b**) or Dunnett's test (**c**).

various tumor xenograft mouse models (Fig. 7; refs. 2, 27). Since tumor accumulation levels of both liposomal YM155 and YM155 solution were similar among the models (data not shown), the free drug concentration profiles were likely comparable in each model. Moreover, less toxicity than the continuous infusions of drug solution can be expected in the bolus injections of liposomal formulation, considering the lower free drug concentration profile in plasma predicted by the simulation (Fig. 3b, $t_{1/2} = 48$ h). These results suggest the potential of the liposomal formulation as a delivery tool that enables bolus injections of YM155 also in the clinical settings.

The antitumor activity of DSPC/PB was the most potent among the formulations with different drug release rates (Fig. 8), which supports the validity of the simulation. DPPC/PB, the formulation with faster release than DSPC/PB, showed attenuated antitumor activity and greater weight loss. Furthermore, deaths were observed in both DPPC/PB (2 out of 6 mice) and DMPC/PB (all mice) groups within 4 days after the first injection (data not shown). These results were probably attributed to shorter exposure of the tumor to the free drug and a higher concentration in the plasma. In the case of the slower formulation, DSPC/AS, its antitumor activity was slightly attenuated compared to that of DSPC/PB in terms of CR ratio (0% for DSPC/AS *vs.* 50% for DSPC/PB). The optimal release rate was different from other anticancer agents such as vincristine and mitoxantrone [18, 31]. This difference indicates the importance of formulation design of liposomes based on pharmacological, pharmacokinetic, and toxicological characteristics of the drug to be encapsulated.

While the antitumor activity of DSPC/AS was weaker than that of DSPC/PB, the difference was not so great as predicted by the simulation. The following reasons are considered for the greater-than-expected efficacy of DSPC/AS: 1) a different drug release rate in tumor tissue and plasma, and 2) return of free drug from cells of the reticuloendothelial system (RES) to the systemic circulation after uptake and digestion of liposomes. Gabizon reported that the *in vitro* drug release rate of PEG liposomal doxorubicin was greater in malignant effusions than in plasma [32], which suggests the possibility of faster *in vivo* drug release from liposomes in tumor tissue than in plasma. In our simulation model, the release rate was assumed to be the same at each site. If the release rate was faster than predicted, the free drug concentration in tumor would reach a high enough level to exert a potent antitumor activity. Additionally, the fate of the drug inside liposomes might need to be taken into account in more detail. Liposomes are mainly taken up by the RES in liver and spleen, and then metabolized [11, 33]. Following this uptake and digestion, some of the drug molecules inside the liposomes may be

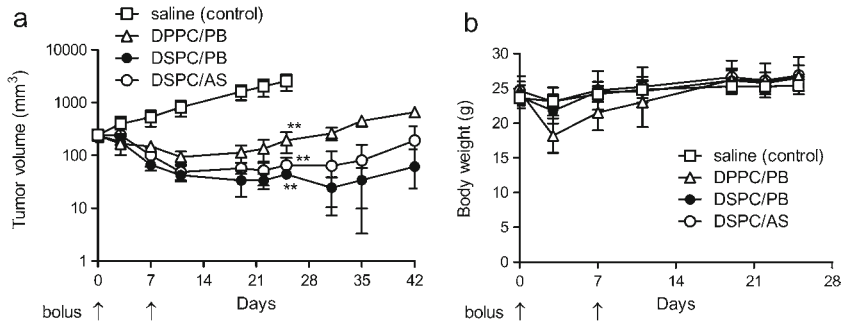

Fig. 8 Antitumor activities of liposomal YM155 with various formulations against Calu-6 xenograft tumors in mice. Liposomal YM155 was administered to the mice on days 0 and 7 at 7 mg/kg as YM155, and (**a**) tumor volumes and (**b**) body weights were measured periodically at the days indicated. Results are expressed as the mean ± SD ($n = 6$). ** $P < 0.01$ *vs.* control by Dunnett's test.

released from the cells of RES into the systemic circulation in a sustained manner [34, 35]. The YM155 concentration in the tumor might be maintained for a sufficient duration, as in the case of continuous infusion, by this mechanism. Further extensive studies such as direct measurement of free drug in the tumor tissue and plasma are necessary to explore these possibilities.

CONCLUSIONS

A simulation model of liposomal YM155 to predict the free drug concentration in the tumor and plasma was developed so that the target drug release rate could be set. The optimal formulation, designed based on the simulation, demonstrated sufficiently potent antitumor activities in tumor xenograft models by weekly bolus injections. These results suggest the possibility of liposomal YM155 to enable more convenient administration instead of continuous infusions in the clinical settings.

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