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Research paper

MMPs-specific PEGylated peptide–DOX conjugate micelles that can contain free doxorubicin

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Abstract

The goal of this study was to develop anti-cancer drug conjugates with increased anti-tumor effect and reduced toxicity. In this regard, we utilized the physiological characteristics of tumors such as angiogenesis, the expression of matrix metalloproteinases (MMPs) and the enhanced permeability and retention (EPR) effect, and designed MMPs-specific PEGylated peptide–DOX conjugate micelles containing doxorubicin. These conjugates were prepared by using two peptides, GPLGV and GPLGVRG (P5D and P7D, respectively), and doxorubicin was loaded into micelles formed by each conjugate. P5D and P7D were specifically cleaved by active MMP-2 and all conjugates showed significantly better cell viability than doxorubicin at equivalent concentrations. *In vivo*, animals treated with PEGylated peptide–DOX conjugate micelles showed approximately 50% of the tumor growth of the control, and doxorubicin-loaded conjugates micelles inhibited tumor growth up to about 72% compared with the control, which matched the effect of doxorubicin. Doxorubicin-loaded PEGylated peptide–DOX conjugate micelles exhibited longer half-lives and maintained higher concentrations of doxorubicin in plasma than PEGylated peptide–DOX conjugate micelles alone. Doxorubicin-loaded PEGylated peptide–DOX conjugate micelles might offer a cancer therapy with an activity that is similar to that of the parent drug but with reduced toxicity.

Keywords: Matrix metalloproteinases (MMPs); Conjugate micelles; PEGylated peptide; Doxorubicin loading; Cancer therapy

1. Introduction

Chemotherapy remains the major systemic treatment for cancer. The therapeutic efficacy of present cancer chemotherapies is, however, limited by narrow therapeutic indexes due to high levels of non-specific toxicity towards normal tissues or organs. Doxorubicin (DOX) is one of the most well-known anti-cancer drugs, and exerts its cytotoxic effect by inhibiting the synthesis of nucleic acids within cancer cells [1,2]. Doxorubicin has been used in chemotherapy for about twenty years and shows broad-spectrum activity against solid tumors such as sarcomas, adenocarcinomas, and melanomas [3]. However, the therapeutic potential of doxorubicin has been restricted by its toxic side effects, mainly its cardiotoxicity and myelosuppression [4], which results in a narrow therapeutic index due to its high toxicity to healthy tissues. To circumvent the concerns of the non-specificity and high systemic toxicity associated with doxorubicin, many researchers have proposed doxorubicin conjugation to hydrophilic synthetic polymers such as *N*-(2-hydroxypropyl)-methacrylamide (HPMA) [5], poly(DL-lactic-co-glycolic acid) (PLGA) [6], poly(ethylene glycol) (PEG) [7]. This method of conjugation results in reduced toxicity whilst increasing or maintaining its therapeutic efficacy compared with free doxorubicin therapy. For many years, poly(ethylene

Abbreviations: MMPs, matrix metalloproteinases; DOX, doxorubicin; IC_{50}, 50% inhibitory concentration; MTD, maximum tolerance dose; LD_{50}, 50% lethal dose.

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glycol) (PEG) has been used to modify peptides and proteins in order to increase solubility, reduce immunogenicity, increase resistance to proteolysis, and in addition, to provide a means of improving pharmacokinetics [8,9]. Recently, PEG conjugates containing anti-cancer drugs such as doxorubicin, paclitaxel, and camptothecin have been described [10]. Polymer-drug conjugates like these are attracting increasing interest as novel anti-cancer agents capable of achieving high drug loadings and providing unique disposition characteristics in the body such as an extended half-life in blood, specific organ targeting, and sustained release at the injection site [11–13]. The possibility of improving the plasma half-life of a polymer-drug conjugate, with maximum accumulation in tumors, can be achieved by loading of a drug in a circulating carrier such as liposomes, nanoparticles, or micelles [14–19]. The distribution of drug-loaded particles in the body is less affected by the properties of the loaded drugs embedded within a particle matrix.

Angiogenesis involves the sprouting of blood vessels from pre-existing blood vessels and the forming of new networks of blood vessels. Angiogenesis is a necessary process for tumor growth because tumor angiogenesis supplies nutrients and oxygen and removes waste products. Matrix metalloproteinases (MMPs) are secreted in excess from cancer cells during tumor angiogenesis and play important roles during tumor progression. They degrade the extracellular matrix (ECM), which is mainly composed of collagen, thereby facilitating tumor invasion and metastasis [20–22]. Type IV collagenases (MMP-2 and MMP-9), in particular, have been reported to play important roles in this process [23,24], as they are observed in several different types of solid tumors such as the tumors of stomach, colorectal, breast, prostate, lung, and ovarian cancers [25,26]. The use of MMP-2 or MMP-9 as targets for cancer diagnosis and therapy has also been reported recently. In terms of this approach, the development of a MMP-2-sensitive tumor imaging probe containing MMP-2 peptides has been undertaken for cancer imaging [27], and an albumin-binding doxorubicin, which has substrate specificity for MMP-2 and MMP-9 [28,29], and a MMP-2 cleavable melittin/avidin conjugate [30] have been developed. In previous study, we concentrated on the environmental characteristics of cancers, such as, angiogenesis and MMP expression and designed MMPs-specific peptide–doxorubicin conjugates as potential anti-cancer therapies with reduced systemic toxicity and increased specificity. Of our peptide–doxorubicin conjugates, GPLGV–DOX was found to be cleaved by MMPs secreted by cancer cells and was shown to have significantly lower cytotoxicity compared with doxorubicin. In an *in vivo* study, GPLGV–DOX was found to suppress tumor growth as much as doxorubicin but did not induce mouse body weight loss due to its reduced toxicity, whereas doxorubicin reduced mouse body weight and induced severe side effects. However, GPLGV–DOX did not circulate in the body for a protracted period because of its low MW [31]. Therefore, because of the above-mentioned advantages of polymer-drug conjugates, we undertook to modify peptide–doxorubicin conjugates with PEG to increase circulation time of conjugates in the body while retaining the characteristics of a prodrug activated by MMPs expressed by tumors. Subsequently, we loaded free doxorubicin in PEGylated peptide–DOX conjugate micelles to increase anti-tumor activity. In addition, it is worth mentioning that the conjugate micelle structure was stabilized by increasing the amount of physically-loaded doxorubicin within the micellar core, which reduced systemic leakage of doxorubicin and achieved enhanced doxorubicin accumulation in solid tumors with fewer toxic side effects caused by non-specific organ distribution [32,33].

In the present study, we synthesized PEGylated peptide–DOX conjugates using peptides cleaved specifically by MMPs, namely, Gly-Pro-Leu-Gly-Val (GPLGV) and Gly-Pro-Leu-Gly-Val-Arg-Gly (GPLGVRG). The MMPs-specific peptide sequences were chosen based on previous studies on the cleavage specificities of Type IV collagenase [34–36] and GPLGVRG was expanded from the GPLGV sequence, which is the minimum sequence to match the active site of MMPs, to compare the specificities for MMPs depending on the sequence length. The characteristics of the synthesized PEGylated peptide–DOX conjugates such as their enzymatic degradations and cell viability were also analyzed. Further, we loaded free doxorubicin into PEGylated peptide–DOX conjugate micelles and then evaluated the anti-tumoral activity and longevity of PEGylated peptide–DOX conjugates and doxorubicin-loaded PEGylated peptide–DOX conjugates in the blood circulation *in vivo*.

2. Materials and methods

2.1. Materials

Doxorubicin was provided generously by the Dong-A Pharm. Co. Ltd. (Seoul, Korea) and PEGylated peptide was purchased from Anygen Co. (Gwangju, Korea). The molecular weight of the poly(ethylene glycol) (Sun Bio) used was 2 kDa. Dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (HOSu), triethyl amine (TEA), and anhydrous *N*,*N*-dimethylformamide (DMF) were purchased from Aldrich Chemical Co. (St. Louis, MO). HPLC grade acetonitrile and trifluoroacetic acid (TFA) for spectroscopy were purchased from J.T. Baker (Phillipsburg, NJ) and Merck (Darmstadt, Germany), respectively. Active MMP-2 was obtained from Oncogene Research Products™ (EMD Biosciences, Inc., Darmstadt, Germany).

2.2. Preparation of PEGylated peptide–DOX conjugates

PEGylated peptide was conjugated with doxorubicin (1.2-fold excess of PEGylated peptide) in anhydrous DMF by using DCC and HOSu (1.5-fold excess of PEGylated peptide), where TEA was added to remove the HCl salt in doxorubicin hydrochloride. After filtering, the reaction mixture was dialyzed using a MWCO 2000 membrane

against deionized water and lyophilized. Pure PEGylated peptide–DOX conjugate was obtained by HPLC (Shimadzu Corp., Kyoto, Japan) using a prep-ODS(H) kit column (Shim-pack, 20 × 250 mm) and a linear gradient (eluent A, water with 0.1% TFA; eluent B, acetonitrile; gradient, 35–50% B over 30 min at a flow rate of 5 ml/min). The purity of PEGylated peptide–DOX was quantitatively analyzed by HPLC (Hitachi 7000 Series, Hitachi Ltd., Tokyo, Japan).

2.3. Loading of doxorubicin into PEGylated peptide–DOX conjugate micelles

Doxorubicin was loaded into PEGylated peptide–DOX conjugate micelles using the o/w emulsion method, as previously reported [14,15]. Briefly, 1 ml of a chloroform solution of doxorubicin (10 mg/ml) with 3.0 equivalents of TEA was added dropwise to 20 ml of a stirred aqueous solution of pre-formed PEGylated peptide–DOX conjugate micelles (1 mg/ml), to form the o/w emulsion. The o/w emulsion was kept overnight in the dark open to the atmosphere at 25 °C, which allowed the chloroform to evaporate. Free doxorubicin was removed from the micelles by triplicate ultrafiltrations using a 1000 MWCO membrane (Amicon® bioseparations, Millipore Corp., Bedford, MA). The amount of doxorubicin in the ultrafiltered solution was quantified photometrically at 479 nm and the loading amount and efficiency were calculated based on the weight ratio of incorporated doxorubicin to micelles.

2.4. Micelle size of PEGylated peptide–DOX conjugates and DOX-loaded conjugates

The micelle size of PEGylated peptide–DOX and DOX-loaded conjugates was measured by electrophoretic light scattering (ELS-8000, OTSUKA Electronics Co., Ltd., Osaka, Japan) using a He–Ne Laser system at a wavelength of 488 nm. Sample solutions (1.0 mg/ml) passing through a 0.45 μm filter were transferred to light scattering cells.

2.5. Active MMP-2 cleavage of PEGylated peptide–DOX conjugates

PEGylated peptide–doxorubicin conjugates (100 μl, 0.1 mM) dissolved in 50 mM tricine buffer (pH 7.5), containing 0.2 M NaCl, 0.01 $MCaCl_2$, and 0.05 (v/v) % Brij 35, were mixed with active MMP-2 (100 μl, 10 μg/ml). The mixture was then incubated with gentle stirring at 37 °C for 6, 12, 24, 36, or 48 h. When incubations had been completed, enzyme activity was inhibited by adding 200 μl of 0.02 M EDTA at pH 8.0. Degraded fragments were fractionated by HPLC by gradient elution with acetonitrile/water containing 0.01% TFA at 1.0 ml/min; the acetonitrile was linearly increased from 5% to 85% over 30 min.

2.6. Cytotoxicity of PEGylated peptide–DOX conjugates against LLC cells

The cytotoxicity of PEGylated peptide–DOX conjugates to Lewis lung carcinoma (LLC) cells was evaluated *in vitro* by using MTT assay. LLC cells (5×10^3) were plated in each well of a 96-well tissue culture plate. Medium supplemented with 10% FBS was added, and cells were allowed to adhere for 24 h. Cells were then incubated with serial dilutions of each PEGylated peptide–DOX conjugate (10^{-6}–10^{-4} M) for 24 h in quadruplicate, and then labeled by adding 20 μl of sterile-filtered MTT solution (5 mg/ml in PBS). After incubating cells at 37 °C for 4 h, supernatants were carefully removed from the wells and formazan crystals were dissolved in DMSO. Absorbance was quantified using a microplate fluorescence reader (FL600, BIO-TEK®, Winooski, VT) at 570 nm. The cell viability by PEGylated peptide–DOX conjugates and by doxorubicin was expressed as percentages of that of the control and IC_{50} values were determined by sigmoidal dose–response fitting of cell viability (%) *vs.* doxorubicin equivalent concentration.

2.7. Inhibition effect on LLC growth

All animal experiments were performed in accordance with the procedure described in the Guide for the Care and Use of Laboratory Animals and the animal care and studies were approved by the Institute of Laboratory Animal Resource of the Seoul National University (Seoul, Korea). Tumor bearing mice were prepared by inoculating a suspension of 1×10^6 LLC cells/100 μl into the shaved right dorsa of 7-week-old male C57BL/6J mice (Daehan Biolink Co. Ltd., Chungbuk, Korea). Animals were randomized into six experimental groups of five mice as follows: control, doxorubicin, PEGylated peptide–DOX conjugate micelles, and drug-loaded conjugate micelles. Tumors were allowed to progress up to a volume of 50–60 mm^3 before initiating treatment. The mice were injected i.v. six times at 3-day intervals. On each treatment day, doxorubicin was administered at 3 mg/kg, and PEGylated peptide–DOX conjugate micelles and DOX-loaded conjugate micelles were administered at an equivalent doxorubicin dose of 3 mg/kg. Control mice received saline buffer. Tumor sizes were measured using vernier calipers in two dimensions every day; individual tumor volumes were calculated using the formula $V_t = (\text{length}) \times (\text{width}^2)/2$.

2.8. Doxorubicin levels in plasma and pharmacokinetic parameters

Drugs were injected via a lateral tail vein into 20–22 g normal male C57BL/6J mice at the dose mentioned in the above animal study, i.e., at an equivalent doxorubicin dose of 3 mg/kg. With a certain time interval after i.v. administration of drug, 450 μl of blood was collected from a capillary in the retroorbital plexus and directly mixed

with 50 μl of sodium citrate (3.8% solution). Plasma was then immediately centrifuged at 2500*g* at 4 °C for 20 min. Plasma doxorubicin concentrations were measured using a microplate fluorescence reader (FL600, BIO-TEK®, Winooski, VT) with fluorescence detection. The excitation wavelength used was 480 nm and the emission wavelength was 560 nm. The pharmacokinetic parameters of doxorubicin, PEGylated peptide–DOX conjugate micelles, and DOX-loaded conjugate micelles were calculated. Elimination half-lives ($t_{1/2}$) were determined by linear regression analysis after log transforming concentrations. From the concentration profiles of doxorubicin in plasma, we calculated volumes of distribution (V_d) and clearances (CL).

2.9. Statistical analysis

Statistical differences in tumor growth and weight between each group were determined using the standard Student's *t*-test. *p* values of <0.005 were considered significant.

3. Results and discussion

3.1. Preparation of PEGylated peptide–DOX conjugates

PEGylated peptides containing the GPLGV or GPLGVRG peptide sequences were conjugated with doxorubicin according to the synthetic scheme illustrated in Fig. 1, and the final products were purified by prep-HPLC to remove unreacted doxorubicin. The doxorubicin conjugation in PEGylated peptide–DOX conjugates was confirmed by HPLC with monitoring at 479 nm.

3.2. Loading of doxorubicin into PEGylated peptide–DOX conjugate micelles

In order to increase doxorubicin content in conjugate micelles, doxorubicin was additionally loaded into the hydrophobic core of PEGylated peptide–DOX conjugate micelles. These loaded samples were named P5D/D and P7D/D, respectively. In both cases, free doxorubicin was entrapped in the conjugate micelles at about 99% loading efficiency and the amounts of loaded doxorubicin in P5D/D and P7D/D were 63.7% and 64.2% own weight, respectively. The amount of loaded doxorubicin was about 10 times more than the amount of conjugated doxorubicin, such that the ratio of loaded DOX to conjugated DOX was 9.7 for P5D/D and 10.6 for P7D/D. The characteristics of P5D/D and P7D/D are listed in Table 1. In general, micelles, which are formed by simple hydrophobic interactions in aqueous condition, have been known to be less stable in physiological system than other conjugate forms such as nanoparticles, aggregates, and micro(nano)spheres [11,18]. But it has been found that the stability of a micelle is also strongly influenced by properties of the micelle core and the quantity of drug-loaded in the inner core as well as the content of chemically conjugated hydrophobic drug [33,37,38].

3.3. Micelle size of the conjugate micelles before and after doxorubicin loading

The conjugation of doxorubicin with a hydrophobic anthracycline moiety and a hydrophilic PEGylated peptide gives an amphiphilic character to the PEGylated peptide–DOX conjugate and thus it can form micelles in water. Micelle sizes ranged from 73 to 121 nm with a narrow distribution, as determined by dynamic light scattering. After loading free doxorubicin, the mean conjugate micelle size increased slightly from 73 to 88 nm for P5D/D and from

Table 1
DOX loadings of PEGylated peptide–DOX conjugate micelles and particle size

	Loading efficiency (%)	Content of loaded DOX (wt%)	Loaded DOX/ conjugated DOX	Mean diameter (nm)
P5D				99 ± 1
P7D				201 ± 2
P5D/D	98.8 ± 0.1	63.1 ± 0.6	9.5 ± 0.2	137 ± 7
P7D/D	98.8 ± 0.1	64.1 ± 0.3	10.6 ± 0.2	347 ± 8

The values are expressed as means ± SD of three experiments.

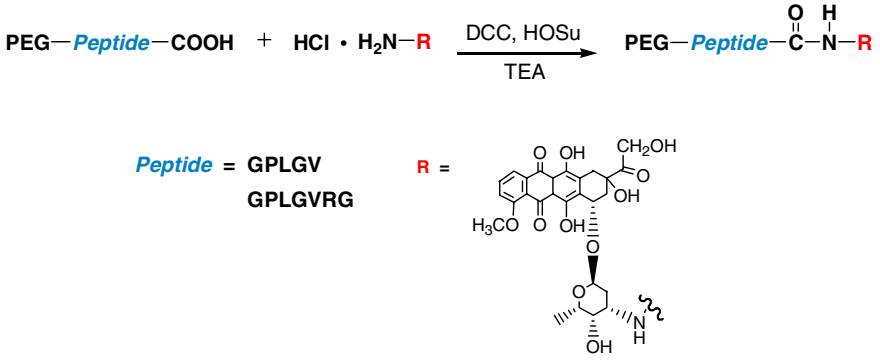

Fig. 1. Synthesis of PEGylated peptide–DOX conjugates.

121 to 148 nm for P7D/D, suggesting that the free doxorubicin was entrapped in the PEGylated peptide–DOX conjugate micelle core. In this study, we found that a large amount of doxorubicin could be loaded into conjugate micelles due to the hydrophobic interaction between the conjugated and the added doxorubicin. Therefore, the PEGylated peptide–DOX conjugate micelles could serve as anti-cancer agents and as anti-cancer drug delivery systems.

3.4. Cleavage of PEGylated peptide–DOX conjugates by active MMP-2

We investigated whether PEGylated peptide–DOX conjugates containing different peptide sequences are degraded by active MMP-2. As shown in Fig. 2, both conjugates were degraded by active MMP-2 in a time-dependent manner. During incubation for 12 h, the two PEGylated peptide–DOX conjugates showed similar degradation rates. However, after 12 h of incubation, P5D degraded slightly faster than P7D. After 48 h of incubation, 54% of the P5D and 42% of the P7D had degraded. Consequently, these results confirmed that both conjugates are substrates of MMP-2. The peaks for fragments after degradation of each conjugate were not in exact agreement with the peak for pure doxorubicin. Hence, we concluded that the degradation products were not doxorubicin itself but amino acid or di-/tri-peptide derivatives linked with doxorubicin.

3.5. Cytotoxicity of PEGylated peptide–DOX conjugates in LLC cells

Next, the cytotoxicity of PEGylated peptide–DOX conjugates was investigated by using MTT assays. In this experiment, we believed that the effect of MMPs secreted from LLC cells on the degradation of conjugates could be negligible *in vitro*. Although it is true that the LLC cells express pro- and active MMP-2 and pro-MMP-9 *in vitro*, the amount of MMPs secreted by LLC cells was not enough to cleave conjugates because only 5000 cells were plated in each well. As shown in Fig. 3, PEGylated peptide–DOX conjugates showed much lower cytotoxicity than doxorubicin against LLC cells. At the doxorubicin equivalent concentration of 10^{-6} M, cell viability was about 93% for P5D or P7D treated cells, while the cell viability of doxorubicin treated cells decreased to 33%. And, IC_{50} values increased from 0.24 μM for doxorubicin to 112.9 and 136.7 μM for P7D and P5D, respectively. However, when the cells were exposed to DOX-loaded PEGylated peptide–DOX conjugates, their ability to damage cells increased and the viability of treated cells decreased moderately compared to that of PEGylated peptide–DOX conjugates treated cells. At a doxorubicin equivalent concentration of 10^{-6} M, all conjugates showed cell viability of more than 90% irrespective of doxorubicin loading, whereas at a doxorubicin equivalent concentration of 10^{-5} M, cell viability decreased from 90% to 27% for P5D and P5D/D and from 88% to 26% for P7D and P7D/D. After doxorubicin loading, the IC_{50} values decreased from 136.7 to 4.0 μM for P5D and from 112.9 to 3.8 μM for P7D. Thus, the cytotoxicity of P5D/D and P7D/D was significantly higher than that of P5D and P7D. In spite of such decrease of cytotoxicity, P5D/D

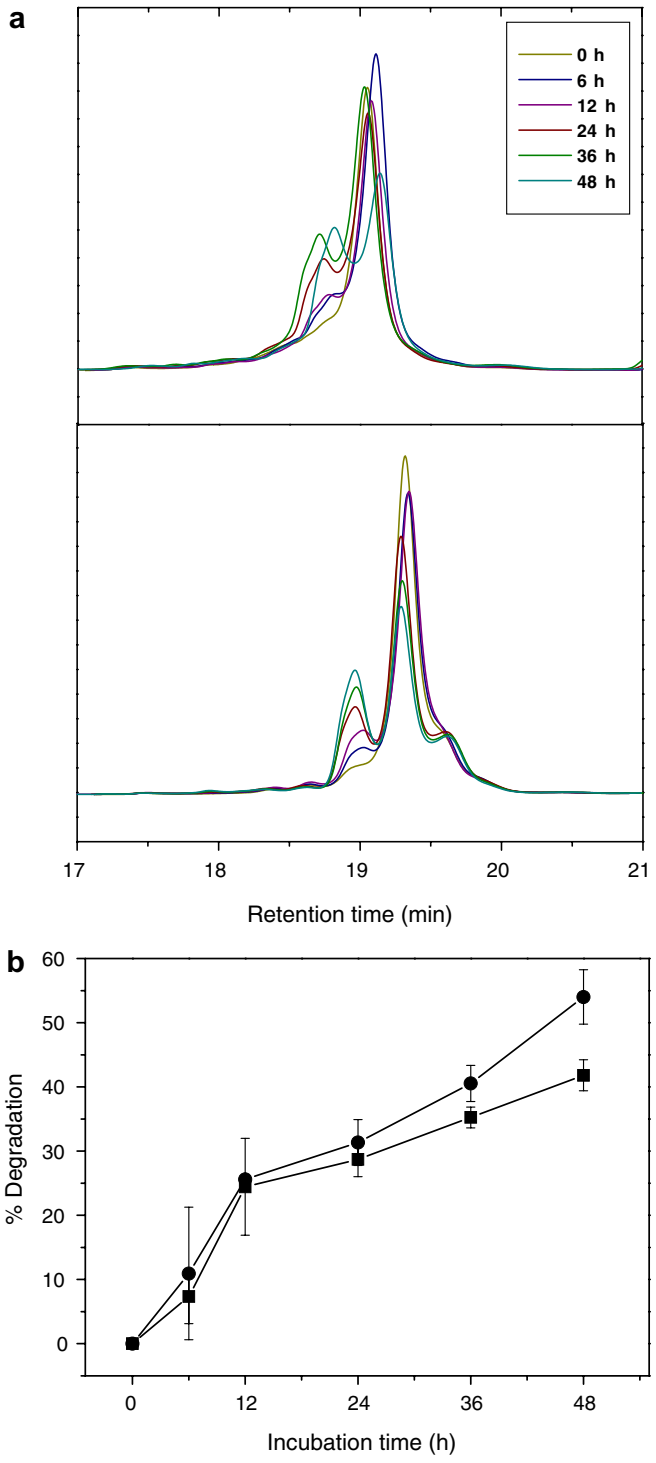

Fig. 2. (a) Cleavage of P5D (upper) and P7D (lower) measured by HPLC after incubation with active MMP-2. (b) Percentage degradation of P5D (●) and P7D (■) and *vs.* incubation time.

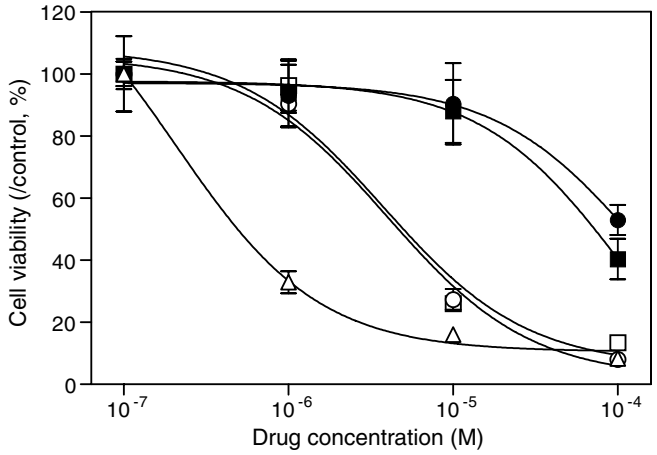

Fig. 3. Cytotoxicity of cells incubated with serial dilutions of doxorubicin (△), P5D (●), P7D (■), P5D/D (○), or P7D/D (□) (10^{-6}–10^{-4} M) after an incubation time of 24 h, as determined using the MTT assay. Data are expressed as percentages of the untreated control. The values shown are means ± SD of quadruplicate samples.

and P7D/D did not surpass the cytotoxicity of doxorubicin and were about 16 times less toxic than doxorubicin.

The IC_{50} values for doxorubicin or PEGylated peptide–DOX conjugates before and after doxorubicin loading are summarized in Table 2. We would expect that if the PEGylated peptide–DOX conjugates were less toxic than doxorubicin against cells, then their therapeutic indexes would be more widened than that of doxorubicin, thereby reducing severe side effects of doxorubicin. Incidentally, they do not show comparable therapeutic effect as doxorubicin because of their reduced cytotoxicity, although they show specificity for MMPs secreted by tumors. Therefore, the loading of doxorubicin into PEGylated peptide–DOX conjugate micelles should augment the therapeutic effect of PEGylated peptide–DOX conjugates but retain the inability to induce the severe side effects of doxorubicin because the loaded doxorubicin is embedded within the conjugate micelles.

3.6. Tumor growth inhibition effect

The tumor growth inhibition effect of PEGylated peptide–DOX conjugate micelles and DOX-loaded PEGylated peptide–DOX conjugate micelles was studied using male C57BL/6J mice bearing Lewis lung carcinoma. An intravenous route through a tail vein was chosen to inject doxorubicin, PEGylated peptide–DOX conjugate micelles, or DOX-loaded conjugate micelles. Drugs were administered five times at 3-day intervals; as represented by arrows in Fig. 4. The dose of doxorubicin was determined based on the MTD of doxorubicin (7–8 mg/kg) and the dose of PEGylated peptide–DOX conjugate micelles was calculated based on doxorubicin-equivalence. PEGylated peptide–DOX conjugate micelles showed approximately 50% tumor growth compared to the untreated control and doxorubicin-loaded conjugate micelles inhibited tumor growth by up to about 75% versus the control, showing comparable effect as doxorubicin. Specifically, P5D and P7D showed tumor inhibitions of 41.8% and 49.3%, respectively, whereas P5D/D and P7D/D suppressed tumor growth by 72.0% and 63.3%, respectively, versus the control. Free doxorubicin was found to inhibit tumor growth by 73.6%. There were no statistically significant differences between P5D and P7D ($p = 0.149$), between P5D/D and P7D/D ($p = 0.205$), between doxorubicin and P5D/D ($p = 0.786$), and between doxorubicin and P7D/D ($p = 0.073$), respectively. We conclude that this significant tumor inhibition effect of DOX-loaded PEGylated peptide–DOX conjugate micelles resulted from the peptide specificity for MMPs and the additive effect of the loaded doxorubicin. As a result of the cytotoxicity and tumor inhibition effect, PEGylated peptide–DOX conjugate micelles have the ability to reduce the toxicity of doxorubicin, but not to work as effectively as doxorubicin despite their MMP-specificities. To improve this situation, free

Table 2
IC_{50} values of doxorubicin, PEGylated peptide–DOX conjugate micelles and DOX-loaded PEGylated peptide–DOX conjugates micelles against LLC cells *in vitro*

Drugs	IC_{50} (μM) of doxorubicin equivalent
Doxorubicin	0.24 ± 0.03
P5D	136.7 ± 5.3
P7D	112.9 ± 2.5
P5D/D	4.0 ± 0.2
P7D/D	3.8 ± 0.5

The values are expressed as means ± SD of three experiments.

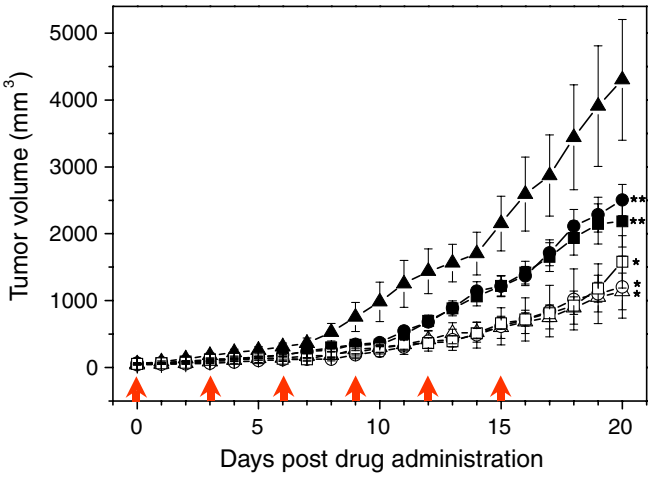

Fig. 4. Tumor growth inhibition effect of doxorubicin, PEGylated peptide–DOX conjugates, and doxorubicin-loaded PEGylated peptide–DOX conjugates on LLC cell growth in mice. Tumor bearing mice were treated i.v. with saline (▲), doxorubicin (△), P5D (●), P7D (■), P5D/D (○), or P7D/D (□) six times every 3 days. Tumor sizes and body weights were measured daily. Individual tumor volumes were calculated using the formula $V_t = (\text{length}) \times (\text{width}^2)/2$. Arrows indicate the drug injection day and the values shown are means ± SD for five mice. *Statistically significant difference *vs.* the control group, $p < 0.001$. **Statistically significant difference *vs.* the control group, $p < 0.005$.

doxorubicin was loaded into PEGylated peptide–DOX conjugate micelles and was found to increase the anti-tumor effect of PEGylated peptide–DOX conjugate micelles to the level of doxorubicin. Summarizing, this means that PEGylated peptide–DOX conjugate micelles containing doxorubicin have lower toxicity than doxorubicin but yet retain the same therapeutic effect as doxorubicin owing to both MMPs specificity at the tumor site and augmentation by the loaded doxorubicin.

3.7. Doxorubicin level in plasma/pharmacokinetics

After injecting doxorubicin, PEGylated peptide–DOX conjugate micelles, or DOX-loaded conjugate micelles, doxorubicin concentrations in blood were evaluated and all pharmacokinetic parameters of each drug were calculated. This experiment was carried out to confirm whether PEGylated peptide–DOX conjugate micelles could circulate in the body for a longer time than doxorubicin and whether DOX-loaded conjugate micelles release free doxorubicin and maintain higher drug levels in blood than PEGylated peptide–DOX conjugate micelles. As shown in Fig. 5, the plasma level of doxorubicin in the doxorubicin group reached a nadir within 5 min whereas the plasma levels of other conjugate micelle groups declined more slowly. Actually, the plasma levels of P5D and P7D were retained for up to 2 h in the body and the plasma levels of P5D/D and P7D/D were maintained for more than 2 h. We would expect that PEGylated peptide–DOX conjugate micelles and DOX-loaded conjugate micelles produce different drug levels in plasma. In the case of DOX-loaded conjugate micelles, we supposed that the loaded doxorubicin shows drug activity faster than the conjugated doxorubicin. The loaded doxorubicin might be easily released from the inner core of the micelles in a freely active form, whereas the conjugated doxorubicin could be released and become active after degradation by MMPs around tumor sites. The doxorubicin concentration profiles observed in groups administered with DOX-loaded conjugate micelles were in accordance with our expectation. After injecting P5D/D or P7D/D, the plasma level of doxorubicin was maintained at 2.5-fold higher and reduced more slowly than for P5D or P7D. This result explains why DOX-loaded conjugate micelles show more tumor growth inhibition effect than PEGylated peptide–DOX itself. The pharmacokinetic parameters (V_d, CL and $t_{1/2}$) of P5D, P7D, P5D/D, and P7D/D are summarized in Table 3. The half-life of all conjugate micelles was higher than doxorubicin but no significant difference in the volume of distributions of doxorubicin and the conjugate micelles was observed. The clearance values of PEGylated peptide–DOX conjugate micelles and DOX-loaded conjugate micelles were remarkably lower than the 712.2 ml/min of doxorubicin, thereby implying that the conjugate micelles in blood are cleared much more slowly from the body than doxorubicin. By comparing PEGylated peptide–DOX conjugate micelles and DOX-loaded conjugate micelles, we are able to show that DOX-loaded conjugate micelles maintain higher concentrations of doxorubicin in plasma and have slightly longer half-lives and mildly higher volume of distribution and clearances than PEGylated peptide–DOX conjugate micelles. It demonstrates that DOX-loaded conjugate micelles can show higher doxorubicin level in plasma than PEGylated peptide–DOX conjugate micelles, because in the case of DOX-loaded conjugate micelles, the loaded free doxorubicin acts prior to the action of the conjugated doxorubicin unlike in the case of PEGylated peptide–DOX conjugate micelles where only the conjugated doxorubicin shows drug activity.

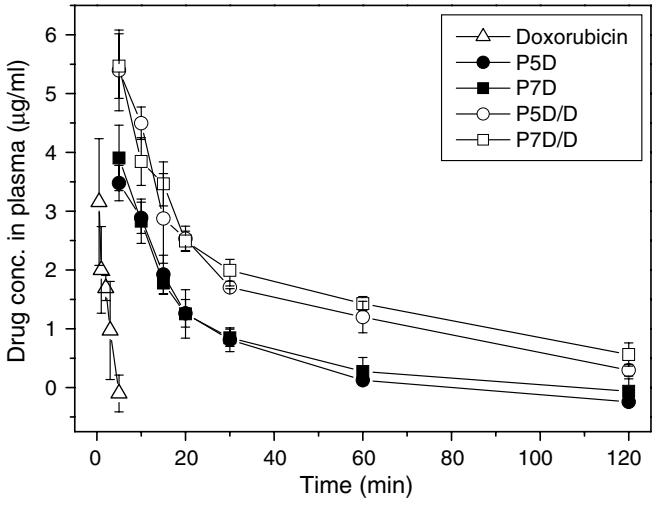

Fig. 5. Concentration profiles of doxorubicin in plasma for 2 h after i.v. injection of doxorubicin (△), P5D (●), P7D (■), P5D/D (○), or P7D/D (□). Plasma concentrations of doxorubicin were measured using a microplate fluorescence reader. The excitation wavelength used was 480 nm and the emission wavelength 560 nm. Values are means ± SD for four mice.

Table 3
Pharmacokinetic parameters

Drugs	Doxorubicin	P5D	P7D	P5D/D	P7D/D
$t_{1/2}$ (min)	1.6	11.4	14.8	13.5	15.2
V_d (l/kg)	1.67	1.24	1.50	1.95	2.06
CL (ml/min)	712.2	75.8	70.4	100.2	93.8

4. Conclusions

MMPs-specific PEGylated peptide–DOX conjugates were synthesized using peptides GPLGV and GPLGVRG (P5D and P7D, respectively) and about 99% of free doxorubicin was loaded into micelles formed by each conjugate. These loaded samples were named as P5D/D and P7D/D, respectively. Both conjugates were specifically cleaved by active MMP-2 in an incubation time dependent manner. PEGylated peptide–DOX conjugates showed significantly lower cytotoxicity than doxorubicin at doxorubicin-equivalent concentrations and the cytotoxicity of DOX-loaded PEGylated peptide–DOX conjugate micelles moderately

decreased without exceeding that of doxorubicin. *In vivo*, P5D and P7D showed 41.8% and 49.3% tumor inhibition, respectively, whereas P5D/D and P7D/D suppressed tumor growth by 72.0% and 63.3%, respectively, compared with the untreated control. Free doxorubicin, as such, inhibited tumor growth by 73.6%. All conjugate micelles regardless of doxorubicin loading exhibited longer half-lives and were more slowly cleared from the body than doxorubicin and DOX-loaded PEGylated peptide–DOX conjugate micelles maintained higher concentrations of doxorubicin in plasma than the PEGylated peptide–DOX conjugate micelles alone. DOX-loaded PEGylated peptide–DOX conjugate micelles offer the advantage of a simple anti-cancer delivery system, and could become a practical system when combined with the process of free drug loading. Our ongoing studies include the *in vivo* evaluation of LD_{50} values and the toxicity of DOX-loaded PEGylated peptide–DOX conjugate micelles; the distribution of doxorubicin in the body after the injection of conjugate micelles will be followed up as further work. In conclusion, we propose DOX-loaded PEGylated peptide–DOX conjugate micelles as a potent cancer therapy that retains the anti-cancer activity of the parent drug but reduces toxicity.

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