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Liposome encapsulation reduces cantharidin toxicity

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ABSTRACT

Several reports have demonstrated that cantharidin is a strong anticancer compound in vitro; however, its in vivo usefulness is often limited due to its high systemic toxicity. In this study, we encapsulated cantharidin into pegylated liposomes and studied its activity against human breast cancer MCF-7 cells in vitro and its systemic toxicity in mice. Another two methods were also used to reduce the dosage of cantharidin, including labeling liposomal cantharidin with octreotide and exposing cells to hyperbaric oxygen. The cytotoxic activity of pegylated liposomal cantharidin was drastically reduced compared with free cantharidin in vitro. Octreotide-labeled pegylated liposomal cantharidin induced cell death by specifically targeting somatostatin receptors in MCF-7 cells. Cell death was augmented with a low dose of cantharidin under hyperbaric oxygen. Liposomal cantharidin had significantly less systemic toxicity than free cantharidin in vivo and also exhibited a high efficacy against antitumor growth in nude mice. These results suggest that the systemic toxicity of cantharidin can be mitigated by liposome encapsulation; however, that did not decrease its antitumor activity.

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1. Introduction

Cantharidin (exo, exo-2,3-dimethyl-7-oxobicyclo[2,2,1]heptane-2,3-dicarboxylic acid anhydride) is a vesicant produced by Chinese blister beetles and Spanish flies (Oaks et al., 1960; Wang, 1989). All body fluids of blister beetles have cantharidin, and the dried bodies have been used as an anticancer agent for a long time in traditional Chinese medicine. In addition, cantharidin has been used to treat warts and molluscum through topical application and it has also found use as an abortifacient and aphrodisiac. Cantharidin demonstrates strong activity against cancer cells in vitro, but several severe side-effects limit its clinical application. The clinical signs of cantharidin poisoning are nonspecific and include dysphagia, hematemesis, dysuria, liver congestion, and renal toxicity (Moed et al., 2001). The median lethal dose is about 1 mg/kg, and fatalities usually result from renal failure and injury to the gastrointestinal tract (Matsuzawa et al., 1987; Karras et al., 1996). Like other natural toxins, cantharidin is a strong protein phosphatase 1 (PP1) and phosphatase 2A (PP2A) inhibitor (Graziano et al., 1988; Li et al., 1993). Several reports have demonstrated that cantharidin exhibits anticancer activity in spite of its toxicity to the body (Rauh et al., 2007). In human hepatoma Hep3B cells, a low dose of cantharidin induced cell cycle arrest in the G2/M phase, and a high dose of antharidin induced acute cell lysis (Wang et al., 2000). Cantharidin also induces G2/M phase arrest and apoptosis in other tumor types, such as C1210, HCT116, and HT29 cells (Sakoff et al., 2002, 2004). Another study indicated that cantharidin induced apoptosis by a p53-dependent mechanism in human leukemia cells, which was associated with the production of reactive oxygen species (ROS) in oxidative stress-resistant thymic lymphoma-derived WEHI7.2 variants with cantharidin (Efferth et al., 2005). In human U937 leukemic cells, cantharidin also induced cell apoptosis by activation of the p38 and JNK MAPK pathways and

Abbreviations: CAN, cantharidin; Oct, octreotide; PEG, poly(ethyleneglycol); Oct-PEG-liposome, octreotide-labeled PEG-liposome; PEG-liposomal CAN, PEG-liposomal cantharidin; Oct-PEG-liposomal CAN, octreotide-labeled PEG-liposomal cantharidin; DPPC, 1,2-palmitoyl-*sn*-glycero-3-phosphatidylcholine; DiI, 1,1′-dioctadecy-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; MTT, thiazolyl blue tetrazolium bromide; PP1, protein phosphatase 1; PP2A, phosphatase 2A; ROS, reactive oxygen species; HBO, hyperbaric oxygen; HBA, hyperbaric air; SSTR, somatostatin receptor; RES, reticuloendothelial system.

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caspase-3, and downregulation of p53 (Huh et al., 2004). The molecular mechanisms of cantharidin induced tumor cell death are still not well understood, and the complexity of its action has impeded its development as an anticancer drug. Therefore, we were interested in reducing the toxicity of cantharidin and used liposomal-encapsulated cantharidin to decrease its toxicity to the human body in this study.

2. Materials and methods

2.1. Materials

The synthetic lipid 1,2-palmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, 1,1′-dioctadecy-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil), cantharidin, and chloroform were purchased from Sigma Chemical (St. Louis, MO, USA). Octreotide was purchased from Bachem AG (Bubendorf, Switzerland). DSPE-PEG and DSPE-PEG-NHS were purchased from Shearwater Polymers (Huntsville, AL, USA).

2.2. Preparation of pegylated octreotide

DSPE-PEG-octreotide (DSPE-PEG-Oct) was synthesized by a combination of octreotide (10 mg/ml in 0.1 M phosphate buffer, pH 5.0) with an equimolar amount of DSPE-PEG-NHS in the presence of 20 mM sodium cyanoborohydride in water at 4 °C for 24 h. The reaction mixture was further loaded onto reverse-phase high-performance liquid chromatography (HPLC), and DSPE-PEG-Oct was isolated and freeze-dried.

2.3. Preparation of liposome-encapsulated cantharidin and Dil dye

Liposomes were prepared by a conventional rotary evaporation method. Briefly, DPPC, cholesterol, and DSPE-PEG (or DSPE-PEG-Oct) was dissolved in an appropriate volume of chloroform according to the ratio, DPPC: cholesterol: DSPE-PEG (or DSPE-PEG-Oct) of 74: 25: 1. The mixture was dried to a thin lipid film under a vacuum using a rotary evaporator. This film was kept under high vacuum for at least 24 h to remove any traces of chloroform. The dry lipid film was hydrated with 10 ml PBS containing 500 μM cantharidin or 1.68 mg Dil dye to obtain a lipid suspension (multilamellar vesicles, MLVs), and the MLV particles were then sonicated for 10 min using a Bandelin HD-200 sonication probe at 50 °C to prepare small unilamellar vesicles (SUVs). The formation of liposomes did not require further washing out the free cantharidin, and the encapsulation rate was about 80% as determined by the HPLC method. The liposome retained a similar size, and the drug release rate was <5% under storage for 1 month at 50 °C.

2.4. Measurement of the particle size and zeta potential

The mean diameter and zeta potential of various kinds of liposomes were determined by photon correlation spectroscopy with a BI 90 particle sizer and Zeta Plus, a zeta potential analyzer (Brookhaven Instruments, Holtsville, NY, USA). The zeta potential was measured by diluting liposomes with phosphate-buffered saline (PBS).

2.5. Cell culture

Human MCF-7 breast carcinoma cells (BCRC 60436, Food Industry Research and Development Institute, Hsinchu, Taiwan) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) and incubated at 37 °C in humidified 5% CO_2.

2.6. MTT assay

Cells were cultured in 24-well plates for 24 h, then treated with drugs as described in the figure legends. When the cell viability had been determined, each well was removed from the medium and another 200 μl of fresh medium was added as well as 50 μl MTT (2 mg/ml) at 37 °C in the dark. After 2 h, the medium was removed, and 200 μl DMSO and 25 μl Sorensen's glycine buffer were added. The supernatant (100 μl) was added to a 96-well plate, and the absorbance at OD 570 nm was measured with an enzyme-linked immunosorbent assay (ELISA) plate reader.

2.7. Hyperbaric oxygenation

The hyperbaric chamber (YI LIN Enterprise, Taipei, Taiwan) was filled with 97.9% O_2 and 2.1% CO_2 and pressurized to 2.5 atmospheres absolute (ATA) over 3 min. Control cells were placed in an incubator at 37 °C, with 21% O_2 and 5% CO_2 at 1 ATA. To rule out the effect of high pressure, cells were incubated at 37 °C, with 21% O_2 and 5% CO_2 at 2.5 ATA. The chambers and incubator were humidified.

2.8. Animals

Female Balb/cByJNarl mice (4–5 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and kept in an animal facility for 1–2 weeks before use. All animal experimental procedures were conducted and approved by the Institutional Animal Care and Use Committee of Taipei Medical University. For determining the systemic toxicity of drugs, mice (>6 animals/group) were intraperitoneally (i.p.) injected with free cantharidin or PEG-liposomal cantharidin. On days 5 and 7, surviving mice were i.p. injected with drugs again. To determine the efficacy of drugs, mice (six animals/group) were subcutaneously injected between the scapulae with 1×10^7 of MCF-7 cells and i.p. injected with β-estradiol 3-benzoate (25 μg/mice) to promote tumor growth. On days 11 and 13 after implantation, mice received an i.p. injection of either PEG-liposome (control), 5 mg/kg free cantharidin, or 5 mg/kg PEG-liposomal cantharidin. On day 19, mice were sacrificed by cervical dislocation, and the tumor volume was estimated according to the following formula: tumor volume (mm^3) = $L \times W^2/2$, where L is the length and W is the width.

3. Results

3.1. Cantharidin caused MCF-7 cell death in a dose- and time-dependent manner

Human breast cancer MCF-7 cells were treated with various concentrations of cantharidin for 24 h, and the cell viability was determined by an MTT assay. As shown in Fig. 1A, cantharidin significantly caused cell death in a dose-dependent manner, and the 50% effective dose (ED_{50}) was about 50 μM. When cells were treated with 50 μM of cantharidin for various time periods, cells died in a time-dependent manner, and the viability was about 15% after 72 h of treatment (Fig. 1B).

3.2. Liposomal cantharidin decreased the cytotoxicity activity and octreotide-labeled liposomal cantharidin increased the cytotoxicity activity in MCF-7 cells

Cantharidin was encapsulated by PEG-liposomes or octreotide-labeled PEG-liposomes (Oct-PEG-liposomes) as described in "*Materials and Methods*", and their particle diameter and surface potential were characterized. As shown in Table 1, the particle sizes of octreotide-labeled PEG-liposomes (Oct-PEG-liposomes) and octreotide-labeled PEG-liposomal cantharidin (Oct-PEG-liposomal CAN) exceeded those of PEG-liposomes or PEG-liposomal CAN, and their diameters ranged from ~101.0 to ~171.6 nm. The surface potentials of all liposomal reagents ranged from −19.95 to −28.9 mV, indicating that all liposomal reagents had similar surface potentials and therefore the same physical affinity to the cell membrane.

Next, we examined the toxicity of PEG-liposomes and octreotide-labeled PEG-liposomes in MCF-7 cells. The results indicated that neither reagent by itself caused any toxicity even at 48 h of treatment (Fig. 2A). However, cantharidin caused time-dependent cell deaths of about 60% and 80% at 24 and 48 h of treatment, respectively (Fig. 2B). PEG-liposomal CAN, by contrast, displayed significantly less cytotoxicity than cantharidin, causing cell deaths of about 15% and 38% at 24 and 48 h of treatment, respectively. Octreotide has been demonstrated to have a specific high-affinity to binding with the somatostatin receptor (SSTR) in MCF-7 cell membranes (Huang et al., 2000). When MCF-7 cells were treated with octreotide-labeled PEG-liposomal CAN, cell death significantly increased. In comparison with PEG-liposomal CAN, octreotide-labeled PEG-liposomal CAN increased cell death to about 70% and 50% at 24 and 48 h treatment, respectively. These results suggest that the high cytotoxicity of cantharidin can be mitigated by liposome encapsulation, and octreotide-labeled liposomal CAN restored the toxicity mediated by the SSTR.

In order to evaluate whether octreotide specifically targets MCF-7 cells, which present it with high-affinity binding sites, we examined the targeting of octreotide-labeled liposomal Dil

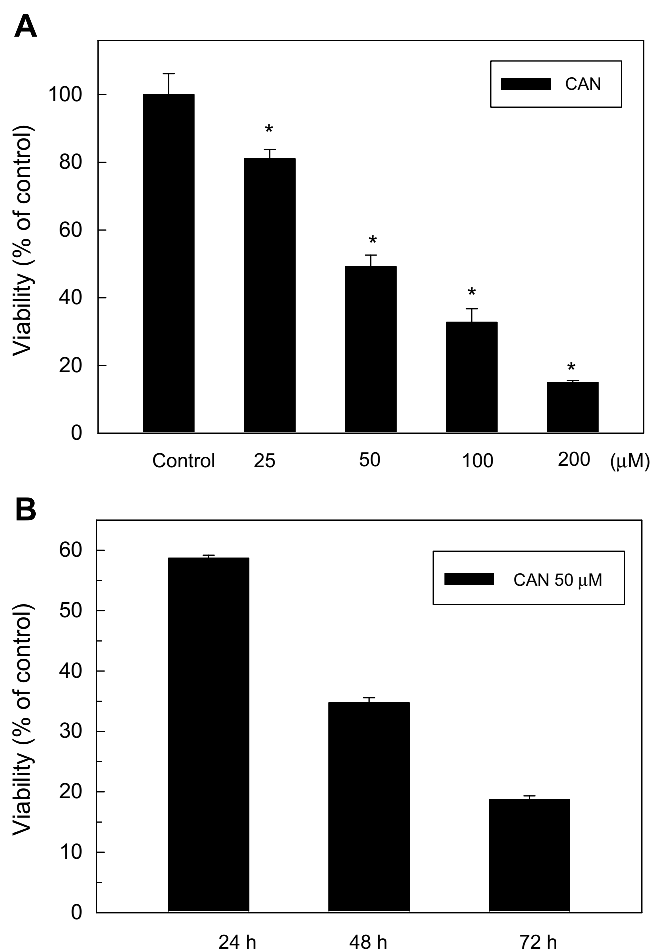

Fig. 1. Dose- and time-dependent effects of cantharidin on the viability of MCF-7 cells. Cells were treated with various concentrations of cantharidin for 24 h (A), or with 50 μM cantharidin for different time periods as indicated (B). Cell viability was determined by the MTT assay. Data are presented as the mean ± S.E. $^{*}p < 0.05$ compared to the control, $n = 3$.

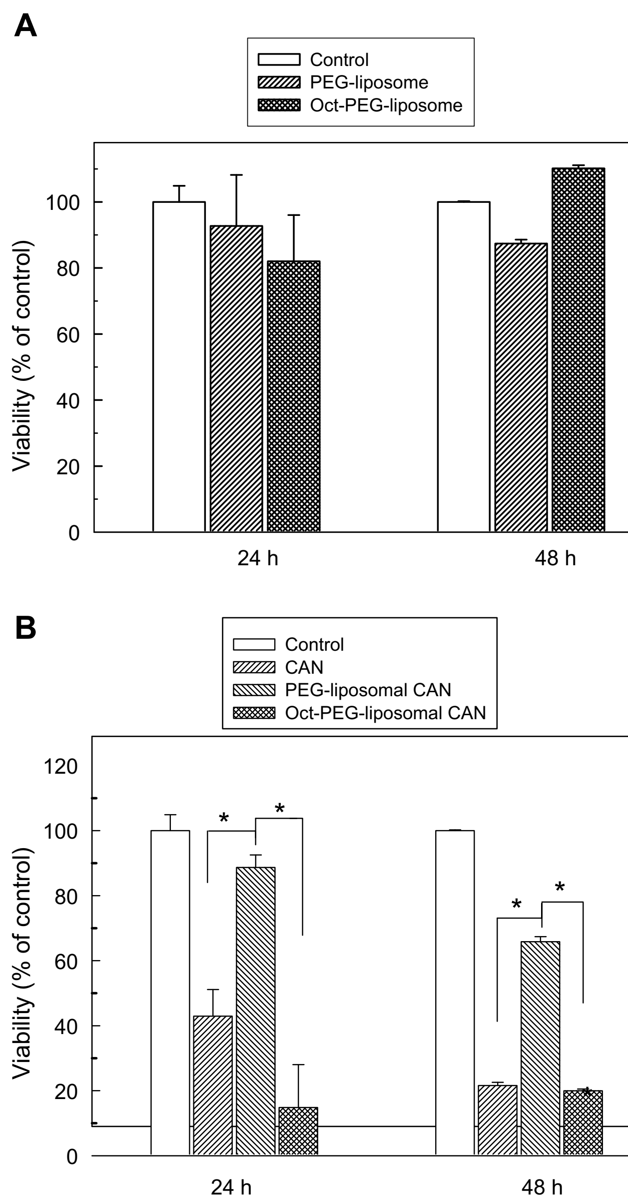

Fig. 2. Liposomal cantharidin decreased cytotoxicity activity, and octreotide-labeled liposomal cantharidin increased it in MCF-7 cells. (A) Cells were treated with PEG-liposome or octreotide-PEG-liposome for 24 and 48 h, and viability was determined by the MTT assay. (B) Cells were treated with cantharidin (50 μM), PEG-liposomal cantharidin, or octreotide-PEG-liposomal cantharidin for 24 and 48 h, and viability was determined by the MTT assay. $^{*}p < 0.05$, $n = 3$.

Table 1
Physical characteristics of various kinds of liposomal reagents

	Average particle diameter (nm)	Average zeta potential (mV)
CAN	0	0
PEG-liposome	101.0	−28.9
Oct-PEG-liposome	171.6	−21.99
PEG-liposomal CAN	113.3	−19.95
Oct-PEG-liposomal CAN	156.8	−22.27

CAN, cantharidin; Oct-PEG-liposome, octreotide-labeled PEG-liposome; PEG-liposomal CAN, PEG-liposomal cantharidin; Oct-PEG-liposomal CAN, octreotide-labeled PEG-liposomal cantharidin.
Numbers are the mean of three independent determinations.

(a fluorescent dye) in MCF-7 cells. Incubation of MCF-7 cells with liposomal DiI for 48 h resulted in a little DiI dye in the cytosol of the cell (Fig. 3C and D). When MCF-7 cells were incubated with octreotide-labeled liposomal DiI, DiI dye was distributed throughout the cytosol of the cell (Fig. 3A and B). These results suggest that octreotide-labeled liposomes can bind with MCF-7 cell membranes and become internalized via SSTR-mediated endocytosis. In addition, these results prove how much more toxic octreotide-labeled liposomal CAN is.

3.3. Hyperbaric oxygen enhanced drug cytotoxicity in MCF-7 cells

Deep inside a tumor mass, there is usually a low vessel and hypo-oxygen environment, and drugs that reach it are usually of a low concentration. To overcome this problem, several experiments have combined anticancer drugs and hyperbaric oxygen (HBO) to augment the drug efficiency and kill tumor cells. HBO increases the drug concentration and the production of oxygen free radicals in a tumor; therefore HBO can enhance tumor cell death (Daruwalla and Christophi, 2006). In order to evaluate whether HBO can reduce the use of cantharidin, we assessed the use of various kinds of cantharidin under HBO, compared to various kinds of cantharidin under normobaric conditions in vitro. As shown in Fig. 4A, additive HBO treatment enhanced cell toxicity in cells with free cantharidin, PEG-liposomal CAN, or octreotide-labeled

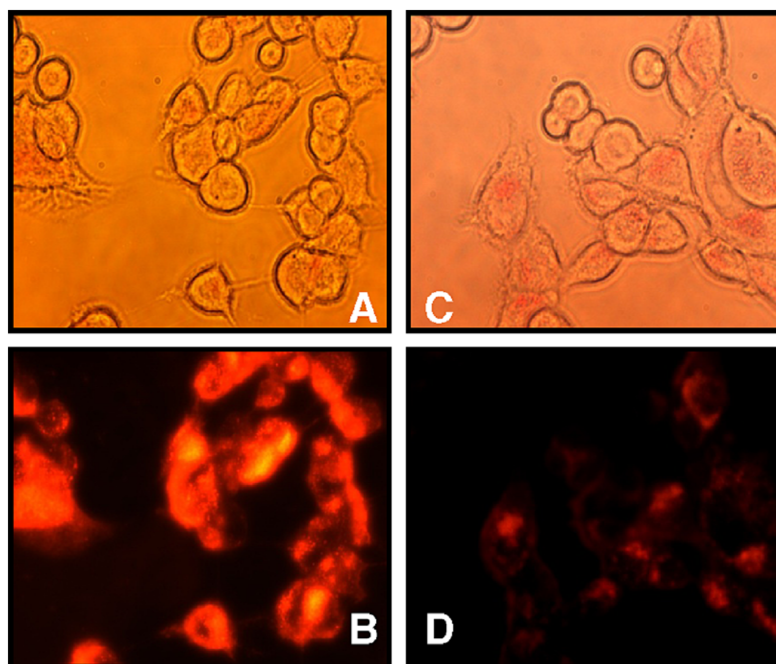

Fig. 3. Octreotide-labeled liposomal DiI specifically targeted MCF-7 cells. Cells were treated with octreotide-labeled liposomal DiI or liposomal DiI for 48 h, then cells were photographed under phase-contrast microscopy (A, C) or under fluoromicroscopy (B, D). Magnification, 200×.

liposomal CAN for 24 h. After 48 h of drug treatment, the survival rate between HBO and normobaric groups was indistinguishable (Fig. 4B). To rule out the possibility of high pressure-induced cell death, we assessed cell viability under hyperbaric air (HBA). As shown in Fig. 4C, both normobaric and HBA conditions showed similar cell survival rates in MCF-7 cells with drugs, indicating that cell death under HBO was due to hyper-oxygen, not to high pressure.

3.4. Liposomal cantharidin exhibited low toxicity in vivo and significantly inhibited tumor growth in nude mice

To evaluate whether liposomal cantharidin resulted in lower mortality than free cantharidin in vivo, mice were thrice i.p. injected with free cantharidin or PEG-liposomal CAN, and the surviving mice were counted. As shown in Table 2, all mice expired after receiving 5 or 10 mg/kg of free cantharidin once. On the other hand, only one mouse died on day 2 after receiving 5 mg/kg of PEG-liposomal CAN, and the other mice survived until day 60 even after two additional PEG-liposomal CAN injections. The 60-day survival rate was 0% for the free cantharidin group and 83.3% for the PEG-liposomal CAN group. However, a high dose of PEG-liposomal CAN (10 mg/kg) also exhibited high toxicity to mice. These results suggest that the toxicity of cantharidin can be mitigated by liposome encapsulation. To determine the antitumor efficacy of the drug, tumor-bearing nude mice were i.p. injected with 5 mg/kg of free cantharidin or PEG-liposomal CAN. At the end, administration of free cantharidin resulted in the death of all tumor-bearing nude mice; however, nonfatal PEG-liposomal CAN significantly inhibited tumor growth by about 75% (Fig. 5). The results suggest that PEG-liposomal CAN not only has low systemic toxicity but is also highly efficacious in antitumor growth.

4. Discussion

Cantharidin is an active compound against tumor cells, but it is too toxic to be directly used in tumor-bearing animals or human patients. The toxicity of cantharidin is believed to be mediated by the inhibition of serine/threonine protein phosphatase A2. Several studies used effectors to modify cantharidin in order to discover novel analogues for anticancer drugs with fewer side-effects and systemic toxicities (Kawamura and Casida, 1990; Kok et al., 2006a, b). They focused on the screening of cantharidin analogues for their ability to inhibit PP1 and PP2A (McCuskey et al., 2000, 2003; Sakoff et al., 2002; Shan et al., 2006). However, norcantharidin, the demethylated derivative analogue, is the only successful anticancer drug at present (Liu et al., 1995; Chen et al., 2002, 2003, 2005). Based on previous findings, we prepared various forms of liposomal cantharidin in an effort to reduce the toxicity. We observed that encapsulation of cantharidin into liposomes was a good strategy in vitro and in vivo. Although liposomal cantharidin might possess lower anticancer activity than free cantharidin, this could be compensated for by using liposomal cantharidin labeled with a specific ligand, octreotide, or by treating cells under HBO. Our results suggest that delivering cantharidin in pegylated liposomes has the potential to effectively treat tumor cells.

Liposomes and other nanoparticles have been used as carrier systems to deliver active drugs in animals and humans. The main advantage of using liposomes to deliver drugs is that they offer the potential to improve the therapeutic index while markedly reducing the side-effects (Nobs et al., 2004). However, liposomal carriers are subject to easy uptake by phagocytic cells of the reticuloendothelial system (RES) and lack targeting specificity, which results in a reduction in the therapeutic efficacy. In this study, we coated liposomal cantharidin with amphipathic polyethyleneglycol (PEG) to overcome the rapid clearance by the RES and increase their ability to extravasate in tumor tissues. In addition, the specific ligand, octreotide, was linked to the distal end of PEG chains to enhance active targeting. Several studies have reported on the ability of liposomal anticancer drugs to mitigate toxicity, such as liposomal anthracyclines with reduced cardiac toxicity and a higher therapeutic index. Gabizon et al. recently reported increased circulation time kinetics, reduced toxicity, and an improved therapeutic index for mitomycin C in a mouse carcinoma (Gabizon et al., 2006). Another study by Arndt et al. demonstrated that liposomal bleomycin showed increased therapeutic activity

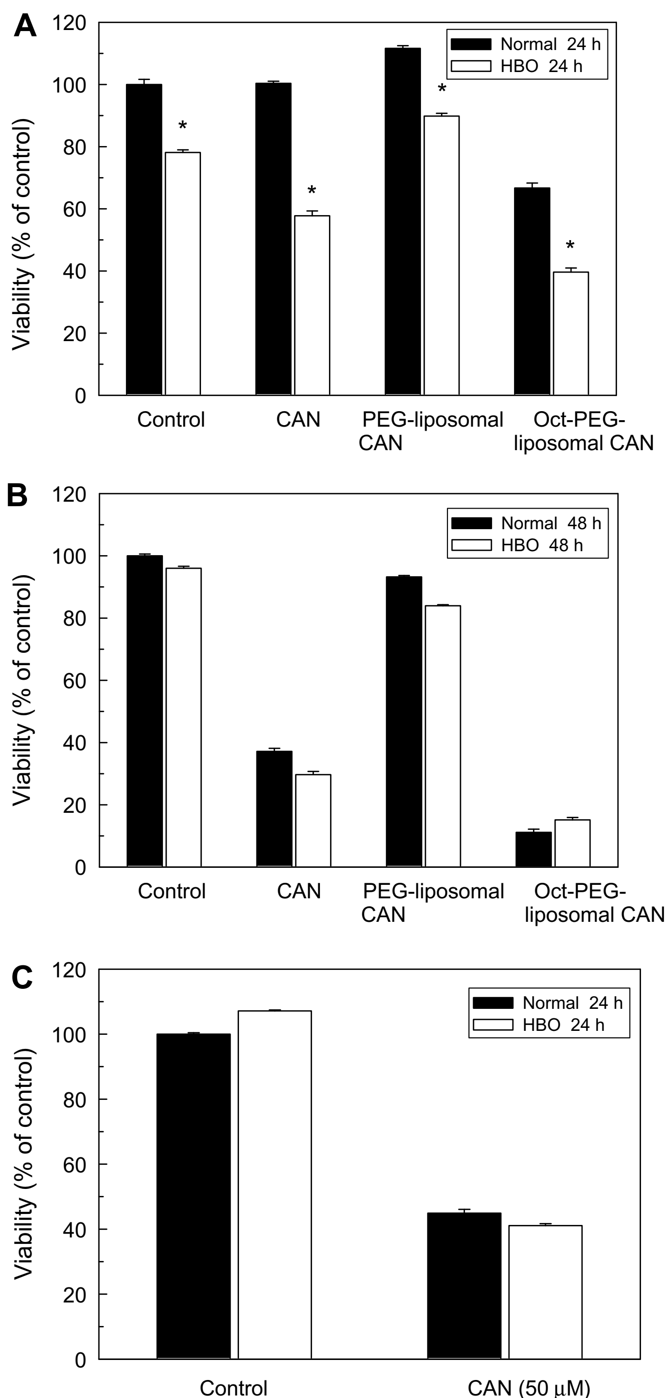

Fig. 4. Hyperbaric oxygen enhanced drug cytotoxicity in MCF-7 cells. (A) Cells were treated with cantharidin (30 μM), PEG-liposomal cantharidin, or octreotide-PEG-liposomal cantharidin under normoxia or hyperbaric oxygen (HBO) for 24 (A) or 48 h (B), and viability was determined by the MTT assay. (C) Cells were treated with cantharidin under normoxia or hyperbaric air for 24 h, and viability was determined by the MTT assay. Data are presented as the mean ± S.E. $^{*}p < 0.05$ compared to individual normoxia, $n = 3$.

and decreased pulmonary toxicity in mice with P388 leukemia and Lewis lung sarcoma (Arndt et al., 2001).

It is well known that solid tumors possess hypoxic regions, which contribute to angiogenesis. In wound healing, HBO therapy can promote the proliferation of fibroblasts, epithelial cells, and blood vessels. Therefore, some studies have suggested that HBO treatment can enhance malignant growth or metastases.

Table 2
Improvement of survival rate by liposomal cantharidin in mice

	Survival rate (%)		
	Day 2 (%)	Day 5 (%)	Day 60 (%)
Control	100	100	100
CAN (5 mg/kg)	0	0	0
CAN (10 mg/kg)	0	0	0
PEG-liposomal CAN (5 mg/kg)	100	83.3	83.3
PEG-liposomal CAN (10 mg/kg)	0	0	0

CAN, cantharidin; PEG-liposomal CAN, PEG-liposomal cantharidin.
The number of each group was above six.

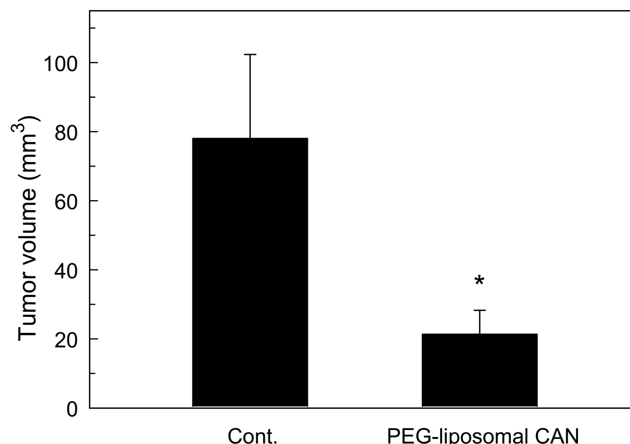

Fig. 5. Effects of PEG-liposomal cantharidin on MCF-7 tumor xenografts in nude mice. MCF-7 cells were subcutaneously injected between the scapulas of athymic nude mice, and the mice received two i.p. injections of 5 mg/kg PEG-liposomal cantharidin. The tumor volume was measured at the end of the experiment. Values were obtained from six samples and are represented as the mean ± S.E. $^{*}p < 0.05$ vs. the control.

Recent studies have produced contrary results indicating that the clinical application of HBO does not promote tumor growth, but results in the induction of cancer cell apoptosis. A possible reason for this finding is that HBO alters tumor hypoxia, a potent stimulator of angiogenesis, and HBO produces a high level of reactive oxygen species (ROS), which can damage tumor cells and induce apoptosis in the tumor region. Our results demonstrated that HBO treatment significantly decreased the viability of MCF-7 cells with cantharidin treatment. The possible mechanism was high ROS production due to HBO treatment (data not shown). Our findings are in agreement with other previous studies suggesting that HBO increases the sensitivity to chemotherapy.

In summary, the encapsulation of cantharidin into liposomes may be an interesting alternative therapeutic approach, particularly in combination with octreotide ligand and HBO treatment. The data presented here support the use of liposomal cantharidin to reduce the toxicity in vitro and in vivo. To our knowledge, this is the first study combining cantharidin with a pegylated liposomal carrier as well as a specific ligand and HBO to show clear advantages over free cantharidin. Further studies in animal tumor xenografts are required to determine the overall spectrum of activity of octreotide-labeled liposomal cantharidin.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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