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Combination antitumor effects of micelle-loaded anticancer drugs in a CT-26 murine colorectal carcinoma model

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ABSTRACT

Experiments were designed to evaluate the *in vitro* cytotoxic interactions of anticancer drugs in combination, evaluate synergistic activity *in vivo* and utilize micelle-forming polymeric drugs as drug carriers in a murine cancer model. Antitumor effects of 5-fluorouracil, cisplatin, CPT-11, oxaliplatin, etoposide, mitomycin-C, doxorubicin and paclitaxel were evaluated by determination of *in vitro* cytotoxicity to CT-26 colorectal tumor cells or *in vivo* following a subcutaneous transplant in BALB/c mice. Single agent and combination *in vivo* studies were also performed using drug-loaded polymeric micelles composed of poly(γ-benzyl L-glutamate) and poly(ethylene oxide) (GEG) or poly(L-lactide)/poly(ethylene glycol) (LE) diblock copolymer. After 3 days exposure, the mean IC_{50} (μg/mL) for 5-fluorouracil, cisplatin, CPT-11, oxaliplatin, etoposide, mitomycin-C, doxorubicin and paclitaxel were 0.95, 2.01, 4.47, 3.34, 3.5, 1.96, 1.8 and 2.1, respectively. When tumor cells were exposed to doxorubicin concurrently with etoposide or paclitaxel, evidence of synergy was observed in CT-26 cells *in vitro*. Doxorubicin and paclitaxel loaded into GEG or LE copolymers at a high concentration (19.5 and 16.7 wt%, respectively) were almost completely released (83.2% and 93.7%, respectively) by day 3. When tumor-bearing mice were treated in combination with doxorubicin–paclitaxel or doxorubicin–etoposide, substantial antitumor activity was evident compared with single therapy. These data suggest that *in vitro* cytotoxicity of anticancer drugs is related to *in vivo* results, and chemotherapy using micelle-loaded anticancer drugs represents a promising potential as a carrier system in modulating drug delivery.

1. Introduction

The efficacy of cancer chemotherapy is considerably limited by toxic side effects of anticancer drugs. This limitation results from the fact that conventional chemotherapy exposes both normal and neoplastic cells to identical doses of cytotoxic agents and relies upon the enhanced sensitivity of rapidly dividing cancer cells to achieve preferential killing (Hardman et al., 1999; Ridwelski et al., 2001). When used on their own, the drugs are not as effective in the treatment of cancer (Neijt, 1996). However, when used in combination, the drugs have synergistic cytotoxicity and high success rates in the treatment of both murine and human neoplasms (Ferraresi et al., 2005; Saltz et al., 2000). There is, however, an upper limit to the concentration of the drugs that may be used in the treatment. Above this threshold, the drugs impose such severe toxic side effects that their use is limited as an effective chemotherapeutic agent. This becomes an important issue when using combinations in the treatment of cancer (Das et al., 2007; Nagai et al., 2008). Therefore, therapeutic efficacy could be enhanced and side-toxicity greatly diminished if a sufficiently high concentration of the tumoricidal agent could be selectively focused on malignant cells. This approach, known as drug targeting, is a novel means of killing dangerous cells, while leaving normal cells unharmed (Sarkar and Yang, 2008; Sofou, 2008; Torchilin, 2008). Aimed at delivering a target drug to the desired site of action in the body in the most efficient way, studies have sought to develop systems for site-specific delivery (Cryan, 2005; Hruby et al., 2005; Petrak, 2005; Ravi Kumar, 2000). One possible means of reaching this goal may be delivery via particulate drug delivery systems (Bussemer et al., 2003; Croy and Kwon, 2006; Eniola and Hammer, 2003).

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By virtue of their small size, particulate drug delivery systems based on nano-sized carriers may be useful as sustained-release injections or for the delivery of a drug to a specific or target site. In particular, the most promising application of polymeric nanoparticulate carriers is their use as carriers for anti-cancer drugs (Yokoyama et al., 1990). When compared to low-molecular weight (MW) anticancer drugs, polymeric nanoparticulate carriers or macromolecular drugs can accumulate more in tumor tissues than in normal tissues due to their enhanced permeability and retention (EPR) effect (Noguchi et al., 1998). In addition, polymeric drug carriers can prolong antitumor activity because of a controlled release of the drug (Bussemer et al., 2003; Gref et al., 1994; Hruby et al., 2005). Especially, block copolymers composed of hydrophilic and hydrophobic domains can form core–shell micellar structures that consist of a polymeric micelle with a hydrophobic inner core surrounded by a hydrated outer shell in aqueous solution. The hydrophobic inner core acts as a drug incorporation site, especially for hydrophobic drugs, and the hydrated outer shell helps avoid uptake by the reticuloendothelial system (RES). Polymeric micelles have the advantages of small particle size, good structural stability, favorable biodistribution, easy sterilization and ready solubilization of hydrophobic drugs (Croy and Kwon, 2006; Kim et al., 2008; Kwon et al., 1995; Yokoyama et al., 1990). Studies with polymeric micelles have focused on their application as novel drug carrier systems because of their superiority as an injectable form of drug delivery (Croy and Kwon, 2006; Jeong et al., 1999; Kim et al., 2001). We previously reported that polymeric micelles based on block copolymers are acceptable vehicles for targeting specific tumor cells *in vitro* and suppress growth of solid tumors in an *in vivo* animal tumor model (Jeong et al., 2005, 2009).

Doxorubicin (DOX) is a widely used anticancer anthracyclin drug that acts as a DNA intercalating agent. Its cardiac toxicity is a serious limitation for its clinical use besides hematological and gastrointestinal disorders (Gianni et al., 2001). Paclitaxel (PCL) is an anticancer cytotoxic that stabilizes cellular microtubules. PCL has been approved in the United States for the adjuvant treatment of early stage, node-positive breast carcinoma. Adverse effects of PCL include myelosuppression, neuropathy, myalgias, fatigue, alopecia, diarrhea, mucosal toxicity and skin and nail changes (Rowinsky and Donehower, 1995). One significant drawback of both DOX and PCL concerns their poor aqueous solubility. To improve solubility, surfactant or solvent (such as an ethanol/cremophor mixture for PCL) is normally used with these drugs. However, most surfactants and solvents are not fully biocompatible, and so can be toxic to the human body. From these points of view, polymeric micelles represent a promising means for solubilizing anticancer drugs and enhancing drug targeting.

The goal of the present study was to assess the combination effect of various anticancer agents, and DOX- or PCL-incorporated polymeric micelles, on the proliferation of tumor cells *in vitro* and *in vivo*. *In vitro* cytotoxic interactions of anticancer drug combinations was ascertained and compared with the combination effects *in vivo*, with the goal of revealing synergism. To circumvent the possibility that the observed interactions might be applied to drug delivery/targeting, parallel studies were conducted to utilize the micelle-forming polymeric drugs as drug carriers with controlled release in a murine cancer model.

2. Materials and methods

2.1. Materials

PCL was a gift from Samyang Pharmaceuticals (Daejeon, Korea). 5-Fluorouracil (5-FU) and mitomycin-C were gifts of Choongwae Pharmaceutical (Seoul, Korea). Cisplatin, oxaliplatin, eloxatin, and etoposide (ETP) were gifts of Boryung Pharmaceutical (Seoul, Korea). CPT-11 (camptothecin) was a gift of Cheiljedang Pharmaceutical (Seoul, Korea). DOX was a gift of Ildong Pharmaceutical (Seoul, Korea). Stock solutions were prepared in 20 mM ethanol and aliquots were stored frozen at −20 °C. Immediately before use, stock solutions were diluted at least 1:1000 (v/v) in growth medium and rediluted thereafter as required. The final concentration of ethanol was <0.1% and was not toxic to the cell line. Bis[poly(ethylene oxide) bis(amine)] (BPEOBA: MW = 20,000), monomethoxy poly(ethylene glycol) (MW = 2000 g/mol) and γ-benzyl L-glutamate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Triphosgene was purchased from Aldrich (Milwaukee, WI, USA). All chemicals were of reagent or spectrometric grade. N-hexane and methylene dichloride were stored with 4 Å molecular sieves and used without further purification.

2.2. Cell line and culture conditions

The CT-26 murine colorectal carcinoma cell line was purchased from American Type Culture Collection (Rockville, MD, USA) and maintained at 37 °C in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with gentamicin, 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were cultured in humidified incubators in an atmosphere of 5% CO_2 and were passaged twice a week by removing the adherent cells with trypsin/EDTA in buffered saline. Cell viability was assessed by means of a standard Trypan Blue exclusion method.

2.3. Single agent cytotoxicity studies

The relationship between drug concentration and tumor cell killing was determined using an assay measuring the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) into insoluble formazan (Alley et al., 1988). Cells (1 and 2×10^4) were exposed to various concentrations of drugs (0.02–400 μg/mL) for 2–3 days. After the incubation period, tumor cells were exposed to MTT for 4 h. Formazan crystals that formed were solubilized with dimethyl sulfoxide or acid/alcohol and the absorbance was measured 570 nm (test samples) or 630 nm (reference samples) using an automated computer-linked microplate reader (Molecular Devices, Sunnyvale, CA, USA). Each drug concentration was assessed in triplicate. The amount of formazan present was proportional to the number of viable cells, as only living cells are capable of reducing MTT to blue formazan. Results were expressed as a percentage of the absorbance present in drug-treated cells compared to that in the control cells. The relationship (IC_{50}) between drug concentration and tumor cell killing was determined by regression analysis.

2.4. Flow cytometry analysis of cytotoxicity

Cultured tumor cells were stained with PKH26 dye (Sigma–Aldrich) following the manufacturer's instructions. Cells (3×10^6) were dispensed in individual wells of a 24-well tissue culture plate. In the presence of anticancer drugs, the plates were incubated at 37 °C in a 5% CO_2 incubator for 4 days. Flow cytometry data files were analyzed with the Proliferation Wizard module of ModFit LT Verity Software (Modfit, Topsham, MA, USA).

2.5. Combination in vitro cytotoxicity studies and statistical analysis for synergy

When tumor cells were exposed to cytotoxic agents concurrently, tumor cytotoxicity was determined using the MTT assay for the single agent studies. A model-free method was applied to select sample points along the expected additive e-isobol and to test

whether any of several dose combinations exhibited better than additive effects. An estimated single agent dose–response curve for each drug and cell line was determined. Seven different dose combinations using various proportions of the IC_{50} of each agent were then selected. Assuming additivity, the same cytotoxicity was expected for each combination (i.e., 100% of IC_{50} of drug A combined with 0% of IC_{50} of drug B; 75% of IC_{50} of drug A combined with 25% of IC_{50} of drug B, etc.) of each single agent. The outcome of each combination was compared with each single agent in two sets of one-sided *t*-tests. After set-wise adjustment for multiple comparisons, drug combinations in which the maximum of the two one-tailed adjusted *P*-values was <0.05 were deemed synergistic.

2.6. Synthesis of block copolymers

Poly(L-lactide) (PLLA)/poly(ethylene glycol) (PEG) (abbreviated as LE) diblock copolymer was synthesized as reported previously (Yu et al., 2002). Briefly, LE diblock copolymer was synthesized by ring-opening polymerization of L-lactide to one terminal hydroxyl group of monomethoxy poly(ethylene glycol) (mPEG). Pre-weighed amounts of L-lactide and mPEG were mixed in a round-bottomed flask and melted at 100 °C in an oil bath. Stannous 2-ethylhexanoate (0.5%, w/w) was added to the flask, which was then evacuated with a vacuum pump. The flask was then placed in an oil bath at 180 °C to start the polymerization. After 6 h, the resulting product was dissolved in methylene chloride and precipitated into diethyl ether several times. The precipitants were harvested by filtration and the product was dried in a vacuum oven at 40 °C for 3 days. The MW and composition was estimated by ^{1}H nuclear magnetic resonance (NMR) measurements using $CDCl_3$ as described previously (Yu et al., 2002). From the characteristic peaks of PLLA (5.1 and 1.5 ppm of methine and methylene proton, respectively) and PEG (3.7 ppm of methylene proton), the copolymer composition and number-averaged molecular weight were estimated. The calculated results of the MW and composition of LE diblock copolymer were about 3470 g/mol, translating into a PLA block length of approximately 1470 g/mol, since the mPEG block length was 2000 g/mol.

Poly(γ-benzyl L-glutamate)/poly(ethylene oxide) hexablock copolymer (abbreviated as GEG): γ-benzyl L-glutamate N-carboxyanhydride (BLG-NCA) and hexablock copolymer were synthesized as described previously (Jeong et al., 1999). Briefly, the hexablock copolymer was obtained by polymerization of BLG-NCA using BPEOBA as an initiator in methylene chloride, at a total concentration of BLG-NCA and BPEOBA of 3% (w/v) at room temperature for 72 h. The resulting mixture was precipitated into a large excess amount of diethyl ether and precipitates were vacuum-dried for at least 3 days. MW and composition of GEG block copolymer was estimated by ^{1}H NMR spectra using $CDCl_3$. As the number-average MW (20,000) of PEO is known, the number-average MWs of the PBLG block and the copolymer composition calculated from the peak intensities in the spectrum assigned to both polymers, respectively, could be estimated (Jeong et al., 1999). MW estimates were based on the peak intensities of the methylene proton signal (5.0 ppm) of the PBLG block and the methylene proton signal (3.7 ppm) of the PEO block. The copolymer MW was 33,100 g/mol and PBLG block length was estimated as 13,100 g/mol, since PEO MW was 20,000 g/mol.

2.7. Analysis of polymers

^{1}H NMR spectra was measured using a JEOL FX 90 Q NMR spectrometer (JEOL, Tokyo, Japan). Particle size was measured by photon correlation spectroscopy using a Zetasizer 3000 apparatus (Malvern Instruments, Worcestershire, UK) with helium-neon laser irradiation at a wavelength of 633 nm at 25 °C (scattering angle of 90°). A nanoparticle solution prepared by diafiltration was used for particle size measurements (concentration 1 mg/mL) in the absence of filtering.

2.8. Preparation of drug-loaded polymeric micelles

To form DOX-loaded polymeric micelles, 50 mg of GEG block copolymer was dissolved in 5 mL of DMF, prior to the addition of 10–20 mg of DOX in 1 mL DMF with 1.3 equivalent of triethylamine. The solution was stirred at room temperature to facilitate solubilization. To form drug-free polymeric micelles, the solution was dialyzed using dialysis tubing with a MW cut-off of 12,000 g/mol against 1.0 L of acetate buffer (pH 5.5, 0.1 M) for 2 h and then 1 L × 4 of distilled water for 9 h with magnetic stirring. The solution was then analyzed or freeze-dried.

For PCL loading, LE diblock copolymer was used to make polymeric micelles as previously described (Zhang et al., 1997). LE diblock copolymer and PCL were completely dissolved in 2 mL of acetonitrile and then organic solvent was evaporated using a rotary evaporator under reduced pressure at 60 °C. Hot deionized water (60 °C) was added to the obtained transparent solid film and stirred magnetically at 60 °C until the solid film was completely reconstituted. The resulting solution was filter-sterilized using a 0.2 μm syringe filter. This solution was cooled to room temperature (20–25 °C) and then analyzed or used for the experiments. For evaluation of drug loading content, DOX-loaded GEG polymeric micelles were measured using a UV-1201 spectrophotometer (Shimadzu, Tokyo, Japan) at 479 nm. Empty GEG polymeric micelles were used as blanks.

2.9. In vitro release studies

To assess *in vitro* release, 10 mg of DOX-loaded polymeric micelles and 1 mL of phosphate buffered saline (PBS; 0.1 M, pH 7.4) were added to dialysis tubing, which was then added to a vial containing 10 mL PBS. At specific times, the whole medium was withdrawn and replaced with fresh PBS. The concentration of the released drug was determined by UV spectrophotometry (Shimadzu UV-1201; Shimadzu) at 479 nm for DOX, 284 nm for ETP and 273 nm for PCL.

2.10. In vivo evaluation in CT-26 mouse tumor models

BALB/c mice were implanted subcutaneously with cultured CT-26 cells (5×10^4 cells/mouse). When tumor dimensions reached approximately 3 mm × 3 mm, the animals were pair-matched into treatment and control groups (day 14). Each group consisted of 8–10 tumor-bearing mice that were ear-tagged and followed-up individually throughout the study. The intravenous administration of drugs or vehicle began on day 14. Each drug was administered at doses of 5 mg/kg for combination therapy or 10 mg/kg for single use twice weekly for four times in total. The control group received the vehicle in PBS. Mortality was monitored daily and tumor growth was measured at 2 day intervals by caliper measurement. Tumor volume was calculated using the following formula: tumor volume (mm^3) = (length × width^2)/2.

When more than half of mice were dead, the measurement was discontinued. All experiments using mice were followed the guideline approved by the Committee for the Care and Use of Laboratory Animals at Chonnam National University.

2.11. Statistical analyses

Wilcoxon's statistics and Student's *t*-test (Stat-graphics, SigmaPlot) were used to assess the differences between experimental groups. $P < 0.05$ was taken as being statistically significant. The statistical significances of differences in survival between

groups was analyzed by Kaplan–Meier method (Kaplan and Meier, 1958) using log rank statistics.

3. Results

3.1. Single agent in vitro studies

The result of single agent studies with CT-26 is shown in Table 1. The concentration range of each anticancer drug used to determine the IC_{50} was 0.13–1000 μM or 0.01–200 μg/mL. After 3 days exposure, a dose-dependent cytotoxicity was apparent for each anticancer drug. The mean IC_{50} for 5-fluorouracil, cisplatin, CPT-11, oxaliplatin, ETP, mitomycin-C, DOX, and PCL were 0.95, 2.01, 4.47, 3.34, 3.5, 1.96, 1.8 and 2.1 μg/mL, respectively.

Table 1
IC_{50} values obtained for CT-26 cells using anti-cancer drugs.

Anticancer drug	IC_{50} (μg/mL)
5-fluorouracil	0.95
Cisplatin	2.01
CPT-11	4.47
Oxaliplatin	3.34
Etoposide	3.50
Mitomycin-C	1.96
Doxorubicin	1.80
Paclitaxel	2.10

3.2. Combination in vitro studies

Since only hydrophobic drugs can be used to make polymeric micelles with GEG or LE for further *in vivo* experiments, tumor cells were exposed to DOX concurrent with other anticancer drugs. When the cells were exposed to each anticancer drug alone or in the presence of 50 μM or 20 μM DOX, a synergistic interaction was observed between DOX/ETP or DOX/PCL (data not shown). To confirm these preliminary results, cells were exposed again to anticancer drug combinations at various ratios of their individual IC_{50}s.

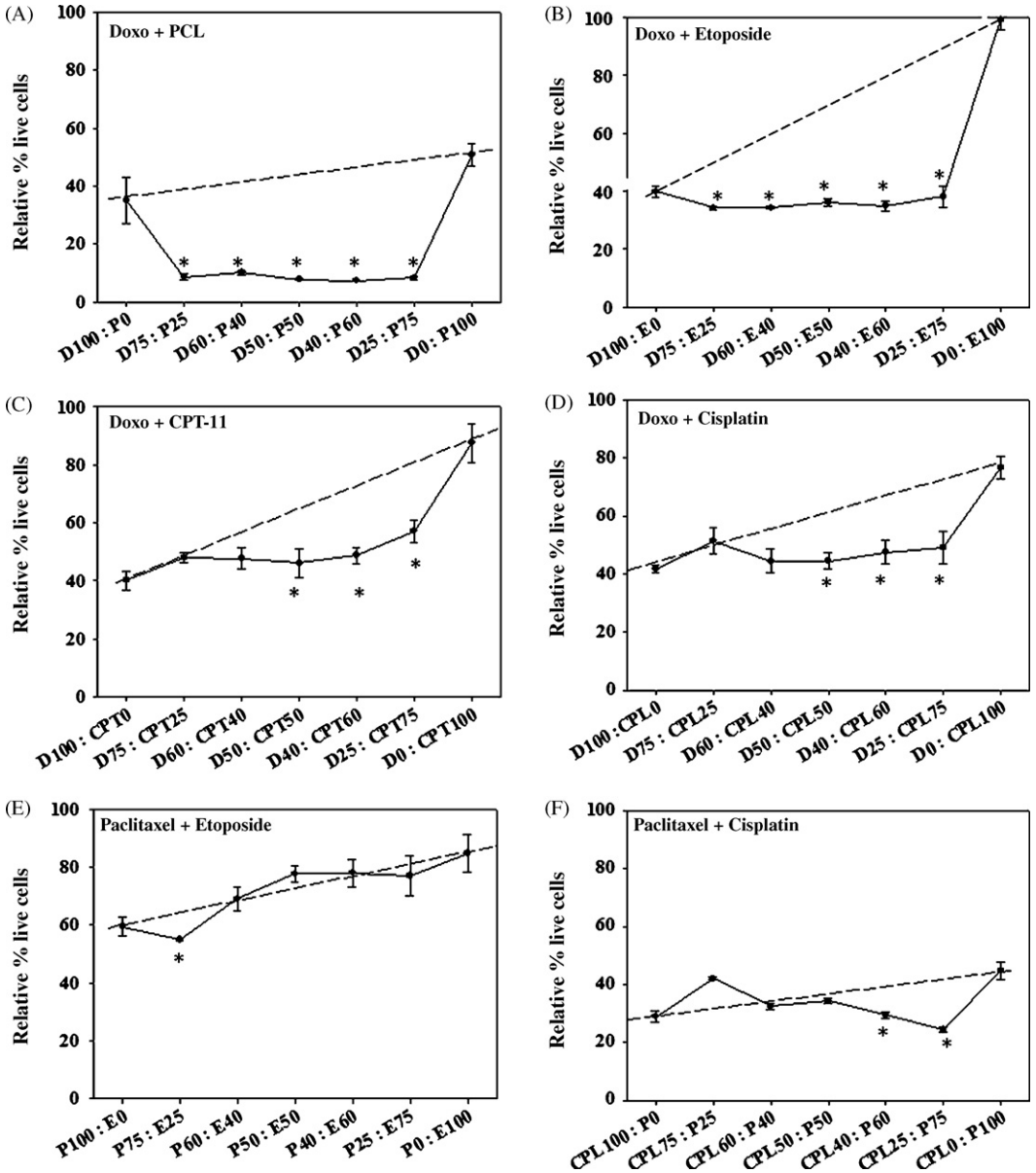

Fig. 1. Determination of additive drug effects. CT-26 cells were exposed to anticancer drug combinations at various ratios to their individual IC_{50}. Cytotoxicity greater than an additive effect of the two drugs is denoted by *, which is significantly ($P < 0.05$) below the isoeffect line (dotted line).

Table 2
Characterization of LE polymeric micelles containing paclitaxel.

Polymer weight (mg)	Initial amount of drug (mg)	Drug contents (% w/w)	Particle size (nm)		
			Intensity (%)	Volume (%)	Number (%)
100	10	9.1	40.7 ± 8.7	40.7 ± 16.9	40.4 ± 16.5
100	20	16.7	51.9 ± 1.9	52.2 ± 1.6	52.1 ± 1.2

MW of LE block copolymer was about 3470 (MW of PEO was 2000) measured by ^{1}H NMR. Drug contents = [(drug remained in the polymeric micelle)/total weight of polymeric micelle] × 100.

Table 3
Characterization of GEG polymeric micelles containing doxorubicin and etoposide.

Polymer weight (mg)	Initial amount of drug (mg)	Drug contents (% w/w)	Loading efficiency (% w/w)	Particle size (nm)		
				Intensity (%)	Volume (%)	Number (%)
Doxorubicin						
50	20	14.9	43.8	77.8 ± 7.1	78.7 ± 2.5	78.6 ± 0.7
50	30	19.5	40.4	81.3 ± 10.4	81.2 ± 13.6	80.9 ± 13.3
Etoposide						
50	10	7.1	38.2	71.5 ± 10.1	69.2 ± 9.1	72.5 ± 5.9
50	20	11.7	33.1	79.1 ± 12.1	80.2 ± 10.6	83.9 ± 9.7

MW of GEG block copolymer was 33,100 (MW of PEO was 20,000) measured by ^{1}H NMR. Critical micelle concentration of GEG block copolymer was 2.8×10^{-7} mol. Drug contents = [drug remained in the polymeric micelle/total weight of polymeric micelle] × 100. Loading efficiency = [drug remained in the polymeric micelle/initial amount of drug] × 100.

Synergy, evident as cytotoxicity significantly ($P < 0.05$) below the isoeffect line, was observed for DOX + PCL, DOX + ETP, DOX + CPT, and DOX + cisplatin, while additive interaction was noted between PCL/cisplatin and PCL/ETP. Antagonism was not evident between any of these combinations (Fig. 1).

3.3. Characterization of polymeric micelles

PCL-incorporated polymeric micelles were prepared using LE block copolymer as previously described (Zhang et al., 1997). Acetonitrile was concluded to be the optimal solvent because acetone, chloroform, dichloromethane and tetrahydrofuran did not produce a clear solution, and most of the polymers and drugs aggregated (data not shown). In contrast, acetonitrile resulted in a clear micelle solution free of aggregates. Table 2 shows characteristics of PCL-incorporated polymeric micelles of LE block copolymer. The particle size of PCL-loaded polymeric micelles of LE block copolymer was 40–50 nm, which is acceptable for intravenous injection and drug targeting. The particle size was slightly increased according to the drug loading contents. The drug loading contents in LE core–shell type nanoparticles were 9.1% (w/w) and 16.7% (w/w) according to the initial drug amount. When drug contents were measured, almost of the initial amount of drug was incorporated into the polymeric micelle; i.e., loading efficiency was close to 100% since loss of drug did not occur in the polymeric micelle preparation process. Furthermore, these results indicated that drug was not degraded in the preparation process of polymeric micelles.

DOX or ETP-incorporated polymeric micelles were prepared using GEG block copolymer by a previously described dialysis procedure (Jeong et al., 2009). Table 3 shows characteristics of DOX- or ETP-incorporated GEG polymeric micelles. GEG block copolymers produced small particles with a narrow size distribution. The size ranges were similar to other block copolymeric micelles such as PBLA (or poly(aspartate))-PEO diblock copolymer (Kwon et al., 1995; Yokoyama et al., 1990). The relationships between particle size and drug contents using DOX and ETP as model drugs are shown in Table 3. Particle size distribution for GEG containing DOX or ETP was 70–90 nm according to the drug contents. Higher drug contents induced formation of larger polymeric micelles. Loading efficiency was 40–44% (w/w) of DOX and 33–38% of ETP. Compared to PCL-incorporated LE polymeric micelles, DOX- or ETP-incorporated polymeric micelles were prepared by dialysis method, and a significant amount of drug was liberated from the micelles during dialysis. These drug contents as a physical state were relatively higher than those of other polymeric micelle systems such as PBLA-PEO (Kwon et al., 1995).

3.4. Drug loading and release study in vitro

The release of DOX from GEG polymeric micelles to the outer aqueous phase is summarized in Fig. 2a. DOX release was slower at higher drug contents than at lower drug contents. An initial burst release was evident for the first day, after which DOX was continuously released from the micelles for about 4 days. Similar to the observations with DOX, ETP was released more slowly from GEG polymeric micelles at higher drug contents than lower drug contents (Fig. 2c). ETP release demonstrated an initial burst of 12 h and then adopted pseudo-zero-order release kinetics until day 3. PCL-incorporated LE polymeric micelles were readily prepared by melting in hot water or PBS (pH 7.4, 0.1 M), and an *in vitro* drug release study was performed. There was a significant initial burst release of drug from polymeric micelles of LE diblock copolymer during the first 12 h prior to pseudo-zero-order release kinetics for up to 3 days (Fig. 2b). Release rate decreased as drug content increased.

3.5. In vitro antitumor activity of micelle-loaded DOX

The *in vitro* cytotoxicity of DOX was evaluated as either the free drug or by means of micelle-loaded form using a PKH cell proliferation assay to determine the viability of tumor cells. The growth-inhibitory activities of free DOX and DOX-incorporated polymeric micelles of GEG block copolymer were compared for the CT-26 murine cell line. Both samples showed dose-dependent cytotoxicity and polymeric GEG/DOX exhibited weaker cytotoxicity with little difference (Fig. 3). These results might be due to the sustained release properties of GEG polymeric micelle as shown in Fig. 2, indicating that cancer cells are exposed to the whole amount of drug in a closed environment such as *in vitro* cell culture system, while they are exposed to a lower amount of drug during treatment of DOX-incorporated polymeric micelles. On the other hand,

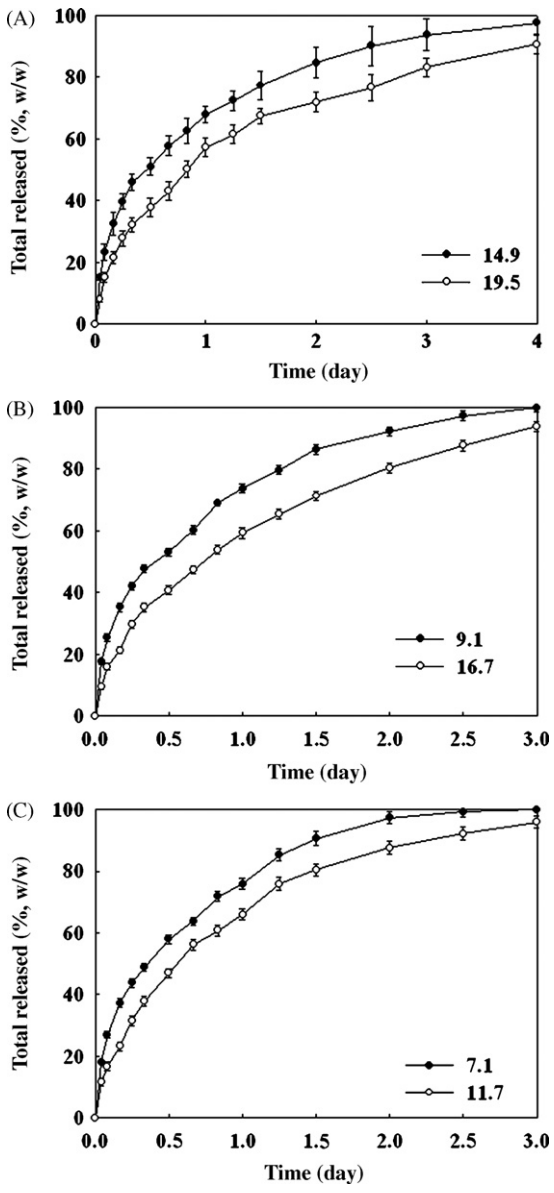

Fig. 2. Release of DOX and ETP from GEG (A and C) and paclitaxel from LE polymeric micelles (B). The drug loading content of DOX and ETP in GEG was 14.9% and 19.5% (w/w), and 7.1% and 11.7% (w/w), respectively, and that of paclitaxel in LE was 9.1% and 16.7% (w/w).

drug may be rapidly cleared from the blood circulation at *in vivo* environment.

3.6. In vivo antitumor activity

The *in vivo* antitumor activity of DOX-loaded GEG micelles (DOX-GEG) and PCL-loaded LE micelles (PCL-LE) against CT-26 murine tumor cells is summarized in Figs. 4 and 5 . Although polymeric DOX-GEG exhibited weaker cytotoxicity against CT-26 cells *in vitro*, all DOX + PCL-loaded nanoparticle-treated mice survived for 42 days after tumor implantation, and showed the highest survival ratio compared with DOX-GEG only ($P < 0.05$), PCL-LE only ($P < 0.05$) and the control group ($P < 0.05$) (Fig. 4b). When tumor-bearing mice were treated with DOX-GEG with ETP-GEG from day 5 following tumor implantation, all DOX + ETP-loaded nanoparticle-treated mice survived for 56 days after tumor implantation, and showed the highest survival ratio compared with DOX-GEG only ($P < 0.01$), ETP-GEG only ($P < 0.01$) and the control group ($P < 0.01$) (Fig. 5). These combinations had substantial antitumor activity compared with single therapy in their survival suggesting that *in vitro* cytotoxicity of anticancer drugs is related to *in vivo* results, and that chemotherapy using micelle-loaded anticancer drugs is a promising carrier system to modulate drug delivery.

4. Discussion

Various anticancer agent are applied to clinical use and, especially, DOX, PCL, and ETP are frequently used to various kinds of cancers. DOX is known to interact with DNA by intercalation and this inhibits the progression of the enzyme topoisomerase II, which unwinds DNA for transcription (Momparler et al., 1976). However, cardiac toxicity of DOX is one of serious limitation for its clinical use besides hematological and gastrointestinal disorders (Gianni et al., 2001). ETP is the enzyme topoisomerase II inhibitor and is often used in combination with other kind of anticancer drug. Side-effects such as low blood pressure, decreased white blood cell count, low red blood cell counts, and bone marrow suppression are known. Otherwise, PCL, a mitotic inhibitor, is known to stabilize microtubule and then this destroys the cell's ability to use its cytoskeleton in a flexible way (Kumar, 1981). Adverse effects of PCL include myelosuppression, neuropathy, myalgias, fatigue, alopecia, diarrhea, mucosal toxicity and skin changes (Rowinsky and Donehower, 1995). These anticancer agents have different mechanism of action and unwanted side-effects. Furthermore, strategies for clinical use of these drugs are varied according to the types of cancers and their stages. Combination of these drugs could contribute synergistic increase of therapeutic effects with decrease of unwanted specific side-effects by using less toxic concentrations of each drug (Gianni et al., 2001; Hardman et al., 1999; Nagai et al., 2008; Neijt, 1996; Ridwelski et al., 2001; Saltz et al., 2000).

The present study was undertaken to analyze the interactions among anticancer drugs with the aim of achieving synergistic activity and enhanced antitumor effects *in vivo* by coupling drugs to polymeric micelles. The CT-26 colorectal cancer cell line was used in BALB/c mice as an *in vivo* model because they are syngeneic. In a preliminary study, we compared the cytotoxic activity of each drug combination *in vitro* (Fig. 1). These experiments were intended to identify the most active agents in combination with DOX or PCL, which are hydrophobic, because polymeric micelles could only be applied with hydrophobic drugs. Multiple drug effect/combination index (CI) isobologram analysis was applied to combinations of DOX or PCL with other anticancer drugs. Drug concentrations were limited to the ranges achievable in humans *in vivo*, and the drugs were applied simultaneously at fixed molar ratios for each drug combination. Interactions were assessed at multiple effect levels (IC_{10}–IC_{90}). Drug interactions were strongly dose-related in CT-26 cells. In an *in vitro* study, combinations of DOX + PCL or DOX + ETP showed that surviving tumor cells were significantly lower than the isoeffect line, indicative of a synergistic antitumor effect at these combinations. Compared to this, other combinations such as DOX + CPT11, DOX + cisplatin, PCL + ETP and PCL + cisplatin did not produce a significant synergistic effect on the growth inhibition of tumor cells. Combinations of DOX + PCL or DOX + ETP, and DOX + PCL or DOX + ETP demonstrated synergy, and other combinations were relatively additive interactions as shown in Fig. 1. As shown in Fig. 1a and b, the present data indicates the superior *in vitro* cytotoxicity of DOX/PCL and DOX/ETP and favorable drug interactions *in vivo* (Figs. 4 and 5), suggesting that *in vitro* cytotoxicity of anticancer drugs is related to *in vivo* outcome.

Taxol is a complex diterpenoid natural product that is being investigated for therapy of colon, ovarian, lung and breast cancer, as well as for melanoma and lymphoma. One problem associated with the administration of taxol is its low aqueous solubility (Singla et al., 2002). PCL isolated from the bark of the Paci Yew

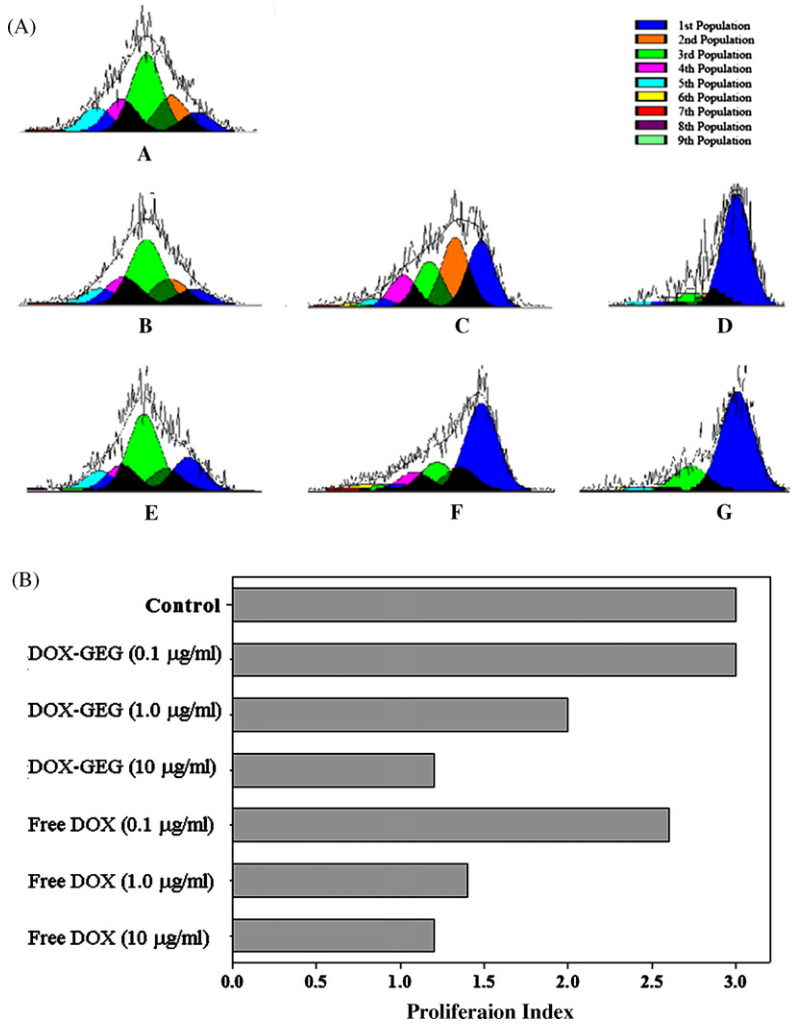

Fig. 3. Effect of free DOX and GEG-DOX on cell division of CT-26 cells. Quantitation of cell proliferation using PKH26 method. After 4 days of culture, CT-26 cells were stained with PHK-26 and the fluorescence of proliferated cells was compared with that of undivided cells. PKH-26 dye fluorescence was read at a wavelength of 575 nm (5000 events were collected). (a) Representative flow cytometric analysis of CT-26 cell proliferation: PKH-26-stained cells were cultured in the presence of 0 μg/mL (A), 0.1 μg/mL (B), 1 μg/mL (C), 10 μg/mL (D) GEG-DOX; 0.1 μg/mL (E), 1 μg/mL (F), 10 μg/mL (G) free-DOX. (b) Cytometric analysis of tumor cell cytotoxicity based on proliferation index. The proliferation cycles were analyzed using cell cycle analysis software.

tree (*Taxus brevifoli*) displays promising anticancer activity against most solid tumors, and was approved as an anticancer agent by the United States Food and Drug Administration in 1992. However, side effects of PCL include myelosuppression, neuropathy, myalgias, fatigue, alopecia, diarrhea, mucosal toxicity and skin and nail changes (Rowinsky and Donehower, 1995). Currently, taxol is intended for clinical use in a 50:50 mixture of cremophore–EL (polyethoxylated caster oil) and ethanol, due to the poor solubility of taxol in water. This preparation is diluted with normal saline or 5% dextrose solution prior to administration. However, in this formulation, the mixture of the surfactant and ethanol is physically incompatible as an intravenous infusion system; serious hypersensitivity reactions have occurred (Coudore et al., 1999; Panchagnula, 1998). DOX is also a widely used anticancer anthracyclin drug, which acts by intercalating with DNA. However, its cardiac toxicity is a serious limitation for clinical use besides hematological and gastrointestinal disorders (Gianni et al., 2001).

The utility of cancer chemotherapy is limited by undesirable toxic side effects to normal cells and tissues, and by the development of multidrug resistance. These limitations are the result of a lack of selectivity to malignant cells and ready excretion from the blood circulation. To overcome these problems, various kinds of anticancer agent carriers have been developed. These include nanoparticles, liposomes and polymer–drug conjugates (Duncan et al., 1987; Papahadjopoulos et al., 1991; Ravi Kumar, 2000). However, conventional carriers such as nanoparticles and liposomes have some disadvantages as injectable drug carriers because of their large particle size, rapid clearance during blood circulation by clearable organs, uptake by the RES and structural instability in blood. Also, polymeric drug carriers have been designed by conjugation of anticancer drugs to the water-soluble homopolymer, alternating polymer and natural polysaccharide (Duncan et al., 1987). Presently, coupling of hydrophobic drugs to polymers led to reduced water solubility, consistent with the hydrophobic character of most anticancer drugs. From this point of view, polymeric micelles have been extensively investigated as a carrier of the hydrophobic drug DOX; DOX-conjugated poly(ethylene glycol)(PEG)-poly(aspartic acid) block copolymer micelles display enhanced tumor accumulation, long blood circulation times and can effectively treat solid tumors (Kwon et al., 1995; Yokoyama et al., 1990).

Polymeric micelles or core–shell type nanoparticles based on block copolymers have been extensively investigated to attain effective drug targeting to the desired site of action (Croy and Kwon, 2006; Gref et al., 1994; Kwon, 2003; Kwon et al., 1994). Due to their amphiphilic properties, block copolymers self-assemble into

polymeric micelles with core–shell structure, and slowly dissociate to free polymeric chain in contrast with low MW surfactants (Malmsten and Lindman, 1992). Polymeric micelles have interesting structural characteristics such as a hydrophobic inner core and hydrophilic outer shell. A hydrophobic block forms the inner core of the structure, which acts as a drug incorporation site especially for the hydrophobic drugs, via hydrophobic interactions (Jeong et al., 1999, 2009; Kwon, 2003), and hydrophilic blocks form a hydrated outershell, which plays a role in avoiding the uptake by the RES, which is major obstacle to the targeting of drugs to specific sites in the body. The advantages of this system include reduced toxic side effects of anticancer drug by micelle formation with block copolymer and selective targeting, solubilization of hydrophobic drugs, stable storage for a long period, protracted blood circulation, favorable biodistribution and lower RES interactions (Gref et al., 1994; Kwon et al., 1994; Yokoyama et al., 1991). In general, polymeric micelles have reduced particle size similar to viruses, which are natural biomolecule vehicles. Since the major factors determining the fate of nanoparticulate carriers in blood circulation are particle size and surface chemistry (Adams et al., 2003; Kwon et al., 1994), polymeric micelles may be appropriate vehicles for site-specific drug targeting of anticancer agents. Polymeric micelles as hydrophobic drug carriers have been studied for use with the anticancer agent DOX (Kwon et al., 1994, 1995; Yokoyama et al., 1990). These studies have demonstrated that polymeric micelles are associated with enhanced tumor accumulation, long blood circulation times and effective treatment of solid tumor by micelle-forming block copolymer-DOX conjugate (Yokoyama et al., 1990, 1991). Similarly, we presently observed that polymeric micelles composed of PBLG and PEO physically entrapped the hydrophobic drug DOX with high stability and exhibited controlled released with pseudo-zero-order kinetics. Even though DOX-GEG showed most effectiveness for suppression of tumor growth (Fig. 4a), survivability of mice was highest at combination of DOX-GEG and PCL-LE (Fig. 4b).

Resultantly, combination of DOX-GEG + PCL-LE or DOX-GEG + ETP-GEG has synergistic effect on the enhanced antitumor activity *in vitro* and enhanced survivability of mice *in vivo*.

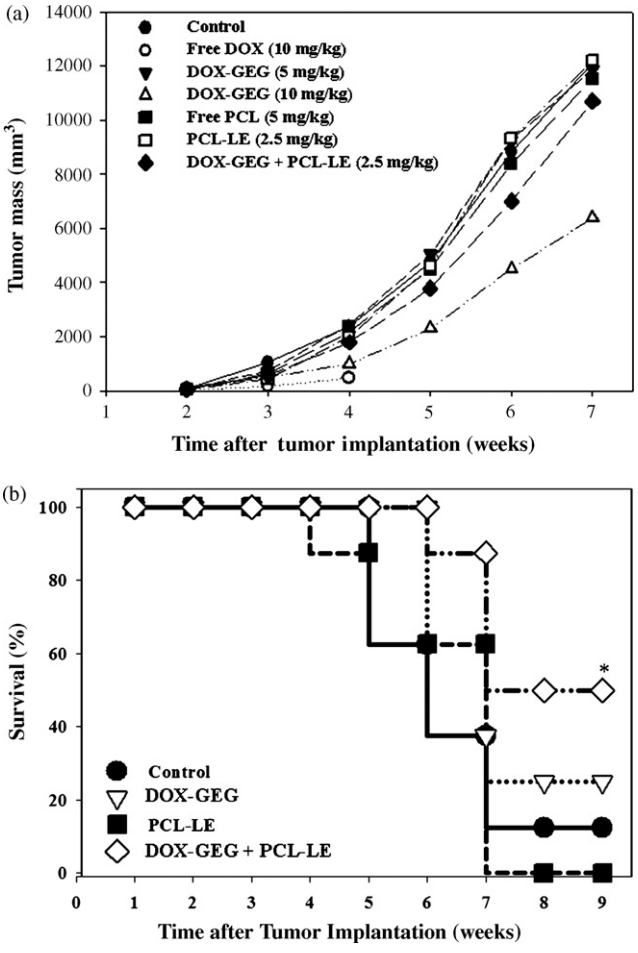

Fig. 4. Growth of tumors and survival of mice. (A) Growth of tumor in mice treated with anticancer drugs. CT-26 murine tumor cells (10^4/mouse) were implanted subcutaneously in BALB/c mice and treated as described. (B) Survival of tumor-bearing mice treated with anticancer drugs. CT-26 murine tumor cells (10^4/mouse) were implanted subcutaneously in BALB/c mice. Treatment was initiated when the primary tumor reached approximately 2 mm × 2 mm (day 14). DOX-incorporated GEG polymeric micelle (DOX-GEG) and PCL-incorporated LE polymeric micelle (PCL-LE) were administered alone at dose of 5 mg/kg and in combination intravenously at a dose of 2.5 mg/kg, respectively. $^{*}P < 0.05$ for DOX + PCL versus control, DOX-GEG or PCL-LE. When more than half of mice were dead, the measurement of tumor volume was discontinued.

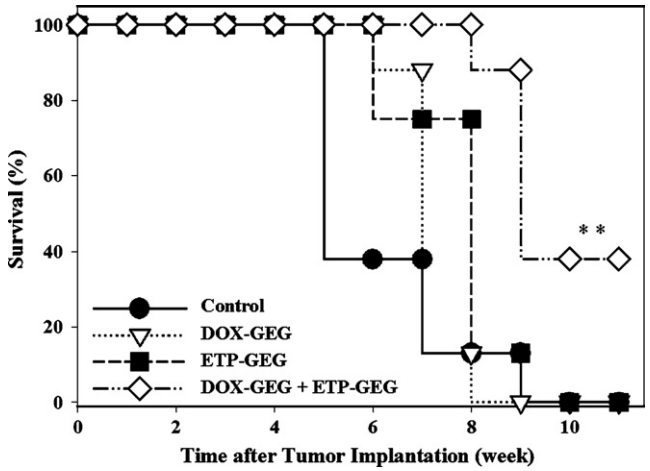

Fig. 5. Survival of tumor-bearing mice treated with anticancer drugs. CT-26 murine tumor cells (10^4/mouse) were implanted subcutaneously in BALB/c mice. Treatment was initiated when the primary tumor reached approximately 2 mm × 2 mm (day 14). DOX (DOX-GEG) and ETP (ETP-GEG) were administered alone at dose of 5 mg/kg and in combination intravenously at a dose of 2.5 mg/kg, respectively. $^{**}P < 0.01$ for DOX + ETP-GEG versus control, DOX-GEG or ETP-GEG. Etop-GEG is ETP-incorporated GEG polymeric micelle.

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