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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.

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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

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