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Anticancer Effectiveness of Polymeric Drug Nanocarriers on Colorectal Cancer Cells

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***Abstract*— Doxifluridine, a prodrug of 5-fluorouracil (5-FU), was used as the initiator directly in ring-opening polymerization of ε-caprolactone to form hydrophobic doxifluridine-poly(ε-caprolactone) (doxifluridine-PCL) that was further grafted with hydrophilic chitosan to synthsize amphiphilic doxifluridine-PCL-chitosan copolymer. This amphiphilic copolymer was self-assembled into micellar nanoparticles. After HT-29 colon cancer cells were treated with the polymeric drug nanocarrier, prodrug doxifluridine was converted into 5-fluorouracil by endogenous thymidine phosphorylase (TP) and thereby resulting in cell death. Chemotherapy drug 7-ethyl-10-hydroxy-camptothecin (SN-38), an active water insoluble metabolite of irinoetcan hydrochloride, was further encapsulated in the hydrophobic core of the polymeric drug nanocarriers and treated with HT-29 cells. The anticancer effectiveness of the polymeric drug nanocarriers was extensively enhanced by synergistic anticancer activity of slowly released cytotoxic drugs (i.e., 5-FU and SN-38). HT-29 cells transfected with TP-encoding plasmids were selected by antibiotic G418 to obtain HT-29/TP cells. These cells overexpressed with TP enzyme were challenged with doxifluridine-PCL-chitosan polymeric prodrug micelles. The viability of HT-29/TP cells were dropped significantly after 72-h treatment.**

I. Introduction

COLORECTAL cancer is among the leading causes of cancer-related morbidity and mortality in industrialized countries. The main treatment for patients in early stages with localized disease involves resection of the primary tumor, which may offer a chance of cure. However, for metastatic colorectal cancer, chemotherapy is currently the main treatment. 5-FU, synthesized around half century ago [1], is one of the anti-tumor agents most frequently used for treating colorectal cancers. To optimize the efficacy of 5-FU, it is often administered by continuous infusion, because of its short plasma half-life [2]. However, 5-FU is poorly tumor selective and its therapy causes high incidences of toxicity in the bone marrow, gastrointestinal tract, central nervous system and skin. To minimize these side effects, several oral 5-FU prodrugs, such as doxifluridine and capecitabine, have been developed [3]. The metabolic conversion of 5-FU from its prodrug doxifluridine is modulated by the TP level in tumors [4].

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TP represents the rate-limiting enzyme in the activation of doxifluridine, which suggests that the sensitivity of tumor cells to this prodrug might be enhanced by increasing TP expression. Indeed, transfection experiments have provided evidence that TP mediates the sensitivity of human KB epidermal carcinoma and MCF-7 human breast cancinoma to doxifluridine [5-6]. One strategy for cancer therapy could involve the overexpression of TP in cancer cells, increasing the metabolic activation of 5-FU (converted from doxifluridine) and thus the sensitivity of cancer cells to 5-FU. To facilitate tumors susceptible to enzyme-prodrug cancer therapy, prodrug-activating exogenous enzyme can be induced by first delivering genes into tumor cells and subsequently administrating non-toxic prodrug which is converted into toxic drug by the activating enzyme. This is so-called gene-directed enzyme prodrug therapy (GDEPT) [7-8].

Poly(ε-caprolactone) (PCL) was used as the core-forming hydrophobic segment of polymeric micelles for GDEPT study. PCL is commonly synthesized by ring-opening polymerization of ε-caprolactone using alcohol as an initiator and stannous (II) octoate ($Sn(Oct)_2$) as a catalyst. Anticancer prodrug doxifluridine possessing hydroxyl groups has recently employed as the initiator to synthesize doxifluridine-PCL by ring-opening polymerization of ε-caprolactone [9]. In this study, the reported liphophilic doxifluridine-PCL was further grafted with hydrophilic biodegradable chitosan which has been widely accepted as a biocompatible material for drug delivery [10-11]. Water-soluble chitosan will be grafted with doxifluridine-PCL to form the amphiphilic polymer used for the preparation of polymeric prodrug micellar nanoparticles.

The doxifluridine-PCL-chitosan polymeric prodrug micelles were further used to encapsulate SN-38. After parental colorectal cancer cells (HT-29 cells) and TP-transfected HT-29 (HT-29/TP) cells were targeted with polymeric drug nanocarriers, doxifluridine was released from polymeric micelles internalized by cancer cells. After that, slowly released doxifluridine was intracellularly converted to 5-FU by TP, which was originally expressed in HT-29 and up-regulated in HT-29/TP cells. Our results showed that maintaining high concentrations of cytotoxic drugs (i.e., 5-FU and SN-38) for longer times inside the cell led to a greater malignant cell death.

II. Materials and Methods

A. Ring-opening polymerization of doxifluridine-PCL

First, doxifluridine (0.05 g) and ε-caprolactone (ε-CL; 2 g)

were well mixed in a dried glass flask and added one drop $Sn(Oct)_2$. Second, the ring-opening polymerization was reacted under nitrogen at 140°C for 24 h. Finally, doxifluridine-PCL polymer was dissolved in tetrahydrofuran (THF) and then precipitated by adding ethyl ether.

B. Synthesis of doxifluridine-PCL-chitosan copolymer

Doxifluridine-PCL polymer (2 g) and succinic anhydride (0.11 g) were dissolved in THF, and then well mixed in a dried flask. Pyridine (88 μL) was added into the flask and reacted under nitrogen at room temperature for two days. Then, doxifluridine-PCL-COOH was precipitated by ethyl ether, air dried, and freeze dried. The obtained doxifluridine-PCL-COOH (2 g) and N-hydroxysuccinimide (NHS; 0.16 g) were dissolved in 20 mL of dichloromethane (DCM) in a flask. The solution was reacted under nitrogen and stirred at room temperature for 24 h. Doxifluridine-PCL-NHS was precipitated into ethyl ether, air dried, and freeze dried. The synthesized doxifluridine-PCL-NHS (1 g) was dissolved in 10 mL acetone and added with 0.5 g of chitosan solution (average MW of 6-8 kD) and stirred under nitrogen for two days. For purification of doxifluridine-PCL-chitosan copolymers, the reaction solution was freeze-dried and dissolved in acetone then filtrated by a 0.45-μm filter.

C. Characterization of doxifluridine-PCL-chitosan copolymers

The ^{1}H-NMR spectra of doxifluridine-PCL and doxifluridine-PCL-chitosan with $CDCl_3$ as the solvent and 10% of TMS as the standard was obtained by a Varian UNITY Inova 400 MHz instrument. The FT-IR spectra of doxifluridine-PCL and doxifluridine-PCL-chitosan were measured by a FT-IR-4200 spectrometer. The average molecular weight of doxifluridine-PCL-chitosan was determined by gel permeation chromatography (GPC) using toluene as the carrying solvent and different molecular weights of polystyrenes (300 - 1.7×10^5 KDa) as the standard.

D. Preparation of polymeric micelles

Doxifluridine-PCL-chitosan copolymer (10 mg) was dispersed in acetone (2 mL) by sonication. The obtained organic solution was added dropwise into deionized water (10 mL) with stirring at room temperature and then dispersed by sonication. Afterwards, acetone was removed by a rotary evaporator. The resulting aqueous solution was filtered through a 0.45-μm filter membrane to collect polymeric micelles.

E. Determination of critical micelle concentration (CMC)

The CMC was measured by fluoresce spectroscopy using pyrene as a hydrophobic probe. As first, the 6×10^{-6} M pyrene solution was prepared in acetone. Concurrently, aliquots of doxifluridine-PCL-chitosan copolymer with concentrations varied from 1.22×10^{-6} to 5 mg/mL were prepared in each of 100 μL stock solution of pyrene in acetone and incubated for 4 h. Then, each of 100 μL pyrene/copolymer acetone solution were dropped in each vial containing 1 mL DI water and incubated at room temperature for 8 h. For each sample, the two emission peaks (I_1 and I_3) were measured at 374 nm and 395 nm. The intensity ratio of I_1/I_3 was plotted against the logarithm of copolymer concentration to find the inflection point. The corresponding concentration at this point was considered to be the copolymer's CMC.

F. Size and zeta potential of polymeric micelles

The size and the zeta potential of fabricated polymeric micelles were obtained by the Zetasizer (Nano ZS, Malvern Instruments, USA).

G. Drug loaded doxifluridine-PCL-chitosan micelles

Encapsulation of SN-38 (7-ethyl-10-hydroxy-camptothecin) in doxifluridine -PCL-chitosan micelles was achieved using a co-solvent evaporation method. doxifluridine-PCL-chitosan and SN-38 was dissolved in acetone. This solution was added to deionized water in a drop-wise manner. After stirring at room temperature, the remaining acetone was removed by vacuum. The aqueous solution of the micellar formulation was centrifuged to remove free SN-38 precipitates.

H. Amplification of plasmid encoding TP cDNA

Full-length human TP cDNA was provided by Dr. Tatsuhiko Furukawa (Kagoshima University, Japan). A KpnI-EcoRI fragment from pPL8 that encompassed the TP coding region was cloned between the KpnI and EcoRI restriction sites of pT7T318U (Pharmacia, Sweden). A XbaI-EcoRI fragment from pT7T318U was then cloned between the NheI and EcoRI restriction sites of mammalian expression vector pBK-RSV (Stratagene, La Jolla, CA) to make the expression vector encoding TP cDNA (RSV/TP). To generate sufficient amount of TP cDNA plasmids, RSV/TP vector was transformed into competent cells (E. coli, DH5α) and the plasmids were purified using the Qiagen Maxiprep kit. The purification and concentration of isolated plasmid DNA were determined by measuring absorption at 260 and 280 nm using UV spectrophotometry.

I. Cell lines

Human colorectal adenocarcinoma cell line (HT-29) and Murine fibroblast cell line (NIH 3T3) were cultivated in DMEM with 10% FBS at 37°C in a humidified incubator with 5% CO_2. 6×10^5 HT-29 human colon carcinoma cells were incubated with 10 μg RSV/TP plasmid and 50 μl Lipofectamine. After 48 h, cells were trypsinized and suspended in a fresh culture medium containing 0.4 mg/ml G418 antibiotic for seven weeks to select stable TP expressing HT-29 cells (named as HT-29/TP).

J. Cytotoxicity study

1×10^4 of NIH 3T3, HT-29 and HT-29/TP cells pre-incubated in a 24-well culture plate were replaced separately with 5, 25, and 50 μg/mL doxifluridine-PCL-chitosan polymeric micelles for 24, 48, and 72 h. After challenged with various amounts of polymeric prodrug micelles, cells were replaced with 2 mL culture

medium containing MTT assay reagent (4 mg/mL) and incubated for additional 4 h. The formed purple crystals were dissolved by 2 mL dimethyl sulfoxide (DMSO). 200 µL of the DMSO solutions from the culture wells were loaded into a 24-well plate and had the absorbance measured at 590 nm. The aforementioned procedure was used again to determine the cytotoxic effect of doxifluridine-PCL-chitosan polymeric micelles loaded with SN-38.

III. RESULTS AND DISCUSSION

A. Characterization of doxifluridine-PCL-chitosan copolymer

Doxifluridine-PCL hydrophobic polymer was synthesized directly by ring-opening polymerization of ε-CL with doxifluridine as the initiator and $Sn(Oct)_2$ as the catalyst. The average molecular weight of doxifluridine-PCL polymer was approximately 15 kDa determined by GPC with polydispersity of 1.2. Hydrophobic doxifluridine-PCL was further grafted with chitosan to obtain doxifluridine-PCL-chitosan amphiphilic copolymer with molecular weight of 23 kDa and polydispersity of 1.21.

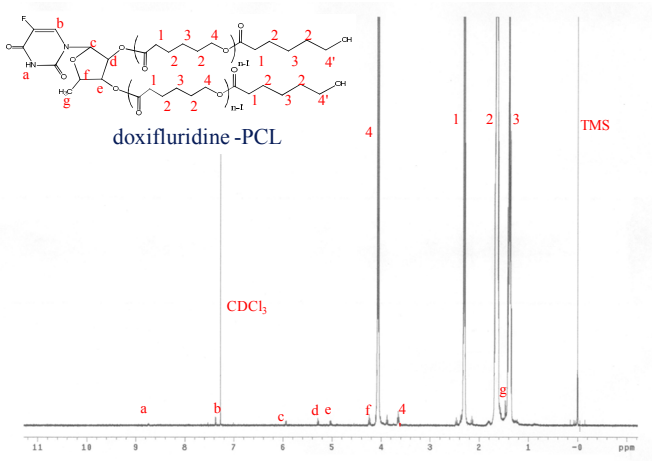

Fig. 1. ^{1}H-NMR spectrum of doxifluridine-PCL polymer (in $CDCl_3$).

Fig. 1 shows the structure of doxifluridine-PCL polymer characterized by ^{1}H-NMR spectra. The result clearly indicated that typical chemical shifts ($CDCl_3$) at 1.4 (3-CH_2), 1.65 (2-CH_2), 2.34 (1-CH_2), 3.62 (4'-CH_2) and 4.05 (4-CH_2) ppm were associated with PCL. The peaks at δ = 1.42 (g-CH_3), 4.21 (f-CH), 5.02 (e-CH), 5.3 (d-CH), 5.95 (c-CH), 7.29 (b-CH) and 8.78 (a-NH) ppm were assigned to the protons in doxifluridine. The NMR analyses demonstrated that the ring-opening polymerization of ε-CL was done by the hydroxyl groups of doxifluridine.

Fig. 2 shows the FT-IR spectra of chitosan, doxifluridine-PCL, and doxifluridine-PCL-chitosan, respectively. Fig. 2A reveals that the characteristic peaks of chitosan at 1585 and 1655 cm^{-1} were attributed to the amino I and amino II group vibration. The absorption band between 3200~3500 cm^{-1} is attributed to the NH and OH stretching vibration. Fig. 2B illustrates that the absorption peak of doxifluridine-PCL at 1726 cm^{-1} was attributed to the C=O stretching vibration. The absorption bands peaked at 2925 and 2851 cm^{-1} were attributed to the C-H stretching vibration. Fig. 2C, representing the spectrum of doxifluridine-PCL-chitosan, indicates that the characteristic peaks at 1537 and 1625 cm^{-1} were attributed to the amino I and amino II group vibration. The amide linkage stretching vibration shown at 1578 cm^{-1} clearly demonstrates that chitosan was grafted onto PCL. The absorption bands peaked at 2925 and 2851 cm^{-1} were same as the ones shown in the spectrum of doxifluridine-PCL. The absorption bands at 1044, 1092, 1236 and 1398 cm^{-1} were attributed respectively to the C-N, C-O, C-O-C and C-N stretching vibration groups of doxifluridine.

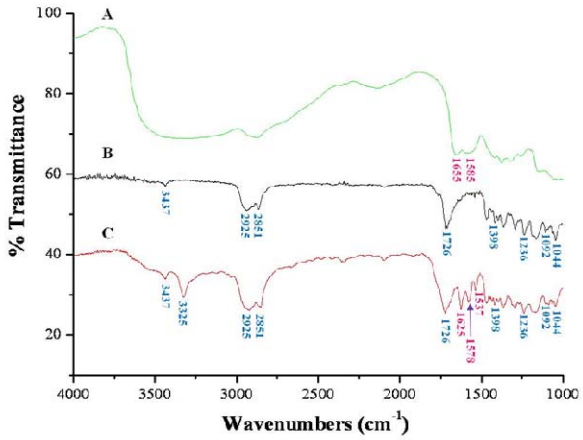

Fig. 2. FT-IR spectra of (A) chitosan, (B) doxifluridine-PCL polymer, and (C) doxifluridine-PCL-chitosan copolymer.

The CMC of doxifluridine-PCL-chitosan copolymer was measured to be 40 mg/mL. The average size and zeta potential of doxifluridine-PCL-chitosan polymeric micelles were detected to be 163.7 nm and 38.8 mV, respectively.

B. Effects of doxifluridine-PCL-chitosan drug nanocarriers

Cell viabilities of NIH 3T3 cells treated with various dosages of doxifluridine-PCL-chitosan polymeric nanoparticles for 24, 48, and 72 h were performed (data not shown). Since there is no endogenous TP expression in NIH 3T3 cells, it is obvious that no cell death was triggered by the polymeric prodrug micelles.

It was demonstrated by Fig. 3 that doxifluridine-PCL-chitosan polymeric micelles could impose detrimental effects on HT-29 cells. Since parental HT-29 cells express endogenous TP enzyme, it is reasonable to interpret that polymeric prodrug micellar nanoparticles endocytosed by HT-29 cells were able to release prodrug doxifluridine via cleavage of the ester bond between

doxifluridine and PCL by acid hydrolysis and/or intracellular esterase. The slowly released doxifluridine was converted to 5-FU by intracellular TP enzyme, thereby leading to anticancer effectiveness via 5-FU.

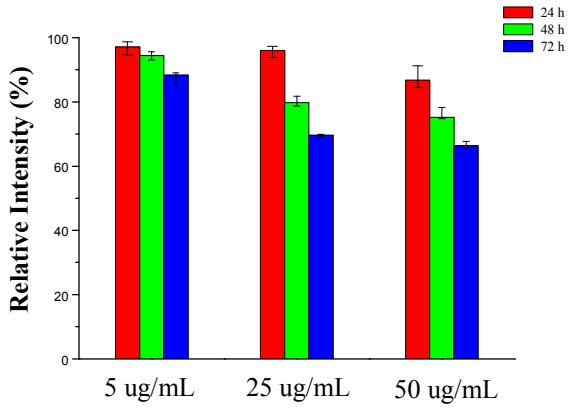

Fig. 3. Cell viability of HT-29 cells treated separately with 5, 25, and 50 µg/mL doxifluridine-PCL-chitosan polymeric micelles for 24, 48, and 72, respectively.

The doxifluridine-PCL-chitosan polymeric micellar nanoparticles further loaded with hydrophobic anticancer drug SN-38 were employed to treat parental HT-29 cells. The results given in Fig. 4 clearly show that the anticancer efficacy was augmented by the polymeric drug nanocarriers.

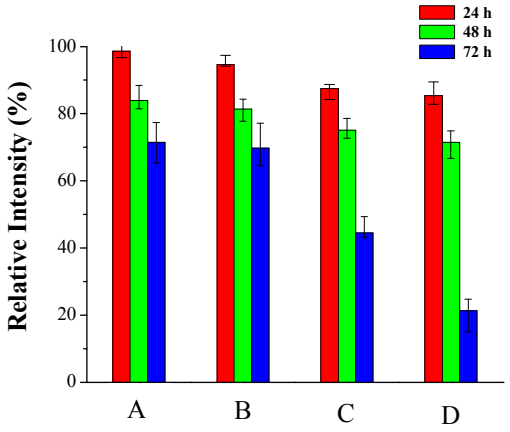

Fig. 4. Cell viability of HT-29 cells treated separately with 25 µg/mL doxifluridine-PCL-chitosan polymeric micelles encapsulated with (A) 0, (B) 0.025, (C) 0.05, and (D) 0.1 mg/mL of SN-38 for 24, 48, and 72, respectively.

HT-29 cells transfected with TP-encoding plasmid were successfully selected by G418. These cells with overexpressed TP enzyme revealed very similar growth kinetics compared to parental HT-29 cells. After challenged with various dosages of doxifluridine-PCL-chitosan polymeric micelles, the degree of cell death (shown in Fig. 5) was dramatically increased in comparison with the data shown in Fig. 3 where the cells used were parental HT-29 cells rather than the modified HT-29/TP cells.

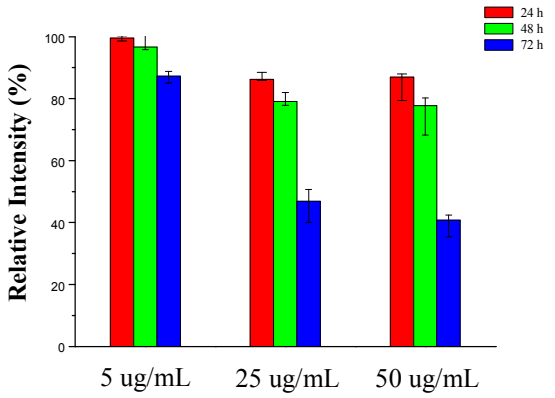

Fig. 5. Cell viability of HT-29/TP cells treated separately with 5, 25, and 50 µg/mL doxifluridine-PCL-chitosan polymeric micelles for 24, 48, and 72, respectively.

IV. Conclusion

Our results indicate that polymeric drug nanocarriers have the potential of killing colorectal cancer cells to a large extent. Developing similar drug nanocarriers could enhance the effectiveness of GDEPT.

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