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Redox-responsive microbeads containing thiolated pectin-doxorubicin conjugate inhibit tumor growth and metastasis: An *in vitro* and *in vivo* study

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ABSTRACT

The objective of this study was to investigate the *in vitro* cytotoxicity and *in vivo* anticancer efficacy of redox-responsive microbeads containing thiolated pectin–doxorubicin (DOX) conjugate. Oral microbeads were coated with an enteric polymer to protect the drug from release in the upper gastrointestinal (GI) tract and allow redox-triggered drug release in the colon. Morphology, particle size, drug content, and *in vitro* drug release behavior of the microbeads were characterized; *in vitro* cytotoxicity was tested on mouse colon carcinoma, human colorectal adenocarcinoma, and human bone osteosarcoma cell lines. *In vivo* anticancer efficacy of coated microbeads was examined in BALB/c mice with murine colon carcinoma. These coated microbeads significantly inhibited the growth of all cell lines. The *in vivo* study confirmed delivery of DOX to the colorectal tumor site, redox-responsiveness, and anticancer efficacy of coated microbeads. Coated microbeads also effectively inhibited primary tumor growth and suppressed tumor metastases without gross toxicity to the non-target tissue. No noticeable damage was found in mouse GI tissues, indicating lack of DOX toxicity. These novel coated microbeads containing thiolated pectin–DOX conjugate may be a promising vehicle for targeted clinical delivery of DOX to the colorectal cancer site by oral administration.

1. Introduction

Colorectal cancer is the third most common cancer in men (10% of the total) and the second most common in women (9.2% of the total) worldwide. Approximately 95% of colorectal cancers are adenocarcinomas (Ferlay et al., 2015; Torre et al., 2015). Decisions about treatment depend on the stage of the cancer. Treatments are usually local therapies, i.e., they treat the tumor without affecting other healthy sites of the body. Surgery and therapies, such as radiation and embolization, are more likely to be effective before metastases. Colorectal cancer can also be treated with chemotherapy, given orally or directly into the bloodstream. Depending on the stage of the cancer and other factors, such as overall patient health and concurrent use of medications for other conditions, different types of treatment may be combined at the same time or used successively (Cunningham et al., 2010; Labianca et al., 2010; Wilkes and Hartshorn, 2009). Patient convenience is one of the several benefits of orally administered drugs. Further, the non-invasive nature of oral administration helps maintain higher quality of life. Compared with intermittent intravenous infusion, oral administration prolongs drug exposure and this sustained exposure is critical for successful therapy as the mutated cancer cells are in a state of constant hyperproliferation.

Doxorubicin (DOX), an anthracycline antibiotic drug, is one of the most widely used frontline chemotherapeutics for a range of cancers, including breast cancer, small-cell lung cancer, colorectal cancer and ovarian carcinoma. DOX is currently available only as a solution for injections, but the intravenous route most often results in toxicity to normal cells, particularly cells of the heart (El-Agamy et al., 2016; Jain et al., 2012; Minotti et al., 2004; Tacar et al., 2013).

Stimuli-responsive systems for the delivery of anticancer drugs to a given target location at a specific time have been pursued with great interest because of potential advantages, such as controlled release, improved pharmacokinetics, enhanced pharmacodynamics at the target site, and reduced side effects to the normal tissue. For delivery to solid tumors, the characteristics of an ideal system will naturally depend on the nature of the stimulus present in the local cancerous environment

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exploited to trigger drug release. Redox-active anticancer drug delivery has been extensively investigated because of the presence of redox-activated stimuli in tumors. Multiple studies have already been conducted using this technology, facilitated by the usually simple chemical nature of the activation process and the ease of fabricating a carrier with an incorporated redox-responsive trigger group (McCarley, 2012; Singh et al., 2016). In our previous work, thiolated pectin–DOX conjugate was fabricated by coupling thiolated pectin to a DOX derivative (i.e., DOX-3,3′-dithiopropionic acid conjugate) by disulfide bond formation or disulfide bond exchange (Cheewatanakornkool et al., 2017b). In the reducing environment of active solid tumors, thiolated pectin–DOX conjugate could uncouple because of cleavage of the disulfide linkers and consequently release DOX.

There is noticeable interest in targeted delivery of chemotherapeutic drugs specifically to the affected site of the colon in a predictable and reproducible manner. However, the main challenges with drugs targeted to the colon are prevention of drug absorption in the upper gastrointestinal (GI) tract and prevention of drug degradation before reaching the colon (Izadi et al., 2016). There are several methods by which oral drugs can be selectively delivered to the colon. For example, microbeads made of alginate and carboxymethyl guar gum (Alg–CMGG) have been used for orally administering DOX, and these provided excellent stability at acidic pH with no measurable drug release and a fivefold slower drug release under simulated intestinal conditions (Bosio et al., 2014). Poly(ethylene glycol) di-acrylate microbeads containing fluorouracil (5-FU) exhibited relatively fast release in the first 12 h and sustained release over the next 156 h, and these microbeads also effectively inhibited Huh-7 tumor cells *in vitro* (Xue et al., 2015). Paharia et al. (2007) found that encapsulated pectin microspheres for colon-selective delivery of 5-FU showed lower accumulation and toxicity in non-target organs than free 5-FU but higher accumulation in the colon tissue of rats.

Pectin is naturally present in plant cell walls and can be efficiently extracted from edible plant materials, such as sugar beet, citrus peel, and apple pomace, followed by selective precipitation with alcohol or salts. Pectin consists of partial methyl esters of polygalacturonic acid and their salts, with molecular weights as high as approximately 200 kDa. The percentage of esterified galacturonic acid units to total galacturonic acid units in a molecule of pectin is referred to as its degree of esterification (DE). Commercial pectin is categorized according to its DE and whether it forms gels quickly or slowly. Pectin can be divided into high methoxy pectin (> 50% DE) and low methoxy pectin (< 50% DE) (Sriamornsak, 2003, 2011). Pectin has great potential for use as a colon-selective drug delivery carrier because it can prolong retention in the upper GI tract and be degraded by colonic enzymes. Pectin shows promise in this regard, as indicated by the large number of studies that have been published since the 1990s (Mohnen, 2008; Sriamornsak, 2011; Zouambia et al., 2009).

The objective of this study was to evaluate the *in vitro* and *in vivo* anticancer efficacies of redox-responsive microbeads containing thiolated pectin–DOX conjugate and coated with an enteric polymer. Furthermore, the histopathology of organs along the GI tract and at the tumor site was also evaluated to assess specificity for the target site.

2. Materials and methods

2.1. Materials

Low methoxy pectin (LMP; type CU701; MW, 70 kDa; lot number: 00412079) with DE of 38% and high methoxy pectin (HMP; type CU201; MW, 200 kDa; lot number: 00501087) with DE of 70% were obtained from Herbstreith & Fox AG (Germany). DL-dithiothreitol (DTT) (lot number: SLBK 4951 V) and DOX (hydrochloride salt) (lot number: SLB 1340 V) were purchased from Sigma-Aldrich (MO, USA). Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (lot number: J069H11) was obtained from Rankem Ltd. (India). Zinc acetate (lot number: 1501186173) was purchased from Ajax Finechem (Australia), and Eudragit® S100 (lot number: B141205008) was a gift from Evonik Industries (Germany). All other chemicals used were of reagent or analytical grade.

2.2. Cell lines and animals

Mouse colon carcinoma (CT26), human prostate cancer (PC3), human bone osteosarcoma (143B), human colorectal adenocarcinoma (HT29), and human epithelial colorectal adenocarcinoma (Caco-2) cell lines were purchased from ATCC (Manassas, VA). Four-week-old male Balb/c mice were purchased from the Animal Resources Centre (Perth, Australia).

2.3. Fabrication of coated microbeads containing thiolated pectin − DOX conjugate

Microbeads containing thiolated pectin and thiolated pectin–DOX conjugate were fabricated by the ionotropic gelation method (Cheewatanakornkool et al., 2017a; El-Gibaly, 2002; Jung et al., 2013; Sriamornsak et al., 2010), which uses zinc acetate as a crosslinking agent. Briefly, lyophilized thiolated pectin–DOX conjugate was dissolved in distilled water and mixed with raw material pectin solution to a final concentration of 2% w/w. This mixture was dropped into 0.3 M zinc acetate and stirred for 20 min. The obtained microbeads were filtered, washed three times with distilled water, dried on a polytetrafluoroethylene tray at ambient temperature (28 °C) for 12 h, and then dried in a hot-air oven at 40 °C for 2 h. The dried microbeads were spray-coated with a methanolic solution of the enteric coating material (2% w/w Eudragit® S100). Finally, the coated microbeads were collected and dried on a polytetrafluoroethylene tray at room temperature for 2 h.

2.4. Microbead characterization

2.4.1. Morphology and particle size determination

The morphology of coated microbeads was examined using a light microscope (model IX51, Olympus, Japan). Light images were processed using ImageJ software (National Institute of Health, USA). For scanning electron microscopy (SEM), all samples were fixed on SEM stubs with double-sided adhesive tape and then coated with a thin gold layer in vacuum before imaging. SEM images were taken using a scanning electron microscope (model Maxim-2000, CamScan Analytical, England) at 15 kV.

2.4.2. Drug content

The DOX content of the coated microbeads was determined by fluorescence spectrometry (model RF 1501, Shimadzu Corporation, Japan) using a method that was described previously (Kumar et al., 2017; Mohan and Rapoport, 2010; Zhao et al., 2015). Briefly, coated microbeads were dissolved in pH 7.4 phosphate buffer containing 0.25 M EDTA and 0.01 M DTT. The mixture was continuously stirred for 4 h and then filtered through a 0.22-μm filter before DOX detection by emission (Em) intensity at 555 nm from 485 nm excitation (Ex). All measurements were performed in triplicate. DOX concentration was then calculated based on a standard fluorescence curve constructed using known amounts of DOX in distilled water. The DOX content was defined as follows:

$$\text{DOX content (\%)} = \frac{\text{Weight of loaded DOX(mg)}\times 100}{\text{Weight of coated microbeads(mg)}}. \quad (1)$$

2.5. In vitro drug release

The dialysis method was used to monitor DOX release from coated