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Complete regression of breast tumour with a single dose of docetaxel-entrapped core-cross-linked polymeric micelles

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ABSTRACT

Treatment with chemotherapy such as docetaxel (DTX) is associated with significant toxicity and tumour recurrence. In this study, we developed DTX-entrapped core-cross-linked polymeric micelles (DTX-CCL-PMs, 66 nm size) by covalently conjugating DTX to CCL-PMs via a hydrolysable ester bond. The covalent conjugation allowed for sustained release of DTX under physiological conditions *in vitro*. *In vivo*, DTX-CCL-PMs demonstrated superior therapeutic efficacy in mice bearing MDA-MB-231 tumour xenografts as compared to the marketed formulation of DTX (Taxotere®). Strikingly, a single intravenous injection of DTX-CCL-PMs enabled complete regression of both small (~150 mm^3) and established (~550 mm^3) tumours, leading to 100% survival of the animals. These remarkable antitumour effects of DTX-CCL-PMs are attributed to its enhanced tumour accumulation and anti-stromal activity. Furthermore, DTX-CCL-PMs exhibited superior tolerability in healthy rats as compared to Taxotere. These preclinical data strongly support clinical translation of this novel nanomedicinal product for the treatment of cancer.

1. Introduction

Despite many marketed anti-cancer agents, treatment of solid tumours still represents a major medical challenge. Conventional chemotherapeutics suffer from a narrow therapeutic index as a result of poor pharmacokinetic and tissue distribution profiles. Besides that, biological barriers at the tumour site such as abnormal blood supply, abundant tumour stroma and high intratumoural pressure limit intratumoural drug penetration, leading to suboptimal therapeutic drug levels [1,2]. To improve the therapeutic index of chemotherapeutics, nanoparticulate systems offer a set of tools to achieve enhanced intratumoural drug accumulation, sustained intratumoural drug release and reduced side effects [3–5].

Compared to normal tissues, tumour tissues generally have hyperpermeable vasculature and poor lymphatic drainage, which allow extravasation and greater retention of nanoscale medicines in tumours, the phenomenon known as the Enhanced Permeability and Retention (EPR) effect [6]. By exploiting the EPR effect, nanoparticles can preferentially localize in tumours and enhance local drug concentration [7–9]. A few passively targeted anti-cancer nanomedicines such as Doxil® (liposomal doxorubicin) and DaunoXome® (liposomal daunorubicin) are already in the market [10] and others, such as polymeric micelles (e.g. NK105 for paclitaxel delivery) and polymer conjugates (e.g. Opaxio™ for paclitaxel delivery), are in advanced clinical trials [11–13]. Although the currently marketed nanomedicines have shown benefits in subsiding the side effects, a gain at the level of antitumour activity has only marginally been achieved [12,14–16]. Also in preclinical studies with nanomedicines, complete regression of solid tumours has hardly been reported. The latter shortcoming is likely attributed to a poor EPR effect and/or insufficient drug release from the extravasated nanoparticles, leading to sub-therapeutic drug levels. Moreover, the delivery of anti-cancer agents can also be significantly limited by the physical barrier of stroma in tumour tissues [17]. Tumour stroma (including cancer-associated fibroblasts, immune cells and extracellular matrix) is the supporting tissue

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adjacent to tumour cells, which plays a pivotal role in tumour growth and progression [18]. Elimination of activated stroma has been considered as a potential approach to anti-cancer therapy [17,18]. Altogether, the development of a nanomedicine with efficient tumour accumulation, sufficient intratumoural drug release and anti-stromal activities is very likely mandatory for achieving optimal antitumour activity.

Docetaxel (DTX), a potent anti-mitotic chemotherapeutic agent, acts by binding to microtubules and thereby interfering with cell division. DTX is approved for the treatment of locally advanced or metastatic breast cancer, gastric cancer, hormone-refractory prostate cancer and non-small cell lung cancer [19–21]. In spite of its wide clinical use, serious side effects are often observed in patients such as acute hypersensitivity reactions, cumulative fluid retention, neurotoxicity, febrile neutropenia, myalgia, nasolacrimal duct stenosis and asthenia [22,23]. Several nanosized vehicles have been developed in recent years to improve the therapeutic index of DTX, including polymeric nanoparticles (NPs) [24], drug-polymer conjugates [25], polymeric micelles [26], lipid-based nanocarriers [27] and inorganic NPs [28]. Many of these nanoparticulate systems demonstrated superior antitumour activity compared to the marketed formulation in preclinical models, yet complete tumour regression was rarely reported and most of them were not (fully) evaluated for their tolerability profiles.

Core-crosslinked polymeric micelles (CCL-PMs) have shown prolonged circulation kinetics upon intravenous (i.v.) administration and enhanced tumour accumulation in various tumour models [29–31]. In the present study, we developed CCL-PMs composed of poly(ethylene glycol)-b-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-*b*-p(HPMAm-Lac$_n$)) copolymers to deliver DTX to tumours after i.v. administration. To assure sufficient drug release from the extravasated CCL-PMs, we conjugated DTX covalently to CCL-PMs via a hydrolysable ester linker to allow controlled drug release [32]. In the present study, the antitumour effect of DTX-CCL-PMs and Taxotere was compared after multi-dose or a single-dose i.v. administration at various doses to tumour-bearing mice. Furthermore, to obtain the safety profile of DTX-CCL-PMs for future clinical translation, the pharmacokinetics (PK) and tolerability profile of DTX-CCL-PMs were examined in healthy rats.

2. Materials and methods

2.1. Materials

Docetaxel (DTX) was obtained from Phyton Biotech GmbH (Ahrensburg, Germany). N,N′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 4-methoxyphenol, methacrylic anhydride, ammonium acetate, formic acid, Mukaiyama's reagent (2-chloro-1-methylpyridinium iodide), oxone, potassium persulfate (KPS), lactic acid, tetramethylethylenediamine (TEMED) and trifluoroacetic acid (TFA) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Dichloromethane (DCM), N,N-dimethylformamide (DMF) and acetonitrile (ACN) were purchased from Biosolve (Valkenswaard, The Netherlands). Absolute ethanol and triethylamine were purchased from Merck (Darmstadt, Germany). The initiator (mPEG$_{5000}$)$_2$-ABCPA was synthesized as described previously [33]. 2-(2-(Methacryloyloxy)ethylthio)acetic acid (linker) was synthesized as described previously [32]. Taxotere® was purchased from Sanofi-Aventis (Berlin, Germany). The other chemicals were used as received.

2.2. Preparation of docetaxel-entrapped core-cross-linked polymeric micelles

First, DTX-derivative (DTXL) was synthesized in a two-step procedure, as shown in Fig. 1A. The detailed synthesis, purification and analysis of DTXL are described in supplementary methods. A methacrylated block copolymer containing monomethoxy poly(ethylene glycol) (mPEG, M_n = 5000) as hydrophilic block and a random copolymer of N-2-hydroxypropyl methacrylamide monolactate (HPMAm-Lac$_1$) and N-2-hydroxypropyl methacrylamide dilactate (HPMAmLac$_2$) as thermosensitive block was synthesized as described previously [29,34]. Docetaxel-entrapped core-cross-linked polymeric micelles (DTX-CCL-PMs) were prepared essentially using the fast heating method [35]. In brief, an ice-cold aqueous solution of methacrylated mPEG-*b*-pHPMAmLac$_n$ block copolymer (830 μL, 24 mg/mL) was mixed with TEMED (25 μL, 120 mg/mL) dissolved in ammonium acetate buffer (150 mM, pH 5). Subsequently, DTXL (100 μL, 20 mg/mL DTX equivalents, dissolved in ethanol) was added, followed by rapid heating to 60 °C while stirring vigorously for 1 min to form polymeric micelles. The micellar dispersion was then transferred into a vial containing KPS (45 μL, 30 mg/mL) dissolved in ammonium acetate buffer (150 mM, pH 5). The polymeric micelles were covalently stabilized by crosslinking the methacrylate moieties in DTXL and block polymer in a N_2 atmosphere for 1 h at RT, to obtain DTX-CCL-PMs. The final feed concentrations of block copolymer and DTXL (DTX equivalents) were 20 and 2 mg/mL, respectively. Next, the DTX-CCL-PMs dispersion was filtered through a 0.2 μm cellulose membrane filter to remove potential aggregates. DTX-CCL-PMs dispersions were purified and concentrated for 10 times using a KrosFlo Research II*i* Tangential Flow Filtration (TFF) System equipped with modified polyethersulfone (mPES) MicroKros® filter modules (MWCO 500 kDa). Ammonium acetate buffer (20 mM, pH 5) containing 130 mM NaCl was used as the washing buffer for TFF and referred to as "vehicle" in the following sections.

2.3. Characterization of DTX-CCL-PMs by DLS, TEM and UPLC

The size of DTX-CCL-PMs was measured by dynamic light scattering (DLS) using a Malvern ALV/CGS-3 Goniometer. DLS results are given as a z-average particle size diameter (Z_{ave}) and a polydispersity index (PDI).

Transmission electron microscopy (TEM) analysis of DTX-CCL-PMs was conducted using a Philips Tecnai 12 microscope equipped with a Biotwin lens and a LaB6 filament, operated at 120 kV acceleration voltage. Glow discharged grids (copper 200 mesh grid with a carbon-coated thin polymer film, Formvar on top) were used for sample preparation and 2% uranyl acetate (w/v) was used as a negative stain. Images were captured with a SIS Megaview II CCD camera and processed with AnalySIS software.

The contents of free DTX, free DTXL, total DTX and polymer in DTX-CCL-PMs dispersions were determined by ultra-performance liquid chromatography (UPLC) as described in supplementary methods. The drug entrapment efficiency (EE) and drug loading (DL) were calculated using the UPLC data as follows:

$$EE = \frac{\text{Amount of drug entrapped} \times 100\%}{\text{Amount of drug added}}$$

$$DL = \frac{\text{Amount of drug entrapped} \times 100\%}{\text{Amount of polymer} + \text{Amount of drug entrapped}}$$

The amount of drug entrapped was calculated as: amount of drug entrapped = amount of total DTX content − amount of free DTX − amount of free DTXL (DTX equivalents).

2.4. In vitro docetaxel release from docetaxel-entrapped core-crosslinked polymeric micelles

The *in vitro* release of DTX from DTX-CCL-PMs was measured in phosphate buffer (100 mM, pH 7.4) containing 15 mM NaCl, whole rat blood and whole human blood at 37 °C, respectively. DTX-CCL-PMs were incubated at 37 °C in different matrices and the samples were collected at different time points and analysed for released DTX content using UPLC. In brief, DTX-CCL-PMs were diluted in phosphate buffer (100 mM, pH 7.4) containing 15 mM NaCl and 1% polysorbate 80 (v/v). The concentration of released DTX was determined by injecting 7 μL of the mixture into a UPLC system (Waters, USA) equipped with an ultraviolet/visible light detector (TUV, Waters). An Acquity HSS T3 1.8 μm column (50 × 2.1 mm) (Waters) was used with a gradient from 100% eluent A (70% H_2O/30% ACN/0.1% formic acid) to 100% B (10% H_2O/90% ACN/0.1% formic acid) in 11 min with a flow of 0.7 mL/min and UV-detection at 227 nm. DTX standards dissolved in ACN were used to prepare a calibration curve (linear between 0.5 and 110 μg/mL). In the case of whole blood, rat or human whole blood was first incubated at 37 °C for 10 min. Next, blood (85 μL) was spiked with DTX-CCL-PMs (15 μL) and incubated at 37 °C for various lengths of time. After incubation, water (100 μL) was added to the mixture, followed by ACN (600 μL). The reaction mixture was vortexed for 30 s and centrifuged at 10,000 × *g* for 5 min at 20 °C. Thereafter, the supernatant (500 μL) was added to water (100 μL) and 7 μL of the resulting mixture was injected into the UPLC system. An Acquity HSS T3 1.8 μm column (50 × 2.1 mm) (Waters) was used with an isocratic run of 3.5 min (mobile phase: 55% H_2O/45% ACN/0.1% formic acid) with a flow of 0.7 mL/min and UV-detection at 227 nm. Only DTX and 7-epi-DTX (the major degradation product of DTX [36,37]) were taken into account for the calculation of the percentage release of DTX, so not the other degradation products of DTX [38]:

$$\%\,\text{Release of DTX} = \frac{\text{Amount of DTX} + \text{Amount of 7} - \text{epi} - \text{DTX} \times 100\%}{\text{Amount of total DTX}}$$

2.5. Efficacy studies in MDA-MB-231 xenografts

All animal experiments were approved by the local ethical committee. All animals were housed in a temperature-controlled room (21 ± 3 °C), with 55 ± 15% relative humidity, and a photoperiod of 12/12 h. Female NCr nu/nu mice (8–12 week old, Charles River) were used to induce MDA-MB-231 breast tumour model.