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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.

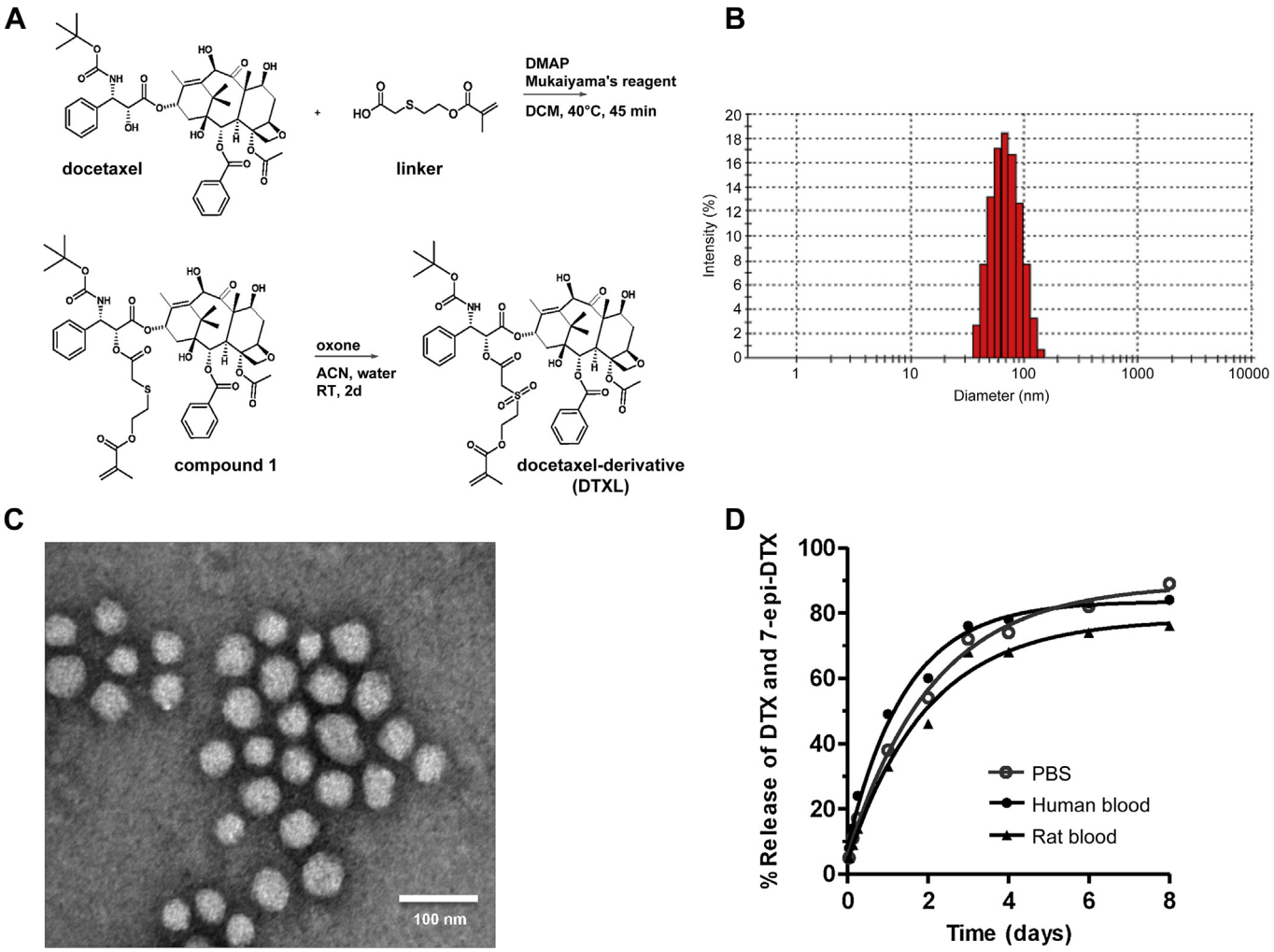

Fig. 1. Synthesis and characterization of DTX-CCL-PMs. (A) Synthesis scheme of DTX-derivative (DTXL), linker = 2-(2-(methacryloyloxy)ethylthio)acetic acid; (B) Particle size distribution of DTX-CCL-PMs as determined by dynamic light scattering; (C) Transmission electron microscopical image of DTX-CCL-PMs and (D) Representative *in vitro* release of DTX and 7-epi-DTX (a major degradation product of DTX) from DTX-CCL-PMs in PBS (pH 7.4), rat blood and human blood at 37 °C. The first measurement time point was at 1 h after the onset of incubation at 37 °C.

Tap-water and pelleted rodent food (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Germany) were provided to the animals. To induce tumours, 5×10^6 MDA-MB-231 human breast tumour cells were subcutaneously implanted in the mammary fat pad of the mice. When tumours attained ~150 mm^3 size (small, early stage) or ~550 mm^3 size (established, late-stage), mice received either a single i.v. injection or multiple injections (weekly i.v. bolus injections for 3 weeks) of either Taxotere, DTX-CCL-PMs or vehicle in the tail vein. The details of the doses, injections and duration of the experiment are specified in the figure (captions). Tumour volume was analysed by caliper measurement biweekly. Animals were monitored individually. The measurement was terminated when a tumour volume of 1500 mm^3 was attained. In the case of animals exiting the study prematurely, the tumour volume data were carried forward until the endpoint (i.e. when ≤50% of the animals remained in the study), which was the point when data plotting stopped. % Survival was calculated using a cut-off tumour volume of 1500 mm^3 as a surrogate for mortality.

2.6. Effect of the DTX-CCL-PMs on the tumour stromal proteins

In the MDA-MB-231 tumour model, when the mean tumour volume reached ~150 mm^3, a single i.v. injection of Taxotere (30 mg DTX/kg), DTX-CCL-PMs (125 mg DTX/kg) or vehicle was administered. Tumours were isolated from mice 4 days after the injections. The collected tumours were frozen at −80 °C till analysis for biomarkers for tumour stroma using Western Blot analyses, as described in the supplementary methods.

2.7. Tumour accumulation of DTX-CCL-PMs in MDA-MB-231 xenografts

In the MDA-MB-231 tumour model, when the mean tumour volume reached ~150 mm^3, mice received a single dose i.v. bolus injection of Taxotere (30 mg/kg) or DTX-CCL-PMs (30 mg/kg). Tumours were collected on day 2 or day 4 post-administration and homogenized to determine total and released DTX contents using HPLC-MS/MS (see supplementary methods).

2.8. Pharmacokinetics and tolerability studies in rats

Charles River Crl:CD (SD) rats were used for the *in vivo* studies. For pharmacokinetic studies, rats were randomly divided into groups of six (three female and three male). A single i.v. bolus injection of DTX-CCL-PMs was given into the tail vein of rats at escalating doses of 1.5, 7.5 or 24 mg/kg. Blood samples (~200 μL) were collected at different time points in EDTA vials and meanwhile systemic tolerance was observed. The content of total DTX in rat whole blood was determined using HPLC-MS/MS (see supplementary methods). Pharmacokinetic evaluation of blood data was performed using WinNonlin, version 6.3 (Pharsight Corporation, Mountain View, CA, USA).

For tolerability studies, acute toxicity and 5-day repeated dose toxicity studies were performed in healthy rats. For the acute toxicity study, a single i.v. bolus injection of DTX-CCL-PMs was administered at a dose of 7.5 mg/kg or 24 mg/kg and clinical observations were recorded systemically for 2 weeks. In addition, the change of body weight and food consumption were also monitored. For 5-day repeated dose toxicity study, an i.v. injection of DTX-CCL-PMs (9.7 mg/kg/day) or Taxotere (6.7 mg/kg/day) was administered into the tail vein of rats daily for five consecutive days. Animals were observed for any signs of behavioural changes, reaction to treatment and illness before and after each dosing. On test day 6, the animals were sacrificed, dissected and inspected microscopically. The size and weight of these organs, as well as any abnormalities in the appearance of these organs were recorded. In addition, clinical haematological parameters and serum biochemistry parameters in these animals were analysed.

3. Results

3.1. Synthesis and purification of DTX-CCL-PMs

DTX-CCM-PMs were prepared in three main steps: (i) derivatization of DTX; (ii) synthesis of methacrylated block copolymer and (iii) preparation of DTX-CCL-PMs. The methacrylated DTX derivative (DTXL) was synthesized in a two-step reaction (Fig. 1A). First, DTX was esterified at its C-2′ hydroxyl group with a methacrylated linker containing a sulfide ester. Next, the sulfide bond was oxidized to a sulfone to obtain DTXL. The synthesized DTXL was purified by column chromatography and obtained as a white solid with high purity (>95%). The identity and purity of the compound were confirmed by ^{1}H NMR, LCMS-UV and UPLC-UV (Fig. S1). Methacrylated block copolymer composed of a hydrophilic mPEG block and a random block of pHPMAm-Lac$_1$/Lac$_2$ was prepared via radical polymerization (75% yield) and its characteristics were in good agreement with previous data [30] (Table S1). DTXL was covalently linked to CCL-PMs upon polymerization of the methacrylate moieties in DTXL as well as in the polymer lactate side chain to obtain DTX-CCL-PMs as an opalescent dispersion. By means of tangential flow filtration (TFF), DTX-CCL-PMs were purified and concentrated to 20 mg DTX equiv. per mL. The mean particle size and polydispersity index (PDI) of DTX-CCL-PMs as determined by dynamic light scattering were 66 nm and <0.1, respectively (Table S2), which are typical for CCL-PMs prepared from this type of block copolymer [32]. A transmission electron microscope (TEM) image showed spherical morphology and confirmed the homogenous size distribution of DTX-CCL-PMs (Fig. 1C). DTX entrapment efficiency and loading were ca. 75% (w/w) and ca. 12% (w/w) respectively.

3.2. In vitro DTX release from DTX-CCL-PMs

Hydrolysis of the ester bond linking DTX to the CCL-PMs allows native DTX to be released under physiological conditions (pH 7.4, 37 °C) following first-order kinetics (Fig. 1D). Due to the degradation of DTX itself [36], the *in vitro* drug release did not reach 100% (Fig. S2). In addition to PBS (pH 7.4), the drug release profile of DTX-CCL-PMs was also evaluated in fresh rat and human blood, in which similar drug release kinetics was observed (Fig. 1D). Accordingly, DTX release kinetics is likely solely dependent on chemical hydrolysis of the ester linkage and is not influenced by e.g. enzymes present in biological fluids, enabling a predictable drug release profile *in vivo*.

3.3. Dose-dependent effect of DTX-CCL-PMs on breast tumour growth

To establish the therapeutic efficacy, the MDA-MB-231 human tumour xenograft model was used as an established *in vivo* model for breast cancer [39].

3.3.1. Multiple dose study

The therapeutic efficacies of Taxotere and DTX-CCL-PMs were first assessed in MDA-MB-231 xenografts following three weekly i.v. injections in nude mice at a dose of 30 mg DTX/kg (referred as 30 mg/kg later), i.e. the maximum tolerated dose (MTD) of Taxotere in nude mice upon weekly i.v. administrations [40]. As shown in Fig. S3A, both Taxotere and DTX-CCL-PMs inhibited tumour growth effectively. However, Taxotere induced a significant ($P < 0.05$) loss in body weight as compared to vehicle group while treatment with DTX-CCL-PMs did not induce any body weight loss (Fig. S3B).

3.3.2. Single dose studies

Since the multiple dose study with DTX-CCL-PMs demonstrated high therapeutic efficacy and good tolerability in terms of body weight loss, studies with different but single doses were carried out to find the best therapeutic outcome. When a tumour size of 150–200 mm^3 was attained, mice were treated with a single i.v. injection of equivalent dose of DTX (30 or 60 mg/kg) in Taxotere or DTX-CCL-PMs. As shown in Fig. 2A, at the dose of 30 mg/kg, both Taxotere and DTX-CCL-PMs exhibited comparable antitumour activity till day 51 post-administration. After that, animals from the Taxotere-treated group had to be sacrificed due to the attainment of humane end point (i.e. larger tumour volumes than allowed) in ≥50% animals. On the other hand, at a dose of 60 mg/kg, DTX-CCL-PMs significantly inhibited the tumour growth as opposed to Taxotere which did not show additional benefit compared to the 30 mg/kg dose, reaching the endpoint at day 54 (Fig. 2A).

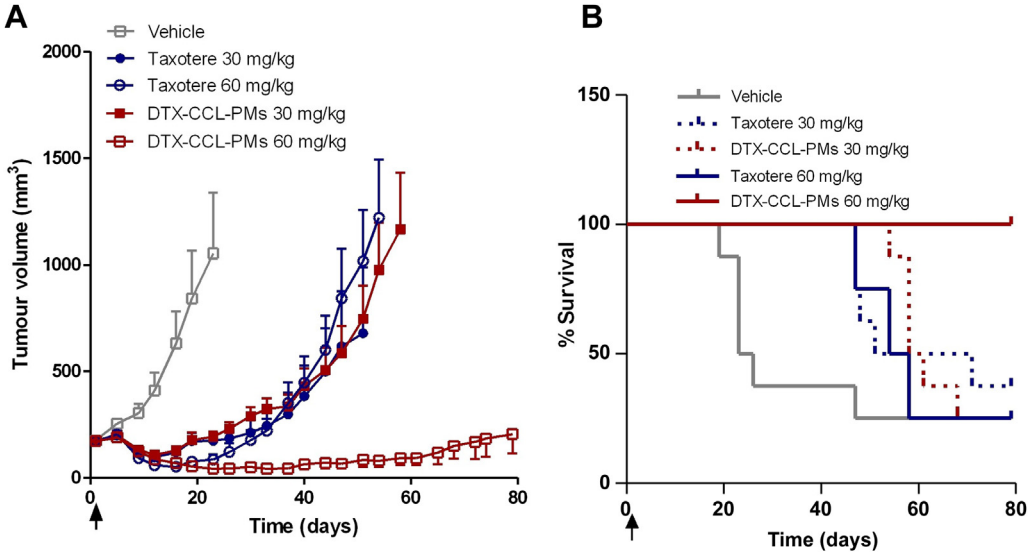

Fig. 2. Antitumour effect of DTX-CCL-PMs at a single dose of 30 and 60 mg DTX/kg. (A) Tumour growth curve and (B) % survival of mice bearing MDA-MB-231 xenografts after a single i.v. injection of Taxotere or DTX-CCL-PMs at equivalent doses (30 and 60 mg DTX/kg). The vehicle group received ammonium acetate buffer (20 mM, pH 5) containing 130 mM NaCl. The duration of the study was 79 days. Data are expressed as the mean ± SEM (n = 8).

Remarkably, by virtue of the substantial tumour regression, 100% survival was achieved with a single dose of DTX-CCL-PMs (60 mg/kg) over the 79-day period of study (Fig. 2B). Regarding tolerability, a single i.v. injection of DTX-CCL-PMs did not result in body weight loss at both doses. In contrast, significant body weight loss was observed at day 5 and 9 with Taxotere treatments ($P < 0.05$), indicating acute toxicity of Taxotere at these doses (Fig. S4). Yet, there was no benefit with Taxotere in inhibition of tumour growth after an increase of the dose from 30 to 60 mg/kg.

3.4. A single dose of DTX-CCL-PMs supresses tumours completely

Although a single dose of DTX-CCL-PMs at 60 mg/kg markedly inhibited tumour growth, mice were not completely cured. As this dose was well tolerated, we examined the antitumour activity of DTX-CCL-PMs at a higher dose of 125 mg/kg. For Taxotere, a dose of 125 mg/kg was not approved by the local experimental animal committee given its known single dose MTD (i.e. 98 mg/kg) [41]. Considering the lack of benefit in antitumour effects and yet acute toxicity with Taxotere after doubling the dose to 60 mg/kg, a single dose of 30 mg/kg was selected for assessing comparative efficacy. Interestingly, we found that a single dose of DTX-CCL-PMs at 125 mg/kg completely abolished tumour growth in mice bearing tumours of 150–200 mm^3 size with a 100% tumour-free survival after the 62-day period of study (Fig. 3A and B).

One essential point in preclinical evaluation is that tumours are generally treated at early stage (i.e. small tumours that may not represent the clinical situation), which may overestimate the potency of a new anti-cancer therapy. Taking this aspect into account, we also assessed DTX-CCL-PMs in mice bearing established tumours of approximately 550 mm^3 size. As shown in Fig. 3, Taxotere exhibited only a moderate antitumour effect in both early and advanced tumour models. Remarkably, a single dose of DTX-CCL-PMs (125 mg/kg) induced complete regression of established tumours leading to 100% tumour-free survival of these mice for 62 days (Fig. 3C and 3D). Moreover, no significant loss in body weight was observed in both treatment groups (Fig. S5).

3.5. Intratumoural effect of DTX-CCL-PMs

To investigate the intratumoural mechanisms for the tumour growth inhibition, we set up an experiment to study the intratumoural effect of the DTX-CCL-PMs within short duration of 4 days. A single dose of Taxotere (30 mg/kg) or DTX-CCL-PMs (125 mg/kg) was injected intravenously into mice bearing tumours of approximately 150–200 mm^3 size. After 4 days, tumours were isolated and analysed for biomarkers for tumour stroma with Western Blot analyses. The data revealed that treatment with DTX-CCL-PMs significantly reduced tumour stroma markers, as shown by the reduction in the protein expression of NG2 (a pericyte marker), α-SMA (a pericyte and cancer-associated fibroblast marker) and collagen-1 (a major extracellular matrix protein) (Fig. 4). In addition, there was also a clear reduction in β-tubulin expression (a marker for microtubules) after the treatment with

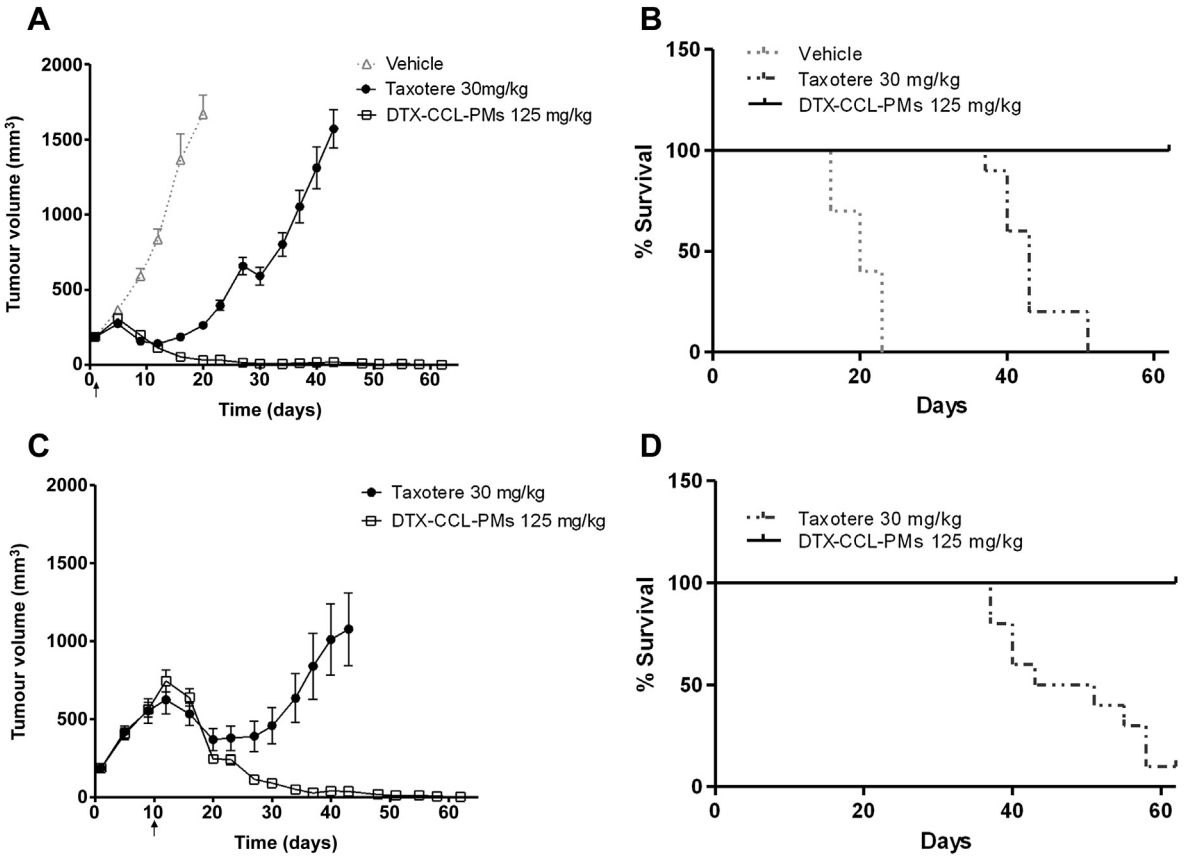

Fig. 3. Antitumour effect of DTX-CCL-PMs (125 mg DTX/kg) in early and established MDA-MB-231 xenografts tumours. (A) Tumour growth curve and (B) % survival of tumour-bearing mice after a single i.v. injection of Taxotere (30 mg DTX/kg), DTX-CCL-PMs (125 mg DTX/kg) or vehicle (when tumours attain ~150 mm^3 size, depicted as day 1). The vehicle group received ammonium acetate buffer (20 mM, pH 5) containing 130 mM NaCl. (C) Tumour growth curve and (D) % survival of tumour-bearing mice after a single i.v. injection of Taxotere (30 mg DTX/kg) or DTX-CCL-PMs (125 mg DTX/kg) (when tumours attain ~550 mm^3 size, depicted as day 10). The duration of the study was 62 days. Data are expressed as the mean ± SEM (n = 10).

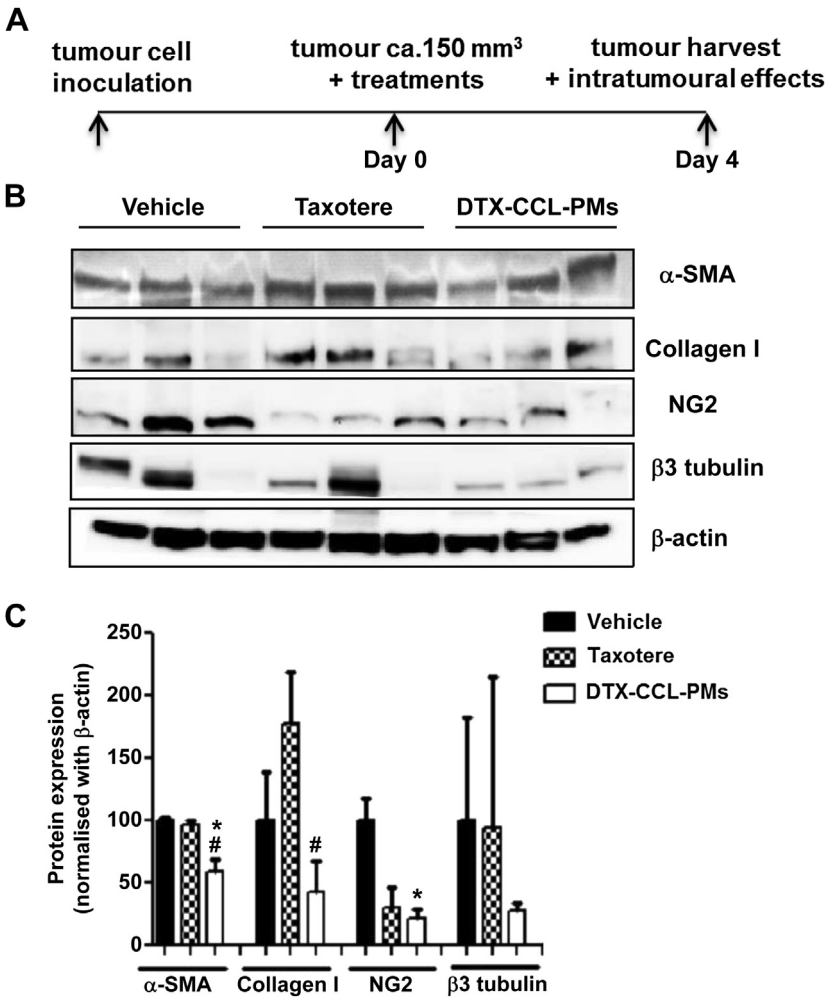

Fig. 4. Intratumoural effect of DTX-CCL-PM on tumour stroma. Western blot analyses in tumour samples collected 4 days after a single i.v. injection of Taxotere (30 mg DTX/kg), DTX-CCL-PMs (125 mg DTX/kg) or vehicle. The vehicle group received ammonium acetate buffer (20 mM, pH 5) containing 130 mM NaCl. (A) Treatment scheme; (B) Protein bands of Western blot and (C) Semi-quantitative analyses of the bands. Data are expressed as the mean ± SEM (n = 3). *P < 0.05 versus vehicle, #P < 0.05 versus Taxotere.

DTX-CCL-PMs. In contrast, treatment with Taxotere showed no significant effects on any of these markers. These data suggest that the antitumour effects of DTX-CCL-PMs are not only caused by the direct inhibitory effects on tumour cells but also by anti-stromal effects.

3.6. Tumour accumulation of DTX-CCL-PMs in mice

To characterize the tumour distribution of DTX-CCL-PMs in mice, the intratumoural levels of released DTX and total DTX (released plus entrapped) in tumour-bearing mice were measured at 2 and 4 days after a single i.v. administration of DTX-CCL-PMs or Taxotere at equivalent dose of 30 mg/kg. As illustrated in Fig. 5A, a single i.v. administration of DTX-CCL-PMs (30 mg/kg) provided a 20-fold (2 days, $P < 0.01$) and 59-fold (4 days, $P < 0.001$) higher total DTX level as compared to Taxotere (30 mg/kg). In addition to the significantly enhanced total DTX levels, 2-fold (2 days) and 4-fold (4 days, $P < 0.05$) higher released DTX levels were found in mice treated with DTX-CCL-PMs. Having expressed as the percentage of injected dose (%ID) in tumour, DTX-CCL-PMs rendered 5-fold ($P < 0.05$) higher released DTX levels and 77-fold ($P < 0.001$) higher total DTX levels in tumour as compared to Taxotere 4 days after the onset of treatment.

3.7. Pharmacokinetic studies in healthy rats

In the present study, the superior efficacy of DTX-CCL-PMs as well as enhanced tumour accumulation was demonstrated in tumour-bearing mice. To continue the development of DTX-CCL-PMs towards clinical evaluation, the PK and tolerability profile were evaluated in healthy rats (as required by regulatory authorities).

PK studies with a single i.v. administration of DTX-CCL-PMs were conducted in healthy rats at the escalating doses (Fig. 5B). The PK evaluation at various doses is given in Table 1. These studies demonstrated that DTX-CCL-PMs had an elimination half-life of 15.9 ± 0.7 h and the extrapolated AUC from zero to infinity ($AUC_{0-\infty}$) at different doses linearly correlated with the administered dose of DTX-CCL-PMs (Fig. 5C, $R^2 = 0.997$).

3.8. Tolerability studies in healthy rats

To investigate the potential toxicity of DTX-CCL-PMs, both acute and 5-day repeated dose toxicities were examined in healthy rats. With respect to acute toxicity, a single i.v. administration of DTX-CCL-PMs at 7.5 mg/kg or 24 mg/kg was well tolerated albeit a slightly reduced (transient) motility for both male and female animals at 24 mg/kg. To establish repeated dose toxicity, DTX-CCL-PMs

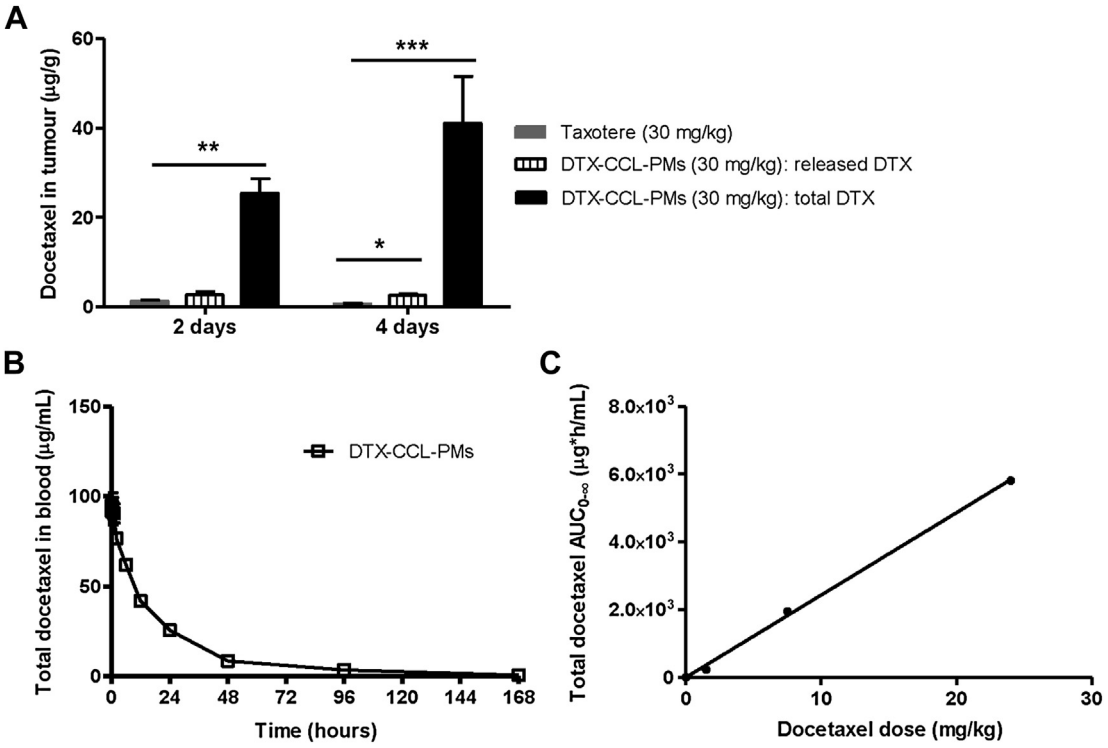

Fig. 5. Pharmacokinetics and tumour accumulation of DTX-CCL-PMs. (A) Intratumoural levels of released DTX and total DTX (released + entrapped) after a single i.v. injection of Taxotere (30 mg DTX/kg) or DTX-CCL-PMs (30 mg DTX/kg) in mice bearing MDA-MB-231 xenografts. Data are expressed as the mean ± SEM (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001. (B) Total DTX (released + entrapped) levels in blood after a single i.v. injection of DTX-CCL-PMs (7.5 mg DTX/kg) in healthy female rats. Data are expressed as the mean ± SEM (n = 6) and (C) Correlation between the injected dose and $AUC_{0-\infty}$ for DTX-CCL-PMs in healthy rats after a single i.v. injection of DTX-CCL-PMs at 1.5, 7.5 and 24 mg DTX/kg, respectively ($R^2 = 0.997$).

Table 1
Pharmacokinetic evaluation for total docetaxel levels in whole blood after a single i.v. administration of DTX-CCL-PMs to female rats at different doses (n = 6).

	DTX-CCL-PMs 1.5 mg/kg	DTX-CCL-PMs 7.5 mg/kg	DTX-CCL-PMs 24 mg/kg
C_0 (μg/mL)	12.6	114.2	364.6
$t_{1/2}$ (h)	15.1	16.2	16.4
$AUC_{0-\infty}$ (μg*h/mL)	229.8	1948.1	5808.9
$AUC_{0-\infty}$/Dose (g*h/mL)	158.5	266.9	240.0
Vz (mL/kg)	137.2	60.0	67.9
Vss (mL/kg)	129.5	56.1	62.3

The values are calculated by PK analysis using a non-compartmental model.
C_0 = concentration at t = 0, extrapolated, $t_{1/2}$ = elimination half-life, $AUC_{0-\infty}$ = extrapolated area under the curve from zero to infinity, Vz = volume of distribution and Vss = volume of distribution at steady state.

or Taxotere was administered intravenously to male and female rats daily for 5 consecutive days. Taxotere was administered intravenously at a dose of 6.7 mg/kg/day (i.e. 40 mg/m^2), the dose often used for toxicity evaluation e.g. by Burstein et al. [42] whereas a 45% higher dose of DTX-CCL-PMs (9.7 mg/kg/day) was administered to the animals considering the superior tolerability of the DTX-CCL-PMs as demonstrated in the acute toxicity study.

Although no mortality occurred, DTX-related target organ toxicities were observed in both groups as reflected by food consumption, diarrhea, size and weight of thymus as well as weight of spleen (Table S3). However, compared to Taxotere, these toxicities were substantially reduced in animals that received DTX-CCL-PMs despite a 45% higher dose given. In addition, haematology and serum biochemistry parameters were also examined (Table S4 and Table S5). Compared to DTX-CCL-PMs, the haematological changes such as panleukopenia, thrombocytopenia and reduction in reticulocytes were significantly higher in rats that received Taxotere.

4. Discussion

Long-circulating nanoparticles such as polymeric micelles are exploited with the aim to improve solubility, stability, pharmacokinetics and tumour accumulation of drugs by means of their capacity to encapsulate and target drugs to tumours via the EPR effect [3,43–45]. However, low stability of micelles in circulation and uncontrolled drug release rate remain critical issues [29,44]. As demonstrated in the present study, covalent conjugation of docetaxel (DTX) to CCL-PMs not only provided small-sized (66 nm) and stable micellar nanoparticles but also enabled prolonged systemic circulation with enhanced tumour accumulation and sustained release of DTX. Convincingly, treatment with a single administration of DTX-CCL-PMs led to complete regression of the human xenograft MDA-MB-231 breast tumours in mice. This remarkable antitumour efficacy was confirmed by the significantly enhanced tumour accumulation of targeted DTX, attributed to the prolonged systemic circulation of DTX-CCL-PMs and the EPR effect [29].

The synthesis of DTXL is straightforward. Although there are four hydroxyl groups in DTX, the methacrylated linker was selectively conjugated to DTX at its C-2′ hydroxyl group, the most amenable and sterically available group for structural modifications [46,47]. Such selective conjugation was also demonstrated in the work of Liu et al. in which PEG was selectively conjugated to DTX at the 2′-hydroxyl position [41]. Importantly, the manufacturing of DTX-CCL-PMs is a well-controlled step with confirmed scalability (up to multi-liters under Good Manufacturing Practices conditions), excellent batch-to-batch reproducibility and tailorable concentration (e.g. 5–20 mg DTX

equiv. per mL) (data not shown). Drug release from polymeric micelles is generally dependent on the degradation of the polymers and/or diffusion of the drug from the micelles, which leads to uncontrolled release of the encapsulated drug [48]. However, as shown in this study, a hydrolysis-sensitive covalent linkage of DTX to the CCL-PMs resulted in sustained release of the drug under physiological conditions (Fig. 1D). As indicated in Fig. 1D, the release of DTX from DTX-CCL-PMs is solely dependent on ester hydrolysis. Such hydrolysis from the CCL-PMs was also reported by Crielaard and coworkers with dexamethasone [32]. Compared to dexamethasone with the same derivatized unit ($t_{1/2} = 8.9 \pm 0.1$ days), DTX was released from the CCL-PMs at a much faster rate ($t_{1/2} = 1.5 \pm 0.3$ days). Ester hydrolysis kinetics is influenced by the electron-density of the surrounding moieties of the ester bond. Unlike dexamethasone, the derivatization of DTX occurred at a secondary hydroxyl group, which gave rise to a reduced electron density at 2′ carbon and therefore faster hydrolysis kinetics under physiological conditions.

With respect to the tumour accumulation, a single i.v. administration of DTX-CCL-PMs (30 mg/kg) rendered not only significantly greater total DTX levels but also higher released DTX levels in tumours as compared to Taxotere administration at the equivalent dose (Fig. 5A). The enhanced released DTX levels in tumour could be ascribed to the release of DTX from the CCL-PMs within the tumour microenvironment and possibly the accumulation of native DTX released from the circulating DTX-CCL-PMs. High tumour accumulation of DTX-CCL-PMs and intratumoural release of DTX obviously attributed to the strong antitumour effect of DTX-CCL-PMs.

At the reported MTD of DTX, i.e. 30 mg/kg (weekly injections) [40], DTX-CCL-PMs and Taxotere showed similar tumour inhibitory effects after three weekly i.v. injections. However, DTX-CCL-PMs had a clear benefit in terms of body weight in comparison to Taxotere (Fig. S3B). The absence of body weight loss in mice that received DTX-CCL-PMs was most likely attributed to the covalent entrapment of DTX within the CCL-PMs and thereby the lower 'active' DTX concentration in systemic circulation and lower exposure of normal tissues as compared to Taxotere.

Based on the high efficacy and good tolerability of DTX-CCL-PMs after multiple dosing, single dose studies were carried out to determine the therapeutic superiority over the marketed formulation. Compared to Taxotere, a clear gain in therapeutic efficacy with an increased dose of DTX-CCL-PMs (from 30 to 60 mg/kg) underlined the benefit of the targeted DTX. The lack of the benefit with Taxotere can be explained by the short plasma half-life and thereby no substantial gain in the tumour accumulation with the increase of the dose. Importantly, a further increase in the dose of DTX-CCL-PMs to 125 mg/kg induced complete regression of the tumours leading to 100% tumour-free survival. These antitumour effects were not only limited to the early stage tumours but also observed with the late-stage (established) tumours, which were completely suppressed with a single dose of DTX-CCL-PMs (Fig. 3). Complete regression of established tumours is highly challenging and rarely achieved in preclinical studies. Huang et al. reported profound regression of MDA-MB-231 tumours after multiple injections of DTX-loaded self-assembled nanoparticles, yet complete tumour regression was not achieved [24]. Similarly, substantial tumour regression was observed in the same breast cancer xenografts, although three i.v. injections of NC-6301 (polymeric micelles of DTX) at a 4-day interval were required [49]. In the present study, the remarkable therapeutic effects already obtained with a single dose of DTX-CCL-PMs highlight the potent antitumour activity of DTX-CCL-PMs and its potential for clinical application.

Since DTX is an anti-mitotic drug, it is primarily assumed to display its antitumour activity through tumour cell growth inhibition. However, interestingly we found that treatment with DTX-CCL-PMs also led to the reduction in tumour stromal components such as pericytes (α-SMA, NG2), fibroblasts (α-SMA) and extracellular matrix (collagen-1) at 96 h after a single dose administration (Fig. 4). In the tumour microenvironment, fibroblastic cells produce excessive extracellular matrix such as collagen, which induces tumour rigidity, less perfusion and increase in interstitial fluid pressure [50]. In addition, pericytes support endothelial cells and thereby the maturation of blood vessels during angiogenesis leading to enhanced nutrition supply to tumours. Inhibition of these components is highly essential to achieve complete regression of tumours. In contrast to DTX-CCL-PMs, treatment with Taxotere showed only a mild reduction in pericyte marker (NG2). These data corroborate with the recent findings of Murakami and coworkers who showed a reduction in collagen and α-SMA in orthotopic MDA-MB-231 tumour model after the treatment with a DTX-conjugate nanoparticle formulation (PEGylated acetylated carboxymethylcellulose-docetaxel conjugate) named Cellax [51]. Our data suggest that the observed antitumour effects of DTX-CCL-PMs are at least partially mediated through the depletion of tumour stroma in addition to the direct effect on tumour cells.

In addition to the studies in tumour-bearing mice, preclinical PK and tolerability studies were carried out in healthy rats as part of the clinical translation program of DTX-CCL-PMs. In the PK studies, the blood levels in healthy rats revealed that DTX-CCL-PMs remained in the circulation for extended periods of time ($t_{1/2} = 16.2$ h) and total DTX was detected in blood up to 7 days after a single dose of DTX-CCL-PMs (7.5 mg/kg) (Fig. 5B). Importantly, this demonstrates that DTX remained entrapped in PMs for several days due to the transiently stable covalent linkage. With respect to tolerability, DTX-CCL-PMs at 9.7 mg/kg was much better tolerated by rats as compared to a lower dose of Taxotere (6.7 mg/kg) (Table S3–S5). The superior tolerability of DTX-CCL-PMs is likely attributed to the blood circulation profile of the intact nanoparticles and thereby the absence of high DTX blood levels and significantly improved volume of distribution at steady state (0.06 L/kg) as compared to Taxotere (4 L/kg) [52]. It is of interest that major DTX dose limiting toxicities observed in the clinic such as diarrhea, pan-leukopenia and effects on immunologically related tissues occurred at a lesser severity and/or incidence in the DTX-CCL-PMs treated rats as compared to animals that received Taxotere. Together with other assays as required by regulatory authorities, these preliminary results of toxicology evaluation advocate the clinical translation of DTX-CCL-PMs.

5. Conclusions

In conclusion, this study demonstrates the development of a novel docetaxel-containing nanomedicine of which a single dose can regress both early and established human xenograft tumours completely, providing 100% tumour-free survival to these animals. Importantly, DTX-CCL-PMs were well tolerated by animals at the examined doses, showing superior tolerability to the marketed Taxotere formulation. Altogether, the improved therapeutic index of DTX-CCL-PMs and straightforward manufacturability strongly support its clinical development.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.02.085

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