HHS Public Access

Author manuscript

J Control Release. Author manuscript; available in PMC 2016 June 28.

Published in final edited form as:

J Control Release. 2015 June 28; 208: 67–75. doi:10.1016/j.jconrel.2015.02.024.

Poly(2-oxazoline) based Micelles with High Capacity for 3rd Generation Taxoids: preparation, *in vitro* and *in vivo* evaluation

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Abstract

The clinically and commercially successful taxanes, paclitaxel and docetaxel suffer from two major drawbacks, namely their very low aqueous solubility and the risk of developing resistance. Here, we present a method that overcomes both drawbacks in a very simple manner. We formulated 3rd generation taxoids, able to avoid common drug resistance mechanisms with doubly amphiphilic poly(2-oxazoline)s (POx), a safe and highly efficient polymer for the formulation of extremely hydrophobic drugs. We found excellent solubilization of different 3rd generation

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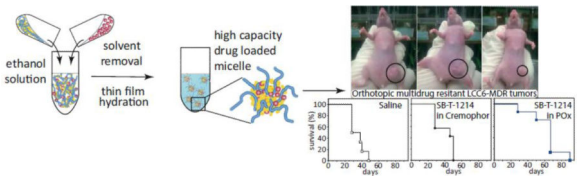
taxoids irrespective of the drug's chemical structures with essentially quantitative drug loading and final drug to polymer ratios around unity. The small, highly loaded micelles with a hydrodynamic diameter of less than 100 nm are excellently suited for parenteral administration. Moreover, a selected formulation with the taxoid SB-T-1214 is about one to two orders of magnitude more active *in vitro* than paclitaxel in the multidrug resistant breast cancer cell line LCC6-MDR. In contrast, in wild-type LCC6, no difference was observed. Using a q4d x 4 dosing regimen, we also found that POx/SB-T-1214 significantly inhibits the growth of LCC6-MDR orthotropic tumors, outperforming commercial paclitaxel drug Taxol and Cremophor EL formulated SB-T-1214.

Abstract



Keywords

paclitaxel, taxoids; drug delivery; nanomedicine; in vitro; in vivo; LCC6; orthotopic tumor model; multi-drug resistance; poly(2-oxazoline)

1. Introduction

Taxanes can arrest cells in the G2/M phase upon binding to the β-tubulin subunits to promote their polymerization and stabilize microtubules, which leads to apoptosis through cell-signal cascades. Several commercially successful and clinically significant taxoids have been developed, such as paclitaxel (PTX), docetaxel (DTX), and cabazitaxel (CBZ)[1]. They are heavily used in the treatment of breast, lung and ovarian cancer as well as other malignancies [2,3]. Unfortunately, these taxoids suffer from two major setbacks.

The first problem is that these compounds are very poorly water soluble [4] and require the use of toxic excipients in their clinical formulations, such as Cremophor EL (now commercialized as Kolliphor EL) and ethanol in Taxol (PTX), or Polysorbate 80 and ethanol in Taxotere (DTX), or Jevtana (CBZ). These excipients can cause severe hypersensitivity reactions [5]. Therefore, to reduce this toxicity the patients receiving these medications must be pre-treated with antihistamine, corticosteroid and H2 antagonist and be immediately removed from therapy if the hypersensitivity reactions are observed. Although many, potentially safer formulations have been developed for PTX and other taxanes [5-22], including the protein-drug nanoparticle, Abraxane and the polymeric micelle drug, Genexol-PM, the drug payload in these formulations remains relatively low and does not exceed 10% [23] for Abraxane, 17% [24] for Genexol-PM and 23% [25] for NK105. Also, significant improvement of the therapeutic outcome and patient survival, for example for treatment with Abraxane, remains to be verified [26].

The second problem is the development of drug resistance in response to the therapy with taxanes. Specifically, cancer cells resistance to PTX, DTX and CBZ can involve overexpression of ABC transporters (i.e. P-glycoprotein, Pgp; multidrug resistance-associated protein 1, MRP1), or point mutations in tubulin [27]. This problem was extensively discussed in the literature [27,28]. Thus, the "2nd-generation taxoids" were developed in which the C-3-phenyl group in taxoids was replaced with an alkenyl or alkyl group and the C-10 position was modified with various acyl groups [29]. Such 2nd-generation taxoids were shown to be one to two orders of magnitude more potent than the parent drugs against drug-resistant human breast cancer cells [29]. Additional substitution (*t*-Boc group) at the C-3'N position of the 2nd-generation taxoids further enhanced their potency against drug-resistant cancer cell lines (specifically Pgp+ mediated MDR and tubulin mutations). Examples of such compounds, termed "3rd generation taxoids", include SB-T-1213, SB-T-121302, SB-T-121303, SB-T-1214, SB-T-121402, SB-T-1216, and SB-T-121602 (Figure 1A,B) and were investigated in the present contribution. The new-generation taxoids were shown to be effective in LCC6-MDR (Pgp+ human breast cancer cell line); NCI/ADR-RES (Pgp+ human ovarian cancer cell line); 1A9PTX10 and 1A9PTX22 (human ovarian cancer cells originated from A2780 cell line possessing point mutations in class I-tubulin), as well as CFPAC-1, PANC-1, MIA PaCa-2 and BxPC-3 (human pancreatic cancer cell lines overexpressing multidrug resistance genes *mdr1*, *mrp1*, *mrp2*, and *lrp*). Furthermore, the taxoid SB-T-1214 was evaluated and demonstrated its efficacy against Pgp+ DLD-1 human colon tumor xenografts in severe combined immune-deficient (SCID) mice [29].

Unfortunately, the 3rd generation taxoids remain very poorly water soluble and require the use of appropriate drug carrier systems. We have recently discovered a novel polymeric drug carrier system, based on block copolymers of hydrophilic poly(2-methyl-2-oxazoline) (PMeOx) and mildly hydrophobic poly(2-butyl-2-oxazoline) (PBuOx). Interestingly, despite the low hydrophobic character of PBuOx, we have found that block copolymers (in particular triblock copolymers) of PMeOx and PBuOx exhibit a surprisingly high efficacy (both relative and absolute) for the solubilization of extremely hydrophobic drugs, including taxanes [14,21,22]. The capacity of POx micelles with respect to such taxanes is unprecedented. For example, POx/PTX micelles have ca. 4 to 5 times higher loading and ca. 10 to 20 times higher drug concentration in injectable formulations than the clinical alternatives of Taxol, Genexol-PM, and Abraxane. Poly(2-oxazoline)s (POx) in general have received increasing attention recently as alternatives to polyethylene glycol based systems [30-34] and first-in-man studies are expected to commence in 2015 [35,36]. Here, we combine the possibilities of our POx-based drug delivery platform for safe and efficient drug formulation and delivery with the advantages of 3rd-generation taxoids, which can overcome multidrug resistance mechanisms. Thereby we set out to develop a formulation, which safely addresses the pressing clinical challenge of drug resistance in cancer patients.

2. Materials and methods

2.1. Materials

Reagents and monomers for polymer synthesis as well as (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) was obtained from Sigma-Aldrich Inc. (St. Louis, MO or Steinheim, Germany). PTX and DTX were purchased from LC Laboratories (Woburn, MA). All other materials were from Fisher Scientific Inc. (Fairlawn, NJ), and all reagents were HPLC grade. MDA435/LCC6 (LCC6-WT) and MDA/LCC6^{mdr1} (LCC6-MDR) cells were obtained from Dr. R. Clarke, Georgetown University Medical School, Washington, DC. LCC6-MDR cells, which express high levels of Pgp, were derived from LCC6-WT cells transfected with a retrovirus engineered to constitutively express the mdr1 gene [37]. Cells were cultured in DMEM medium (Gibco 11965-092) supplemented with 10% FBS and 1% penicillin–streptomycin. The T11 orthotopic syngeneic transplant model is derived from the p53 null strain described by Medina et al. [38]. The T11 tumors were originally developed by serial orthotopic transplantation of a murine breast tumor derived from a p53-null mouse into a syngeneic p53 competent recipient, carrying sporadic, somatic K-Ras mutation and exhibiting an RNA expression pattern characteristic of the human claudin-low disease. Tumors growing out of this GEM were evaluated through RNA Microarray analysis as described recently [39]. Most tumors were determined to be of the triple-negative phenotype and T11 was chosen as the most representative claudin-low. T11 cells were cultured in RPMI medium containing 10% FBS and 1% penicillin/streptomycin. Nude mice were purchased from NCI and housed in UNC DLAM animal facility.

2.2. Preparation and characterization of POx/taxoids micelles

The amphiphilic triblock copolymers [P(MeOx$_{33}$-b-BuOx$_{26}$-b-MeOx$_{45}$), M_n = 10.0 kg/mol, PDI ($Đ_M$ = 1.14)], were synthesized by living cationic ring-opening polymerization as described previously [22] and used to prepare formulations of the 3rd-generation taxoids in polymeric micelles. Drug loaded POx micelles were prepared using the thin film hydration method [14]. Briefly, predetermined amounts of POx and drugs (stock solution 10–20 g/L in ethanol) were combined with small amount of ethanol and mixed well. Following a complete removal of ethanol (first, by drying the solution under a stream of air and second, *in vacuo)*, the formed thin film was rehydrated with appropriate amounts of deionized (DI) water or saline and heated at 50 –60 °C for 5–20 min (heating time was dependent on the drug concentration). Samples were allowed to cool to room temperature (r.t.) and centrifuged at 10,000 rpm for 3 min (Sorvall Legend Micro 21R Centrifuge, Thermo Scientific) to remove residual solid (if present). Only the transparent supernatant solution was used for the subsequent experiments. The hydrodynamic diameter and polydispersity index (PDI) of the micelles were determined by dynamic light scattering (DLS) using a Malvern Nanosizer and monitored for up to 9 days at r.t. for stability test.

2.3. HPLC Analysis of drugs in POx micelles

The amounts of drugs solubilized in POx micelles were quantified via reverse-phase high-performance liquid chromatography (HPLC) using an Agilent Technologies 1200 series HPLC system using a Nucleosil C18 5μm column (250 mm × 4.6 mm). The sample was diluted 20 times using mobile phase (specified below) and injected (20 μL) into the HPLC

system. A mixture of acetonitrile (ACN)/water (55/45, v/v) was used as the mobile phase. The flow rate was 1.0 mL/min, and column temperature 30° C. Detection wavelength was 228 nm.

The following equations were used to calculate the drug loading capacity (*LC*), loading efficiency (*LE*):

$$LC = \frac{m_{drug}}{m_{drug} + m_{excipient}} \cdot 100\%, \quad (1)$$

$$LE = \frac{m_{drug}}{m_{durg \quad added}} \cdot 100\%, \quad (2)$$

where m_{drug} and $m_{excipient}$ are the weight amounts of the solubilized drug and polymer excipient in the solution, while $m_{drug\ added}$ is the weight amount of the drug added to the dispersion. Drug concentration (*DC*) was determined by HPLC and calculated against free PTX standards.

2.4. *In vitro* drug release

The drug release from POx micelles was studied using the membrane dialysis method against phosphate buffered saline (PBS), pH 7.4 at 37 °C. Briefly, the drug loaded POx micelle formulations were diluted with PBS to yield solutions of approximately 0.1 mg/mL of each drug. Then the resulting solutions (100 μL) were placed in 100 μL floatable Slide-A-Lyzer MINI dialysis devices with a MWCO of 3.5 kDa (Thermo Scientific) and suspended in 20 mL of PBS. One device was used for every time point. At each time point the sample was withdrawn from the dialysis device and the remaining drug amount of sample was quantified by HPLC.

2.5. *In vitro* cytotoxicity assay

In vitro cytotoxicity of drug-loaded POx micelles was determined using MTT assay. Four formulations, namely Taxol, Abraxane, POx/PTX and POx/SB-T-1214 were compared using each cell line. Briefly, cells were seeded in 96-well plates at a density of 4000 cells/well 48 h prior to drug treatment. Cells were treated for 24 h with respective drug formulations each prepared at series of dilutions in the full medium. After this incubation, medium was removed and cells were further incubated with fresh medium for another 72 h. Subsequently, the medium was again removed and 100 μL of fresh medium with MTT (100 μg/well) reagent was added for additional 4 h incubation at 37 °C. Finally, the medium was discarded, and the formed formazan salt was dissolved in 100 μL of DMSO and absorbance was read at 562 nm using a plate reader (SpectraMax M5, Molecular Devices). Cell survival was calculated as normalized to control untreated wells. Data is presented as means (n = 6) ± standard error means (SEM). The mean drug concentration required for 50% growth inhibition (IC_{50}) was determined using Graphpad Prism 5 software.

2.6. *In vivo* maximum tolerated dose (MTD) of drug-loaded POx micelles

All animal experiments were carried out with approval of the University of North Carolina Institutional Animal Care and Use Committee. MTD evaluation for POx/SB-T-1214 micellar formulations was performed in dose escalation study in 6-8 week old female NCI nu/nu mice. Animals (n = 3 per group) received i.v. injections (tail vein) of 20, 40, 60, 90, and 120 mg/kg of SB-T-1214 in POx micelles using a q4d x 4 regimen (total 4 times repeated dosing, every 4th day with saline as a control). Mice survival and changes in body weight were observed daily over two weeks in all groups following the last injection. The highest dose that did not cause animal death or noticeable toxicity (as defined by a median body weight loss of 15% of the control or abnormal behavior including hunched posture and rough coat) was used as MTD for efficacy experiment.

2.7. *In vivo* efficacy study

The efficacy of POx/SB-T-1214 polymeric micelles was evaluated in LCC6-MDR orthotopic breast cancer model. Briefly, 100 μL of cell solution containing 50 % (v/v) 8×10^6 LCC6-MDR cells suspended in DMEM medium (vide supra) and 50 % (v/v) Matrigel are implanted into mammary fat pad of 8-week-old female nude mice using a 25 G needle. Every 4 days, perpendicular tumor diameters were measured by digital caliper and used to calculate tumor volume according to the formula: volume = $Dd^2/2$, where D equals larger diameter and d equals smaller diameter. When tumor volumes reached about 300 mm^3, animals were treated with all formulations by q4d x 4 regimen. Following treatment groups (n = 7) were compared: 1) Saline; 2) POx Polymer; 3) Taxol (20 mg/kg PTX); 4) Abraxane (80 mg/kg PTX); 5) Cremorphor EL(CRE)/SB-T-1214 (20 mg/kg); and 6) POx/SB-T-1214 micelles (20 mg/kg). Tumor volume and animal survival were monitored 2 times per week. Mice were sacrificed when the tumor reached a volume of 2000 mm^3 or developed ascites metastasis.

The efficacy of POx/SB-T-1214 micelles was also investigated in T11 murine breast cancer orthotopic syngeneic transplant (OST) model (claudin-low subtype). Tumor volumes reached about 10-50 mm^3 on 5^{th} day following T11 cell transplant. This was defined as day 0. On day 4, we started to treat animals with all formulations by q4d regimen until tumor remission or experimental endpoints. The following treatment groups (n = 10) were compared: 1) Saline; 2) Taxol (20 mg/kg PTX); 3) Cremophor EL (CRE)/SB-T-1214 (20 mg/kg, MTD dose); and 4) POx/SB-T-1214 micelles (20 mg/kg). Tumor volume and survival were monitored 3 times per week. Mice were sacrificed when the tumor reached a volume of 3500 mm^3 or upon signs of ulceration.

3. Results

3.1. Formulation of new taxoids in POx micelles

Here, we employed an amphiphilic triblock copolymer [$P(MeOx_{33}$-b-$BuOx_{26}$-b-$MeOx_{45})$, M_n = 10.0 kg/mol, $Đ_M = M_w/M_n$ = 1.14)] for polymeric micelle formulations of 3^{rd} generation taxoids using a thin-film approach. The chemical structures of investigated taxoids and PTX are depicted in Fig. 1A-C. Stock solutions of these drugs and the polymer were prepared and combined in appropriate ratios. The solvent was removed and the

resulting polymer film was hydrated using deionized water or USP saline (Fig. 1D), resulting in the formation of the drug loaded polymer micelles. The polymer concentration in the final formulation was set to 10 g/L, while the drug concentration was varied from 5 g/L to 15 g/L. The actual maximum loading capacity, *LC* that was achieved for the different drugs was between 40 and 50 wt.% for 10 g/L or 12 g/L (15 g/L in one case (SB-T-121602)), respectively (Fig. 1E). Similar to previous studies [14,22], the drug loading efficiency, *LE* were high, until the maximal *LC* values were achieved, after which *LC* dropped considerably for all taxoids investigated (Table 1). Due to the limited amounts of compounds available, no extensive stability tests were performed. However, during our experiments, we did not encounter any stability issues and the stability of PTX in POx micelles has been shown to be extraordinarily high in previous studies [14,22].

3.2. Physicochemical characterization and drug release of POx/SB-T-1214 micelles

According to previous studies [27,29], SB-T-1214, a 3rd generation taxoid, is an excellent candidate to overcome drug resistance. It exhibited high activity *in vitro* against many drug resistant cancer cell lines including LCC6-MDR, NCI/ADR-RES, 1A9PTX10, 1A9PTX22, CFPAC-1, PANC-1, MIA PaCa-2, BxPC-3, and CFPAC-1. Furthermore, its anti-tumor activity was also evaluated *in vivo* using a Pgp+ human colon cancer DLD-1 xenograft tumor model [29]. In this study, Tween 80/ethanol was used as excipients for solubilizing the drug.

Here, we solubilized SB-T-1214 in POx polymeric micelles for further *in vitro* and *in vivo* activity studies. In order to scale up the formulation for *in vivo* studies, we set the polymer concentration at 50 g/L and used 10, 20 and 45 g/L as the initial drug feeding concentrations. Even at such high concentrations, the drug incorporation into the micelles was nearly quantitative and the *LC* values achieved were excellent with 16 wt% (10 g/L), 28 wt.% (19.2 g/L) and 46 wt.% (41.8 g/L), respectively. These formulations will be denoted 50/10, 50/20 and 50/40, respectively. Noteworthy, the highest achieved drug concentration of 41.8 g/L in the POx micelles is ca. 9500 fold of the intrinsic solubility of SB-T-1214 in water at room temperature (4.4 mg/L, determined by optimized shake-flask method). The size of the drug-loaded POx micelles was determined by DLS. The data suggested that the size (z-average hydrodynamic diameter D_z) of the drug-loaded micelles depends on the loading but remains below 100 nm: 15 nm, 26 nm and 75 nm for 50/10, 50/20 and 50/40 formulations, respectively. Moreover, the drug-loaded micelles were found to be well defined with a relatively small PDI (<0.2) (Fig. 2A) and of spherical morphology as evidenced by transmission electron microscopy (TEM) (Fig. 2B,C). These results correspond well to our previous results with PTX formulations [22]. We monitored the formulations by DLS for 9 days at r.t. and observed no significant changes in the particle size and PDI (Fig. 2D,E), suggesting that the micelles were stable at r.t. for at least 9 days. Also, no drug crystallization or precipitation was observed by visual inspection of the micellar nanoformulation. This is important to note, since taxoids typically exhibit a tendency for crystallization and it is often difficult to obtain formulations that are stable in aqueous media.

In contrast, as we applied sink conditions in a dialysis experiment to test the potential release of the drug from the micelles, the drug was continuously released, with > 80% of released drug after 24 h. Under the present experimental conditions, a burst release was observed. The release was essentially identical for both 50/20 and 50/40 formulations. However, the release of the drug from the 50/10 formulation was significantly slower with less of a burst release character (Fig. 2F). Accordingly, there was little concern that the drug would not be released, despite the excellent stability in the absence of sink conditions.

3.3. *In vitro* cytotoxicity of POx/SB-T-1214 micelles in cancer cells

Since SB-T-1214 was known to be effective against multidrug resistance cell lines that overexpress Pgp [29], we evaluated the *in vitro* drug efficacy of the 50/40 formulation and compared it to Abraxane, Taxol and POx/PTX formulations in wild-type LCC6-WT and multidrug resistant LCC6-MDR cells using MTT assay.

In multidrug resistant LCC6-MDR cells, the cytotoxicity profile of POx/SB-T-1214 clearly shifted to lower concentrations as compared to the other three formulations of PTX. IC_{50} value was determined as 34.6 ng/mL for POx/SB-T-1214, which was much lower than 769, 536, and 1385 ng/mL, determined for Abraxane, Taxol and POx/PTX, respectively (Fig. 3A). We also performed MTT assays in LCC6-WT cells and observed IC_{50} values of the same order of magnitude for all four formulations, specifically 5.8 for POx/SB-T-1214 and 9.4, 18.4 and 17.8 ng/mL for Abraxane, Taxol and POx/PTX, respectively (Fig. 3B). When comparing the effectiveness of the drug formulations in resistant vs. wild-type cells using the Resistance factor (R/S) = (IC_{50} for drug resistant cell line, R)/(IC_{50} for drug-sensitive cell line, S), we found that POx/SB-T-1214 had R/S value of ca. 6 while for Abraxane, Taxol and POx/PTX values of around 82, 29 and 78, respectively were obtained. This result indicates that POx/SB-T-1214 is very potent against both non-resistant wild-type and MDR cells, while the other three formulations containing PTX are only active against wild-type and are less efficient against MDR cells (Table 2).

In addition, we tested the extremely aggressive T11 murine cancer cell line, which is characterized as a claudin-low subtype of triple negative breast cancer (TNBC) and known for its extremely poor prognosis. Similar to the result observed using LCC6-MDR cells, the IC_{50} was about 23 times lower for POx/SB-T-1214 (43 ng/mL) as compared to POx/PTX (983 ng/mL) (Fig. 3C; Table 2).

3.4. *In vivo* MTD studies

The MTD evaluation was performed in a dose escalation manner in healthy 6-8 week old female nude mice, which received 20, 30, 40, and 60 mg/kg of POx/SB-T-1214 (50/40) micelles using a q4d x 4 regimen. At 30 mg/kg dose, the animal lost more than 15% of their body weight after the second dosing. Therefore, the MTD was determined as 20 mg/kg at this dosing regimen (Fig. 4A).

We hypothesized that at higher polymer content, the drug release might be slower (analogous to our *in vitro* results) and thus, the MTD might be higher. Therefore, we investigated whether changes in the formulation or the dosing regimen would lead to an

increase of MTD. However, adjustments of the formulations were not successful in this respect. Since changes of the polymer/drug ratio in the formulation (50/10 or 50/20 at 40 mg/kg, Fig. 4B) did not improve the MTD in any way, we modified the treatment regimen by increasing dose intervals to a weekly injection for 4 weeks (q7d x 4). This improved the situation slightly. Mice body weight loss remained below 15% until the third injection at day 21. Thus, we concluded that MTD was less than 40 mg/kg under this weekly dosing regimen (Fig. 4B).

3.5. *In vivo* efficacy in the LCC6-MDR model

In vivo efficacy of POx/SB-T-1214 was evaluated in the orthotopic LCC6-MDR mouse model using MTD doses for all groups to achieve the best therapeutic effects possible (Fig. 5 and Supporting Fig. S1). The Cremophor EL(CRE)/SB-T-1214 treatment group displayed a similar growth rate as groups treated with saline and POx polymer alone groups - all showing a tumor volume increase from ca. 300 to 2,000 mm^3 within 4 weeks. Taxol slightly decreased the rate of the tumor growth but the difference was not statistically significant as compared to saline, CRE/SB-T-1214 and POx groups. In contrast, in the POx/SB-T-1214 treatment group, the tumor volume reached only ca. 700 mm^3 on the 28 day (Fig. 5A). Abraxane at 80 mg/kg (MTD dose) also showed significant tumor inhibition compared to CRE/SB-T-1214. However, while the tumor growth for the Abraxane-treated group was similar to that in the POx/SB-T-1214 group, no statistically significant difference in survival was observed between the Abraxane and saline groups (Fig. 5B). In contrast, treatment with POx/SB-T-1214 significantly extended the survival time with a median survival of 67 days ($p = 0.0003$). Median survival in Abraxane, CRE/SB-T-1214, Taxol and saline groups were 37, 46, 37 and 33 days, respectively. Representative images of mice at day 26 with orthotopic tumors clearly show the differences in the tumor burden between these groups with a visibly reduced tumor burden in the POx/SB-T-1214-treated mice (Fig. 5C).

Body weight loss over 15% or other signs of severe toxicity were not observed in any treatment group, although the animals in the CRE/SB-T-1214 and POx/SB-T-1214 groups showed about 10% weight loss after four injections, which was regained after 3 weeks (Fig. 5D). Injection site inflammation and sometimes prompt shock upon injections (animals eventually recovered) were seen in Taxol and CRE/SB-T-1214 groups, which was probably associated with the excipient Cremophor EL.

We also tested our POx/SB-T-1214 (50/40) formulation in the very aggressive T11 orthotopic syngeneic transplant (OST) model. At 20 mg/kg, POx/SB-T-1214 was able to suppress the tumor growth to some extent. The treatment outcomes in the CRE/SB-T-1214 and Taxol groups differed significantly. In the CRE/SB-T-1214 treatment groups the tumors rapidly proliferated to 3,000 mm^3 within 20 days and no effect of the treatment was observed compared to the control (Fig. 6A). The Kaplan-Meier survival plots (Fig. 6B) show that 90% of mice in this treatment group needed to be sacrificed at day 19, which is a worse outcome as compared to saline control. The animals treated with Taxol fared only little better, if at all. In contrast, POx/SB-T-1214 significantly improved the survival time such that, at day 20, only one mouse had to be sacrificed. The survival curve declined gradually and 30% of mice survived until day 27.

4. Discussion

New and clinically relevant formulations of taxanes and taxoids remain a matter of considerable interest. For instance, an amphiphilic block copolymer consisting of poly(ethylene glycol) (PEG) and 4-phenyl-1-butanol modified poly(aspartate) was designed to physically entrap PTX. During the self-assembly process, the polymer forms micelles, which incorporate PTX into their core through hydrophobic interactions between the drug and modified poly(aspartate) (hydrophobic segment). This formulation, designated as NK105, can incorporate 23% (w/w) PTX and has shown less toxicity and enhanced efficacy compared to free drug in the preclinical and clinical development [40,41]. Another example is the targeted PEG-poly($_{D,L}$-lactide-coglycolide) (PLGA) polymer formulation of DTX using an RNA aptamer A10 as targeting moiety that binds to the extracellular domain of the prostate-specific membrane antigen on the surface of prostate cancer cells [42]. It has been shown that the targeted nanoparticles enhance cellular uptake compared to their non-targeted counterparts *in vitro* and *in vivo*. However, use of this delivery system may be limited because of its rather large particle size (160-290 nm) and its very low drug loading (<1%).

Similar to our previous work on POx polymeric micelles for PTX [14,21,43], we used the triblock copolymer with a central hydrophobic BuOx block and two flanking hydrophilic MeOx blocks in this study. The molar mass of the polymer is approximately 10 kg/mol. Therefore, while the polymer micelles (> 10 nm) are likely to be well above the renal excretion threshold, the unimers are well below this threshold and thus expected to be rapidly excreted by renal filtration [44]. Since the synthesis of POx is based on a living cationic ring-opening polymerization, the polymers are well defined and accessible in a reproducible manner. In addition, POx (co)polymers alone displayed very little, if any, toxicity up to concentrations of 10 g/L in various cell lines [14,45-48]. We have previously demonstrated the extremely high loading capacity of the POx micelles for several hydrophobic drugs [14,21,22]. In the present work, for all taxoids studies *LC* values between 45 and 50% were achieved while SB-T-121303 could be loaded at over 50%.

Similar to previous studies [21,22] the drug loaded spherical micelles were found to be well defined (PDI <0.2) and relatively small with D_z of 15 to 75 nm, which did not change in size over 9 days at r.t.. The morphology and size of the loaded micelles during drug release has not been determined so far. In a recent study [22], we investigated the morphology of the micelles in dependence of different PTX concentrations. Based on these results, it can be expected that for the low drug concentrated formulation (50/20 and 50/10) no change in micelle size should occur during drug release. Comparing the results of Schulz et al. and present study, we deduce that the micelle size and likely the morphology are very similar, whether PTX or other taxoids are loaded. On the other hand, for the high loaded micelles a decrease in size might be likely with decreasing drug concentration. For the formulation 50/20 and 50/40 there might be also a change in morphology upon complete release of the drug. In the above mentioned study a change of morphology of the micellar core towards a raspberry-like shape was observed via SANS at 9wt% PTX and higher. There is also the possibility of formation of wormlike micelles at very low PTX concentration. However, we would also like to note that such studies of size and morphology were obviously not performed *in vivo*. Such an endeavor would be virtually impossible at the current state of art.

Relevance of *in vitro* release and size and morphology for in vivo performance is in any way questionable.

In the present study, the POx micelle formulation of taxoid SB-T-1214 improved the drug efficacy in the LCC6-MDR model as compared to PTX formulation or SB-T-1214 formulated with Cremophor EL and ethanol, the vehicle used in the commercial Taxol formulation. The reason for this increased efficacy remains unknown to date. A detailed pharmacokinetic study may help to elucidate this matter, but this was outside the scope of the present study. We also found that Abraxane effectively reduced the tumor growth, but did not prolong the survival. It is noteworthy that Abraxane was much less effective than SB-T-1214 *in vitro* but at MTD exhibited similar tumor growth inhibition as SB-T-1214 *in vivo*. In this regard we would like to point out that a correlation between *in vitro* tumor growth inhibition and *in vivo* efficacy is not straightforward and generally should not be expected. In this regard *in vitro* experiments performed on cell monolayers may only reflect that a researched compound is pharmacologically active. In contrast, the *in vivo* efficacy accounts for a much more complex set of factors including tumor a drug distribution to the tumor, cancer cells heterogeneity, interactions with the tumor microenvironment and contribution of the off-target side effects at the level of the whole organism. The “mismatch” between *in vitro* and *in vivo* activities is well documented in the literature for many drugs. For example, two highly selective progesterone receptor modulators showed 4-fold potency difference *in vitro*, while exhibiting similar efficacy in rats against mutagen 7,12-dimethylbenz[*a*]anthracene induced breast tumor [49]. Another example involves the derivatives of statin-class drugs. One such derivative cerivastatin, is 6 to 7 times more potent than another derivative, pitavastatin *in vitro* in U87 glioma and MDA-432 breast cancer cell lines. However, cerivastatin demonstrated similar, if not worse, tumor inhibition compared pitavastatin *in vivo* [50]. Moreover, a mismatch between *in vitro* and *in vivo* efficacy was also reported for different formulations of paclitaxel, specifically, Taxol and Abraxane. Thus, Demeure et al. reported that Abraxane has similar *in vitro* inhibition (IC_{50} = 0.33 μM = 282 ng/mL) in H295R cells, as Taxol (IC_{50} = 0.35 μM = 299 ng/mL). However, Abraxane showed a significantly better efficacy than Taxol *in vivo* in H295R xenograft adrenocortical cancer model. Another example also suggests that Abraxane has similar *in vitro* activity as Taxol but outperforms the latter in tumor inhibition *in vivo* in pediatric solid tumors [51]. Overall, the improved efficacy and higher response rate to Abraxane in preclinical and clinical studies compared to other drug formulations [23] may be attributed to the gp60 mediated transport of paclitaxel-loaded albumin into tumor cells or its binding to an extracellular matrix protein, SPARC (secreted protein acidic rich in cysteine), which increases Abraxane accumulation in the tumor. We also would like to point out that in our work Abraxane is used *in vivo* at its MTD dose (80 mg/kg), and is much more efficient in tumor inhibition than Taxol at 20 mg/kg while having comparable efficacy to POx/SB-T-1214 micelles at 20 mg/kg. At the same time the effect of Abraxane on the animal's lifespan non-significant compared to Taxol and both agents are much less effective in this regard than POx/SB-T-1214 micelles.

In addition to LCC6-MDR tumors, we also evaluated our POx/SB-T-1214 formulation using the T11 murine cancer model. This is an extremely aggressive cancer model that faithfully

recapitulates claudin-low breast cancer, a subtype of TNBC recently classified via gene expression profiling, exhibiting particularly poor prognosis [39,52]. It is an OST model, which was established via isolating cells from the mammary gland of genetically engineered balb/c mice which were null for p53, and genetically engineering them to tumor cells carrying claudin-low subtype and subsequently transplanting the cells orthotopically. Abraxane was excluded in this set of studies due to expected immunogenicity upon injection of human albumin to immuno-competent mice. It should be noted that most previously tested chemotherapeutic drugs were not effective in this model and typically tumor growth curves of groups treated with a single drug show no difference to control [52].

The *in vitro* MTT results suggested that SB-T-1214 is more active than PTX in T11 cells. The T11 cells might be intrinsically resistant to chemotherapy with agents such as PTX. Despite the inability to produce long-term survivors, the performance of our formulation *in vivo* is very promising when one takes into account the inability for other single drug chemotherapeutic regimens to achieve any efficacy in this cancer model [52]. Although these aggressive tumors will ultimately continue to grow, combination therapies [17] that involve the POx micelle delivery system and new generation taxoids along with other anticancer drugs are worth exploring in the future. The present platform readily allows for combination therapy [21] and is therefore very well suited for exploring new treatments of such challenging cancer models.

5. Conclusion

We presented the first example of formulation of 3rd generation taxoids using amphiphilic POx block copolymer. All taxoids studied could be incorporated at a nearly 1/1 ratio ($w_{taxoid}/w_{polymer}$) resulting in stable formulation with 50 %wt. of active drug. The micellar size of the nanoformulation remained around or below 100 nm as evidenced by DLS and transmission electron microscopy. The efficacy of the selected 3rd generation taxoid SB-T-1214 *in vitro* against MDR cancer cell lines was higher than that of PTX, while no difference was observed in the non-resistant cell line. Although the MTD of SB-T-1214 formulated with Cremophor EL and the POx block copolymer were identical, the tumor inhibition using the POx/SB-T-1214 polymeric micelles was enhanced in two orthotopic multidrug resistant tumor models, LCC6-MDR and T11. The latter model is characterized as a particularly faithful and clinically relevant model TNBC. Survival in this model may be further improved by using synergistic drug combinations, for which the POx polymeric micelle platform is ideally suited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was supported by a Cancer Nanotechnology Platform Partnership grant (U01 CA116591) of the National Cancer Institute Alliance for Nanotechnology in Cancer to AVK, and a grant from the National Cancer Institute (R01 CA 103314) to IO. Zhijian He is grateful for support from GlaxoSmithKline Clinical Research and Drug Development Fellowship. We thank the directors and personnel of the UNC Lineberger Comprehensive Cancer Center's Mouse Phase One Unit (MP1U) for assistance with animal handling, therapeutic studies and tumor serial

assessment as well as the Animal Studies Core (ASC). Work in the MP1U and ASC was supported by the University Cancer Research Fund. Rainer Jordan is grateful for financial support via the Dresden Initiative for Bioactive Interfaces and Materials (DIB).

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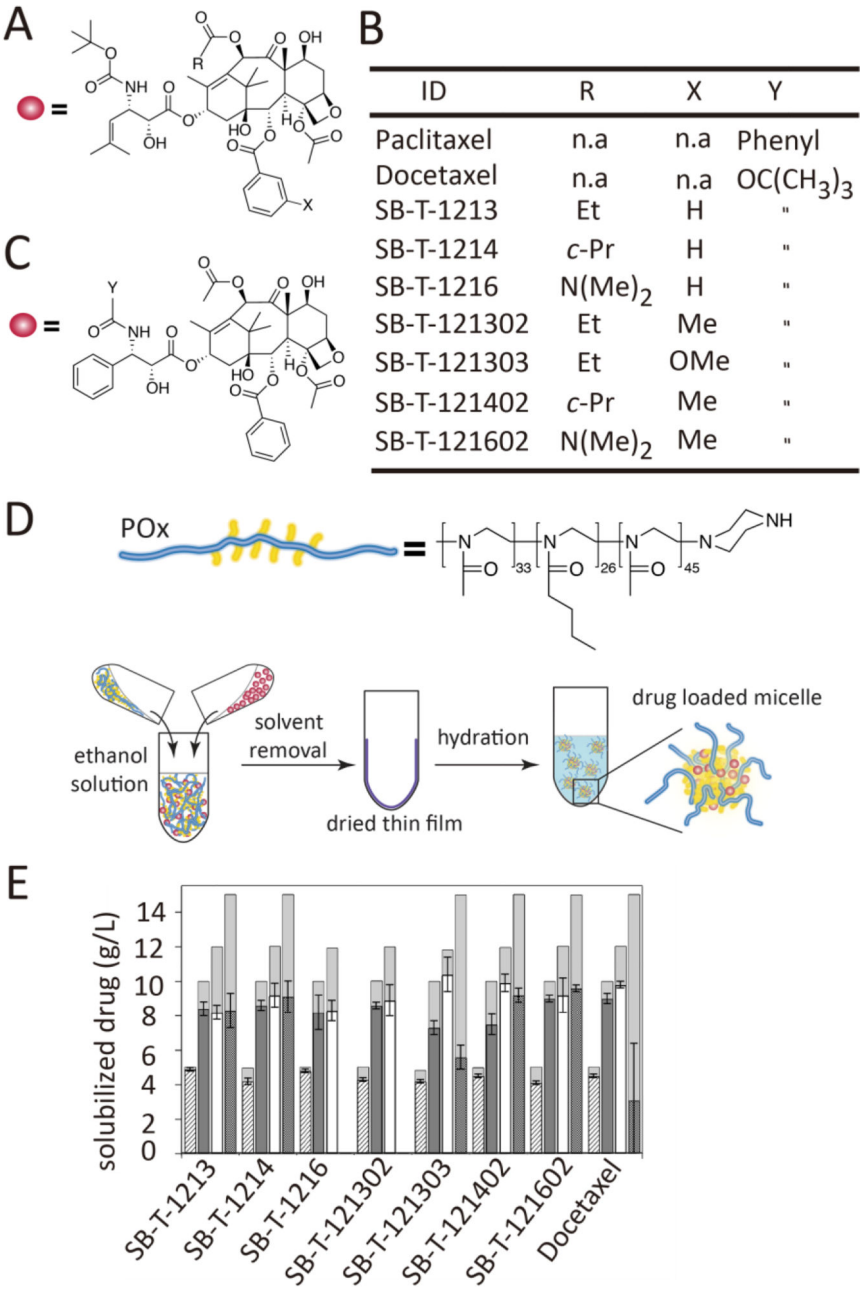

Fig. 1.

Preparation of POx/taxoid micellar nanoformulations. (A,B) Chemical structures of 3rd generation taxoids. (C) Chemical structure of PTX/DTX (D) Schematic showing of formation of POx/taxoid micelles formed through self-assembly. (E) Drug loading of taxoids in POx micelles. POx concentration was fixed at 10 g/L while taxoid feeds were 5, 10, 12 and 15 g/L (light gray bars in background), respectively.

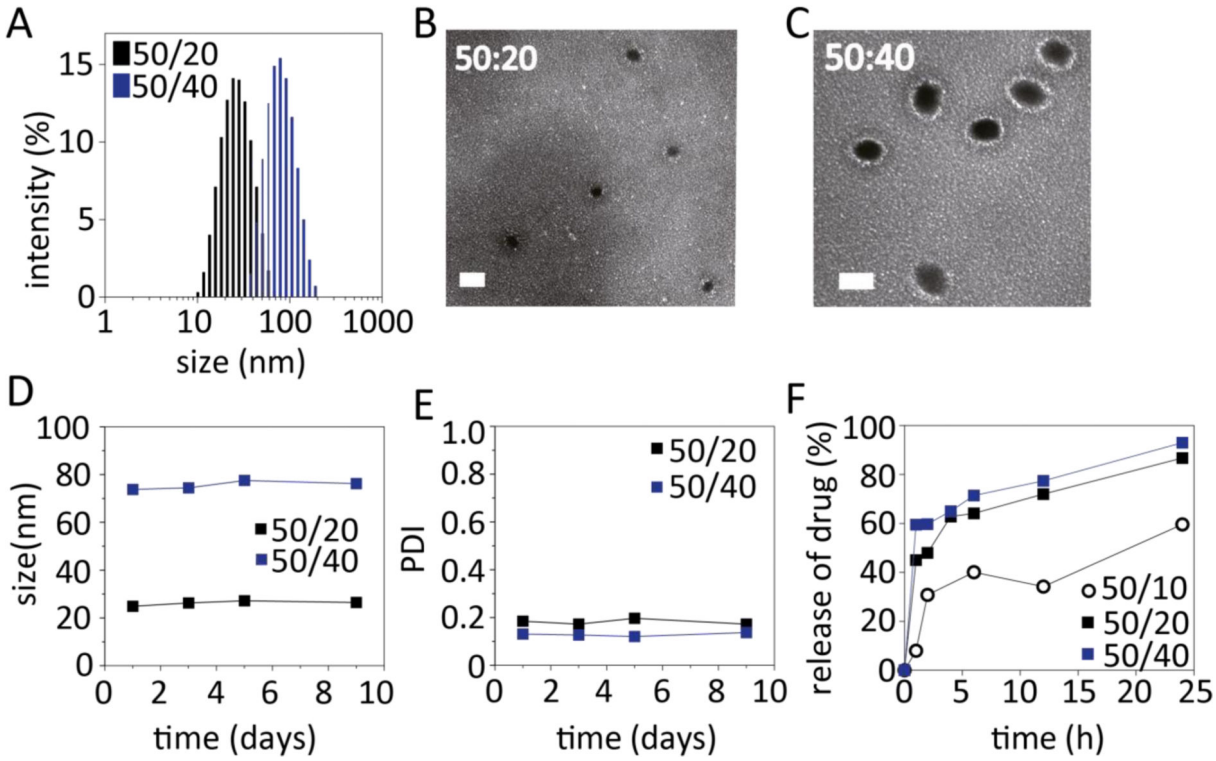

Fig. 2.

Physicochemical properties of various POx/SB-T-1214 polymeric micelle formulations. (A) Size (z-average, D_z) and size distribution of POx/SB-T-1214 at 50/40 and 50/20 ratios measured by DLS. (B,C) TEM micrograph of POx/SB-T-1214 at 50/40 and 50/20 ratios. Scale bar = 100 nm. Stability of the POx/SB-T-1214 micelles at r.t. as determined by the size (D) and PDI (E) measurements over time. (F) Drug release profiles of SB-T-1214 from POx micelles at different polymer/drug ratios of 50/40, 50/20 and 50/10. The drug release study was performed at 37 °C in PBS buffer at pH 7.4.

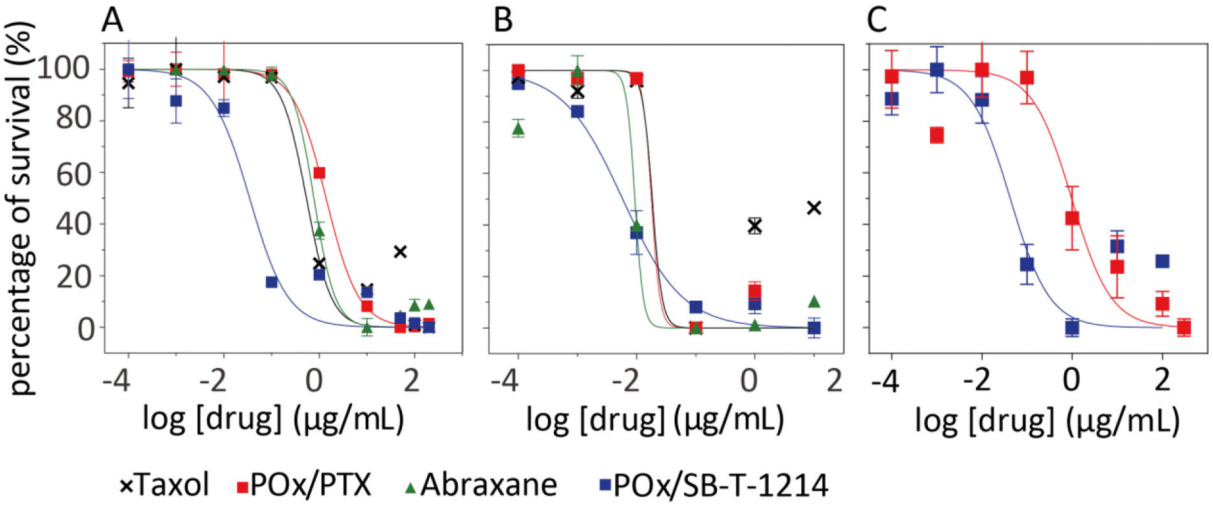

Fig. 3.

In vitro cytotoxicity of various PTX and SB-T-1214 formulation in (A) LCC6-MDR cells, (B) LCC6-WT, and (C) T11 cells (mean ± SEM, n = 6).

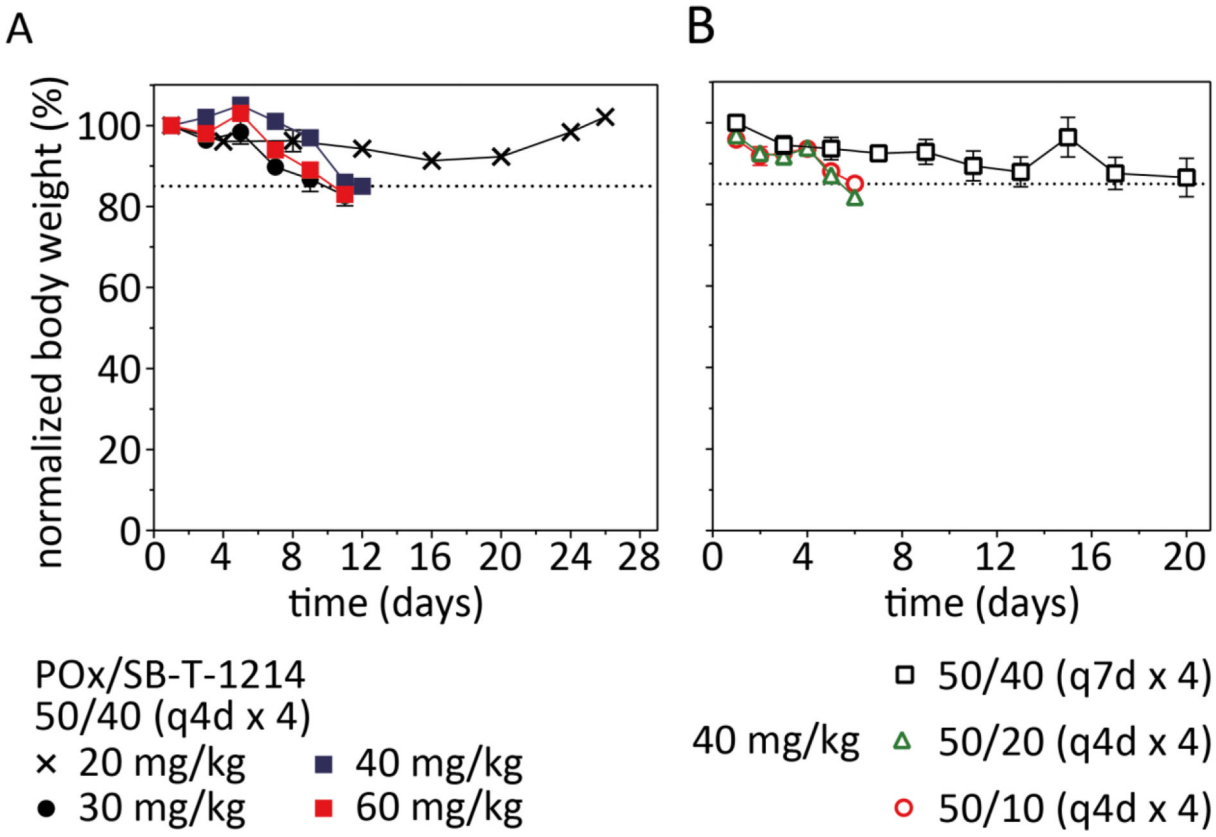

Fig. 4.

Establishment of the safe dose of SB-T-1214 in nude mice. (A) MTD of POx/SB-T-1214 = 50/40 formulation using a q4d x 4 treatment regimen in escalating doses from 20-60mg/kg. (B) MTD of POx/SB-T-1214 = 50/20 and 50/10 using a q4d x 4 regimen or 50/40, 40 mg/kg using a q7d x 4 regimen.

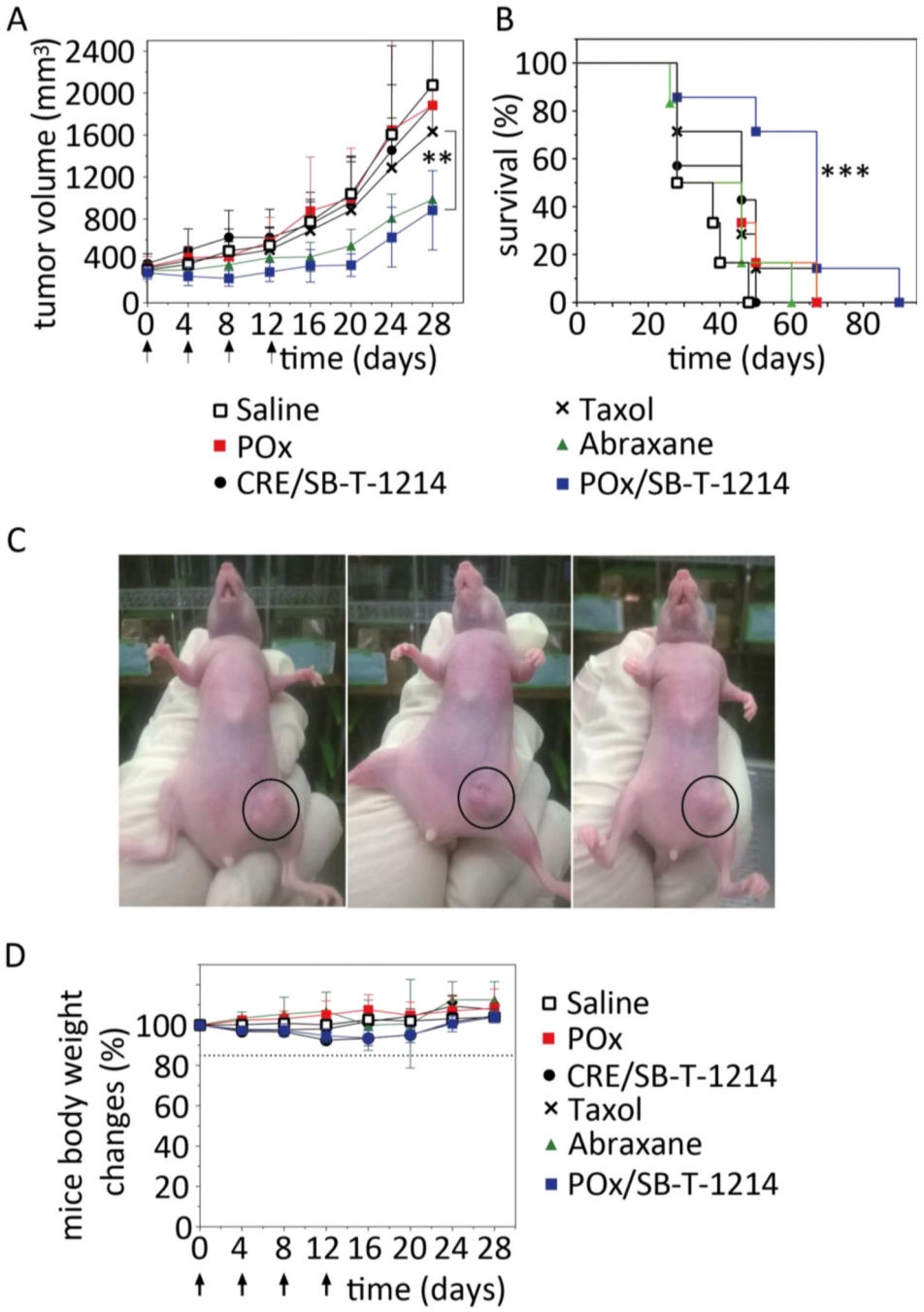

Fig. 5.

Efficacy of various drug formulations at MTD doses in LCC6-MDR tumors. (A) Tumor growth inhibition of POx/SB-T-1214=50/40 formulation (20 mg/kg) compared to Taxol (20 mg/kg), Abraxane (80 mg/kg) and CRE/SB-T-1214 (20 mg/kg), saline as well as POx polymer alone (equivalent polymer amount as POx/SB-T-1214 micelle formulation). Each formulation was injected on days 0, 4, 8, 12. Data is expressed as mean ± SEM, n = 7. *** $p < 0.001$ (vs. saline group). (B) Kaplan-Meier survival plot for all groups. (C) A

representative image of treated mice at day 26. Left, saline group; middle, CRE/SB-T-1214 group; right, POx/SB-T-1214 group. (D) Body weight loss for each treatment group.

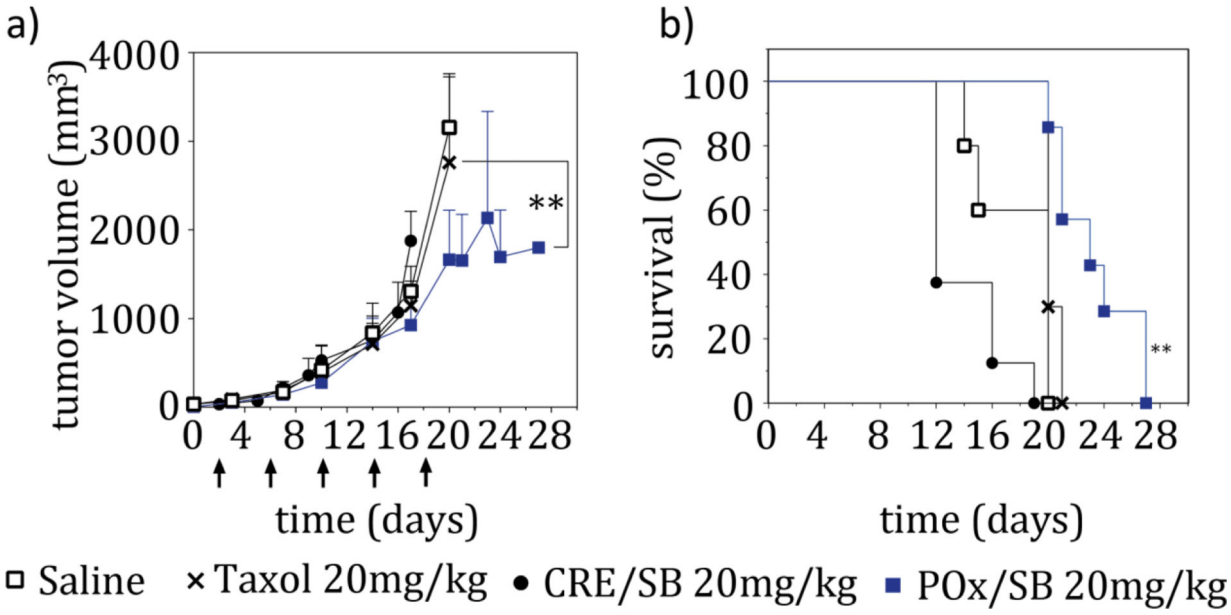

Fig. 6.

Efficacy of various drug formulations in T11 OST tumors. (A) Tumor growth inhibition of POx/SB-T-1214 = 50/40 formulation compared to Taxol, CRE/SB-T-1214 as well as saline. Each formulation was injected on days 2, 6, 10, 14 and 18. Data is expressed as mean ± SEM, n = 10, ** $p < 0.01$. (B) Kaplan-Meier survival plot for all groups.

Table 1

Data of solubilization experiments. Polymer concentration was set to 10 g/L. Data is presented in means ± standard deviation (n = 3).

	5 g/L			10 g/L		
Drug ID	**DC (g/L)**	**LE (%)**	**LC (%wt)**	**DC (g/L)**	**LE (%)**	**LC (%wt)**
SB-T-1213	4.9±0.1	98	33	8.4±0.4	84	46
SB-T-1214	4.2±0.2	84	30	8.6±0.3	86	46
SB-T-1216	4.8±0.1	96	32	8.2±1.0	82	45
SB-T-121302	4.3±0.1	86	30	8.6±0.2	86	46
SB-T-121303	4.2±0.1	84	30	7.3±0.4	73	42
SB-T-121402	4.5±0.1	90	31	7.5±0.6	75	43
SB-T-121602	4.1±0.1	82	29	9.0±0.2	90	47
Doxetaxel	4.5±0.1	88	31	9.0±0.3	90	47

Drug ID	**12 g/L**			**15 g/L**		
SB-T-1213	8.2±0.4	68	45	8.3±1.0	55	45
SB-T-1214	9.2±0.7	77	48	9.1±0.9	61	48
SB-T-1216	8.3±0.6	69	45			
SB-T-121302	8.9±0.9	74	47			
SB-T-121303	10.4±1.C	87	51	5.6±0.7	37	36
SB-T-121402	9.9±0.5	83	50	9.2±0.4	61	48
SB-T-121602	9.2±1.0	77	48	9.6±0.2	64	49
Doxetaxel	9.8±0.2	82	49	3.1±3.3	71	24

Note: DC, final loaded drug concentration; LE, loading efficiency (loaded drug concentration/initial drug feeding concentration*100%); LC, loading capacity (final drug wt./total micelles wt.*100%).

Table 2

IC_{50} values of POx/SB-T-1214 micelles vs. other PTX formulations

Formulations	IC_{50} (ng/mL)		Resistance factor (R/S)	IC_{50} (ng/mL) T11
	LCC6-MDR	LCC6-WT		
P0x/SB-T-1214	34.6	5.8	6	43
POx/PTX	1385	17.8	78	983
Abraxane	769	9.4	82	N/A
Taxol	536	18.4	29	N/A

Note: Resistance factor R/S=(IC50 for drug resistant cell line LCC6-MDR, R)/(IC50 for drug-sensitive cell line LCC6-WT, S)