In vivo comparative study of distinct polymeric architectures bearing a combination of paclitaxel and doxorubicin at a synergistic ratio

Hemda Baabur-Cohen a, 1, Laura Isabel Vossen b, 1, Harald Rune Krüger b, Anat Eldar-booock a, Eilam Yeini a, Natalie Landa-Rouben c, Galia Tirama d, Stefanie Wedepohl d, Ela Markovsky a, Jonathan Leor c, Marcelo Calderón b, *, Ronit Satchi-Fainaro a, *

a Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
b Institut für Chemie und Biochemie, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany
c Neufeld Cardiac Research Institute, Sheba Medical Center, Tel-Aviv University, Tel-Hashomer, 5262000, Israel

Abstract

Nowadays, combination therapy became a standard in oncology. In this study, we compare the activity of two polymeric carriers bearing a combination of the anticancer drugs paclitaxel (PTX) and doxorubicin (DOX), which differ mainly in their architecture and supramolecular assembly. Drugs were covalently bound to a linear polymer, polyglutamic acid (PGA) or to a dendritic scaffold, polyglycerol (PG) decorated with poly(ethylene glycol) (PEG), forming PGA-PTX-DOX and PG-PTX-bz-DOX-PEG, respectively. We explored the relationship between the polymeric architectures and their performance with the aim to augment the pharmacological benefits of releasing both drugs simultaneously at the tumor site at a synergistic ratio. We recently designed and characterized a PGA-PTX-DOX conjugate. Here, we describe the synthesis and characterization of PG dendritic scaffold bearing the combination of PTX and DOX. The performance of both conjugates was evaluated in a murine model of mammary adenocarcinoma in immunocompetent mice, to investigate whether the activity of the treatments is affected by the immune system. Drug conjugation to a nano-sized polymer enabled preferred tumor accumulation by extravasation-dependent targeting, making use of the enhanced permeability and retention (EPR) effect. Both PGA-PTX-DOX and PG-PTX-bz-DOX-PEG nano-sized conjugates exhibited superior anti-tumor efficacy and safety compared to the combination of the free drugs, at equivalent concentrations. However, while PGA-PTX-DOX was more efficient than a mixture of each drug conjugated to a separate PGA chain, as was previously shown, PG-PTX-bz-DOX-PEG had similar activity to the mixture of the PG-PTX-bz-PEG and PG-DOX-PEG conjugates. Our results show that both conjugates are potential candidates as precision combination nanomedicines for the treatment of breast cancer.

© 2016 Elsevier B.V. All rights reserved.

Keywords:
Polymeric nanomedicines
Combination therapy
Polyglutamic acid
Polyglycerol
Paclitaxel
Doxorubicin
Dendritic polymer

1. Introduction

Nanotechnology and combination therapy are two major fields that show great promise in the treatment of cancer. The use of polymers as targetable drug carriers helps to improve drug’s therapeutic effectiveness while reducing adverse side effects associated with high dosage by improving their pharmacokinetics [1].

The rationale for using polymers as carriers for the delivery of antitumor agents, even if they are not conjugated to cell-specific molecules, is based on the work of Matsumura and Maeda defined as the enhanced permeability and retention (EPR) effect [2]. We and others utilize the EPR effect in order to design appropriate nano-sized agents, which enable a blood circulation and extravasation-dependent targeting to different types of cancer and believe that it is sufficiently prevalent in human tumors [3,4]. However, distinct tumors differ in the pathophysiological status of their vasculature enabling (or not) the EPR phenomenon of macromolecules. Therefore, using distinct supramolecular polymeric architectures enabled us to explore their suitability to extravasate from diverse hyperpermeable angiogenic vasculature in heterogeneous tumor types. The optimal size of a nano-sized agent, which can deliver an adequate dose of drugs distributed homogeneously and thus induce the most efficient therapeutic effect, is reportedly between 10 nm and 200 nm in diameter [4]. However, the supramolecular structure of the conjugates, whether they are dendritic, hyperbranched or linear, and their biodegradability, can have a major impact on the nanomedicine’s performance [5].
An important advantage of polymeric nanomedicines is their versatility, enabling to tailor different compounds with controlled loading percentage on a single polymeric backbone [6,7]. Paclitaxel (PTX) and doxorubicin (DOX) are potent anticancer drugs used in the clinic as monos—therapies [8,9] or in combination with other modalities to treat various neoplasms [10–13]. However, both drugs suffer from side effects, like neurotoxicity for PTX and dose-dependent cardiotoxicity for DOX, as well as poor pharmaco kina tics. These two drugs have dissimilar physico-chemical properties, pharmaco kinetics and distinct mecha nisms of action, toxicity and drug resistance [14]. As was already shown, binding two synergistic drugs to the same polymeric backbone will most likely result in improved anti-cancer activity and reduced toxicity [15–18].

A chemical conjugation of PTX and DOX to nanocarriers could offer pharmacodynamic and pharmacokinetic advantages by enhanced blood circulation and extravasation-dependent targeting, delivering both drugs to the tumor site at the required ratio for synergism. Undesired toxicity could be overcome, the dose reduced and multiple targets reached, by combining two therapeutic agents resulting in an increased therapeutic effect and an overall increased anticancer activity [11,19]. Such combination of polymer-drug conjugates can be achieved by (a) two polymeric systems, each carrying a different drug, or (b) one single polymeric carrier that is administered bearing two or more different drugs. It has been shown that both approaches yield superior properties as compared to the administration of the two pristine drugs [18,20,21].

Since the polymeric structure can have a major impact on the therapeutic efficacy, we have chosen to compare polymer-drug conjugates based on a linear or a dendritic nanocarrier. Polyglutamic acid (PGA) was chosen as the linear carrier, whereas dendritic polyglycerol (PG) decorated with poly(ethylene glycol) (PEG) was chosen as the dendritic counterpart. It is known that PGA-based conjugates when conjugated to hydrophobic drugs tend to form intramolecular aggregation when dissolved in water [22] yielding surface properties mainly driven by the negatively charged PGA backbone. In the case of PEGylated dendritic polymer-drug conjugates with a core-shell architecture, the surface properties in aqueous solution are mainly driven by the PEG shell [23]. We therefore hypothesize that the two types of conjugates would have different properties in solution due to the differences in their surface properties.

PGA is a potent polymeric carrier which is synthesized by ring-opening co-polymerization of the corresponding N-carboxyanhydrides (NCA), initiated by amines or nucleophilic agents. It is water-soluble, non-toxic, biodegradable and non-immunogenic at the required concentrations to exert its anticancer activity when bound to anticancer drugs. Cysteine proteases, particularly cathepsin B, an enzyme that is highly expressed in most tumor tissues, play a key role in the lysosomal degradation of PGA [24]. In addition, PGA has a γ-carboxyl group in each repeating unit of l-glutamic acid that offers multiple attachment of drugs [18,25]. Those features make PGA an attractive drug carrier, and indeed PGA-PTX (OPAXIO®) — former names XYOTAX and CT-2103 — is the most progressed polymer-drug conjugate in the pipeline for market approval [26,27].

Our second selected polymeric carrier, PG, has outstanding properties as a drug delivery system regarding its structure, biocompatibility and water solubility. PG can be prepared in a controlled synthesis via anionic ring-opening multibranching polymerization (ROMBP) [28]. It is characterized by the combination of highly branched, stable, with very low polydispersity, consisting of a biocompatible polyester scaffold, and a compact, well-defined dendrimer-like architecture that has great impact on their physical and chemical properties [29]. A significant amount of research has been realized towards the design of many different architectures, where the PG hydroxyl groups have been modified into different functionalities, demonstrating a promising therapeutic approach. Drug conjugation to PG through pH-sensitive [30] or enzymatically-cleavable linkers [31] have demonstrated the great potential of PG conjugates against cancer cells in vitro as well as in vivo. Recently, we reported the therapeutic potential of a multifunctional drug immunoconjugate for targeting cancer cell lines which express the epidermal growth factor receptor (EGFR) in vitro [32]. All these polymer drug conjugates showed optimal properties for in vitro and in vivo applications because of their appropriate size for passive tumor targeting, their high water solubility, pronounced environment responsive properties, a high stability at physiological conditions, cellular internalization, and a favorable tolerability profile.

The aim of this study was to synthesize two different nanocarriers bearing a combination of synergistic drugs at an appropriate ratio, and to determine their advantage in vivo over drugs conjugated to separate polymer chains and to free drugs, as well as to evaluate the effect of the polymeric architecture (linear PGA versus dendritic PG) on the anti-tumor activity and safety profile of the conjugates.

2. Materials and methods

2.1. Chemical data

All chemicals were of analytical grade and purchased from Fluka (Germany), Aldrich (Germany), or Merck (Germany), and used as received unless otherwise stated. Chemical reagents included: N,N-dioisopropylcarbodiimide (DIC), 1-hydroxybenzotriazol (HOBt), diisopropylmethylethylamine (DIPEA), N-hydroxysuccinimide (OHSuc), N,N-dimethylaminopyridine (DMAP), anhydrous N,N-dimethylformamide (DMF), and anhydrous tetrahydrofuran (THF). Maleimido-poly(ethylene glycol) (PEG-mal) with MW = 2 kDa was purchased from Rapp Polymer, Germany. The hydrazone derivative of DOX (DOX-EMCH, i.e., DOX bound to 3,3′-N-[e-maleimidocaprylic acid]) as well as the hydrazone derivative of paclitaxel (PTX-bz-EMCH) were prepared as described previously [33–35]. The Indocarboxyanine maleimide dye (IDCC) was obtained from Epios Therapeutics GmbH and used to fluorescently label the PG-PTX-bz-DOX-PEG.

Dendritic PG (average MW 10 kDa, PDI = 1.6, approximately 135 OH groups) was prepared according to published procedure [36]. PG amine with 30% of the total hydroxyl groups converted to amino groups (ca. 95 OH and 40 NH2 groups per PG scaffold) was synthesized according to previously reported methodologies [37]. Briefly, PG amine was prepared by a three-step reaction starting from PG and a conversion of OH groups into azide (N3) functionalities, and OH groups into mesyl (Ms) groups, followed by transformation of Ms. to free drugs, as well as to evaluate the effect of the polymeric architecture (linear PGA versus dendritic PG) on the anti-tumor activity and safety profile of the conjugates.

2. Materials and methods

2.1. Chemical data

All chemicals were of analytical grade and purchased from Fluka (Germany), Aldrich (Germany), or Merck (Germany), and used as received unless otherwise stated. Chemical reagents included: N,N-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazol (HOBt), diisopropylmethylethylamine (DIPEA), N-hydroxysuccinimide (OHSuc), N,N-dimethylaminopyridine (DMAP), anhydrous N,N-dimethylformamide (DMF), and anhydrous tetrahydrofuran (THF). Maleimido-poly(ethylene glycol) (PEG-mal) with MW = 2 kDa was purchased from Rapp Polymer, Germany. The hydrazone derivative of DOX (DOX-EMCH, i.e., DOX bound to 3,3′-N-[e-maleimidocaprylic acid]) as well as the hydrazone derivative of paclitaxel (PTX-bz-EMCH) were prepared as described previously [33–35]. The Indocarboxyanine maleimide dye (IDCC) was obtained from Epios Therapeutics GmbH and used to fluorescently label the PG-PTX-bz-DOX-PEG.

Dendritic PG (average MW 10 kDa, PDI = 1.6, approximately 135 OH groups) was prepared according to published procedure [36]. PG amine with 30% of the total hydroxyl groups converted to amino groups (ca. 95 OH and 40 NH2 groups per PG scaffold) was synthesized according to previously reported methodologies [37]. Briefly, PG amine was prepared by a three-step reaction starting from PG and a conversion of OH groups into mesyl (Ms) groups, followed by transformation of Ms. groups into azide (N3) functionalities, and finally reduction of the N3 groups to amine (NH2) groups by using triphenylphosphine as reducing agent. Water of Millipore quality (resistivity - 18 MΩ cm⁻¹, pH 5.6 ± 0.2) was used in all experiments and for preparation of all samples. If not otherwise specified, sodium phosphate buffer (50 mM) was used for the pH of 7.4, and acidic pH values were reached by a sodium acetate buffer (pH 4, 50 mM) and a Britton-Robinson buffer (pH 2.0). All measurements were carried out with freshly prepared solutions at 25 °C. pH values were measured with a Scott instruments HandyLab pH meter at 25 °C.

Size exclusion chromatography (SEC) of PG conjugates was performed with Sephacryl G-25 superfine or Sephadex LH-20 (GE Healthcare) respectively under ambient pressure and temperature. All reactions that involved air or water sensitive compounds were carried out in dried flasks under an argon atmosphere and dried solvents from the solvent purification system MB SPS 800, M. Braun Inertgas-Systeme GmbH, Garching, Germany. Size exclusion chromatography analysis was performed using UltiMate 3000 LC System (Thermo Scientific) with photodiode array (PDA)-UV detector and Shodex RI-101 detector (Showa Denko America, Inc.), with Xenix SEC-100 (Sepax) column in phosphate buffer pH = 7.0, flow 1 mL min⁻¹. Reversed phase (RP) high pressure liquid chromatography (HPLC) analysis was performed using UltiMate 3000 LC system (Thermo Scientific) with PDA-UV detector and C18 LiChroCART® Purospher® STAR 250 × 4.6 mm column (5 μm) (Merck Millipore). The mobile phase was a gradient of water (A) and acetonitrile (ACN) (B) both containing...
0.1% (v/v) trifluoroacetic acid (TFA), 20% to 100% solvent B in 15 min. Chromelion software was employed for data analysis. PGA conjugates were purified by SEC on Sephacryl S-200 HR (GE Healthcare, Buckinghamshire, UK), using water as eluent.

2.2. Synthesis of PGA-PTX-DOX conjugate

The synthesis was repeated according to a protocol that was recently published [18]. Briefly, the synthesis included three main steps. First, PTX was bound directly to PGA by an ester bond. In this case, PTX was not a pH-sensitive prodrug, but cleavable from the biodegradable PGA nanocarrier, upon enzymatic cleavage by cathepsin B. Then, cysteamine was added in order to introduce free thiol groups, for coupling to DOX-EMCH, forming an acid-sensitive hydrazide bond similar to the linker used in the PG-DOX-PTX conjugate. Physico-chemical characterization and drug release profiles were previously described [18].

2.3. Physico-chemical characterization of PGA-PTX-DOX conjugate

2.3.1. Surface charge measurements

Zeta potential measurements were performed with a Zetasizer Nano ZS analyzer with an integrated 4 mW He–Ne laser (λ = 633 nm; Malvern Instruments Ltd., Malvern, Worcestershire, U.K.). To elucidate the surface charge of the conjugates, PGA-PTX, PGA-DOX and PGA-PTX-DOX potentials were measured in 20% phosphate buffered saline (PBS), pH = 7.4. The samples were freshly prepared at a concentration of 0.1 mg mL$^{-1}$, and filtered through 0.2 μm filter. All measurements were performed at 25 °C using folded capillary cells (DTS 1070).

2.3.2. Hydrodynamic diameter measurements

Hydrodynamic diameters were measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS equipped with a He–Ne laser (λ = 633 nm; Malvern Instruments Ltd., Malvern, Worcestershire, U.K.). The samples were freshly prepared in PBS at a concentration of 1 mg mL$^{-1}$. Prior to the measurements, all samples were filtered through a 0.45 μm pore size filter. All measurements were performed at 25 °C using quartz cells.

2.4. Synthesis of PG-PTX-bz-DOX-IDCC-PEG dendritic conjugate

Polyglycerolamine with 30% of the hydroxyl groups converted to amino groups was dissolved in MeOH. 2-iminothiolane (1.5 eq. per NH$_2$ group) was added in minimal amount of MeOH. After stirring 20 min at room temperature the reaction mixture was directly applied on a Sephadex LH-20 column and eluted with MeOH. The polymeric fraction was collected and to this solution a solution of PTX-bz-EMCH (1.5 eq. per PG) was added and stirred for 15 min at room temperature. Next, a solution of PTX-bz-EMCH (1.5 eq. per PG) was added and stirred 10 min at room temperature. Then, a solution of IDCC-maleimide (1.5 eq. per PG) MeOH was added and the mixture was stirred for 10 min. Afterwards, a solution of poly(ethylene glycol) (PEG) 2 kDa-maleimide (50 eq. per PG in phosphate buffer, pH 7.4) was added and the mixture was stirred for another 2 h. Finally, the solution was concentrated and purified by size exclusion chromatography, to give the final conjugate, PG-PTX-bz-DOX-IDCC-PEG.

2.5. Physico-chemical characterization of PG-PTX-bz-DOX-IDCC-PEG dendritic conjugate

2.5.1. Surface charge measurements

Zeta potential measurements were performed with a Zetasizer Nano-ZS 90 (Malvern Instruments) with an integrated He–Ne laser (λ = 633 nm). The samples were freshly prepared in Milli-Q water at a concentration of 1 mg mL$^{-1}$. Prior to the measurements, all samples were filtered through a 0.45 μm pore size filter. All measurements were performed at 25 °C using folded capillary cells (DTS 1070).

2.5.2. Hydrodynamic diameter measurements

DLS measurements were performed using a Malvern Zetasizer Nano-ZS 90 (Malvern Instruments) equipped with a He–Ne laser with a wavelength of λ = 633 nm under a scattering angle of 173°. The samples were freshly prepared in Milli-Q water at a concentration of 1 mg mL$^{-1}$. Prior to the measurements, all samples were filtered through a 0.45 μM pore size filter. All measurements were performed at 25 °C using quartz cells.

2.6. Drug release profile determination of PG-PTX-bz-DOX-IDCC-PEG conjugate

2.6.1. Analysis of DOX release by SEC and UV/Vis spectroscopy

The drug release of PG-PTX-bz-DOX-IDCC-PEG was studied by separating the conjugate from the free drug fraction on a SEC (Sephadex G-10) and collecting the free drug fraction, followed by determination of the concentration of the free drug photometrically by UV/Vis spectroscopy. The cleavage study was performed at pH 4.0 and 7.4 respectively. Initially the conjugate was dissolved in the buffer (150 μL) and incubated for more than 20 h in total. At specific time points (0, 1, 6 and 21 h) a defined volume (15 μL) was taken out of the mixture, applied on a 1 mL Sephadex G-25 column and eluted with phosphate buffer (PB) (pH 7.4). The larger band corresponds to the conjugate and the slower band which increased intensity over time corresponds to the released drug. The free drug band was collected into a graduated flask which was filled up to 1 mL with PB pH 7.4. The amount of DOX in the solution was directly determined by UV/Vis spectroscopy at 495 nm (ε$_{495}$ = 10.645 M$^{-1}$ cm$^{-1}$) and plotted as amount of total DOX loaded on conjugate (% released) against time.

2.6.2. Analysis of PTX release by HPLC

The cleavage study of the conjugate PG-PTX-bz-DOX-IDCC-PEG was performed at pH 7.4 and pH 4.0. It was carried out using a Knauer Smartline-HPLC system with an internal UV absorption detector (λ = 227 nm) and EZiChrom Software. A Hypersil™ ODS C18 column (Thermo Scientific, 100 mm × 4.6 mm, particle Size: 5 μm) with a direct connected guard column C18 was employed. Acetonitrile–water (65:35) was used as the mobile phase at a flow rate of 1.0 mL min$^{-1}$ under isocratic regime. The injection volume was 20 μL. The conjugates were dissolved, incubated in the different buffer systems and aliquots were taken at specific time intervals (1, 2.5, 6, 15 and 21 h). A defined volume was taken out from the mixture and extracted with a defined DCM/Et$_2$O (1:1) mixture, centrifuged, and dried in vacuum. The residue was redissolved in acetonitrile and analyzed by reversed phase HPLC (RP-HPLC). Stock solutions of PTX-bz in acetonitrile were prepared and assessed by RP-HPLC in order to obtain a calibration curve for PTX-bz (5.0–50 μg, R = 0.998) (Retention time: 2.9–3.0 min) (Fig. S2).

2.7. Cell culture

MDA-MB-231 and MCF-7 human breast cancer cells and DA3 and 4T1 murine mammary adenocarcinoma were obtained from the American Type Culture Collection (ATCC). mCherry-labeled 4T1 cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg mL$^{-1}$ penicillin, 100 μg mL$^{-1}$ streptomycin, 12.5 μg mL$^{-1}$ nystatin (PSN), and 2 mM L-glutamine (L-Glu). 4T1 cells were grown in Dubelcco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg mL$^{-1}$ penicillin, 100 μg mL$^{-1}$ streptomycin, 12.5 μg mL$^{-1}$ nystatin, and 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer and 2.5 g L$^{-1}$ d-glucose. Cells were grown at 37 °C; 5% CO$_2$. Please cite this article as: H. Baabur-Cohen, et al., In vivo comparative study of distinct polymeric architectures bearing a combination of paclitaxel and doxorubicin at a synergistic ratio..., J. Control. Release (2016), http://dx.doi.org/10.1016/j.jconrel.2016.06.037
2.7.1. Cell proliferation assay

Cells were plated onto 24-well plates (1 × 10^4 cells/well for MDA-MB-231, 5 × 10^3 cells/well for 4T1) and allowed to attach for 24 h. Cells were incubated with the conjugates and free drugs, dissolved in appropriate cell culture medium at serial concentrations, for 72 h. For PGA-PTX-Dox treatments containing PTX concentrations ranged from 0.01 nM to 1000 nM, according to PTX-equivalent concentration. For treatments containing DOX concentrations ranged from 0.025 nM to 2500 nM, according to DOX-equivalent concentration. These concentrations were selected in order to have the same molar ratio of PTX to DOX in all the controls as in the PGA-PTX-Dox conjugate (1:2.5 PTX:DOX, respectively). In the case of PG-PTX-bz-Dox-IDCC-PEG, treatments containing PTX, concentrations ranged from 0.002 nM to 2500 nM, according to DOX-equivalent concentration. For treatments containing DOX, concentrations ranged from 0.01 nM to 10,000 nM, according to DOX-equivalent concentrations.

PGA and PG-PEG were used at concentrations equivalent to carrier content in the conjugates. Following incubation, cells were washed, detached by trypsin and counted by using a Coulter Counter® (Beckman Coulter).

2.7.2. Isobolograms of PTX and DOX drug combination treatments

IC_{50} represents the concentration of a drug that is required for 50% inhibition in vitro. The IC_{50} values of treatment with PTX, DOX and their combinations were calculated from the proliferation assays. IC_{30}, 50, 70 values of PTX and DOX were marked on X, Y axes respectively and a line which represents additive effect was drawn between each inhibition concentration (IC). The combination index (CI) of each treatment was calculated according to the classic isobologram equation combination index = [(D)1 / (Dx)1] + [(D)2 / (Dx)2] as previously described[38]. Area on the right side of each IC additive line represents antagonistic effect and the left side represents synergistic effect.

2.7.3. Migration (scratch) assay

The migration of MDA-MB-231 cells in the presence of PG-PTX-bz-Dox-IDCC-PEG and their controls were evaluated using the scratch assay. A scratch was done on a confluent cell monolayer, and cells were incubated with the conjugates and the free drugs at PTX-equivalent concentrations of 20 nM and DOX-equivalent concentrations of 100 nM for 21 h. Plates were imaged and the gap width was measured in the beginning and end of the experiment, at the same reference point.

2.7.4. Intracellular uptake of the conjugates by confocal microscopy

MDA-MB-231 cells were seeded on cover glasses placed in 24-well plates and allowed to attach for 24 h. PG-DOX-IDCC or free DOX (DOX-equivalent concentration of 400 nM) were incubated with the cells for 0.5, 4, 8, 24 h. After the desired incubation time, the cells were washed with PBS, fixed with 4% paraformaldehyde (PFA) for 20 min at RT, followed by permeabilization with 0.1% Triton-X for 10 min. Fixed cells were blocked with 2% bovine serum albumin (BSA) in PBS for 30 min. Cells were stained with rabbit anti-LysoSosomal-associated membrane protein 1 (LAMP-1) as primary antibody for 60 min (Cell Signaling, Technology, Massachusetts, US). After being washed 3 times with PB, cells were incubated with Goat anti rabbit IgG-Rhodamine secondary antibody (Santa Cruz, Heidelberg, Germany). Slides were mounted with Prolong Gold® antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Cellular uptake was imaged with a Leica SP8 Confocal Imaging system.

2.8. Biocompatibility evaluation

2.8.1. Biodistribution of the conjugate and dendritic conjugate

PG-PTX-bz-Dox-IDCC-PEG was injected intravenously (i.v.) via the tail vein to nu/nu mice bearing mammary tumors. Mice were killed following 1, 6, or 24 h. Then, mice were perfused with saline and organs and tumors were taken for analysis. Organs and tumor were homogenized and lyophilized. ACN (1 mL) was added to extract the free drugs, suspension was vortexed well and centrifuged. Supernatant (0.5 mL) was dried by SpeedVac and the obtained residue dissolved in 100 μL ACN, to concentrate the solution. To the remaining organs, suspension 1 mL DDW was added to extract the water-soluble conjugate. Same procedure as for ACN was repeated. The resulting 100 μL solutions were injected to a HPLC to determine the amount of the conjugate and the released drugs. In addition, the amount of PG-PTX-bz-Dox-IDCC-PEG was measured using a SpectraMax M5e plate reader (Molecular Devices), by measuring the absorbance of IDCC (λ = 650 nm).

2.9. Evaluation of antitumor activity and toxicity of the conjugates and dendritic conjugates in mouse model of murine cancer

Balb/c female mice were inoculated to the mammary fat pad with 0.5 × 10^6 mCherry-labeled 4T1 mammary adenocarcinoma cells. Mice bearing ~70 mm³ tumors were treated i.v. with conjugates PGA-PTX-Dox (n = 5), PGA-PTX (n = 5), PGA-DOX (n = 5) and combination of PGA-PTX and PGA-DOX (n = 4), dendritic conjugates (PG-PTX-bz-Dox-IDCC-PEG) (n = 4), PG-PTX-bz-IDCC-PEG (n = 5), PG-DOX-IDCC-PEG (n = 4) and combination of PG-PTX-bz-IDCC-PEG and PG-DOX-IDCC-PEG (n = 5), free drugs PTX (n = 5), PTX-bz (n = 5), DOX (n = 4), PTX + DOX combination (n = 5) and PTX-bz + DOX (n = 5) combination, all carriers alone – PGA (n = 5) and PG-IDCC-PEG (n = 5) and saline (n = 5) were defined as control (n = 15). All drugs and conjugates solutions were prepared with PBS, beside PTX and PTX-bz that were dissolved according to the clinical protocol (e.g. ETOH:Cremophore EL:Saline, 1:1:8). The dendritic conjugates were dissolved in diluted saline (×4), since the dendritic conjugates were lyophilized in PB, in order to result with isotonic solution (salt content was converted to NaCl equivalents). All treatments were administered at 5 mg kg⁻¹ DOX-equivalent dose and 3 mg kg⁻¹ PTX-equivalent dose. This drug’s ratio represents the average ratio as on the combined conjugate and dendritic conjugate (e.g., PGA-PTX-DOX, PG-PTX-bz-DOX-IDCC-PEG). Mice were treated three times a week, starting at day 9 post tumor inoculation (5 treatments in total). Tumors were measured by a caliper and tumor volume was calculated as: width² × length × 0.52. Body weight and tumor size were monitored every other day. Mice were euthanized when tumor volume reached 1500 mm³ or when they lost 15% of their body weight.

2.9.1. Intravital non-invasive fluorescence imaging

IVIS Spectrum CTR™ non-invasive fluorescence imaging system was used to follow tumor progression of mice bearing mCherry-labeled tumors. Mice were treated with a depilatory cream (Veet®) before each imaging and were kept anesthetized under continuous isoflurane flow. Multispectral image-cubes were acquired using excitation (605 nm) and emission (660, 680, 700, 720) filter set. Autofluorescence and undesired background signals were eliminated by spectral analysis and unmixing, using a control mouse without tumor as background.

2.9.2. Cardiotoxicity evaluation

In order to evaluate DOX-related cardiotoxicity, when animals were euthanized the hearts were perfused with 4% formaldehyde for 10 min. Hearts were embedded in paraffin, sectioned into 5-μm slices and stained with hematoxylin-eosin. To evaluate pathological changes in cardiac morphology and structure, hearts were stained with Masson’s Trichrome.
2.10. Statistical methods

Data were expressed as mean ± SD for in vitro assays or ± SEM for in vivo. Statistical significance was determined using an unpaired t-test or ANOVA with repeated measurement. All statistical tests were two-sided.

3. Results

3.1. Evaluation of the synergistic activity of PTX and DOX combination on the proliferation of DA3 and MCF-7 cells

In order to determine the combination index and the ratio of Dox and PTX at which the drugs exert synergistic activity, we evaluated the ability of the combined drugs to inhibit tumor cell growth. To that end, we based the concentrations tested here on murine DA3 and human MCF-7 mammary adenocarcinoma cells on our previous studies performed on MDA-MB-231 and ES-2 cells. The resulting synergistic ratio was originally used for the design of PGA-PTX-DOX conjugate [18]. In this study, we set to strengthen the use of the combination of PTX and DOX in additional breast cancer cell lines, including murine and human ones. Several concentrations of PTX:DOX resulting in different ratios were studied until the optimal ratio was found. In this case, the combination treatment of PTX and DOX decreased the inhibitory concentration (IC) of the drugs as single treatments. Fig. 1 shows the optimal results for both cells. PTX inhibited the proliferation of DA3 cells at IC_{40, 60, 80} of 15, 25, 90 nM and DOX at 9, 25, 90 nM (Fig. 1A). PTX inhibited the proliferation of MCF-7 cells at IC_{30, 50, 70} of PTX at 4, 13, 40 nM and DOX at 9, 35, 120 nM (Fig. 1B). The combination treatment of PTX and DOX decreased the inhibitory concentration (IC) of the drugs as single treatments. Fig. 1 shows the optimal results for both cells. PTX inhibited the proliferation of DA3 cells at IC_{40, 60, 80} of 15, 25, 90 nM and DOX at 9, 25, 90 nM (Fig. 1A). PTX inhibited the proliferation of MCF-7 cells at IC_{30, 50, 70} of PTX at 4, 13, 40 nM and DOX at 9, 35, 120 nM (Fig. 1B). In order to evaluate the combined treatments’ effect, data from combination treatment results were collected, analyzed and calculated according to combination index (CI) equations [38] (Fig. S1A, S1B, S1C). Combination treatments I (PTX + DOX 5 nM) and II (DOX + PTX 10 nM) inhibited DA3 cells proliferation at IC_{40, 60, 80} at 8, 30, 100 and 0.01, 10, 80 nM respectively (Fig. S1A). Combination treatment I (PTX + DOX 1 nM) and II (DOX + PTX 1 nM) inhibited MCF-7 cells proliferation at IC_{30, 50, 70} at 3, 9, 25 and 5, 20, 50 nM respectively (Fig. S1B). Combination treatments I (PTX + DOX 20 nM) and II (DOX + PTX 10 nM) inhibited 4T1 cells proliferation at IC_{40, 60, 80} at 15, 25, 55 and 15, 30, 65 nM respectively (Fig. S1C). Combination treatments of PTX and DOX exhibited synergistic effect on DA3 cells’ proliferation when combined as DOX + PTX 10 nM and additive effect when combined as PTX + DOX 5 nM. Combination treatments of PTX and DOX have a synergistic effect on MCF-7 cells’ proliferation when combined as DOX + PTX 1 nM and additive effect when combined as PTX + DOX 1 nM. Combination treatments of PTX and DOX exhibited synergistic effect on DA3 cells’ proliferation when combined as DOX + PTX 10 nM and additive effect when combined as PTX + DOX 5 nM. Combination treatments of PTX and DOX have a synergistic effect on MCF-7 cells’ proliferation when combined as DOX + PTX 1 nM and additive effect when combined as PTX + DOX 1 nM. Combination treatments of PTX and DOX have a synergistic effect on 4T1 cells’ proliferation when combined as DOX + PTX 10 nM and additive effect when combined as PTX + DOX 20 nM.

3.2. PGA-PTX-DOX synthesis and characterization

PGA conjugate bearing PTX and DOX (PGA-PTX-DOX) and control conjugates (PGA-PTX and PGA-DOX) have been synthesized and characterized, as previously described in literature [18]. Physico-chemical properties of the conjugates are presented in Table 1. Zeta potential of PGA and the conjugates was measured. As expected, PGA is negatively charged, and all other conjugates have zeta potential values in the same range, as was already published [18]. This demonstrates that the surface of the conjugates is mainly comprised of PGA.
Hydrodynamic diameter of the conjugates was measured by DLS and gave similar values as in the previous study (41.4 nm compared to 37.5 nm, respectively) [18]. However, as opposed to our previous study, in which PGA-DOX had a diameter of 17 nm, in this study PGA-DOX had a diameter of 128.7 nm. The explanation for this discrepancy lies in the difference of DOX loading (2.8% compared to 8.8% in this study), which influence the spatial structure of the PGA-DOX conjugate stacked together by hydrophobic forces through π-electron interaction [39]. In addition, the reduction in polarity of the highly loaded conjugate, might also contribute to the hydrophobic interactions. PGA-PTX had the smallest diameter of all conjugates (4.6 nm).

PTX loading was determined indirectly by HPLC, by determining the unbound drug amount. DOX loading was determined by UV/Vis spectroscopy, using the molar absorption coefficient of DOX-EMCH. For the PGA-PTX conjugate, PTX loading was 3.9 mol% (14.4% w/w) and DOX loading was 6.2 mol% (14.6% w/w). For the PGA-PTX conjugate, loading was 13.5 mol% (44.0% w/w) and for the PGA-DOX conjugate, loading was 8.8 mol% (31.0% w/w).

3.3. PG-PTX-bz-DOX-IDCC-PEG synthesis and characterization

A PG-based dendritic conjugate bearing PTX and DOX (PG-PTX-bz-DOX-IDCC-PEG) and control dendritic conjugates (PG-PTX-bz-IDCC-PEG and PG-DOX-IDCC-PEG) were synthesized in a one-pot synthesis according to Scheme 1. The first step comprised thiolating the PG-NH₂ (1) (10 kDa, 30% amine functionalization) using 2-iminothiolane hydrochloride as thiolation reagent. The highest thiol concentration was achieved after 20 min reacting time at r.t. which was proven by following reaction kinetics with an Ellman’s test. The thiolation was followed by a purification step by SEC using Sephadex LH-20. Afterwards, prodrugs (3 and 4), IDCC-mal (5) and PEG-mal (6) were sequentially added in order to perform a selective Michael addition between the thiols and the maleimide groups. The precursor of prodrug 4, PTX-bz, was previously synthesized in a three-step reaction according to literature procedures [35].

The reaction conditions were adapted to previously published literature [32]. The synthesis was performed in methanolic solution to which PB (pH 7.4) was added. The final reaction mixture was stirred for 2 h followed by a reduction of the volume by ultracentrifugation using a Centriprep. The residual volume was directly applied to a SEC column packed with Sephadex G-25 gel. Conjugation formation was proven by a faster band on a SEC column. The collected polymeric fraction was lyophilized and characterized after reconstitution in PB.

3.3.1. Hydrodynamic diameter and zeta potential measurements of the dendritic conjugates

Hydrodynamic diameters of the conjugates were measured by DLS. All conjugates showed sizes between 20 and 28 nm with PG-DOX-IDCC-PEG having the largest diameter (28.1 nm) and PG-IDCC-PEG the smallest diameter (20.1 nm). The sizes of PG-PTX-bz-IDCC-PEG and PG-DOX-PTX-bz-IDCC-PEG were very similar (23.4 nm versus 24.2 nm, respectively). These results represent size distribution measurements by volume, since all conjugates showed some aggregation and relatively high PDIs (0.3–0.5) (Fig. S3) Moreover, zeta potential of the conjugates was measured and as expected the PEGylated surface shows almost no charge. The physico-chemical parameters of the conjugates are summarized in Table 2.

3.3.2 Drug and dye loading of the dendritic conjugates

The DOX and IDCC concentrations were determined photometrically using the molar absorption coefficients of Dox-EMCH and the IDCC dye at 495 nm (ε = 10.645 M⁻¹ cm⁻¹) and at 650 nm (ε = 208.000 M⁻¹ cm⁻¹) respectively after reconstitution of the lyophilized samples in PB (pH 7.4). The concentration of bound PTX-bz was determined by determining the released amount of PTX-bz by RP-HPLC at low pH. For this purpose, the conjugate was incubated in Britton-Robinson buffer (pH 2.0) for 8 h and the concentration of released PTX-bz was
measured. The obtained loadings are summarized in Table 2. The in vitro stability studies performed by SEC for DOX release and by RP-HPLC for PTX-bz release showed a minimal release of the drug at pH 7.4 after 20 h (less than 6% and 2%), while at acidic pH the half-lives of the conjugates were below 3 h for DOX release and around 5 h for PTX-bz release. These properties should enable the drugs to be released in the slightly acidic tumor microenvironment and intracellular environment of tumor cells (e.g. pH 5–6 in the endosomes and down to 4–5 in thelysosomes) (Fig. 2).

3.4 Cell culture experiments with PGA-PTX-DOX conjugate

3.4.1. Evaluation of the cytotoxic effect of PGA-PTX-DOX conjugate on mCherry-labeled 4T1 cells

Cytotoxicity of the free drugs, PGA-PTX-DOX conjugate, PGA-PTX and PGA-DOX conjugates was evaluated on mCherry-labeled 4T1, murine breast cancer cells. Cells were incubated with the conjugates (PGA-PTX-DOX, PGA-PTX, PGA-DOX and combination of PGA-PTX and PGA-DOX at the same ratio as on the PGA-PTX-DOX conjugate), free drugs (PTX, DOX and their combination in the same ratio as on the PGA-PTX-DOX conjugate) and PGA for 72 h. Treatment with free PTX and free DOX had an IC_{50} value of 20 and 25 nM in mCherry-labeled 4T1. The combined free drugs (PTX and DOX) exhibited similar activity as the free drugs alone. We have shown before, that PGA-PTX and PGA-DOX had a higher IC_{50} value as compared to free drugs in MDA-MB-231, since as opposed to the free drugs, the conjugates cannot internalize rapidly by diffusion [18]. Interestingly, in mCherry-labeled 4T1 cells, similar IC_{50} values were seen in PGA-PTX and PGA-DOX treatments of 20 and 40 nM, respectively. Combination of PGA-PTX with PGA-DOX, and the final conjugate PGA-PTX-DOX had IC_{50} of 15 and 40 nM (Fig. 3). PGA alone exhibited no toxicity up to the concentrations relevant for the in vivo study. IC_{50} values of the treatments are summarized in Table 3.

3.5. Cell culture experiments with PG-PTX-bz-DOX-IDCC-PEG dendritic conjugate

3.5.1. Evaluation of the cytotoxic effect of PG-PTX-bz-DOX-IDCC-PEG conjugate on MDA-MB-231 cells

The conjugate activity was determined in vitro on the proliferation of MDA-MB-231 human breast cancer cells and mCherry-labeled 4T1, murine breast cancer cells. Cells were incubated with the conjugates (PG-PTX-bz-DOX-IDCC-PEG, PG-PTX-bz-IDCC-PEG, PG-DOX-IDCC-PEG and combination of PGA-PTX-bz-IDCC-PEG and PG-DOX-IDCC-PEG at the same ratio as on the PG-PTX-bz-DOX-IDCC-PEG conjugate), free drugs (PTX-bz, DOX and their combination in the same ratio as on the PG-PTX-bz-DOX-IDCC-PEG conjugate) and PG-IDCC-PEG for 72 h. Treatment with free PTX-bz and free DOX had an IC_{50} value of 20 and 5 nM in MDA-MB-231 and 80 and 35 nM in mCherry-labeled 4T1, respectively. The combined free drugs (PTX-bz and DOX) exhibited similar activity as the free drugs alone in both cell lines. As expected, PG-PTX-bz-IDCC-PEG and PG-DOX-IDCC-PEG had a higher IC_{50} value of 100 and 400 nM in MDA-MB-231 and 1000 and 250 nM in mCherry-labeled 4T1, respectively, since as opposed to the free drugs, the conjugates cannot internalize rapidly by diffusion. Such a considerable difference between the cytotoxicity of the pristine drug and drug conjugated to PG was reported previously. Combination of PG-PTX-bz-IDCC-PEG with PG-DOX-IDCC-PEG, and the final conjugate PG-PTX-bz-DOX-IDCC-PEG had IC_{50} of 400 and 150 nM in MDA-MB-231, and 300 and 350 nM in mCherry-labeled 4T1, respectively (Fig. 4). IC_{50} values of the treatments are summarized in Table 4. PG-PTX-bz-DOX-IDCC-PEG conjugate showed high cytotoxic activity, which indicates that drugs are released from the polymer and retain their activity. Similarly, to our results with

Table 2

<table>
<thead>
<tr>
<th>Size (diameter) [nm]</th>
<th>Zeta potential (mV)</th>
<th>Mw (theoretical) [kDa]</th>
<th>PTX loading (w/w)</th>
<th>DOX loading (w/w)</th>
<th>IDCC loading (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG-IDCC-PEG</td>
<td>20.1</td>
<td>+0.65</td>
<td>51</td>
<td>–</td>
<td>0.30</td>
</tr>
<tr>
<td>PG-DOX-IDCC-PEG</td>
<td>28.6</td>
<td>–0.83</td>
<td>55</td>
<td>–</td>
<td>1.60</td>
</tr>
<tr>
<td>PG-PTX-bz-IDCC-PEG</td>
<td>23.4</td>
<td>–1.72</td>
<td>52</td>
<td>0.75</td>
<td>0.47</td>
</tr>
<tr>
<td>PG-PTX-bz-DOX-IDCC-PEG</td>
<td>24.2</td>
<td>–2.89</td>
<td>56</td>
<td>0.76</td>
<td>1.70</td>
</tr>
</tbody>
</table>
PGA-PTX-DOX [18], we expect that the advantage of the PG conjugates will be seen in vivo, since the drugs will be released mainly in the tumor area, due to the EPR effect [2] and reach the same target cell simultaneously.

3.5.2. Evaluation of the effect of PG-PTX-bz-IDCC-PEG dendritic conjugate on migration of cancer cells

In order to evaluate further the ability of the conjugate to inhibit tumor progression, we set to determine its ability to inhibit the migration of tumor cells, a hallmark of cancer leading to metastasis. Furthermore, we and others have previously shown that PTX inhibits the motility of cancer cells [18,40,41,42], and we wanted to determine whether this activity was retained following its modification to PTX-bz and its conjugation to PG. The conjugate, PG-PTX-bz-DOX-IDCC-PEG, at PTX-equivalent concentrations of 20 nM and DOX-equivalent concentrations of 100 nM, inhibited the migration of MDA-MB-231 cells by 49% of gap closure (Fig. 5).

3.5.3. Intracellular uptake of PG-PTX-bz-DOX-IDCC-PEG conjugate

Internalization and accumulation of DOX in the nucleus of MDA-MB-231 cells was evaluated by confocal microscopy. In all samples, DOX was seen inside the cells already after 30 min of incubation, indicating rapid internalization both for the free drug and the conjugates. Free DOX is seen in the nucleus of the cells after 4 h. In the PG-PTX-bz-DOX-IDCC-PEG sample, DOX is colocalized with IDCC (which is bound to the carrier) at 0.5, 4 and 8 hour time points. After 24 h, DOX that was released from PG was co-localized with the lysosomes (Fig. 6). In addition, internalization of the conjugates was studied in HeLa cells over 24 h, showing a co-localization of DOX with the nucleus and hence a successful release of DOX to its intracellular target site (Fig. S4).

3.6. Biocompatibility evaluation

3.6.1. Biodistribution of PG-PTX-bz-DOX-IDCC-PEG conjugate

Biodistribution of the PG-PTX-bz-DOX-IDCC-PEG conjugate in mammary adenocarcinoma-bearing mice was evaluated following i.v. administration. Amounts of conjugate in organs and tumors were evaluated by measuring IDCC absorbance using a SpectraMax M5e multi-detection reader. The conjugate accumulated mainly in the tumor (Fig. 7). Some lower amount of the conjugate was also detected in the kidneys and in the spleen.

3.7. Evaluation of antitumor activity and toxicity of the conjugates and dendritic conjugates in a model of murine cancer

3.7.1. Efficacy

Treatment with the linear PGA-PTX-DOX conjugate and the dendritic PG-PTX-bz-DOX-IDCC-PEG conjugate led to substantial antitumor effect in murine mammary adenocarcinoma mouse model, as compared to the controls. Both combined polymeric conjugates were potent and safe, with a minor superiority to PGA-PTX-DOX. Interestingly, the combined dendritic conjugate PG-PTX-bz-DOX-IDCC-PEG inhibited tumor growth to a similar extent as the mixture of individually-conjugated drugs [PG-PTX-bz-IDCC-PEG + PG-DOX-IDCC-PEG]. Mice treated with each of the linear conjugates exhibited a stable body weight gain compared to free drugs (i.e., PTX and DOX), as was already shown [18]. Similarly, mice treated with dendritic conjugates also exhibited a stable body weight gain as compared to free drugs (i.e., PTX-bz and DOX), besides a minor decrease in PG-DOX-IDCC-PEG group, in which the trend was altered on day 26 where the weight loss was completely reversible (data not shown). As expected from the clinic, combination of free drugs (PTX + DOX, PTX-bz + DOX) is potent, however toxic, as was also shown previously for PTX + DOX mixture [18] (data not shown). On
day 22, when all groups were still included in the study, a 52% inhibition of tumor growth was seen compared to saline. On day 32, we found 63% tumor growth inhibition compared to free PTX. On day 32, all conjugates were present in the study. The graph representing data of each group was terminated on the day at which the initial number of the mice was reduced due to toxicity or tumor size. However, data collection of remaining mice in the group continued and is shown in the Kaplan-Meier survival curve. Treatment efficacy, defined as tumor growth inhibition ratio between treated versus control groups (T/C), was calculated on day 22 following treatment initiation, when the first mouse was euthanized due to large tumor size (Table 5). Mice treated with PGA-PTX-DOX conjugate, PG-PTX-bz-DOX-IDCC-PEG dendritic}

---

**Fig. 5.** Inhibition of cancer cell migration (scratch assay) by PG-PTX-bz-IDCC-PEG dendritic conjugate. PG-PTX-bz-IDCC-PEG inhibited the migration of MDA-MB-231 cells better than other controls. *p < 0.05, **p < 0.01, ***p < 0.001 (compared to untreated control).

**Fig. 6.** Internalization of PG-PTX-bz-IDCC-PEG dendritic conjugate into breast cancer cells. Internalization of PG-PTX-bz-IDCC-PEG conjugate into MDA-MB-231 cells was examined by confocal microscopy after incubation with the drug for 0.5, 8, 24 h. The nucleus is stained with DAPI (blue), IDCC is bound to PG (cyan), the lysosome was stained with rabbit anti-LAMP-1 and goat anti rabbit IgG-Rhodamine as primary and secondary antibodies, respectively (green), and DOX is marked in red. After 30 min, the conjugate was internalized into the cells, and DOX is co-localized with its carrier. After 24 h, co-localization of DOX in the lysosome is observed (marked in white arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Please cite this article as: H. Baabur-Cohen, et al., In vivo comparative study of distinct polymeric architectures bearing a combination of paclitaxel and doxorubicin at a synergistic ratio..., J. Control. Release (2016), http://dx.doi.org/10.1016/j.jconrel.2016.06.037
conjugate, PG-PTX-bz-IDCC-PEG + PG-DOX-IDCC-PEG survived for the longest period of all groups (Fig. 8). PTX-bz + DOX had similar survival, but mice in this group started dying on day 24.

On day 17, following a complete dosing regimen for all treatments, representative CT images were taken from all groups treated with both PTX and DOX (PTX + DOX, PTX-bz + DOX, PGA-PTX-DOX and PG-PTX-bz-DOX-IDCC-PEG and saline as control). Each image represents the average tumor volume of the group at that time point. Tumor size in the groups with the free drugs was larger compared to the groups treated with the conjugated drugs.

3.7.2. Cardiotoxicity evaluation

When mice were sacrificed due to large tumor size (above 1500 mm³) or due to toxicity (more than 15% body weight loss), hearts were perfused with PFA. To evaluate pathological changes in cardiac morphology and structure, hearts were stained with Masson’s Trichrome. Fig. 9 presents results of histological analysis of heart of mice treated with PGA-PTX-DOX conjugate, PG-PTX-bz-DOX-IDCC-PEG dendritic conjugate and controls, showing adverse remodeling (enlarged left ventricle volume, diminished thickness of the ventricular wall) of the heart treated with DOX, and especially with the combination of both free drugs (PTX-bz + DOX and PTX + DOX) as opposed to the heart of mice treated with the different conjugates (Fig. 9).

4. Discussion

Despite considerable advances in breast cancer chemotherapy, mortality rates have remained relatively unaffected over the span of three decades. Throughout this period, anthracyclines and taxanes have dominated the chemotherapeutic landscape, proving indispensable in several combination regimens [43].

The main aim of this study was to compare the anti-cancer activity of two polymer therapeutics combining two synergistic drugs at an appropriate ratio, and to determine their advantage over drugs conjugated to separate polymer chains, as well as over the combination of free drugs. PTX and DOX were chosen because they are widely used in the clinic for different types of cancer, especially as sequential combination for the treatment of MBC [12,13], and their exceptional synergistic effect was already shown in immunodeficient mouse model [18].

Before coupling PTX and DOX to the carrier, we thoroughly investigated the optimal ratio of drugs for synergism and the different types of breast cancer cells in which synergism occurs. We already showed that these drugs have a synergistic cytotoxic activity on MDA-MB-231 cells, when DOX is at higher concentration than PTX, according to calculated isobolograms [18]. In order to strengthen this finding, we also showed that PTX and DOX have a synergistic cytotoxic activity on murine mammary adenocarcinoma, DA3 and 4T1 cells and human breast cancer cells, MCF-7.

In addition to the design and synthesis of the PGA-PTX-DOX conjugate [18], we designed, synthesized and characterized a dendritic conjugate bearing both PTX and DOX, namely, PG-PTX-bz-DOX-IDCC-PEG. All dendritic conjugates were synthesized to contain PTX and/or DOX in a molar ratio of 1:5, respectively. Furthermore, all conjugates were characterized by DLS, demonstrating that all are nano-sized conjugates (~40 nm for PGA-PTX-DOX and 24 nm for PG-PTX-bz-DOX-IDCC-PEG). It should be noted that attaching drugs at the periphery of the dendrimer can lead to unpredictable aggregation. However, the attachment of PEG chains to the periphery of the conjugates would decrease the aggregation and avoid uptake by the reticuloendothelial system and enhance the plasma residence.

In these conjugates, the drugs were linked via a hydrazide bond (EMCH linker) which is an acid-labile linker. Cleavage of the drugs should occur upon internalization of the conjugate in cells and will not be reversed by the acidic pH of the cytoplasm.

### Table 5

<table>
<thead>
<tr>
<th>T/C ratio</th>
<th>DOX</th>
<th>PTX</th>
<th>PTX + DOX</th>
<th>PGA-DOX</th>
<th>PGA-PTX</th>
<th>PGA-DOX + PGA-PTX</th>
<th>PGA-PTX-DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.65</td>
<td>1.02</td>
<td>0.59</td>
<td>0.81</td>
<td>0.88</td>
<td>0.66</td>
<td>0.57</td>
<td>0.48</td>
</tr>
<tr>
<td>1.00</td>
<td>0.54</td>
<td>0.48</td>
<td>0.52</td>
<td>0.54</td>
<td>0.57</td>
<td>0.54</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Please cite this article as: H. Baabur-Cohen, et al., *In vivo* comparative study of distinct polymeric architectures bearing a combination of paclitaxel and doxorubicin at a synergistic ratio..., J. Control. Release (2016), http://dx.doi.org/10.1016/j.jconrel.2016.06.037
result in the potential release of free PTX and DOX in acidic environment as suggested by the in vitro drug release studies in pH 4.0 (Fig. 2), such as that in the endolysosomal compartments. It appears that PTX and DOX are efficiently released from the conjugates, as suggested by their strong inhibitory effect. We have previously demonstrated such pH mediated intracellular cleavage and release from PG conjugates through the use of fluorescent smart probes [44,45]. PG-PTX-bz-IDCC-PEG was highly cytotoxic to both MDA-MB-231 and mCherry-labeled 4T1 cells, suggesting that PTX and DOX are released from the dendritic polymer and retain their activity within 72 h. PG-PTX-bz-IDCC-PEG had a higher inhibitory effect compared to the free drugs (one order of magnitude) and as expected, similar inhibitory effect compared

Fig. 8. Antitumor activity and safety of PGA-PTX-DOX and PG-PTX-bz-DOX-IDCC-PEG in mammary tumor bearing mice. (A) The combined conjugates (i.e., PGA-PTX-DOX and PG-PTX-bzDOX-IDCC-PEG) inhibited tumor volume growth more than the combination of PTX and DOX conjugated to separate polymer chains (PGA-PTX + PGA-DOX and PG-PTX-bz-IDCCPEG + PG-DOX-IDCC-PEG) and the combination of free drugs (PTX + DOX and PTX-bz + DOX) in equivalent concentrations. Data represents mean ± SEM. Statistical significance was determined compared to Control group, on day 22 when all groups were still present, using two-tailed t-test, (**p < 0.005, *p < 0.01). (B) Kaplan-Meier survival curve. Mice in the PGA-PTX-DOX, PG-PTX-bz-DOX-IDCC-PEG and PG-PTX-bz-IDCC-PEG + PG-DOX-IDCC-PEG groups had the longest overall survival. (C) CT images of tumor-bearing mice representing the average tumor size of each group. Images were taken 18 days after tumor inoculation.
to PG-PTX-bz-IDCC-PEG, PG-DOX-IDCC-PEG and their combination (same order of magnitude). PG-PTX-bz-DOX-IDCC-PEG is less cytotoxic in vitro compared to PGA-PTX-DOX (IC₅₀ of 250 nM and 15 nM, respectively).

Cell internalization and nuclear accumulation of the conjugated DOX was evaluated by confocal microscopy. Both free DOX and the dendritic conjugate (PG-PTX-bz-DOX-IDCC-PEG) internalized into the cells, but not into the nucleus, by 30 min. At this time point, DOX was still bound to its carrier. Only after 24 h DOX was co-localized with the lysosome and there was no co-localization with IDCC, indicating the release of the drug from its carrier PG.

In addition, PG-PTX-bz-DOX-IDCC-PEG moderately inhibited the migration of MDA-MB-231 cells. Following 15 h of incubation with the dendritic conjugate and the controls, the combined dendritic conjugate showed the highest inhibition of cell migration, presenting 49% of gap closure, compared to the initial time (t₀). It seems that PTX-bz is less active in inhibiting the migration of the cells compared to PTX. Although, in this scratch assay, lower concentration of the anti-migratory agent was used (20 nM PTX-bz compared to 100 nM PTX), similar results were achieved when the same concentration was used (i.e., 100 nM PTX-bz) (data not shown). Since the molar ratio between PTX-bz and DOX on the dendritic conjugate is higher (1:5) compared to the ratio on the polymer-drug conjugate, PGA-PTX-DOX (1:2.5), a higher concentration of PTX-bz was not used in order to avoid increased toxicity to the cells. Moreover, the anti-migratory effect occurs by PTX and not by DOX.

Biodistribution analysis of PG-PTX-bz-DOX-IDCC-PEG demonstrated preferred accumulation of the conjugate in tumors at all-time points, similarly to the biodistribution that was seen with PGA-PTX-DOX conjugate [18]. The accumulation of the conjugate in the kidneys is most probably due to renal excretion that was already apparent 1 h following injection of the dendritic conjugate. As already shown for PGA-PTX-DOX [18], some amount of the PG-PTX-bz-DOX-IDCC-PEG was also detected in the spleen suggesting splenic clearance of particles by the reticuloendothelial system.

Treatment with PGA-PTX-DOX conjugate and PG-PTX-bz-DOX-IDCC-PEG dendritic conjugate led to substantial antitumor effect on murine mammary adenocarcinoma mouse model, as compared to the controls. As opposed to the human mammary model (human breast cancer cells, MDA-MB-231), the follow-up was shorter (39 days), since the tumor type was more aggressive (murine mammary cancer cells, mCherry-labeled 4T1). Interestingly, the combined dendritic conjugate PG-PTX-bz-DOX-IDCC-PEG inhibited to a similar extent tumor growth as the mixture of individually-conjugated drugs (PG-PTX-bz-IDCC-PEG + PG-DOX-IDCC-PEG). This was a major difference compared to PGA-PTX-DOX, which was much more potent than the combination of the separate conjugates PGA-PTX + PGA-DOX, in this study in murine model of cancer and in previous study in human model of cancer in immunodeficient mice [18]. We attribute this observation to the different conformation of PGA conjugates and PG dendritic conjugates. Moreover, we assume that when administrated together, PG-PTX-bz-IDCC-PEG and PG-DOX-IDCC-PEG share similar pharmacokinetics, which enables them to reach at the same time to the tumor site, and release the drugs there simultaneously. As opposed to PGA-PTX and PGA-DOX, which apparently differ in their pharmacokinetic profile, and when administrated together, result in lower efficacy compared to PGA-PTX-DOX. One parameter that might contribute to that is the different loading of the drugs on each polymer (i.e., 13.5% in PGA-PTX and 2.8% or 8.8% in PGA-DOX). Furthermore, the low polydispersity of dendritic polymers should provide a more reproducible pharmacokinetic behavior than in linear polymers [46].

It is noteworthy that in contrast to the xenogeneic mouse model of cancer, PGA-DOX did induce tumor growth inhibition in vivo compared to PBS-treated mice (T/C = 0.81). In this experiment, there was higher loading of DOX on PGA-DOX conjugate (i.e., 8.8% vs. 2.8%), which might have improved the release kinetics and the delivery efficiency [18]. All groups treated with free DOX suffered from extreme weight loss, and several mice were euthanized due to a reduction of 15% in body weight, whereas treatment with the conjugates at equivalent concentrations did not.

In our study, DOX-related cardiotoxicity was observed in all treatments with free DOX (alone or in combination with PTX or PTX-bz). The combination of PTX and DOX was more toxic compared to DOX alone. Several reports demonstrate that PTX potentiates DOX-induced cardiotoxicity following DOX/PTX combination treatment. The authors attributed the potentiated DOX-induced cardiotoxicity to an increase in cardiac tissue concentration of the drug or its active metabolite doxorubicin, and/or pharmacokinetic interference of DOX elimination by PTX, an effect that is highly dependent on the interval between administration of the drugs and the duration of PTX infusion [47,48]. Gianni et al. showed that PTX enhances the nonlinearity of DOX pharmacokinetics and significantly decreases the systemic clearance of DOX [49]. Since free drugs, as opposed to our conjugates, diffuse rapidly through normal blood vessels, heart’s accumulation of both PTX and DOX is more likely to occur, and then the aforementioned decrease in the systemic clearance will result in higher cardiotoxicity.

Both polymer therapeutics showed optimal properties for in vitro and in vivo applications. Table 6 summarizes the major differences between the linear conjugate, PGA-PTX-DOX and the dendritic conjugate, PG-PTX-bz-DOX-IDCC-PEG. The nanocarriers exhibit some of the critical features (e.g., suited size, solubility, and molecular mass) required for a delivery system in order to achieve enhanced efficacy and safety compared to its free counterparts.

PGA and PG polymers are widely explored in the field of polymer-drug conjugates [29,50], in which we see an increasing focus on delivering a combination of two drugs on the same polymeric carrier [15]. However, several nanoparticle platforms are currently being explored clinically in breast cancer [43]. The clinically approved PEGylated-liposomal DOX (i.e., Doxil) was combined with docetaxel (DTX) in a phase III clinical trial in patients with advanced breast cancer, resulting in improved progression-free survival from 7 to 9.8 months [51]. In Phase I/II trial involving patients with locally advanced or metastatic breast
cancer, non-PEGylated liposomal doxorubicin was administered with trastuzumab and paclitaxel, after which the response rate was found to be 98.1% with a median time to progression of 22.1 months in metastatic patients [52]. Another nanoparticle platform is found in the work of Ferrari et al. [53]. They examined the therapeutic potential of delivering rapamycin and paclitaxel preferentially to breast tumors using nanoparticles. Polymer micelles, core-shell nanoparticles formed from poly(ethylene glycol)-poly(ε-caprolactone) block copolymers, were used as drug carriers given their biocompatibility and lack of adverse effects. Synergy following nanoparticle delivery was shown to be ratio dependent both in vitro and in vivo, due to increased delivery of both rapamycin and paclitaxel preferentially to tumors and precise preservation of drug ratios for extended time periods.

To conclude, our studies showed that PG-PTX-bz-DOX-IDCC-PEG had a similar activity to the mixture of PTX and DOX bound to separate PG conjugates (PG-DOX-IDCC-PEG + PG-PTX-bz-IDCC-PEG). It will be very interesting to follow this observation in the future, since it is synthetically easier to modify PG with only one drug and one could have a higher control on tuning the ratios. On the other hand, as was previously shown, PGA-PTX-DOX was more efficient than a mixture of the free drugs and the separate conjugates suggesting that the superiority of the combined conjugate depends on many factors including supramolecular structure, linkers, and their mode of activation, as well as the drug release profiles.

An important advantage of linear polymeric carriers over spherical particles is their flexible random coil structure, which allows them to “snake” into gaps smaller than their hydrodynamic diameter and penetrate into tissues better than the more structured spherical and particulated carriers. This, perhaps, makes them more effective in the conditions of angiogenic vasculature characterized by gaps of variable size and of dense tumor tissue [54]. A recent study on PEG-based particles showed that rod-shaped nanoparticles are internalized faster than spherical particles, suggesting that linear polymers might internalize faster than other spherical carriers [55]. However, dendritic polymers hold several advantages as well. Unlike linear polymers, properly designed high-generation dendrimers or hyperbranched polymers with high degree of branching exhibit a distinct “interior” that is sterically shielded within the polymer branches and enables their use as nanoscale transport systems. The synergy between their multivalency and size in the nanoscale has a range of options to place them as an appropriate competitor to the linear polymers in the therapeutic practice [29].

These characteristics of both PGA and PG can be used to generate new material for biomedical applications, to create extremely high local concentrations of drugs, molecular labels, or probe moieties, or to modulate therapeutic efficacy of the active molecules. Taken together, our work provides in vivo evidence for the potential of polymer-based multi-drug targeting and strengthens its prospective clinical translation based on systematically- and rationally-designed combinations.

Acknowledgements

This study was partially supported by the European Research Council (ERC) Consolidator Award (617445 - PolyDorm), the Israel Science Foundation (grant no. 918/14), and by grants from the Israeli National Nanotechnology Initiative (INNI), Focal Technology Area (FTA) program: Nanomedicine for Personalized Theranostics, and by the Leona M. and Harry B. Helmsley Nanotechnology Research Fund. MC gratefully acknowledges the financial support from the Focus Area Nanoscale and the Bundesministerium für Bildung und Forschung (BMBF) through the NanoMatFutur award (13N12561).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.06.037.

References


Please cite this article as: H. Baabar-Cohen, et al., In vivo comparative study of distinct polymeric architectures bearing a combination of paclitaxel and doxorubicin at a synergistic ratio..., J. Control. Release (2016), http://dx.doi.org/10.1016/j.jconrel.2016.06.037.


