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Integrin-Targeted Nano-Sized Polymeric Systems for Paclitaxel Conjugation: A Comparative Study

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ABSTRACT

The generation of rationally-designed polymer therapeutics via the conjugation of low molecular weight anti-cancer drugs to water-soluble polymeric nanocarriers aims to improve the therapeutic index. Here, we focus on applying polymer therapeutics to target two cell compartments simultaneously - tumor cells and angiogenic endothelial cells. Comparing different polymeric backbones carrying the same therapeutic agent and targeting moiety may shed light on any correlation between the choice of polymer and the anti-cancer activity of the conjugate. Here, we compared three paclitaxel (PTX) bound conjugates with poly-L-glutamic acid (PGA, 4.9 mol%), 2-hydroxypropylmethacrylamide (HPMA, 1.2 mol%) copolymer, or polyethyleneglycol (PEG, 1:1 conjugate). PGA and HPMA copolymer are multivalent polymers that allow the conjugation of multiple compounds within the same polymer backbone, while PEG is a commercially-available Food and Drug Administration (FDA)-approved polymer. We further conjugated PGA-PTX and PEG-PTX with the integrin $\alpha_v\beta_3$-targeting moiety RGD (5.5 mol% and 1:1 conjugate respectively). We based our selection on the overexpression of integrin $\alpha_v\beta_3$ on angiogenic endothelial cells and several types of cancer cells. Our findings suggest that polymer structure has significant effect on the conjugate's activity on different tumor compartments. A multivalent PGA-PTX-E-[c(RGDfK)$_2$] conjugate displayed a stronger inhibitory effect on the endothelial compartment, with a 50% inhibition of cell migration in HUVEC cells, while a PTX-PEG-E-[c(RGDfK)$_2$] conjugate possessed enhanced anti-cancer activity in MDA-MB-231 tumor cells (IC_{50} = 20 nM vs IC_{50} 300 nM for the PGA conjugate).

INTRODUCTION

The conjugation of chemotherapeutic drugs to polymeric carriers aims to improve drug pharmacokinetic and pharmacodynamic profiles, increase efficacy, reduce toxicity, and enable easier administration to improve patient compliance [1]. Furthermore, these conjugates, known as polymer therapeutics, demonstrate tumor specific accumulation via the enhanced permeability and retention (EPR) effect [2]. However, the particular polymeric backbone used can influence both the pharmacokinetics and pharmacodynamics of the conjugated drug. Polymer characteristics, such as average molecular weight (Mw), molecular weight distribution (dispersity, Đ = Mw/Mn), architecture, charge, and hydrophilicity can affect drug solubility, biodistribution, excretion, and interaction with the immune system [3-5]. Among the different polymers in clinical use are 2-*N*-hydroxypropylmethacrilamide (HPMA) [6, 7], poly-L-glutamic acid (PGA) [8], and polyethyleneglycol (PEG) [9].

PGA is a water soluble, non-toxic, and biodegradable polymer that can be synthesized by ring-opening polymerization (ROP) of the corresponding N-carboxyanhydrides (NCA) [10]. PGA contains a γ-carboxyl group in each repeating unit of L-glutamic acid that offers multivalent attachment points for drugs and can be degraded in the lysosome by cysteine proteases such as cathepsin B [8]. These features make PGA an attractive drug carrier. Reinforcement is given by the PGA-PTX formulation (Opaxio™, formerly known as XYOTAX), which is currently in Phase II/III clinical trials [11-13]. Encouragingly, Opaxio™ has demonstrated promising results in different types of cancer alone or in combination with radiotherapy [14-16].

PEG, while not biodegradable, is water-soluble, biocompatible, and is Food and Drug Administration (FDA)-approved for clinical use [17]. PEG has been traditionally used in the polymer therapeutics field in polymer-protein conjugates for its anti-immunogenic and stabilizing properties as well as for being an excellent time-of-circulation enhancer. More

recently, PEG has also been conjugated to certain drugs in order to improve their biocompatibility and pharmacokinetics profile. In fact, Naloxegol (NKTR-118 - also known as Movantik®), the first polymer-drug conjugate to achieve market approval, uses PEG as a carrier [18-20].

The third polymer selected for this study, HPMA copolymer, represents one of the most studied platforms for polymer-drug conjugates. HPMA copolymer is water-soluble, non-charged, and non-immunogenic, and has been developed for site-specific delivery of anti-cancer drugs [21].

HPMA copolymer, PGA, and PEG have all been used in polymer conjugates with chemotherapeutic drugs such as paclitaxel (PTX) [22-25]. PTX, an anti-microtubule and anti-angiogenic agent [26-29], has proven to be an effective treatment for metastatic breast cancer and ovarian cancer, among notable others [30, 31]. However, poor water solubility necessitates a formulation with Cremophor EL, which causes anaphylactic and hypersensitivity reactions [32]. Conjugation of PTX with PEG, PGA, or HPMA-copolymer preserves the anti-cancer potential of PTX while also abrogating associated side effects [22-25].

Another strategy employed to improve the efficiency of polymer-drug conjugates is the addition of a targeting moiety that targets specific cell types within or proximal to tumor sites. This gains significance given the synergistic anti-cancer outcomes observed when targeting angiogenesis, the generation of new blood vessels from pre-existing vasculature [33, 34], and cancer cells at the same time. Tumor angiogenic blood vessels overexpress certain unique molecular markers, such as the integrin $\alpha_v\beta_3$ heterodimeric cell surface receptors, which enhance tumor cell proliferation, migration, invasion, and survival. The tripeptide sequence Arg-Gly-Asp (RGD) targets integrin $\alpha_v\beta_3$ [35], while cyclization of linear RGD improves binding properties and stability [36, 37]. Synthetic RGD-containing

molecules are miss-recognized and captured by these integrins in their search for extracellular fibrinogen, which is RGD-motive rich in its structure. The successful application of cyclic RGD as a targeting peptide has seen success in breast [25, 38] and prostate [39] cancer models with enhanced anti-angiogenic and anti-cancer effects and reduced toxicity compared to the unconjugated (or free) chemotherapeutic drug used in each study. Furthermore, the RGD-based molecule Cilengitide (EMD121974) acts as a potent integrin $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor and was the first agent reported to display anti-angiogenic clinical potential [40, 41]. Cilengitide is currently being tested in several clinical trials as a monotherapy or in combination with conventional chemotherapy [42-44].

In order to fully understand and compare each polymer-drug conjugate, we proposed a two-step comparison. First, we conjugated PTX to three different polymers (PGA, HPMA copolymer, and PEG) and compared each conjugate in terms of drug release and anti-cancer activity. Following this, we compared PGA-PTX and PEG-PTX conjugates with the addition of RGD moieties in the hope of specifically targeting integrin-expressing cancer cells and endothelial cells, while sparing healthy tissues.

MATERIALS AND METHODS

Materials

PTX was purchased from Alcon Biosciences Ltd. (Mumbai, India; Petrus Chemicals and Materials Ltd., Israel). Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Penicillin, Streptomycin, Nystatin, L-glutamine, HEPES buffer, sodium pyruvate, and fibronectin were from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). EGM-2 medium was purchased from Cambrex (Walkersville, MD, U.S.A). Matrigel matrix was purchased from BD Biosciences, USA. E-$[c(RGDfK)]_2$ and c(RADfK) were purchased from

Peptides International (Louisville, KY, USA); Trt-S-C_2H_4-NHCO-PEG-C_3H_6COO-NHS (MW 11.7 kDa) and Trt-S-C_2H_4-NHCO-PEG-C_3H_6COOH (MW 9.7 kDa) were purchased from Rapp Polymers (Germany); PGA (MW 17 kDa, Đ = 1.3) was purchased from Polypeptide Therapeutic Solutions SL (PTS, Spain). HPMA copolymer-Gly-Phe-Leu-Gly-ONp (Mw = 31.6 kDa, Đ = 1.66) incorporating 10 mol% of the methacryloyl-Gly-Phe-Leu-Gly-p-nitrophenol ester monomer units were obtained from Polymer Laboratories (Church Stretton, UK). Organic solvents were High-performance liquid chromatography (HPLC) grade (Labscan Ltd., Dublin, Ireland; Roth, Karlsruhe, FRG; Merck, Darmstadt, FRG). All other chemicals used were reagent grade and obtained from Sigma-Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany) and used without further purification; buffers were vacuum-filtered through a 0.2 μm membrane (Sartorius, Germany) and thoroughly degassed with nitrogen prior to use. Mass spectra were obtained using a Thermo Electron LCQ Advantage with the associated MAT SS 200 data system using electron spray ionization. UV/VIS-spectrophotometry was carried out with a double-beam spectrophotometer U-2000 from Hitachi.

Synthesis of PGA-PTX conjugates

Synthesis of PGA-PTX, PGA-PTX-E-[c(RGDfK)$_2$] and PGA-PTXc(RADfK) conjugates was previously reported by Eldar-Boock *et al.* [25]**.**

a) Synthesis of PGA-PTX (1)

Briefly, PGA-PTX was conjugated to PGA (Mw = 17.7 kDa, Đ = 1.3) by carbodiimide coupling (DIC/HOBT). The reaction was allowed to proceed at room temperature (RT) for 24 h. The conjugate was precipitated in a $CHCl_3$:Acetone mixture (1:4), filtered, and dried under vacuum and after lyophilization, PGA-PTX (**1**) formed a white powder (70-80% yield) (Figure 1). PTX content was determined by ultraviolet (UV) (λ = 227 nm and 230 nm in

MeOH) and HPLC (indirect analysis determining PTX content in reaction residues λ = 227 nm, gradient 35- 80% (v/v) water:CH_3CN) and obtained residue was dissolved in 1.0 M $NaHCO_3$ and purified by aqueous Size Exclusion Chromatography (SEC) in a Sephadex G25 column.

b) Synthesis of PGA-PTX-c(RADfK) (3) and PGA-PTX-E-[c(RGDfK)$_2$] (4):

Briefly, PGA (Mw = 17.7 kDa, Đ = 1.3) was conjugated to PTX by carbodiimide coupling (DIC/HOBT). The reaction was allowed to proceed at RT for 24 h. Then, without product isolation, N-hydroxysuccinimide (SuccOH, 30 mol %) was added to the reaction mixture and the reaction was allowed to proceed for another 24 h. The mixture was then poured into a mixture of $CHCl_3$:Acetone (1:4). The resulting precipitate was collected and washed again with acetone and MeOH and dried in vacuum to yield a white solid powder (**2**). The solid was intermediately dissolved once more in anhydrous dimethylformamide (DMF) and then c(RADfK) or E-[c(RGDfK)$_2$] were added together with catalytic amounts of 4-dimethylaminopyridine (DMAP). The pH was adjusted to 8 with DIEA. The reaction was allowed to proceed at RT for 48 h and then the mixture was poured into $CHCl_3$:Acetone. The resulting precipitate was collected and dried in vacuum to yield a white powder that was then dissolved in 1.0 M $NaHCO_3$. This aqueous solution was run through an SEC (Sephadex G25) column and lyophilization of the fractions yielded the product as a white powder (70-80% yield). PTX content was analyzed as described above for conjugate **1**. The total peptide content in these polymeric conjugates was determined by UV (λ = 254 nm and 260 nm in MeOH) and HPLC (indirect analysis determining peptide content in reaction residues, gradient: CH_3CN:0.1% (v/v) trifluoroacetic acid (TFA) (10-90% (v/v)) λ = 220 nm). Peptide content was also determined by amino acid analysis.

Synthesis of PEG-PTX conjugates:

a) Synthesis of PTX-EMCH (6)

In order to generate an acid sensitive linker, the non-commercial PTX-derivative Ketotaxol (**5**) was synthesized following the protocol from Rodrigues *et al.* [45]. Afterwards, as described by Moktan *et al.* [46], 400 mg of (**5**) (0.4 mmol) and 272 mg of 6-maleimidocaproic acid hydrazidetrifluoroacetate (0.8 mmol) were dissolved in 12 mL anhydrous ethanol and stirred for 90 min at RT. The solution was incubated overnight at +4°C. The white precipitate was isolated by centrifugation, washed with 15 mL diethylether, and isolated by centrifugation. Drying under high vacuum produced 283 mg of **6**.

b) Synthesis of PTX-PEG-COOH conjugate (10)

Trt-S-C_2H_4-NHCO-PEG-C_3H_6COOH (154 mg, 16.2 µmol, 1 equiv., Mw = 9.7 kDa) in 400 µL TFA/ dichloromethane (DCM) 1/1, v/v (containing 2.5 % (v/v) *i*Pr_3SiH, 5 % thioanisole/anisole, 1/1, (v/v); 5 % (v/v) H_2O) was sonicated for approximately 30 seconds (clear yellow solution turned into clear colorless solution) and stirred at RT for 20 min. The solvent was removed in high vacuum (approximately 20 min) and petrol ether/ether (1.5-2 mL, 1/1, v/v) was next added to the residue and sonicated for ~30 seconds. The supernatant was removed after centrifugation. A final wash with dry ether was performed to yield conjugate **7**. In order to remove the protecting group on the thiol, conjugate **7** was dissolved in 500 µL of 50 mM sodium phosphate buffer pH 7. The pH of the remaining solution was readjusted to 7-7.5 with 0.1 N NaOH. PTX-EMCH was immediately added (19 mg, 1 eq), sonicated for 20 min, and stirred on ice for 4 h. The conjugate was purified by SEC (Sephadex G25 in phosphate-buffered saline (PBS) pH=7) to yield conjugate **10**.

c) Synthesis of HS-PEG- E-[c(RGDfK)$_2$] (8) and HS-PEG-c(RADfK) (9)

Conjugates (**8**) and (**9**) were obtained following an already described protocol by Polyak *et al.* [38]. Briefly, *N*,*N*-Diisopropylethylamine (DIEA) (10.2 or 24 µL respectively) was added to a solution of the Trt-S-C_2H_4-NHCO-PEG-C_3H_6COO-NHS (700 mg, 59.8 mmol, 1 equiv., Mw = 11.7 kDa) and, depending on the case, E-[c(RGDfK)$_2$] (78.9 mg, 59.8 mmol, 1 equiv.) or c(RADfK) (10 mg, 16.2 µmol, 1 equiv.) in dry DMF (15 mL). The solution was stirred at RT for 48 h, then precipitated in dry diethyl ether (300 mL), washed with pentane (150 mL), and filtered and dried *in vacuum* to obtain a white powder (694 mg, Mw = 13.0 kDa in the case of conjugate **8** and 103 mg, 16 µmol Mw = 12.3 kDa for conjugate **9**). The material was stored at -20 °C. The protected Trt-S-PEG-E-[c(RGDfK)$_2$] (25 mg) in 400 µL TFA/DCM 1/1, v/v (containing 2.5 % (v/v) *i*Pr$_3$SiH, 5 % (v/v) thioanisole/anisole, 1/1, v/v; 5 % (v/v) H_2O) was sonicated for 30-60 seconds (clear yellow solution turned into clear colorless solution) and then stirred at RT for 20 min. The solvent was removed in high vacuum (approximately 20 min). Petrol ether/ether (1.5-2 mL, 1/1, v/v) was added to the residue and sonicated for approximately 30 seconds. The supernatant was removed after centrifugation and dry ether was added to the residue. After centrifugation, the residue was dissolved in 500 µL of a 50 mM sodium phosphate buffer pH 7. The pH of the remaining solution was readjusted to 7-7.5 with 0.1 N NaOH. The amount of SH groups was determined by means of an Ellman assay (3 x 10 µL solution; λ= 412 nm, ε = 13600 M^{-1} cm^{-1}). The solution was used directly for coupling to the PTX prodrug-EMCH (**6**).

d) Synthesis of PTX-PEG-E-[c(RGDfK)$_2$] (11) and PTX-PEG-c(RADfK) (12)

PTX prodrug-EMCH (1.05 mg, 0.8 µmol, 1 eq.) was added to a solution of freshly deprotected HS-PEG-E-[c(RGDfK)$_2$] (**8**) or HS-PEG-c(RADfK) (**9**) (10 and 9.3 mg, 1 eq., respectively) in 50 mM sodium phosphate buffer pH 7. The resulting solution was sonicated

and stirred on ice for 4 h. The PEG conjugate was isolated by SEC (G25 Sephadex in PBS buffer, pH=7). The fractions were freeze dried to yield conjugate **11** and **12** respectively.

Synthesis of HPMA copolymer-GFLG-FK-PTX conjugate (18)

The conjugation of PTX with HPMA copolymer through a GFLG-FK peptidic linker was performed as previously described [24]. The synthetic strategy comprises the attachment of PTX to a FK-PABC linker (**15**) and then its conjugation to HPMA copolymer–GFLG-ONp (Figure 3).

a) Synthesis of Boc-Phe-Lys (alloc)-OH (13)

L-Boc-Phe-ONp (208.6 mg, 0.54 mmol) was dissolved in 3 mL DMF and commercially available L-lys(alloc)-OH (124 mg, 0.54 mmol) and Et3N (200 µL) were then added. The reaction mixture was stirred for 12 h and was monitored by thin layer chromatography (TLC) (AcOH:MeOH:EtOAc 0.5:10:89.5). Upon completion of the reaction, the solvent was removed under reduced pressure and the crude product was purified by using column chromatography on silica gel (AcOH:MeOH:EtOAc 0.5:10:89.5) to give conjugate **13** (214 mg) as a white solid (Figure 3).

b) Synthesis of Boc-Phe-Lys (alloc)-PABA (14)

Boc-Phe-Lys (alloc)-PABA **13** (208 mg, 0.435 mmol) was dissolved in dry tetrahydrofuran (THF) and the solution was cooled to -15° C. Then, N-Methylmorpholine N-oxide (NMM) (475 µL, 0.435 mmol) and isobutyl chloroformate (675 µL, 0.522 mmol) were added. The reaction was stirred for 20 min and a solution of 4-aminobenzyl alcohol (80.46 mg, 0.65 mmol) in dry THF was added. The reaction mixture was stirred for 2 h and was monitored by TLC (EtOAc). Upon completion of the reaction, the solvent was removed under reduced

pressure and the crude product was purified by using column chromatography on silica gel (EtOAc) to give conjugate **14** (208 mg) as a yellow solid (Figure 3).

c) Synthesis of Boc-Phe-Lys (alloc)-PABC-ONp (15)

Boc-Phe-Lys (alloc)-PABA **(14)** (186.1 mg, 0.315 mmol) was dissolved in dry THF and the solution was cooled to 0° C. Then N, N-Diisopropylethylamine (DIPEA) (221 µL, 1.273 mmol), PNP-chloroformate (193.1 mg, 0.526 mmol), and a catalytic amount of pyridine were added. The reaction was stirred for 2 h and monitored by TLC (EtOAc:Hex 3:1). Upon completion of the reaction, the solvent was removed under reduced pressure. The crude product was diluted with EtOAc and washed with saturated NH_4Cl. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by using column chromatography on silica gel (EtOAc:Hex 3:1) to give conjugate **15** (238.5 mg) as a white solid (Figure 3).

d) Synthesis of Boc-Phe-Lys (alloc)-PABC-PTX (16)

Boc-Phe-Lys (alloc)-PABC-ONp **15** (212 mg, 0.28 mmol) was dissolved in dry DCM. Then PTX (123.5 mg, 0.33 mmol) and DMAP (17.65 mg, 0.33 mmol) were added. The reaction mixture was stirred for 8 h at RT and monitored by TLC (EtOAc). Upon completion of the reaction, the solvent was removed under reduced pressure and the crude product was purified by using column chromatography on silica gel (EtOAc) to give conjugate **16** (389.4 mg) as a white solid (Figure 3).

e) Synthesis of HPMA copolymer-GFLG-Phe-Lys(alloc)-PABC-PTX conjugate (17)

Boc-Phe-Lys (alloc)-PABC-PTX (Conjugate **16)** (12 mg, 7.57 μmol) was dissolved in 0.5 mL TFA and stirred for 2 min at 0°C. The excess of acid was removed under reduced pressure and the crude amine salt was dissolved in 0.5 mL DMF. HPMA copolymer-GFLG-ONp (26.3 mg, ONp = 8.32 μmol, 31.6 kDa, Đ =1.66) was added followed by the addition of Et_3N (3 μL). The reaction mixture was stirred for 12 h and the solvent was removed under reduced pressure. Free FK-PTX and ONp were removed by fast protein liquid chromatography (FPLC) using an XK26/70 column with Sephadex LH20 column (MeOH, 1 mL/1 min) to give the alloc protected HPMA copolymer-GFLG-FK-PTX **(17)** as a white solid (20 mg) (Figure 3).

f) Synthesis of HPMA copolymer –GFLG-FK-PTX (18)

Alloc protected HPMA copolymer –GFLG-FK(alloc)-PTX (30 mg, alloc = max. 9.9 μmol) was dissolved in DMF (1 mL). Then, acetic acid (2.71 μL, 47.4 μmol), Bu_3SnH (30.6 μL, 113 μmol), and a catalytic amount of Pd $(PPh_3)_4$ were added. The reaction mixture was stirred for 2 h at RT and was concentrated under reduced pressure, followed by addition of 10 mL of acetone. The precipitate was filtered out and washed with acetone several times. The crude product was purified by FPLC using XK26/70 column with Sephadex LH20 (MeOH, 1 mL/1 min) to give HPMA copolymer-GFLG-FK-PTX (20 mg) as a white solid (Figure 3). Content of PTX in the conjugate was determined by HPLC analysis according to calibration curve of free PTX-FK.

Determination of mean hydrodynamic diameter of the conjugates

The mean hydrodynamic diameter of the nanocarriers was evaluated by dynamic light scattering (DLS) using a ZetaSizer Nano ZS instrument with an integrated 4mW, He-Ne laser

(λ=633 nm; Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were prepared by dissolving 1 mg of polymer conjugate in 1 ml of 155 mM phosphate buffer, pH=7.4. All measurements were performed at 25°C using polystyrol/polystyrene (10×4×45) mm cell.

Drug release profile and polymer degradation kinetics

a) Degradation in the presence of cathepsin B

Degradation of PTX catalyzed by cathepsin B was studied for PGA-PTX and HPMA copolymer-GFLG-FK-PTX. In the case of PGA-PTX, a previously reported protocol was followed [25]. Briefly, cathepsin (5 U) was added to a solution of conjugate or free drug (3 mg/mL) in a fresh prepared buffer made of 20 mM sodium acetate, 2 mM EDTA and 5 mM DTT, pH 5.5) and incubated at 37°C. Aliquots (100 µL) were taken at different times up to 72 h, immediately frozen in liquid nitrogen, and stored in darkness. The amount of released compound was assayed by HPLC (analysis after extraction procedure by Poros50 resin drug content analysis (λ=227 nm)) using doxycycline as internal standard. The release of PTX from HPMA-PTX-FK conjugate was monitored by reversed phase (RP) HPLC using Phenomenex Jupiter 5 µm 250 X 4.60 mm C-18 300A column. Samples (50 µL) were collected every 24 h, until a plateau was observed (up to 72 h). For PTX extraction, sodium carbonate buffer solution (0.2 M, pH=9.6) was added to each sample, followed by ethyl acetate. Samples were vigorously vortexed and centrifuged and the organic layer carefully removed and evaporated. The residue was dissolved in MeCN and analyzed by HPLC. For non-enzymatic hydrolytic cleavage assessment, polymers were incubated in buffer alone (pH 5.5) in the absence of cathepsin B. Additionally, an LC-MS analysis of the released compounds was carried out to determine the major metabolites released.

b) Degradation in acidic pH

PEG-PTX or PTX-PEG-RGD conjugate were dissolved at a concentration of 5 mg/mL in PBS at pH 5.5 or pH 7.4. Samples were incubated at 37°C and aliquots (50 µL) were taken at several time-points up to 72 h. Samples were stored at -20°C in darkness until analysis. The amount of released drug was assayed by HPLC against a calibration curve of free PTX. Samples (50 µL) were injected to HPLC equipped with C18 column (Jupiter, 300A, 250x4.6 mm, 5 micron).

Cell culture

The MDA-MB-231 human mammary adenocarcinoma cell line and 4T1 murine breast cancer cell lines were purchased from the American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 12.5 U/mL Nystatin, and 2 mM L-glutamine. 4T1 cells were grown in RPMI-1640 supplemented with 10% (v/v) FBS, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 12.5 U/mL Nystatin, 2 mM L-glutamine, 1% (w/v) HEPES 1M, 22.5% (w/v) Glucose and 1 mM Sodium Pyruvate. Human umbilical vein endothelial cells (HUVEC) were isolated in our laboratory and grown in EGM-2 medium (Cambrex). Cells were grown at 37°C; 5% CO_2.

Isolation of endothelial cells from human umbilical cord veins (HUVEC)

Umbilical cords were collected at Lis Maternity Hospital, Sourasky Medical Center, Tel Aviv, Israel. The protocol was approved by the Institutional Review Board (IRB), as previously described [47]. Umbilical cords were washed with 50 mL PBS containing 100 U/mL Penicillin, 100 µg/mL Streptomycin ,12.5 U/mL Nystatin. A blunt end needle was inserted into one edge and both edges were tightened using a clip. Following 15 min

incubation with 0.25 mg/mL collagenase II (Sigma) at 37˚C, HUVEC were washed into tubes using 50 mL PBSx1. Cells were centrifuged for 10 min at 1100 rpm, resuspended in EGM-2, and cultured on fibronectin-coated tissue culture plates. Cells were grown according to the manufacturer's protocol in EGM-2 MV medium (Cambrex). All Cells were grown at 37°C; 5% CO_2.

Cell viability assay

HUVEC were plated onto 24-well plate (1.5 x 10^4 cells/well) in growth factor-reduced media (EBM-2, Cambrex, USA) supplemented with 5% (v/v) FBS. Following 24 h of incubation (37°C; 5% CO_2), medium was replaced with EGM-2 (Cambrex, USA). 4T1 cells (3,000 cells/well), were plated onto 24-well culture plates in RPMI supplemented with 5% (v/v) FBS and incubated for 24 h (37^0C; 5% CO_2). The medium was then replaced with RPMI 1640 supplemented with 10% (v/v) FBS. MDA-MB-231 cells were plated onto 96 well plate (5 x 10^3 cells/well) in DMEM supplemented with 5% (v/v) FBS and incubated for 24 h (37°C; 5% CO_2). Following 24 h of incubation, medium was replaced with full medium. Cells were exposed to PTX and PTX bounded conjugates at serial dilutions, at equivalent dose of the free PTX for up to 72 h. Following incubation, HUVEC and 4T1 cells were counted using a Coulter Counter **(**Beckman **Coulter®**). MDA-MB-231 cell viability was measured using MTT assay.

$\alpha_v\beta_3$ integrin expression

As was previously described [25], cells were harvested with 2.5 mM ethylenediaminetetraacetic acid (EDTA), re-suspended in serum-free medium and incubated for 30 min. Cells were then re-suspended in PBS (containing Mg^{2+} and Ca^{2+}) with an MAB1976-anti-$\alpha_V\beta_3$ integrin antibody (Chemicon) and incubated for 1 h at RT. Control

samples were antibody-free. Cells were then washed and incubated with FITC-donkey anti-mouse IgG antibody (Jackson) for 30 min at RT in the dark. Cells ($1x10^5$) were collected by fluorescence-activated cell sorter (FACS) and analyzed using Cyplogic software (6Cytek DxP 6-Color Upgrade, Facscan™).

Capillary-like tube formation assay

The surface of 24-well plates was coated with Matrigel matrix (50 µL/well) (BD Biosciences, USA) on ice and was then allowed to polymerize at 37°C for 30 min. HUVEC (3×10^4 cells) were challenged with free PTX (20 nM), PTX produg, PTX-PEG, PTX-PEG-[cRADfK], and PTX-PEG-E-[c(RGDfK)$_2$] conjugates or with PGA-PTX, PGA-PTX-[cRADfK], and PGA-PTX-E-[c(RGDfK)$_2$] conjugates at PTX-equivalent concentrations, and were seeded on coated plates in the presence of complete EGM-2 medium. After 8 h of incubation (37°C; 5% CO_2), wells were imaged using Nikon TE2000E inverted microscope integrated with Nikon DS5 cooled CCD camera by 4X objective, brightfield technique.

Endothelial cell adhesion assay

The ability of free and PGA or PEG conjugated E-[c(RGDfK)$_2$] to bind $\alpha_v\beta_3$ integrin on the cell surface was evaluated by HUVEC adherence to fibrinogen following incubation with the conjugates, as previously described [48]. Shortly, flat bottom 96-well culture plates were coated with 0.5 µg/well fibrinogen (overnight, 4ºC). Following three washes with PBS, the wells were blocked with 1 % (w/v) bovine serum albumin (BSA) for 1 h at 37ºC. HUVEC were harvested in PBS with 2.5 mM EDTA, re-suspended in EBM-2 serum-free media and were incubated with the different treatments for 30 min at RT. Treated HUVEC were then plated at 5×10^4 cells/well and allowed to attach to fibrinogen-coated plates for 1 h at 37ºC.

Following incubation, unattached cells were removed by rinsing the wells with PBS. Attached cells were fixed with 3.7% (v/v) formaldehyde, stained with 0.5% (w/v) crystal violet, and imaged using a Nikon TE2000E inverted microscope integrated with a Nikon DS5 cooled CCD camera by 6X objective, bright field technique. The number of attached cells was quantified with NIH ImageJ processing and analysis software. Non-specific binding was determined by adhesion to BSA-coated plates.

Migration assay

Cell migration assay was performed as previously described [49]. HUVEC ($1.5x10^5$) were exposed to PTX, PTX prodrug, PTX-PEG, PTX-PEG-[cRADfK], and PTX-PEG-E-[c$(RGDfK)_2$] conjugates or to PGA-PTX, PGA-PTX-[cRADfK], and PGA-PTX-E-[c$(RGDfK)_2$] conjugates at PTX-equivalent concentrations, that were added to the upper chamber of transwells for 2 h of incubation prior to migration towards vascular endothelial growth factor (VEGF). Migration was normalized so that 100% represents migration to VEGF alone without exposure to any compound.

Statistical methods

Data is expressed as mean ± s.d. Statistical significance was determined using an unpaired *t*-test. $P<0.05$ was considered statistically significant. All statistical tests were two-sided.

RESULTS

Multiple polymers, each with their own advantages, have been employed as integral parts of targeted polymer therapeutics for efficient drug delivery. However, we lack a detailed comparison between different polymers in a similar system. Therefore, we evaluated drug release and cytotoxicity of three polymeric carriers (PGA, PEG, and HPMA-copolymer)

following conjugation to PTX in two mammary adenocarcinoma cell lines (human MDA-MB-231 and murine 4T1). To address the fundamental question regarding the influence of polymeric scaffold multivalency on the anti-angiogenic activity of PTX conjugates, we also decorated PGA-PTX and PEG-PTX with E-c(RGDfK)$_2$, an $\alpha_v\beta_3$ integrin targeting moiety. We assessed the anti-angiogenic behavior (*i.e.* cell proliferation, capillary-like tubular-structure formation, cell migration, and cell adhesion to fibrinogen) of these conjugates in an integrin-overexpressing cell line (HUVEC), as well as each conjugate's cytotoxic effect on integrin-overexpressing cancer cell lines.

Synthesis, characterization, and drug release profiles for PGA, PEG, and HPMA bearing PTX

a) Synthesis of PGA-PTX conjugate (1)

We conjugated PGA-PTX, as previously described [25], via PTX introduction by esterification of the 2'-hydroxyl with DIC/HOBT activated carboxylic residues of PGA (Figure 1). We determined average molecular weight (Mw) and dispersity (Mw/Mn, Đ) by SEC as 17.7 kDa (Đ = 1.3) for PGA and 25.3 kDa (Đ = 1.3) for PGA-PTX conjugate. Drug loading determination via UV-Vis and HPLC demonstrated a range of 4.9 mol% (around 6-7 molecules of PTX per chain) with a free-drug content of less than 2% of the total payload.

b) Synthesis of PEG-EMCH-PTX conjugate (10)

In order to conjugate PTX to PEG (Mw = 9.7 kDa), we used 6-maleimidocaproyl hydrazine (EMCH) as a pH-responsive linker and achieved efficient conjugation via a Thiol-Maleimide reaction. Therefore, the resulting conjugate (Mw = 10.9 kDa) contains a hydrazone bond that is cleaved at the acidic pH of endosomes and lysosomes resulting in intracellular drug release [46] (Figure 2).

c) Synthesis of HPMA copolymer-GFLG-FK-PTX conjugate (18)

Figure 3 displays the synthetic pathway and the chemical structure of HPMA copolymer-GFLG-FK-PTX conjugate 3. We conjugated PTX to the HPMA copolymer through a cathepsin B-hydrolyzable Gly-Phe-Leu-Gly (GFLG) peptidic sequence followed by a Phe-Lys-PABC carbonate linker (referred in the text as *FK*). The conjugation to the HPMA copolymer used a three-step procedure: 1) PTX attachment to the FK–PABC linker, 2) PTX-linker conjugation to HPMA copolymer–GFLG–ONp (Mw = 31.6 kDa, Đ = 1.66), and finally, 3) removal of the alloc protecting group. The resulting conjugate **18** (Mw (theoretical) = 34.1 kDa,) was water-soluble with a PTX loading (determined by HPLC) of 1.12 mol% (around 2 PTX molecules per polymeric chain).

c) Hydrodynamic diameter of the conjugates

We characterized the hydrodynamic diameter and size distribution of our conjugates, finding a diameter of ~6.8 nm for PGA-PTX and 4.9 nm for PEG-PTX in physiological PBS, indicating a behavior as monomolecular micelles. However, we found a larger diameter for the HPMA copolymer-GFLG-FK-PTX conjugate (~19.51 nm), possibly due to some degree of aggregation. Irrespective of this small aggregation effect, all conjugates demonstrated the appropriate size to take full advantage of the EPR effect and enhanced tumor accumulation. Table 1 summarizes the physico-chemical features of the different conjugates.

Table 1. Physico-chemical characteristics of the different PTX-bearing conjugates

	Mw[†]	Size[§]	Payload[¶]
PGA-PTX	25.3	6.8	4.9
PEG-PTX	10.9	4.9	1[‡]
HPMA-PTX	34.1	19.51	1.12

‡ 1 molecule of PTX per chain. † Mw = molecular weight (in kDa). Measured by GPC in PBS (20 mM, pH= 7). § Diameter, determined by DLS, in nm. Distribution by volume. ¶ Determined by HPLC against PTX calibration curve in acetonitrile-water gradient and by UV (λ = 227 nm) in mol%.

d) Drug release from the conjugates

In order to assess linker degradation, we performed a preliminary drug release assay using an incubation with acetate buffer (pH 5.5) and cathepsin B for the PGA and HPMA conjugates. As expected, both conjugates exhibited defined time-dependent PTX release (Figure 4A and 4C respectively). Ester hydrolysis in the case of PGA and a 1,6-elimination followed by a decarboxylation after the hydrolysis of the FK linker for HPMA yielded PTX as the main metabolite released from the polymer, as determined by liquid chromatography–mass spectrometry (LC-MS) experiments using a MALDI-TOF as the MS detector. An incubation of 48 h led to a 16.5% PTX release from PGA-PTX conjugate, and similarly, a 13.2% release from the HPMA-GFLG-FK-PTX conjugate. In the absence of cathepsin B, we observed a negligible amount of PTX release from the PGA conjugate and only 3% of the total payload for the HPMA conjugate. This observation confirmed that cathepsin B effectively hydrolyzed the peptide-base backbone or GFLG linker in PGA and HPMA copolymer, respectively.

The non-peptidic EMCH linker in PEG-PTX conjugate contains an acid labile hydrazone bond and is insensitive to cathepsin B hydrolysis. Hence, we employed a drug release assay upon incubation at pH 5.5 in this case (Figure 4B). Similar to the previous conjugates, PTX represented the main released metabolite from the polymer, as determined by analytical HPLC. Following 48 h incubation at pH 5.5, the conjugate released 19.7% PTX. PTX was not released at pH 7.5 and remained under 2%.

Cell growth inhibition of the non-targeted PTX-bearing conjugates

To evaluate whether PTX retained its cytotoxic activity following polymer conjugation, we determined potential inhibitory activity on the proliferation of human MDA-MB-231 and murine 4T1 mammary adenocarcinoma cell lines after 72 h of incubation.

a) Inhibition of MDA-MB-231 cells

Initial results suggested that all PTX conjugates mediated a decrease in the proliferation of human MDA-MB-231 cells. PGA-PTX conjugate exhibited an IC_{50} value of ~80 nM (Figure 5A), PEG-PTX exhibited a lower IC_{50} value of ~8 nM PTX-equivalent concentrations (Figure 5B), while HPMA-PTX-FK exhibited higher IC_{50} of ~150 nM (Figure 5C). We found that the IC_{50} of free PTX to be ~10 nM.

b) Inhibition of 4T1 cells

Interestingly, the conjugates displayed considerable differences concerning the proliferation of murine 4T1 cells. Free PTX exhibited an IC_{50} of ~50 nM and PGA-PTX exhibited a similar level of inhibition with an IC_{50} of ~150 nM (Figure 5D), while PEG-PTX exhibited a less effective IC_{50} of ~9000 nM (Figure 5E) and HPMA-PTX-FK exhibited an IC_{50} of ~1000 nM (Figure 5F). Overall, this suggests a clear advantage for PGA conjugation.

Conjugates for anti-angiogenic and cytotoxic combination therapy: $\alpha_v\beta_3$ integrin-targeted PGA-PTX and PEG-PTX conjugates

We wanted to address the fundamental question of whether the conjugation of an integrin-targeting moiety (E-[c(RGDfK)$_2$]) to the polymeric backbone could influence drug release and cytotoxicity of PTX conjugates. In addition, we also aimed to assess whether the introduction of c(RGDfK), whose structure is very similar to the reported anti-angiogenic

molecule Cilengitide (c(RGDfV)), can confer an additional anti-angiogenic activity on the conjugate.

Based on our initial results, we chose PGA and PEG conjugates for further conjugation with E-[c(RGDfK)$_2$]. PGA-PTX and PEG-PTX displayed similar sizes (6.8 and 4.9 nm) and both exhibited time-dependent release of PTX after 48 h. In addition, while the PEG conjugate displayed the greatest inhibitory effect on human MDA-MB-231 mammary cancer cells, the PGA conjugate was the best on murine 4T1 mammary cancer cells.

Another unresolved issue in polymer therapeutics is the influence of the number of targeting moieties attached to one single backbone. Although the ratio between PTX and RGD in both conjugates was equal to 1, PGA contains more than 6 molecules per side-chain while PEG enables the conjugation of only one moiety at one end of its chain.

Synthesis and characterization of RGD and PTX containing conjugates

a) Synthesis of PGA-PTX-E-[c(RGDfK)$_2$] and control conjugates

We synthesized PGA-PTX-E-[c(RGDfK)$_2$] (**4**) carrying PTX and E-[c(RGDfK)$_2$] at similar loadings, 4.9 and 5.5 mol% respectively (and also a conjugate with c(RADfK) (**3**) to be used as a control in all the experiments) following reported protocols [25] (Figure 1). In order to avoid crosslinking reactions with the carboxylate present in E-[c(RGDfK)$_2$], we separately activated the remaining glutamic carboxylates in PGA-PTX (**1**) with N-hydroxysuccinimide and coupled via amide bond to the E-[c(RGDfK)$_2$] α-free amino group in its linker glutamic amino acid (E).

b) Synthesis of PTX-PEG-E-[c(RGDfK)$_2$] and control conjugates

Similarly to PGA-PTX-E-[c(RGDfK)$_2$], we conjugated the cyclic peptides to the NHS activated carboxylated of Trt-S-PEG-CO-NHS forming an amide (**8** and **9** for RGD and RAD

respectively, Figure 2). We deprotected the Thiol groups of these conjugates and immediately conjugated them to the maleimide moiety of EMCH-PTX prodrug (**11** and **12**) [46].

c) Hydrodynamic diameter of E-[c(RGDfK)$_2$] targeted conjugates

In order to evaluate the change in size of our conjugates after the introduction of the targeting moiety, we determined hydrodynamic diameter by DLS. PGA-PTX-E-[c(RGDfK)$_2$] conjugate exhibited a diameter of ~8.6 nm, while PTX-PEG-E-[c(RGDfK)$_2$] conjugate exhibited a diameter of ~10.44 nm (Figure 6).

d) PTX release from integrin-targeted PGA and PEG conjugates

We next evaluated drug release kinetics upon incubation with either cathepsin B or acidic pH for PGA-PTX-E-[c(RGDfK)$_2$]. Cathepsin B was responsible for the observed time-dependent release profile, with ~60% of the total PTX detached after 72 h (Figure 7A). Moreover, we found that a pH of 5.5 did not hydrolyze the ester bond during the same time-scale.

The synthetic linker in the PEG-PTX conjugate was not expected to be cleaved by cathepsin B; therefore, we focused on pH-dependent drug release. As expected, we found a progressive degradation of the hydrazone linker at acidic pH (Figure 7B). Following 72 h of incubation at pH 5.5, the conjugate released ~25% of PTX, while we observed no significant release at neutral pH.

Cell growth inhibition of $\alpha_v\beta_3$ integrin-expressing cell lines by RGD-targeted PTX-bearing conjugates

In order to evaluate whether conjugation of RGD peptides to the polymeric backbone has any effect on the internalization and cytotoxic activity of PTX, we determined cell-

growth inhibitory efficiency on the proliferation on two $\alpha_v\beta_3$-expressing cell lines, namely MDA-MB-231 [50] and HUVEC [51].

Inhibition of MDA-MB-231 cell growth

We incubated $\alpha_v\beta_3$ integrin-expressing MDA-MB-231 (Figure 8A) with different conjugates and used free PTX as a reference. PGA-PTX conjugates inhibited the proliferation of MDA-MB-231 cells and exhibited IC_{50} values of ~300 nM PTX-equivalent concentrations for PGA-PTX-E-[c(RGDfK)$_2$] and PGA-PTX-c(RADfK) and ~1000 nM for PGA-PTX (Figure 8B). However, PTX-PEG-E-[c(RGDfK)$_2$], PTX-PEG-c(RADfK), and PTX-PEG exhibited an IC_{50} in the range of 10-20 nM PTX-equivalent concentrations, which was very similar to the powerful activity of free PTX. Surprisingly, the PTX-EMCH prodrug showed a higher IC_{50} of ~150 nM (Figure 8C).

Anti-angiogenic cascade inhibition by PGA-PTX-E-[c(RGDfK)$_2$] and PTX-PEG-E-[cRGDfK)$_2$]

With the initial results at hand, we evaluated the anti-angiogenic properties of PGA-PTX-E-[c(RGDfK)$_2$] and PTX-PEG-E-[c(RGDfK)$_2$] compared to free PTX. To that end, we carried out endothelial cell proliferation, capillary-like tube formation, cell adhesion, and migration assays using HUVECs.

a) Inhibition of the Endothelial cell proliferation

We incubated $\alpha_v\beta_3$ integrin-expressing HUVECs (Figure 8D) with different conjugates and used free PTX as a reference. After 72 h of exposure, free PTX exhibited an IC_{50} value of ~2 nM. In the same range, PGA-PTX-E-[c(RGDfK)$_2$] conjugate inhibited HUVEC proliferation at an IC_{50} of ~8 nM (Figure 8E). PTX-PEG and PTX-PEG-E-[c(RGDfK)$_2$] presented an IC_{50} of ~20 nM, and ~25 nM, respectively, while PTX-EMCH

prodrug exhibited a higher IC_{50} of ~50 nM (Figure 8F). However, a shorter time exposure (15 min) of the cells to the conjugates revealed a significant difference between the two targeted polymer conjugates. While PGA-PTX-E-[c(RGDfK)$_2$] exhibited an IC_{50} of ~2000 nM (Figure 8G), we could not establish an IC_{50} value for PTX-PEG-E-[c(RGDfK)$_2$], probably due to its slow release (Figure 8H). Table 4 summarizes the different IC_{50} values.

b) Capillary-like tubular structures inhibition

Next, we examined the effect of the conjugates on capillary-like tubular structure-forming ability of HUVEC in Matrigel, an additional crucial step in the angiogenic cascade of events (Figure 9A). Free PTX and PGA-PTX-E-[c(RGDfK)$_2$] conjugate inhibited the formation of tubular structures of HUVEC by ~25%.

Interestingly, while PTX-PEG-E-[c(RGDfK)$_2$] displayed a similar activity to free PTX at the same PTX-equivalent (~20%), neither the PTX-PEG conjugate or the PTX prodrug (PTX- EMCH) inhibited the formation of the tubular structures under the same conditions (Figure 9C) [25, 52]].

c) Cell adhesion assays

We also determined the ability of the conjugates to adhere to fibrinogen in order to evaluate the targeting specificity of conjugated E-[c(RGDfK)$_2$] binding to $\alpha_v\beta_3$ integrin. As expected, a solution of 50 μM of free E-[c(RGDfK)$_2$] abrogated HUVEC adhesion by ~60%, while at the same concentration, the free c(RADfK) peptide displayed no significant effect on the adhesion of endothelial cells to fibrinogen. RGD-equivalent doses of PGA-PTX-E-[c(RGDfK)$_2$] or PTX-PEG-E-[c(RGDfK)$_2$] inhibited the adhesion by 70% and 60%, respectively (Figure 9B). Free PTX, PTX-PEG, and PTX-PEG-c(RADfK) control conjugates

(at PTX-equivalent concentration) displayed negligible effects on endothelial cell adhesion. Unexpectedly, PTX- prodrug also inhibited HUVEC adhesion by ~30% (Figure 9D).

d) Cell migration assays

To complete the evaluation of the anti-angiogenic activity, we finally assessed the effect of conjugates on HUVEC migratory activity. We carried out all assays in non-cytotoxic PTX-equivalent concentrations while using short incubation times. Using these conditions, we could evaluate the anti-angiogenic effect of PTX and the conjugates while eliminating PTX cytotoxic activity towards HUVECs.

Under these conditions, PGA-PTX-E-[c(RGDfK)$_2$] inhibited HUVEC migration by 50% [25]; however, PTX-PEG-E-[c(RGDfK)$_2$], PTX-PEG-[cRADfK], PTX-PEG conjugates, and the PTX-EMCH prodrug did not significantly affect cell migratory ability (Data not shown).

DISCUSSION

Emerging strategies for the improvement of cancer treatment include the conjugation of potent cytotoxic chemotherapeutic drugs, such as paclitaxel (PTX) to a hydrophilic polymeric backbone to improve pharmacokinetics and reduce harmful side effects and the targeting of $\alpha_v\beta_3$ integrin receptors. Encouragingly, both of these strategies are separately under evaluation in clinical trials. Additionally, the conjugation of both anti-angiogenic and cytotoxic agents to the same polymer chain to gain a synergistic effect has also proven to be a successful strategy. This is exemplified by our previous studies of an anti-$\alpha_v\beta_3$ and PTX bi-specific macromolecule targeting tumor cells and their endothelial microenvironment using a PGA polymer [25], the promising *in vivo* results of Doxorubicin-PEG-E-[c(RGDfK)$_2$] [38],

and the potent anti-cancer activity of an HPMA copolymer conjugated with RGDfK and docetaxel [39].

Our aim in this manuscript was to study the influence of the polymeric backbone and the linker in the anti-angiogenic and cytotoxic performance of conjugates. In addition to the above-mentioned PGA-PTX-E-[c(RGDfK)$_2$] conjugate, other conjugates using the same rational design and bearing different backbones have been reported [53]. However, to the best of our knowledge, there are no published studies that directly compare conjugates containing these particular polymeric chains under the same conditions.

In this manuscript, we have synthesized, characterized, and compared the cytotoxic activity of PGA-PTX, PEG-PTX, and HPMA copolymer-PTX (Figures 1, 2, and 3, respectively). The choice of linker between the polymer and the drug also represents an important decision in the total design of a nanomedicine/polymer therapeutic. While PGA is biodegradable and displays adequate drug release kinetics in the presence of cathepsin B, without the need of any specific linker other than a simple ester bond, the methacrylic backbone of HMPA copolymer is not degradable. Therefore, HPMA copolymerized during synthesis included a GFLG sequence within the side chains. Studies have reported that the GFLG tetrapeptide is recognized and hydrolyzed by cathepsin B to ensure sustainable and time-dependent drug release within the tumor cell (Figure 3). PEG is also non-degradable, and for PEG, we chose an established hydrolyzable linker, 6-maleimidocaproyl hydrazine, which reacts with PTX to yield a PTX-EMCH prodrug (**6**). This prodrug contains an acid-pH labile hydrazone bond in its structure and can be linked to thio-modified PEG by a thio-maleimide reaction (Figure 2). We next compared the three conjugates in terms of drug loading, size, drug release, and anti-cancer *in vitro* activity (Table 2).

Table 2. Comparison of PEG, PGA and HPMA copolymer- Drug conjugates

Conjugate	Linker	Trigger	BD†	Size§	Max. Drug Release¶	IC50 (nM)*	
						MDA-MB-231	4T1
PGA-PTX	Amide/Ester	Cath B / pH	Y	6.8	16	80	150
PEG-PTX	Hydrazide	pH	N	4.9	20	8	9000
HPMA-PTX	GFLG	Cath B	N	19.51	13	150	1000

† stands for Biodegradability of the polymeric backbone. § Hydrodynamic diameter determined by dynamic light scattering, in nm. ¶ Percentage of drug released after 48 h. * IC_{50} values were calculated at PTX-equivalent concentrations.

The first requirement for a biocompatible polymer-drug conjugate is solubility in water. Free PTX only dissolves in organic solvents and it is co-administered in a formulation together with CremophorEL, which can cause irritation and hypersensitivity [32]. Conjugation of PTX to our polymers yielded water-soluble nanoconstructs up to, at least, 10 mg/mL.

PGA-PTX (25 kDa) and PEG-PTX (11 kDa) conjugates demonstrated a similar hydrodynamic diameter corresponding to monomolecular micelles (5-7 nm). HMPA copolymer-PTX (34.1 kDa) displayed a degree of aggregation (19 nm), which slows down target cell internalization.

The multivalence of PGA and HPMA enables different drug loading and in this study, PGA conjugate loading was 4.9 mol%, which ensures 6-7 molecules of PTX/conjugate, while HPMA copolymer loading was lower, at 1.12%, containing around 2 molecules of PTX/conjugate. Both nano-constructs demonstrated cathepsin B-dependent drug-release (Figure 4A and 4C) with a release after 48 h of 16% and 13% of total drug, respectively, with minimal release at pH 5.5. In contrast, as every chain of PEG contains a PTX prodrug, the release at 48 h in an acid lysosomal-mimicking pH was ~20% (Figure 4B).

We tested the cell proliferation inhibition properties of the three conjugates in two breast cancer cell lines: human MDA-MB-231 and murine 4T1 adenocarcinomas. Curiously, at equivalent doses of PTX, PEG-PTX displayed 10-fold greater potency than the PGA-PTX conjugate in inhibiting MDA-MB-231 proliferation (IC_{50} = 8 *versus* IC_{50} = 80 nM).

Conversely, we observed opposite findings with 4T1 (IC_{50} = 150 *versus* IC_{50} = 9000 nM for PGA and PEG, respectively). This observation might be explained by the difference in cathepsin B expression in different cells [51] and pH levels, which affect the balance between the kinetic constants of enzymatic degradation and pH-dependent hydrolysis. Our findings with the HPMA copolymer-PTX suggested lower potency when compared to PGA-PTX in both cell lines. It is possible that both the PGA and HPMA backbone may wrap PTX in a very efficient fashion and, therefore, access of cathepsin B to the conjugate core may be very restricted. However, while HPMA is a non-degradable methacrylate, PGA can be progressively degraded by cathepsin B and this way, PTX may be released in a more rapid manner (Figure 4).

The main conclusion of this part of our work was that the ability of the conjugates to inhibit cell growth, at PTX-equivalent concentrations, depends amongst other characteristics, on the cell type and the cathepsin B level of expression in every type of cell and not only on the architecture or drug loading of the conjugates. Therefore, we recommend cathepsin B level quantification of neoplastic cells for each patient in order to inform on the administration of the most relevant polymer therapeutic construct. These conclusions are in line with the European Medicines Agency (EMA) recommendations towards the need of personalized medicine [54].

We next focused our interest on the introduction of RGD-containing molecules to actively drive our conjugates towards endothelial cells as part of an anti-angiogenic strategy. We conjugated E-[c(RGDfK)$_2$] to PGA at an equimolar ratio with respect to PTX (around 5 mol% each, Figure 1) to enable a direct comparison between the multivalent PGA-PTX-E-[c(RGDfK)$_2$] and the monovalent PTX-PEG-E-[c(RGDfK)$_2$] (Figure 2). Although the introduction of the bulky and charged E-[c(RGDfK)$_2$] molecule can double the size of the

final conjugate, we expect that this size change will not influence the internalization pathway utilized as the diameter of both conjugates remained below 11 nm (Figure 6, Table 3).

Size changes may affect drug release by the conjugate (Figure 7 and Table 3); however, we found that targeted constructs permitted almost double the level of drug release after 48h (42% *versus* 16% for PGA, 32 *versus* 20% in the case of PEG). After 72 h, this percentage was even greater for PGA (60%) and remained stable for PEG (35%) conjugates. We note a remarkable lack of hydrolysis of the PGA conjugate at lysosomal pH in the absence of cathepsin B. Again, insufficient PGA backbone degradation might lead to the effective shielding of the acid-labile ester bond by hampering the access of hydronium cations.

Table 3: Summary of the comparison of PEG and PGA conjugates

Conjugate	Ratio		Trigger	BD[†]	Size[§]	Max. Drug Release[¶]		IC_{50} (nM)[*]
	PTX	RGD				Cathepsin B	pH 5.5	MDA-MB-231
PTX	-	-	-	-	-		-	10
PGA-PTX-RGD	4.9	5.5	Cathepsin B / pH	Y	8.6	42 (60)	2 (2)	300
PTX-PEG-RGD	1	1	pH	N	10.4	NA	33 (35)	20

[†] stands for biodegradability of the polymeric backbone. [§] Hydrodynamic diameter determined by dynamic light scattering. [¶] Percentage of drug released after 48 h or in brackets, 72 h. [*] IC_{50} values were calculated at PTX-equivalent concentrations.

We used MDA-MB-231 cells to compare the cytotoxicity of PGA and PEG-PTX conjugates in the presence and absence of the integrin targeting moiety (Table 2 for untargeted conjugates and Table 3 for the targeted ones). The cytotoxicity profile observed for the untargeted conjugates was maintained when the RGD molecule was introduced in the backbone.

Table 4: Summary of the comparison of anti-angiogenic properties of PGA- and PEG-based conjugates

Conjugate	HUVEC proliferation[†]		Tubules inhibition[§]	HUVEC Cell adhesion[¶]	Cell migration[*]
	Long time	Short time			
PTX	~1	~10	20	no effect	
PGA-PTX				no effect	
PGA-PTX-RGD	8	2000	25	70	50
EMCH-PTX	50		no effect		no effect
PEG-PTX	20		no effect	No effect	no effect
PTX-PEG-RGD	25	>10000	20	60	no effect

[†] IC_{50} value in nM. Long and short time stands for 72 h and 15 min experiment respectively. § On Matrigel. Values are presented as percentage, visually assessed from the images with respect the same experiment in non-treated HUVEC. ¶ Percentage of adhesion inhibition to a fibrinogen matrix with respect the same experiment in non-treated HUVEC. * Percentage of cell migration inhibition with respect the same experiment in non-treated HUVEC.

In order to compare the anti-angiogenic activity of our conjugates on HUVEC overexpressing $\alpha_v\beta_3$ integrin and interacting with E-[c(RGDfK)$_2$], we tested four different pathways in which angiogenesis can be blocked: inhibition of cell growth, capillary-like tubular structures formation, cell adhesion to fibrinogen matrixes, and cell migration towards the VEGF. Table 4 summarizes the main results of this study. The PGA conjugate inhibited HUVEC proliferation 2-fold greater than the PEG conjugate (IC_{50} = 8 and 20 nM, respectively). In addition, both conjugates inhibited endothelial cell adhesion and capillary-like tube formation. We found the most noticeable difference between both conjugates in the cell migration assay. While the PEG conjugate did not inhibit the migratory ability of HUVEC, PGA-PTX-E-[c(RGDfK)$_2$] blocked 50% of cell migration, possibly due to the combined action of the 6 molecules of RGD present in a single chain of the conjugate [55, 56]. Therefore, we propose that the multivalent PGA conjugate might be used as treatment against metastases, as we already demonstrated using the metastatic 4T1 mammary adenocarcinoma mouse model [25].

CONCLUSIONS

In this study, we compared three different polymeric conjugates of PTX, namely PGA, HPMA copolymer, and PEG. The backbones of PGA and HPMA copolymer are multivalent and due to their degradable backbone or peptidic linkers, respectively, drug release from these conjugates is cathepsin B-dependent. PEG conjugates are monovalent and demonstrate a clear pH-dependent drug release. We observed that the anti-cancer potency of the conjugates also depended on the cancer cell type employed, suggesting that the choice of the (degradable) backbone/linker and its cleavage upon environmental stimuli is just as important as its supramolecular structure and drug loading on the conjugate itself.

Following RGD conjugation to PGA-PTX or PEG-PTX conjugates, both acquired enhanced anti-angiogenic properties. Both conjugates inhibited endothelial cell growth, adhesion, and tubular structure formation in a similar manner. However, we identified a clear difference between the RGD multivalent PGA conjugate with respect to the homologous monovalent PEG conjugate in the inhibition of HUVEC migration towards a chemoattractant (VEGF). Therefore, we encourage the development of PGA-PTX-E-[c(RGDfK)$_2$] conjugates as an efficient and effective treatment for aggressive metastatic tumors as it targets the two tumor compartments more efficiently.

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