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Dendritic Poly(ethylene glycol) Bearing Paclitaxel and Alendronate for Targeting Bone Neoplasms

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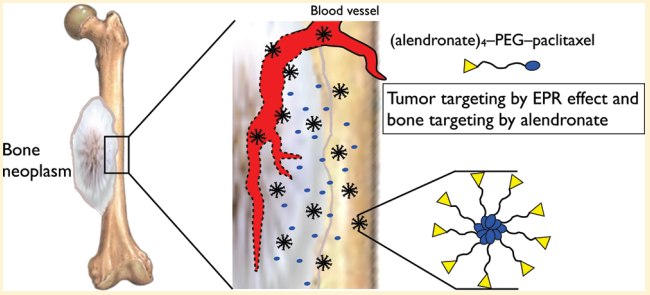
Supporting Information

ABSTRACT: Poly(ethylene glycol) (PEG) is the most popular polymer for protein conjugation, but its potential as carrier of low molecular weight drugs has been limited by the intrinsic low loading, owing to its chemical structure. In fact, only the two end chain groups of PEG can be modified and exploited for drug coupling. We have demonstrated that by synthesizing a dendrimer structure at the polymer end chains, it is possible to increase the drug payload and overcome this limitation. Furthermore, this approach can be improved by using heterobifunctional PEG. These polymers allow the precise linking of two different drugs, or a drug and a targeting agent, on the same polymeric chain. Heterobifunctional PEG-dendrimers have been obtained with defined chemical structures leading to their attractive use as drug delivery systems. In fact, they offer a double benefit; first, the possibility to choose the best drug/targeting agent ratio, and second, the separation of the two functions, activity and targeting, which are coupled at the opposite polymer end chains. In this study, we investigated the role of a PEG−dendrimer, H_2N−PEG−dendrimer−$(COOH)_4$, as carrier for a combination of paclitaxel (PTX) and alendronate (ALN). PTX is a potent anticancer drug that is affected by severe side effects originating from both the drug itself and its solubilizing formulation, Cremophor EL. ALN is an aminobiphosphonate used for the treatment of osteoporosis and bone metastases as well as a bone-targeting moiety. The PTX-PEG-ALN conjugate was designed to exploit active targeting by the ALN molecule and passive targeting through the enhanced permeability and retention (EPR) effect. Our conjugate demonstrated a great binding affinity to the bone mineral hydroxyapatite *in vitro* and an IC_{50} comparable to that of the free drugs combination in human adenocarcinoma of the prostate (PC3) cells. The PTX-PEG-ALN conjugate exhibited an improved pharmacokinetic profile compared with the free drugs owed to the marked increase in their half-life. In addition, PTX-PEG-ALN could be solubilized directly in physiological solutions without the need for Cremophor EL. The data presented in this manuscript encourage further investigations on the potential of PTX-PEG-ALN as treatment for cancer bone metastases.



KEYWORDS: poly(ethylene glycol), paclitaxel, alendronate, bone metastases

INTRODUCTION

Polymer−anticancer drug conjugates have been investigated for decades, as improved therapies against cancer aimed to address the relevant limitations of current protocols using low molecular weight drugs.[1−3] It is well-known that most of the chemotherapeutics are potent drugs. On the other hand, they lack a mandatory selectivity to direct the cytotoxicity to tumor cells, thus causing severe and dramatic adverse effects to normal healthy cells. The coupling of these agents with water-soluble polymers has been demonstrated to strongly improve both the safety profile and antitumor efficacy by acting on several aspects, such as: (i) increasing the solubility;[4] many antineoplastic drugs are almost insoluble in biological fluids requiring a formulation for clinical administration, (ii) improving the biodistribution; conjugates have restricted distribution, due to their large sizes, thus reducing the concentration in sites of dose-limiting toxicity, (iii) providing passive targeting to solid tumors; the enhanced permeability and retention (EPR) effect[5] promotes the extravasation of macromolecules into tumors due to the abnormally leaky blood vessels in cancers with respect to healthy tissues, (iv) bypassing P-glycoprotein-mediated drug resistance;[6] due to alternative cellular entry and trafficking, and (v) preventing drug inactivation and degradation.[7]

So far, most of the studied anticancer polymer−drug conjugates and polymer therapeutics in general have based their tumor selectivity mainly on the EPR effect. Despite of the advantages of this approach, it is now recognized that the future generation of polymer therapeutics should move a step forward in active targeting and specific activity. Recent studies have been

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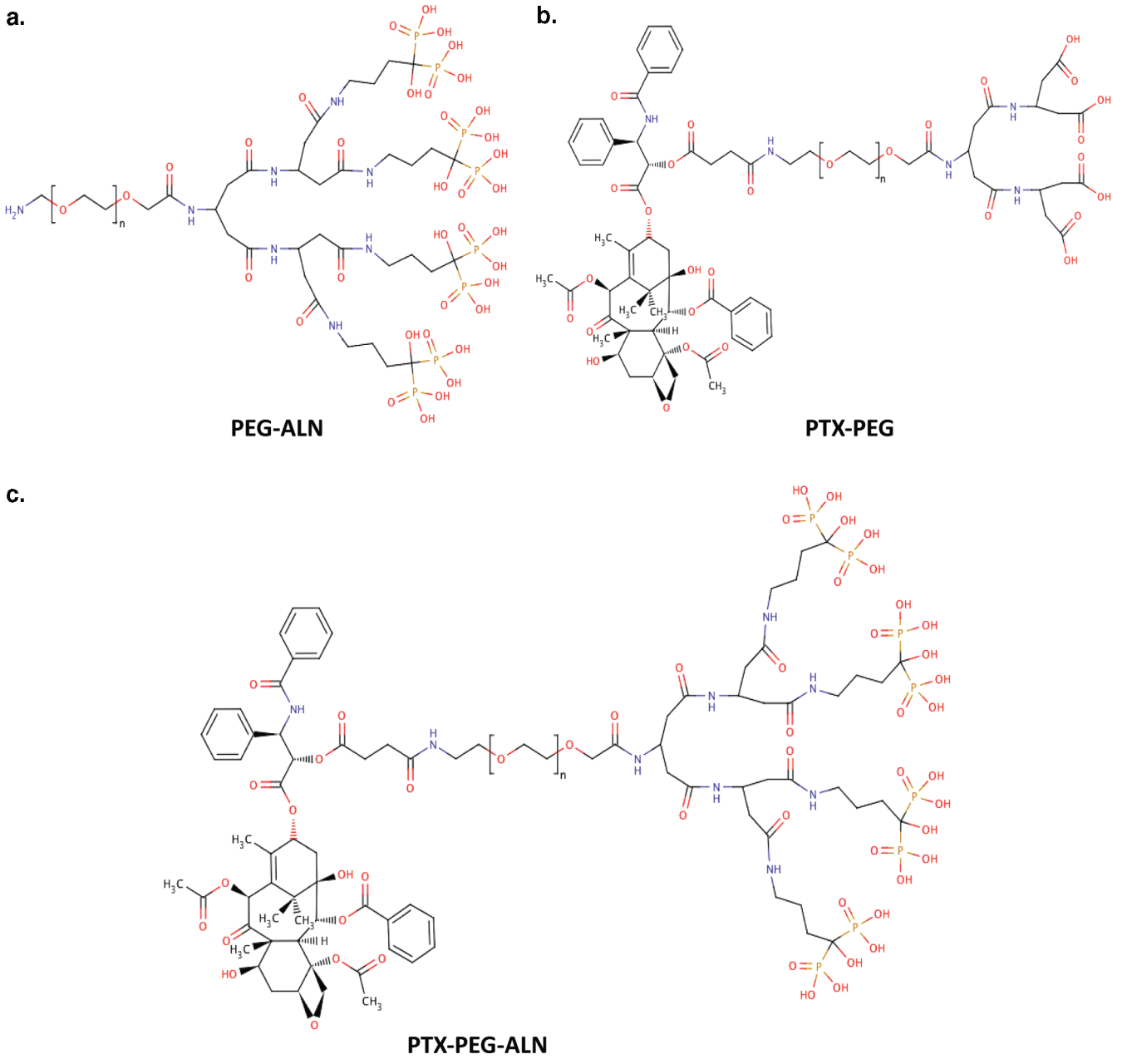


Figure 1. Chemical structure of (a) PEG-ALN, (b) PTX-PEG, and (c) PTX-PEG-ALN.

directed to either synthesizing targeted conjugates[8−11] or polymers bearing two anticancer drugs for combination therapy.[12−14] Satchi-Fainaro et al. reported one of the first studies in which both concepts, targeting and combination therapy, are conveyed in a unique conjugate by linking paclitaxel (PTX) and alendronate (ALN) to HPMA copolymer.[15] PTX is a potent anticancer drug, used for the treatment of several cancers; however, it is associated with severe side effects due to both its scarce tumor selectivity and the formulation in Cremophor EL.[16−18] The HPMA copolymer-PTX-ALN conjugate was designed to target bone metastases through ALN, a bisphosphonate that exhibits both a high affinity for the bone mineral hydroxyapatite (HA) and an effective antiangiogenic activity.[10,15,19−21] The conjugate exhibited increased anticancer and antiangiogenic activity with respect to the free drugs and, remarkably, reduced toxicity. Starting from these promising results, in this manuscript, we have investigated the role of a different polymeric carrier, poly(ethylene glycol) (PEG). PEG, especially in its heterobifunctional functionalization that bears two different reactive groups at the polymer ends, offers the possibility to design precise polymer architecture for increased conjugate homogeneity. Heterobifunctional BOC-NH-PEG-COOH was exploited to prepare a PEG-dendrimer, H_2N-PEG-β-Glu-(β-Glu)$_2$-(COOH)$_4$, presenting a single amino group and four carboxylic groups for PTX and ALN coupling, respectively. The driving idea was that a well-defined conjugate in terms of both PTX/ALN ratio and chemical structure (i.e., the hydrophobic PTX and the hydrophilic ALN linked at the opposite end chains of the polymer, see Figure 1), might present better targeting and enhanced activity compared to the combination of the free drugs. The synthesized conjugate, PTX-PEG-ALN,

was water-soluble overcoming the need for the toxic Cremophor EL, currently used to solubilize PTX. The conjugate was physicochemically characterized and then tested in vitro against PC3 human prostate adenocarcinoma cells showing a high cytotoxicity, comparable to that of free PTX/ALN combination. The pharmacokinetic profile in mice demonstrated a great half-life prolongation proving the effectiveness of this conjugation approach.

EXPERIMENTAL SECTION

Materials and Methods. PTX was from Indena (Milan, IT). The poly(ethylene glycol) Boc-NH-PEG-NHS was from Iris Biotech GmbH (Marktredwitz, Germany). *N*-Hydroxysuccinimide (NHS), *N*,*N*-dicyclohexylcarbodiimmide (DCC), succinic anhydride, β-glutamic acid (β-Glu), silica gel (SiO_2), sodium sulfate anhydrous (Na_2SO_4), triethylamine (TEA), trifluoroacetic acid (TFA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), dimethylsulfoxide-d_6, and D_2O were purchased from Sigma-Aldrich. ALN was purchased from Alcon Biosciences Ltd. (Mumbai, India; Petrus Chemicals, Israel). Glycil-glycine (Gly-Gly) was obtained from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, fetal bovine serum (FBS), penicillin, and streptomicin were from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). Dextran (MW 70 000 Da) and all other chemical reagents, including salts and solvents, were purchased from Sigma-Aldrich.

Synthesis of the PTX-PEG, PEG-ALN, and PTX-PEG-ALN Conjugates. Synthesis of 2′-succinyl-paclitaxel (SPTX): To 1 g (1.17 mmol) of PTX, dissolved in 30 mL of anhydrous pyridine, 585 mg (5.85 mmol) of succinic anhydride was added. The reaction was stirred at room temperature for 48 h. The SPTX was purified by chromatography on a SiO_2 column (30 × 2.5 cm) eluted with a chloroform−methanol mixture (97:3 to 90:10) and determined by thin-layer chromatography (TLC; Rf 0.5 in chloroform−methanol, 90:10).

^{1}H-NMR of SPTX ($CDCl_3$, δ ppm): 1.15 (s, 3H, C16), 1.24 (s, 3H, C17), 1.68 (s, 3H, C18), 1.79 (s, 3H, C19), 2.24 (s, 3H, C31), 2.38 (s, 3H, C29), 2.5−2.7 (m, 4H, $-CH_2-CH_2-$ succinic spacer), 4.9 (d, 1H, C5), 5.66 (d, 1H, C2′), 6.27 (s, 1H, C10), 7.25 (s, 3′-Ph), 7.4 (m, 3′-NBz), 7.5 (m, 2-OBz), 7.75 (d, 3′NBz), 8.1 (d, 2-OBz).

Synthesis of PTX-PEG Conjugates. Boc-NH-PEG-(β-Glu)-$(COOH)_2$ (**1**): 3.5 g (0.71 mmol) of Boc-NH-PEG-NHS (MW 4928 Da) was added to 313 mg (2.13 mmol) of β-Glu, dissolved in 150 mL of 0.1 M borate buffer/CH_3CN (3:2) mixture at pH 8.0. The reaction was let to proceed for 5 h under stirring. Then, the pH was adjusted to about 4.5 with 0.2 N HCl, and product **1** was purified from the excess of β-Glu by extractions with $CHCl_3$ (6 × 300 mL). The organic phase, dried over anhydrous Na_2SO_4, was concentrated under vacuum and dropped into 1 L of cold diethyl ether under stirring. After 1 h at −20 °C, the precipitate of **1** was filtered and dried under vacuum (yield: 3.345 g, 95%). The absence of free β-Glu in the conjugate was verified by a TNBS test according to the Snyder and Sabocinsky assay.[22]

Boc-NH-PEG-(β-Glu)-$(NHS)_2$ (***2***). To 3.33 g (0.67 mmol) of **1**, dissolved in 100 mL of anhydrous CH_2Cl_2, 469 mg (4.07 mmol) of NHS and 1.114 g (5.4 mmol) of DCC were added. The reaction was stirred at room temperature overnight. Then the mixture was filtered and dropped into 1 L of cold diethyl ether. After 1 h at −20 °C, the precipitate of product **2** was recovered by filtration and dried under vacuum (yield: 3.1 mg, 89.5%). The degree of activation was 91%, determined on the basis of the amino group modification of an equimolar solution of Gly-Gly as reported elsewhere.[23]

Boc-NH-PEG-(β-Glu)-$(β\text{-Glu})_2$-$(COOH)_4$ (***3***). To 532 mg (3.6 mmol) of β-Glu, previously dissolved into 200 mL of 0.1 M borate buffer/CH_3CN (3:2) mixture at pH 8.0, 3.09 mg (0.6 mmol) of **2** was added. The reaction was conducted as for **1**, and the product was purified by the same method (yield: 2.9 g, 92%).

Boc-NH-PEG-(β-Glu)-$(β\text{-Glu})_2$-$(NHS)_4$ (***4***). A total of 1.7 g (0.32 mmol) of 3 was activated with NHS and DCC as reported above. The degree of activation was 81% (yield: 1.52 g, 89%).

Boc-NH-PEG-(β-Glu)-$(β\text{-Glu})_2$-$(ALN)_4$ (***5***). To 802 mg (2.46 mmol) of ALN dissolved in 0.1 M borate buffer at pH 8.0, 1.45 g (0.25 mmol) of **4** was added, and the reaction proceeded for 5 h under stirring. The product was purified by the same method as product **1** (yield: 1.3 g, 83%).

H_2N-PEG-(β-Glu)-$(β\text{-Glu})_2$-$(COOH)_4$ (***6***) *and H_2N-PEG-(β-Glu)-$(β\text{-Glu})_2$-$(ALN)_4$ (PEG-ALN;* ***7***). A total of 1.2 g of product **3** or 1.3 g of **5** was dissolved in 4 mL of $CH_2CH_2/CF_3COOH/H_2O$ (55.4:45.4:0.1%) mixture for 3 h to remove the protecting group t-Boc. The reaction mixture was evaporated to remove the TFA, and the obtained oil was solubilized in CH_2Cl_2 and dropped into 400 mL of diethyl ether. The product was recovered by filtration and dried under vacuum (yield: 1.17 g, 97% for **6** and 1.1 g, 91% for **7**).

PTX-PEG-(β-Glu)-$(β\text{-Glu})_2$-$(COOH)_4$ (PTX-PEG) and PTX-PEG-(β-Glu)-$(β\text{-Glu})_2$-$(ALN)_4$ (PTX-PEG-ALN). The two conjugates were synthesized and purified using the same procedure. Here we report the procedure for PTX-PEG-ALN as an example. To 190 mg (0.2 mmol) of SPTX, dissolved in anhydrous DMF, 40.5 mg (0.3 mmol) of HOBT and 40.2 mg (0.22 mmol) of EDC, already dissolved in anhydrous DMF, were added. The reaction was stirred for 5 h at room temperature, and then product **7**, previously dissolved in DMF, was added and let to react for 24 h. The product was purified from the excess of SPTX by gel-filtration chromatography using Sephadex LH-20 resin eluted with DMF. The fractions containing PTX-PEG-ALN were collected in a round-bottom flask, and DMF was evaporated under vacuum. The product was dissolved in anhydrous CH_2Cl_2 and dropped into 500 mL of cold diethyl ether under stirring. After 1 h at −20 °C, the precipitate was filtered and dried under vacuum. The amounts of free and total contents of PTX in PTX-PEG-ALN conjugate were evaluated as reported in the dedicated section.

Determination of Free and Total PTX Contents in the Conjugates. The amount of PTX in the conjugates was evaluated by reverse phase HPLC (RP-HPLC) using an Agilent 300-Extend C18 (4.6 × 250 mm; 5 μm) column, with the UV detector settled at 227 nm. The eluents A and B were H_2O and CH_3OH, respectively. The elution was performed by the following gradient: from 5% B to 50% B in 5 min, from 50% B to 80% B in 14 min, from 80% B to 100% B in 5 min, and from 100% B to 5% B in 5 min at a flow rate of 1 mL/min.

The total drug content was evaluated by RP-HPLC following the release of PTX from the conjugates. A total of 3 mg of conjugate was dissolved in 1 mL of MeOH. Following the addition of 2% (v/v) of 0.2 N NaOH, the solution was incubated at 50 °C for 2 h. The drug was then extracted by ethyl acetate. The organic phase was evaporated, and the residue was solubilized in methanol. The elution was performed as reported above. The amount of PTX was calculated using the PTX calibration

curve obtained using the same method. The standard error for this analysis, calculated using solutions of PTX at known concentrations, is ±1.89%.

Determination of ALN Content Bound to PEG. The formation of chromophoric complex between ALN and Fe^{3+} ions in perchloric acid solution was used to determine the ALN content by spectrophotometry.[24] Briefly, conjugates (2.5, 5, and 10 mg) were dissolved in a mixture of 0.1 mL of 4 mM $FeCl_3$ and 0.8 mL of 0.2 M perchloric acid ($HClO_4$). The content of ALN in the conjugates was determined against a calibration graph of serial dilutions of 0−3 mM ALN. Sample absorbance was measured spectrophotometrically at λ = 300 nm.

Dynamic Light Scattering (DLS) of Conjugates. The mean hydrodynamic diameter of PTX-PEG and PTX-PEG-ALN conjugates was evaluated using a real time particle analyzer (NanoSight LM20). PTX-PEG and PTX-PEG-ALN (5 mg/mL) were injected into the chamber, allowed to equilibrate for 30 s, and analyzed by a nanoparticle tracking analysis (NTA) software.

Drug Release from the Conjugates in Buffer Solution at Different pH Values and in Plasma. Each conjugate (3 mg/mL) was incubated at 37 °C for 48 h in PBS at pH 5 and 7.4 to evaluate the drug release. Samples of 50 μL were withdrawn at predetermined times and analyzed by RP-HPLC using the same conditions reported above, evaluating the decrease of the conjugate peak in the chromatographic profile.

The conjugates were also incubated at 37 °C for 48 h in mouse plasma, obtained after centrifugation of blood sample at 2000*g* for 10 min. Samples of 60 μL were withdrawn at predetermined times, and 60 μL of CH_3CN was added to achieve plasma protein precipitation. Samples were centrifuged at 15 000*g*, and the supernatant was withdrawn and analyzed by RP-HPLC using the same conditions reported above.

Stability of Polymeric Structures in Buffer Solutions at Different pH Values. The stability of the conjugates was also evaluated by DLS. A solution of each conjugate (7 mg/mL) was obtained by solubilization in PBS at pH 5 and 7.4. These solutions were immediately extruded with manual extruder (Liposofast Avestin) at 200 nm and analyzed using a light scattering instrument (Malvern Nano-S, Worcestershire, United Kingdom). The instrument was settled at 37 °C, the detector position was at 173°, and the analysis was performed every 20 min (the first measurement was performed after 5 min of equilibration) for 4 h; after storage in similar conditions, the sample was analyzed at 24 h.

HA Binding Assay. PEG, PEG-ALN, and PTX-PEG-ALN conjugates were dissolved in phosphate buffered saline (PBS), pH 7.4 (5 mg/mL). The conjugate solution (600 μL) was incubated with HA powder (30 mg), in 600 μL of PBS, pH 7.4. NH_2-PEG-$COOH_4$ was used as the control. Incubated samples were centrifuged at 7000 rpm for 3 min and a sample from the upper layer (100 μL) was collected after 0, 2, 5, 10, and 60 min. Fast protein liquid chromatography (FPLC, AKTA Purifier, Amersham Biosciences) analysis using a HiTrap desalting column (Amersham) was used for the detection of unbound conjugates in the samples (FPLC conditions: AKTA Purifier, mobile phase 100% DDW, 2 mL/min, λ = 215 nm). HA-binding kinetic analysis of the conjugates was performed using the Unicorn AKTA software. Areas under the curve (AUC) were calculated from chromatographs at each time point. AUC of each HA-incubated conjugate chromatogram was normalized to the percent AUC of conjugate sample in the absence of HA.

Red Blood Cells (RBC) Lysis Assay. Rat RBC solution (2% w/w) was incubated with serial dilutions of the combination of PTX plus ALN, PEG, and PTX-PEG-ALN conjugate at equivalent PTX and ALN concentrations, for 1 h at 37 °C. Negative controls were PBS and Dextran (MW ∼70000 Da), while positive controls were 1% (w/v) solution of Triton X100 (100% lysis) and poly(ethylenimine) (PEI). Following centrifugation, the supernatant was drawn off and its absorbance measured at 550 nm using a microplate reader (Genios, TECAN). The results were expressed as percent of hemoglobin released relative to the positive control (Triton X100).

Cell Culture. A PC3 human prostate adenocarcinoma cell line was purchased from the American Type Culture Collection (ATCC). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, 100 U/mL streptomycin, 12.5 U/mL nystatin, and 2 mM L-glutamine. Cells were grown at 37 °C; 5% CO_2.

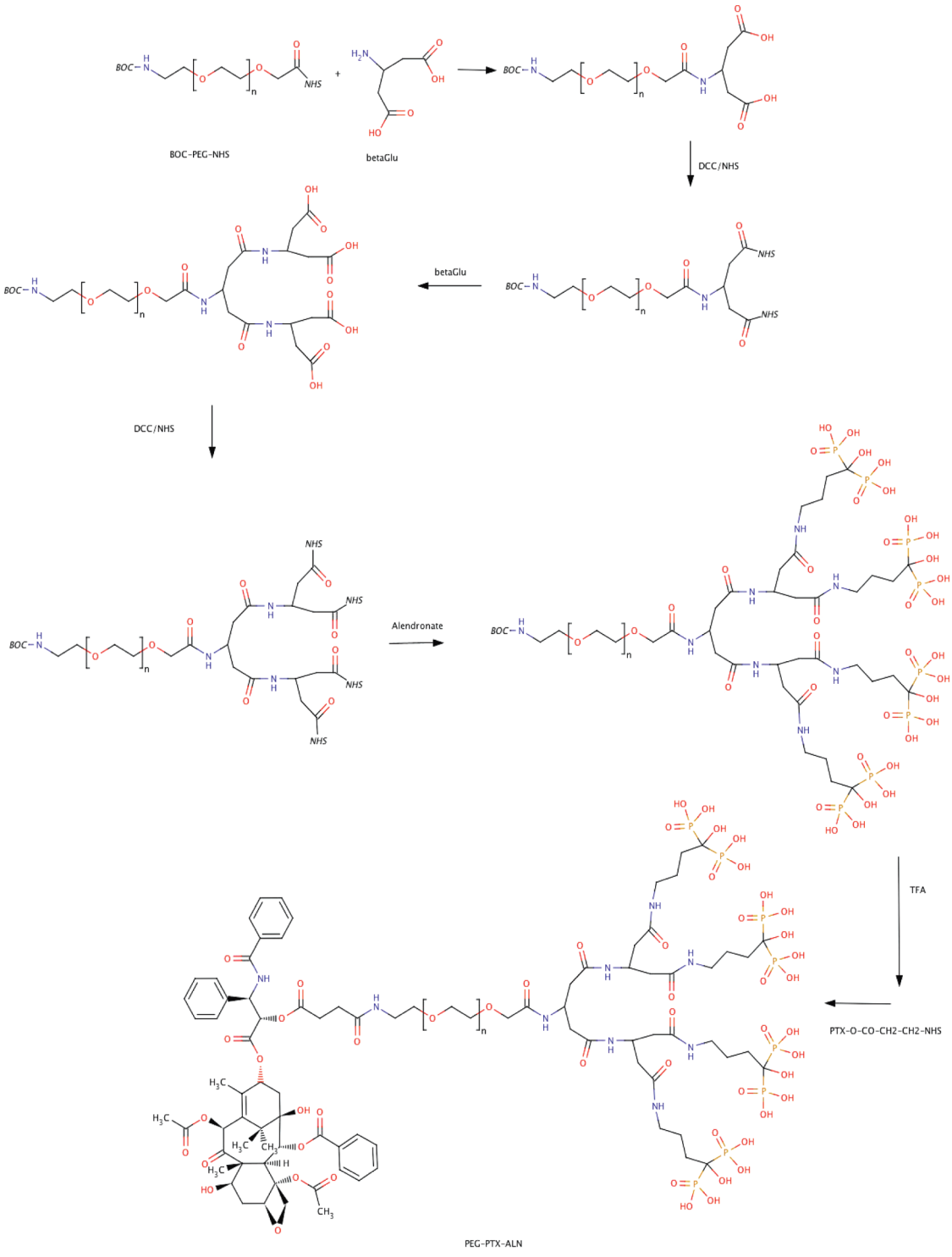
Cell Proliferation Assay. PC3 cells were plated onto a 96 well plate (5×10^3 cells/well) in DMEM supplemented with 5% FBS and incubated for 24 h (37 °C; 5% CO_2). Following 24 h of incubation, the medium was replaced with DMEM containing 10% FBS. Cells were exposed to the combination of PTX and ALN, each drug alone, and with PEG, PEG-ALN, PTX-PEG, and PTX-PEG-ALN conjugates at serial concentrations for 72 h. Following incubation, PC3 cells were counted by MTT.

Migration Assay. A cell migration assay was performed using modified 8 μm Boyden chambers Transwells (Costar Inc., USA) coated with 10 μg/mL fibronectin (Biological Industries, Beit Haemek, Israel). PC3 (15×10^4 cells/100 μL) were challenged with the combination of free PTX (10 nM) and ALN (46 nM), each free drug alone, and with PEG, PEG-ALN, PTX-PEG, and PTX-PEG-ALN conjugates at equivalent PTX and ALN concentrations and were added to the upper chamber of the transwells for 2 h incubation prior to migration toward DMEM containing 10% FBS. Following incubation, cells were allowed to migrate to the underside of the chamber for 4 h in the presence or absence of 10% FBS in the lower chamber. Cells were then fixed and stained (Hema 3 Stain System; Fisher Diagnostics, USA). The stained migrated cells were imaged using Nikon TE2000E inverted microscope integrated with Nikon DS5 cooled CCD camera by 10× objective, brightfield illumination. Migrated cells from the captured images per membrane were counted using NIH image software. Migration was normalized to percent migration, with 100% representing migration to medium containing FBS.

Ethics Statement. All animal procedures were approved by the Ethics Committee of University of Padua and the Italian Health Ministry, and all animals received care according to the DLGS 116/92 and in compliance with the "Guide for the Care and Use of Laboratory Animals".

Pharmacokinetic Studies in Mice. Pharmacokinetics of PTX, PTX-PEG, and PTX-PEG-ALN were determined in female Balb/C mice (23−25 g). The 30 mice were randomly divided in three groups of 10 animals. A total of 150 μL of PTX in 1:1:8 ethanol/Cremophor EL/saline, PTX-PEG in PBS pH 6, or PTX-PEG-ALN in PBS pH 6 (dose: 10 mg/kg PTX equiv.) were administered via a tail vein to mice anaesthetized with 5% isoflurane gas (mixed with O_2 in enclosed cages). At predetermined times, two blood samples (150 μL) were withdrawn from the retro-orbital plexus/sinus of two animals, with a heparinized capillary, and then centrifuged at 1500*g* for 15 min. To 50 μL of plasma, 350 μL of CH_3CN was added for protein precipitation,

Scheme 1. Synthesis of (a) PEG-ALN, (b) PTX-PEG, and (c) PTX-PEG-ALN



and the resulting mixture was centrifuged at 20 000*g* for 5 min. A total of 300 μL of the supernatant was collected and freeze-dried. The residue was dissolved in 50 μL of CH_3OH and analyzed by RP-HPLC under conditions reported above. For PTX-PEG and PTX-PEG-ALN the residues after freeze-drying were also hydrolyzed with 2N NaOH as reported above.

Statistical Methods. In vitro and in vivo data are expressed as mean ± SD. The statistical significance was determined using an unpaired *t*-test. $P < 0.05$ was considered statistically significant. All statistical tests were two-sided.

RESULTS

Synthesis of PTX-PEG, PEG-ALN, and PTX-PEG-ALN Conjugates. Three derivatives of a PEG-(β-Glu) dendrimer, bearing ALN and/or PTX, were synthesized (Figure 1). The synthesis of PTX-PEG was performed in three main steps: synthesis of SPTX,

synthesis of PEG-dendrimer, and binding of SPTX to PEG-dendrimer (see Scheme 1). SPTX was characterized by ^{1}H NMR spectroscopy, showing the characteristic signals of PTX together with those of the succinic spacer. The PEG-dendrimer was built at carboxylic activated terminus of commercial Boc-NH-PEG-NHS using β-Glu as symmetric bicarboxylic branching unit. PEG-ALN was obtained by first linking the ALN targeting residues to the PEG dendrimer carboxylic group and then by removing the Boc protecting group. The coupling of SPTX to PEG-ALN yielded PTX-PEG-ALN.

Table 1. PEG-(β-Glu) Dendrimer, PEG-ALN, PTX-PEG, and PTX-PEG-ALN Molecular Weight and w/w Loading % of ALN and PTX

compound	PTX loading (w/w) %	ALN loading (w/w) %
PEG	0.0	0.0
PEG-ALN	0.0	11.9
PTX-PEG	6.0	0.0
PTX-PEG-ALN	4.7	11.0

Physicochemical Characterization. The content of ALN on PTX-PEG-ALN and on PEG-ALN conjugates (reported in Table 1) was determined spectrophotometrically via the chromophoric complex formed between ALN and Fe^{3+} ions in perchloric acid and against a calibration graph of ALN.

The content of free PTX on PTX-PEG-ALN and PTX-PEG conjugates (below 0.6% w/w) was determined directly by RP-HPLC analysis of the conjugates solubilized in DMSO. The total PTX amount determination was performed by RP-HPLC after hydrolysis of the conjugates to release the linked drug. The w/w % loading of ALN and PTX and the molecular weight of each of the PEG-(β-Glu) dendrimer is summarized in Table 1.

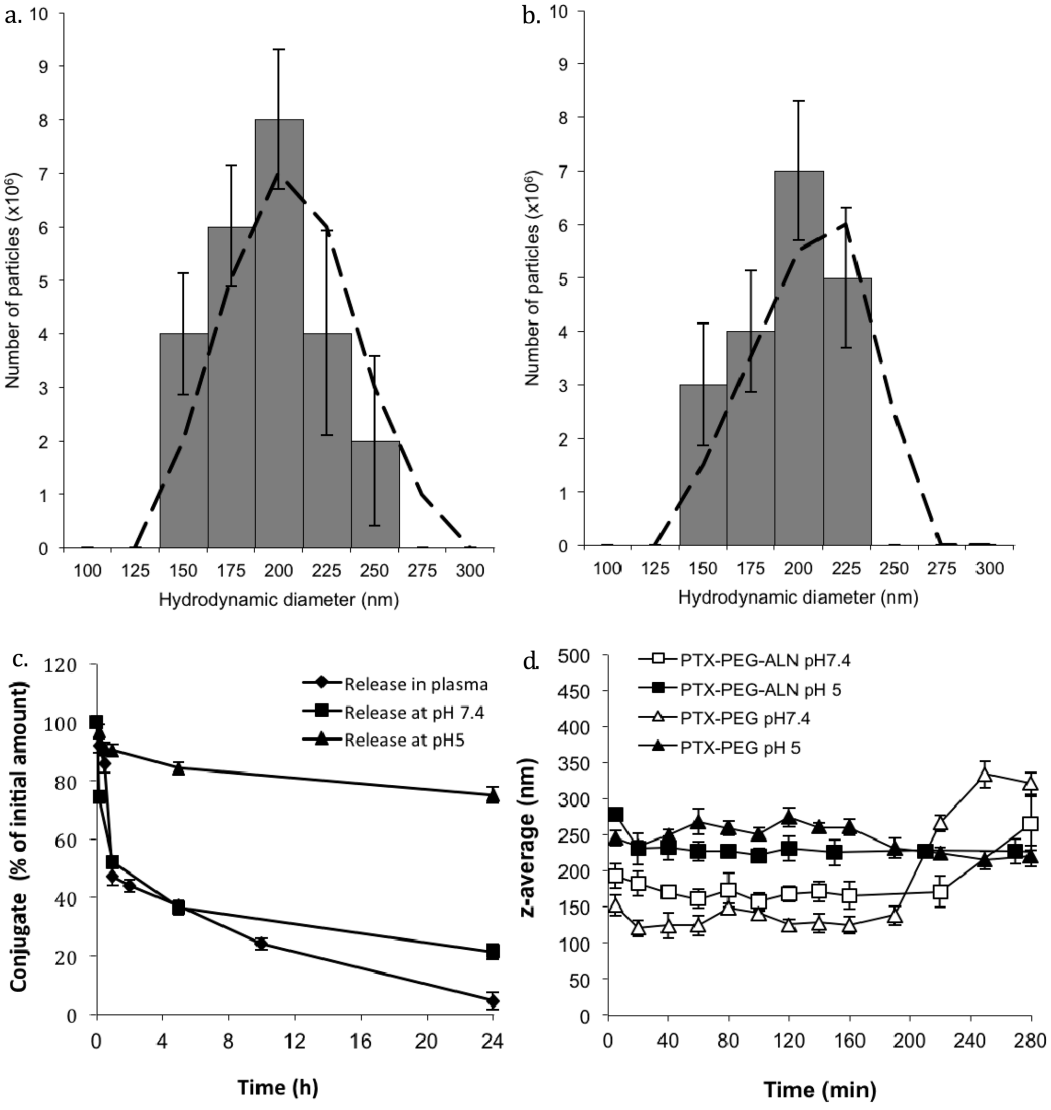


Figure 2. In vitro characterization of PEG-ALN, PTX-PEG, and PTX-PEG-ALN conjugates. The mean hydrodynamic diameter and size distribution of PTX-PEG (a) and PTX-PEG-ALN (b) conjugates was demonstrated as ~200 nm using a real time particle analyzer (NanoSight LM20). (c) 50% of PTX-PEG-ALN conjugate is degraded by 1 h at PBS pH 7.4 and in the plasma. PTX-PEG-ALN conjugate was incubated at PBS pH 7.4, PBS pH 5, and in plasma. Samples were taken at the indicated time points and analyzed by HPLC. (d) PTX-PEG-ALN and PTX-PEG conjugates stability. Conjugates were incubated in buffers at pH 5 and pH 7.4 at 37°. The size of the micelles was monitored using a DLS (Malvern Nano-S). At acidic pH the micelles were stable for up to 24 h, whereas after 3 h at pH 7.4 the size of the samples start to increase owing to the release of PTX from the conjugates, which is insoluble in the aqueous buffer and precipitates forming a suspension.

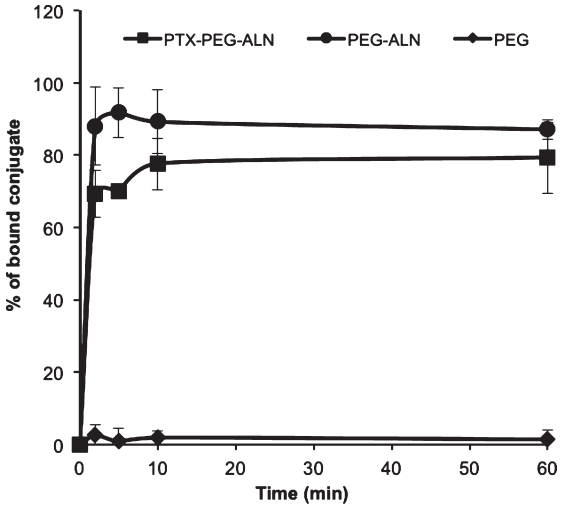


Figure 3. Binding kinetics of PEG-ALN and PTX-PEG-ALN conjugates to the bone mineral HA. PTX-PEG-ALN and PEG-ALN conjugates were incubated with the bone mineral HA for 0, 2, 5, 10, and 60 min. Samples were taken at the indicated time points and analyzed by FPLC. PEG-ALN and PTX-PEG-ALN was rapidly adsorbed to HA and reached a plateau after 5 min of incubation time with ~80% of bound conjugate.

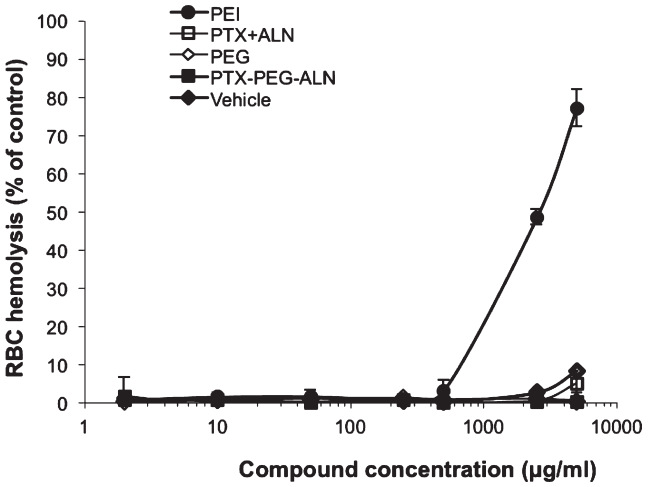


Figure 4. PTX-PEG-ALN conjugate is not hemolytic to red blood cells (RBC). RBC were incubated for 1 h with PTX-PEG-ALN (close squares), PEG (close diamonds), PEI (close circles), PTX vehicle (1:1:8 ethanol/Cremophor EL/saline, open diamonds), and the combination of free PTX plus ALN (open squares) at serial concentrations. Results are presented as % of hemoglobin release produced by the different compounds ± sem. Due to similar values, some symbols overlap.

The hydrodynamic diameter and size distribution of PTX-PEG-ALN and of PTX-PEG conjugates were evaluated using laser light scattering microscopy with nanoparticle tracking analysis (NTA) technology (NanoSight LM20, Salisbury, UK). The mean hydrodynamic diameter of both PTX-PEG-ALN and of PTX-PEG conjugates in PBS pH 7.4 was ~190 nm (Figure 2a,b).

The stability of PTX-PEG-ALN was evaluated in buffer solutions at physiological pH (7.4), at lysosomal pH (pH 5), and in mice plasma (Figure 2c). At pH 7.4 and in plasma, about 50% of the PTX-PEG-ALN conjugate was degraded within the first 1 h, the remaining conjugate was degraded within 24 h. Similar results were found with PTX-PEG.

The stability of the conjugates micelles, monitored at 37 °C for 24 h by DLS, was in line with the kinetics of PTX release. The micelles of PTX-PEG-ALN and PTX-PEG conjugates preserved the same size for 24 h when incubated in buffer at pH 5, whereas at pH 7.4 the same micelles were stable for 3 h (Figure 2d), then the PTX release from the conjugates destabilized the system.

Next, the binding capacity of PTX-PEG-ALN and PEG-ALN conjugates to bone mineral through ALN was evaluated. HA was used as a model mineral mimicking bone tissue. An in vitro HA binding assay and FPLC analysis using a HiTrap desalting column was performed. Following 5 min of incubation, 80% or 90% of PTX-PEG-ALN or PEG-ALN conjugates, respectively, were bound to HA and reached a plateau (Figure 3).

PTX-PEG-ALN Conjugates Demonstrate a Biocompatible Profile in Vitro on Red Blood Cells. The biocompatibility of PTX-PEG-ALN was evaluated using rat red blood cell (RBC) hemolysis assay.[25] Rat RBC solution was incubated with serial concentrations of the combination of PTX and ALN, PEG, or PTX-PEG-ALN conjugate at equivalent PTX and ALN concentrations, PTX vehicle (1:1:8 ethanol/Cremophor EL/saline), and poly(ethylene imine) (PEI) which served as control for hemolysis.[26] As shown in Figure 4, PTX-PEG-ALN conjugate did not exhibit detectable RBC hemolysis at all concentrations up to 5 mg/mL (the estimated blood concentration after in vivo administrations is about 0.5 mg/mL). PTX vehicle cytotoxicity is known on normal nonproliferating cells,[27] and indeed, a slight RBC hemolysis of ~8% was observed in RBCs incubated with PTX vehicle. About 5% hemolysis was observed in RBCs incubated with the combination of PTX plus ALN at the highest equivalent to the conjugate concentration of 5 mg/mL. This hemolysis observed is probably caused by the Cremophor EL vehicle in which these drugs were dissolved.

PTX-PEG-ALN Conjugate Inhibits the Proliferation of PC3 Human Prostate Adenocarcinoma Cells. The taxane PTX is a potent cytotoxic agent approved as first line of therapy for metastatic breast cancer, and it is being tested in the clinic in combination with other chemotherapeutic agents for the treatment of metastatic prostate cancer.[28−30] To evaluate whether PTX retained its cytotoxic activity following conjugation with PEG polymer, a proliferation assay of PC3 human prostate adenocarcinoma cells was performed. The proliferation of PC3 cells was similarly inhibited by PTX-PEG and PTX-PEG-ALN conjugates, by PTX and combination of free PTX plus ALN, exhibiting an IC_{50} of 25−60 nM (Figure 5a and Figure S1 of the Supporting Information).

PEG-(β-Glu) dendrimer served as the control and was nontoxic at any of the concentrations tested. ALN alone was found to be toxic only at the highest concentration tested of 10 μM; however, ALN bound to PEG at equivalent concentration was not toxic at any of the concentrations tested.

PTX-PEG-ALN Conjugate Inhibits the Migration of PC3 Human Prostate Adenocarcinoma Cells. The effect of PTX-PEG and PTX-PEG-ALN on the ability of PC3 to migrate toward FBS was evaluated (Figure 5b). PC3 cells were incubated with each free PTX (10 nM) or ALN (46 nM), the combination of the free drugs, and with PEG conjugates at equivalent concentrations for 6 h. The migration of PC3 incubated with both PTX-PEG and PTX-PEG-ALN conjugates and the combination of free PTX plus ALN toward PBS was inhibited by ~70% (Figure 5b).

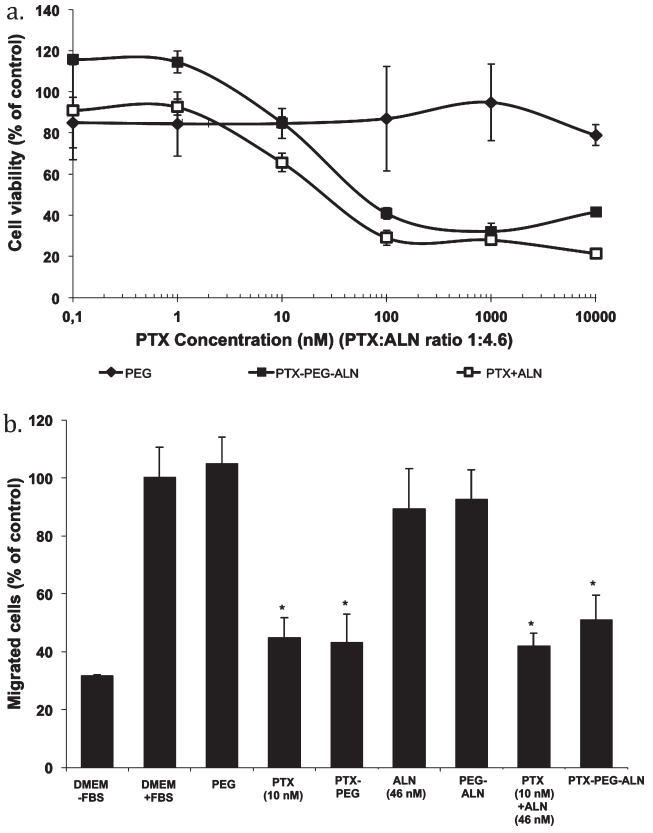


Figure 5. (a) PTX-PEG-ALN and PTX-PEG conjugates inhibit the proliferation of the human adenocarcinoma of the prostate PC3 cells. PC3 cells were incubated with PTX-PEG-ALN, PTX-PEG, and PEG-ALN conjugates, free PTX, or ALN, and the combination of free PTX plus ALN at equivalent concentrations for 72 h. All treatments at equivalent concentrations had a similar cytotoxic effect on the proliferation of PC3 cells. Data represent the mean ± SD (standard deviation). The *X*-axis is presented at a logarithmic scale (the graph of PTX-PEG and PEG-ALN conjugates, free PTX and ALN, is reported in Figure S1 of the Supporting Information). (b) PTX-PEG-ALN and PTX-PEG conjugates inhibit the migration of PC3 cells by 50% compared with control untreated cells. PC3 cells were incubated with the combination of free PTX plus ALN, PTX, ALN, PEG, PTX-PEG-ALN, PTX-PEG, and PEG-ALN conjugates for 2 h. Following incubation, cells were allowed to migrate to the underside of the chamber for 4 h in the presence or absence of 10% FBS in the lower chamber. Migration was normalized to percent migration while 100% representing migration to DMEM containing 10% FBS. The quantitative analysis of the number of migrated cells is presented. Data represent the mean ± SD.

PTX-PEG-ALN Conjugate Showed an Improved Pharmacokinetic Profile in Mice. The pharmacokinetics of PTX dissolved in 1:1:8 ethanol/Cremophor EL/saline, PTX-PEG, and PTX-PEG-ALN were determined in mice. The serum levels of PTX were evaluated by RP-HPLC. As shown in Figure 6, after administration of free PTX, high levels of the drug were recorded, however at 5 min postinjection, the PTX concentration decreased dramatically, and it was not detectable at 60 min. On the contrary, the two conjugates showed a marked half-life prolongation, with detectable levels of PTX after 3 h for PTX-PEG and after 24 h for PTX-PEG-ALN. In particular, elimination half-lives ($T_{1/2\beta}$) were 15.1, 77.9, and 85.5 min for PTX, PTX-PEG, and PTX-PEG-ALN, respectively.

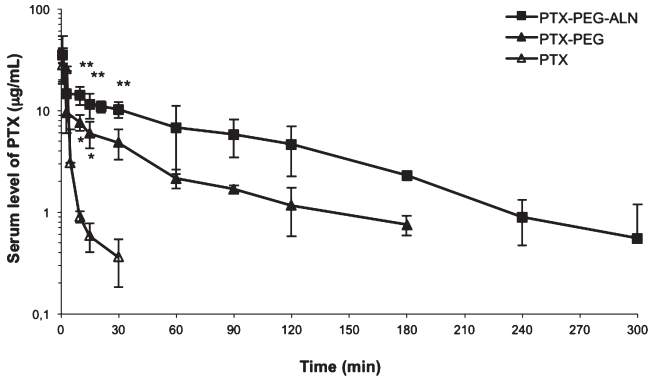


Figure 6. PTX-PEG-ALN and PTX-PEG display an improved pharmacokinetic profile in mice. Female Balb/C mice were administered with PTX in 1:1:8 ethanol/Cremophor EL/saline, PTX-PEG in PBS pH 6, or PTX-PEG-ALN in PBS pH 6 (dose: 10 mg/kg PTX equiv., n = 10 animals per group). Each point is the mean of PTX serum level in animals (* = $p < 0.05$ of PTX-PEG vs PTX; ** = $p < 0.05$ of PTX-PEG-ALN vs PTX). *Y*-axis is presented at a logarithmic scale.

DISCUSSION

This study investigates the effects of a PEG carrier on the cytotoxicity of PTX/ALN combination and on ALN-mediated bone targeting. PTX is a potent anticancer drug used for the treatment of breast, ovarian, nonsmall-cell lung, and prostate cancers.[28−30] ALN is a bisphosphonate,[19−21] used for the treatment of osteoporosis and bone metastases and also investigated as a bone targeting agent.[10,15,19,31,32] The PTX/ALN combination has been already proposed by coupling the two active agents to HPMA copolymer.[15] HPMA copolymer PTX-ALN conjugate showed an increased anticancer and antiangiogenic activity with respect to the free drugs and, at the same time, a reduced toxicity.

Here, we hypothesized that, by synthesizing a heterobifunctional PEG-dendrimer, namely, H_2N-PEG-(β-Glu)-(β-Glu)$_2$-(COOH)$_4$, it could be possible to obtain a conjugate with a high degree of homogeneity, thus impacting positively on both the targeting and the activity properties. Indeed, this polymeric structure can offer a great control over PTX/ALN ratio and on the chemical structure of the conjugate, as reported for different drug combinations.[11] The optimal ratio can be selected simply by growing the dendrimer structure based on β-Glu as a branching unit,[23] whose carboxylic groups have been exploited for ALN coupling. We decided to exploit the four carboxylic groups of H_2N-PEG-(β-Glu)-(β-Glu)$_2$-(COOH)$_4$ for the coupling of ALN because, in a previous study with conjugates having different drug/targeting moiety ratios, we demonstrated that the selective cytotoxicity of the conjugates increases with the number of targeting molecules per polymer chain.[11] Furthermore, the heterobifunctional PEG allows the subdivision of targeting and activity functions by linking PTX and ALN at the two different end chains of the polymers. This design leads to the obtainment of an amphiphilic conjugate, being PTX highly hydrophobic and ALN hydrophilic. The spatial separation of these drugs, besides offering the possibility to form self-assembled micelles, will maintain all ALN molecules exposed to the water, promptly available for binding to the bone mineral HA. As opposed to the architectures obtained from these PEG-dendritic conjugates, it is conceivable that conjugates obtained with multivalent polymers, like polyglutamic acid (PGA) and HPMA copolymer, can present some ALN units embedded in a PTX cluster, which could reduce its targeting properties.

Starting from these considerations, we achieved the synthesis of PTX-PEG-ALN conjugate, as reported in Scheme 1. The derivative can target bone neoplasms by dual-targeting as follows: (1) through ALN (active mechanism), and (2) by exploiting the EPR effect (passive mechanism), which is due to the atypically leaky tumor blood vasculature[5] that enhances tumor accumulation of the conjugate thanks to its increased size with respect to the free drug. The loading of ALN is also a relevant parameter. Rapid and elevated targeting to bone tumors and enhanced antiangiogenic activity are favored by high ALN loadings. Wang et al. and Segal et al. prepared ALN-targeted HPMA copolymer conjugates having an ALN loading of 1% and 7% (mol), respectively.[19,15] In our study, thanks to a great control over the conjugate chemical structure, we could achieve a higher percent of ALN loading (11% w/w). PTX-PEG-ALN conjugate showed great affinity to HA. This result confirms that the favorable PEG/ALN ratio (Table 1) and the dendrimer structure, bearing ALN molecules, ensure high and fast binding to the bone mineral HA. The building blocks of the conjugate (succinic acid, PEG and β-Glu) are all nontoxic. Indeed the in vitro RBC lysis assay demonstrated that both nonconjugated PEG and PTX-PEG-ALN conjugate had no hemolytic activity at up to 5 mg/mL. In contrast, the commercial solubilizing vehicle for PTX that contains Cremophor EL had significant hemolytic activity on RBC.

Lysosomotropic drug release has always been seen as the preferred choice because it can reduce the side effects of the drug. Indeed, most examples of anticancer drug conjugates in the literature, either for mono or combination therapy, have the drug/s coupled to the polymer by cathepsins-cleavable linkers.[12,33−38] In our case, PTX-PEG-ALN conjugate was designed for a strong bone tropism and a faster drug release compared to that from our previously described conjugate, HPMA copolymer-PTX-ALN.[15] We hypothesized that, with PTX-PEG-ALN conjugate, a cathepsin B-cleavable linker might not be suitable because the derivative in vivo will bind to the bone HA matrix. The high affinity to the bone originating from the presence of a bisphosphonate in the conjugate can affect the conjugate internalization into cancer cells and consequently slow the rate of PTX release, if a cathepsin B-cleavable linker is used. Cathepsin B is overexpressed in lysosomes of many types of tumor cells, but also secreted to the extracellular matrix. In general, enzymatic cleavage is efficient when slow and controlled drug release is required. When a fast release is desired, a different mechanism, such as hydrolysis, is necessary. Therefore, in our case, a PTX-polymer hydrolysis at physiological conditions has been preferred because it allows drug release in the surroundings of bone metastasis, where the conjugate will fast accumulate. PTX was linked to PEG through an ester linkage exploiting a succinimidyl spacer, which releases the drug at physiological pH. The PTX release was investigated in vitro for PTX-PEG-ALN under different conditions (Figure 2c). As expected, the hydrolysis rate of the ester bond between the drug and the polymer was higher in buffer at pH 7.4 than in pH 5. Interestingly, the incubation in plasma showed a drug release comparable to that in buffer at pH 7.4, suggesting that PTX is released by a hydrolytically based mechanism without a significant contribution of esterases. The faster drug release at pH 7.4 affected also the stability of PTX-PEG-ALN and PTX-PEG micelles, which at this pH were less stable than at pH 5. Micelles at pH 7.4 were stable up to about three hours although half of the PTX amount was released within 1 h. This behavior can be explained with the high hydrophobicity of PTX that limits the PTX escape from the hydrophobic inner core of the micelles, thus prolonging the micelles stability. Probably, this process holds only in simple in vitro models, whereas in vivo these micelles might be less stable due to both the interaction with blood components and the dilution in the bloodstream. Nevertheless, the pharmacokinetic profiles of PTX-PEG and PTX-PEG-ALN conjugates in mice showed marked half-lives increase with respect to free PTX solubilized in Cremophor EL (about 5 and 6 times longer, respectively). Interestingly, in the case of PTX-PEG-ALN conjugate, the serum level of PTX was still detectable after 24 h, whereas with PTX-PEG the concentration dropped below the detection limit after 3 h. Likely, this behavior of PTX-PEG-ALN conjugate can be explained by the targeting action of ALN, which induces a strong binding of the PTX-PEG-ALN conjugate to the bones from which PTX is released. Therefore, in the case of PTX-PEG the half-life prolongation is based only on the increased size of the conjugates, whereas for PTX-PEG-ALN, beside the role of the carrier, also the ALN targeting effect might reduce the clearance as seen for other targeted conjugates.[11] Also the increased negative net charge of PTX-PEG-ALN with respect to PTX-PEG (Figure 1) might play a role in reducing the kidney excretion, owing to repulsion with negatively charged capillary wall in the glomerulus.[39]

In this study the cytotoxic in vitro evaluation was directed toward PC3 prostate cancer cell line. PTX-PEG-ALN showed similar IC_{50} compared with free PTX, suggesting that PTX can be released from the conjugates and achieve tumor cell killing efficacy.

CONCLUSIONS

A well-defined nontoxic conjugate of PTX and ALN, based on a PEG-(β-Glu)-dendrimer, was successfully designed and characterized. The architecture of polymeric carrier offered the possibility to obtain an optimal PTX/ALN ratio. This parameter, together with the separation of PTX from ALN (coupled at the opposite PEG end chains), ensured a high HA binding for a strong bone targeting. At the same time, these features allowed the preservation of a cytotoxic activity comparable to that of free PTX/ALN combination against prostate cancer cells.

ASSOCIATED CONTENT

Supporting Information. Proliferation inhibition of the human adenocarcinoma of the prostate PC3 cells (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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