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Poly(ethylene glycol)–paclitaxel–alendronate self-assembled micelles for the targeted treatment of breast cancer bone metastases

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ABSTRACT

Paclitaxel (PTX) and alendronate (ALN) are effective drugs used for the treatment of breast cancer bone metastases. Growing evidence suggests that low-dose taxanes and bisphosphonates possess anti-angiogenic properties. However, PTX is water-insoluble and toxic, even if administered at anti-angiogenic dosing schedule. Polymer conjugation of PTX will increase water-solubility and improve its pharmacokinetic profile directing it to the tumor site. We further propose to combine it with ALN for active bone targeting. We conjugated ALN and PTX with poly(ethylene glycol) (PEG) forming self-assembled micelles where PTX molecules are located at the inner core and the water-soluble ALN molecules at the outer shell. PTX–PEG–ALN micelles exhibited similar *in vitro* cytotoxic and anti-angiogenic activity as the free drugs. Biodistribution analysis demonstrated preferential tumor accumulation of FITC-labeled PTX–PEG–ALN micelles. Pharmacokinetic studies revealed longer $t_{1/2}$ of the conjugate than free PTX. PTX–PEG–ALN micelles achieved improved efficacy and safety profiles over free PTX in syngeneic and xenogeneic mouse models of mCherry-infected mammary adenocarcinoma in the tibia, as monitored intravitally non-invasively by a fluorescence imaging system. The described data warrants the potential use of PTX–PEG–ALN as bone-targeted anticancer and anti-angiogenic therapy for breast cancer bone metastases.

1. Introduction

Breast cancer is the second leading cause of cancer–related deaths among women worldwide [1]. Despite extensive exploration for novel anticancer drugs or therapeutic strategies, the success of current treatments is far from satisfying, and the side effects can be particularly harsh [2,3]. Bone is an especially favored metastatic site for breast and prostate cancers. More than 25% of breast cancer patients with invasive cancer will develop bone metastases [4]. At autopsy, more than 80% of women who die from the disease show evidence of skeletal involvement [5].

Paclitaxel (PTX) has been proved to be a potent drug used to treat metastatic breast cancer, ovarian cancer, and other forms of cancer. PTX's mechanism of action on cancer cells is well known, however its anti-angiogenic activity was reported only in the last decade [6–9]. Administration of PTX at both maximum tolerated dose (MTD), that targets cancer cells and at low metronomic (anti-angiogenic) dosing schedule that targets endothelial cells is associated with several toxic side-effects and clinical complications [10,11]. In addition, due to poor water-solubility of PTX, it is formulated in Cremophor EL which causes anaphylactic and hypersensitivity reactions [11]. Therefore, potential therapeutic strategies to improve the efficacy of PTX and reduce its side effects are needed.

The aminobisphosphonate, alendronate (ALN) has emerged in recent years as a highly effective therapeutic option for the prevention of skeletal complications caused by bone metastases. Although ALN is mostly known for its potent antiresorptive activity due to specific osteoclasts induction of apoptosis, it also demonstrates anticancer and anti-angiogenic activity [12–14]. Like all bisphosphonates, it exhibits exceptionally high affinity to the bone-mineral hydroxyapatite (HA) [12,14–16]. Because of the high bone affinity of bisphosphonates, much effort has been made to

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conjugate them with non-specific bone therapeutic agents in order to obtain osteotropicity [16–18]. Recently, ALN was successfully conjugated with polymeric drug delivery systems [15,19,20].

Here, we developed an approach which attempts to target PTX selectively to bone metastases, thus reducing its side effects, improving its pharmacokinetics, modifying its biodistribution and enhancing its efficacy. Our strategy rests upon the conjugation of the specific bone targeting moiety ALN, and PTX with poly(ethylene glycol) (PEG) bearing a β-Glutamic acid dendron at one end of the polymeric backbone, as an aim to increase the loading of ALN.

We have previously shown that an HPMA copolymer-PTX–ALN conjugate enhanced the anti-angiogenic and anticancer effect of the free drugs, and reduced their toxicities [20,21]. ALN facilitated the delivery of PTX to the bones. Here, we hypothesized that the conjugation with PEG, similar to HPMA copolymer, would target PTX mostly to the metastatic sites within the bones and scarcely to normal healthy bones, due to the enhanced permeability and retention (EPR) effect, by virtue of its size [22].

The water-soluble PEG is a non-toxic, non-immunogenic, and commercially-available FDA-approved polymer [23]. Furthermore, conjugation with PEG should restrict the passage through the blood brain barrier thus abrogating neurotoxicity associated with free PTX and would prolong the circulating half-life of the free drugs ALN and PTX. Consequently, the inhibitory effect on the growth of tumor endothelial and epithelial cells would be enhanced, by exposing the cells to the conjugated drugs in the circulation for a longer time [24–26]. Furthermore, to achieve stronger targeting to the bone, we designed and synthesized a PTX–PEG–ALN conjugate with an increased loading of ALN molecules per polymer chain. This approach yields a conjugate with a well-defined chemical structure resulting in improved batch to batch reproducibility and biological outcomes [27,28].

We previously reported the design, synthesis and physicochemical characterization of PTX–PEG–ALN that showed a marked affinity for the bone mineral HA and an IC_{50} towards human prostate cancer cells (PC3) comparable to that of the free drugs combination [29]. Here, we evaluated the anti-angiogenic and anticancer activity *in vitro* and *in vivo*, the biodistribution and pharmacokinetics of FITC-labeled PTX–PEG–ALN conjugate.

2. Materials and methods

2.1. Materials

PTX and ALN were purchased from Alcon Biosciences Ltd. (Mumbai, India; Petrus Chemicals and Materials Ltd., Israel). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, 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 from Cambrex (Walkersville, MD, U.S.A). Matrigel matrix was from BD Biosciences, USA. Peroxidase Block was purchased from Merck, Germany. Primary rat anti-murine CD34 antibody (MEC 14.7) was from Abcam, (Cambridge, MA). Rabbit anti-rat antibody, anti-rabbit horseradish peroxidase-conjugated antibody (ABC detection kit) and ImmPACT™ DAB diluent kit were from Vector Laboratories (Burlingame, CA, USA). pEGFPLuc plasmid was from Clontech (Mountain View, CA, USA). Nuclear staining was from Procount, BD Pharmingen (San Jose, CA, USA). 7-aminoactinomycin D (7AAD) was from Chemicon (Billerica, MA). Dextran (MW ~70,000) and all other chemical reagents, including salts and solvents were purchased from Sigma–Aldrich, Israel. All reactions requiring anhydrous conditions were performed under an $Ar_{(g)}$ or $N_{2(g)}$ atmosphere. Chemicals and solvents were either A.R. grade or purified by standard techniques.

2.2. Synthesis of PTX–PEG, PEG–ALN, PTX–PEG–ALN and FITC labeled conjugates

The conjugates were synthesized and characterized in terms of their drug loading and hydrodynamic diameter as previously described [29]. Synthesis of FITC-labeled conjugates was performed exploiting the same chemical strategy for the preparation of non-labeled conjugates. The new steps are described in the supplemental materials.

2.3. Cell culture

MDA-MB-231 human mammary adenocarcinoma and 4T1 murine mammary adenocarcinoma cell lines were purchased from the American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS, 100 μg/ml Penicillin, 100 U/ml Streptomycin, 12.5 U/ml Nystatin and 2 mM L-glutamine. 4T1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 μg/ml Penicillin, 100 U/ml Streptomycin, 12.5 U/ml Nystatin, 2 mM L-glutamine, 10 mM Hepes buffer, and 1 mM sodium pyruvate. Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex (Walkersville, MD, U.S.A) and grown according to the manufacturer's protocol in EGM-2 medium (Cambrex). Cells were grown at 37 °C; 5% CO_2.

2.4. Generation of mCherry-infected human MDA-MB-231 and murine 4T1 mammary adenocarcinoma cell lines

4T1 and MDA-MB-231 cells were infected with mCherry as previously described [19].

2.5. Cell viability assay

HUVEC were plated onto 24-well plate (1.5×10^4 cells/well) in growth factors reduced media, (EBM-2, Cambrex, USA) supplemented with 5% FBS. Following 24 h of incubation (37 °C; 5% CO_2), medium was replaced with EGM-2 (Cambrex, USA). 4T1 and MDA-MB-231 cells were plated onto 96 well plate (5×10^3 cells/well) in DMEM or RPMI, respectively, supplemented with 5% FBS and incubated for 24 h (37 °C; 5% CO_2). Following 24 h of incubation, medium was replaced with DMEM, or RPMI containing 10% FBS. Cells were challenged with the combination of free PTX plus ALN, each free drug alone, and with PEG, PEG–ALN, PTX–PEG, and PTX–PEG–ALN conjugates at serial concentrations for up to 72 h. Following incubation, HUVEC were counted by Coulter Counter. 4T1 and MDA-MB-231 cells viability was measured using the MTT assay.

2.6. 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) were challenged with the combination of free PTX (5 nM) plus ALN (23 nM), each drug alone, and with PEG, PEG–ALN, PTX–PEG and PTX–PEG–ALN conjugates at 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 4× objective, brightfield technique.

2.7. Migration assay

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). HUVEC (15×10^4 cells/100 μL) were challenged with the combination of PTX (100 nM) plus ALN (460 nM), each 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 to vascular endothelial growth factor (VEGF). Following incubation, cells were allowed to migrate to the underside of the chamber for 4 h in the presence or absence of VEGF (20 ng/mL) 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 VEGF alone.

2.8. Measurement of circulating endothelial cells (CEC) and circulating endothelial progenitor (CEP) by flow cytometry

Blood was obtained from anaesthetized mice by retro-orbital sinus bleeding. CEC and CEP were quantitated using flow cytometry, as described previously [30]. Briefly, 24 h after treatment, blood was collected in tubes containing EDTA to avoid clotting. Monoclonal antibodies were used to detect CEC and CEP population with the following antigenic phenotypes: CD13+/VEGFR2+/CD45−/dim. CEP population was also CD117+. Nuclear staining was used in some experiments to exclude platelets or cellular debris. 7-aminoactinomycin D (7AAD) was used to distinguish apoptotic and dead cells from viable cells. After red cell lysis, cell suspensions were analyzed and at least 200,000 cells per sample were acquired. Analyses were considered informative when an adequate number of events (i.e. >50, typically 50–150) were collected in the CEC and CEP enumeration gate in untreated control animals. Percentages of stained cells were determined and compared with appropriate negative controls. Positive staining was defined as being greater than non-specific background staining. Flow cytometry studies were performed on Cyan ADP flow