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Targeting Bone Metastases with a Bispecific Anticancer and Antiangiogenic Polymer–Alendronate–Taxane Conjugate**

*Keren Miller, Rotem Erez, Ehud Segal, Doron Shabat, and Ronit Satchi-Fainaro**

Bone metastases derived from cancers at advanced stages, such as breast and prostate cancer, are devastating and incurable. Chemotherapy agents and bisphosphonates are the common treatments for advanced metastatic disease. However, the disease progresses with time to a phase in which standard therapy fails to control the malignancy, and then progresses further to a highly chemotherapy resistant state.[1,2]

Angiogenesis is a critical step in tumor development and metastasis formation.[3–5] Tumor endothelial cells are sensitive to drugs for long time periods of drug treatment and may be treated with cytotoxic agents in an "antiangiogenic dosing schedule".[6] This schedule involves the administration of chemotherapy in low doses, well below the maximum tolerated dose (MTD), at close intervals ("metronomic dosing").[7,8] As a result, the drugs may be administered for extended periods of time,[6,8] and acute toxicity is avoided.

The taxane paclitaxel (PTX) is a known potent cytotoxic agent approved as a first line of therapy for metastatic breast cancer. It is being tested in the clinic in combination with other chemotherapeutic agents for the treatment of metastatic prostate cancer.[9–11] Despite its potent anticancer activity, PTX exhibits serious dose-limiting toxicities owing to its lack of selectivity for the target tissue. Furthermore, because of the poor water solubility of PTX, it is formulated in cremophor EL, which causes hypersensitivity.[12] At low doses, PTX has antiangiogenic properties.[13,14] For these reasons, we chose PTX as the model chemotherapeutic agent.

We have now developed a new approach in an attempt to target bone metastases selectively with PTX and thus decrease the side effects caused by the drug. Our strategy rests upon the conjugation of the specific bone-targeting agent alendronate (ALN) and PTX with an *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer.

ALN, which is generally used to treat osteoporosis and bone metastases as well as to prevent bone fractures, was chosen as the bone-targeting moiety and antiangiogenic model agent. Like all bisphosphonates, it exhibits an exceptionally high affinity for the bone mineral hydroxyapatite (HA).[15,16] This unique feature of bisphosphonates makes them good candidates for the bone targeting of antineoplastic compounds, radionucleotides, and nucleoside analogues.[16,17]

ALN should facilitate the delivery of PTX to the bones. Conjugation with the HPMA copolymer should cause PTX to mostly target the metastatic sites within the bones: Passive extravasation of the conjugate should occur through the leaky tumor vessels, whereas normal blood vessels in healthy bones should be poor targets owing to the size of the conjugate. The water-soluble HPMA copolymers are biocompatible, nonimmunogenic, and nontoxic carriers that enable selective delivery into tumor tissue.[18] These macromolecules (diameter of 10–200 nm) do not diffuse through normal blood vessels but rather accumulate selectively in the tumor site because of the enhanced permeability and retention (EPR) effect.[19] Furthermore, conjugation with the HPMA copolymer should restrict the passage through the blood–brain barrier and thus eliminate the neurotoxicity associated with free PTX and prolong the circulation half-life of the free drugs ALN and PTX. Consequently, the inhibitory effect on the growth of tumor endothelial and epithelial cells should be enhanced by the exposure of the cells to the conjugated drugs in the circulation for a longer time.[20–22]

PTX has already been conjugated to polymers, such as polyglutamic acid (opaxio) and an HPMA copolymer (PNU166945), and to proteins, such as albumin (abraxane), with the aim of improving drug solubility and the subsequent controlled release of PTX.[23] Indeed, the resulting conjugated forms of PTX were more soluble than free PTX and diminished the need for chemical solvents.[24–27] However, in the case of PNU166945, neurotoxicity and neuropathy were observed at early stages of clinical trials.[27] In PNU166945, PTX was attached to the HPMA copolymer through an ester bond, which was relatively unstable under physiological conditions. As a result of spontaneous hydrolysis and/or the activity of endogenous esterases, PTX was released from the polymer prematurely, and therefore induced the commonly observed toxicities of free PTX.[27] In this study, we chose to conjugate PTX with HPMA copolymer–Gly-Phe-Leu-Gly–*p*-nitrophenol (HPMA copolymer–GFLG–ONp) through a Phe-Lys–*p*-aminobenzyl carbonate (FK–PABC) spacer. This dipeptide–PABC linker enables the stable conjugation of

[*] K. Miller,[+] E. Segal, Dr. R. Satchi-Fainaro
Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978 (Israel)
Fax: (+972) 3-640-9113
E-mail: ronitsf@post.tau.ac.il
R. Erez,[+] Prof. D. Shabat
Department of Organic Chemistry, School of Chemistry
Raymond and Beverly Sackler Faculty of Exact Sciences
Tel Aviv University (Israel)
[+] These authors contributed equally.

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PTX with the HPMA copolymer through a carbonate linkage.[28,29] The GFLG and FK linkers are both cleaved by the lysosomal enzyme cathepsin B, an overexpressed and secreted enzyme in tumor endothelial and epithelial cells[30–36] (conjugate **1**, Scheme 1). The cleavage of the FK dipeptide by cathepsin B releases an amine intermediate (PABC–PTX), which disassembles spontaneously through 1,6-elimination and decarboxylation to release free PTX (Scheme 1).

PTX was conjugated to the HPMA copolymer through a two-step procedure in which PTX was first attached to the FK–PABC linker and then conjugated to HPMA copolymer–GFLG–ONp (Scheme 2). A detailed description of the synthesis can be found in the Supporting Information.

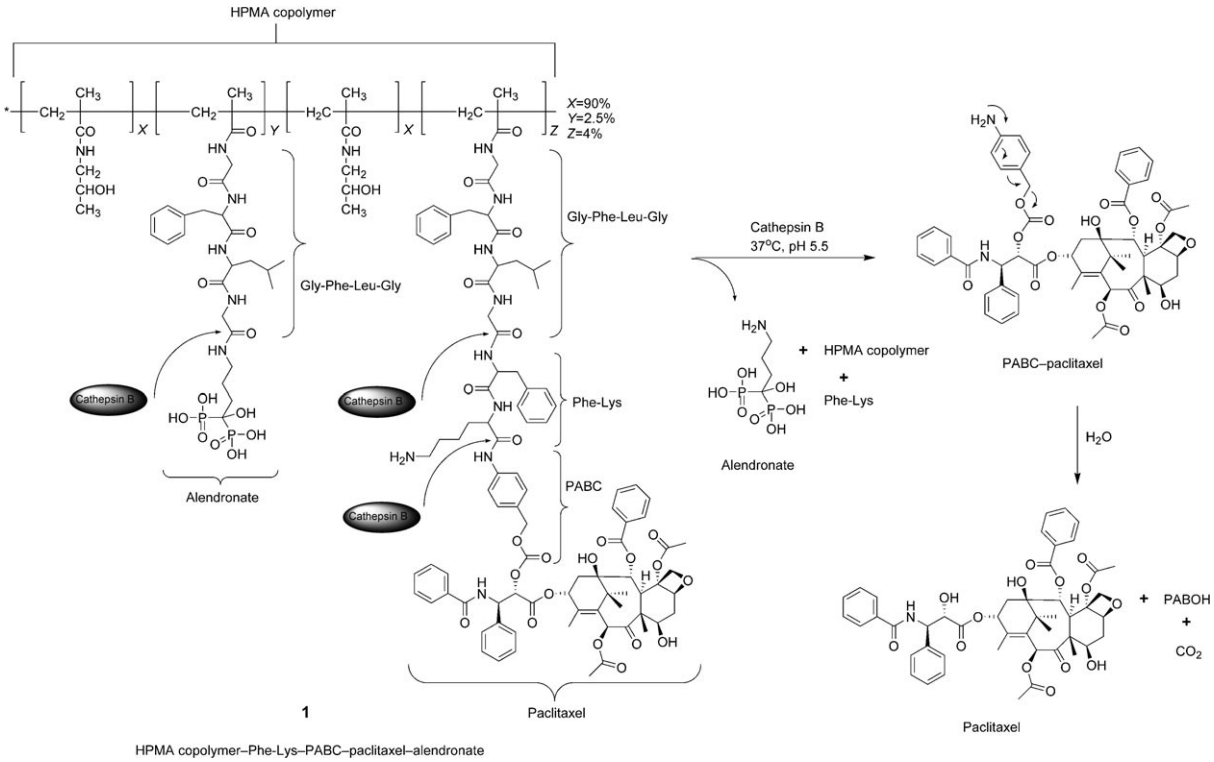
The hydrodynamic diameter and size distribution of our polydispersed nanoscale HPMA copolymer–PTX–FK–ALN conjugate were then characterized by laser light scattering microscopy with nanoparticle tracking analysis (NTA) technology (NanoSight LM20, Salisbury, UK). The mean hydrodynamic diameter of the conjugate was 95 nm (Figure 1a).

Next, we evaluated the binding capacity of the conjugate to bone mineral through its ALN moiety. HA was used as a model mineral to mimic bone tissue. We carried out an in vitro HA-binding assay and analysis by fast protein liquid chromatography (FPLC) with a HiTrap desalting column. After incubation for 5 min, approximately 50% of the conjugate in the solution was bound to HA, and a plateau was reached (Figure 1b). We suggest two possible explanations for these results: Either 50% of the HPMA copolymer–PTX–FK conjugate chains were bound to the ALN moiety, or partial steric hindrance prevented the attachment of all polymer-conjugate chains to HA.

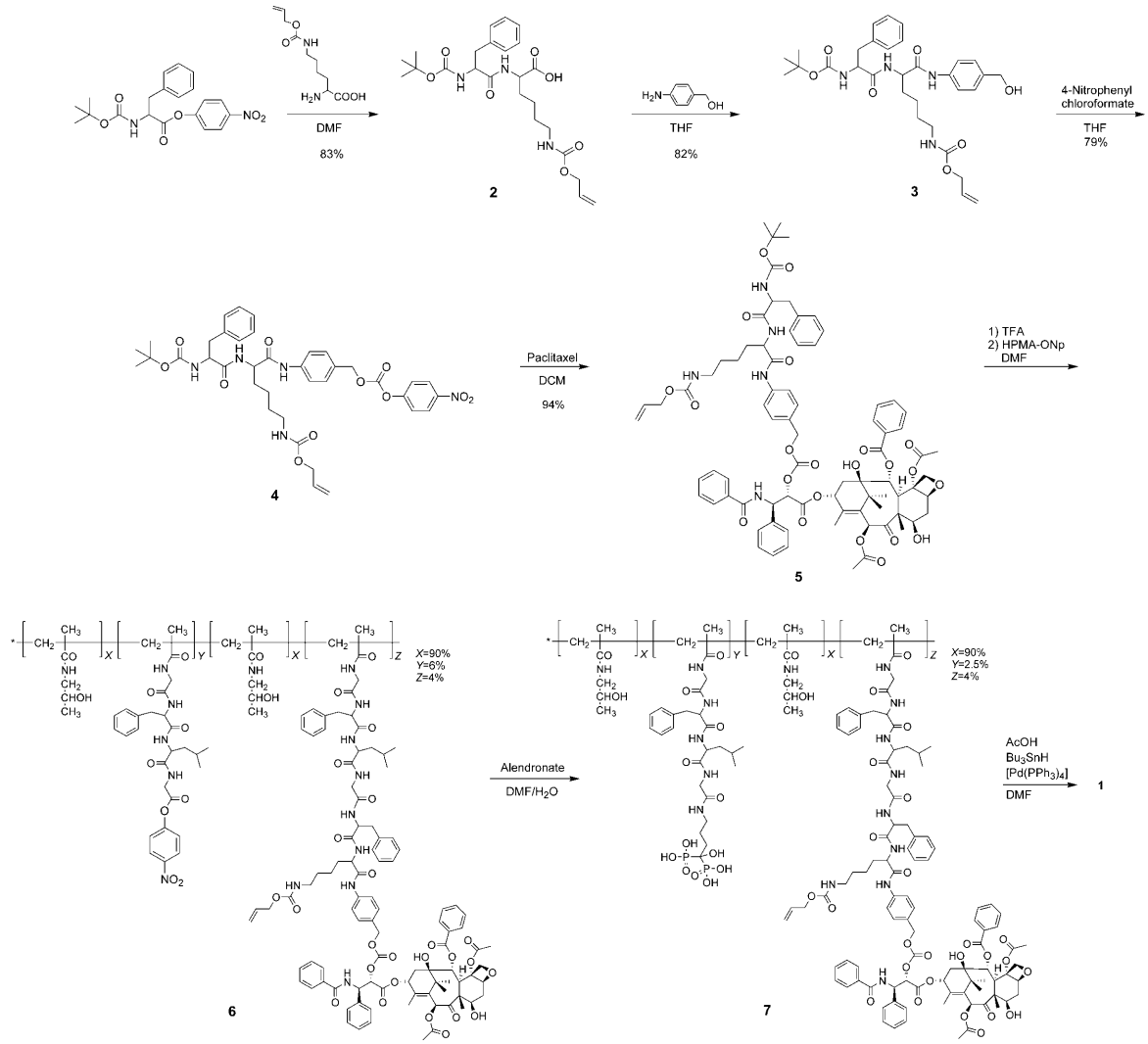
The ALN content in the HPMA copolymer–PTX–FK–ALN conjugate was determined spectrophotometrically on the basis of the chromophoric complex formed between ALN and Fe^{3+} ions in perchloric acid, and against a calibration graph for ALN. Of the functional GFLG–ONp chains (10 mol%), 2.5 mol% were bound to ALN. This percentage content of ALN on the HPMA copolymer surpasses the amount needed for bone targeting, as indicated previously.[15]

Cathepsin B was used in vitro to cleave compound **1** at the two cleavable linker sites, GFLG and FK, at 37 °C and pH 5.5. Samples were taken after 12, 24, 48, and 72 h, and the PTX content of the HPMA copolymer–PTX–FK–ALN conjugate was determined by HPLC analysis. The PTX content was determined against a calibration curve for free PTX. Of the functional GFLG–ONp chains (10 mol%) on the HPMA copolymer, 4 mol% were bound to PTX (Figure 1c).

To prove that the HPMA copolymer–PTX–FK–ALN conjugate is active mainly upon the release of PTX by cleavage with cathepsin B, and not by spontaneous hydrolysis, we synthesized an HPMA copolymer–Gly-Gly-Gly-Gly–PTX conjugate containing the noncleavable Gly-Gly (GG) linker (Scheme 3a) and compared it with the cleavable HPMA copolymer–GGFK–PTX conjugate (Scheme 3b). The HPMA copolymer–GGGG–PTX conjugate inhibited the proliferation of human umbilical-vein endothelial cells (HUVECs) with an IC_{50} value of approximately 10000 nM, that is, at a concentration two orders of magnitude higher than that required for the HPMA copolymer–GGFK–PTX conjugate ($IC_{50} \approx 100$ nM), which is cleaved by cathepsin B (see the Supporting Information). This finding further supports the notion that PTX–FK bound to the HPMA copolymer is released mainly through cleavage by cathepsin B.



Scheme 1. Mechanism for the cleavage of the HPMA copolymer–PTX–FK–ALN conjugate, **1**, by cathepsin B.



Scheme 2. Synthesis of the HPMA copolymer–PTX–FK–ALN conjugate. DCM = dichloromethane, DMF = *N*,*N*-dimethylformamide, TFA = trifluoroacetic acid.

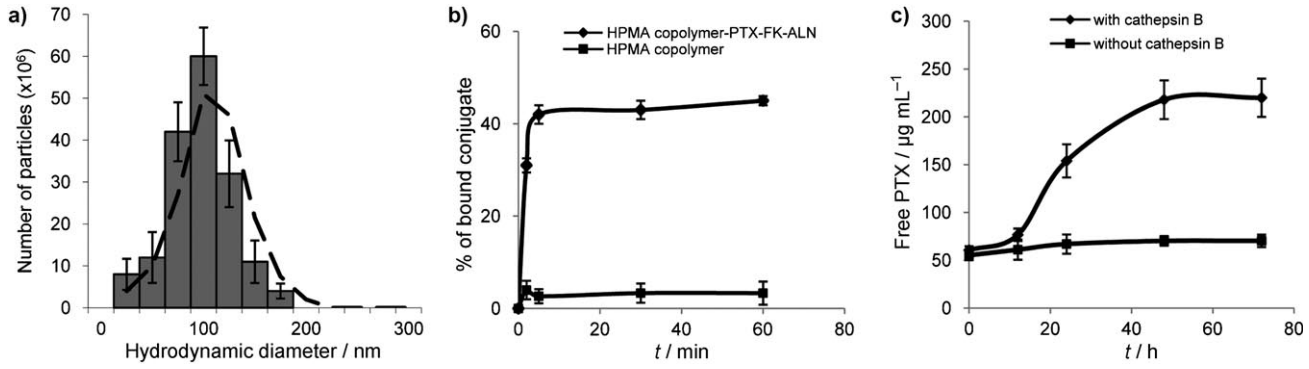
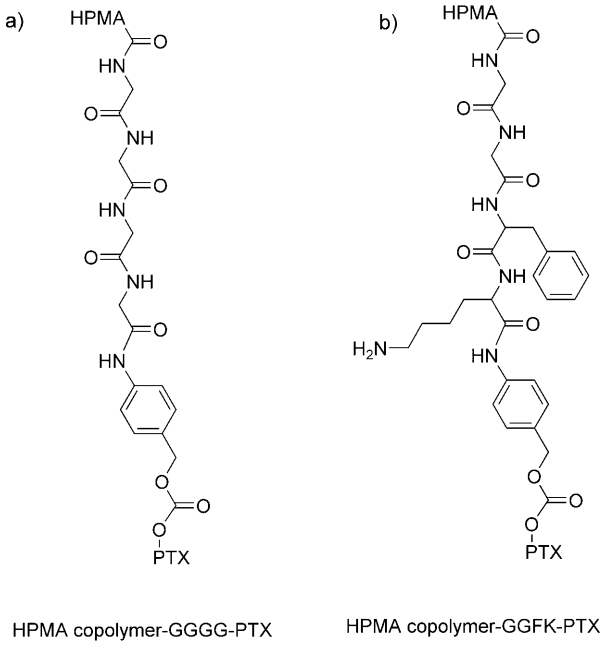


Figure 1. In vitro characterization of the HPMA copolymer–PTX–FK–ALN conjugate. a) Mean hydrodynamic diameter and size distribution of the HPMA copolymer–PTX–FK–ALN conjugate. b) Binding kinetics for the binding of the HPMA copolymer–PTX–FK–ALN conjugate to the bone mineral HA. c) Cleavage of the HPMA copolymer–PTX–FK–ALN conjugate by the enzyme cathepsin B.

Several reports have indicated PTX as an effective agent that could be used to treat advanced metastatic prostate cancer.[10,37] To evaluate whether PTX and ALN retained their anticancer activity following conjugation with the HPMA copolymer, we carried out a proliferation assay of the human prostate cell line PC3. The proliferation of PC3 cells was inhibited similarly by the HPMA copolymer–PTX–FK–ALN conjugate and by a combination of PTX–FK and



Scheme 3. a) Chemical structure of the noncleavable conjugate HPMA copolymer–GGGG–PTX. b) Chemical structure of the cleavable conjugate HPMA copolymer–GGFK–PTX.

ALN at equivalent concentrations (see the Supporting Information).

To assess whether, like PTX, the HPMA copolymer–PTX–FK–ALN conjugate possesses antiangiogenic properties, we carried out endothelial cell proliferation assays, capillary-like tube formation assays, and migration assays of endothelial cells treated with the conjugate. The proliferation of HUVECs was inhibited to a similar extent by a combination of PTX–FK and ALN and by the HPMA copolymer–PTX–FK–ALN conjugate at equivalent PTX/FK/ALN concentrations. IC_{50} values of approximately 10 nM and approximately 6 nM were found for PTX–FK and ALN, respectively (Figure 2a). The HPMA copolymer alone was inert, as expected on the basis of previously published data.[38]

Next, the effect of the HPMA copolymer–PTX–FK–ALN conjugate on the ability of HUVECs to migrate towards vascular endothelial growth factor (VEGF) was tested. The HPMA copolymer–PTX–FK–ALN conjugate and a combination of PTX–FK and ALN at equivalent concentrations of 100 and 60 nM, respectively, inhibited the migration of HUVECs towards VEGF by approximately 35% (Figure 2b).

Having shown that free and conjugated PTX/FK/ALN have antiangiogenic potential by inhibiting the proliferation and migration of HUVECs, we examined the effect of these drugs on the ability of HUVECs to form capillary-like tube structures on matrigel. The formation of such structures is an additional crucial step in the angiogenic cascade of events (Figure 2c). The HPMA copolymer–PTX–FK–ALN conjugate and a combination of PTX–FK and ALN at equivalent concentrations of 0.5 and 0.3 nM, respectively, inhibited the formation of tubular structures of HUVECs by approximately 65% (Figure 2d).

We have described the design and development of a new strategy for the treatment of neoplastic bone metastases. The aim of our approach is the selective targeting of bone metastases with PTX and aversion of the side effects associated with the free drug. PTX and ALN were conjugated to the HPMA-copolymer macromolecule. The targeting agent ALN should provide selective delivery to bones. This assumption is based on previously published data from an in vivo study that demonstrated the selective accumulation of an HPMA copolymer–ALN conjugate in the bones.[15,39,40] We hypothesize that there are three possibilities for the fate of the conjugate in the body, all of which will occur following intravenous (i.v.) administration of the conjugate: 1) The conjugate will be directed to the bone marrow and then extravasate from the leaky tumor vessels into the tumor metastases, which are usually situated in the bone marrow. There, it will internalize by endocytosis into the tumor endothelial cells and tumor cells and release both ALN and PTX in the lysosome in the presence of cathepsin B. 2) The conjugate will be directed to the bone as a result of the high affinity of ALN for HA. Cathepsin B, which is overexpressed in the lysosome of tumor endothelial and epithelial cells and secreted by these cells, will first cleave the GFLG linker extracellularly and release HPMA–GFLG–FK–PTX from the ALN–HA complex in the vicinity of the tumor. HPMA–GFLG–FK–PTX will internalize slowly into both tumor endothelial cells and tumor cells by endocytosis and release PTX in the lysosome. 3) Some free PTX will also be released extracellularly by the secreted cathepsin B in the tumor microenvironment and will enter all cells in the vicinity by simple diffusion.

A few HPMA copolymer–drug conjugates with the tetrapeptide linker GFLG (e.g. HPMA copolymer–TNP-470, named caplostatin, HPMA copolymer–doxorubicin conjugates, named PK1 and PK2, HPMA copolymer–doxorubicin–aminoglutethimide, and HPMA copolymer–doxorubicin–bisphosphonate) have been shown to have antitumor activity with improved efficacy and decreased toxicity.[18,20,21,41–46] This study demonstrates the full release of PTX from the HPMA copolymer by the enzyme cathepsin B within 48 h. The release kinetics of PTX are slow enough for it to be accumulated in the tumor and still fast relative to those of other conjugated forms of PTX (e.g. opaxio, which is more stable and releases PTX slower).[47] The faster release of PTX from our conjugate is due to the additional FK linker. Furthermore, ALN changes the pharmacokinetics of the conjugate, which is sent promptly to the target: bone neoplasms.

This delivery system is water-soluble and therefore could be administered in aqueous solution. This property is itself a major improvement with respect to insoluble PTX and removes the need for the solubilizing agent cremophor EL. The administration of this novel conjugate at a low metronomic dose to target tumor endothelial cells should help to avoid side effects and drug resistance.[6,8]

Assays of the proliferation and migration of endothelial cells, and the formation of capillary-like tubes by these cells, revealed that the newly synthesized HPMA copolymer–PTX–FK–ALN conjugate possesses antiangiogenic properties and

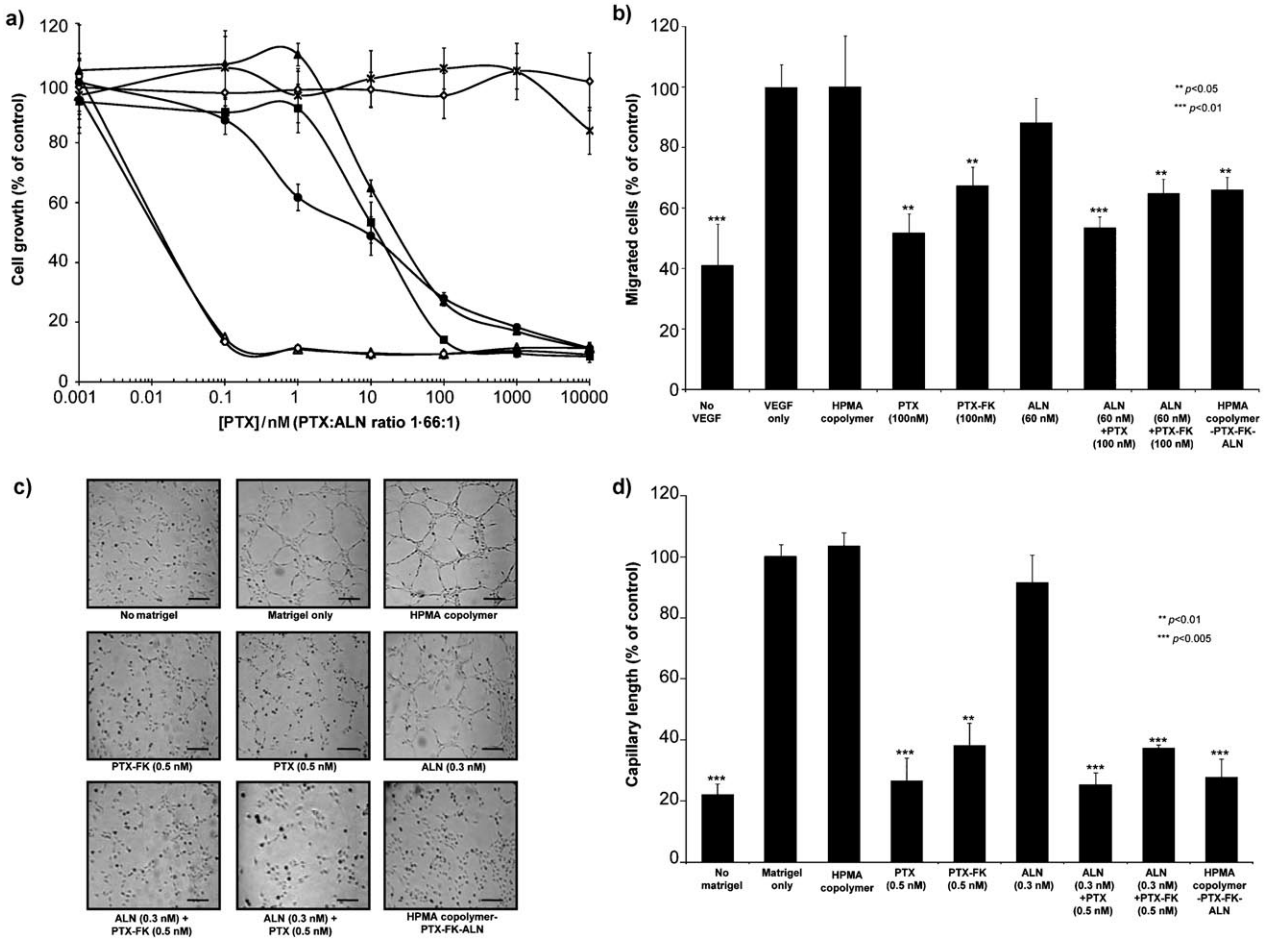


Figure 2. Inhibition of steps in the angiogenic cascade by the HPMA copolymer–PTX–FK–ALN conjugate. a) Inhibition of the proliferation of HUVECs by the HPMA copolymer–PTX–FK–ALN conjugate. HUVECs were incubated with PTX (○), ALN (×), PTX–FK (▲), ALN+PTX (△), PTX–FK+ALN (●), the HPMA copolymer (◇), and the HPMA copolymer–PTX–FK–ALN conjugate (■) for 72 h. The scale on the *x*-axis is logarithmic. b) Inhibition of the migration of HUVECs towards the chemoattractant VEGF by the HPMA copolymer–PTX–FK–ALN conjugate. Migration was normalized to percent migration with 100% representing migration to VEGF alone. A quantitative analysis of the number of migrated cells is presented. c,d) Inhibition of the ability of HUVECs to form capillary-like tube structures by the HPMA copolymer–PTX–FK–ALN conjugate. c) Representative images of capillary-like tube structures of HUVECs seeded on matrigel following treatment (scale bars: 100 μm). d) Quantitative analysis of the mean length of the tubes. Data corresponds to the mean ± SD (standard deviation).

is as potent as a combination of free ALN and PTX–FK at equivalent concentrations.

In conclusion, we have demonstrated the stable conjugation of PTX and ALN with an HPMA copolymer. The resulting HPMA copolymer–PTX–FK–ALN conjugate is an effective cytotoxic and antiangiogenic agent and may be used as a bone-delivery system. If successful in further in vivo assays, this novel approach of targeting tumor endothelial cells with a polymer-based drug-delivery system for PTX and ALN could be a realistic strategy for the treatment of prostate- and breast-cancer bone metastases and osteosarcomas. The treatment of cancer with nontoxic polymer-based antiangiogenic agents could make an incurable disease chronically manageable.

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