Medicinal Chemistry

DOI: 10.1002/anie.200805133

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.

[**] We thank Paula Ofek for critical appraisal of the manuscript and Simon Benita for helpful discussions. This research was supported by The Israel Science Foundation (grant No. 1300/06), The Cancer Biology Research Center of Tel Aviv University, and a grant from the Igal Alon Foundation (R.S.F.). We are grateful to the European Association for Cancer Research for a travel fellowship and the Marian Gertner Institute for Medical Nanosystems for a PhD fellowship (K.M.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805133.

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.

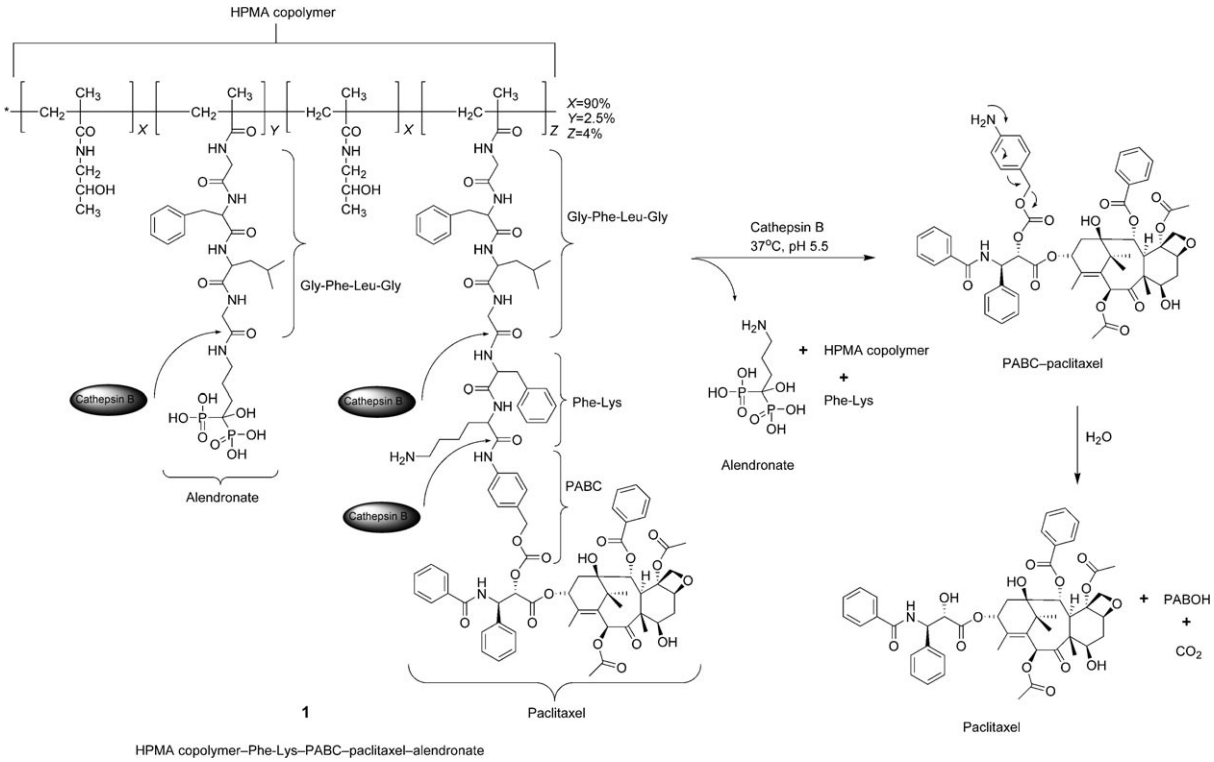
The hydrodynamic diameter and size distribution of our polydispersed nanoscale HPMA copolymer–PTX–FK–ALN conjugate were then characterized by laser light scattering microscopy with nanoparticle tracking analysis (NTA) technology (NanoSight LM20, Salisbury, UK). The mean hydrodynamic diameter of the conjugate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

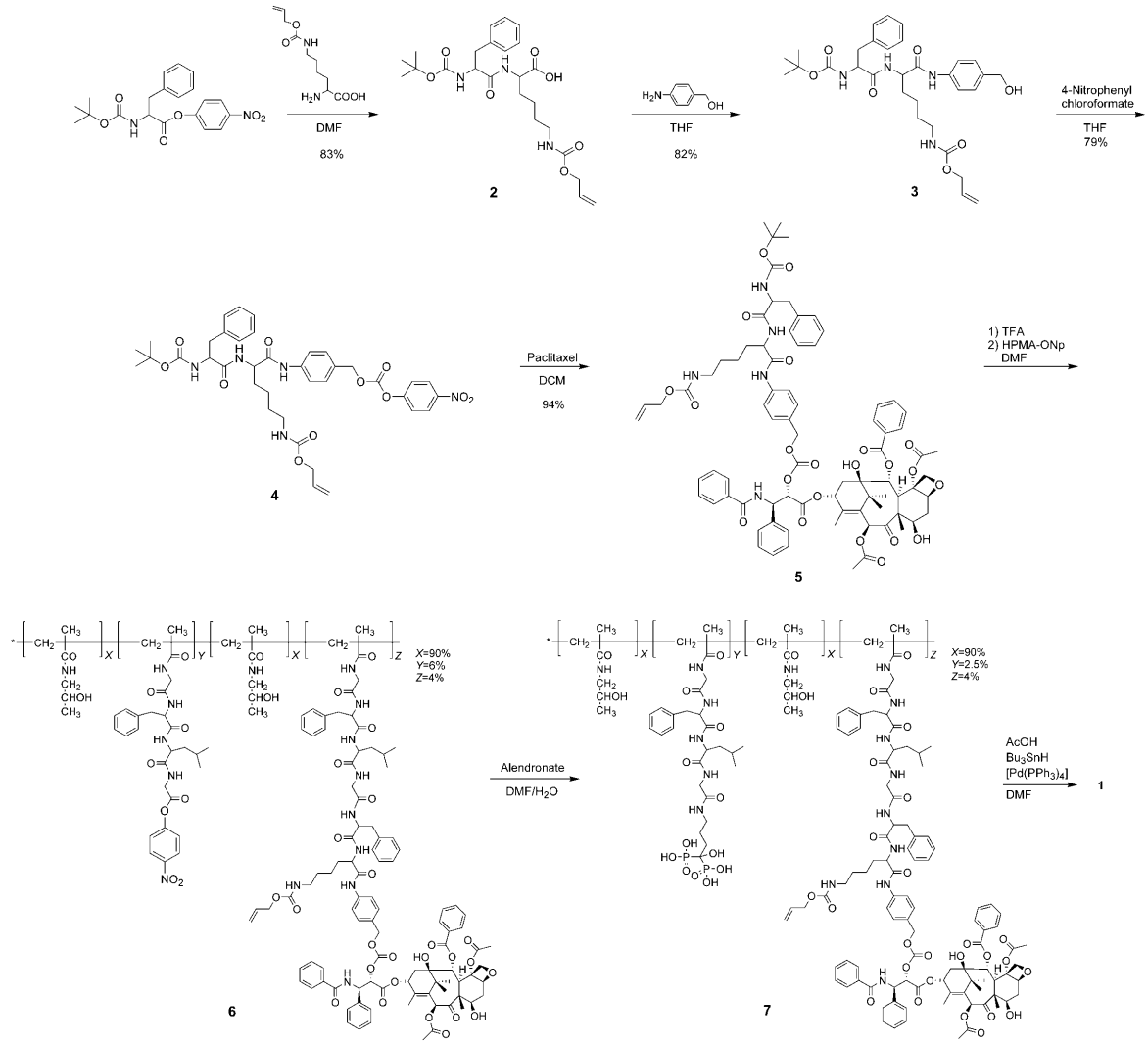
The ALN content in the HPMA copolymer–PTX–FK–ALN conjugate was determined spectrophotometrically on the basis of the chromophoric complex formed between ALN and Fe^{3+} ions in perchloric acid, and against a calibration graph for ALN. Of the functional GFLG–ONp chains (10 mol%), 2.5 mol% were bound to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

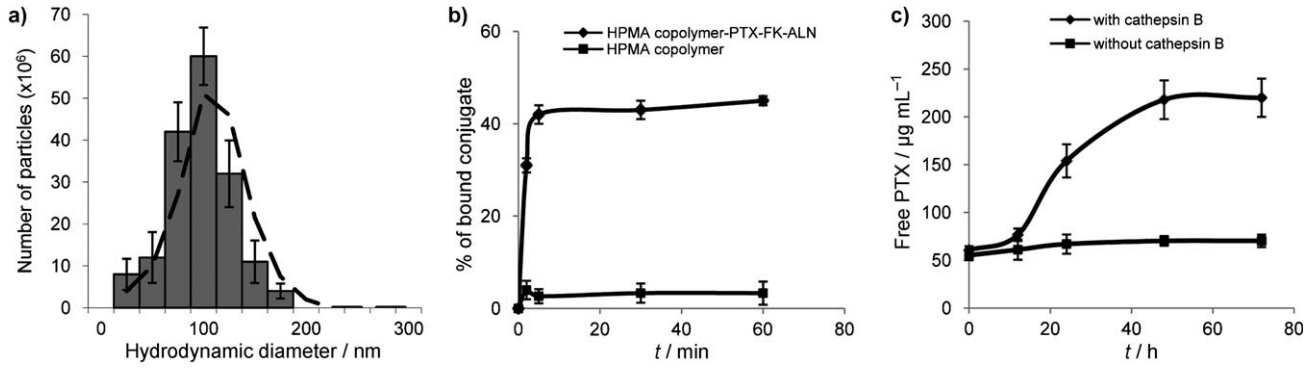
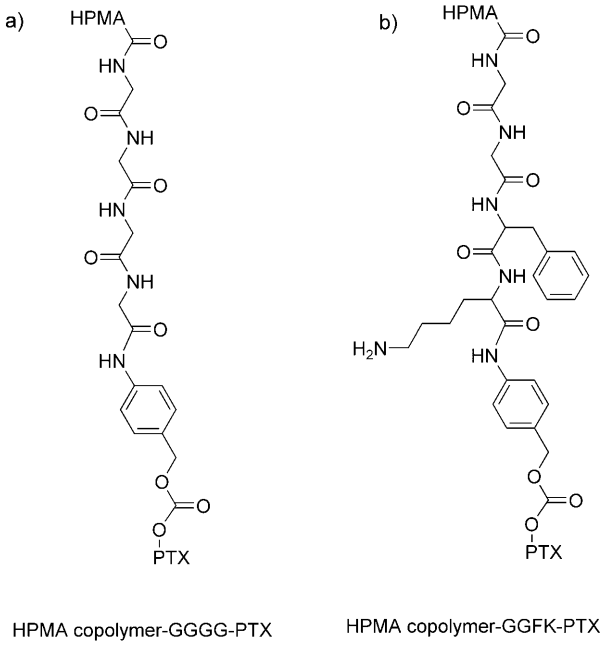


Figure 1. In vitro characterization of the HPMA copolymer–PTX–FK–ALN conjugate. a) Mean hydrodynamic diameter and size distribution of the HPMA copolymer–PTX–FK–ALN conjugate. b) Binding kinetics for the binding of the HPMA copolymer–PTX–FK–ALN conjugate to the bone mineral HA. c) Cleavage of the HPMA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

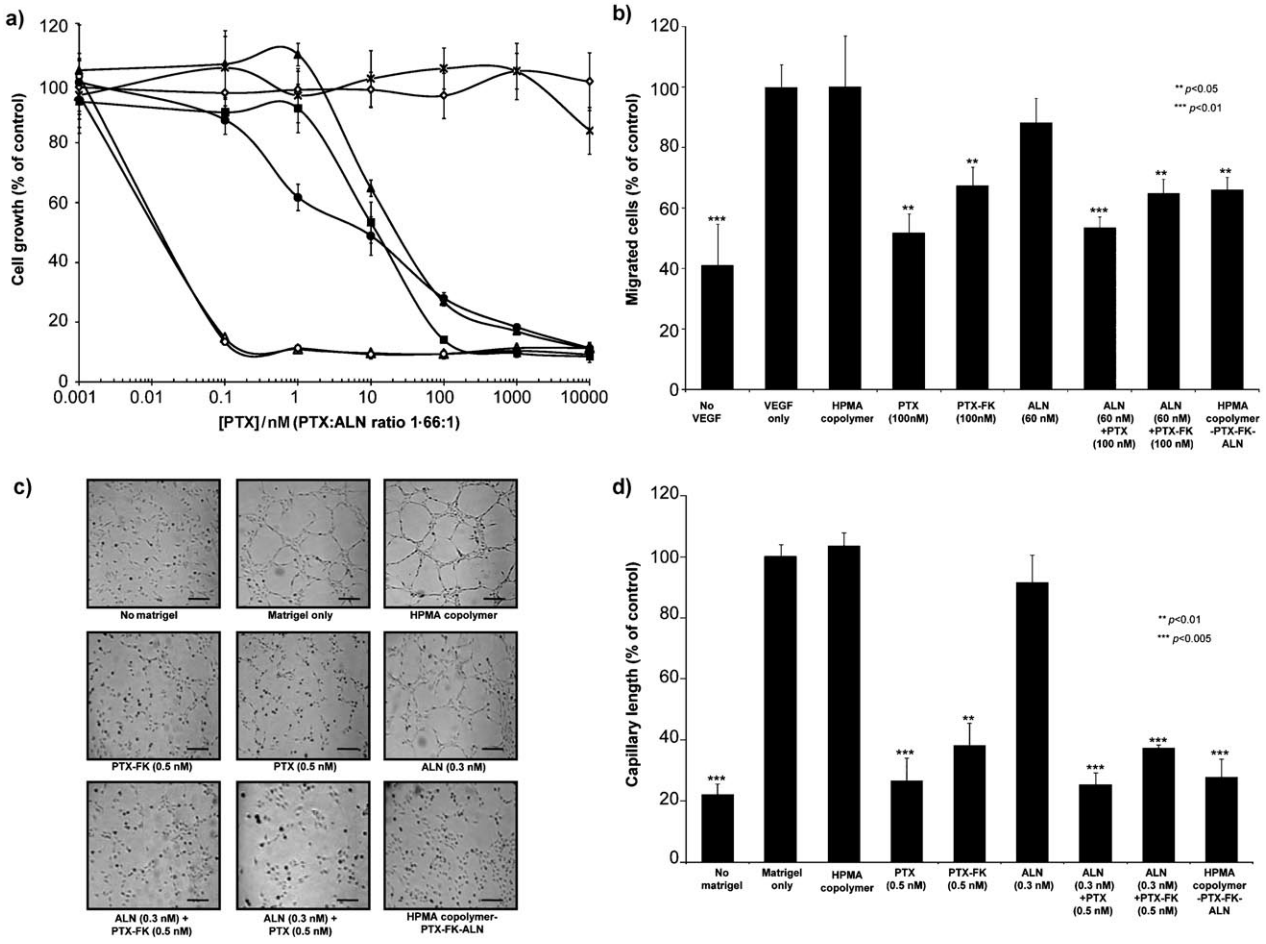


Figure 2. Inhibition of steps in the angiogenic cascade by the HPMA copolymer–PTX–FK–ALN conjugate. a) Inhibition of the proliferation of HUVECs by the HPMA copolymer–PTX–FK–ALN conjugate. HUVECs were incubated with PTX (○), ALN (×), PTX–FK (▲), ALN+PTX (△), PTX–FK+ALN (●), the HPMA copolymer (◇), and 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.

Received: October 20, 2008
Revised: January 4, 2009
Published online: March 17, 2009

Keywords: angiogenesis · antitumor agents · bone metastases · nanoconjugates · polymers

[1] G. Sonpavde, T. E. Hutson, W. R. Berry, *Cancer Treat. Rev.* **2006**, *32*, 90.
[2] M. Caraglia, D. Santini, M. Marra, B. Vincenzi, G. Tonini, A. Budillon, *Endocr.-Relat. Cancer* **2006**, *13*, 7.
[3] D. Hanahan, J. Folkman, *Cell* **1996**, *86*, 353.
[4] L. Chesler, D. D. Goldenberg, I. T. Seales, R. Satchi-Fainaro, M. Grimmer, R. Collins, C. Struett, K. N. Nguyen, G. Kim, T. Tihan, Y. Bao, R. A. Brekken, G. Bergers, J. Folkman, W. A. Weiss, *Cancer Res.* **2007**, *67*, 9435.
[5] J. Folkman, *N. Engl. J. Med.* **1971**, *285*, 1182.
[6] T. Browder, C. E. Butterfield, B. M. Kraling, B. Shi, B. Marshall, M. S. O'Reilly, J. Folkman, *Cancer Res.* **2000**, *60*, 1878.
[7] G. Klement, S. Baruchel, J. Rak, S. Man, K. Clark, D. J. Hicklin, P. Bohlen, R. S. Kerbel, *J. Clin. Invest.* **2000**, *105*, R15.
[8] R. Munoz, Y. Shaked, F. Bertolini, U. Emmenegger, S. Man, R. S. Kerbel, *Breast* **2005**, *14*, 466.
[9] R. S. Herbst, F. R. Khuri, *Cancer Treat. Rev.* **2003**, *29*, 407.

[10] J. P. Cetnar, S. B. Malkowicz, S. C. Palmer, A. J. Wein, D. J. Vaughn, *Urology* **2008**, *71*, 942.
[11] K. N. Bhalla, *Oncogene* **2003**, *22*, 9075.
[12] H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom, *Eur. J. Cancer* **2001**, *37*, 1590.
[13] J. Wang, P. Lou, R. Lesniewski, J. Henkin, *Anticancer Drugs* **2003**, *14*, 13.
[14] E. Pasquier, M. Carre, B. Pourroy, L. Camoin, O. Rebai, C. Briand, D. Braguer, *Mol. Cancer Ther.* **2004**, *3*, 1301.
[15] D. Wang, S. Miller, M. Sima, P. Kopeckova, J. Kopecek, *Bioconjugate Chem.* **2003**, *14*, 853.
[16] H. Uludag, *Curr. Pharm. Des.* **2002**, *8*, 1929.
[17] A. A. El-Mabhouh, C. A. Angelov, R. Cavell, J. R. Mercer, *Nucl. Med. Biol.* **2006**, *33*, 715.
[18] R. Satchi-Fainaro, M. Puder, J. W. Davies, H. T. Tran, D. A. Sampson, A. K. Greene, G. Corfas, J. Folkman, *Nat. Med.* **2004**, *10*, 255.
[19] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, *J. Controlled Release* **2000**, *65*, 271.
[20] R. Duncan, *Nat. Rev. Cancer* **2006**, *6*, 688.
[21] R. Duncan, *Biochem. Soc. Trans.* **2007**, *35*, 56.
[22] R. Satchi-Fainaro, *J. Drug Targeting* **2002**, *10*, 529.
[23] K. Miller, R. Satchi-Fainaro in *Polymer Therapeutics: From Novel Concepts to Clinical Applications: Wiley Encyclopedia of Chemical Biology* (Ed.: T. P. Begley), Wiley, Hoboken, **2009** (in press).
[24] J. W. Singer, *J. Controlled Release* **2005**, *109*, 120.
[25] J. W. Singer, B. Baker, P. De Vries, A. Kumar, S. Shaffer, E. Vawter, M. Bolton, P. Garzone, *Adv. Exp. Med. Biol.* **2003**, *519*, 81.
[26] N. Desai, V. Trieu, Z. Yao, L. Louie, S. Ci, A. Yang, C. Tao, T. De, B. Beals, D. Dykes, P. Noker, R. Yao, E. Labao, M. Hawkins, P. Soon-Shiong, *Clin. Cancer Res.* **2006**, *12*, 1317.
[27] J. M. Meerum Terwogt, W. W. ten Bokkel Huinink, J. H. Schellens, M. Schot, I. A. Mandjes, M. G. Zurlo, M. Rocchetti, H. Rosing, F. J. Koopman, J. H. Beijnen, *Anticancer Drugs* **2001**, *12*, 315.
[28] S. Q. Gao, Z. R. Lu, B. Petri, P. Kopeckova, J. Kopecek, *J. Controlled Release* **2006**, *110*, 323.
[29] G. M. Dubowchik, K. Mosure, J. O. Knipe, R. A. Firestone, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3347.
[30] E. Skrzydlewska, M. Sulkowska, M. Koda, S. Sulkowski, *World J. Gastroenterol.* **2005**, *11*, 1251.
[31] J. A. Foekens, J. Kos, H. A. Peters, M. Krasovec, M. P. Look, N. Cimerman, M. E. Meijer-van Gelder, S. C. Henzen-Logmans, W. L. van Putten, J. G. Klijn, *J. Clin. Oncol.* **1998**, *16*, 1013.
[32] T. T. Lah, E. Kalman, D. Najjar, E. Gorodetsky, P. Brennan, R. Somers, I. Daskal, *Hum. Pathol.* **2000**, *31*, 149.
[33] T. Strojnik, J. Kos, B. Zidanik, R. Golouh, T. Lah, *Clin. Cancer Res.* **1999**, *5*, 559.
[34] T. Strojnik, I. Zajc, A. Bervar, B. Zidanik, R. Golouh, J. Kos, V. Dolenc, T. Lah, *Pflugers Arch.* **2000**, *439*, R122.
[35] J. Decock, N. Obermajer, S. Vozelj, W. Hendrickx, R. Paridaens, J. Kos, *Int. J. Biol. Markers* **2008**, *23*, 161.
[36] A. A. Sinha, M. J. Wilson, D. F. Gleason, P. K. Reddy, M. Sameni, B. F. Sloane, *Prostate* **1995**, *26*, 171.
[37] N. J. Sanfilippo, S. S. Taneja, A. Chachoua, H. Lepor, S. C. Formenti, *J. Clin. Oncol.* **2008**, *26*, 2973.
[38] J. W. Hopewell, R. Duncan, D. Wilding, K. Chakrabarti, *Hum. Exp. Toxicol.* **2001**, *20*, 461.
[39] H. Pan, M. Sima, P. Kopeckova, K. Wu, S. Gao, J. Liu, D. Wang, S. C. Miller, J. Kopecek, *Mol. Pharm.* **2008**, *5*, 548.
[40] D. Wang, M. Sima, R. L. Mosley, J. P. Davda, N. Tietze, S. C. Miller, P. R. Gwilt, P. Kopeckova, J. Kopecek, *Mol. Pharm.* **2006**, *3*, 717.
[41] M. Hruby, T. Etrych, J. Kucka, M. Forsterova, K. Ulbrich, *J. Appl. Polym. Sci.* **2006**, *101*, 3192.
[42] M. J. Vicent, F. Greco, R. I. Nicholson, A. Paul, P. C. Griffiths, R. Duncan, *Angew. Chem.* **2005**, *117*, 4129; *Angew. Chem. Int. Ed.* **2005**, *44*, 4061.
[43] R. Duncan, H. Ringsdorf, R. Satchi-Fainaro, *J. Drug Targeting* **2006**, *14*, 337.
[44] R. Haag, F. Kratz, *Angew. Chem.* **2006**, *118*, 1218; *Angew. Chem. Int. Ed.* **2006**, *45*, 1198.
[45] R. Satchi-Fainaro, R. Mamluk, L. Wang, S. M. Short, J. A. Nagy, D. Feng, A. M. Dvorak, H. F. Dvorak, M. Puder, D. Mukhopadhyay, J. Folkman, *Cancer Cell* **2005**, *7*, 251.
[46] R. Satchi-Fainaro, R. Duncan, C. M. Barnes in *Polymer Therapeutics II: Polymers as Drugs, Conjugates and Gene Delivery Systems, Vol. 193* (Eds.: R. Satchi-Fainaro, R. Duncan), Springer, Berlin, **2006**, p. 1.
[47] C. Li, R. A. Newman, Q. P. Wu, S. Ke, W. Chen, T. Hutto, Z. Kan, M. D. Brannan, C. Charnsangavej, S. Wallace, *Cancer Chemother. Pharmacol.* **2000**, *46*, 416.

Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Targeting Bone Metastases with a Novel Bi-Specific Anticancer and Anti-Angiogenic Polymer-Alendronate-Taxane Conjugate

Keren Miller[1¥], Rotem Erez[2¥], Ehud Segal[1], Doron Shabat[2] and Ronit Satchi-Fainaro[1*]

[¥] Authors contributed equally to this work.

Supporting Information

1. Abbreviations: **AcOH**- Acetic acid, **Alloc**- Allyl alcohol, **ALN**- Alendronate, **AUC**- Area under the curve, **Boc**- Butyl-O-carbonyl, **Bu_3SnH**- Tributyltinhydride, **DCM**- Dichloromethane, **DIPEA**- Diisopropylethylamine, **DMAP**- Dimethylaminopyridine, **DMF**- Dimethylformamide, **DMSO**- Dimethyl sulfoxide, **EPR effect**- Enhanced permeability and retention effect, **Et_3N**- Triethylamine, **EtOAc**- Ethylacetate, **Gly**- Glycine, **HA**- Hydroxyapatite, **Hex**- Hexane, **HPMA**- N-(2-Hydroxypropyl)methacrylamide, **HUVEC**- Human umbilical vein endothelial cells, **Leu**- Leucine, **Lys**- Lysine, **MeOH**- Methanol, **MTD**- Maximum tolerated dose, **NaOH**- Sodium hydroxide, **NH_4Cl**- Ammonium chloride, **NMM**- N-Methylmorpholine, **ONp**- O-Nitrophenyl, **PABC**- *p*-Aminobenzyl carbonate, **PABOH**- *p*-Aminobenzyl alcohol, **PBS**- Phosphate buffered saline, **Phe**- Phenylalanine, **PNP**- *p*-Nitrophenyl, **PTX**- Paclitaxel, **TFA**- Trifluoroacetic acid, **THF**- Tetrahydrofuran, **TLC**- Thin layer chromatography**.**

2. Materials

All reactions requiring anhydrous conditions were performed under an Ar or N_2 atmosphere. HPMA copolymer-Gly-Gly-*p*-nitrophenol (ONp) incorporating 5 mol% of the methacryloyl-Gly-Gly-*p*-nitrophenol ester monomer units, and HPMA copolymer-Gly-Phe-Leu-Gly-ONp incorporating 10 mol% of the methacryloyl-Gly-Phe-Leu-Gly-*p*-nitrophenol ester monomer units were obtained from Polymer Laboratories (Church Stretton, UK). The HPMA copolymer-GFLG-ONp has a molecular weight of 31,600 Da and a polydispersity of 1.66. Chemicals and solvents were either A.R. grade or purified by standard techniques. Thin layer chromatography (TLC): silica gel plates Merck 60 F_{254}; compounds were visualized by irradiation with UV light and/or by treatment with a solution of phosphomolybdic acid (20% wt. in ethanol), followed by heating. Flash chromatography (FC): silica gel Merck 60 (particle size 0.040-0.063 mm), eluent given in parentheses. ^{1}H NMR: Bruker AMX 200 or 400 instrument. The chemical shifts are expressed in δ relative to TMS (δ=0 ppm) and the coupling constants *J* in Hz. The spectra were recorded in $CDCl_3$, as a solvent at room temp. 400 Mesh copper grid SPI Supplies, West Chester, PA. PTX and ALN were purchased from Petrus Chemicals and Materials Ltd. Bovibe spleen cathepsin B, Hydroxyapatite (HA), cathepsin B inhibitor (CA-074 methyl ester) and all chemical reagents, including salts and solvents, were purchased from Sigma-Aldrich, Israel.

3. Methods

3.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza, Switzerland. Cells were cultured in EGM-2 medium (Lonza, Switzerland) and were grown at 37°C; 5% CO_2. The human prostate cell line PC3 was purchased from the American Type Culture Collection. PC3 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-glutamin. Cells were grown at 37°C; 5% CO_2.

3.2 Synthesis of HPMA copolymer-PTX-FK-ALN conjugate

The synthesis of conjugate **1** is illustrated in Scheme 2. Previously synthesized L-Boc-Phe-ONp [48], was reacted with L-Lys(alloc)-OH to give dipeptide **2**. The latter was conjugated with 4-aminobenzyl alcohol to generate alcohol **3**. Activation of alcohol **3** with 4-nitrophenyl chloroformate afforded carbonate **4**, which was reacted with PTX to yield compound **5**.

Deprotection of Boc-Phe-Lys(alloc)-PABC-PTX **5** with TFA, followed by conjugation with HPMA copolymer-Gly-Phe-Leu-Gly-ONp gave compound **6**. Both Gly-Phe-Leu-Gly and Phe-Lys cathepsin B-cleavable peptides were used in order to provide convenient conjugation chemistry, longer spacer and higher probability of cleavage. Amidation of ALN with HPMA copolymer was performed according to Wang *et al.* [15]. Briefly, the remaining activated sites of the copolymer were conjugated with excess of ALN generating compound **7**. Deprotection of the alloc residue of **7** afforded the desired conjugate **1** (Scheme 1). Similarly, a non-cleavable HPMA copolymer-GG-PTX-GG conjugate, and the cleavable HPMA copolymer-GG-PTX-FK conjugate were synthesized using HPMA copolymer Gly-Gly-ONp, conjugated to PTX-Gly-Gly, and PTX-Phe-Lys, respectively. (Figure 2a, b).

3.3 Enzymatic release of PTX from HPMA-PTX-FK-ALN by cathepsin B and PTX content determination

PTX and ALN were released from HPMA-PTX-FK-ALN conjugate by bovine spleen cathepsin B at conditions mimicking lysosomal intracellular drug release. HPMA copolymer-PTX-FK-ALN conjugate (7 mg/mL, 0.5 mM PTX-equivalent) was incubated at 37ºC in phosphate buffer (0.1 M sodium phosphate, 0.05 M NaCl, 1 mM EDTA, pH 6), reduced glutathione (5 mM), and cathepsin B (0.5 µM). Aliquots of 300 µL were taken after 12, 24 and 48 hr. Free PTX was extracted at 0.2 M Na_2CO_3/$NaHCO_3$, pH 9.8 by 100% EtOAc. Free PTX concentration was analyzed by high pressure liquid chromatography (HPLC, AKTA™ Purifier®, Amersham Biosciences, µBondapak™ C18 3.9 x 150 mm column, Waters, acetonitrile-water gradient of 30-100% acetonitrile, at 1 mL/min, λ=245 nm) against calibration curve of PTX which was extracted at the same conditions.

3.4 Hydroxyapatite binding assay

HPMA copolymer-PTX-FK-ALN conjugate was dissolved in phosphate buffered saline (PBSX1), pH 7.4 (1 mg/mL). The conjugate solution (500 µL) was incubated with HA powder (15mg), in 500 µL PBS, pH 7.4. HPMA copolymer-Gly-Phe-Leu-Gly was used as control. Incubated samples were centrifuged at 6000 RPM for 3 min and a sample from the upper layer (100 µL) was collected at selected time points. Fast protein liquid chromatography (FPLC, AKTA™ Purifier®, Amersham Biosciences) analysis using HiTrap™ desalting column (Amersham®) was used for detection of unbound conjugate in the samples (FPLC conditions: AKTA™ Purifier®, mobile phase 100% DDW, 2 mL/min, λ=215 nm). HA-binding kinetic analysis of the conjugate 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 percent AUC of conjugate sample in the absence of HA.

3.5 Determination of ALN content

HPMA copolymer-PTX-FK-ALN conjugate (3.4, 1.7 and 0.85 mg) was dissolved in a mixture of 0.8 mL of 0.2 M perchloric acid ($HClO_4$) and 0.1 mL of 4 mM $FeCl_3$. The content of ALN in HPMA copolymer-PTX-FK-ALN conjugate was determined against a calibration graph of serial dilutions of 0-3 mM ALN. Samples absorbance was measured spectrophotometrically at λ=300 nm.

3.6 Quantitative evaluation of HPMA copolymer-PTX-FK-ALN conjugate hydrodynamic diameter and size distribution

The mean hydrodynamic diameter of the conjugate was evaluated using a real time particle analyzer (NanoSight LM20™) containing a solid-state, single mode laser diode (< 20 mW, 655 nm) configured to launch a finely focused beam through a 500 µL sample chamber. HPMA copolymer-PTX-FK-ALN conjugate was dissolved in PBS to final concentrations of 2, 1, 0.5 mg/mL. The samples were then injected into the chamber by syringe and allowed to equilibrate to unit temperature (23^0C) for 30 sec. The particles dynamics were visualized at 30 frames per second (fps) for 60 sec at 640 x 480 resolution by the device CCD camera. The paths the particles take under Brownian motion over time were analyzed using Nanoparticle Tracking Analysis (NTA) software. The diffusion coefficient and hence sphere equivalent hydrodynamic radius of each particle was separately determined and the particle size distribution profiles were generated. Each sample was measured three times in triplicates, and the results represent the mean diameter.

3.7 Cell proliferation assay

HUVEC were plated onto 24-well plate (1 x 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). PC3 cells were plated onto 96 well plate (2 x 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 medium was replaced with DMEM containing 10% FBS. Cells were challenged with the combination of PTX and ALN, combination of PTX-FK and ALN, each dug alone, and with HPMA copolymer-PTX-FK-ALN conjugate at serial concentrations for 72 hr. After incubation HUVEC and PC3 were counted by Coulter Counter or by XTT respectively.

3.8 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 x 10^4) were challenged with the combination of PTX and ALN, combination of PTX-FK and ALN, each dug alone, and with HPMA copolymer-PTX-FK-ALN conjugate, 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.

3.9 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 x 10^4 cells/100 µL) were challenged with the combination of PTX and ALN, combination of PTX-FK and ALN, each dug alone, and with HPMA copolymer-PTX-FK-ALN conjugate and were added to the

upper chamber of the transwells for 2 h incubation prior to migration to 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 10X 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.

3.10 Statistical methods

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

4. Synthesis of HPMA copolymer-PTX-FK-ALN conjugate

Compound 2

L-Boc-Phe-ONp (104.3 mg, 0.27 mmol) was dissolved in 2 mL DMF. Then commercially available L-Lys(alloc)-OH (62 mg, 0.27 mmol) and Et_3N (100 μL) were added. The reaction mixture was stirred for 12 h and was monitored by 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 compound **2** (107 mg, 83%) as a white solid (Scheme 2).

^{1}H NMR (200MHz, $CDCl_3$): δ = 7.24-7.11 (5H, m); 5.83 (1H, m); 5.45-5.11 (3H, m); 4.50-4.48 (3H, m); 3.08-2.92 (4H, m); 1.84-1.64 (2H, m); 1.44-1.36 (4H, m); 1.30 (9H, s). ^{13}C NMR (100MHz, $CDCl_3$): δ = 177.09, 164.91, 158.52, 157.61, 138.45, 134.82, 131.26, 130.50, 128.83, 120.24, 82.27, 67.51, 57.59, 53.98, 42.37, 38.60, 33.53, 33.44, 30.14, 22.63. MS (FAB): *m/z*: 478.3 $[M+H]^+$, 500.3 $[M+Na]^+$.

Compound 3

Compound **2** (832.1 mg, 1.74 mmol) was dissolved in dry THF and the solution was cooled to -15° C. Then NMM (0.19 mL, 1.74 mmol) and isobutyl chloroformate (0.27 mL, 2.09 mmol) were added. The reaction was stirred for 20 minutes and a solution of 4-aminobenzyl alcohol (321.85 mg, 2.61 mmol) in dry THF was added. The reaction mixture was stirred for 2 h and was monitored by TLC (EtOAc 100%). 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 100%) to give compound **3** (835 mg, 82%) as a yellow solid (Scheme 2).

^{1}H NMR (200MHz, MeOD): δ = 7.56 (2H, d, J=8 Hz); 7.29 (2H, d, J=8 Hz); 7.21-7.07 (5H, m); 5.86 (1H, m); 5.29-5.10 (2H, m); 4.83 (2H, s); 4.49-4.46 (4H, m); 3.17-3.08 (4H, m); 1.88-1.70 (2H, m); 1.44 (4H, m); 1.34 (9H, s). ^{13}C NMR (100MHz, $CDCl_3$): δ = 174.08, 171.45, 158.61, 157.79, 139.17, 138.87, 138.03, 134.79, 131.12, 130.71, 130.49, 129.90, 129.08, 122.15, 82.71, 67.48, 66.83, 58.08, 55.74, 42.09, 39.84, 32.70, 31.31, 30.16, 24.31. MS (FAB): *m/z*: 583.3 $[M+H]^+$, 605.3 $[M+Na]^+$.

Compound 4

Compound **3** (353.6 mg, 0.60 mmol) was dissolved in dry THF and the solution was cooled to 0° C. Then DIPEA (0.42 mL, 2.42 mmol), PNP- chloroformate (367 mg, 1.82 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 compound **4** (453.2 mg, 79%) as a white solid (Scheme 2).

^{1}H NMR (200MHz, $CDCl_3$): δ = 8.26 (2H, d, J=8 Hz); 7.64 (2H, d, J=8 Hz); 7.40-7.34 (4H, m); 7.22-7.14 (5H, m); 5.83 (1H, m); 5.24 (2H, s); 5.18-5.06 (2H, m); 4.56-4.37 (4H, m); 3.19-3.05 (4H, m); 1.95-1.73 (2H, m); 1.59-1.46 (4H, m); 1.39 (9H, s). ^{13}C NMR (100MHz, $CDCl_3$): δ = 174.11, 171.51, 158.63, 157.60, 157.46, 154.36, 147.32, 140.70, 137.91, 134.76, 131.75, 131.53, 131.08, 130.78, 129.17, 127.21, 123.72, 122.05, 119.61, 82.86, 72.65, 67.50, 58.17, 55.84, 41.95, 39.71, 32.42, 31.40, 30.16, 24.27. MS (FAB): *m/z*: 770.4 $[M+Na]^+$.

Compound 5

Compound **4** (360.3 mg, 0.48 mmol) was dissolved in dry DCM. Then PTX (494.06 mg, 0.57 mmol) and DMAP (70.61 mg, 0.57 mmol) were added. The reaction mixture was stirred for 8 h and monitored by TLC (EtOAc 100%). 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 100%) to give compound **5** (662 mg, 94%) as a white solid (Scheme 2).

MS (FAB): *m/z*: 1463.7 [M], 1486.9 $[M+Na]^+$.

Compound 6

Compound **5** (82.1 mg, 56.1 µmol) was dissolved in 1.5 mL TFA and stirred for 2 minutes at 0° C. The excess of acid was removed under reduced pressure and the crude amine salt was dissolved in 2 mL DMF. HPMA copolymer-Gly-Phe-Leu-Gly-ONp (198 mg, ONp = 66.0 µmol) was added followed by the addition of Et_3N (100 µL). The reaction mixture was stirred for 12 h and the solvent was removed under reduced pressure. The crude product was used for the next step without further purification.

Compound 7

ALN (100 mg, 30.8 µmol) was dissolved in water (1 mL). While stirring the sample, a solution of crude product **6** (80 mg, ONp = max. 26.6 µmol) in DMF (350 µL) was added dropwise into the aqueous solution. NaOH (0.2 M) was then dropped into the solution. Slowly, the pH value was increased to 7. Then, in 1 h, it was further increased to pH 8. Afterward, the pH value was quickly raised to 9 to finish the reaction. Free amine **6,** ONp and ALN were removed by FPLC using XK26/70 column with Sephadex LH20 column (MeOH 100%, 1 mL/1 min) to give compound **7** as a white solid (47 mg) (Scheme 2).

Compound 1

Compound **7** (47 mg, alloc = max. 15.6 µmol) was dissolved in DMF (1.5 mL). Then acetic acid (4.46 µL, 78 µmol), Bu_3SnH (25.17 µL, 93.6 µmol) and a catalytic amount of Pd $(PPh_3)_4$ were added. The reaction mixture was stirred for 2 h 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 HPLC using XK26/70 column with Sephadex LH20 (MeOH 100%, 1 mL/1 min) to give compound **1** (32 mg) as a white solid (Scheme 1).

5. Cathepsin B inhibitor reduces PTX-FK and HPMA copolymer-PTX-FK-ALN conjugate cytotoxicity.

In order to further prove that HPMA copolymer-PTX-FK-ALN conjugate is active mainly upon the release of PTX by cathepsin B cleavage, and not by spontaneous hydrolysis, we performed a proliferation assay on HUVEC in the presence and absence of cathepsin B inhibitor. As shown in Figure 1S, HUVEC were incubated with HPMA copolymer-PTX-FK-ALN conjugate and with PTX-FK+ALN at equivalent concentrations of 500 nM and 300 nM respectively. Following 48 hours HPMA copolymer-PTX-FK-ALN conjugate inhibited the proliferation of HUVEC by ~27% and ~40% in the presence or absence of cathepsin B inhibitor, respectively. Similarly, the proliferation of HUVEC was diminished by ~25% and ~50% by PTX-FK+ALN with or without cathepsin B inhibitor correspondingly.

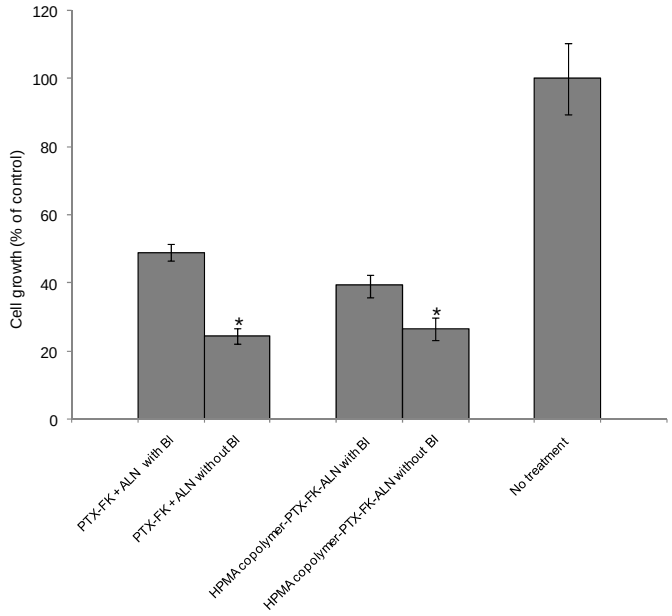


Figure 1S. Cathepsin B inhibitor reduces HPMA copolymer-PTX-FK-ALN conjugate and PTX-FK cytotoxicity. HUVEC were incubated with PTX-FK+ALN or with HPMA copolymer-PTX-FK-ALN conjugate in the presence or absence of cathepsin B inhibitor (BI) for 48 hr. Data represents mean ± SD. *, $P<0.05$.

6. HPMA copolymer-GGGG-PTX is less active than the cathepsin B-cleavable HPMA copolymer-GGFK-PTX conjugate.

To further reinforce the fact that HPMA copolymer-PTX-FK-ALN conjugate is active mainly upon the release of PTX by cathepsin B cleavage mechanism, and not by spontaneous hydrolysis, we synthesized an HPMA copolymer-GGGG-PTX conjugate bearing the non-cleavable Gly-Gly linker (Figure 2Sa), and compared its cytotoxic activity to that of the cleavable HPMA copolymer-GGFK-PTX conjugate (Figure 2Sb). HPMA copolymer-GGGG-PTX conjugate inhibited the proliferation of HUVEC at a 2-logs higher concentration than the cathepsin B-cleavable HPMA copolymer-GGFK-PTX conjugate. Following 48 hours, there was probably some free PTX released hydrolytically from HPMA copolymer-GGGG-PTX which led to the inhibition of proliferation of HUVEC at

concentrations higher than 1000 nM PTX-equivalent concentrations (Figure 2Sc). As can be seen in Figure 1S, the addition of cathepsin B inhibitor to HPMA copolymer-PTX-FK-ALN conjugate and to PTX-FK+ALN reduces the cytotoxic effect of these agents, however, the inhibition of cathepsin B did not fully block the PTX effect. These observations could be explained by the fact that cathepsin B inhibitor has only a partial inhibitory effect. Furthermore, it is well known that other cathepsins such as cathepsin H, D and L also cleave the GFLG linker. These alternative cathepsins can partially compensate for the loss of cathepsin B activity by the inhibitor.

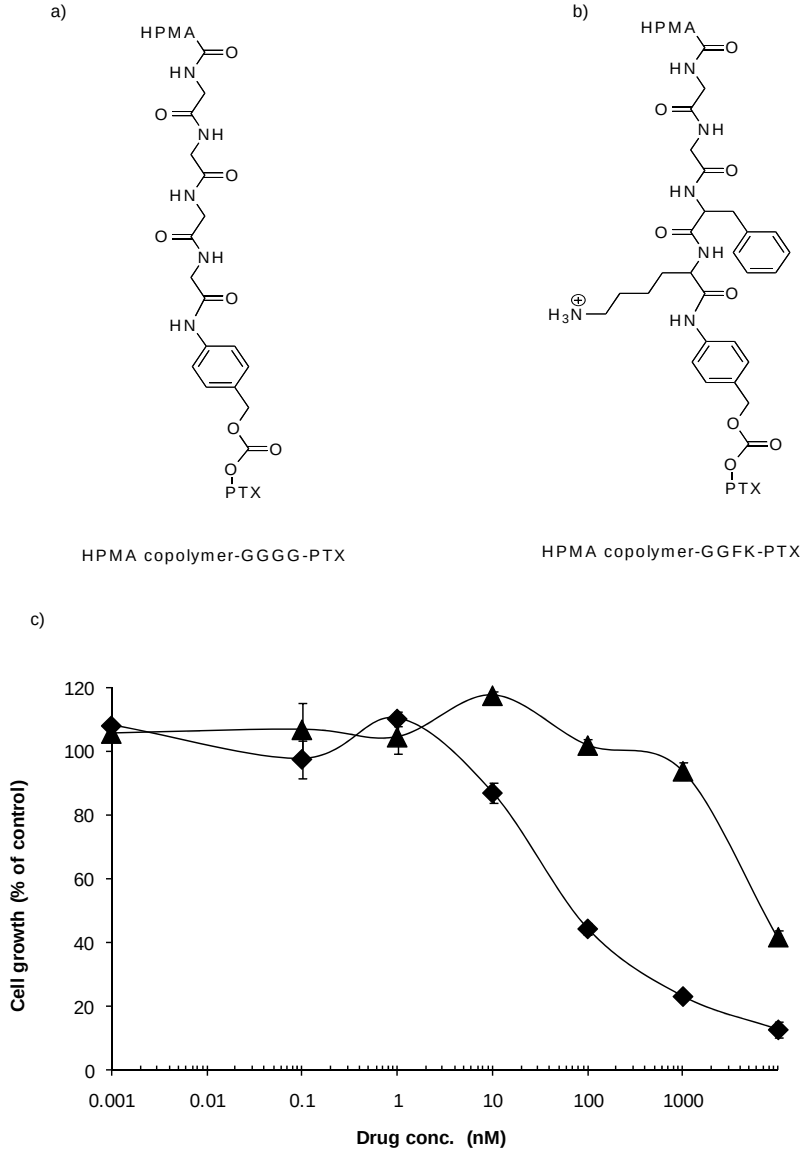


Figure 2S. HPMA copolymer-GGGG-PTX is less active than the cathepsin B-cleavable HPMA copolymer-GGFK-PTX conjugate. a) Chemical structure of the non-cleavable conjugate HPMA copolymer-GGGG-PTX b) Chemical structure of the cleavable conjugate HPMA copolymer-GGFK-PTX. c) Proliferation assay of HUVEC. HUVEC were incubated with HPMA copolymer-GGGG-PTX (close triangle) or with HPMA copolymer-GGFK-PTX (close square) conjugates for 48 hr. HPMA copolymer-GGGG-PTX conjugate inhibited the proliferation of HUVEC at a 2-logs higher concentration than the cathepsin B cleavable HPMA copolymer-GGFK-PTX conjugate. Data represents mean ± SD. X axis in logarithmic scale.

7. HPMA copolymer-PTX-FK-ALN conjugate inhibits the proliferation the human prostate cell line PC3.

There have been several reports indicating PTX as an effective agent that could be used to treat advanced metastatic prostate cancer. We evaluated whether PTX and ALN, when bound to HPMA copolymer, retained their cytotoxic activity on the proliferation of the human prostate PC3 cell line. The proliferation of PC3 cells was similarly inhibited by HPMA copolymer-PTX-FK-ALN conjugate and the combination of PTX-FK plus ALN at equivalent concentrations, exhibiting an IC_{50} of ~10 μM PTX-FK and ~6 μM ALN (Figure 3S). PTX alone exhibited an IC_{50} of 1 μM. ALN, alone or when combined with PTX, or with PTX-FK at the concentrations tested of 0.01 nM - 10 μM had no effect on the proliferation of PC3 cells.

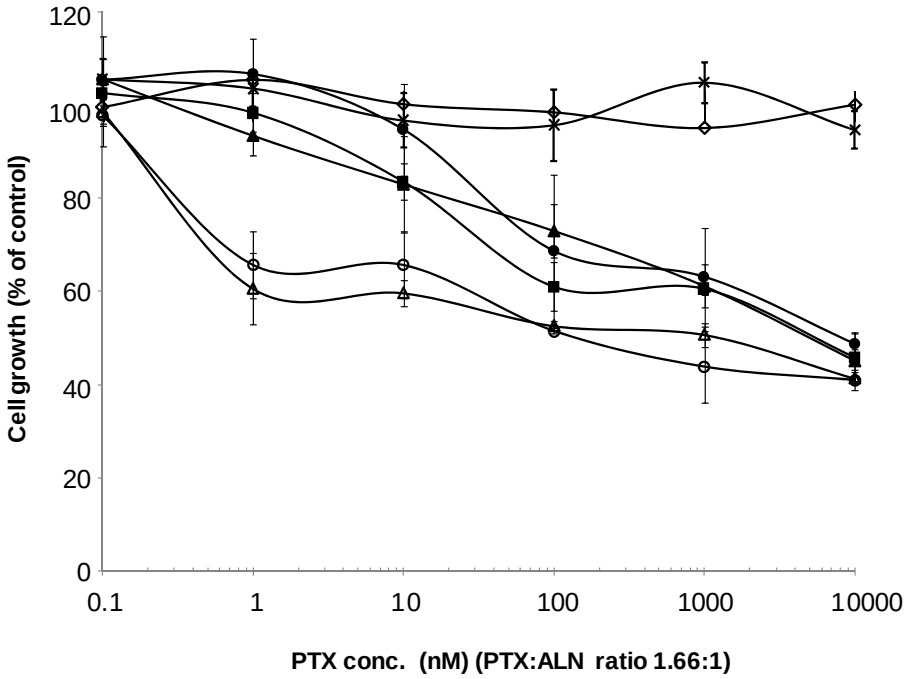


Figure 3S. HPMA copolymer-PTX-FK-ALN conjugate inhibits the proliferation of the prostate cell line PC3. PC3 cells were incubated with PTX (open circle), ALN (cross), PTX-FK (close triangle), ALN+PTX (open triangle), PTX-FK +ALN (close circle), HPMA copolymer (open diamond), and HPMA copolymer-PTX-FK-ALN conjugate (close square) for 72 hr. Data represents mean ± SD. X axis in logarithmic scale.