PDEPT: Polymer-Directed Enzyme Prodrug Therapy. 2. HPMA Copolymer-β-lactamase and HPMA Copolymer-C-Dox as a Model Combination

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Polymer-directed enzyme prodrug therapy (PDEPT) is a novel two-step antitumor approach that uses a combination of a polymeric prodrug and polymer−enzyme conjugate to generate a cytotoxic drug rapidly and selectively at the tumor site. Previously we have shown that *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer-bound cathepsin B can release doxorubicin intratumorally from an HPMA copolymer conjugate PK1. Here we describe for the first time the synthesis and biological characterization of a PDEPT model combination that uses an HPMA-copolymer-methacryloyl-glycine-glycine−cephalosporin−doxorubicin (HPMA-*co*-MA-GG−C-Dox) as the macromolecular prodrug and an HPMA copolymer conjugate containing the nonmammalian enzyme β-lactamase (HPMA-*co*-MA-GG−β-L) as the activating component. HPMA-*co*-MA-GG−C-Dox had a molecular weight of ∼31 600 Da and a C-Dox content of 5.85 wt %. Whereas free β-L has a molecular weight of 45 kDa, the HPMA-*co*-MA-GG−β-L conjugate had a molecular weight in the range of 75−150 kDa, and following purification no free enzyme was detectable. Against the cephalosporin C or HPMA-*co*-MA-GG−C-Dox substrates, the HPMA-*co*-MA-GG−β-L conjugate retained 70% and 80% of its activity, respectively. In vivo ^{125}I-labeled HPMA-*co*-MA-GG−β-L showed prolonged plasma concentration and greater tumor targeting than ^{125}I-labeled β-L due to the enhanced permeability and retention (EPR) effect. Moreover, administration of HPMA-*co*-MA-GG−C-Dox iv to mice bearing sc B16F10 melanoma followed after 5 h by HPMA-*co*-MA-GG−β-L led to release of free Dox. The PDEPT combination caused a significant decrease in tumor growth ($T/C = 132\%$) whereas neither free Dox nor HPMA-*co*-MA-GG−C-Dox alone displayed activity. The PDEPT combination displayed no toxicity at the doses used, so further evaluation of this approach to establish the maximum tolerated dose (MTD) is recommended.

INTRODUCTION

Polymer therapeutics, including polymer−protein conjugates (e.g., PEG-L-asparaginase) and polymer−drug conjugates are emerging as a new class of anticancer agents (reviewed in *1*, *2*). PEGylated proteins display prolonged plasma circulation times and reduced immunogenicity (*3*, *4*), and the *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates (*5*, *6*) and polyglutamate−paclitaxel (*7*) display significant passive tumor targeting (>70-fold) due to the enhanced permeability and retention (EPR) effect (*8*). Once within the tumor interstitium such polymeric conjugates must usually be internalized by endocytosis to allow lysosomotropic delivery and cathepsin B-mediated liberation of active drug. Polymer-directed enzyme prodrug therapy (PDEPT) is a new two-step antitumor approach which uses a combination of a polymeric prodrug and polymer−enzyme conjugate to generate cytotoxic drug rapidly outside the tumor cells, i.e., within the tumor interstitium (*9*). PDEPT offers the advantage that drug liberation can be externally triggered, and thus release of active drug is not reliant on the rate of conjugate internalization or the intracellular level of activating enzyme.

The first PDEPT combination described used HPMA-*co*-MA-GFLG-doxorubicin (PK1), a conjugate currently in Phase II clinical development (*10*), and HPMA-*co*-MA-GG-cathepsin B (*9*) to obtain in vivo proof of concept. Following endocytosis within tumor tissue, PK1 is normally degraded intracellularly by the lysosomal thiol-dependent protease cathepsin B to liberate doxorubicin (Dox) slowly. However, using the PDEPT approach it was clearly shown that subsequent (5 h) administration of HPMA-*co*-MA-GG-cathepsin B led to a dramatic and rapid (within 1 h) additional Dox release (*9*) consistent with interstitial liberation. We now describe the first PDEPT combination that uses a PDEPT combination that uses a nonmammalian enzyme. An HPMA copolymer-methacryloyl-Gly-Gly-cephalosporin−doxorubicin (HPMA-*co*-MA-GG−C-Dox) conjugate was synthesized as a substrate and HPMA copolymer-methacryloyl-Gly-Gly-β-lactamase (HPMA-*co*-MA-GG−β-L) synthesized as the activating enzyme conjugate (Figure 1).

The β-lactamases are small (30−45 kDa), soluble monomeric enzymes that have varying specificity. All are capable of hydrolyzing β-lactams to substituted β-amino acids (*11*). Some act more readily on penicillins, while others have greater activity against cephalosporins, and

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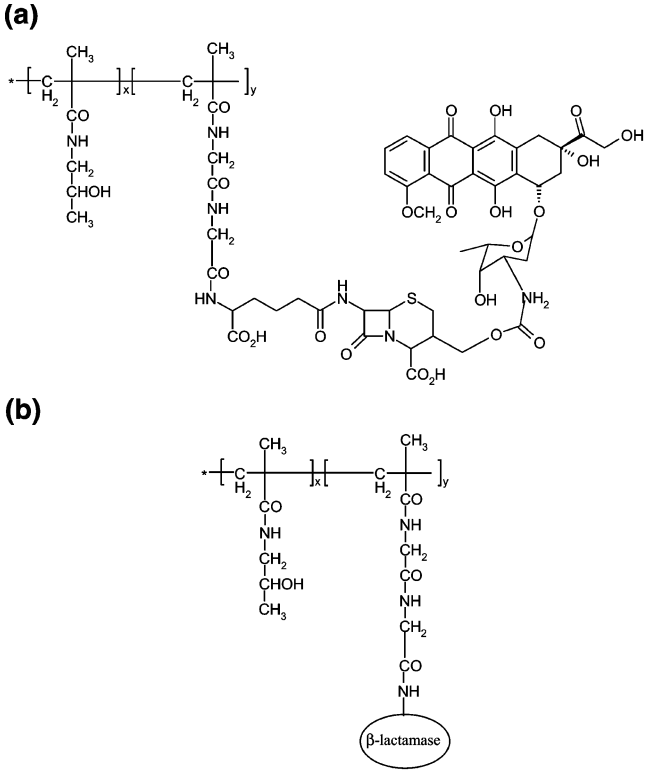


Figure 1. Structure of (a) HPMA-*co*-MA-GG−C-Dox and (b) HPMA-*co*-MA-GG−*β*-L.

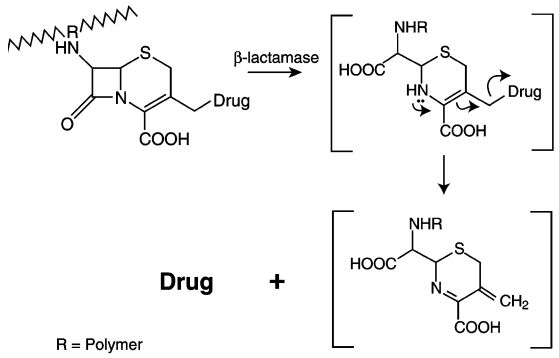


Figure 2. Mechanism of Dox release from HPMA-*co*-MA-GG−C-Dox by *β*-L.

the mechanism of hydrolysis involves expulsion of a 3′ leaving group. *β*-Lactamases are particularly tolerant to a wide variety of substituents at this position. Cleavage of the *β*-lactam ring leads to chemical rearrangement (Figure 2) and the terminal residue, i.e., the anticancer drug, is liberated. A wide variety of cytotoxic agents bearing NH_2 or OH groups can be used to create prodrugs. Cephalosporin derivatives of various mustards (*12*), taxol (*13*), and doxorubicin (*14*, *15*) have already been described as low molecular weight prodrugs for the antibody-directed enzyme prodrug (ADEPT) approach (*16*). As *β*-L is not a mammalian enzyme, it should display minimal interference from mammalian inhibitors, physiological enzyme substrates, and competing endogenous enzyme systems. However, it does have the potential disadvantage of immunogenicity. Use of an HPMA copolymer conjugate is particularly attractive, as conjugation of poly(ethylene glycol) (PEG) and HPMA copolymers is well-known to reduce immunogenicity of bound proteins (*4*, *17*, *18*).

Here we describe the synthesis and characterization of the HPMA-*co*-MA-GG−C-Dox and HPMA-*co*-MA-GG−*β*-L conjugates. The biological properties, including retained enzyme activity against low and high molecular weight substrates and cytotoxicity of the PDEPT combination, were studied in vitro. Finally, the pharmacokinetics of ^{125}I-labeled HPMA-*co*-MA-GG−*β*-L and ^{125}I-labeled *β*-L and the antitumor activity of this PDEPT combination were determined in vivo using a sc B16F10 model.

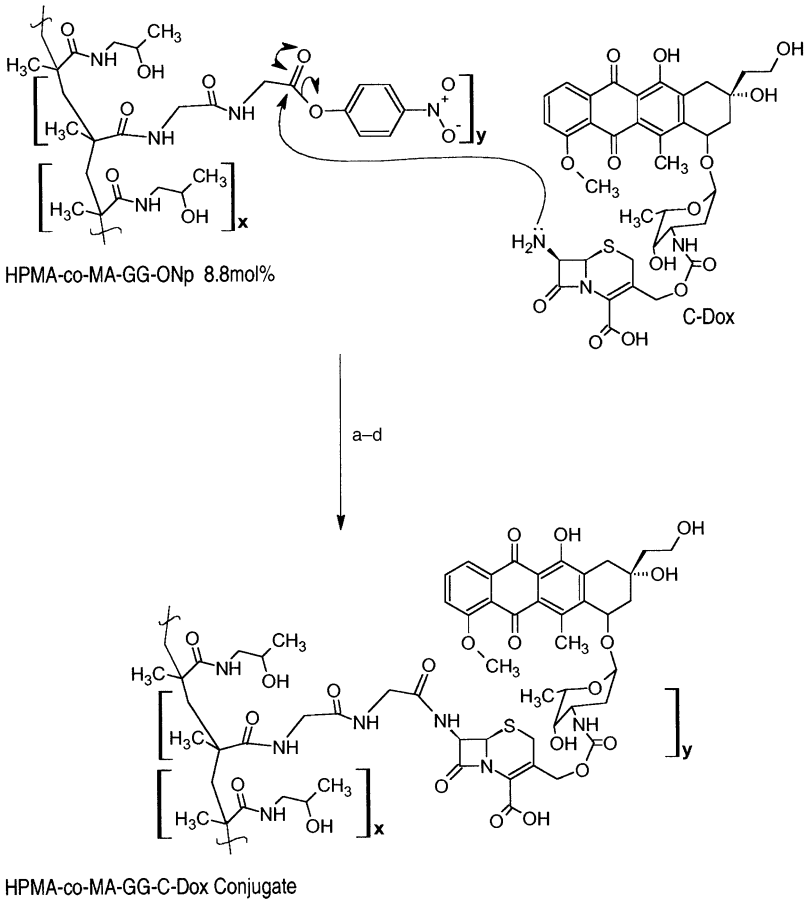
EXPERIMENTAL PROCEDURES

Materials. A random copolymer of HPMA copolymerized with methacryloyl-Gly-Gly-*p*-nitrophenyl ester (HPMA-*co*-MA-GG-ONp) containing 4.4 mol % of the MA-GG-ONp monomer units and with a M_w 34 700 and M_n 21 100 g/mol as a polymeric precursor was used for enzyme conjugation. The HPMA-*co*-MA-GG-ONp precursor used for cephalosporin−doxorubicin (C-Dox) conjugation containing 8.8 mol % of the MA-GG-ONp monomer units and had a M_w of 31 600 and M_n of 19 000 g/mol. Both were from Polymer Laboratories (UK). *β*-L from *Enterobacter cloacae* P99 was purchased from CAMR (UK). Cephalosporin C, ammonium formate, and bovine serum albumin (BSA) were from Sigma. 2-Propanol, methanol, orthophosphoric acid, and chloroform were from J. T. Baker (Israel) (all HPLC grade). Ammonia solution (32%) was from Merck (Germany). A cell proliferation assay kit with XTT reagent was from Biological Industries (Israel). C-Dox was from Ultrafine (UK). Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were from Aldrich (UK). All other chemicals were of analytical grade from Aldrich (UK), Fisher Chemicals (UK), or BDH Ltd. (UK) unless otherwise stated.

M109 murine lung carcinoma cells were kindly donated by Professor A. Gabizon (Hadassah Medical Center, Israel). DA3 murine mammary carcinoma cells were donated by I. Witz (Tel Aviv University, Israel) and B16f10.9 murine melanoma cells were from Y. Keisari (Tel Aviv University, Israel). All cells were maintained in DMEM media supplemented with 10% fetal bovine serum (FBS, Life Technologies, Israel), 2 mM L-glutamine, and penicillin/streptomycin antibiotics at 37 °C, 5% CO_2.

Synthesis of HPMA-*co*-MA-GG−C-Dox. C-Dox (200 mg, 0.25 mmol) was dissolved in dry DMF (7 mL) and added to a stirred solution of the HPMA-*co*-MA-GG-ONp (662 mg) in dry DMF (5 mL) in the dark, under an argon atmosphere and at ambient temperature (25 °C). Dry triethylamine (139 *μ*L, 1 mmol) was then added, and the reaction was followed by TLC and HPLC. After 21 h, HPLC indicated that the quantity of free C-Dox remaining in the reaction mixture had fallen to 43%. TLC at this time also showed a faint trace of Dox aglycone. The reaction was terminated after 22 h by addition of DL-1-amino-2-propanol (40 *μ*L, 0.5 mmol) with stirring for a further 1 h. The reaction mixture was then added dropwise (under argon) to stirred dry diethyl ether (130 mL) which had been precooled to ca. 10 °C. The crude product precipitated as a fine reddish solid, and it was collected by filtration using a Grade 3 sintered-funnel. The crude product was then washed on the filter with small aliquots of dry diethyl ether, dry acetone, and again with dry diethyl ether. The final product was dried overnight in a vacuum oven at ambient temperature. The crude product was dissolved in dry DMF (12 mL) in the dark under an argon atmosphere. TLC at this stage showed the presence of substantial free C-Dox and a very small quantity of free Dox. PL-FDMP (150−300 *μ*m, 1.5 mmol/g aldehyde-functionalized) resin (811 mg) in the

Scheme 1 [a]



[a] (a) 4 equiv of Et_3N/DMF/25 °C/21 h; (b) 2 equiv DL-1-amino-2-propanol, 1 h (quench remaining activated ester); (c) precip into cold Et_2O, filter, wash, dry; (d) dissol DMF/PL-FDMP resin/trace AcOH/3 h (scavenge resid C-Dox), filter resin, reprecip filtrate into cold Et_2O, filter product, wash, dry.

presence of a trace of acetic acid was used to scavenge this remaining free C-Dox and free Dox from solution. Within 3 h TLC showed negligible free C-Dox or Dox remained. The resin beads were removed by filtration through a sintered-funnel and quickly washed on the funnel with dry DMF (6 mL). The combined filtrates were added dropwise, under argon, to stirred dry diethyl ether (180 mL) precooled to 10 °C. After the precipitate was stirred for a further 30 min, the purified product was isolated by filtration on a Grade 3 sintered-funnel. The filter-cake was washed on the funnel successively with small aliquots of dry diethyl ether until the washings were colorless (ca. 100 mL of diethyl ether in total). The final product was collected from the sintered funnel and dried overnight in a vacuum oven at ambient temperature until constant weight. The synthesis is summarized in Scheme 1. A sample of the final HPMA-*co*-MA-GG−C-Dox was taken and its C-Dox content determined spectrophotometrically (UV).

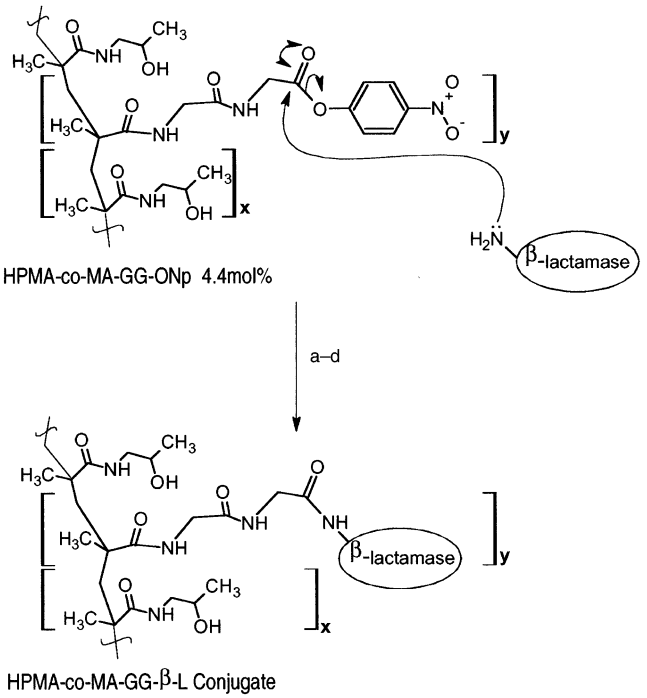
Characterization of HPMA-*co*-MA-GG−C-Dox during the Reaction. *HPLC.* The HPMA-*co*-MA-GG−C-Dox conjugation reaction was monitored at 254 nm at ambient temperature by HPLC using a Techsphere 5 ODS column (250 × 4.6 mm) with gradient elution using eluent (A) 0.05 M triethylammonium formate, adjusted to pH 2.8 with HCl and eluent (B) acetonitrile. The flow rate used was 1 mL/min, and the gradient used was 0−8 min 30% B, 8−15 min 30% to 80% B, 15−20 min 80% to 30% B, 20−25 min 30% B. Samples applied to the column were made up using 10 μL of the reaction mixture added to 495 μL of eluent A and 495 μL of eluent B. The injection loop volume was 20 μL.

TLC. TLC plates (Merck, aluminum sheets, 5 × 10 cm on Silica gel 60 F_{254}) were spotted with reaction mixture and prerun with diethyl ether (to shift the DMF reaction solvent away from sample spot). After being allowed to air-dry in fume-hood, the plates were then rerun using a dichloromethane/methanol/glacial acetic acid/water (80:20:7:3) mixture as eluent.

Synthesis of the HPMA-*co*-MA-GG−*β*-L Conjugate. HPMA-*co*-MA-GG-ONp (containing 4.4 mol % MA-GG-ONp) was dissolved in DMSO (5 mg/mL), and a solution of *β*-L in DMSO (5 mg/mL) was added dropwise under stirring. The reaction mixture was stirred in dark for 20 min and then terminated by adding DL-1-amino-2-propanol (20 μL) to quench any remaining unreacted ONp groups remaining on the copolymer. The final yellow solution was transferred to a VivaSpin (10 kDa MW cutoff) column and centrifuged at 4 °C at 3000*g* for 30 min to remove the low MW contaminants present and replace the solvent with aqueous solution. This centrifugation was repeated, with addition of phosphate buffer each time, until no visible ONp (yellow) remained. The mixture was concentrated to a final volume of 500 μL. The synthesis of HPMA-*co*-MA-GG−*β*-L is summarized in Scheme 2.

Purification and Characterization of HPMA-*co*-MA-GG−*β*-L by FPLC. The final HPMA-*co*-MA-GG−*β*-L conjugate was purified and characterized by FPLC and SDS−PAGE electrophoresis (gradient gels 5−15%

Scheme 2 [a]



[a] (a) DMSO/25 °C/20 min; (b) DL-1-amino-2-propanol, 1 h (quench remaining activated ester); (c) ultrafiltration 10 kDa MWCO/30 min/4 °C, then aq buffer washes (repeat until visible NpOH removed); (d) concentrate and purify by FPLC.

acrylamide), and the protein content in the conjugate was determined by the Bradford assay. FPLC using a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) with a UV (280 nm) detector and FPLC director version 1.10 software was used to analyze the final HPMA-*co*-MA-GG−*β*-L and purify it. Samples recovered from the VivaSpin (200 μL) were injected onto the FPLC column and were eluted using 0.01 M phosphate buffer containing 0.15 M NaCl, pH 7.4, at a flow rate of 0.5 mL/min. The column was calibrated using free *β*-L (1 mg/mL). Fractions (1 mL) were also collected and analyzed for *β*-L activity. The final conjugate was lyophilized and stored at −20 °C.

Measurement of *β*-L Activity Using Cephalosporin C as Substrate. All reagents were prepared in PBS, pH 7.4, containing 12.5 μg/mL BSA. Cephalosporin C was dissolved in the PBS/BSA buffer at a concentration of 300 μM. *β*-L was dissolved in the PBS/BSA at a concentration of 30 μg/mL. The cephalosporin solution (335 μL) and the PBS/BSA buffer (635 μL) were added to a 1.0 mL cuvette and placed in a spectrophotometer. Baseline absorbance was monitored at 260 nm for 5 min at 25 °C. Then the free or conjugated *β*-L (enzyme or FPLC fraction) solution (40 μL) was added to the cuvette, and activity (initial rate) was measured by monitoring the fall in absorbance at 260 nm. The specific activity was calculated as μmol/s/mg.

Measurement of *β*-L Activity Using HPMA-*co*-MA-GG−C-Dox as Substrate. HPMA-*co*-MA-GG−C-Dox was dissolved in the PBS/BSA buffer at a concentration of 300 μM cephalosporin C-equivalent. Either free or conjugated *β*-L was dissolved in the PBS/BSA buffer at 30 μg/mL *β*-L-equivalent. To a polypropylene tube were added the PBS/BSA buffer (665 μL) and the HPMA-*co*-MA-GG−C-Dox solution (335 μL); or 630 μL of PBS/BSA and 335 μL of HPMA-*co*-MA-GG−C-Dox solution together with free or conjugated *β*-L solution (35 μL) were added. All tubes were incubated at 37 °C. Samples (100 μL) were taken regularly over a 5 h period. They were snap frozen in liquid nitrogen and stored at −20 °C in the dark until analyzed by HPLC.

To measure Dox release, free Dox was first extracted from each sample and then analyzed by HPLC using methods previously described (*9*). Briefly, daunomycin (100 ng) was added to each sample as an internal standard followed by ammonium formate buffer 1 M, pH 8.5 (100 μL), and the extraction mixture (chloroform:propan-2-ol 4:1) (5 mL). The tubes were then vortex-mixed (three times) for 20 s and centrifuged (1000*g*) for 30 min at 10 °C. The aqueous layer was carefully removed and the organic layer evaporated to dryness using the Techne nitrogen sample concentration at room temperature under $N_{2(g)}$. The residue was then dissolved in methanol (100 μL) before analysis by HPLC.

All samples from the degradation studies and Dox calibration standards were analyzed by HPLC using a Waters μBondapak C18 125 Å 10 μm reverse phase HPLC column (3.9 × 150 mm) with a Waters Sentry Guard Column (μBondapak C18 125 Å, 10 μm, 3.9 × 20 mm) with eluent at a flow rate of 1 mL/min. Results were analyzed using a Millenium[32] Login Version 3.05.01 Chromatography Manager.

Evaluation of in Vitro Cytotoxicity. *Colony Formation Assay.* B16F10.9 melanoma cells (100) were seeded into a 24-well plate containing DMEM medium with 10% fetal calf serum (FCS) and Pen/Strep antibiotics. HPMA-*co*-MA-GG−C-Dox (50 μg/well Dox-equiv) alone, or the combination of HPMA-*co*-MA-GG−C-Dox (50 μg Dox-equiv) plus HPMA-*co*-MA-GG−*β*-L (20 μL at a 1 mg/mL concentration) were added to the cells. Treatment with free Dox (50 μg/well) or PBS were added to wells as positive and negative controls. The cells were then incubated for 8 days, fixed to the plate with 100% methanol for 20 min, and then washed with running water. The plate wells were then filled with 10% aqueous Giemsa solution. The plates were left at room temperature for 20 min and then washed again with running water and dried, and the number of colonies were counted. The average number of colonies seen following each treatment was expressed as a percentage of the untreated control.

Cell Proliferation Assay with XTT Reagent. The tetrazolium dye XTT (*19*) was used to assess cell viability. B16F10.9 cells (10^3 per well in 100 μL) were seeded in a flat-bottomed 96-well plate and incubated at 37 °C for 24 h. Then various concentrations of HPMA-*co*-MA-GG−C-Dox or HPMA-*co*-MA-GG−C-Dox and HPMA-*co*-MA-GG−*β*-L or saline or Dox were added (n = 8). The cells were then incubated for 72 h, XTT reagent (50 μL) was added to each well, and the plate was incubated for 2 h. Spectrophotometric absorbance of the formazan product was measured at a wavelength of 450 nm using 690 nm as reference wavelength.

Evaluation of the Body Distribution of ^{125}I-Labeled HPMA-*co*-GG-*β*-L and Free *β*-L in Mice Bearing B16 Melanoma sc. All animal experiments were conducted according to the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Guidelines. Male C57BL/6J mice were inoculated with 10^5 viable B16F10 cells sc, and the tumor was allowed to establish until the area was approximately 50−70 mm^2 as measured by the product of two orthogonal diameters. Animals were injected iv with free or conjugated ^{125}I-labeled *β*-L (5×10^5 CPM/mouse) and animals euthanized at times up to 72 h. The main organs were dissected, and the blood was collected. The tumor, organs, and blood

samples were homogenized and read in a γ-counter. Results were calculated as % of administered dose/g.

Evaluation of Antitumor Activity of the PDEPT Combination: HPMA-*co*-MA-GG−C-Dox and HPMA-*co*-MA-GG−*β*-L. Male C57BL/6J mice (∼9 weeks, ∼20 g) were inoculated with 10^5 viable B16F10 melanoma cells sc. The tumor was allowed to establish until the area was approximately 50−70 mm^2 as measured by the product of two orthogonal diameters. Animals (five per group) were injected iv with HPMA-*co*-MA-GG−C-Dox (10 mg/Kg Dox-equiv.) or HPMA-*co*-MA-GG−C-Dox (10 mg/kg Dox-equiv) followed after 5 h by HPMA-*co*-MA-GG−*β*-L. As a comparison mice (five per group) were injected with either saline (100 μL iv) or free Dox (10 mg/kg). Animals were weighed and the tumors measured daily. Mice were euthanized when the tumor reached or surpassed the size of 300 mm^2. Animals were weighed daily and observed twice a day for signs of tumor progression. They were euthanized if their body weight decreased below 80% of the starting weight. Animals were monitored for general health, weight loss, and tumor progression and at termination were examined by post-mortem; tumors were dissected and weighed. Experimental data were expressed as the mean survival time, *T*/*C* defined as the ratio of the mean survival time of the treated animals (*T*) divided by the mean survival of the untreated control group (*C*) expressed as a percentage.

Statistical Methods. All the in vitro data are expressed as the mean ± standard deviation of the mean (SD). All the in vivo data are expressed as the mean ± standard error of the mean (SE). Statistical significance was assessed using the Student's *t*-test. *P* values of 0.05 or less were considered statistically significant.

RESULTS AND DISCUSSION

Novel treatment strategies are badly needed if cancer chemotherapy is to be significantly improved. Polymer−drug conjugates are emerging as one promising option (*21*). They display passive tumor targeting by the EPR effect, and lysosomotropic delivery results in marked reduction in toxicity of the bound drug. For example HPMA-*co*-MA−GFLG-doxorubicin (PK1) was ∼5-fold less toxic than Dox in a Phase I study (*10*). Eleven such conjugates have now progressed into clinical development, and activity has been repeatedly seen in chemotherapy refractory patients (reviewed in *21*). Clinically, combination chemotherapy is routinely used in an attempt to increase efficacy, bypass resistance, and minimize the peripheral toxicity that is often dose-limiting. The PDEPT concept was developed with the aim of further improving the tumor selectivity of polymer-drug conjugates (*9*) and also to circumvent some of the disadvantages of ADEPT (discussed in *9*, *16*). A model combination of HPMA-*co*-MA-GG-cathepsin B and HPMA-*co*-MA-GLFG-Dox demonstrated the practicality of the approach (*9*).

PDEPT involves two steps. First, systemic administration of a polymeric prodrug containing a linker designed for cleavage by the activating enzyme. This conjugate typically has a molecular weight of ∼30 000 g/mol. Once the circulating polymer−drug conjugate has cleared (after 1−2 h due to renal elimination), a polymer−enzyme conjugate can then be administered as a second step. This conjugate has a higher molecular weight (∼50 000−100 000 g/mol), and thus it circulates for longer. Both conjugates display tumor targeting by the EPR effect and when they meet, activation of the prodrug leads to localized drug release in the tumor interstitium and therefore minimal nonspecific toxicity. The model combination of HPMA-*co*-MA-GG-cathepsin B and PK1 showed that a polymer−enzyme would indeed gain access to the drug conjugate in the tumor interstitium (*9*) and promote drug release.

The full potential of PDEPT will, however, only be fully realized if a nonmammalian enzyme is used in combination with a polymer prodrug incorporating the appropriate matching linker. The combination described here was

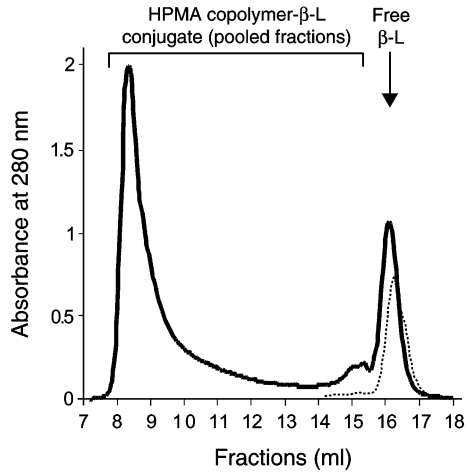


Figure 3. FPLC traces of free β-L (- - -) and HPMA-*co*-MA-GG−β-L (—) showing the fractions pooled to give the final product.

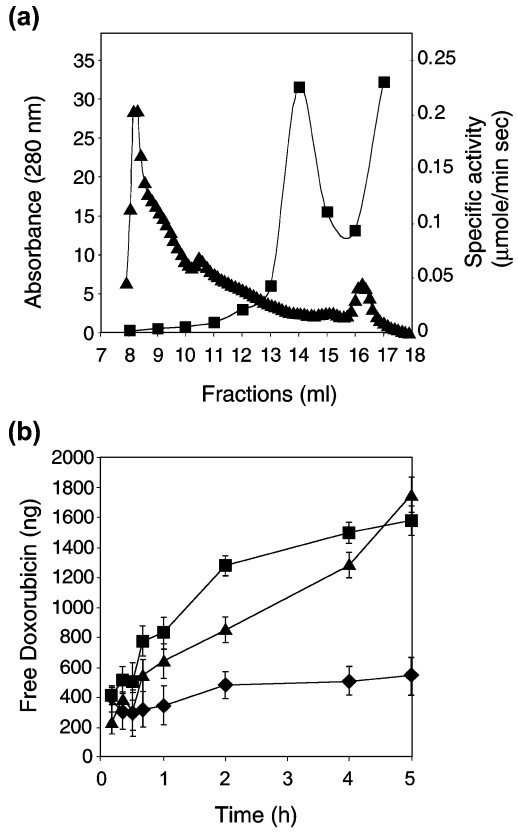


Figure 4. Comparison of enzymatic activity of free β-L and HPMA copolymer−β-L *in vitro.* Panel a shows the activity of β-L (■) in HPMA copolymer−β-L fractions (▲) seen using cephalosporin C as a substrate. Panel b shows the release of Dox from HPMA copolymer−C-Dox when incubated with β-L (■) or HPMA copolymer−β-L (▲). Release of Dox from HPMA copolymer−C-Dox in the absence of enzyme is included as a control (♦). Data represent mean ± SD.

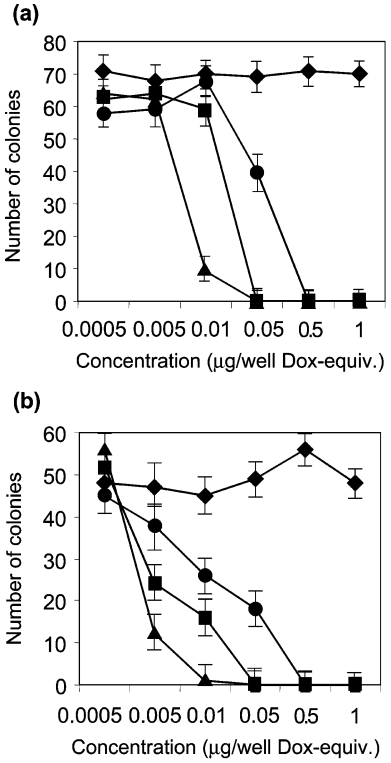


Figure 5. In vitro antitumor activity. Inhibition of colony formation after 24 h using (a) B16F10 cells and (b) DA3 cells. The data show the effect of Dox (▲), HPMA copolymer−C-Dox (●); HPMAcopolymer−C-Dox + HPMA copolymer−β-L (■) and the untreated control cells (♦). Data represent mean ± SD.

the first attempt to achieve this goal. First the polymer prodrug, HPMA-*co*-MA-GG−C-Dox conjugate (Figure 1) was synthesized having a C-Dox content of 5.85 wt % (as determined by UV) and a M_w of ∼31 600 g/mol. The conjugate contained neither contaminating free C-Dox nor Dox according to TLC. Synthesis of antibody− and polymer−enzyme conjugates is often problematic. Conjugation typically has a low yield (10−15%) (*22*, *23*) and results in reduced enzyme activity. The semirandom aminolysis method used to bind the HPMA copolymer precursor to cathepsin B gave conjugates with a relatively high yield (30−35%) and also with retained enzyme activity in vitro against both a low molecular weight and macromolecular substrate (20−25%) (*9*). Interestingly, the HPMA-*co*-MA-GG−β-L described here had a much higher yield in respect of the bound protein (80−90%). This could be due to carrying out the aminolysis reaction in DMSO and not PBS, thus minimizing nonspecific hydrolysis. Whereas free β-L had a band at 45 kDa on SDS PAGE, the HPMA-*co*-MA-GG−β-L conjugate had a molecular weight in the range of 75−150 kDa. Following purification by FPLC (Figure 3) and pooling of conjugate fractions, no free enzyme was detectable in the final product by SDS PAGE (results not shown). During purification, all FPLC fractions were assayed for activity using cephalosporin C as substrate (Figure 4a). The recovered conjugate retained 70% of enzyme specific activity (mean: ∼15 μmol/sec/mg) against cephalosporin C. Incubation of HPMA-*co*-MA-GG−C-Dox with HPMA-*co*-MA-GG−β-L led to release of Dox, and the conjugate retained 80% of the activity of free enzyme against the macromolecular substrate (Figure 4b). No nonspecific cleavage of the β-lactamic linker occurred in buffer solutions lacking β-L over 5 h. However, slow hydrolytic release of free Dox was seen on storage of HPMA-*co*-MA-GG−C-Dox in buffer over several days. The high retention of enzymatic activity following the relatively harsh conjugation conditions is a puzzle. It might be due to very high stability of β-L. Furthermore, it is known that this enzyme is able to undergo renaturation following denaturation (*11*).

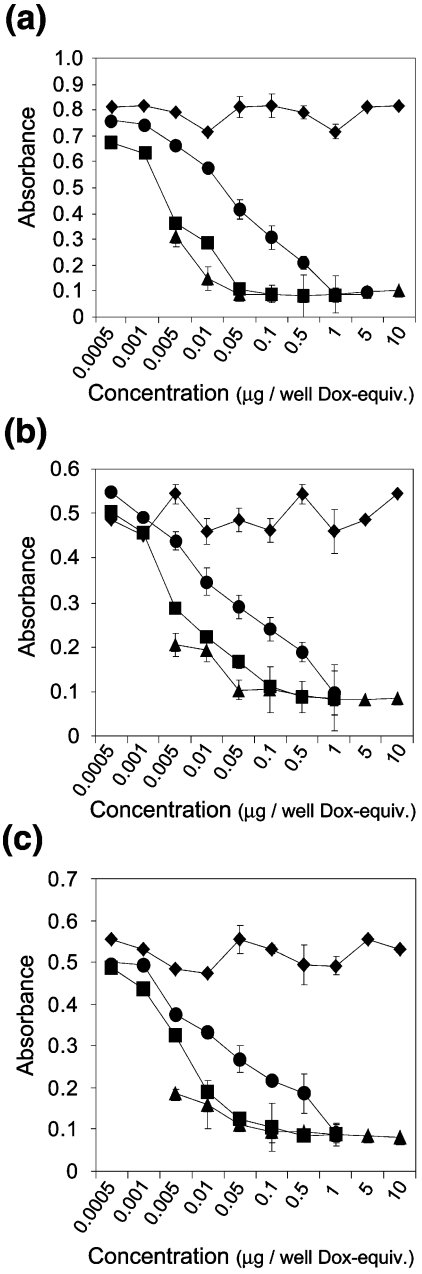


Figure 6. In vitro antitumor activity measured using the XTT cytotoxicity assay: (a) DA3 murine mammary carcinoma; (b) M109 murine lung carcinoma; (c) B16F10 murine melanoma. The data show the effect of Dox (▲),HPMA-*co*-MA-GG−C-Dox (●); HPMA-*co*-MA-GG−C-Dox + HPMA-*co*-MA-GG−β-L (■); and untreated control cells (♦). Data represent mean ± SD.

Ability of HPMA-*co*-MA-GG−β-L to liberate pharmacologically active Dox from the HPMA-*co*-MA-GG−C-Dox conjugate is essential for antitumor activity. Experiments were first performed in vitro using B16F10 and DA3 cells. The plating efficiency assay revealed inhibition of colony formation when cells were treated with either the PDEPT combination or free Dox (Figure 5). Activation of HPMA-*co*-GG−C-Dox by HPMA-*co*-MA-GG−β-L resulted in a cytotoxicity profile that was similar to that seen for free Dox. In contrast, in the absence of activating enzyme, HPMA-*co*-MA-GG−C-Dox was 10-fold less cytotoxic than

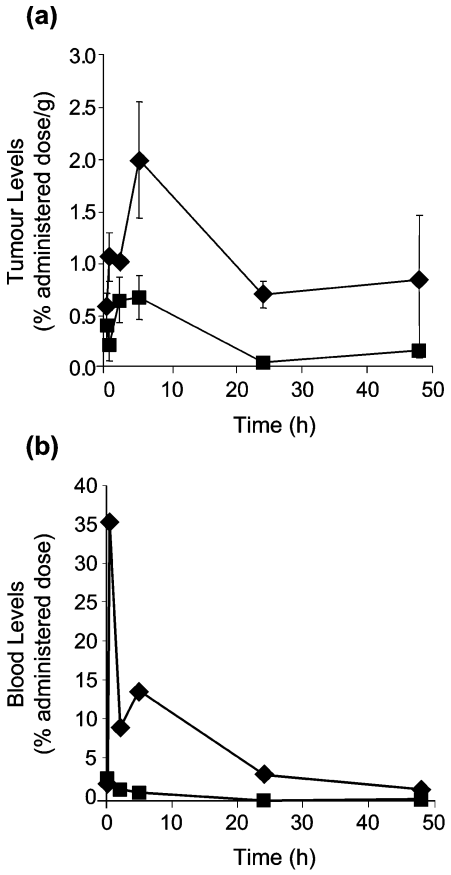


Figure 7. Body distribution of ^{125}I-labeled β-L and ^{125}I-labeled HPMA copolymer−β-L in mice bearing sc B16F10 tumors. Panel a shows the tumor levels of radioactivity and panel b the blood clearance. Symbols represent ^{125}I-labeled β-L (■) and ^{125}I-labeled HPMA-coMA-GG−β-L (♦), and data are mean ± SE.

the PDEPT combination. To further investigate this phenomenon, XTT cytotoxicity assay was conducted using three cell lines. In these experiments the PDEPT combination was also ~10-fold more active than HPMA-*co*-MA-GG−C-Dox alone; 13.3-fold in DA3 cells, 7.5-fold in M109 cells, and 10-fold in B16F10.9 (Figure 6). These assays involve a 3 day incubation; thus, the cytotoxicity of HPMA-*co*-MA-GG−C-Dox alone can be explained by the hydrolytic Dox release occurring in the meantime. Since HPMA-*co*-MA-GG−C-Dox is only present in the circulation for a short time (<5 h), the importance of this nonspecific release in vivo would be minimal.

The above-mentioned in vitro observations justified evaluation of this PDEPT combination in vivo. It was considered important first to determine the blood clearance and tumor accumulation of the novel enzyme conjugate. It is likely that the pharmacokinetics of HPMA-*co*-MA-GG−C-Dox will be similar to PK1 as reported previously in preclinical (*24*) and clinical studies (*10*). Free and bound β-L were radiolabeled with [^{125}I]-iodide using the Bolton and Hunter reagent (*20*). The products had a specific activity of 37 μCi/mg and 99.4 μCi/mg for β-L and HPMA copolymer-GG−β-L, respectively, and their free [^{125}I]iodide content was <1%, making them suitable for in vivo use. ^{125}I-Labeled β-L had a $t_{1/2\alpha}$ = 1.9 h; the conjugate had a longer blood residence time ($t_{1/2\alpha}$ = 4.7 h) consistent with the reduced cellular clearance and/or increased resistance to proteolysis (*4, 25*). The plasma AUC of the conjugate was 8.2-fold greater than seen for free enzyme. The increased circulation time led to a higher conjugate tumor accumulation (2.9-fold increase in AUC, Figure 7) attributable to passive targeting by the EPR effect.

Table 1. Antitumor Activity of HPMA-*co*-MA-GG−C-Dox and the PDEPT Combination Administered iv to C57 Black Male Mice Bearing sc B16F10

treatment	Dox dose (mg/kg)	time to progression (days ± SE)	*T*/*C* (%)	no. of toxic deaths
saline		8.6 ± 2		0/5
doxorubicin	10	10 ± 4	116	0/5
HPMA-*co*-MA-GG−C-Dox	10	9 ± 1	110	0/5
HPMA-*co*-MA-GG−C-Dox + HPMA-*co*-MA-GG−β-L	10	12 ± 2	133[a]	0/5

[a] $p \leq 0.03$.

Preliminary antitumor studies were conducted using a sc B16F10 model, which has been widely used to evaluate other polymer−drug conjugates (*26*). Mice showed a significantly increased survival when treated with the PDEPT combination (Table 1) and did elicit significant decrease in tumor growth rate compared with that seen in the control mice group. The PDEPT combination caused neither toxic deaths nor animal weight loss. Previous studies investigating ADEPT and using PEGylated enzymes clearly showed that antitumor activity improves dramatically following dose optimization of both the enzyme conjugate and the polymeric prodrug (*27*). Future studies with this new PDEPT combination will optimize these parameters and also evaluate the effect of repeated cycles of administration.

In summary, the feasibility of PDEPT strategy using a nonmammalian enzyme and the corresponding polymer prodrug has been demonstrated. The use of nonhuman proteins for parenteral therapy raises the issue of the immunogenicity. Indeed, this has been seen in clinical studies involving ADEPT (*28*). Cyclosporin can be used to combat the problem (*29*), but immunosuppression in cancer patients is not desirable. Polymer conjugation of an enzyme, for example PEGylation (*30, 31*), does bring the advantage of reduced protein immunogenicity. The HPMA-*co*-MA-GG-cathepsin B and HPMA-*co*-MA-GG−β-L conjugates do show a significant reduction or even abrogation of the immunogenicity of the conjugated parent enzymes (*32*). Thus, with optimization, this PDEPT combination may provide a new and potentially immunosilent EPR-targeted approach for improved cancer chemotherapy.

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