Published in final edited form as:

Bioconjug Chem. 2008 September ; 19(9): 1813–1821. doi:10.1021/bc800154f.

Peptide-Targeted Polyglutamic Acid Doxorubicin Conjugates for the Treatment of $\alpha_v\beta_6$ -Positive Cancers

Huili Guan[§], Michael J. McGuire[§], Shunzi Li, and Kathlynn C. Brown^{*}

Division of Translational Research, Department of Internal Medicine and The Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Abstract

Most chemotherapeutics exert their effects on tumor cells as well as their healthy counterparts, resulting in dose limiting side effects. Cell-specific delivery of therapeutics can increase the therapeutic window for treatment by maintaining the therapeutic efficacy while decreasing the untoward side effects. We have previously identified a peptide, named H2009.1, which binds to the integrin $\alpha_v\beta_6$. Here, we report the synthesis of a peptide targeted polyglutamic acid polymer in which the high affinity $\alpha_v\beta_6$ -specific tetrameric H2009.1 peptide is incorporated via a thioether at the N-terminus of a 15 amino acid polymer of glutamic acid. Doxorubicin is incorporated into the polymer via an acid-labile hydrazone bond. Payloads of four doxorubicin molecules per targeting agent are achieved. The drug is released at pH 4.0 and 5.6 but the conjugate is stable at pH 7.0. The conjugate is selectively internalized into $\alpha_v\beta_6$ positive cells as witnessed by flow cytometric analysis and fluorescent microscopy. Cellular uptake is mediated by the H2009.1 peptide, as no internalization of the doxorubicin-PG polymer is observed when it is conjugated to a scrambled sequence control peptide. Importantly, the conjugate is more cytotoxic toward a targeted cell than a cell line that does not express the integrin.

INTRODUCTION

Most chemotherapeutics exert their effects on tumor cells as well as their healthy counterparts, resulting in dose limiting side effects. Cell-specific delivery of therapeutics can increase the efficacy of the drug while decreasing its side effects, thus opening the therapeutic window for treatment. Despite its advantages, tumor targeting of therapeutic molecules remains a major challenge in biomedicine. The challenges in developing targeted drug delivery systems can be broken into three main areas. First, cell specific binding reagents that display high affinity and specificity for the desired cell type must be identified. Additionally, if the drug mediates its cytotoxic effect by an intracellular activity, the ligand must mediate both binding and uptake by the targeted cell. Second, these targeting moieties must be incorporated into drug systems that allow delivery of molecular cargo specifically to

the tumor. This takes into account factors such as size, vascular permeability, tissue retention, and stability. Third, the cargo needs to be released in an active form within the desired cellular compartment.

Toward the goal of targeted therapies for non-small cell lung carcinoma (NSCLC), we have isolated a peptide, named H2009.1, which binds to a large number of adenocarcinoma (AD) lung cancer cell lines (1). When placed on a tetrameric trilycine scaffold, this peptide has half-maximal binding affinity of 40–60 pM for its target cell. Additionally, the peptide can mediate cellular uptake (2). The cellular receptor for the H2009.1 peptide is the restrictively expressed integrin, $\alpha_v\beta_6$ (3). Expression of $\alpha_v\beta_6$ is widespread in early stage NSCLC, and it is associated with poor patient survival. Furthermore, this integrin is up-regulated in NSCLC tumors compared to normal lung tissue. The restricted expression suggests that it may be a good receptor for targeted therapies for lung cancer.

We have previously demonstrated that the H2009.1 peptide can be conjugated to doxorubicin to affect cell-specific death (2). While this experiment established proof-of-concept, only one molecule of doxorubicin was delivered per molecule of peptide. Increasing the drug load is expected to improve the efficacy. Polyglutamic acid (PG) has recently been used as a polymeric drug carrier (4). This water soluble biopolymer is biodegradable and is well tolerated at high doses. The pendant carboxyl groups of the glutamic acid provide a reactive moiety to which chemotherapeutics can be conjugated. As such, greater drug loads can be achieved based on a per molecule basis. This carrier has been used for doxorubicin (5–7), paclitaxel (8–11), camptothecin (12, 13), and retinoids (14, 15). In addition, MRI contrast reagents have been coupled to PG for tumor imaging (16–18). The PG-paclitaxel conjugate, XYOTAX (CT-2103, Cell Therapeutics Inc.) is in clinical trials for NSCLC and ovarian cancer (19–21).

Most of the PG-drug conjugates are large molecular weight polymers, which increase the circulation time of the drug when compared to free drug. Accumulation in the tumor is the result of passive targeting due to the enhanced permeability and retention (EPR) effect which results from the disordered, leaky vasculature of the tumor coupled with a dysfunctional lymphatic system (22). Active targeting of the PG-drug conjugates can be achieved by the incorporation of a targeting moiety. To date, few reports of targeted-PG conjugates have been reported, and they have relied on antibody-based targeting reagents (5).

Here, we report the synthesis of a peptide targeted polyglutamic acid polymer in which the high affinity $\alpha_v\beta_6$ -specific tetrameric H2009.1 peptide is incorporated via a thioether at the N-terminus of a 15 amino acid polymer of glutamic acid. Doxorubicin is incorporated into the polymer via an acid-labile hydrazone bond. Payloads of four doxorubicin molecules per targeting agent are achieved and the drug is released in acidic conditions. The conjugate is selectively internalized into $\alpha_v\beta_6$ positive cells as witnessed by flow cytometric analysis and fluorescent microscopy. Cellular uptake is mediated by the H2009.1 peptide, as minimal internalization of the doxorubicin- PG polymer is observed when it is conjugated to a scramble sequence control peptide. Importantly, the conjugate is preferentially cytotoxic toward a targeted cell when compared to a cell line that does not express the $\alpha_v\beta_6$ integrin.

EXPERIMENTAL SECTION

General Peptide Synthesis and Characterization

All peptides were synthesized on a Symphony Peptide Synthesizer (Rainin Instruments, Protein Technologies, Inc. Woburn, MA) using standard Fmoc chemistry. All Fmoc-protected amino acids, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), were purchased from EMD Biosciences Inc. (San Diego, CA), anhydrous 1-hydroxybenzotriazole (HOBt) was purchased from SynBioSci (Livermore, CA), *N*-methylmorpholine (NMM) was purchased from Acros Organics (Geel, Belgium), and piperidine was purchased from Sigma-Aldrich Inc. (St. Louis, MO). The crude peptides were dissolved in 20% methanol/8 M guanidinium hydrochloride and purified with RP-HPLC using eluents of H₂O/0.1% TFA (eluent A) and acetonitrile/0.1% TFA (eluent B). The programmed elution profile 0–1 min, 100% A; 1–80 min, B is increased from 0–75% at a flow rate of 10 mL/min on preparative column (Spirit Peptide C18, 5 μ m column, 25 $\times$ 2.12) (referred to as method A) or 0–1 min, 100% A; 1–70 min, B is increased from 0–75% at a flow rate of 1 mL/min on analytical column (Spirit Peptide C18, 5 μ m column, 15 $\times$ 0.4) (referred to as method B). Peptide purity was determined by analytical HPLC monitoring peptide elution by absorbance at 220 nm. The peptide mass was characterized by matrix assisted laser desorption/ionization (MALDI) on a Voyager DE Pro (Applied Biosystems Inc., Foster City, CA) using α -cyano-4-hydroxycinnamic acid as matrix in the reflector mode or in linear mode using sinapinic acid as matrix, respectively. N-terminal sequence analysis of peptide was carried out by Edman protein sequencers (Applied Biosystems 494) in the Protein Chemistry Technology Center of University of Texas Southwestern Medical Center.

Cell Lines

Human lung cancer cell lines were obtained from the Hamon Center for Therapeutic Oncology Research (UTSW) and were maintained according to standard protocols (23). RPMI1640 was purchased from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was purchased from Gemini Bioproducts (Woodland, CA).

Synthesis of Tetrameric-10mer H2009.1 and a Corresponding Scrambled Sequence Peptide

The peptides were synthesized by standard Fmoc chemistry using the Fmoc₄-Lys₂-Lys-Cys(Acm)- β -Ala-CLEAR Acid Resin (loading 0.21 mmol/g, Peptides International, Louisville, KY). Fmoc-protected amino acids were doubly coupled at 5-fold molar excess using HBTU/HOBt/NMM (5/5/10) as an activator system. Coupling times of 40–50 min were employed. A single coupling of Fmoc-NH-(PEG)₁₁-COOH (C₄₂H₆₅NO₁₆, Polypure, Oslo, Norway) was performed overnight. The removal of the Fmoc group was carried out with 20% piperidine in DMF. The peptide was cleaved by treating the final resin with a cocktail of TFA/TIS/H₂O (95%/2.5%/2.5%) for 3 h. The cleavage solution was concentrated by passing Ar into the solution and the peptide was subsequently precipitated by addition of cold ether. The crude peptide was purified with HPLC using method A. The purified product was analyzed by method B. MALDI mass spectra were obtained in linear mode using sinapinic acid as matrix. N-terminal sequence was analyzed by Edman protein sequencer.

Tetramer-10mer-H2009.1 (RGDLATLRQL; C₃₂₇H₆₀₆N₈₂O₁₁₄S): MALDI MH⁺ (average mass calculated/found: 7543.86/7544.20), purity >98.4%, (RP-HPLC, retention time of 34.37 min using method B). Scramble Tetramer-10mer-H2009.1 (DALRLQGTLR; C₃₂₇H₆₀₆N₈₂O₁₁₄S): MALDI MH⁺ (average mass calculated/found: 7543.86/7544.83), purity >98.1% (RP-HPLC, retention time of 34.58 min using method B).

Deprotection of the Acetamidomethyl-Cysteine To Reveal a Free Thiol on the Tetrameric Peptides

The tetrameric-10mer H2009.1 peptide with the acetamidomethyl-cysteine (22 mg, 3 μmol) was dissolved in TFA (1.5 mL) containing 1% anisole to which silver acetate (80 mg, 480 μmol) was added. The solution was stirred for 2 h at 4 °C and then concentrated under Ar to 0.5 mL. The peptide was precipitated by the addition of 5 mL of cold ether and collected by centrifugation, and then treated with dithiothreitol (1 mL, 0.2 M in 1 M acetic acid) at RT for 3 h. The solution was filtered and purified with HPLC using method A. The purified product was analyzed by method B. MALDI mass spectra were obtained in linear mode using sinapinic acid as matrix. The same reaction and characterization conditions were employed on scrambled sequence tetrameric peptide. Tetrameric-10mer-H2009.1 (C₃₂₄H₆₀₁N₈₁O₁₁₃S): MALDI MH⁺ (average mass calculated/found: 7472.78/7473.99), purity >98.4% (RP-HPLC, retention time of 34.71 min using method B). Scramble tetrameric-10mer-H2009.1 (C₃₂₄H₆₀₁N₈₁O₁₁₃S): MALDI MH⁺ (average mass calculated/found: 7472.78/7473.84), purity >98.1% (RP-HPLC, retention time of 33.55 min using method B).

Synthesis of Maleimido-PEG-polyGlu₁₅

Synthesis of maleimido-PEG-polyGlu₁₅ was performed by sequential coupling of Fmoc-Glu(O-*t*-Bu)-OH (5-fold molar excess) on Rink Amide AM resin (200–400 mesh, 0.43 mmol/g, EMD Biosciences Inc.). A double coupling was performed for each addition of glutamic acid to the growing peptide chain. A PEG moiety was incorporated by a single coupling of Fmoc-NH-(PEG)₁₁-COOH (5-fold molar excess). The peptide was capped with maleimide functionality at the amino terminus by the reaction of *N*-β-maleimidopropionic acid (BMPPA, 5-fold molar excess) for 4 h. HBTU/HOBt/NMM (5/5/10) was employed as an activator system for all couplings. The removal of the Fmoc groups was performed in 20% piperidine with DMF. The linear peptide was cleaved from the final resin in a cocktail of TFA/TIS/H₂O (95%/2.5%/2.5%) for 3 h. Upon concentrating the cleavage cocktail by passing Ar into the solution, the peptide was precipitated in cold ether. The crude product was purified with HPLC using method A. The final product was analyzed by method B. The MALDI mass spectrum was obtained in reflector mode using α-cyano-4-hydroxycinnamic acid as matrix. Maleimido-PEG-polyGlu₁₅ (compound 1, C₁₀₉H₁₆₆N₁₈O₆₁): MALDI MNa⁺ (monoisotopic mass calculated/found: 2726.487/2726.46), purity >98.3% (RP-HPLC, retention time of 27.95 min using method B).

Incorporation of H₂NNHBoc to Pendant Carboxyl Group of Maleimido-PEG-polyGlu₁₅

The hydrazide groups were conjugated to the pendant carboxyl groups of compound 1 via acid anhydride reaction. Triethylamine (TEA, 0.2 mL) was added to maleimido-PEG-

polyGlu₁₅ (20 mg, 7.4 μ mol polymer, 111 μ mol COOH groups) dissolved in anhydrous DMF (1.5 mL), followed by dropwise addition of isobutyl chloroformate (0.2 mL, 1.5 mmol) under an Ar atmosphere at 4 °C. After 5 min, *tert*-butyl carbazate (H₂NNHBoc, 1.5 mmol in 0.8 mL DMF) was added. The reaction was performed for 30 min at 4 °C and then continued for 3 more hours at RT. The resultant salts were removed by filtration and low molecular weight impurities were removed by dialysis in DMF using a dialysis membrane with a molecular weight cutoff (MWCO) of 1 kDa. The final product was precipitated from cold ether and dried in vacuum. The number of carboxylate groups converted to CONHNHBoc was calculated by comparing intensity of characteristic resonance of the Boc protecting group at 1.41 ppm and maleimide resonance at 6.93 ppm (CH=CH) by ¹H NMR in DMSO-*d*₆. The results showed that 71% of the COOH groups were converted to CONHNHBoc groups. This corresponds to an average of 10–11 hydrazide moieties per polymer molecule. MALDI mass spectrometry of the resultant maleimido-PEG-polyGlu(NHNHBoc)₁₅ (compound 2) showed a broad mass distribution centered around 4400 Da (α -cyano-4-hydroxycinnamic acid as matrix). This is consistent with a distribution of products due to incomplete modification of the carboxylate.

Removal of the Boc Protecting Group from Maleimido- PEG-polyGlu(NHNHBoc)₁₅

Compound 2 (15 mg) was treated by TFA (1 mL) to remove protective BOC groups. The resultant maleimido-PEG-polyGlu(NHNH₂) was purified with HPLC using method A. The removal of Boc groups is indicated by the complete disappearance of methyl proton at 1.41 ppm in ¹H NMR. MALDI mass spectrometry of the deprotected product maleimido-PEG-polyGlu(NHNH₂)₁₅ (compound 3) showed a broad mass distribution centered around 3288 Da (α -cyano-4-hydroxycinnamic acid as matrix). This is consistent with the removal of approximately 10–11 Boc protecting groups.

Conjugation of Peptides to Maleimido-PEG-polyGlu(NHNH₂)₁₅

A typical protocol for the reaction of thiol group of the peptide with maleimide is described below. Compound 3 (3.8 mg) was dissolved in PBS (0.5 mL, pH 7.0) containing EDTA (0.1 M) degassed by passing Ar into the solution for 5 min. The tetrameric 10mer H2009.1 peptide (9.6 mg, 1.3 μ mol) was added, and the reaction was carried out at RT for 3 h. The product was isolated with HPLC using method A. The purified product was analyzed by method B and found to be a single peak with a retention time of 33.48 min for the tetrameric 10- mer H2009.1-PEG-PolyGlu(NHNH₂)₁₅ (compound 4). A retention time of 33.55 min was observed for the corresponding product coupled to the sequence scrambled version of the peptide. MALDI mass spectra were obtained in linear mode using sinapinic acid as matrix. A major product was observed with a MH⁺ of 10 389.9 (compound 4).

Synthesis of Tetrameric 10mer H2009.1-PEG-polyGlu-(NHN=Dox)

Compound 4 (5 mg, 0.48 μ mol) and doxorubicin HCl (15 mg, 26 μ mol, Shanco International, Inc., South Plainfield, NJ) were dissolved in anhydrous methanol (1.5 mL) with TFA as an acid catalyst. The reaction solution was stirred at RT in the dark for 48 h. After removal of the solvent, the resultant solid was dried overnight under vacuum. The crude product was purified by size exclusion chromatography (Ultrahydrogel 500, 7.8 $\times$ 300

mm) in PBS/CH₃CN (70/30). The proper fraction was collected and lyophilized to yield final product as wine red powder. The product was evaluated by HPLC (elution profile of 0–1 min, eluent B is increased from 0–15%; 1–40 min, B is increased from 15–80% at a flow rate of 1 mL/min using the Spirit Peptide C18 column) monitoring the presence of doxorubicin at 485 nm to confirm the absence of unbound free doxorubicin. The conjugate has a retention time of 17.8 min and is well separated from the free drug which elutes at 16.3 min. The loading percentage of doxorubicin in the conjugates was 24% as determined by comparing the concentration of Dox obtained by absorption at 481 nm ($\epsilon = 10\,410\text{ M}^{-1}\text{ cm}^{-1}$) (24) and the concentration of peptide moiety obtained by Edman sequencing analysis.

Evaluation of pH Dependent Drug Release

Tetrameric 10mer H2009.1-M-PEG-polyGlu(NHN=Dox) (50 μM Dox equivalents) was dissolved in phosphate buffered saline (pH 7.0, 0.1 mL) or acetate buffer (pH 4.0, 0.1 mL and pH 5.6, 0.1 mL). The samples were incubated at 37 °C for the indicated times. The release of free doxorubicin was determined by RP-HPLC using a C18 column and monitoring doxorubicin absorbance at 485 nm.

Flow Cytometric Analysis

Integrin $\alpha_v\beta_6$ -positive H2009 cells and $\alpha_v\beta_6$ -negative H1299 cells (3) were seeded in 12 well culture plates at approximately 100 000 cells/well in a volume of 1 mL RPMI-1640 supplemented with 5% bovine serum (R5 medium) and allowed to adhere overnight. Cells were incubated for 120 min with 30 μM unconjugated doxorubicin, tetrameric scrambled peptide- or tetrameric 10mer-H2009.1-M-PEG-polyGlu(NHN=Dox). Cells were washed four times with PBS⁺ containing 0.1% BSA, followed by 2 brief rinses with 20 mM HCl-glycine, pH 2.2 containing 150 mM NaCl. Following the washes, the cells were rinsed with PBS and 1 mL/well of enzyme-free cell dissociation buffer (Invitrogen, Grand Island, NY) was added. Cells were incubated for 30 min on ice in cell dissociation buffer, scraped from the plate, and prepared as a single cell suspension by passage through a 27 gauge needle. Doxorubicin uptake was assessed for 10 000 cells per treatment group by flow cytometry in a CellQuanta flow cytometer. Cells were gated by size and side scatter properties and doxorubicin assayed by fluorescence in channel 2 (excitation 488 nm, emission 550–600 nm).

Cell Viability Assay

Integrin $\alpha_v\beta_6$ -positive H2009 cells and $\alpha_v\beta_6$ -negative H1299 cells were seeded in 96 well culture plates at 1000 cells/well in a volume of 50 μL R5 medium and allowed to adhere overnight. Unconjugated doxorubicin or tetrameric 10mer H2009.1-M-PEG-polyGlu(NHN=Dox) was added to quadruplicate wells in 50 μL R5 media to produce the final concentrations indicated (ranging from 200 nM to 5 μM , all based on total doxorubicin concentration). Untreated control cells received 50 μL R5 only and were cultured as a set of eight replicate wells per plate. After 24 h of exposure to the drug, media was aspirated from all wells, and the wells were washed four times with 200 μL R5 before continuing culture in 100 μL R5 for 72 h. At the end of the post-treatment incubation, cell viability was assessed using CellTiter Glo reagent (Promega, Madison, WI). Luminescence was detected using a plate reader 30–60 min after the addition of the CellTiter Glo reagent.

Fluorescent Microscopy

Images of individual cells were captured from wells of 96 well plates from the viability assays detailed above. Cells were observed 2 h after removal of the drug and addition of fresh media using an inverted Leica 6500 microscope. Cell images were captured with phase contrast illumination to visualize the cells and fluorescence in the red channel to visualize doxorubicin uptake. Untreated control cells had no detectable intrinsic fluorescence under these conditions. Figures are displayed as overlays of the phase contrast and fluorescence microscopy. Images in an individual experiment were captured under the same settings (gain, time of exposure, contrast) so that fluorescence intensity is proportional to drug accumulation by the cells.

RESULTS

Synthesis of Tetrameric 10mer H2009.1-PEG-polyGlu-(NHN=Dox) (Compound 5)

We sought to develop a targeted-doxorubicin conjugate that met three requirements. First, it must contain a high affinity targeting reagent that can be incorporated in a chemospecific fashion that allows for the targeting moiety to retain its activity. Second, the ratio of targeting reagent to drug must exceed the 1:1 ratio that can be achieved by direct conjugation. Third, the doxorubicin linker must be labile once the conjugate is internalized into the target cell so that doxorubicin can exert its cellular effects.

Toward these objectives, we synthesized the tetrameric 10mer H2009.1-polyGlu(NHN=Dox) (compound 5) according to Scheme 1. A defined length PG polymer serves as the drug carrier. Synthesis of the polyglutamic acid was accomplished by linear Fmoc peptide chemistry. A PEG moiety was incorporated at the amino terminus of the peptide to improve solubility and provide a flexible linker between the drug carrier and the targeting moiety. The amino terminus was capped with a maleimide so that the targeting reagent could be introduced by a chemoselective reaction with a free thiol.

While doxorubicin can be conjugated directly to the carboxylic acid of glutamic acid via an amide (5), we wished to incorporate the drug through an acid labile functionality. We and others have conjugated doxorubicin to a carrier by the formation of a hydrazone utilizing the ketone of 2-hydroxyacetyl group of doxorubicin (2, 25–28). To accomplish this, the carboxylic acid functionalities of the glutamic acid side chains were converted to hydrazide groups by treatment with *tert*-butyl carbazate (25). The number of converted carboxylate groups was determined using ¹H NMR by integrating the characteristic resonance of the Boc protecting group (9 protons per modification) at 1.41 ppm and maleimide resonance at 6.93 ppm (2 protons per polymer chain). Accordingly, 71% of the COOH groups were converted to CONHNHBoc groups, corresponding to 10–11 modified side chains. The Boc group was removed by treatment with trifluoroacetic acid to reveal the hydrazide.

The targeting reagent chosen was the peptide H2009.1 which binds specifically to the uniquely expressed integrin, $\alpha_v\beta_6$. This peptide was synthesized on a trylisine scaffold, as tetramerization has been demonstrated to increase affinity of this peptide for its cellular receptor (2). Although this peptide was originally selected as a 20 amino acid peptide, only the 10 N-terminal amino acids are necessary for binding to the integrin (S.L., M.J.M., and

K.C.B., unpublished results). As such, we chose to focus on the smaller peptide to ease the difficulty of the peptide synthesis. Importantly, in this format, the peptide can mediate cellular uptake. Synthesis was performed using linear Fmoc chemistry using Fmoc₄-Lys₂-Lys-Cys(Acm)- β -Ala-CLEAR Acid Resin. An acetamidomethyl (Acm) protected cysteine was incorporated before the branch point. This allows for a reactive thiol to be revealed upon treatment with Ag(I). Additionally, a PEG linker was placed between the core and the peptide to improve solubility, increase flexibility between the peptide branches, and prevent peptide aggregation. As a control, a peptide that contains the same amino acid composition but a different sequence was synthesized as well. This scrambled sequence peptide has been shown to have no binding affinity for $\alpha_v\beta_6$ (data not shown). These peptides were integrated into the polyGlu(NH₂) by reaction of the unique thiol of the peptide with the maleimide at the N-terminus of the polyGlu peptide.

The final step of the synthesis is the coupling of doxorubicin to the hydrazide to form the hydrazone linkage between the side chain of the glutamic acid and the ketone of doxorubicin. As the ketone functionality is chemically orthogonal to reactive groups found in naturally occurring amino acids, there was no need to protect the peptide side chains. Free drug was removed from the polymer by size exclusion chromatography under neutral pH conditions. The mole amount of doxorubicin in the conjugate was determined by UV absorbance at 481 nm. The concentration of compound 5 was determined by Edman sequencing analysis. The average drug loading is found to be 25% based on the number of glutamates or approximately 4 doxorubicin molecules per polymer unit.

Doxorubicin Is Released from the Carrier at Low pH

For the doxorubicin to be active, it must be released from the carrier so that it can exert its cellular effects within the nucleus. However, premature release of the drug will result in a decrease of the efficacy of the targeted therapeutic and will have similar side effects observed with the free drug. Internalization via a clatherin mediated endocytosis results in peptides entering the endosomal pathway with eventual localization in the lysosome (29–31). As the vesicles involved in this pathway have an acidic pH, pH sensitive linkers can be used for drug release. It is important to note that the pH drops at each sequential step of the internalization process, with early endosomes having a pH of 5.5–6.0; late endosomes exhibit a pH of approximately 5.0, whereas the lysosome's pH can drop to as low as 4.5. As such, if the drug conjugate becomes stalled at any one of these stages, drug release must occur at that pH. For this reason, the doxorubicin was coupled to the polyglutamic acid polymer via an acid labile hydrazone. To ensure that the drug conjugate is stable at the neutral pH but is released at the pH found within the lysosomal compartment of the cells, compound 5 was incubated at pH 7.0, pH 5.6, and pH 4.0 for defined times. Aliquots were removed and the release of doxorubicin was determined by HPLC. As shown in Figure 1, minimal drug was released at pH 7.0, even after 48 h. However, at pH 4.0, free doxorubicin was observed at 2 h. By 4 h, 25% of the drug has been released. This increases to 75% drug release at 51 h. As pH 4.5 is believed to be the minimum pH that would be reached in the cell, we also determined doxorubicin release at pH 5.6. The kinetics of drug release at pH 5.6 and pH 4.0 are the same (Figure 1). These results suggest that the drug will remain

conjugated to the carrier in an inactive form in the serum but will be released intracellularly due to the low pH environment found in the endosome or lysosome.

Tetrameric 10mer H2009.1-PEG-polyGlu(NHN=Dox) Selectivity Binds to $\alpha_v\beta_6$ Expressing Cells

Taking advantage of doxorubicin's fluorescence, the association of the conjugate with an $\alpha_v\beta_6$ expressing cell line was determined by flow cytometric analysis. Specifically, the conjugate was incubated with the human adenocarcinoma cell line H2009, which expresses $\alpha_v\beta_6$, or with H1299 cells, a human large cell lung cancer cell line which does not express the cellular receptor. As observed in Figure 2, the H2009 cells demonstrate 2-fold greater binding of targeted conjugate than the H1299 cells which do not bind the peptide. This binding is sequence specific, as the compound conjugated to the scrambled sequence peptide does not demonstrate significant binding to either cell line. Free doxorubicin internalizes into both cell lines equally. It should be noted that more doxorubicin is seen associated with the cells when they are treated with free drug versus the same concentration of targeted drug (based on total moles of doxorubicin). This phenomenon is not uncommon for drug conjugates and is most likely the result of receptor based transport of the drug versus the inherent cell permeability of the free drug (32, 33).

Of note, the fluorescence of doxorubicin is approximately 2-fold less when conjugated to the polymer compared to free doxorubicin (data not shown). As these studies were performed at 2 h of exposure to the polymer conjugate, in which time little doxorubicin is released from the polymer, the total uptake of the polymer conjugated drug is going to be underestimated by fluorescence experiments when compared to the free drug. However, the mean fluorescence intensity (MFI) determined by flow cytometry is greater than 4-fold higher for free doxorubicin than the conjugate on H2009 cells (Figure 2, panel A). As such, the data suggest that less drug is being delivered to the cells when conjugated to the polymer than when free doxorubicin is employed. Nonetheless, the differential binding of compound 5 to its target cell type compared to a nontargeted cell type is anticipated to reduce side effects, thus expanding the therapeutic window.

Tetrameric 10mer H2009.1-PEG-polyGlu(NHN=Dox) Is More Cytotoxic toward $\alpha_v\beta_6$ Expressing Cells

To determine if compound 5 could deliver doxorubicin selectivity to $\alpha_v\beta_6$ positive cells, H1299 and H2009 cells were incubated in the presence of varying concentrations (200 nM to 5 μ M) of unconjugated doxorubicin or doxorubicin linked to the polyglutamic acid-H2009.1 peptide conjugate. After 24 h, the drug was removed. Cells were incubated in fresh complete media for 72 h, after which the cell viability was determined. The results are shown in Figure 3. Both cell types are sensitive to free doxorubicin; pairwise comparison of cell viability after free doxorubicin treatment of both cell types did not demonstrate any statistical difference in the viability of these two cell types. However, after treatment with compound 5, more cell death was observed for the $\alpha_v\beta_6$ positive H2009 cells when compared to the $\alpha_v\beta_6$ negative H1299 cells at all concentrations ($p = 0.033$). Comparing the viability after treatment at 2 μ M with the doxorubicin-peptide conjugate, the difference in viability of the two cell types is 2-fold ($p = 0.024$).

There is a significant reduction in the potency of the doxorubicin when it is conjugated to the polymer. This could be due to reduced uptake of the drug and/or inefficient release of the drug from its carrier. H1299 and H2009 cells were incubated in the presence of 5 μ M unconjugated doxorubicin or doxorubicin linked to the polyglutamic acid-H2009.1 peptide conjugate. After 24 h, the drug was removed from the cells. Cells were then observed by phase contrast and fluorescence microscopy. Overlays of the resulting images are shown in Figure 4. Similar levels of unconjugated doxorubicin accumulate in the nuclei of both cell types (panel A and panel C). In contrast, higher levels of doxorubicin accumulate in $\alpha_v\beta_6$ -integrin positive H2009 cells (panel D) than in H1299 cells (panel B) when treated with compound 5. However, most of the drug appears to be perinuclear in the H2009 cells, suggesting that the release kinetics of the drug from the conjugate may be slower in living cells than expected from the *in vitro* incubation at pH 4.0 and 5.6.

DISCUSSION

Targeted therapeutic strategies for the treatment of solid tumors are dependent on the availability of high affinity and specificity ligands for cell surface cancer biomarkers as well as appropriate drug carriers which can deliver potent therapeutics in an active form. Toward this goal, we have synthesized a targeted polyglutamic acid-doxorubicin conjugate. This compound incorporated the high affinity $\alpha_v\beta_6$ -specific tetrameric H2009.1 peptide at the N-terminus of a 15 amino acid polymer of glutamic acid. Four doxorubicin molecules per peptidic targeting reagent are attached via an acid labile hydrazone. The conjugate is preferentially internalized into $\alpha_v\beta_6$ positive cells as witnessed by flow cytometric analysis and fluorescent microscopy. Cellular uptake is mediated by the H2009.1 peptide as less internalization of the doxorubicin-PG polymer is observed when it is conjugated to a scrambled sequence control peptide. Importantly, the conjugate is 2-fold more cytotoxic toward a targeted cell than a cell line that does not express the integrin.

Water soluble polymers as drug carriers have received much attention for the treatment of solid tumors (34, 35). These large molecular weight polymers offer several advantages over the use of nonconjugated therapeutics. First, the polymers can overcome the poor solubility associated with hydrophobic compounds. Second, the blood half-life of the drugs is often extended from minutes to hours or days, allowing the compound time to reach its target. Last, the polymer accumulates preferentially in the tumor because of the inherent leakiness of the tumor vasculature and is retained at this site because of the lack of a functional lymphatic system within the tumor. Passive targeting as a result of EPR effect increases the drug concentration in the tumor relative to other tissues.

Several different polymers have been explored as drug carriers (34, 35). Most prevalent are poly(ethylene glycol) (PEG), *N*-(2-hydroxypropylmethacrylamide) (HPMA) (36–38), and polyglutamic acid (PG) (4). Of these, only PG is biodegradable. As such, PEG and HPMA polymers must be kept under the size required for renal clearance. A variety of polymer–drug conjugates are in different stages of clinical trials (35). In addition to the nature of the polymer, the linkage between the drug and polymer plays an important role in the efficacy of the treatment (28, 37). Typically, one of three approaches have been employed: conjugation of the drug through a peptide linkage that is cleaved by endosomal and

lysosomal proteases such as cathepsin-B (37, 39), attachment via an acid labile hydrazone that is cleaved in the acidic environment of the endosome and lysosome (28, 37), or by esterification (4). In the case of the hydrazone or peptidyl linkers, release of the drug requires cellular uptake of the polymer. However, in the case of the ester conjugate found in XYOTAX, release of the drug occurs in the extracellular space; the antitumor efficacy is greater due to the long dwell time of the polymer within the tumor (4).

Toward the goal of cell-targeted therapeutics, cell-specific targeting agents have been incorporated into polymeric drug carrier compounds (5, 33, 40–43). Most have conjugated an antibody to a tumor associated biomarker into the polymer itself. Increased efficacy is observed for the targeted polymeric carrier when compared to the corresponding nontargeted polymers. In some cases, extended survival is observed in animal models, albeit with nonsolid tumor models (37, 42). However, conjugation of antibodies or other targeting agents such as peptides or small molecules through the side chains of the polymers can be problematic; it is difficult to control the coupling efficiency of the antibody, it reduces the total drug load by using available coupling sites, and aggregation can occur. To address these problems, Li and co-workers attached an antibody to the epidermal growth factor receptor (EGFR) to the N-terminus of a PG-doxorubicin polymer (5). A PEG moiety was placed between the PG and the antibody. As such, a single targeting agent was incorporated in a chemospecific fashion. Uptake of the drug carrier was observed in an EGFR positive cell line, while minimal internalization was observed in NIH3T3 fibroblasts. Importantly, the construct was able to affect cell death. It should be noted that doxorubicin was conjugated via an amide in this PG polymer, yet nuclear localization of doxorubicin was observed. The mechanism for the nuclear accumulation is unknown, but because of the rapid time frame in which it occurs, it is unlikely to be due to hydrolysis from the polymer.

While the larger polyglutamic acid polymers are often retained in tumors due to the EPR effect and have potentially higher drug capacity, we chose to focus on a defined 15 amino acid polymer. Smaller conjugates are expected to demonstrate better tumor penetration. Additionally, the smaller peptide is easier to modify in a regiospecific fashion. However, the tradeoff is lower drug loading capacities. Additionally, the tumor accumulation due to EPR may be less. On the other hand, the ease in which a tumor targeting agent can be incorporated into the polymer is a benefit, and active targeting can potentially be enhanced by more efficient escape from the vasculature and increased diffusion into the core of the tumor. Further *in vivo* experiments will be required to address the role of passive versus active targeting and tumor penetration of the smaller polymers.

The tumor targeting agent employed in this case is the tetrameric peptide H2009.1 which displays high affinity for $\alpha_v\beta_6$ (2, 3). This peptide does not bind to other RGD-binding integrins and has a half-maximal cell binding affinity in the 40–60 pM range. The integrin $\alpha_v\beta_6$ is emerging as an appealing cancer biomarker for targeted therapy. Expression of the β_6 subunit is restricted to epithelial cells, and its expression is low or undetectable in most normal adult primate tissues (44). This integrin is up-regulated in a variety of epithelial derived malignancies, including lung, oral, colon, breast, and ovarian cancers (3, 45–49). Expression of $\alpha_v\beta_6$ correlates with features found in more aggressive tumors, such as enhanced cell migration and invasion (50–53). β_6 expression is predictive of reduced

survival time for patients with nonsmall cell lung carcinoma (3) and colorectal cancer (54). Thus, this targeting peptide is anticipated to have utility in molecular guided therapies for these aggressive tumor types.

While uptake of compound 5 is greater for cell line expressing $\alpha_v\beta_6$, the difference is only 2-fold, as observed by flow cytometry. Additionally, the conjugate is only 2-fold more cytotoxic toward H2009 cells compared to H1299 cells. However, it should be noted that there is a 60 fold difference in binding of the H2009.1 peptide for H2009 cells compared to the H1299 cells, when comparing binding of the individual peptide-displayed phage clones (1). This suggests that the polyGlu-doxorubicin portion of the molecule may dampen the selective uptake of the peptide by contributing to non- $\alpha_v\beta_6$ dependent cellular uptake. The flow cytometry data supports this, as some uptake of the conjugate containing the scramble control peptide is observed for both H1299 and H2009 cells. However, the cellular uptake of the nontargeted conjugate is substantially less than the freely cell permeable doxorubicin, and as such, there is a widening of the therapeutic index.

The cytotoxicity of compound 5 is less than that of free doxorubicin. The reduced efficacy of the conjugate is most likely due to a combination of reduced uptake of the conjugate compared to a cell permeable small molecule and poor release of the drug from the polymer scaffold. Flow cytometry shows that uptake of the conjugate is less than that of the free drug for the targeted cells. This is not uncommon for targeted drug conjugates. This limitation can be overcome by increasing the drug loads further or by moving to more cytotoxic agents. For example, the first line treatment of NSCLC usually employs paclitaxel whose IC_{50} is 40-fold lower than that of doxorubicin for H2009 cells (personal communication, Drs. John D. Minna and Michael Peyton). However, it should also be noted that the fluorescence of doxorubicin is 2-fold less when conjugated to the polymer compared to free doxorubicin (data not shown). As these studies were performed at 2 h, in which time little doxorubicin is released from the polymer, the total uptake of the polymer conjugated drug will be underestimated by fluorescence experiments. However, the differences in MFI cannot be accounted for by this effect alone, suggesting that the targeted polymer cannot deliver as much doxorubicin to H2009 cells as when the cells are treated with free drug.

Perhaps more importantly, microscopy reveals that the drug is perinuclear. This suggests that the drug is not being efficiently released from the conjugate and moving into the nucleus where it exerts its primary effect. While others have shown that doxorubicin hydrazones are hydrolyzed within the cell (28, 37,55), hydrolysis from this conjugate may not occur within the appropriate time frame. The linker type, linker length, and mechanism of cellular uptake have been shown to influence the release of the drugs from polymeric carriers (28). This suggests that further optimization of the drug tether is required before progressing to *in vivo* experiments.

In summary, we have completed the synthesis of an integrin targeted polyglutamic acid-doxorubicin conjugate using a peptide that is highly specific for the tumor biomarker, $\alpha_v\beta_6$. To our knowledge, this is the first targeted polyglutamic acid drug conjugate which employs a peptidic targeting agent, and the only one that utilizes an acid labile linker between the therapeutic and polymer. This compound exhibits integrin specific cytotoxicity. Future work

in lowering the background uptake of the polyglutamic acid-doxorubicin conjugates and improvement of the drug release by optimizing the linker will be required to improve the therapeutic properties of this compound.

ACKNOWLEDGMENT

We thank Bethany Gray for insightful discussions and critical reading of the manuscript. This work was supported by the NIH (1R01CA106646) and the Welch Foundation (I1622). This paper represents publication # CSCN034 from the Cell Stress and Cancer Nanomedicine Program of the Simmons Comprehensive Cancer Center.

LITERATURE CITED

1. Oyama T, Sykes KF, Samli KN, Minna JD, Johnston SA, Brown KC. Isolation of lung tumor specific peptides from a random peptide library: generation of diagnostic and cell-targeting reagents. *Cancer Lett.* 2003; 202:219–230. [PubMed: 14643452]
2. Zhou X, Chang Y, Oyama T, McGuire MJ, Brown KC. Cell-specific delivery of a chemotherapeutic to lung cancer cells. *J. Am. Chem. Soc.* 2004; 129:15656–15657. [PubMed: 15571383]
3. Elayadi AN, Samli KN, Prudkin L, Liu YH, Bian A, Xie XJ, Roth JA, Wistuba II, McGuire MJ, Brown KC. A peptide selected by biopanning identifies the integrin $\alpha_v\beta_6$ as a prognostic biomarker for non-small cell lung cancer. *Cancer Res.* 2007; 67:5889–5895. [PubMed: 17575158]
4. Li C. Poly(l-glutamic acid)-anticancer drug conjugates. *Adv. Drug Delivery Rev.* 2002; 54:695–713.
5. Vega J, Ke S, Fan Z, Wallace S, Charnsangavej C, Li C. Targeting doxorubicin to epidermal growth factor receptors by site-specific conjugation of C225 to poly(l-glutamic acid) through a polyethylene glycol spacer. *Pharm. Res.* 2003; 20:826–832. [PubMed: 12751641]
6. Zunino F, Pratesi A, Micheloni A. Poly(carboxylic acid) polymers as carriers for anthracyclines. *J. Controlled Release.* 1989; 10:65–73.
7. Hoes CJT, Potman W, van Heeswijk WAR, Mud J, de Grooth BG, Greve J, Feijen J. Optimization of macromolecular prodrugs of the antitumor antibiotic adriamycin. *J. Controlled Release.* 1985; 2:205–213.
8. Li C, Yu FF, Newman RA, Cabral F, Stephens LC, Hunter N, Milas L, Wallace S. Regression of well-established tumors using a novel water-soluble poly(l-glutamic acid)-paclitaxel conjugate. *Cancer Res.* 1998; 58:2404–2409. [PubMed: 9622081]
9. Li C, Price JE, Milas L, Hunter NR, Ke S, Yu DF, Charnsangavej C, Wallace S. Antitumor activity of poly(l-glutamic acid)-paclitaxel on syngeneic and xenografted tumors. *Clin. Cancer Res.* 1999; 5:891–897. [PubMed: 10213226]
10. Milas L, Mason KA, Hunter N, Li C, Wallace S. Poly(l-glutamic acid)-paclitaxel conjugate is a potent enhancer of tumor radiocurability. *Int. J. Rad. Oncol. Biol. Phys.* 2003; 55:707–712.
11. Auzenne E, Donato NJ, Li C, Leroux E, Price RE, Farquhar D, Klostergaard J. Superior therapeutic profile of poly-l-glutamic acid-paclitaxel copolymer compared with taxol in xenogeneic compartmental models of human ovarian carcinoma. *Clin. Cancer Res.* 2002; 8:573–581. [PubMed: 11839679]
12. Singer JW, de Vries P, Bhatt R, Tulinsky J, Klein P, Li C, Milas L, Lewis RA, Wallace S. Conjugation of camptothecins to poly-(l-glutamic acid). *Ann. N.Y. Acad. Sci.* 2000; 922:136–150. [PubMed: 11193889]
13. Zou Y, Wu QP, Tansy D, Chow MC, Hung C, Charnsangavej C, Wallace S. Effectiveness of water soluble poly(l-glutamic acid)-camptothecin conjugate against resistant human lung cancer xenografted in nude mice. *Int. J. Oncol.* 2001; 18:331–336. [PubMed: 11172600]
14. Zou C, Brewer M, Cao X, Zang R, Lin J, Deng Y, Li C. Antitumor activity of 4-(N-hydroxyphenyl)-retinamide conjugated with poly(l-glutamic acid) against ovarian cancer xenografts. *Gynecol. Oncol.* 2007; 107:441–449. [PubMed: 17854871]
15. Zhu G, Cao X, Chang JY, Milas L, Li C. Polymeric retinoid prodrug PG-4HPR enhances the radiation response of lung cancer. *Oncol. Rep.* 2007; 18:645–651. [PubMed: 17671714]

16. Wen X, Jackson EF, Price RE, Kim E, Wu Q, Wallace S, Charsangavej C, Gelovani JG, Li C. Synthesis and characterization of poly(L-glutamic acid) gadolinium chelate: A new biodegradable MRI contrast reagent. *Bioconjugate Chem.* 2004; 15:1408–1415.
17. Melacon MP, Wang Y, Wen X, Bankson JA, Stephens LC, Jasser S, Gelovani JG, Myers JN, Li C. Development of macromolecular dual-modality MR-optical imaging for sentinel lymph node mapping. *Invest. Rad.* 2007; 42:569–578.
18. Jackson EF, Esparza-Coss E, Wen X, Ng CS, Daniel SL, Price RE, Rivera B, Charnsangavej C, Gelovani JG, Li C. Magnetic resonance imaging of therapy-induced necrosis using gadolinium-chelated polyglutamic acids. *Int. J. Rad. Oncol. Biol. Phys.* 2007; 68:830–838.
19. Singer JW. Paclitaxel poliglumex (XYOTAX™, CT-2103): A macromolecular taxane. *J. Controlled Release.* 2005; 109:120–126.
20. Nemunaitis J, Cunningham C, Senzer NS, Gray M, Oldham F, Pippen J, Mennel R, Eisenfeld A. Phase I study of CT-2103, a polymer-conjugated paclitaxel, and carboplatin in patients with advanced solid tumors. *Cancer Invest.* 2005; 23:671–676. [PubMed: 16377585]
21. Bonomi P. Paclitaxel poliglumex (PPX, CT-2103): macromolecular medicine for advanced non-small-cell lung cancer. *Expert Rev. Anticancer Ther.* 2007; 74:415–422. [PubMed: 17428162]
22. Jain RK. Delivery of molecular and cellular medicine to solid tumors. *Adv. Drug Delivery Rev.* 2001; 46:149–168.
23. Phelps RM, Johnson BE, Ihde DC, Gazdar AF, Carbone DP, Linnoila P, Matthews MJ, Bunn PA, Carney D, Minna JD, Mulshine JL. NCI-Navy medical oncology branch cell line database. *J. Cell. Biochem.* 1996:32–91.
24. Tian Y, Bromberg L, Lin SN, Hatton TA, Tam KC. Complexation and release of doxorubicin from its complexes with pluronic P85-*b*-poly(acrylic acid) block copolymers. *J. Controlled Release.* 2007; 121:137–145.
25. Bae Y, Fukushima S, Harada A, Kataoka K. Design of environment-sensitive supramolecular assemblies for intracellular drug delivery: Polymeric micelles that are responsive to intracellular pH change. *Angew. Chem., Int. Ed.* 2003; 42:4640–4643.
26. Ulbrich K, Subr V. Polymeric anticancer drugs with pH-controlled activation. *Adv. Drug Delivery Rev.* 2004; 56:1023–1050.
27. Lee CC, Gillies ER, Fox ME, Guillaudeu SJ, Frechet MJM, Dy EE, Szoka FC. A single dose of doxorubicin-functionalized bow-tie dendrimer cures mice bearing C-26 colon carcinomas. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103:16649–16654. [PubMed: 17075050]
28. Hovorka O, Etrych T, Subr V, Strohalm J, Ulbrich K, Rihova B. HPMA based macromolecular therapeutics: Internalization, intracellular pathway and cell death depend on the character of covalent bond between the drug and the peptide spacer and also on spacer composition. *J Drug Targeting.* 2006; 14:391–403.
29. Feener EP, King GL. The biochemical and physiological characteristics of receptors. *Adv. Drug Delivery Rev.* 1998; 29:197–213.
30. Bareford L, Swaan P. Endocytotic mechanisms for targeted drug delivery. *Adv. Drug Delivery Rev.* 2007; 59:748–758.
31. Conner S, Schmid S. Regulated portals of entry into the cell. *Nature.* 2003; 422:37–44. [PubMed: 12621426]
32. Rodrigues PC, Beyer U, Schumacher P, Roth T, Fiebig HH, Unger C, Messori L, Orioli P, Paper DH, Mulhaupt R, Kratz F. Acid-sensitive polyethylene glycol conjugates of doxorubicin: preparation, in vitro efficacy and intracellular distribution. *Bioorg. Med. Chem.* 1999; 7:2517–2524. [PubMed: 10632061]
33. Luo Y, Bernshaw NJ, Lu Z-R, Kopecek J, Prestwich GD. Targeted delivery of doxorubicin by HMPA copolymers-hyaluronan bioconjugates. *Pharm. Res.* 2002; 19:396–402. [PubMed: 12033370]
34. Vicent MJ, Duncan R. Polymer conjugates: nanosized medicines for treating cancer. *Trends Biotechnol.* 2006; 24:39–47. [PubMed: 16307811]
35. Duncan R. Polymer conjugates as anticancer nanomedicines. *Nat. Rev. Cancer.* 2006:688–701. [PubMed: 16900224]

36. Satchi-Fainaro R, Puder M, Davies JW, Tran HT, Sampson DA, Greene AK, Corfas G, Folkman J. Targeting angiogenesis with a conjugate of HPMa copolymer and TNP-470. *Nat. Med.* 2004; 10:255–261. [PubMed: 14981512]
37. Kovar M, Kovar L, Subr V, Etrych T, Ulbrich K, Mrkvan T, Loucka J, Rihova B. HPMa copolymers containing doxorubicin bound by a proteolytically or hydrolytically cleavable bond: comparison of biological properties in vitro. *J. Controlled Release.* 2004; 99:301–314.
38. Kopecek J, Kopeckova P, Minko T, Lu Z. HPMa copolymer-anticancer drug conjugates: design, activity, and mechanism of action. *Eur. J. Pharm. Biopharm.* 2000; 50:61–81. [PubMed: 10840193]
39. Dubowchik GM, Firestone RA, Padilla L, Willner D, Hofstead SJ, Mosure K, Knipe JO, Lasch SJ, Trail PA. Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: Model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. *Bioconjugate Chem.* 2002; 13:855–869.
40. Tang A, Kopeckova P, Kopecek J. Binding and cytotoxicity of copolymer conjugates to lymphocytes mediated by receptor-binding epitopes. *Pharm. Res.* 2003; 20:360–367. [PubMed: 12669954]
41. Kovar M, Mrkvan T, Strohalm J, Etrych T, Ulbrich K, Stastny M, Rihova B. HPMa copolymer-bound doxorubicin targeted to tumor-specific antigen of BCL1 mouse B cell leukemia. *J. Controlled Release.* 2003; 92:315–330.
42. Ulbrich K, Etrych T, Chytil P, Jelinkova M, Rihova B. Antibody-targeted polymer-doxorubicin conjugates with pH-controlled activation. *J. Drug Targeting.* 2004; 12:477–489.
43. Nan A, Ghandehari H, Hebert C, Siavash H, Nititakis N, Reynolds M, Sauk JJ. Water-soluble polymers for targeted drug delivery to human squamous carcinoma of head and neck. *J. Drug Targeting.* 2005; 13:189–197.
44. Breuss JM, Gillette M, Lu L, Sheppard D, Pytela R. Restricted distribution of integrin β_6 mRNA in primate epithelial tissues. *J. Histochem. Cytochem.* 1993;41.
45. Ahmed N, Riley C, Rice GE, Quinn MA, Baker MS. $\alpha_v\beta_6$ Integrin- A marker for the malignant potential of epithelial ovarian cancer. *J. Histochem. Cytochem.* 2002; 50:1371–1379. [PubMed: 12364570]
46. Arihiro K, Kaneko M, Fujii S, Inai K, Yokosaki Y. Significance of $\alpha_9\beta_1$ and $\alpha_v\beta_6$ integrin expression in breast carcinoma. *Breast Cancer.* 2000; 7:19–26. [PubMed: 11029766]
47. Jones J, Sugiyama M, Watt FM, Speight PM. Integrin expression in normal, hyperplastic, dysplastic, and malignant oral epithelium. *J. Pathol.* 1993; 169:235–243. [PubMed: 8445489]
48. Kawashima A, Tsugawa S, Boku A, Kobayashi M, Minamoto T, Nakanishi I, Oda Y. Expression of α_v integrin family in gastric carcinomas: Increased $\alpha_v\beta_6$ is associated with lymph node metastasis. *Pathol. Res. Pract.* 2003; 199:57–64. [PubMed: 12747466]
49. Breuss JM, Gallo J, DeLisser HM, Klimanskaya IV, Folkesson HG, Pittet JF, Nishimura SL, Aldape K, Landers DV, Carpenter W, Gillette M, Sheppard D, Matthey MA, Albelda SM, Kramer RH, Pytela R. Expression of the β_6 integrin in development, neoplasia, and tissue repair suggests a role in epithelial remodeling. *J. Cell Sci.* 1995; 108:2241–2251. [PubMed: 7673344]
50. Thomas GJ, Poomsawat S, Lewis MP, Hart IR, Speight PM, Marshall JF. $\alpha_v\beta_6$ Integrin upregulates matrix metalloproteinase 9 and promotes migration of normal oral keratinocytes. *J. Invest. Derm.* 2001; 116:898–904. [PubMed: 11407978]
51. Thomas GJ, Lewis MP, Whawell SA, Russell A, Sheppard D, Hart IR, Speight PM, Marshall JF. Expression of the $\alpha_v\beta_6$ integrin promotes migration and invasion in squamous carcinoma cells. *J. Invest. Derm.* 2001; 117:67–73. [PubMed: 11442751]
52. Thomas GJ, Lewis MP, Hart IR, Marshall JF, Speight PM. $\alpha_v\beta_6$ integrin promotes invasion of squamous carcinoma cells through up-regulation of matrix metalloproteinase-9. *Int. J. Cancer.* 2001; 92:641–650. [PubMed: 11340566]
53. Agrez M, Gu X, Turton J, Meldrum C, Niu J, Antalis T, Howard EW. The $\alpha_v\beta_6$ integrin induces gelatinase secretion in colon cancer cells. *Int. J. Cancer.* 1999; 81:90–97. [PubMed: 10077158]
54. Bates RC, Bellovin DI, Brown C, Maynard E, Wu B, Kawakatsu H, Sheppard D, Oettgen P, Mercurio AM. Transcriptional activation of integrin β_6 during the epithelial-mesenchymal

- transition defines a novel prognostic indicator of aggressive colon cancer. *J. Clin. Invest.* 2005; 115:339–347. [PubMed: 15668738]
55. Kovar L, Strohalm J, Chytil P, Mrkvan T, Kovar M, Hovorka O, Ulbrich K, Rihova B. The same drug but a different mechanism of action: Comparison of free doxorubicin with two different N-(2-hydroxypropyl)methacrylamide copolymer-bound doxorubicin conjugates in EL-4 cancer cell line. *Bioconjugate Chem.* 2007; 18:894–902.

Figure 1. pH dependent release of doxorubicin from the polyglutamic acid-H2009.1 peptide conjugate. Polyglutamic acid-H2009.1-doxorubicin conjugate was dissolved in aqueous buffer at pH 7.0, pH 5.6, or pH 4.0 and incubated at 37 °C. At the indicated times, aliquots were removed and the ratio of unconjugated to conjugated doxorubicin was assessed by reverse phase HPLC on an analytical C18 column.

Figure 2.

Cell targeted delivery of doxorubicin mediated by a cell-specific peptide. H2009 (panel A) and H1299 (panel B) cells were incubated for 2 h in the presence of 30 μ M unconjugated doxorubicin (red line) or doxorubicin linked to the poly glutamic acid-H2009.1 (blue line) peptide conjugate or scrambled peptide conjugate (black line). At the end of this incubation period, the drug was removed; cells were washed with PBS⁺/0.1% BSA 4 times as well as twice with 20 mM HCl-glycine, pH 2.2. Uptake of doxorubicin into cells was assessed by

flow cytometry, counting 10 000 events per sample. Results obtained with untreated cells are shown in gray shaded area.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

Cell targeted delivery of doxorubicin preferentially kills $\alpha_v\beta_6$ -integrin positive tumor cells. H1299 (■ - black, red) and H2009 (● - black, red) cells were incubated in the presence of varying concentrations (200 nM to 5 μ M) unconjugated doxorubicin (black lines) or doxorubicin linked to the polyglutamic acid-H2009.1 peptide conjugate (red lines). After 24 h, the drug was removed; cells were washed and incubated in fresh complete media for 72 h. Cell viability was determined using the Cell Titer-Glo assay (Promega Corp). The results shown are an average of 3 independent experiments. Pairwise comparison of cell viability after free dox treatment of both cell types did not demonstrate any statistical difference whereas the difference in the viability of these two cell types after poly-glu-dox-H2009.1 conjugate treatment was statistically significant ($p = 0.033$).

Figure 4.

Cell targeted delivery of doxorubicin mediated by a cell-specific peptide. H1299 (A and B) and H2009 (C and D) cells were incubated in the presence of 5 μ M unconjugated doxorubicin (A and C) or doxorubicin linked to the polyglutamic acid-H2009.1 peptide conjugate (B and D). After 24 h, the drug was removed; cells were washed and incubated in fresh complete media for 2 h. Cells were observed by phase contrast and fluorescence microscopy at 400 $\times$ magnification. Overlays of the resulting images are shown.

Scheme 1. Synthesis of Targeted PG-Doxorubicin Conjugates

^a The structure of the tetrameric H2009.1 peptide is shown in the inset.