Targeted Delivery of Paclitaxel to EphA2-Expressing Cancer Cells

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Abstract

Purpose: YSA is an EphA2-targeting peptide that effectively delivers anticancer agents to prostate cancer tumors. Here, we report on how we increased the drug-like properties of this delivery system.

Experimental Design: By introducing non-natural amino acids, we have designed two new EphA2 targeting peptides: YNH, where norleucine and homoserine replace the two methionine residues of YSA, and dYNH, where a D-tyrosine replaces the L-tyrosine at the first position of the YNH peptide. We describe the details of the synthesis of YNH and dYNH paclitaxel conjugates (YNH-PTX and dYNH-PTX) and their characterization in cells and in vivo.

Results: dYNH-PTX showed improved stability in mouse serum and significantly reduced tumor size in a prostate cancer xenograft model and also reduced tumor vasculature in a syngeneic orthotopic allograft mouse model of renal cancer compared with vehicle or paclitaxel treatments.

Conclusion: This study reveals that targeting EphA2 with dYNH drug conjugates could represent an effective way to deliver anticancer agents to a variety of tumor types. Clin Cancer Res; 19(1); 128–37. ©2012 AACR.

Introduction

Targeted delivery of chemotherapeutic agents, such as paclitaxel (PTX; ref. 1), is preferred to systemic administration because such agents are nonselective (2), causing severe adverse side effects. A potential solution to this difficult problem is to take advantage of the growing number of tumor specific cell surface biomarkers to design targeted delivery modules (3, 4). Usage of these targets has resulted in a wide variety of tumor-homing motifs coupled to a variety of anticancer and imaging agents (3–8). Humanized monoclonal antibodies are currently the most advanced tumor specific cell surface biomarkers to design targeted delivery systems (3, 4). Usage of these targets has resulted in a wide variety of tumor-homing motifs coupled to a variety of anticancer and imaging agents (3–8). Humanized monoclonal antibodies are currently the most advanced targeting peptides: YNH, where norleucine and homoserine replace the two methionine residues of YSA, and dYNH, where a D-tyrosine replaces the L-tyrosine at the first position of the YNH peptide. We describe the details of the synthesis of YNH and dYNH paclitaxel conjugates (YNH-PTX and dYNH-PTX) and their characterization in cells and in vivo.

Results: dYNH-PTX showed improved stability in mouse serum and significantly reduced tumor size in a prostate cancer xenograft model and also reduced tumor vasculature in a syngeneic orthotopic allograft mouse model of renal cancer compared with vehicle or paclitaxel treatments.

Conclusion: This study reveals that targeting EphA2 with dYNH drug conjugates could represent an effective way to deliver anticancer agents to a variety of tumor types. Clin Cancer Res; 19(1); 128–37. ©2012 AACR.

The Eph receptors are a family of receptor tyrosine kinases that play a role in neuronal connectivity, blood vessel development, and numerous other physiological processes by influencing cell shape and migration through their effects on the actin cytoskeleton as well as cell survival and proliferation (25–27). They are also involved in numerous pathologic processes, including cancer (28, 29). In tumor tissue, the Eph receptors modulate cell–cell communication involving not only tumor cells but also tumor stroma and vasculature (28, 29), thus differentiating them from traditional oncogenes that function only in tumor cells. Specifically, the family member EphA2 is overexpressed in many types of cancers (1, 26, 27, 30–32), where its presence has risk of an adverse immune response, and high manufacturing costs (9, 10).

Short peptides that target tumor-specific markers also show promise for selective tumor targeting. Tumor-specific peptide sequences able to bind cancer cell-specific motifs have been identified using combinatorial chemistry methods and phage display techniques (11–23). For example, peptides such as iRGD and RC-160 have been used to target neuropilin-1 (5) and the somatostatin receptor (3), respectively. Peptides can be conjugated to anticancer agents in various drug/peptide ratios. Furthermore, some tumor targeting peptides are able to not only target tumor cells but also facilitate cell penetration of both the peptide and the payload (24). The ability to identify tumor cells as well as mediate drug internalization can result in an increase in drug activity and a reduction in drug toxicity, thus making homing peptides attractive drug delivery vehicles.

The Eph receptors are a family of receptor tyrosine kinases that play a role in neuronal connectivity, blood vessel development, and numerous other physiological processes by influencing cell shape and migration through their effects on the actin cytoskeleton as well as cell survival and proliferation (25–27). They are also involved in numerous pathologic processes, including cancer (28, 29). In tumor tissue, the Eph receptors modulate cell–cell communication involving not only tumor cells but also tumor stroma and vasculature (28, 29), thus differentiating them from traditional oncogenes that function only in tumor cells. Specifically, the family member EphA2 is overexpressed in many types of cancers (1, 26, 27, 30–32), where its presence has
Targeted Delivery of Paclitaxel

Translational Relevance

Overexpression of the EphA2 positively correlates with tumor malignancy and poor prognosis. For this reason, EphA2 is an attractive target for cancer cell specific drug delivery. In this study, we report on the development of dYNH, an EphA2 targeting peptide that when coupled to paclitaxel (PTX) has favorable pharmacologic properties and possesses powerful antitumor activity in vivo. dYNH-PTX may allow for an expanded therapeutic index of PTX as well as precluding the need for complex formulations and long infusion times.

been linked to tumor malignancy and poor prognosis (26, 32). In addition, EphA2 is expressed in the tumor vasculature and promotes tumor angiogenesis (33), making it a compelling target to reduce angiogenesis at the tumor site. Thus, because of its association with a wide variety of cancers and its multifaceted role in cancer progression, EphA2 is an attractive target for drug design (25).

Amino acid sequence (YSA; YSAYPDSVPMMS) is a peptide identified by phage display that selectively targets EphA2 and, similarly to the natural EphA2 ligand ephrin-A1, causes receptor activation and internalization (17, 34). A version of this peptide coupled to magnetic nanoparticles was used to capture circulating ovarian cancer cells in vivo (35) and YSA-functionalized nanogels were used to chemosensitize cancer cells expressing EphA2 by mediating siRNA delivery and internalization (36, 37). We recently reported that when YSA is conjugated to PTX, it targets EphA2 overexpressing prostate cancer cells in vivo, inhibiting tumor growth in a prostate cancer xenograft model more effectively than PTX alone (1). Here, we describe our efforts to improve the pharmacologic properties and efficacy of YSA-drug conjugates by introducing non-natural amino acids in the targeting motif.

Materials and Methods

Chemical synthesis and purification

Unless otherwise noted, all reagents and anhydrous solvents were obtained from commercial sources and used without purification. All reactions were conducted in oven-dried glassware. All reactions involving air or moisture sensitive reagents were conducted under a nitrogen atmosphere. silica gel chromatography was conducted using prepacked silica gel or C-18 cartridges (RediSep). All final compounds were purified to more than 95% purity, as determined by a HPLC Breeze from Waters Co. using an Atlantis T3 5.0 μmol/L 4.6 mm × 150 mm reverse phase column. 1D and 2D NMR spectra were recorded on a bruker avance spectrometer equipped with a TCI-cryoprobe. NMR spectra were acquired on a 600 MHz Bruker Avance spectrometer equipped with a TCI-cryoprobe. ITC were measured with Model ITC200 from Microcal/GE Life Sciences.

ELISA-based competition and binding assays

For competition assays, EphA2 Fc (R&D Systems) diluted to 1 μg/ml in Tris-buffered saline and Tween 20 (TBST) buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, with 0.01% Tween 20) was immobilized in protein A-coated wells. The wells were then incubated for 3 hours with 40 μL 0.01 mmol/L ephrin-A5 fused to alkaline phosphatase (ephrin-A5 AP) in culture medium diluted in TBST in the presence of different concentrations of YNH-PTX or dYNH-PTX. The concentration of ephrin-A5 AP was calculated based on AP activity measurements. Bound ephrin-A5 AP was quantified by adding p-nitrophenyl phosphate as the substrate and measuring the absorbance at 405 nm. Absorbance from wells where human Fc (R&D Systems) was immobilized instead of EphA2 Fc was subtracted as the background. Curves for EphA2 Fc binding to YSA-PTX, YNH-PTX, dYNH-PTX, and DYP-PTX were determined by adding different concentrations of EphA2 Fc to high binding capacity ELISA half-well plates (Corning) precoated with streptavidin (Pierce Biotechnology) and 20 μL 1 μmol/L biotinylated peptides. Bound EphA2 Fc was detected with an AP conjugated anti-human IgG.
antibody (Promega, 1:2,000 dilution in TBST). Absorbance at 405 nm was measured following incubation with 0.2 mg/mL 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma-Aldrich) in citric acid as a substrate, and the absorbance in wells without EphA2 was subtracted as background. Inhibition and binding curves were analyzed using nonlinear regression and the program Prism (GraphPad Software, Inc.).

**Fluorescence cell imaging**

PC-3M-luc-C6 Bioware (Caliper) and LNCaP prostate cancer cells (American Type Culture Collection) were grown in RPMI 1640 medium (Mediatech, Inc.) with 10% FBS and Pen/Strep. For quantum dot internalization experiments, PC3M and LNCaP cells were grown overnight on glass coverslips. The conditioned medium was then removed from the cells, supplemented with 10 mmol/L HEPES and stored at 4°C, and the cells were serum starved for 3 hours in serum-free medium. The cells were then treated with 300 μL of 100 μmol/L biotinylated YNH-PTX, dYNH-PTX, YSA-PTX, or DYP-PTX in quantum dot-binding buffer for 20 minutes on ice, followed by 20 nmol/L streptavidin-conjugated Qdot 655 (Invitrogen/Molecular Probes) in binding buffer for 20 minutes on ice. After removing the quantum dot solution, the cells were washed with the binding buffer and incubated with the stored conditioned medium in a 37°C CO2 incubator for 2 hours to allow internalization of the EphA2-peptide-PTX quantum dots complexes. The cells were then washed with ice cold PBS, fixed in 4% formaldehyde for 10 minutes, permeabilized for 3 minutes with 0.5% Triton-X100 in PBS, and incubated for 1 hour with PBS containing 10% goat serum. For EphA2 staining, the coverslips were incubated with a rabbit anti-EphA2 antibody (Life Technologies/Invitrogen) followed by a secondary anti-rabbit antibody conjugated with Alexa Fluor 488 (Life Technologies/Molecular Probes). For staining of lysosomes, the coverslips were incubated with polyclonal rabbit anti-human Lamp1 antibody (38) followed by a secondary anti-rabbit antibody conjugated with Alexa Fluor 568 (Life Technologies/Molecular Probes). The nuclei were counterstained with DAPI. Images were obtained with an Inverted TE300 Nikon fluorescence microscope and processed using Adobe Photoshop.

To image EphA2 internalization and colocalization with lysosomes after stimulation with ephrin-A1 Fc or the PTX-coupled peptides, PC3M cells plated on glass coverslips were serum starved for 1 hour in serum-free medium and then stimulated with 0.2 μg/mL Fc (as a negative control), 0.2 μg/mL ephrin-A1 Fc (as a positive control), or 100 μmol/L PTX-coupled peptides for 2 hours. The cells were then fixed, permeabilized, and labeled as described above. Images were acquired using a Radiance 2100 MP confocal microscope.

**Immunoprecipitation and immunoblotting**

PC-3M-luc-C6 cells were serum-starved for 1 hour in serum-free medium and treated for 1 hour with 0.2 μg/mL human Fc (as a negative control), 0.2 μg/mL ephrin-A1 Fc (as a positive control), 100 μmol/L YSA-PTX, 100 μmol/L DYP-PTX, 100 μmol/L YNH-PTX, or 100 μmol/L dYNH-PTX. The cells were then placed on ice, rinsed once with cold PBS, and incubated for 20 minutes at 4°C with a 0.5 mg/mL EZ-link sulfo-NHS-biotin (Thermo Scientific/Pierce) in PBS. The cells were then washed 3 times with a 100 mmol/L glycine in PBS to quench the biotinylation reaction, followed by PBS. The cells were lysed in modified RIPA lysis buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mmol/L Tris, and pH 8.0) containing protease inhibitors and 1 mmol/L sodium orthovanadate. For immunoprecipitations, the lysates were incubated with 1 μg anti-EphA2 antibody (Millipore-Upstate, Inc.) immobilized on GammaBind Sepharose beads (GE Healthcare Life Sciences). Immunoprecipitates and lysates were probed by immunoblotting with an antiphosphotyrosine antibody (Millipore, Inc.), streptavidin coupled to horseradish peroxidase (HRP; Thermo Scientific/Pierce), or anti-EphA2 antibody (Life Technologies/Invitrogen). Lysates of PC-3M-luc-C6 and RENCA cells were probed by immunoblotting with the EphA2 Millipore antibody and with a GAPDH antibody (AbCam).

**Measurement of peptide stability**

The peptides were incubated at 37°C with cultured PC3 cells or in mouse serum for different times. Medium diluted 1:3 or serum diluted 1:20 (corresponding to a final concentration of 25 μmol/L for YSA-PTX and YNH-PTX and 75 μmol/L for dYNH-PTX) were incubated in ELISA wells precoated with protein A and EphA2 Fc for 2 hours in the presence of 40 μL 0.01 nmol/L ephrin-A5 AP. Inhibition of EphA2-ephrin-A5 binding was measured as described above. Absorbance from wells coated with Fc and incubated with ephrin-A5 AP and culture medium or serum was subtracted as the background. Absorbance obtained from wells incubated with culture medium or mouse serum not containing any peptide was used to determine the 0% inhibition level (0% peptide remaining) and absorbance obtained in the presence of the peptides mixed with culture medium or serum immediately before adding them to the ELISA wells was used for normalization (100% peptide remaining).

**In vivo prostate cancer xenograft studies**

As general procedure, PC-3M-luc-C6 cells (1 × 10⁶) were injected subcutaneously into 6- to 8-week-old female athymic nude mice (Harlan Labs) and the peptide-PTX conjugates were dissolved in a mixture of 84% PBS, 8% dimethyl sulfoxide (DMSO), and 8% water and injected in a 100 μL final volume. For the experiment reported in Fig. 6A, once the tumors reached palpable sizes averaging approximately 100 mm³ per group, the mice were treated 3 times a week for 3 weeks with intravenous doses of vehicle (n = 4), PTX (5 mg/Kg; n = 4), or dYNH-PTX (15.3 mg/kg, hence a dose that is equimolar to the taxol dose; n = 5). Tumor sizes during treatment were measured using calipers. One mouse in
the vehicle group and 1 mouse in the PTX group died at the beginning of the experiment and, therefore, were not included in the analysis. In the experiment reported in Fig. 6B, once the tumors reached palpable sizes, the mice were treated 3 times a week for 3 weeks with intravenous doses of vehicle (n = 5), PTX (5 mg/Kg; n = 5), dYNH-PTX (15.3 mg/Kg; n = 5), DYP-PTX (15.9 mg/Kg; n = 5), YSA-PTX (15.9 mg/Kg; n = 5). The dose of peptide-drug conjugates was equimolar to the taxol dose. Tumors in the dYNH-PTX-treated group became undetectable after 14 days of treatment. Hence, statistical analysis for this group could not be conducted.

The experiment reported in Supplementary Fig. S1 was similarly carried out. Once the tumors reached palpable sizes averaging approximately 230 mm³ per group, the mice were treated 3 times a week for 3 weeks with intravenous doses of vehicle (n = 8), PTX (10 mg/Kg; n = 8), DYP-PTX (31.8 mg/Kg; n = 8), and YSA-PTX (31.8 mg/Kg; n = 7). All doses of peptide-drug conjugates were equimolar to the taxol dose. Tumor sizes during treatment were measured using calipers. In the YSA-PTX treated group, 1 mouse died at the beginning of the experiment and therefore was not included in the analysis.

**In vivo renal cancer allograft studies**

RENCA cells (1 × 10⁶) were harvested from nonconfluent monolayer cell cultures and mixed with 30 μL of rat-tail collagen. The cells were grafted under both renal capsules of BALB/c mice (Harlan Labs) as previously described (39, 40). Three weeks following grafting, the mice were injected every 2 days with intravenous doses of vehicle (n = 4 mice), PTX (20 mg/kg; n = 4 mice), or dYNH-PTX (at a dose that is approximately equimolar to the taxol dose, 60 mg/kg; n = 5 mice) for a total of 3 injections. Both dYNH-PTX and PTX were dissolved in a mixture of 84% PBS, 8% DMSO, and 8% Tween and injected in a 100 μL final volume. After 1-week treatment, the mice were sacrificed. The grafts were then harvested, tumor volume calculated, and photographed. Endothelial cells were visualized by immunohistochemistry with anti-CD31 antibody (1:50, Abcam) using paraformaldehyde-fixed, paraffin-embedded 5 μm tissue sections. CD31-positive vascular outlines were measured from at least five ×10 images per tumor (n ≥ 8 tumors/group) using Image J software (NIH).

**Results**

**Design and synthesis of YNH-paclitaxel and dYNH-paclitaxel**

Previously, we successfully used the YSA-PTX conjugate to target prostate cancer cells in vivo (1). However, the 2 methionine residues in the YSA peptide are susceptible...
to oxidation in vivo and thus represent a likely liability. We, therefore, replaced the 2 methionines with unnatural amino acids to generate the YNH peptide [YSAYPDSVP (L-norleucine)(L-homoserine)S]. With the aim of further improving the stability of the peptide, we also replaced the L-tyrosine at the N-terminus of the YNH peptide with a D-tyrosine to generate the dYNH peptide [ySAYPDSVP(L-norleucine)(L-homoserine)S; Fig. 1]. The subsequent synthesis of the YNH-PTX and dYNH-PTX conjugates was based on our recently reported selective protection/deprotection strategy and click chemistry (Fig. 1; ref. 1). The integrity and purity of the final peptide-drug conjugates was confirmed by high-performance liquid chromatography (HPLC), 1-dimensional (1D) and 2-dimensional (2D) NMR and mass spectrometry (see Supplementary Material).

**Characterization of YNH-PTX and dYNH-PTX**

We initially used $^{15}$N-labeled EphA2 ligand binding domain (residues 27–200) and 2D [$^1$H-$^{15}$N] heteronuclear single quantum coherence (HSQC) spectra to monitor protein–peptide interactions. Chemical shift perturbations in 2D [$^1$H-$^{15}$N] HSQCs, indicative of peptide interactions with residues in the EphA2 ligand-binding domain, were clearly observed in the presence of YNH-PTX or dYNH-PTX, but not with the scrambled control peptide conjugate, DYP-PTX (Figs. 1 and 2). Quantitative isothermal titration calorimetry (ITC) analysis further confirmed that YSA-PTX, YNH-PTX,

![Figure 3](image-url)

**Figure 3.** Binding affinities of the EphA2 targeting peptide-drug conjugates. Dissociation constants for the binding of the peptide-drug conjugates to the EphA2 ligand-binding domain (residues 27–200), as determined by isothermal titration calorimetry, are reported for (A) DYP-PTX, (B) YSA-PTX, (C) YNH-PTX, and (D) dYNH-PTX.
and dYNH-PTX bind to the isolated recombinant ligand-binding domain of EphA2 with apparent K_d values of 9.8, 2.2, and 33 μmol/L, respectively, whereas the control DYP-PTX failed to appreciably bind under the same experimental conditions (Fig. 3). We further confirmed the relative affinities of the PTX-coupled peptides for EphA2 using an ELISA measuring EphA2 Fc binding to the biotinylated peptides immobilized onto ELISA wells. Using this method, relative affinity values of 0.17 and 3.7 were obtained from the comparison of YNH-PTX and dYNH-PTX, respectively, with YSA-PTX. The control DYP-PTX again failed to bind under the same experimental conditions (Fig. 4A).

The relative stability of YSA-PTX, YNH-PTX, and dYNH-PTX is an important, but difficult characteristic to assess. The molecules are not amenable to standard liquid chromatography/mass spectrometry detection because of an inherent inability to ionize. Nonetheless, we were able to determine the relative stability of YSA-PTX, YNH-PTX, and dYNH-PTX in PC3 cell cultures and in mouse serum by assaying the ability of each peptide conjugate to compete with ephrin-A5 AP binding to EphA2 Fc immobilized on ELISA wells. The concentrations of intact peptide-drug conjugates used (25 μmol/L for YSA-PTX and YNH-PTX, 75 μmol/L for dYNH-PTX) inhibit ephrin-A5 AP-EphA4 Fc binding by approximately 80% to 90%. The amount of peptide-drug conjugates remaining was estimated based on the ability to inhibit EphA2-ephrin-A5 interaction, with efficacy = 1 for the inhibition observed with the intact peptide-drug conjugates not incubated in culture medium or serum.

**Figure 4.** Affinity and stability of the EphA2 targeting peptide-drug conjugates. A, streptavidin-coated ELISA wells were incubated with biotinylated YSA-PTX, DYP-PTX, YNH-PTX, or dYNH-PTX, followed by addition of EphA2 Fc at different concentrations. Bound EphA2 Fc was detected using an anti-human Fc antibody conjugated with AP. Apparent K_d values for the binding of the dimeric EphA2 Fc to the peptides were calculated and normalized to the values obtained for YSA-PTX (relative affinity = 1). The graphs show averages ± SE from triplicate measurements in representative experiments, whereas the relative affinity values are averages ± SE calculated from 3 experiments. B, peptide-drug conjugates were incubated with cultured PC3 cells or in mouse serum for the indicated times at 37°C and then tested for their ability to inhibit ephrin-A5 AP binding to EphA2 Fc immobilized on ELISA wells. The concentrations of intact peptide-drug conjugates used (25 μmol/L for YSA-PTX and YNH-PTX, 75 μmol/L for dYNH-PTX) inhibit ephrin-A5 AP-EphA4 Fc binding by approximately 80% to 90%. The amount of peptide-drug conjugates remaining was estimated based on the ability to inhibit EphA2-ephrin-A5 interaction, with efficacy = 1 for the inhibition observed with the intact peptide-drug conjugates not incubated in culture medium or serum.

YNH-PTX and dYNH-PTX target selectively EphA2 overexpressing tumor cells

To investigate EphA2 targeting and selectivity, we visualized YNH-PTX and dYNH-PTX internalization in EphA2 overexpressing tumor cells.
overexpressing PC-3M-luc-C6 (PC3M) cells and in LNCaP cells, which do not express EphA2 (1). Incubation of the C-terminally biotinylated peptides coupled to streptavidin-conjugated red fluorescent quantum dots (Qdots) with the 2 cell types showed that YNH-PTX and dYNH-PTX were internalized only in the EphA2 positive PC3M cells, but not in LNCaP cells (Fig. 5A). The fluorescence of the Qdots overlaps with staining for EphA2 and the lysosomal marker Lamp1 in PC3M cells, suggesting that YNH-PTX and dYNH-PTX mediate cellular uptake of EphA2 and the Qdots into lysosomes (Fig. 5A). Moreover, we confirmed that even when not coupled to Qdots, YNH-PTX and dYNH-PTX cause EphA2 internalization into PC3M cells and receptor colocalization with the lysosomal marker, similar to the ephrin-A1 Fc ligand (Fig. 5B). On the contrary, the DYP-PTX scramble peptide is not internalized into PC3M cells and also fails to trigger EphA2 internalization (Fig. 5A and B). YNH-PTX and dYNH-PTX, but not DYP-PTX, also cause EphA2 tyrosine phosphorylation, which is indicative of receptor activation, and concomitant loss of the receptor from the surface of PC3M cancer cells (Fig. 5C).

These data indicate that the YNH-PTX and dYNH-PTX conjugates can mediate effective EphA2 internalization of PTX into cells, suggesting that they could be useful for targeting cancer cells expressing this receptor.

dYNH-PTX targets tumors in vivo

The combination of the ability of dYNH-PTX to induce EphA2 activation and internalization and its improved stability in mouse serum warranted further evaluation in vivo. Therefore, we tested the effect of dYNH-PTX in PC3M tumor xenografts. Tumor-bearing mice were treated 3 times weekly with an intravenous injection of PTX, dYNH-PTX, or vehicle control for 3 weeks. Significant tumor growth inhibition (p < 0.05) was observed with dYNH-PTX as compared with an equimolar dose of PTX (Fig. 6A). Moreover, mouse body weights were not affected by dYNH-PTX administration in comparison to administration of vehicle control (not shown), and blood chemistry analysis revealed no adverse signs of toxicity in the dYNH-PTX-treated mice (Supplementary Table S1). In a separate repeated experiment, we also compared control vehicle and PTX...
groups with groups treated with dYNH-PTX, YSA-PTX, or DYP-PTX (Fig. 6B). In this experiment, tumors in the dYNH-PTX-treated group become undetectable after 14 days of treatment. The observed tumor regression in the dYNH-PTX-treated group (Fig. 6B) is in agreement with the results reported in Fig. 6A, where 2 of 5 mice treated with dYNH also experienced tumor shrinkage. Moreover, the scrambled peptide-PTX conjugate (DYP-PTX) was ineffective in vivo (Fig. 6B). This latter observation was further confirmed in a separate xenograft study (Supplementary Fig. S1).

dYNH-PTX decreases tumor vascularization in a mouse renal tumor model

Recent studies have shown that overexpression of EphA2 in renal cell carcinoma correlates with poorer prognosis and increased vascularization (41). Thus, we tested the efficacy of dYNH-PTX using an orthotopic renal cancer model where EphA2 expressing RENCA renal cancer cells were grafted in the renal capsule of syngeneic BalbC mice and grown for 4 weeks. The mice were treated with vehicle, PTX, or dYNH-PTX only for the final week before harvesting the tumors. There was no significant difference in tumor growth inhibition between the groups treated with dYNH-PTX as compared with the group treated with an equimolar dose of PTX (Fig. 6C). However, both treatments significantly decreased tumor volume compared with the vehicle control \( (P < 0.003) \). Strikingly, the 3 dYNH-PTX treatments in the fourth week after tumor grafting caused a significant decrease in tumor vasculature, as determined by CD31 staining, compared with vehicle or PTX alone \( (P = 0.001) \) (ANOVA; Fig. 6D). dYNH-PTX did not, however, affect the vasculature of the normal host kidney parenchyma (data not shown). Of note is that while RENCA cells do express EphA2, the levels of EphA2 do not seem to be as high as in the PC3 cells (Supplementary Fig. S2), perhaps explaining why dYNH-PTX was not as efficacious as in the PC3 xenograft in promoting tumor regression.

Discussion

The benefit of chemotherapy is often mitigated by negative side effects linked to a lack of selectivity (2). As a result, recent cancer research has been focused on exploiting tumor specific, cell-penetrating molecules as vehicles for selective anticancer drug delivery. Here, we describe the next generation of EphA2-targeting peptide-drug conjugates: YNH-PTX and dYNH-PTX. Both YNH-PTX and dYNH-PTX effectively target EphA2 and are able to induce receptor activation, internalization, and delivery to lysosomes, which likely causes the release of PTX inside the cancer cells. Our previous work established...
that a YSA-PTX conjugate affords a significant increase in the amount of drug delivered to tumors as compared with administration of free PTX (1). In this study, we show that dYNH-PTX inhibits the growth of EphA2-expressing tumors more effectively than PTX, and even causes the disappearance of some tumors, suggesting that the improved stability of dYNH-PTX compensates for its decrease in affinity. In addition, consistent with EphA2 expression in tumor blood vessels (29), treatment with dYNH-PTX affected the tumor vasculature in an immune competent model of late stage renal cancer progression, suggesting that this peptide could target not only the tumor cells but also the tumor vasculature. However, the specific mechanism underlying the vascular changes remains to be elucidated.

Exploiting EphA2 as a cancer-specific target is attractive due to the fact that EphA2 expression is limited in normal tissues (26, 27, 30, 32). In addition, in normal epithelial cells EphA2 is presumably bound to endogenous ephrin ligands and, as a result, it likely cannot be effectively targeted, whereas in EphA2 overexpressing tumors the receptor is not frequently coexpressed with ephrin ligands (42–45). Therefore, using EphA2-targeting peptides for drug delivery may reduce exposure of normal tissues to cytotoxic drugs, presumably reducing toxic side effects.

The anticancer activity of the EphA2-targeting peptide-drug conjugates can be attributed to tumor tissue penetration and selectivity. PTX is actively transported into cells rather than relying on passive transport, which is the case when a tumor selective molecule lacks the ability to facilitate internalization. In addition, dYNH-PTX affected the tumor-associated vasculature in an immune competent model of late stage renal cancer progression. As a result, dYNH-PTX may expand the therapeutic index of PTX to a point where increased quantities could be safely used. dYNH conjugation may also increase water solubility, making PTX more easily delivered (46) without the use of complex formulations and long infusions.

In summary, by optimizing the sequence of the YSA peptide, we have identified the YNH and dYNH peptides as the next generation of EphA2-targeting peptides. In particular, we have shown that dYNH-PTX has more favorable properties and possesses a powerful antitumor activity in vivo. Future optimization studies will focus on increasing the affinity of dYNH-PTX for EphA2 without compromising its stability to obtain an even more powerful targeting agent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S. Wang, J.L. Siebkins, S. Billet, P. Fisher, E.B. Pasquale, M. Pellecchia


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Wang, R. Noberini, J.L. Siebkins, S.K. Das, Z. Zhang, B. Wu, S. Mitra, S. Billet, A. Fernandez, S. Kitada, P. Fisher, M. Pellecchia

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Wang, R. Noberini, J.L. Siebkins, S.K. Das, Z. Zhang, S. Billet, N.A. Bhowmick, P. Fisher, M. Pellecchia

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