Published in final edited form as:

ChemMedChem. 2014 July ; 9(7): 1403–1412. doi:10.1002/cmdc.201400067.

Design, Synthesis and Bio-evaluation of an EphA2-based Targeted Delivery System

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Abstract

We recently described a new targeted delivery system based on specific EphA2 receptor targeting peptides conjugated with the chemotherapeutic agent paclitaxel. In this manuscript we investigate the chemical determinants responsible for the stability and degradation of these agents in plasma. Introducing modifications in both the peptide and the linker between the peptide and paclitaxel, resulted in drug conjugates that are both long-lived in rat plasma and that markedly reduced tumor size in a prostate cancer xenograft model compared to paclitaxel alone treatment. These studies identify critical rate-limiting degradation sites on the peptide-drug conjugates, enabling the design of agents with increased stability and efficacy. These results provide support for our central hypothesis that peptide-drug conjugates targeting the EphA2 receptor represent an innovative and potentially effective strategy to selectively deliver cytotoxic drugs to cancer cells.

Keywords

Targeted delivery; drug discovery; EphA2; ephrin; chemotherapy

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Authors Contributions

E.B. and S.W. contributed equally to this work. M.P. designed the research strategy for the peptide conjugates and directed research by E.B., S.W., J.L.S., and R.D. S.W. synthesized, purified and characterized the peptide-PTX conjugates. E.B. performed and analyzed detailed stability and solubility experiments by NMR, performed and analyzed *in vivo* toxicity studies with the drug conjugates. R.D. performed the plasma stability data using LC/MS in Table 1. R.N. performed the ELISA and cell culture experiments, under the supervision of E.B.P. S.K.D. performed the tumor xenograft experiments under the supervision of P.B.F., who helped in the design and analysis of the tumor xenografts with PC3M cells. J.L.S. helped with the analysis of the xenograft studies. M.P. and E.B. wrote the manuscript with help from the other authors.

Supporting Information Available. Detailed experimental procedures, spectral and purity analysis of all compounds, supporting figures, are available in the Supporting Information file.

We investigated the chemical determinants responsible for the stability and degradation in plasma of an EphA2-based targeted delivery system that is constituted by receptor targeting peptides conjugated with paclitaxel. We demonstrate that our agents are both long-lived in plasma and markedly reduce tumor size in a prostate cancer xenograft model.

Introduction

Tumor-specific cell surface receptors represent attractive targets for the development of cancer selective drug delivery systems,^[1] allowing the design of agents that target malignant cells while sparing normal cells.^[2] One such cancer-specific target is the EphA2 receptor.^[3] Eph receptor signaling pathways control cell shape, migration, invasion, and formation of tissue boundaries by altering the organization of the actin cytoskeleton, integrins and intercellular adhesion molecules.^[3d, 4] Critical to their function, the Eph receptors have an extracellular domain that binds their ligands, the ephrins, and an intracellular tyrosine kinase domain, in addition to other structural or regulatory domains.^[5] While EphA2 is expressed at low levels in adult normal tissues, it is overexpressed in several cancers, where it has been shown to increase tumor malignancy and it is associated with poor prognosis.^[3a, 3b] Indeed, a high level of EphA2 has been detected in tumor cells including many solid tumors such as melanoma^[4b, 6] and breast,^[3g] brain,^[7] ovary,^[8] urinary bladder,^[9] prostate^[10] pancreas,^[11] esophagus,^[12] lung,^[13] and stomach^[14] cancers. Hence, such differential expression of EphA2 in normal cells compared with cancer cells highlights its importance as a potential drug target.^[2a, 11a]

Various therapeutic approaches targeting EphA2 have been reported, including the use of monoclonal antibodies, RNA interference-mediated EphA2 downregulation, immunotherapy, adenoviral vectors, nanoparticles, and small molecules.^[3b, 3e, 15] For example, dasatinib, a potent small molecule kinase inhibitor that targets EphA2^[16] despite its original design against Abl,^[17] is currently under clinical investigation as single agent for the treatment of squamous cell carcinomas,^[18] and in combination therapy for endometrial cancer, glioblastoma and solid tumors (www.clinicaltrials.gov). Because receptor activation causes its internalization, antibody drug-conjugates (ADCs)^[19] and chimeric ephrin-ligand-toxin constructs^[20] have also been investigated. In particular, a recent Phase I study intended to evaluate increasing doses of MEDI-547, an ADC composed of a human anti-EphA2 monoclonal antibody (1C1) linked to a cytotoxic auristatin derivative (maleimidocaproylmonomethyl auristatin phenylalanine, mMMAF) was carried out in a small cohort of patients with solid tumors that had relapsed or were refractory to standard therapy.^[21] However, the study had to be discontinued due to the adverse side effects of the starting dose, possibly caused by MEDI-547 cross-reaction with other proteins.^[21] Despite the results of this study, EphA2 remains a valid target for delivery of cancer therapeutics by using either more selective ADCs^[21] or peptides that may be even more suitable for this intent. Indeed, short peptides that interact with the ephrin-binding pocket in the extracellular N-terminal domain of EphA2 have been reported.^[6] The amino acid sequence YSAYPDSVPMMS (YSA), identified by using a phage display technique, has been shown to bind the extracellular domain of EphA2 and promote receptor activation and internalization in several cancer cell types.^[22]

Recently, we reported on the conjugation^[23] and optimization^[24] of the YSA peptide linked to the chemotherapeutic agent paclitaxel (PTX), via a triazole-ester linker (YSA-L1-PTX; Figure 1A). We demonstrated that when PTX is conjugated to an EphA2 targeting peptide, the resulting agent efficiently targets EphA2-expressing cancer cells, selectively

delivering PTX at the tumor site and resulting in an increased efficacy *in vivo* compared to equal doses of unconjugated PTX. [23-24]

The stability of both the targeting peptide and the linker between the peptide and the antineoplastic agent play an important role in the selective delivery of a drug conjugate. We previously demonstrated that the replacement of the N-terminal L-tyrosine of YSA-L1-PTX with a D-Tyrosine (dYNH-L1-PTX, Figure 1A) results in an EphA2 targeting agent with improved half-life in mouse serum.^[24] Here we address in greater detail the stability of both the peptide and the linker between the peptide and the cytotoxic drug by LC/MS and NMR spectroscopy. These studies reveal that both the linker and the first amino-acid in YSA-L1-PTX conjugates are rapidly degraded in plasma with an overall half-life for the conjugate of about 20 min. While this half-life is apparently sufficient for the conjugate to deliver the drug at the tumor site^[23-24], we sought here to design novel EphA2 targeting agents drug conjugates with improved stability. We report on new conjugates that are long-lived in plasma, well tolerated *in vivo*, and efficacious in a mouse model of human prostate cancer.

Results

Plasma stability of YSA-L2-PTX and dYNH-L2-PTX as determined by LC/MS and NMR

Previously, we successfully conjugated PTX to different EphA2 targeting peptides using a triazole-ester linker (L1 in Figure 1A). Initially, we focused our efforts on YSA as the EphA2 targeting peptide (YSA-L1-PTX; Figure 1A).^[23] However, we soon recognized that by replacing the two methionine residues of the YSA peptide with L-norleucine and L-homoserine, we could reduce the possibility for its oxidation *in vivo* thus increasing stability of the resulting conjugate (YNH-L1-PTX; Figure 1A).^[24] We sought to further increase the stability of the peptide by replacing the L-tyrosine at the N-terminus of YNH-L1-PTX with a D-tyrosine resulting in dYNH-L1-PTX (Figure 1A).^[24] Despite these advances with the targeting peptide, the presence of two ester bonds in the linker may render the conjugate susceptible to premature degradation in aqueous buffer and blood.

Hence, we decided to investigate the stability and efficacy of a different linker, linker 2 (L2), which is shown in Figure 1B. The synthesis of the peptide-drug conjugates using this new linker (YSA-L2-PTX, dYNH-L2-PTX, and the scrambled version of YSA, namely DYP-L2-PTX, Figure 1B) was carried out according to our recently reported selective protection/deprotection strategy and click chemistry (Figure 1B).^[23] 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) ¹H and ¹³C NMR and mass spectrometry (Supporting Information).

In our recent work, we determined that dYNH-L1-PTX was more stable compared to YNH-L1-PTX, by measuring the amount of active peptide in an EphA2-ephrin-A5 competition assay, following incubation with cultured PC3 cells or mouse serum.^[24] However, because the peptides will interact with the EphA2 receptor independent of the linker and the conjugated drug, this method only addresses the integrity of the peptide and not of the linker. In fact, the dissociation constant for the binding of YSA-L2-PTX to the EphA2 ligand binding domain (residues 27-200), determined by isothermal titration calorimetry

(ITC), is 2.1 μM , similar to 9.8 μM previously reported for YSA-L1-PTX (Supporting Information).^[24]

In order to have a more detailed and direct assessment of the stability of the agents and the linker in particular, we monitored compound degradation in plasma by both LC/MS and by 1D ^1H NMR. To assess the stability of YSA-L1-PTX, YSA-L2-PTX and dYNH-L2-PTX each conjugate was incubated at 37 °C with fresh rat plasma and the reactions were terminated at 15 minutes intervals for 1 hour. Following protein precipitation and centrifugation, the samples were analyzed by LC/MS and the percentage of the compound remaining at each time point was calculated from peak area ratios in relation to an internal standard. The data are reported in Table 1 and, as expected, the conjugate with L1 is less stable in plasma than the L2 correspondent.

To further determine the rate limiting steps in the degradation of these agents, we adopted an NMR approach. In this method, we first collected a complete set of 1D and 2D NMR spectra for PTX alone to obtain its complete resonance assignment (^1H and ^{13}C , Supporting Information). This was necessary for the subsequent ^1H resonance assignments of the peptides conjugated with PTX in the assay solvent (see experimental). The assay conditions, including compound and plasma concentrations as well as choice of deuterated buffer and quenching solvents, were optimized in order to obtain a signal to noise ratio sufficient to monitor by NMR the PTX proton signals that are sensitive to linker hydrolysis (namely H-13, H-2', *p*-Ph2, *o*-Ph3 in Figure 2). Hence, with this approach we were able to detect the degradation events throughout the molecule, including the peptide and the linker portions. In Figure 2, we show the time course of plasma degradation for YSA-L2-PTX. These data show that L2 is very stable over time, as the appearance of the free PTX moiety in solution upon linker hydrolysis is observed only after 8 hours of incubation with plasma under the experimental conditions used (Figure 2). From the same figure the linker was more stable than the peptide portion, which starts to be degraded after only 30 mins, as shown by the diagnostic 2H-*o* and 2H-*m* signals of the Tyr1 residue in the YSA motif (Figure 2). This is supported by similar experiments conducted with dYNH-L2-PTX in which the YSA sequence is replaced with the more stable dYNH motif and in which d-Tyr1 does not appear to be degraded even after 8 hours incubation with plasma, consistent with the notion that D-amino acids are not susceptible to degradation by proteolytic enzymes such as the aminopeptidases that are predominant in plasma^[25] (Figure 3). In contrast, similar experiments conducted with YSA-L1-PTX confirmed that, while it seems very stable in organic solvents such as DMSO or methanol (Supporting Information), it is rapidly degraded in aqueous media, with the degradation of the linker in buffer observed already within the first 10 minutes of incubation (Supporting Information). This rapid hydrolysis precluded direct monitoring of L1-conjugates in plasma with the NMR method, but the LC/MS method confirmed a rapid degradation of these agents (Table 1). These observations provided the impetus to further evaluate the biological properties of our newly derived YSA-L2-PTX and related conjugates, including dYNH-L2-PTX and a scrambled control conjugate DYP-L2-PTX.

YSA-L2-PTX and dYNH-L2-PTX target selectively EphA2 overexpressing tumor cells

We used confocal microscopy to assess the ability of the EphA2 targeting peptide-drug conjugates to be internalized. Conjugate targeting of EphA2 was assessed by visualization of receptor internalization in the human prostate cancer PC-3M-luc-C6 (PC3M) cell line, which expresses high levels of endogenous EphA2. [26] EphA2 activation by the ephrin-A1 ligand results in receptor internalization and subsequent degradation at least in part through a lysosomal pathway. [22, 27] Similar to the ephrin-A1 Fc ligand, YSA-L2-PTX and dYNH-L2-PTX cause EphA2 internalization into PC3M cells and receptor colocalization with the lysosomal marker Lamp1 (Figure 4A). On the contrary, the scrambled DYP-L2-PTX fails to trigger EphA2 internalization (Figure 4A). Moreover, YSA-L2-PTX and dYNH-L2-PTX, but not DYP-L2-PTX, cause EphA2 tyrosine phosphorylation, which is indicative of receptor activation, and concomitant loss of the receptor from the surface of PC3M cancer cells (Figure 4B). These results clearly indicate that the YSA-L2-PTX and dYNH-L2-PTX conjugates can mediate effective EphA2 internalization of PTX into PC3 cells.

Efficacy and toxicity of EphA2 targeting drug conjugates in vivo

The ability of the L2 drug conjugates to induce EphA2 activation and internalization in combination with the improved stability warranted further evaluations *in vivo*. Therefore, we compared the anti-cancer properties of PTX alone and the PTX-conjugates in a PC3 xenograft model (Figure 4C). Intravenous administration of YSA-L2-PTX, dYNH-L2-PTX, or the scrambled DYP-L2-PTX conjugates is facilitated by their increased solubility compared to that of PTX alone (Supporting Information). The effect of these agents on tumor growth was monitored by using PC3M cells subcutaneously implanted in athymic nude mice. Mice received controls or conjugated agents 9 times during the course of a 3 weeks treatment (3 times per week). Mice in the dYNH-L2-PTX and YSA-L2-PTX treatment groups showed significant tumor growth inhibition ($P < 0.05$) as compared with mice treated with an equimolar dose of PTX. Moreover, the scrambled peptide-PTX conjugate (DYP-L2-PTX) was ineffective *in vivo* at the same dose (Figure 4C).

During the 3 weeks of the efficacy study, no signs of adverse toxicity were observed in any of the treated mice. In addition, acute toxicity was also assessed by injecting equimolar doses of PTX (50 mg/Kg) and YSA-L2-PTX (156 mg/Kg), into the tail vein of female Balb/c mice. After 4 hours, whole blood samples collected from the retro-orbital sinus of treated mice were analyzed using a hematology analyzer. PTX administration caused a significant white blood cell (WBC) reduction (4.70 ± 0.39 (SEM) $\times 10^9/L$) while WBC counts for YSA-L2-PTX treated mice ($7.53 \pm 0.61 \times 10^9/L$) were within the normal range reported for mice ($6-15 \times 10^9/L$). [28]

Discussion and Conclusions

PTX is one of the most widely prescribed antineoplastic agents because it effectively inhibits mitosis, motility and intracellular transport mechanisms within tumor cells. [29] It has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of breast, ovarian, non-small-cell lung cancer (NSCLC) and AIDS-related Kaposi sarcoma (www.cancer.gov). Despite its high efficacy, PTX treatment provokes severe adverse side

effects, including hypersensitivity reactions, peripheral neuropathy, arthralgia, myalgia and neutropenia^[29-30] many of which are due to the composition of its formulation. Specifically, because of its high lipophilic nature (log P = 3.96), very poor aqueous solubility (< 0.01 mg/mL) and lack of ionizable functional groups, PTX has been formulated in a vehicle composed of a 1:1 blend of polyoxyethylated castor oil, Cremphor EL® (CrEL), and ethanol to allow parenteral administration. The presence of CrEL requires premedication with antihistamines and corticosteroids to increase safety by reducing hypersensitivity reactions associated with CrEL, which also seems to cause additional toxicity in endothelial cells and vesicular muscles.^[31] In order to ameliorate these issues, innovative formulations have been reported including the use of nanoparticles, nanosuspensions, liposomes, emulsions, micelles, implants, pastes and gels.^[32] Nanoparticle albumin-bound paclitaxel, known as Abraxane® (Celgene Corporation, San Diego), is indeed a CrEL-free formulation recently approved by the U.S. FDA to treat patients with late-stage (metastatic) pancreatic cancer, besides the previously approved use to treat advanced NSCLC and metastatic breast cancer (www.cancer.gov). Nonetheless, as for PTX,^[33] administration of Abraxane also causes a marked reduction of white blood cells in patients (www.abraxane.com/nsclc/patients), further limiting the doses and frequency of treatment with this drug.

We recently described our EphA2 targeting peptide-drug conjugates such as YSA-L1-PTX and reported on their ability to target EphA2 expressing cancer cells *in vivo*.^[23-24] We previously demonstrated that targeting EphA2 receptor via a specific peptide-drug conjugates resulted in EphA2 mediated internalization in prostate and breast cancer cell lines.^[23-24] In addition, we also demonstrated that these drug conjugates can deliver their cargo to tumors *in vivo* using models of prostate^[23] and renal carcinoma,^[24] resulting in tumor shrinkage compared to the unconjugated drugs. Of note is that in these previous studies *in vivo* analysis of PTX levels indicated a greater concentration of the drug in tumors from mice treated with the conjugated drug versus those treated with an equivalent amount of unconjugated compound, thus suggesting that the targeted delivery is concentrating the drug at the EphA2 expressing tumor cells.^[23] In this manuscript we first investigated the chemical determinants responsible for the stability and degradation of these agents in plasma, and subsequently introduced modifications in both the peptide and the linker between the peptide and the drug, and examined solubility, plasma stability, cellular and *in vivo* efficacy and toxicity of the new drug conjugates (YSA-L2-PTX or dYNH-L2-PTX) compared to unconjugated PTX.

PTX aqueous solubility is a major limitation of this drug, as described above. However, when conjugated with a hydrophilic peptide moiety, such as YSA, its solubility is significantly increased, as easily demonstrated by the analysis of ¹H NMR spectra collected in aqueous buffer for YSA-L2-PTX compared to PTX alone (Supplementary Information). From these experiments, we assessed that the solubility of PTX in aqueous buffer is well below 10 μM, while YSA-L2-PTX is soluble well above 50 μM (Supporting Information). A second very important requirement for any drug-conjugate is the stability of the linker. Linker of several nature have been investigated in recent years including esters, thioethers, amides, each providing different levels of stability in various biological media, depending on the conjugated drug and the exact composition of the linker.^[34] Ideally, the linker needs

to be sufficiently stable in the bloodstream so as not to release the cytotoxic drug before reaching the cancer cells, but has to be easily cleaved once internalized, thus allowing the free drug to access its target. By using an NMR approach, we were able to detect the degradation events of both the peptide and the linker portions in our drug-conjugates accurately, a type of information that cannot be easily obtained via LC-MS with these type of complex molecules. Indeed, while LC-MS is routinely performed for drug metabolism of small molecules, in our hands the approach did not provide unambiguous data with peptide-drug conjugates under investigation in determining the exact composition of the metabolic products in serum. Once the complete resonance assignments for the drug-conjugates were obtained, these could be more directly used to determine degradation events in serum. These data indicated that L2 is very stable over time, as the appearance of the free PTX moiety upon linker hydrolysis is observed only after 8 hours of incubation with plasma under the established experimental conditions (Figure 2). Accordingly, because PTX is not released in the bloodstream prior to its internalization via the EphA2 receptor into cancer cells, administration of a relatively high dose of YSA-L2-PTX (156 mg/Kg) did not appreciably reduce the WBC counts, while, as expected, significant WBC reduction was observed in mice treated with an equivalent dose of PTX (50 mg/Kg).

While these data demonstrate that PTX in the L2 conjugates is not prematurely released in serum or in the bloodstream, our cellular (Figure 4A and B) and *in vivo* efficacy studies (Figure 4C) clearly suggest that the drug is effectively internalized in EphA2 expressing cancer cells, such as PC3 cells. As a consequence, a striking effect on tumor growth is observed in mice treated with both YSA-L2-PTX and dYNH-L2-PTX. Tumor volumes (and weights) decreased below detection in 4 out of 5 mice, and 2 out of 5 mice, respectively, after 3 weeks of treatment with YSA-L2-PTX and dYNH-L2-PTX. In comparison, PTX alone or PTX conjugated to a scrambled YSA peptide (DYP-L2-PTX) were significantly less effective (Figure 4C).

Combined with the increased solubility and *in vivo* tolerability of PTX when conjugated with our delivery system, we anticipate that in a clinical setting, such agents would allow for both increased doses of PTX to be delivered to the tumor and/or for an increased frequency of treatment, hence presumably increasing the effectiveness of the drug.

While we found that YSA-L1-PTX undergoes rapid hydrolysis in buffer and in plasma with an overall half-life of 23 min (Table 1), we recently reported that this agent was also effective *in vivo* in reducing tumor volumes in xenograft models of prostate cancer.^[24] It may simply be that the half-life, albeit short, is still sufficiently long for the YSA-L1-PTX drug conjugate to reach the EphA2 receptor hence delivering its cargo to cancer cells.^[24] A second more speculative possibility is that, even if hydrolyzed from the drug, the unconjugated peptide alone can still interact with the EphA2 receptor in tumor cells. The resulting receptor internalization events could render cancer cells more receptive to circulating PTX, by enhancing cellular uptake of the drug, similar to what recently proposed by Ruoslahti and co-workers with the iRGD peptide^[1a, 35] and more recently as observed in related peptide-micelle combinations that displayed better uptake by the cells compared to the corresponding covalently conjugated micelles.^[36] Nonetheless, these non-covalent mixes while effective lose in specificity for cancer cells. While this is an intriguing

observation, our L2 conjugates, being very stable in plasma, leave fewer doubts about the mechanism of action of our agents *in vivo*, which are directly internalized via the EphA2 receptor, hence more specific to cancer cells. Hence, future studies will be centered on assessing the tumor targeting ability of our drug-conjugates against prostate and other EphA2 overexpressing cancers, possibly also using imaging reagents.

In conclusion, together with its increased solubility, plasma stability, efficacy and tolerability in mice, our results strongly suggest that our agents may provide a promising new avenue for the development of more effective chemotherapeutic strategies against a large variety of cancers that overexpress the EphA2 receptor.

Experimental Section

Chemical synthesis and purification

Unless otherwise noted, all reagents and anhydrous solvents were obtained from commercial sources and used without purification, and the peptide motifs were purchased from Abgent Inc. All reactions were performed in oven-dried glassware. All reactions involving air or moisture sensitive reagents were performed under a nitrogen atmosphere. Silica gel chromatography was performed using prepacked silica gel or C-18 cartridges (RediSep). All final compounds were purified to >95% purity, as determined by a HPLC Breeze from Waters Co. using an Atlantis T3 5.0 μ M 4.6 mm $\times$ 150 mm reverse phase column. 1D and 2D NMR spectra were recorded on a Bruker 600 MHz instruments. Chemical shifts are reported in ppm (δ) relative to ^1H (Me_4Si at 0.00 ppm), coupling constant (J) are reported in Hz throughout, and NMR signal assignments were based on DEPT, 2D [^1H , ^1H]-COSY, 2D [^1H , ^1H]-TOCSY, 2D [^1H , ^{13}C]-HSQC and 2D [^1H , ^{13}C]-HMBC experiments. Low resolution and high resolution mass spectral data were acquired on an Esquire LC00066 Mass Spectrometer, an Agilent ESI-TOF Mass Spectrometer, or a Bruker Daltonic Autoflex Maldi-Tof/Tof Mass Spectrometer.

Plasma Stability by LC/MS

A 25 μL aliquot of a 40 μM solution in DMSO of the test compound was added to 975 μL of heparinized rat plasma (Lampire, P1-150N) to obtain a 1 μM final solution (Final DMSO concentration = 2.5%). The mixture was incubated for 1 hour at 37 $^\circ\text{C}$. Aliquots of 100 μL were taken at 15 min intervals and diluted with 100 μL of quenching solution (water : acetonitrile (20 : 80) with 0.5% formic acid). The samples were centrifuged and the supernatant was mixed 1:1 with quenching solution containing an internal standard (2.5 mM indomethacin). The supernatant was transferred into an autosampler vial and the amount of test compound was quantified using a LC-MS/MS system consisting of an Applied Biosystems Sciex (ABSciex) API 3000 triple quadrupole mass spectrometer and a Shimadzu Prominence HPLC in MRM mode. The ratio of the peak AUC of the analyte to the peak AUC of the internal standard was determined using Analyst 1.4.2 software (ABSciex). The change of the ratios in function of time was used as a measure of plasma stability.

Plasma Stability by NMR spectroscopy

A 200 μ L aliquot of a 2 mM solution of test compound in deuterated sodium phosphate buffer (5% d_6 -DMSO) was added to heparinized rat plasma (Pel Freeze Biologicals, Lampire, catalog number 36142-1) to obtain a 400 μ M final concentration in the reaction mixture. The mixture was incubated at 37 °C for several hours under stirring conditions. Aliquots of 200 μ L were taken at different time points and diluted with 400 μ L of methanol- d_4 (Aldrich, catalog number 422878), as quenching solvent. The samples were centrifuged and the supernatant was mixed 2:1 with methanol- d_4 containing sodium-3-trimethylsilylpropionate- d_4 (TMSP) as internal standard. After a second centrifugation step, the supernatant was collected and transferred into NMR tubes. The final concentration of test compounds was approximately 90 μ M and the solvent composition as follows: 77% methanol- d_4 , 18.3% D_2O , 4.5% H_2O , 0.2% d_6 -DMSO. 1H NMR spectra were recorded on a 600 MHz Bruker Avance spectrometer equipped with TCI cryoprobe, at 300 K. NMR data were processed using TOPSPIN 2.1 (Bruker Biospin Corp., Billerica, MA, USA) and analyzed with Mnova 8.1 (Mestrelab Research, Santiago de Compostela, Spain, www.mestrelab.com, 2013).

Compounds degradation was monitored by comparing the spectra of test compounds in presence of plasma enzymes after different incubation times with the one collected in absence of enzymes, used as negative control.

Fluorescence cell imaging

PC3-3M-luc-C6 Bioware® (Caliper) cells were grown in RPMI 1640 medium (Mediatech, Inc, Herndon, VA) with 10% FBS and Pen/Strep.

To image EphA2 internalization and co-localization 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 μ g/mL Fc (as a negative control), 0.2 μ g/mL ephrin-A1 Fc (as a positive control), or 100 μ M PTX-coupled peptides for 2 hours. The cells were then fixed, permeabilized 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^[37] 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.

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 μ g/mL human Fc (as a negative control), 0.2 μ g/mL ephrin-A1 Fc (as a positive control), 100 μ M YSA-L2-PTX or dYNH-L2-PTX or DYP-L2-PTX. The cells were then placed on ice, rinsed once with cold PBS and incubated for 20 min at 4°C with a 0.5 mg/mL EZ-link sulfo-NHS-biotin (Thermo Scientific/Pierce, Rockford, IL) in PBS. The

cells were then washed 3 times with a 100 mM glycine in PBS to quench the biotinylation reaction, followed by PBS. The cells were lysed in modified RIPA lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris, pH 8.0) containing protease inhibitors and 1 mM sodium orthovanadate. For immunoprecipitations, the lysates were incubated with 1 μ g anti-EphA2 antibody (Millipore-Upstate, Inc. Temecula, CA) immobilized on GammaBind Sepharose beads (GE Healthcare Life Sciences). Immunoprecipitates and lysates were probed by immunoblotting with an anti-phosphotyrosine antibody (Millipore, Inc. Temecula, CA), streptavidin coupled to *HRP* (Thermo Scientific/Pierce, Rockford, IL), or anti-EphA2 antibody (Life Technologies/Invitrogen). Lysates of PC-3M-luc-C6 cells were probed by immunoblotting with the EphA2 Millipore antibody and with a GAPDH antibody (AbCam).

In vivo toxicity and prostate cancer xenograft studies

Acute toxicity was assessed by injecting equimolar doses of PTX (50 mg/Kg, $n = 3$) and YSAL2-PTX (156 mg/Kg, $n = 4$), into the tail vein of female Balb/c mice (23 gram as average weight, Harlan Labs, IN). Both PTX and YSA-L2-PTX were dissolved in a mixture of 80% PBS, 10% Tween-20 and 10% DMSO, and injected in a 100 μ L final volume. After 4 hours, a 100 μ L of whole blood sample, from the retro-orbital sinus of treated mice was collected in EDTA tubes and analyzed by the hematology analyzer VetScan HMII (Abaxis, CA). PTX administration caused a significant WBC reduction ($4.70 \pm 0.39 \times 10^9/L$) while WBC counts for YSA-L2-PTX treated mice was $7.53 \pm 0.61 \times 10^9/L$ (the error represents SEM).

For the efficacy studies, PC-3M-luc-C6 cells (1×10^6) were injected subcutaneously into 6-8 week old female athymic nude mice (Harlan Labs, CA) and the peptide-PTX conjugates were dissolved in a mixture of 84% PBS, 8% DMSO, and 8% Tween-20 and injected in a 100 μ L final volume.

For the experiment reported in Figure 4C, once the tumors reached palpable sizes averaging approximately 100 mm³ per groups, the mice were treated three times a week for three weeks with intravenous doses of vehicle ($n = 5$), PTX (5 mg/Kg; $n = 5$), YSA-L2-PTX (15.7 mg/Kg; $n = 5$), dYNH-L2-PTX (15.3 mg/Kg; $n = 5$), DYP-L2-PTX (15.7 mg/Kg; $n = 5$). The dose of peptide-drug conjugates was equimolar to the PTX dose. Tumor sizes during treatment were measured using calipers. Tumors in the YSA-L2-PTX (4 out of 5) and in the dYNH-L2-PTX (2 out of 5) treated group became undetectable after 21 days of treatment. On day 21, all mice were sacrificed and the tumors, where present, were collected and weighed (data not shown). Tumor growth curves for the control and PTX served as controls also in our previous work [24], as L1 and L2 experiments were carried out in parallel to better compare the two molecules.

Supplementary Material

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Acknowledgments

Financial support was obtained in part by NIH grant CA138390 to M.P. and E.B.P., NIH grant CA168517 to M.P. and P.B.F. and the National Foundation for Cancer Research (NFCR) to P.B.F. We thank: Dr. Ziming Zhang for early support on NMR characterization of the synthesized compounds, Dr. Andrey Bobkov for assistance with ITC measurements, Dr. Belal Azab for assistance with the *in vivo* PC3M tumor xenograft study. P.B.F. holds the Thelma Newmeyer Corman Chair in cancer research in the VCU Massey Cancer Center.

Abbreviations Used

EphA2	Erythropoietin-producing hepatocellular carcinoma A2
ADC	Antibody Drug Conjugate
YSA	A linear peptide of sequence YSAYPDSVPMMS
YNH	A linear peptide of sequence YSAYPDSVP(L-Norleucine)(L-Homoserine)S
dYNH	A linear peptide of sequence ySAYPDSVP(L-Norleucine)(L-Homoserine)S
DYP	A linear peptide of sequence DYPSMAMYSPSV
PTX	paclitaxel
ITC	isothermal titration calorimetry
FDA	Food and Drug Administration
PC3M	human prostate cancer PC-3M-luc-C6 cells
CrEL	Cremphor EL®
WBC	White Blood Cell

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Figure 1. EphA2 targeting peptides conjugated with PTX

(A) Chemical structure of EphA2 targeting peptides conjugated with PTX through linker 1 (L1). (B) Chemical structure and synthetic scheme of EphA2 targeting peptide-drug conjugates containing linker 2 (L2). Reagents and conditions: a) TBSCl, imidazole, DMF, rt, 6 h; b) LiHMDS, allyl chloroformate, THF, -70°C , 1 h; c) TBAF, THF, rt, 30 min; d) 6-azido-hexanoic acid, DIPC, DMAP, DCM, rt, 12 h; e) Pd(Ph₃P)₄, 1,3-dimethylbarbituric acid, THF, rt, 6h; f) YSA motif, or dYNH motif or DYP motif, CuSO₄, sodium ascorbate, DMSO/water, rt, 48 h.

Figure 2. Hydrolysis of YSA-L2-PTX by rat plasma enzymes monitored by 1D ^1H NMR
 Bottom and top spectra belong to YSA-L2-PTX and to PTX, respectively, both measured in absence of plasma. All other spectra represent a time course of YSA-L2-PTX degradation in presence of rat plasma. Key PTX resonances corresponding to protons sensitive to linker hydrolysis are labeled and their position highlighted by dotted lines. The appearance of the PTX proton signals (H-13, H-2', *p*-Ph2, *o*-Ph3) upon YSA-L2-PTX hydrolysis after 8 hours is evident as indicated by asterisks. Y1 and Y4 refer to the aromatic peaks of the Tyrosine 1 (7.10 and 6.75 ppm) and Tyrosine 4 (7.05 and 6.75 ppm) in YSA-L2-PTX, respectively. Note that the peak corresponding to H-2' in PTX alone is outside the reported spectral region.

Figure 3. Hydrolysis of dYNH-L2-PTX by rat plasma enzymes monitored by 1D ^1H NMR
 Bottom and top spectra belong to dYNH-L2-PTX and to PTX, respectively, both measured in absence of plasma. All other spectra represent a time course of dYNH-L2-PTX degradation in presence of rat plasma. Contrary to what observed with YSA-L2-PTX in Figure 2, no chemical shift changes suggestive of degradation have been observed for the aromatic resonances corresponding to the D-tyrosine in position 1 (Y1; 7.10 and 6.75 ppm; dotted lines) or the second L-tyrosine in position 4 (Y4; 7.05 and 6.75 ppm; dotted lines). Likewise, even after 8 hr incubation, no degradation of other moieties of the molecule is detected, including the linker.

Figure 4. Drug-conjugates with the stable L2 linker are internalized in cancer cells expressing EphA2 and inhibit *in vivo* PC3M tumor growth

(A) PC3M cells were treated for 2 hours with 0.2 μ g/mL ephrin-A1 Fc or 100 μ M DYP-L2-PTX, YNH-L2-PTX or dYNH-L2-PTX. The cells were stained for Lamp1 (red) and EphA2 (green) and nuclei were labelled with DAPI (blue). Representative confocal microscopy images are shown. Scale bar = 25 μ m. (B) PC3M cells were treated for 1 hour with 0.2 μ g/mL ephrin-A1 Fc or 100 μ M YSA-L2-PTX, DYP-L2-PTX, or dYNH-L2-PTX. Proteins present on the cell surface were then labeled with biotin. EphA2 immunoprecipitates were probed with an anti-phosphotyrosine antibody (PTyr), reprobed with streptavidin-HRP (biotin) and then with an anti-EphA2 antibody. All the lanes are from the same blot at the same exposure. (C) Groups of 5 athymic nude mice bearing pre-established subcutaneous PC3M tumors were treated three times weekly starting at day 0 with intravenous doses of vehicle, PTX (5 mg/Kg) and the indicated peptide-drug conjugates (at a dose equimolar to the PTX dose). Tumor sizes were measured and averages $\pm$ SE are shown. $P < 0.05$ for the comparison of the YSA-L2-PTX or dYNH-L2-PTX group with the PTX control group by repeated measures two-way ANOVA.

Table 1

Stability of selected PTX conjugates in rat plasma measured by LC/MS.

Compound	Plasma stability (t $\frac{1}{2}$)
YSA-L1-PTX	23 min
YSA-L2-PTX	> 1 h
dYNH-L2-PTX	> 1 h