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Targeted inhibition of hedgehog signaling by cyclopamine prodrugs for advanced prostate cancer

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Abstract

A promising agent for use in prostate cancer therapy is the Hedgehog (Hh) signaling pathway inhibitor, cyclopamine. This compound, however, has the potential for causing serious side effects in non-tumor tissues. To minimize these bystander toxicities, we have designed and synthesized two novel peptide-cyclopamine conjugates as prostate-specific antigen (PSA)-activated prodrugs for use against prostate cancer. These prodrugs were composed of cyclopamine coupled to one of two peptides (either HSSKLQ or SSKYQ) that can be selectively cleaved by PSA, converting the mature prodrug into an active Hedgehog inhibitor within the malignant cells. Of the two prodrugs, Mu-SSKYQ-Cyclopamine was rapidly hydrolyzed, with a half-life of 3.2 h, upon incubation with the PSA enzyme. Thus, modulating cyclopamine at the secondary amine with PSA-cleavable peptides is a promising strategy for developing prodrugs to target prostate cancer.

Keywords

PSA; Cyclopamine; Prodrug; Hedgehog signaling; Peptides

1. Introduction

Prostate cancer is the second leading cause of cancer death in American men, exceeded only by lung cancer. The American Cancer Society estimates that 27,050 men will die of prostate cancer and about 218,890 new cases of prostate cancer will be diagnosed in 2007 in the United States. Virtually all the mortality associated with prostate cancer is related to the dissemination of the disease to organs such as the bones. Metastatic prostate cancer initially responds to androgen ablation therapy, however over time, patients generally progress to a hormone-refractory state, which is usually lethal.^{1,2} In addition, both conventional anti-metabolites and androgen ablation are associated with systemic toxicities in various extra-prostatic tissues.^{3,4} Therefore, therapeutic strategies that specifically target aberrant signaling pathways in metastatic prostate cancer could greatly enhance survival, especially in hormone-refractory disease, while at the same time reducing bystander toxicity in non-tumor tissues.

One such pathway is the *hedgehog* (Hh) signaling pathway, which specifies patterns of cell growth and differentiation during embryogenesis in a wide range of tissues. The Hh signaling

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pathway operates as a sequence of inhibitory interactions, where in the basal state, the twelve transmembrane receptor *patched* (PTCH) antagonizes signal transduction by inhibiting the activity of the seven transmembrane receptor *smoothened* (Smo). Upon binding of Hh ligands (e.g., Sonic Hh, Indian, Hh, or Desert Hh being the three known mammalian ligands), the inhibition of *Smo* by *PTCH* is released, and a series of intracellular signal transduction events are initiated, resulting in nuclear translocation of the Hh transcription factor *Gli1*, which then initiates transcription of Hh responsive genes. In addition to its role in developmental patterning, this pathway plays a critical role in mature tissue homeostasis and the maintenance of somatic cell numbers in various organs.^{5,6} In a subset of familial cancers that arise in the context of Gorlin syndrome (specifically, familial basal cell carcinoma, rhabdomyosarcoma, and medulloblastoma), this pathway is constitutively activated by inactivating mutations in PTCH (recall that PTCH in the basal state inhibits the critical Hh signal transducing receptor Smo, and therefore, inactivation of PTCH renders the pathway aberrantly active in these tumors). In addition to Gorlin syndrome associated familial cancers, a much more common event is aberrant activation of the Hh pathway in endoderm-derived cancers, such as cancers of the lung, GI tract, and prostate.^{7,8} In the overwhelming majority of these cancers, Hh activation does not arise because of inactivating somatic mutations PTCH, but rather by endogenous overexpression of Hh ligands such as Shh. In the context of prostate cancers, expression of Hh target genes is a feature restricted to metastatic and not to localized tumors, suggesting a critical role for this pathway in disease progression.⁸

In epithelial cancers with aberrant Hh activation, abrogation of Hh signaling by cyclopamine, a naturally occurring Hh-specific small-molecule inhibitor, causes profound inhibition of tumor growth. Cyclopamine is a lipophilic compound extracted from the lily *Veratrum californicum*.⁹ It antagonizes *Smo* activity by binding to its heptahelical bundle.⁸ Thus, it can block pathway activation resulting in any of the two upstream events of Smo—i.e., either from *PTCH* mutations or from Hh ligand over-expression. Studies in preclinical models of rodent and human prostate cancer have confirmed that blockade of Hh signaling by cyclopamine can inhibit tumor growth as well as tumor progression. Administration of cyclopamine causes both down-regulation of proliferation and initiation of apoptosis, with consequent reduction in tumor size. Administration of cyclopamine in a lethal, metastatic rodent model of prostate cancer completely abrogates systemic metastases and dramatically improves survival.^{8,9}

The specificity of cyclopamine for the Hh pathway is demonstrated by an absence of cytotoxicity in cells that lack Hh signaling. However, given that Hh signaling is required in the stem cell niches of various tissues, such as the gonads, gastrointestinal tract, and bone marrow, a major pitfall of this otherwise promising cancer therapy is its potential for ‘on-target’ toxicity in somatic stem cells occurring as a result of inhibition of the intended target [i.e., Hh] in non-cancerous cells.^{8,10–14} Such ‘on-target’ side effects have been described with other therapies that interfere with stem/progenitor cell function, e.g., in mice receiving systemically administered Notch inhibitors.¹⁵

In order to circumvent these toxicities while harnessing the anti-cancer therapeutic potential of cyclopamine, it would be of great value to devise platforms for targeted delivery and/or activation of this compound within the milieu of prostate cancer. Thus, we hypothesized that the ‘on-target’ systemic side effects of cyclopamine could be decreased or eliminated by the creation of an inactive ‘prodrug,’ in which cyclopamine is coupled to a peptide carrier that is a substrate for tissue- or cancer-specific protease(s) and is only activated when exposed to the protease(s) of interest.

Therefore, we have synthesized a peptide carrier that is designed to serve as a substrate for the unique prostate tissue-specific serine protease, prostate-specific antigen (PSA). PSA is expressed in high levels only in neoplastic and normal prostate cells and not in any significant

amounts by other normal cell types.^{16,17} PSA is synthesized initially as a pro-enzyme that is processed to an active chymotrypsin-like serine protease with unique substrate specificity. Thus, the extracellular fluid around prostate epithelial cells (either normal or neoplastic) contains a remarkably high level (i.e., >100 µg/ml) of enzymatically active PSA. Once PSA reaches the circulation, however, its enzymatic activity is completely inhibited by a >1000-fold molar excess of serum protease inhibitors, with which it rapidly forms complexes.^{17,18} Thus, we reasoned that it would be possible to achieve selective local activity of an anti-prostate cancer agent such as cyclophosphamide by coupling the inhibitor to a PSA-specific carrier substrate, to produce an inactive prodrug that is non-toxic in the circulation and PSA-negative tissues but becomes cytotoxic when processed proteolytically by PSA within the milieu of prostate cancer.

We have previously identified a peptide with the amino acid sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) that is selectively and efficiently hydrolyzed by PSA,^{19,20} and we have successfully linked the HSSKLQ peptide to doxorubicin to produce a prodrug that could be selectively activated by PSA both in vitro and in vivo.^{17,21} Recently, we have also identified a second PSA substrate, Ser-Ser-Lys-Tyr-Gln (SSKYQ), which demonstrates a ~ 10-fold higher K_{cat}/K_m value than that for HSSKLQ.

In the present study, we have evaluated the biological properties of these two cyclophosphamide conjugates, in which cyclophosphamide is coupled with the PSA substrate morpholino- (Mu-) HSSKLQ or Mu-SSKYQ. Our results indicate that the peptide-cyclophosphamide prodrug strategy has considerable potential for yielding a rational, minimally toxic, and efficacious therapy for this lethal disease.

2. Results and discussion

2.1. Chemistry

Compound **2** was synthesized by coupling cyclophosphamide in tetrahydrofuran (THF) with 2.5 equivalents of the peptide Mu-HSSKLQ activated with EDC/DIPEA. Similarly, prodrug compound **3** was synthesized by coupling cyclophosphamide with the peptide Mu-SSKYQ (Scheme 1). The prodrugs were purified by high-performance liquid chromatography (HPLC) and characterized by matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy and Nuclear Magnetic Resonance (NMR) spectroscopy.

2.2. Kinetics of cyclophosphamide release from the prodrug formulations

Hydrolysis of the cyclophosphamide prodrug by PSA was determined by individually incubating the peptide prodrugs **2** and **3** (at 100 µM) with commercially available, enzymatically active PSA (Calbiochem) in 50 mM Tris and 0.1 M NaCl (pH 7.8) at room temperature. The rates of hydrolysis were determined using HPLC. At discrete time points (0, 0.5, 2, 4, 6, 8, 10, 12, and 18 h), aliquots of the reaction mixture were removed and analyzed by HPLC on a reversed-phase C18 Phenomenex analytical column (25 cm × 4.6 mm). A standard curve produced by using purified free peptide and the prodrug was used to convert peak area to free peptide and the prodrug concentrations. Since the peptide prodrugs are to be administered systemically via the blood, hydrolysis rates of the peptide-prodrugs in normal human sera and in sera from nude mice were also determined in order to assess the serum stability of the prodrugs. The percent hydrolysis was determined from the ratio of the peak area of the free drug to the total peak area (free drug + prodrug).

The Mu-SSKYQ-cyclophosphamide prodrug (**3**) appeared to be efficiently hydrolyzed by PSA, with a half-life of 3.2 h (Fig. 2); in contrast, Mu-HSSKLQ-cyclophosphamide (**2**) was less rapidly cleaved, with a half-life of 22 h (Fig. 1).

2.3. Biological effects of the prodrugs

Mechanistic specificity was measured in the PSA-non-expressing DU145 prostate cancer cell line, cultured in the presence and absence of exogenous PSA, in order to compare the cytotoxic effects of the prodrug itself to the Hh pathway-dependent growth inhibition caused by the released cyclopamine. The free cyclopamine alone at 5 and 10 μ M caused a robust suppression of growth at 96 h in the absence of PSA, while Mu-HSSKLQ-cyclopamine **2** in the absence of PSA had only a minimal effect (Fig. 3). In contrast, addition of PSA to the medium led to a sevenfold enhancement in the efficacy of the prodrug, with pronounced growth inhibition being detected at 96 h. All experiments were performed in triplicate (Fig. 3).

Similar results were obtained with Mu-SSKYQ-cyclopamine **3**. Although it is difficult to explain the fact that prodrugs **2** and **3** showed better activity in the presence of PSA than did the cyclopamine alone at similar concentration, we made the interesting observation that there was a significant difference in the activity of prodrugs **2** and **3** in the presence and absence of PSA.

3. Experimental

3.1. General methods

All reactions were carried out in a nitrogen atmosphere under anhydrous conditions, unless otherwise noted. All reagents and solvents were purchased at high commercial quality and used without further purification. NMR spectra were recorded on a 400 MHz Varian NMR spectrometer. Chemical shifts are reported as ppm (δ units) relative to tetramethylsilane as an internal standard. The purity of the prodrugs was checked with RP-HPLC. All compounds were at least 95% pure. HPLC analyses were carried out on a Waters system equipped with photodiode array detector and either a 150 $\times$ 4.60 mm 5 μ C18 (analytical runs) or a 250 $\times$ 10.00 mm 10 μ C18 (preparative runs) reversed-phase column. Mass spectra were obtained with Voyager DE-STR (Applied Biosystems) MALDI-TOF or Bruker LC-MS. Flash column chromatography was performed with Merck Kieselgel 60, and TLC analysis was performed on Analtech precoated silica gel 60 GF-254 plates.

The PSA-specific peptide sequences (with the morpholino- [Mu-] protection group) were prepared in our laboratory by solid-phase peptide synthesis (SPPS) according to the fluorenylmethoxycarbonyl (Fmoc) protocol on a Wang resin. Completion of each coupling was confirmed by the Kaiser ninhydrin test. The synthesis was carried out in a programmable menu-driven SPPS machine; model PS-3 (Rainin Instrument Company-Protein Technologies, Inc., Woburn, MA).

3.2. Synthesis of Mu-HSSKLQ-cyclopamine (**2**)

To a solution of 55 mg (0.06 mmol) of Mu-His-Ser-Ser-Lys(Fmoc)-Leu-Gln-OH, 11.5 mg (0.06 mmol) of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride and 10 mg (0.024 mmol) of cyclopamine (**1**) in DCM (2 ml) were added and stirred for 6 h. DCM was evaporated, acetonitrile and water (1:1) (10 ml) were added, and the product was purified by HPLC to obtain the desired product: 28 mg (80%) of Mu-His-Ser-Ser-Lys(Fmoc)-Leu-Gln-cyclopamine. Deprotection of the Fmoc on the Mu-His-Ser-Ser-Lys(Fmoc)-Leu-Gln-cyclopamine was carried out by stirring with 20% piperidine in DMF for 30 min. Acetonitrile and water (1:1) (10 ml) were then added, and the product was purified by HPLC to give 20 mg (75%) of the desired product, Mu-His-Ser-Ser-Lys-Leu-Gln-cyclopamine (Mu-SSKYQ-cyclopamine). ¹H NMR (400 MHz, CD₃OD) δ 1.01 (6H, s), 1.06 (6H), 1.26 (5H), 1.29 (2H, m), 1.44 (2H), 1.55 (2H, m), 1.59 (2H), 1.65 (2H), 1.67 (3H), 1.71 (3H), 1.75 (4H), 1.79 (2H), 1.83 (1H), 1.92 (4H), 1.94 (1H), 2.07 (2H), 2.11 (2H), 2.18 (2H), 2.65 (3H), 3.05 (2H), 3.25 (1H), 3.47 (4H), 3.67 (4H), 3.93 (1H), 4.03 (2H), 4.36 (1H), 4.53 (3H, m), 4.95 (1H), 5.37

(1H), 6.8 (1H), 7.44 (1H). MS (MALDI) m/e 1205 ($M + H$)⁺, 1227 ($M + Na$)⁺. Anal. (C₆₁H₉₆N₁₂O₁₃) C, H, N.

3.3. Synthesis of Mu-SSKYQ-cyclopamine (3)

To a solution of 48 mg (0.06 mmol) of Mu-Ser-Ser-Lys (Fmoc)-Tyr-Leu-Gln-OH, 11.5 mg (0.06 mmol) of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride and 10 mg (0.024 mmol) of cyclopamine (**1**) in DCM (2 ml) were added and stirred for 6 h. DCM was evaporated, acetonitrile and water (1:1) (10 ml) were added, and the product was purified by HPLC to obtain the desired product: 25 mg (85%) of Mu-Ser-Ser-Lys(Fmoc)-Tyr-Leu-Gln-cyclopamine. Deprotection of the Fmoc on the Mu-Ser-Ser-Lys(Fmoc)-Tyr-Leu-Gln-cyclopamine was carried out by stirring with 20% piperidine in DMF for 30 min. Acetonitrile and water (1:1) (10 ml) were then added, and the product was purified by HPLC to give 19 mg (80%) of the desired product, Mu-Ser-Ser-Lys-Tyr-Gln-cyclopamine (Mu-SSKYQ-cyclopamine). ¹H NMR (400 MHz, CD₃OD) δ 1.05 (6H), 1.25 (5H), 1.3 (2H, m), 1.4 (2H), 1.58 (2H, m), 1.6 (2H), 1.65 (2H), 1.65 (3H), 1.71 (3H), 1.74 (2H), 1.79 (2H), 1.92 (4H), 1.95 (1H), 2.06 (2H), 2.11 (2H), 2.19 (2H), 2.67 (3H), 3.05 (2H), 3.24 (1H), 3.48 (4H), 3.67 (4H), 3.93 (1H), 4.03 (2H), 4.36 (1H), 4.54 (2H, m), 4.9 (1H), 5.37 (1H), 6.69 (2H), 6.97 (2H). MS (MALDI) m/e 1119 ($M+H$)⁺, 1131 ($M+Na$)⁺. Anal. (C₅₈H₈₇N₉O₁₃) C, H, N.

4. Conclusions

In summary, we have synthesized two prodrug formulations, Mu-HSSKLQ-cyclopamine and Mu-SSKYQ-cyclopamine, for potential use in prostate cancer therapy. Both of these prodrug formulations demonstrated efficient release of free cyclopamine in the presence of the enzyme PSA, with compound **3** (Mu-SSKYQ-cyclopamine) being much more efficiently hydrolyzed, with a half-life of 3.2 h. Coupling of this peptide (MuSSKYQ-) to the secondary amine of cyclopamine successfully masked the activity of the cyclopamine at 1 μ M; cleavage of the prodrug by PSA would allow this cyclopamine activity to be unleashed at the desired site of action (neoplastic prostatic tissue). Importantly, this prodrug strategy has the potential to minimize cyclopamine related 'on-target' toxicities in non-neoplastic tissues, which require sustained Hh signaling but do not produce the proteolytic PSA enzyme (e.g., nervous and hematopoietic systems). It is important to note that there remain substantial challenges with this strategy, including the measurement of stability of these peptide prodrugs in the GI tract. However given the tremendous potential of this therapeutic strategy, we are in the process of performing in vivo studies to move this platform close toward clinical translation. We suggest that modulating the cyclopamine at the secondary amine with the PSA-cleavable peptides is a promising strategy for developing prodrugs to target prostate cancer.

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Figure 1.
Kinetics of free peptide release, corresponding to PSA-catalyzed hydrolysis of the Mu-HSSKLQ-cyclopamine prodrug.

Figure 2.
Kinetics of free peptide release, corresponding to PSA-catalyzed hydrolysis of the Mu-SSKYQ-cyclopamine prodrug.

Figure 3. Mu-HSSKLQ-cycloamine leads to growth inhibition in DU145 cells only in the presence of PSA (10 $\mu\text{g/mL}$). Results are means $\pm$ SD of triplicate experiments.