ELSEVIER

Urologic Oncology: Seminars and Original Investigations 30 (2012) 635–645

UROLOGIC ONCOLOGY

Original article

Identification of a bladder cancer-specific ligand using a combinatorial chemistry approach☆

Hongyong Zhang, D.V.M., Ph.D.[a], Olulanu H. Aina, D.V.M., Ph.D.[a], Kit S. Lam, M.D., Ph.D.[a], Ralph de Vere White, M.D.[b], Christopher Evans, M.D.[b], Paul Henderson, Ph.D.[a], Primo N. Lara, M.D.[a,b,c], Xiaobing Wang, Ph.D.[d], James A. Bassuk, Ph.D.[e], Chong-xian Pan, M.D., Ph.D.[a,b,c,*]

[a] *Division of Hematology and Oncology, Department of Internal Medicine, University of California Davis Cancer Center, Sacramento, CA 95817, USA*
[b] *Department of Urology, University of California Davis, Sacramento, CA 95817, USA*
[c] *VA Northern California Health Care System, Mather, CA 95655, USA*
[d] *AnaSpec, Inc., San Jose, CA 95131, USA*
[e] *Program in Human Urothelial Biology, Center for Tissue and Cell Sciences, Seattle Children's Research Institute, Seattle, WA 98101, USA*

Received 15 April 2010; received in revised form 16 June 2010; accepted 17 June 2010

Abstract

Objectives: To develop bladder cancer-specific ligands using a combinatorial chemistry approach.

Materials and methods: We performed a high-throughput one-bead one-compound combinatorial chemistry approach to identify ligands that bound to bladder transitional cell carcinoma cells. The whole-cell binding assay allowed successful identification of a few peptides that bound selectively to bladder cancer cells. Single cell suspensions derived from clinical bladder cancer specimens and cell lines were used to determine the binding specificity. Studies with mouse xenografts were performed to determine the in vivo binding and targeting efficiency, specificity, and biodistribution of one of the ligands.

Results: One cyclic peptide named PLZ4 (amino acid sequence: cQDGRMGFc) was identified that could selectively bind to bladder cancer cell lines and all of the 5 primary bladder cancer cells from human patients, but not to normal urothelial cells, cell mixtures from normal bladder specimens, fibroblasts, and blood cells. Comparison of PLZ4 binding to cell lines of different cancer origins showed that it was bladder cancer-specific ($P < 0.05$). PLZ4 could bind to tumor cells treated with urine at pH 6.0, but not to noncancerous cells collected from the urine of 4 patients actively being treated with intravesical Bacillus Calmette-Guerin therapy. In vivo and ex vivo imaging studies showed that PLZ4 linked to Cy5.5 fluorescent dye administered via tail vein injection was specifically taken up in mouse xenografts developed from excised fresh human bladder cancer specimens. Several ligands contain the same DGR motif, but only PLZ4 was bladder cancer-specific. We performed alanine walk and rainbow bead coding experiments, and found that the C-terminal GF residues were also important for cell binding and modulated the binding specificity.

Conclusions: PLZ4 has the potential to be used for targeted therapy and imaging detection during diagnosis and follow-up/surveillance of noninvasive and advanced bladder cancer. Published by Elsevier Inc.

Keywords: Bladder cancer; Targeted therapy; Combinatorial chemistry

1. Introduction

Bladder cancer is the fourth most common cancer in men and ninth in women [1]. At diagnosis, about 75% of patients are at the noninvasive stages [2]. Noninvasive bladder cancer is ideal for imaging and targeted therapy with cancer-specific ligands because it is easily accessible through intravesical instillation, relatively isolated from the rest of the human body, and has only a few confounding cells. The treatment for noninvasive cancer is usually transurethral resection of bladder tumor (TURBT) followed by intravesical instillation of Bacillus Calmette-Guerin (BCG) or mi-

☆ This publication is based upon work supported in part by University of California Cancer Research Coordinating Committee grant (PI: C.-X.P.), the Career Development Award-II from the Office of Research and Development, Department of Veteran Affairs (PI: C.-X.P.), by a K12 award (Grant Number UL1 RR024146) from the National Center for Research Resources (NCRR) (PI: C.-X. P.), NIH R01DK058881 (J.A.B.) and R01DK062251 (J.A.B.).

* Corresponding author. Tel.: +1-916-734-3771; fax: +1-916-734-7946.
E-mail address: cxpan@ucdavis.edu (C.-X. Pan).

1078-1439/$ – see front matter Published by Elsevier Inc.
doi:10.1016/j.urolonc.2010.06.011

tomycin C to reduce recurrence. Despite this treatment, 20% to 80% of patients will recur and 25% will have disease progression [3–5]. All these patients require long-term follow-up with urine cytology and cystoscopy. The sensitivity of urine cytology ranges between 29% and 74%, with the overall sensitivity of approximately 35% [6–8]. Cystoscopy is intrusive, uncomfortable, and costly. Because of the long-term survival and the need for monitoring over an extended period of time, the cost per case for bladder cancer is the highest among all cancer types, ranging from $96,000 to $187,000 (2001 values) per case [9,10]. Thus, novel alternative diagnostic and monitoring strategies are warranted.

We hypothesized that combinatorial chemistry could be used to develop bladder cancer-specific ligands for imaging and targeted therapy during the diagnosis, treatment, and follow-up of bladder cancer. Screening of a phage display peptide library identified several peptides that have the potential to be used for diagnosis of bladder cancer [11]. However, the in vivo targeting with human primary bladder cancer cells has not yet been determined. We used the one-bead one-compound combinatorial peptide library technology (OBOC) developed by one of us [12,13]. Each bead is ~90 μm in diameter that bears up to 10^{13} copies of ligands with the same chemical identity (hence the name OBOC). In order to develop cancer-specific ligands, millions of beads (each with a unique ligand sequence) can be screened in parallel to identify those ligands binding to cancer cell surface molecules. "Positive beads" that bear ligands specific for the desired target can be selected using an enzyme-linked colorimetric assay similar to the western blot, or by the evidence of cell attachment on the bead surface [14,15]. Unnatural amino acids, D-amino acids, or even non-peptide moieties can be incorporated in the library to make the molecules resistant to proteolysis and increase the binding affinity [16]. The ligand leads identified through screening of OBOC libraries can be further optimized to achieve high affinity and specificity [16]. Here, we used OBOC methodology to develop PLZ4 as a bladder-specific ligand that has the potential use for imaging, targeted therapy of bladder cancer, and for capturing of cancer cells in urine for diagnosis and follow-up.

2. Materials and methods

2.1. Synthesis of the initial and focused OBOC libraries

OBOC libraries were synthesized on solid phase TentaGel S NH2 resin (Rapp Polymere GmbH, Tübingen, Germany). A "split-mix" synthesis method was performed to construct the combinatorial OBOC libraries, each containing random libraries of millions of beads/ligands [12,13,16]. The ligands on the bead surface was synthesized by standard solid-phase peptide synthesis techniques using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and N-hydroxybenzotriazole (HOBt)/N,N'-diisopropylcarbodiimide (DIC) coupling. The completion of coupling was confirmed with a ninhydrin test. The beads were stored in 70% ethanol at 4°C until use.

2.2. Cells

Four human bladder cancer cell lines including 5637 (HTB-9), SCaBER, TCCSUP (HTB-5), and T24 (HTB-4) and other human cell lines were purchased from the American Type Culture Collection (Manassas, VA) and are outlined in detail in Supplement 1, which can be found in the electronic version of this article. The isolation, characterization, and maintenance of normal urothelial cells was performed as previously described [17]. Normal peripheral blood mononuclear cells (PBMC) were prepared by using the Ficoll-Paque gradient method from peripheral blood of healthy donors. Bladder cancer specimens obtained from cystectomy were cut into pieces, digested with collagenase at 37°C for 1 to 2 hours per the manufacturer's protocol, and filtered through 40-μm strainers to make single cell suspensions. Cells (mainly cancer cells) were then isolated with Ficoll-Paque gradient method (800 × g, 30 minutes at 4°C). This protocol was approved by UC Davis IRB before the experiments were started (protocol no. 200614340). Informed consent was obtained from each patient or healthy donor before specimens were collected.

2.3. Screening of OBOC library for bladder cancer-specific ligands

The beads were washed extensively with double-distilled water and phosphate-buffered saline (PBS) before screening. Bladder cancer cells and normal urothelial cells were detached from culture dishes with trypsin/EDTA, washed with their corresponding culture medium, resuspended at 10^6 cells/ml, and incubated with OBOC beads in Petri dishes in a humidified CO_2 incubator at 37°C with shaking (60 rpm). Beads bound by cells appeared as rosettes with a central bead covered by a layer(s) of cells under a microscope (Fig. 1). The positive beads were picked with a pipette under inverted microscope, treated with guanidine-HCl (8M, 20 minutes) to remove cells and proteins on the bead surface, and underwent a second round of screening with the same cells to confirm the binding. Only those beads with cell bindings at both rounds were sent for peptide sequencing as previously described [16].

2.4. Synthesis of peptide and peptide-biotin

The synthetic chemistry of solution phase PLZ4 and PLZ4-biotin for biological testing is similar to that of the library using HOBt/DIC coupling (Supplement 2). Rink amide resin was used as solid support to prepare compounds with carboxyl amide derivatives.

2.5. Fluorescence microscopy

Bladder cancer cells and normal urothelial cells (2×10^4 cells per well) were seeded on chamber slides. When the cells grew to confluent of approximately 70%, they were washed with PBS and blocked with 3% BSA-PBS at 4°C for