



Discovery of PSMA-specific peptide ligands for targeted drug delivery



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ABSTRACT

Prostate-specific membrane antigen (PSMA) has been widely used as a biomarker and targeting receptor for prostate cancer therapy because of its overexpression in most prostate cancer cells. In this study, a novel combinatorial phage biopanning procedure was developed to discover PSMA-specific peptides that can be potentially used as ligands for targeted drug delivery to prostate cancer cells. Five rounds of biopanning against recombinant human PSMA extracellular domain (ECD), PSMA-positive LNCaP cells, and LNCaP xenografts in nude mice were conducted. Various affinity assays were conducted to identify high-affinity peptides for PSMA ECD and PSMA-positive prostate cancer cells. Among them, the GTI peptide shows the highest affinity as well as specificity to PSMA in prostate cancer cells. The apparent K_d values of the GTI peptide to PSMA-positive LNCaP and C4-2 cells are 8.22 μ M and 8.91 μ M, respectively. The GTI peptide can specifically deliver the proapoptotic peptide $D(KLAKLAK)_2$ to LNCaP cells to induce cell death. In the biodistribution study, the GTI peptide shows the highest uptake in C4-2 xenografts, while its uptake in other major organs, such as the liver and spleen, are either low or negligible. Compared to its scrambled control (random permutation of the GTI peptide), the GTI peptide exhibits higher and more specific uptake in C4-2 xenografts. All the results suggest that the GTI peptide is a potentially promising ligand for PSMA-targeted drug delivery for prostate cancer therapy.

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1. Introduction

Prostate cancer is the most commonly diagnosed malignancy and second prevalent cause of cancer death in American men. The estimated death of prostate cancer in 2015 is 27,540, which accounts for about 9% of all male cancer deaths in the United States (Siegel et al., 2015). Conventional therapies for prostate cancer include surgery, radiation and hormone therapy. Despite these treatments are relatively efficient for early stage prostate cancer, most patients with localized prostate cancer ultimately relapse (Mabjeesh et al., 2002). Chemotherapy is currently widely used for advanced prostate cancer treatment, but with limited success. Lack of targeted delivery is one of the major hurdles that limit the effectiveness of chemotherapy (Barve et al., 2014). As a result, a great deal of attention has been paid to the development of targeted drug delivery systems for prostate cancer therapy. Prostate-specific membrane antigen (PSMA), a \sim 100 kDa type II transmembrane glycosylated protein with folate hydrolase activity, is overexpressed not only in nearly all prostate cancer cells but also

in tumor neovasculature of a variety of cancers (Wright et al., 1995; Chang et al., 1999; O'Keefe et al., 1998). By contrast, its expression in normal prostate epithelium tissues and other normal tissues is 100–1000 fold lower (Wright et al., 1995; Silver et al., 1997; Ghosh and Heston, 2004; Sokoloff et al., 2000). PSMA, therefore, is a validated target for prostate cancer therapy and has been adopted as a biomarker for diagnosis and imaging. Aptamers and antibodies targeting PSMA have been discovered for targeted drug delivery to prostate cancer cells in the past few years (Barve et al., 2014). Although aptamers and antibodies retain high binding affinity to PSMA, their drawbacks, such as large size, possible immunogenicity and instability, may limit their applications in targeted drug delivery. In contrast, peptides have a number of advantages, including small molecular weight, high permeability, great stability, less immunogenicity, ease of synthesis and flexibility in chemical conjugation. Moreover, it has been reported that peptides can achieve high binding affinity and specificity that are comparable with antibodies (Pazgier et al., 2009; Huang et al., 2003).

Phage display has been widely used to identify peptide ligands for a wide variety of molecular targets including proteins, cells, or animal tissues. A phage display library contains billions of different phages, and each phage retains a unique inserted peptide sequence on the surface. Phage display technology therefore provides a high

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throughput tool for affinity selection. Protein-based biopanning and cell-based biopanning are the two most common strategies to identify peptide ligands, but both of them have disadvantages when they are used alone (Chen et al., 2015; Qin et al., 2011).

In this study, combinatorial biopannings against recombinant human PSMA extracellular domain (ECD), PSMA-positive LNCaP cells, and LNCaP xenografts in nude mice were conducted. The identified GTI peptide shows high affinity and specificity to PSMA *in vitro* and *in vivo*. The peptide can also deliver a fused proapoptotic peptide to PSMA-positive prostate cancer cells, indicating its capability to deliver other therapeutic cargos. The GTI peptide is therefore a potentially promising ligand for targeted drug delivery to prostate cancer.

2. Materials and methods

2.1. Materials

The Ph.D.TM-12 phage display peptide library and E. coli ER2738 were purchased from New England Biolabs (Beverly, MA). LNCaP, PC-3, C4-2, CWR22Rv1, and HeLa cells were obtained from American Type Culture Collection (Manassas, VA). HSC-T6 cell line was kindly provided by Dr. Scott L. Friedman from New York University. D(KLAKLAK)₂ peptide was purchased from Anaspec, Inc (Fremont, CA). All other peptides including FAM-labeled peptides and GTI-KLA fusion peptides were purchased from United Biosystems Inc (Herndon, VA). Non-enzymatic cell dissociation solution was obtained from MP Biomedicals (Santa Ana, CA). Homozygous nude mice were ordered from The Jackson Laboratory (Bar Harbor, ME).

2.2. Cell culture

LNCaP, C4-2, CWR22Rv1 and PC-3 cells were maintained in RPMI-1640 medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. HSC-T6 and HeLa cells were cultured in DMEM medium with 10% FBS and penicillin/streptomycin. The cells were incubated in a 5% CO₂-humidified atmosphere incubator at 37 °C and passaged when they reached 80% confluence.

2.3. Cloning and expression of PSMA ECD

The plasmid pcDNA3.1-PSMA encoding full-length human PSMA was kindly provided by Dr. Shawn E. Lupold (Johns Hopkins University School of Medicine, Baltimore, MD). PSMA ECD was amplified from the plasmid using the forward primer 5'-ATCAGATCTAAATCCTCCAATGAAGC-3' and reverse primer 5'-ATCAAGCTTCTGCACTGTGAAGGCTGCAACATA-3'. The amplified fragment was excised using *Bgl*III and *Hind*III, purified using a PCR Clean-Up kit, and cloned into the pRSET A vector (Invitrogen, Grand Island, NY) as we described before (Jia et al., 2007). The pRSET A vector encoding PSMA ECD was transformed into BL21 (DE3)pLysS competent cells. The transformed competent cells were cultured in LB medium, and IPTG was added to induce the expression of PSMA ECD when the OD₆₀₀ reached 0.5. After four hours of induction, the cells were harvested, lysed, and the expressed protein was collected.

2.4. Combinatorial phage biopanning against the recombinant human PSMA ECD, LNCaP cells, and xenograft LNCaP tumor

A combinatorial phage biopanning procedure was conducted against recombinant human PSMA ECD (the first, third, and fifth rounds), LNCaP cells (the second and fourth rounds), and xenograft LNCaP tumor (the fifth round). Biopanning against recombinant protein was conducted as we described before (Chen et al., 2015).

Briefly, ten micrograms of the recombinant human PSMA ECD was coated on 24-well plates at 4 °C overnight. Ten microliters of the M13 phage display library (10¹³ pfu/mL) were incubated with PSMA ECD at 4 °C for one hour under gentle shaking. Unbound phages were removed by washing the immobilized PSMA ECD with PBST (0.1% Tween 20) three times. Bound phages were eluted by adding 0.2 M glycine-HCl (pH 2.2) and amplified by infecting ER2738 bacteria.

Biopanning against LNCaP cells was conducted as reported (Qin et al., 2011; Giordano et al., 2001). Briefly, PC-3 and LNCaP cells were harvested with ice cold PBS containing 5 mM EDTA and suspended in RPMI-1640 medium at a density of 1 × 10⁷ cells/mL. Phages from the previous round (10¹¹ pfu) were incubated with PC-3 cells (PSMA negative) at 4 °C for one hour to remove non-specific bound phages. The precleaned phages were incubated with LNCaP cells (PSMA positive) at 4 °C for one hour under gentle rotation. The cell suspension was then mixed with 200 µL of an organic phase composed of dibutyl phthalate and cyclohexane (9:1, v/v). After centrifugation at 10,000 RPM for 10 min, the mixture was snap frozen using liquid nitrogen. The bottom of the tube was sliced off, and bound phages in the cell pellet were recovered by infecting ER2738 bacteria.

Part of the phages from the fourth round were used for an additional biopanning against recombinant PSMA ECD, while the other part were used for *in vivo* biopanning against xenograft LNCaP tumor. The animal experiment was conducted under a protocol approved by the University of Missouri-Kansas City Institutional Animal Care and Use Administrative Advisory Committee. Male nude mice aged 5–6 weeks were subcutaneously inoculated with LNCaP cells to generate prostate cancer xenografts. Once the tumor volume reached 1 cm³, 2 × 10⁹ pfu of the fourth round phages were injected into the nude mouse via tail vein. After one hour, the nude mouse was sacrificed and the heart was perfused with 50 mL PBS to remove unbound phages in the body. The xenograft tumors were harvested and homogenized in TBS buffer. After centrifugation to remove the supernatant, the cell pellet was washed three times with TBS buffer (pH 7.4), followed by the addition of 0.2 M glycine HCl (pH 2.2) to adjust the pH to 3.0. After incubation at room temperature for 10 min, the supernatant containing eluted phages was collected and neutralized to pH 8.5 by Tris buffer (pH 9.1).

Individual phage clones from the fourth and fifth rounds of biopanning were randomly selected, cultured and sequenced as we described before (Chen et al., 2015). Encoded peptides sequences were deduced from the phage DNA sequences.

2.5. Cell phage ELISA

LNCaP and PC-3 cells were seeded in 96-well plates at a concentration of 2 × 10⁴ cells per well. After 24 h, the cells were fixed with cold methanol-acetone (1:1, v/v). Phages suspended in RPMI 1640 medium were incubated with the fixed cells for 1 h, followed by washing with PBS to remove unbound phages. The cells were incubated with HRP conjugated anti-M13 monoclonal antibodies for 1 h. After adding TMB substrate, the absorbance at 450 nm was measured with a Beckman DTX 880 multimode Detector (Beckman coulter, Inc., Brea, CA).

2.6. Binding affinity to various types of cells

LNCaP, PC-3, HSC-T6, and HeLa cells were detached using non-enzymatic cell dissociation solution and suspended in medium with 1% BSA at a density of 1 × 10⁷ cells/mL. The suspended cells were incubated with the GTI phages at 4 °C for 1 h under gentle rotation. The cell suspension was then mixed with an organic phase composed of dibutyl phthalate and cyclohexane (9:1, v/v).

After centrifugation at 10,000 RPM for 10 min, the mixture was snap frozen using liquid nitrogen. The bottom of the tube was sliced off, and bound phages in the cell pellet were recovered by infecting ER2738 bacteria.

2.7. Competitive inhibition assay

Competitive binding inhibition between selected phages and their encoded peptides was examined in this study. LNCaP cells (1×10^7 cells/mL) suspended in RPMI-1640 medium were incubated with encoded peptides at 4 °C for 30 min under gentle rotation. The cells were then washed, resuspended in fresh RPMI-1640 medium containing phage clones, and incubated at 4 °C for 1 h. The bound phages were recovered and tittered as described above.

2.8. Flow cytometry analysis

Cells were detached using non-enzymatic cell dissociation solution and suspended in PBS (pH=7.4) at a concentration of 1×10^6 cells/mL. A series of concentrations (0.1–100 μ M) of FAM-labeled peptides were incubated with 500 μ L suspended cells at 4 °C for 1 h under gentle rotation. The cells were then washed with PBS for three times and subjected to fluorescence analysis on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.9. Cellular uptake of FAM-labeled peptides

Cells were seeded in 4-well chambers at a density of 5×10^4 cells per well and incubated at 37 °C for 24 h. The cells were then washed with PBS and then incubated with 10 μ M FAM-labeled

peptides in Opti-MEM medium at 37 °C for 1 h. After incubation, the cells were gently washed three times with DPBS, fixed with 10% formalin, and mounted with VECTASHIELD® mounting medium containing DAPI. Cellular uptake of the peptides was examined using a laser scanning confocal microscope (Leica TCS SP5).

2.10. DHT (5- α -dihydrotestosterone) treatment

LNCaP cells were cultured in PRMI-1640 medium with 5% charcoal-stripped FBS (DHT free) for 24 h. Subsequently, the cells were incubated in 5% charcoal-stripped FBS PRMI-1640 medium containing 2 nM DHT for 48 h. FAM-labeled GTI peptide (10 μ M) was incubated with DHT-treated LNCaP cells and normal LNCaP cells at 37 °C for 1 h. The cells were washed, fixed with 10% formalin, and mounted with VECTASHIELD® mounting medium containing DAPI. Cellular uptake of the GTI peptide was examined using a laser scanning confocal microscope (Leica TCS SP5).

The expressions of PSMA in the DHT-treated LNCaP and normal LNCaP cells were examined using western blot. Briefly, the cells were lysed on ice with RIPA buffer containing protease and phosphatase inhibitor cocktail. Protein concentrations were determined by a BCA protein assay kit. Equivalent amounts of protein (20 μ g) were separated by a 12% SDS-PAGE gel. The proteins were transferred to PVDF membrane, blocked with 5% non-fat milk at room temperature for 2 h, and probed with the primary anti-PSMA antibody (Abcam, Cambridge, MA). The protein was then visualized with horseradish peroxidase-conjugated secondary antibody and the FluorChem FC2 imaging system (Alpha Innotech, CA).

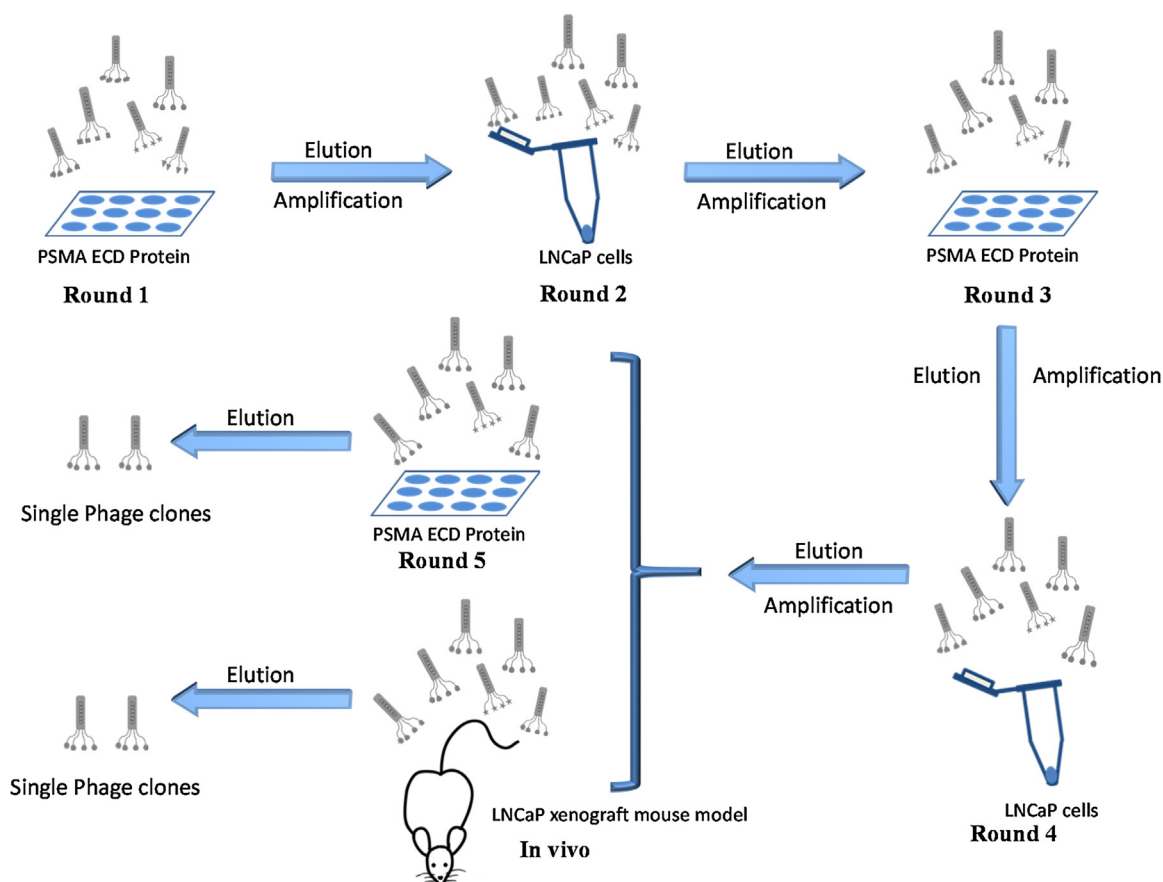


Fig. 1. Scheme of the combinatorial phage biopanning. The biopanning was conducted against recombinant human PSMA extracellular domain (ECD), PSMA-positive LNCaP cells, and LNCaP xenografts in nude mice.

2.11. Biodistribution study

The animal protocol was approved by the University of Missouri-Kansas City, Institutional Animal Care and Use Committee (IACUC). The C4-2 xenograft tumor model was developed by subcutaneous injection of 1×10^6 C4-2 cells with matrigel into each flank of BALB/c nude male mice aged 5–6 weeks. Once the tumor is formed ($\sim 1 \text{ cm}^3$), 20 nmol FAM-labeled GTI peptide and its scrambled peptide (random permutation of the GTI peptide) were injected into the mice via tail vein. After two hours, the mice were sacrificed, and major organs including the tumors, liver, heart, spleen, lungs, kidneys, and muscle were harvested. Fluorescence intensity of these organs was examined using a Xenogen IVIS imaging system (Xenogen, Hopkinton, MA).

2.12. Statistics analysis

Data were presented as the mean \pm standard deviation (SD) or standard error (SE). Difference between any two groups was evaluated by ANOVA. $P < 0.05$ is considered statistically significant.

3. Results

3.1. Identification of PSMA-specific peptides

A novel combinatorial phage biopanning procedure was developed to discover PSMA-specific peptides that can be potentially used as ligands for targeted drug delivery to prostate cancer. As shown in Fig. 1, five rounds of biopanning against recombinant human PSMA extracellular domain (ECD), PSMA-positive LNCaP cells, and LNCaP xenografts in nude mice were conducted. One hundred and twenty-two individual phage clones from the fourth and fifth rounds of biopanning were randomly selected and amplified. Binding affinities of these selected phage clones were examined in PSMA-positive LNCaP and PSMA-negative PC-3 cells using cell phage ELISA. The ratio of absorbance in LNCaP and PC-3 cells (LNCaP/PC-3) was calculated to identify phage clones that show higher binding affinity for LNCaP than PC-3 cells. Fifty phage clones with higher affinity for PSMA-positive LNCaP cells were selected and sequenced. These phage clones express 17 different peptide sequences which are listed in Fig. 2.

As shown in Fig. 2A, GTI phage encoding the peptide GTIQYPFWSGY shows the highest binding affinity to LNCaP cells. Fig. 2B lists the frequency of each peptide sequence in these fifty phages. The NRP phage encoding the peptide NRPDSAQFWLHH repeats 6 times from *in vitro* biopanning and 10 times from *in vivo*

biopanning. The phages encoding the peptides SMAGEQISWALI, GTIQYPFWSGY, QPGHAILAQHPT, YVNSHSILGYTG, TGHLYPTRMEIQ, HSDNHYPADKL, and YPTDWLWHGHNK were also found not only in *in vitro* but also *in vivo* biopannings. We therefore selected these eight phages for further evaluation.

3.2. Competitive inhibition of selected phages and their corresponding peptides

We first evaluated whether these phages bind to LNCaP cells via specific interaction with their encoding peptides. LNCaP cells were pre-incubated with the encoding peptides, followed by washing and incubation with the corresponding phages. The bound phages were then eluted and titered. As shown in Fig. 3A, the GTI peptide shows the highest competitive inhibition to its corresponding phage. The TGH and YVN peptides exhibit relatively weaker competitive inhibition compared to the GTI peptide, while competitive inhibition of the peptides SMA, HSD, QPG, YPT and NRP are negligible. Fig. 3B further illustrates the competitive inhibition effect of the GTI and NRP peptides at various concentrations. The GTI peptide exhibits a concentration dependent inhibition to its corresponding phage, while the NRP peptide does not affect the binding of its corresponding phage to LNCaP cells. This result suggests that the GTI phage binds to LNCaP via the same receptor as its peptide, while the NRP phage may bind to LNCaP via a different moiety as its encoding peptide.

3.3. Binding affinity and specificity of the GTI phage to PSMA-Positive LNCaP cells

We next compared the binding affinity of the GTI phage to PSMA-positive LNCaP and PSMA-negative cells. Compared to insertless phage, which does not encode a peptide, the GTI phage exhibits a much higher binding affinity to LNCaP cells (Fig. 4A). In addition, the GTI phage exhibits higher affinity to LNCaP cells than to PSMA-negative cells (Fig. 4B), which further indicates that the GTI phage specifically bind to PSMA on LNCaP cells.

3.4. Apparent equilibrium dissociation constant (K_d) of the peptides

To quantitate the apparent affinity of phage encoding peptides to prostate cancer cells, FAM-labeled peptides were incubated with PSMA-positive LNCaP cells at different concentrations. Flow cytometry was employed to determine the percentage of cells that were labeled with the peptides. The apparent K_d values were calculated by fitting to a one-site specific binding model using

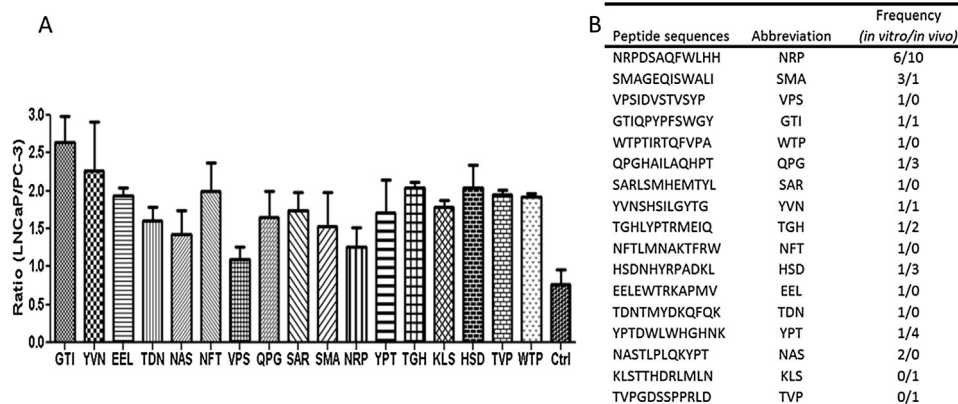


Fig. 2. Binding affinity of selected phage clones in LNCaP cells. (A) Binding of the phages on PSMA-positive LNCaP and PSMA-negative PC-3 cells were examined using cell phage ELISA. The ratio of absorbance in LNCaP and PC-3 cells was calculated. Results are represented as the mean \pm SD ($n = 3$). (B) Peptide sequences of the selected phage clones.

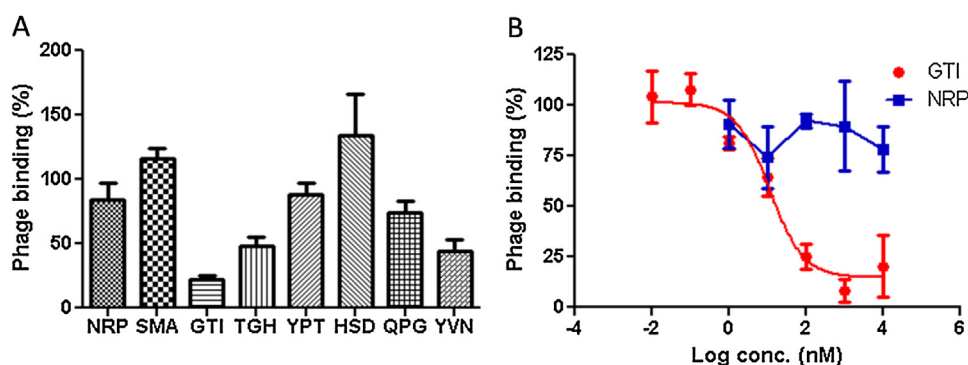


Fig. 3. Competitive inhibition of peptides and their corresponding phages. LNCaP cells were pre-incubated with synthetic peptides and then incubated with their corresponding phages. The bound phages were recovered and tittered. (A) Competitive inhibition of eight selected peptides at 100 μM. (B) Competitive inhibition of the GTI and NRP peptide at a series of concentrations. Results are represented as the mean ± SD (n=3).

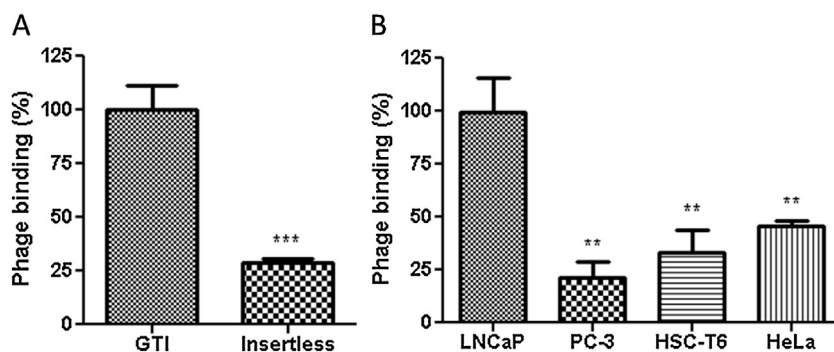


Fig. 4. Binding affinity of the GTI phage in LNCaP and PSMA-negative cells. The cells were suspended and incubated with the GTI phage at 4 °C for 1 h, and the bound phages were eluted and tittered. The number of GTI phages that bound to LNCaP cells was normalized as 100%. (A) Binding of the GTI phage and an insertless phage in LNCaP cells. (B) Binding of the GTI phage in PSMA-positive LNCaP cells and PSMA-negative cells. Results are represented as the mean ± SD (n=3). (***)p < 0.001, (**)p < 0.01

GraphPad Prism 5. As illustrated in Fig. 5A&C, the HSD, GTI, TGH, and YVN peptides exhibit higher binding affinity compared to other peptides. This is in accordance with the results of their corresponding phages in Figs. 2 and 3. Their apparent K_d values against LNCaP cells are 12.80 μM, 8.22 μM, 8.01 μM, and 9.34 μM, respectively. We also examined the apparent K_d values of HSD, GTI, TGH, and YVN in another PSMA-positive prostate cancer C4-2 cell line (Fig. 5B&C). The GTI and YVN peptides show similar affinity to C4-2 as to LNCaP cells, while the HSD and TGH peptides exhibit less affinity to C4-2 cells compared to LNCaP cells. Binding affinities of these FAM-labeled peptides in PC-3 cells were presented in Fig. 5D. The GTI peptide shows the lowest binding affinity in PC-3, indicating its high specificity to PSMA-positive cells. As a result, the GTI peptide was selected as the best PSMA-specific ligand for following studies.

3.5. Cellular uptake of FAM-labeled GTI peptide in various PSMA-Positive cells

We next evaluated cellular uptake of the FAM-labeled GTI peptide in three PSMA-positive cells, including LNCaP, C4-2 and CWR22Rv1. PSMA-negative PC-3 cells were used as a control. As illustrated in Fig. 6, the GTI peptide shows high uptake in all three PSMA-positive cells, while its uptake in PC-3 cells is negligible.

To determine whether cellular uptake of the GTI peptide is mediated by PSMA, we downregulated the expression of PSMA in LNCaP cells using 5-α-dihydrotestosterone (DHT) (Fig. 7A) (McNamara et al., 2006; Israeli et al., 1994) and then examined the uptake of the FAM-labeled GTI peptide. As shown in the confocal images (Fig. 7B), uptake of the GTI peptide in DHT-treated LNCaP cells is

much lower compared to that in untreated LNCaP cells. Flow cytometry assay (Fig. 7C) shows the similar result. The percentage of cells labeled by the GTI peptide is significantly reduced when the expression of PSMA is downregulated by DHT. These results clearly demonstrate that the GTI peptide is a PSMA-specific ligand.

3.6. The GTI Peptide Enhances the Uptake and Apoptotic Effect of a Proapoptotic Peptide

The objective of this study is to identify a PSMA-specific peptide that can be used as a targeting ligand to deliver various therapeutic agents into PSMA-positive prostate cancer cells. It is therefore critical to demonstrate that the GTI peptide can deliver a cargo to prostate cancer cells. The proapoptotic peptide KLAKLAKKLAKLAK (KLA) was therefore used as a model drug in this study. Once inside cells, the KLA peptide can induce mitochondrial disruption and cellular toxicity by triggering permeabilization and swelling of the mitochondria. However, the KLA peptide itself cannot enter cell. Instead, it has to be fused with a protein transduction domain to exert its apoptotic activity (Javadpour et al., 1996; Mai et al., 2001). In our study, we designed a GTI-KLA fusion peptide and evaluated its proapoptotic activity in LNCaP and PC-3 cells. A series of concentrations of the fusion peptide GTI-KLA, the mixture of GTI and KLA peptides, and the KLA peptide were incubated with LNCaP cells and PC-3 cells for 48 h. As Fig. 8A indicates, the GTI-KLA fusion peptide demonstrates cytotoxicity in LNCaP cells, whereas no cytotoxicity was observed in either the mixture of GTI KLA peptides or the KLA peptide alone up to 20 μM. Meanwhile, the GTI-KLA fusion peptide does not show cytotoxicity in PC-3 cells (Fig. 8B), indicating that the fusion peptide enters LNCaP cells via PSMA. The

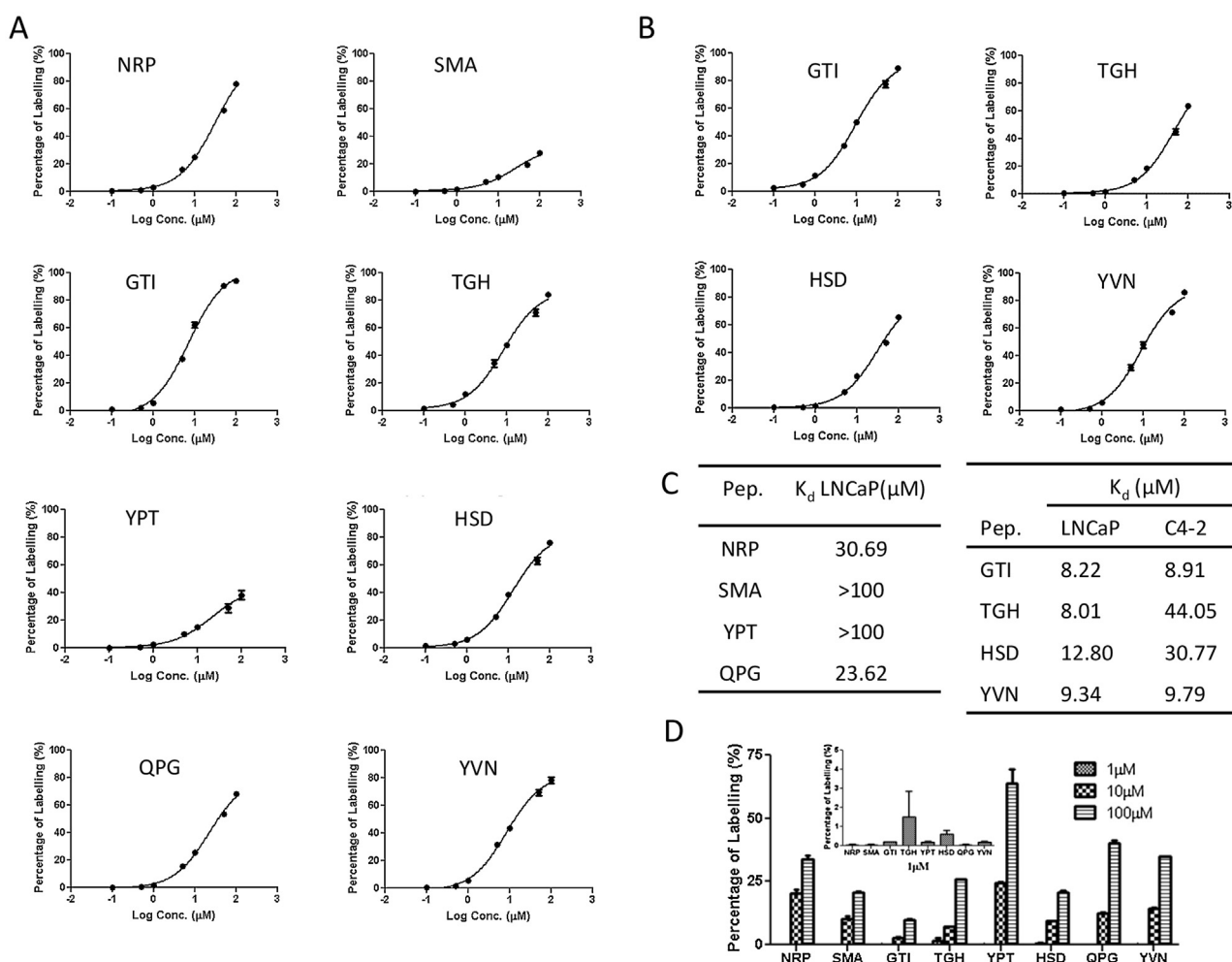


Fig. 5. Binding affinity of selected peptides in LNCaP, C4-2, and PC-3 cells. The cells were suspended and incubated with a series of concentrations of FAM-labeled peptides at 4 °C for 1 h. The cells were then subjected to flow cytometry analysis to determine the percent of cells that take up the peptides. Equilibrium dissociation curves of the selected peptides in LNCaP and C4-2 cells were presented in (A) and (B), respectively. The apparent equilibrium dissociation constants (K_d) were summarized in (C). Binding of the peptides (1 μ M, 10 μ M and 100 μ M) in PC-3 cells was illustrated in (D). Results are represented as the mean \pm SD ($n = 3$).

IC_{50} of the GTI-KLA fusion peptide in LNCaP cells is approximately 12.10 μ M (Fig. 8C). This study demonstrates the potential of using the GTI peptide as a PSMA-specific ligand to deliver therapeutic agents to prostate cancer cells.

3.7. Biodistribution study

In order to evaluate the distribution profile of the GTI peptide *in vivo*, 20 nmol of FAM-labeled GTI peptide (GTIQYPFSGWGY) and its scrambled peptide (random permutation of the GTI peptide, SGYQTFYWP GPI) were injected into nude mice bearing subcutaneous C4-2 xenograft tumor via the tail vein. The GTI peptide exhibits lower apparent K_d in C4-2 prostate cancer cells compared to its scrambled peptide (Fig. 9). It has been reported that peptides achieves its highest uptake in tissues 2 h post-administration (Chen et al., 2004), we therefore sacrificed the mice at 2 h post-administration and harvested major organs including the tumor, liver, kidneys, muscle, heart, lungs, and spleen. As illustrated in Fig. 9, the GTI peptide shows higher uptake in the tumors than other tissues including the liver and kidney, which are the major sites for peptide metabolism (Carone and Peterson, 1980). Uptake of the GTI peptide in other organs, such as the heart, lung and muscle is negligible. Moreover, the GTI peptide exhibits much higher uptake in the tumors in comparison to its scrambled peptide. On the contrary, the scrambled peptide shows the highest

uptake in the liver. These results demonstrate that the GTI peptide can specifically bind to PSMA overexpressing C4-2 xenografts *in vivo*, suggesting its potential promise as a PSMA-specific ligand for prostate cancer targeted drug delivery.

3.8. Comparison of the GTI peptides with other prostate cancer specific peptides

We recently discovered a peptide KYLAYPDSVHIW (KYL) that can specifically bind to LNCaP cells (Qin et al., 2011). WQPDTHH-WATL (WQP) is another peptide that binds to the catalytic site of PSMA and inhibit its enzymatic activity (Aggarwal et al., 2006). We therefore compared the apparent binding affinity of FAM-labeled GTI, KYL, and WQP peptides to LNCaP cells. As shown in Fig. 10, the GTI peptide exhibits the highest binding affinity to LNCaP cells with an apparent K_d of 8.22 μ M, while the apparent K_d values of KYL and WQP peptides are 14.36 and 23.57 μ M, respectively.

4. Discussion

Phage biopanning is a powerful tool to discover peptide ligands for various molecular moieties. Most of the biopannings so far are conducted on a single target, such as a recombinant protein, a cell line, or a tissue. However, each of these methods has its own advantages and disadvantages. For example, a recombinant protein

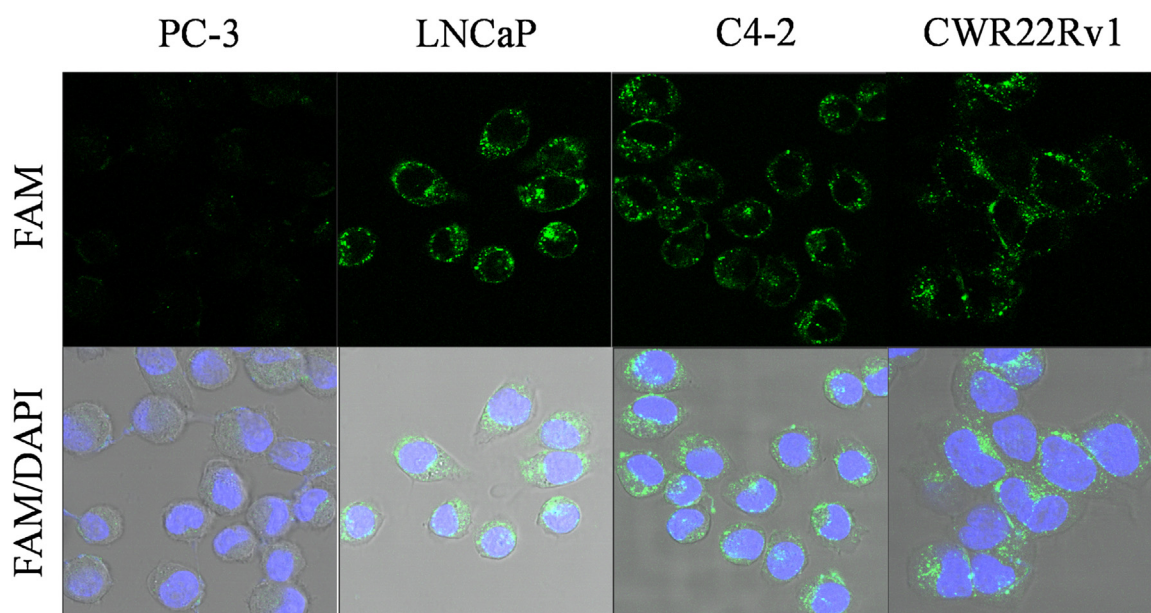


Fig. 6. Cellular uptake of the FAM-labeled GTI peptide in PSMA positive and negative cells. The FAM-labeled GTI peptide was incubated with the cells at 37 °C for 1 h, followed by washing with PBS and examination under a confocal microscope.

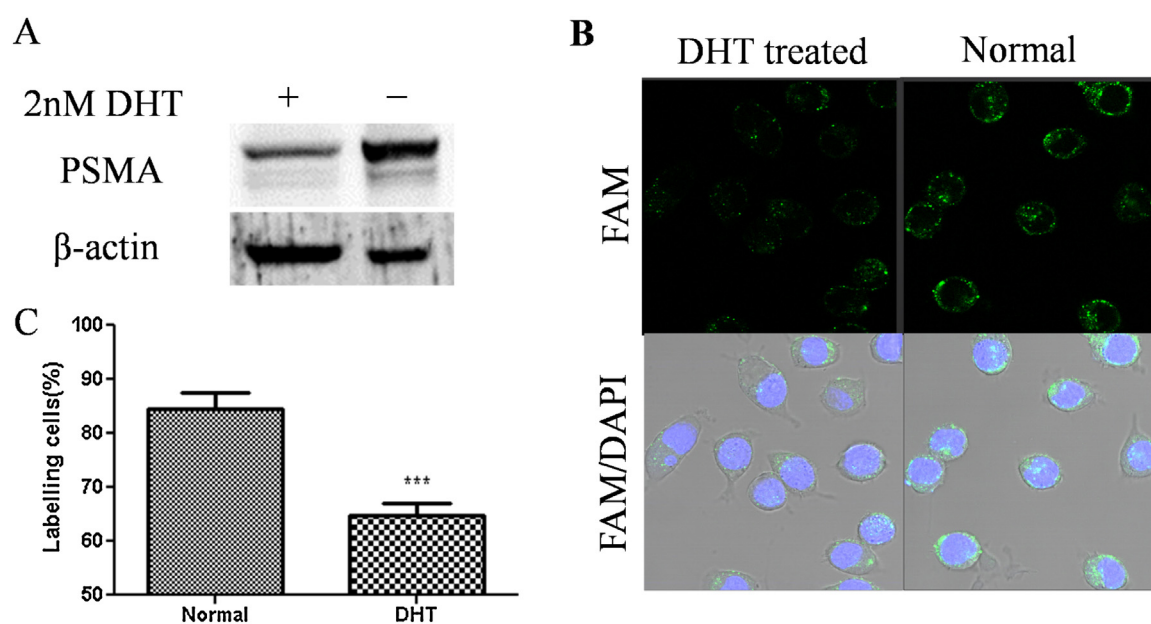


Fig. 7. Downregulating the expression of PSMA inhibits cellular uptake of the FAM-labeled GTI peptides in LNCaP cells. LNCaP cells were treated with 2 nM DHT before the cellular uptake study to downregulate the expression of PSMA. LNCaP cells without DHT treatment were used as the control. (A) The expression of PSMA in normal and DHT treated LNCaP cells was examined using western blot. (B) LNCaP cells were incubated with 10 μ M FAM-labeled GTI peptide at 37 °C for 1 h and then examined under a confocal microscope. (C) Suspended LNCaP cells were incubated with FAM-labeled GTI peptide at 4 °C for 1 h, followed by flow cytometry analysis to determine cellular uptake. Results are represented as the mean \pm SD (n = 3). (***) $p < 0.001$

may exhibit a different conformation structure from its native form in cells. Therefore, a peptide ligand discovered by biopanning on recombinant protein may not exhibit the same affinity to its target cells *in vitro* and *in vivo*. On the other hand, the intricate and complex structures of cell membrane may lead to the discovery of a peptide ligand that binds to an unknown moiety. We recently conducted a whole cell biopanning on PSMA-positive LNCaP cells. Although the identified peptide ligand exhibits very high affinity and specificity to LNCaP cells, the peptide is not PSMA-specific and its target moiety is unknown (Qin et al., 2011). Subsequently, future clinical application of this type of peptide ligands may be

unpredictable. In addition, peptide ligands identified from *in vitro* biopanning may not survive the complex environment in the body after systemic administration. Therefore, we designed a novel combinatorial biopanning against recombinant human PSMA ECD, PSMA-positive LNCaP cells, and LNCaP xenografts in nude mice. The *in vivo* biopanning step was included because it can offer a selection of peptide ligands depending on their pharmacokinetic and tumor targeting properties (Newton et al., 2006). It is our expectation to use this novel combinatorial biopanning procedure to discover a PSMA-specific peptide that can be used *in vivo* for prostate cancer therapy and imaging.

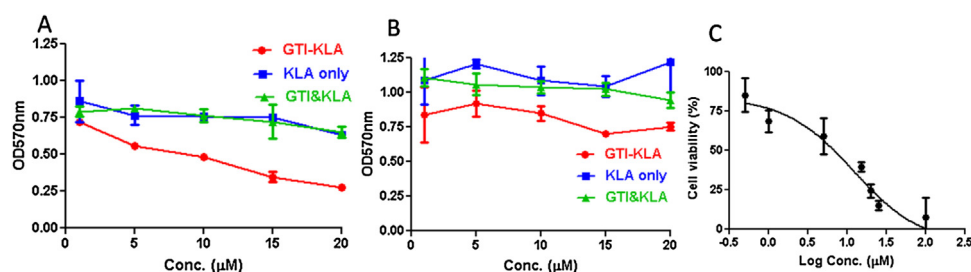
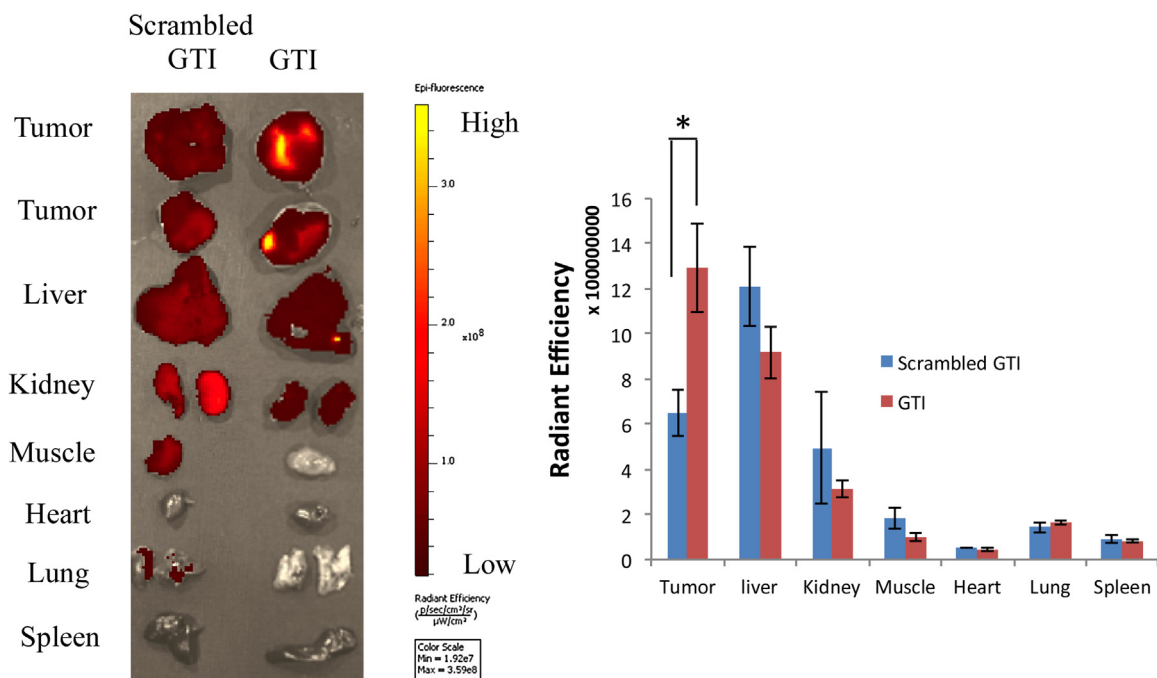


Fig. 8. The GTI peptide enhances the uptake and apoptotic effect of a proapoptotic peptide (KLA) in PSMA-positive cells. The GTI/KLA fusion peptide, the mixture of GTI and KLA peptides, and KLA peptide were incubated with the PSMA-positive LNCaP cells (A) and PAMS-negative PC-3 cells (B) for 48 h, followed by MTT assay to determine the apoptotic effect. The viability concentration-response curve of the GTI/KLA fusion peptide in LNCaP cells was presented in (C). Results are represented as the mean \pm SD ($n = 3$).



Peptide	Sequence	K_d in C4-2 cells (μ M)
GTI	GTIQYPFSWGY	8.91
Scrambled GTI	SGYQTFYWP GPI	>100

Fig. 9. Biodistribution of the GTI peptide and its scrambled peptide in nude mice bearing prostate cancer xenografts. The apparent K_d values of the GTI peptide and its scrambled form in C4-2 cells were determined using flow cytometry. FAM-labeled GTI and its scrambled peptides were injected into nude mice bearing C4-2 xenograft tumors via the tail vein. The mice were sacrificed two hours post-administration, and major organs including the tumor, liver, kidney, muscle, heart, lung, and spleen were harvested for fluorescence imaging using a Xenogen IVIS imaging system. Data are presented as the mean \pm SE ($n = 4$). (* $p < 0.05$)

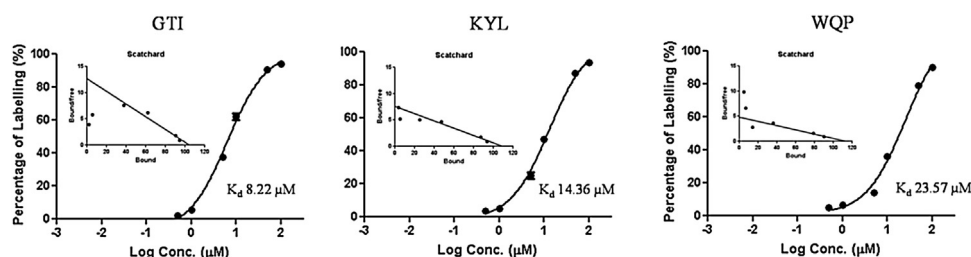


Fig. 10. Binding affinity of the GTI peptide and other prostate cancer specific peptides in LNCaP cells. LNCaP cells were suspended and incubated with a series of concentrations of the peptides at 4 °C for 1 h, followed by flow cytometry analysis to determine cellular uptake. Results are represented as the mean \pm SD ($n = 3$).

Using the combinatorial biopanning, we discovered the GTI peptide which exhibits high affinity to recombinant PSMA, PSMA-positive cells, and xenograft tumors in nude mice. The GTI peptide shows high affinity not only to LNCaP cells but also to other PSMA-positive prostate cancer cells, such as C4-2 and CWR22Rv1 (Figs. 5 and 6). The result in Fig. 7 clearly demonstrates the specificity of the GTI peptide to PSMA. In addition, the GTI peptide shows higher affinity to LNCaP cells compared to two other prostate cancer specific peptides (Fig. 10). Biodistribution study (Fig. 9) also suggests that the GTI peptide can specifically target prostate cancer xenografts *in vivo*. Even compared to its scrambled peptide (random permutation of the GTI peptide), the GTI peptide exhibits higher uptake in the tumors but less uptake in other organs, including the liver, kidneys, muscle, heart, lungs, and spleen. This result demonstrates the promising potential of the GTI peptide as a targeting ligand for prostate cancer targeted therapy *in vivo*.

PSMA, or NAAG peptidase, is a transmembrane glycoprotein containing a large extracellular domain (707 amino acids), an intracellular domain (19 amino acids), and a transmembrane domain (24 amino acids) (Ghosh and Heston, 2004; Israeli et al., 1993; Mesters et al., 2006). PSMA is highly expressed in prostate cancers (Su et al., 1995), but its expression in other tissues is relative low. The expression level of PSMA is 100–1000 fold higher in prostate cancer cells compared to other normal tissues (Ghosh and Heston, 2004; Sokoloff et al., 2000). Moreover, the expression level of PSMA correlates with prostate cancer progression (Wright et al., 1995; Silver et al., 1997; Marchal et al., 2004). As a result, PSMA is a validated targeting moiety in advanced prostate cancer and has been widely employed for targeted drug delivery to prostate cancer. As a carboxyl peptidase, PSMA can cleave the terminal glutamate from NAAG or γ -linked poly glutamate (Ghosh and Heston, 2004). The enzymatic activity of PSMA was found elevated in prostate cancer cells (Lapidus et al., 2000), indicating its important role in prostate cancer progression by regulating angiogenesis (Conway et al., 2006). Therefore, inhibition of the PSMA enzymatic activity could be a potential therapeutic approach for prostate cancer (Dassie et al., 2014). Aggarwal and colleagues identified the peptide WQPDTAHHWATL, which can specifically bind to the catalytic site of PSMA and inhibit its enzymatic activity with an IC_{50} of 23 μ M (Aggarwal et al., 2006). However, the GTI peptide in our study does not inhibit the PSMA enzymatic activity (data has not shown), indicating that the GTI peptide binds to a different site of PSMA ECD rather than the catalytic site.

Binding affinity of peptide ligands to their receptor is generally lower compared to antibodies (Chen et al., 2015; Piotukh et al., 2005; Hao et al., 2008). However, there are several strategies to increase the binding affinity of phage derived peptides. For example, affinity maturation is often employed to improve the binding affinity of peptide ligands by mutagenesis (Yu and Smith, 1996). After additional rounds of selection with these affinity maturation libraries, peptide ligands with higher affinity can be discovered (Yu and Smith, 1996; Molek et al., 2011). In addition, dimerization or tetramerization is a common approach to improve peptides' binding affinity. For instance, we recently demonstrated that dimerization of an IGF2R-specific peptide improves its apparent affinity by nearly 9-fold (Chen et al., 2015). Modification of peptide side-chains and substitution of D-amino acids are other strategies to improve binding affinity. For example, Chen and colleagues replaced glycines with D-form amino acids and significantly improved the binding affinity and stability of the peptide (Chen et al., 2013).

In conclusion, we identified a PSMA-specific GTI peptide through combinatorial biopanning. The GTI peptide exhibits high binding affinity and selectivity to PSMA and PSMA-positive prostate cancer cells. It can mediate internalization of the apoptotic KLA peptide to PSMA-positive LNCaP cells and induce

cell death. Moreover, FAM-labeled GTI peptide shows a high and specific tumor uptake in nude mice bearing human prostate cancer xenografts. All the results demonstrate that the GTI peptide can be employed as a PSMA-specific ligand for prostate cancer targeted drug delivery or imaging.

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