An enzyme-responsive conjugate improves the delivery of a PI3K inhibitor to prostate cancer

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Abstract

An enzyme-responsive peptide drug conjugate was developed for TGX-D1, a promising PI3K inhibitor for prostate cancer therapy. LNCaP-specific KYL peptide was used as the targeting ligand and the prostate specific antigen (PSA) cleavable peptide (SSKYQSL) was used as the enzyme-responsive linker. SSKYQSL is cleaved by recombinant human PSA at $10–250 \, \mu g/mL$. By contrast, the linker is stable in the serum of prostate cancer patients with high PSA levels (>500 ng/mL), indicating that this linker can survive the systemic circulation in prostate cancer patients but be cleaved in the tumor microenvironment. Cellular uptake of the peptide drug conjugate in prostate cancer cells is improved by about nine times. Biodistribution study reveals significant tumor accumulation of the peptide drug conjugate in nude mice bearing C4–2 tumor xenografts. Meanwhile, distribution of the conjugate in other major tissues is the same as the parent drug, indicating a high specificity of the conjugate to prostate cancers *in vivo*.

Prostate cancer is the most common male malignancy and remains the second-leading cause of death due to cancer in American men. While surgery and radiation are effective in prostate cancer therapy at early stages, majority of the patients ultimately relapse and develop advanced prostate cancer. Chemotherapy is still the mainstay of treatment for recurrent prostate cancer, and a great deal of effort has been made to discover novel molecules for advanced prostate cancer.[1,2] The phosphoinositide 3-kinases (PI3Ks) signaling pathway is one of the most important pathways in cancer, and a number of PI3K inhibitors are currently in preclinical and clinical studies for various cancers.[3–8] Specifically, the isoform PI3K-p110β has been proven essential for prostate cancer tumorigenesis and androgen-independent progression.[9,10] TGX-221 is a novel, isoform-specific, and potent small molecule inhibitor of PI3K-110β.[11] It has been successfully used in animals to suppress PI3K-p110β for antithrombotic therapy.[12] However, therapeutic applications of TGX-221 in prostate cancer are currently not feasible because of its poor aqueous solubility and nonspecific expression of PI3K-110β in various tissues. Therefore, it is critical to improve TGX-221's solubility and target-ability to prostate cancers.

We recently developed a derivative of TGX-221, TGX-D1, with a functional group that can be used for peptide conjugation. We conjugated a HER2-specific peptide to TGX-D1 and demonstrated improved solubility as well as enhanced cellular uptake in HER-2 positive prostate cancer cells.[13] In another study, we conducted a whole-cell biopanning procedure using peptide phage display and discovered an LNCaP-specific peptide, KYLAYPDSVHIW (KYL). The KYL peptide exhibits high and specific affinity to LNCaP cell, which is the most widely used cell line in prostate cancer research. Moreover, the KYL peptide can deliver its cargo to prostate cancer cells, indicating its great promise as a targeting ligand for prostate cancer diagnosis and targeted drug delivery.[14]

In addition to actively target tumor tissues, a stimulus-responsive system that can specifically release active agents in tumor microenvironment may further increase the tumor-specific accumulation and penetration of the drug.[2,15] For example, prostate-specific antigen (PSA) is serine protease that is highly expressed in prostate cancers rather than other normal tissues.[16] The interstitial space of prostate cancer contains a high level ($50-500 \, \mu g/mL$) of

enzymatically active PSA.[17] Moreover, PSA is only enzymatically active in the interstitial space of prostate tumors. By contrast, PSA in the blood circulation is not active because of the presence of PSA inhibitors.[18,19] PSA-cleavable linker has therefore been utilized to improve the efficacy of various targeted therapeutics in prostate cancer.[13,18,20,21]

In this study, the LNCaP-specific peptide KYLAYPDSVHIW is linked to TGX-D1 through a PSA-cleavable linker (SSKYQSL). We examined the stability of the PSA-cleavable linker in recombinant human PSA and the sera of prostate cancer patients. We also investigated the cellular uptake, *in vitro* activity and biodistribution of the peptide conjugated TGX-D1 in mice bearing human prostate cancer xenografts.

Methods

Materials

TGX-221 was purchased from Medkoo Biosciences (Chapel Hill, NC). N,N-Diisopropylethylamine (DIPEA), 4-dimethyl-aminopyridine (DMAP), trifluoroacetic acid (TFA), triethylamine, triisopropylsilane (TIPS), HATU, and piperidine were ordered from Fisher Scientific. Unidentified sera of prostate cancer patients were obtained from BioServe Biotechnologies, Ltd. (Beltsville, MD), and recombinant human PSA was ordered from Lee Biosolutions (St. Louis, MO). Amino acids were purchased from Anaspec (Fremont, CA). The N-terminal of peptides is modified by acetyl group to increase peptide stability.

Cleavage study of the PSA-cleavable peptide

The PSA-cleavable peptide Ac-SSKYQSL-NH$_2$ was incubated at a final concentration of 300 µM at 37 °C with PBS, human serum, serum from prostate cancer patient, and recombinant human PSA. PSA levels in the sera from prostate cancer patients were measured using a human PSA ELISA kit (R&D Systems Inc. Minneapolis). Aliquots of 30 µL from each group were collected at various time points (0, 1, 3 and 6 h) and incubated with 90 µL acetonitrile, containing 0.1% TFA at room temperature. After incubation for 5 min, concentration of the intact Ac-SSKYQSL-NH$_2$ in the organic phase was determined using HPLC.

Synthesis of the peptide drug conjugate KYL-TGX

TGX-D1 and NH$_2$-L-TGX-D1 were synthesized and purified as we reported before. KYL-TGX was synthesized using a similar method.[13] Briefly, NH$_2$-L-TGX-D1 (0.01 mmol) and the undeprotected peptide Ac-KYLAYPDSVHIWGGGSSKYQS (0.02 mmol) dissolved in dried DMF were mixed with HATU and DIPEA at room temperature for 12 h. The reaction was then stopped by addition of saturated NH$_4$Cl aqueous solution. After removal of the solvent, the residue was reconstituted in 20 mL of chloroform, washed with water, concentrated under vacuum, and purified using silica gel column. After removing the protecting groups present in the peptide moiety, the peptide drug conjugate KYL-TGX was purified using reverse-phase HPLC and characterized with LC/MS. Ac-KYLAYPDSVHIWGGGSSKYQSL-TGX-D1 (KYL-TGX): ESIMS calculated for C$_{139}$H$_{194}$N$_{32}$O$_{36}$ 2909.433, found 969.5 (M + 3)$^{3+}$, 1455.4 (M + 2)$^{2+}$.

Release of the parent drug from KYL-TGX

Release of the parent drug from KYL-TGX in the presence of recombinant human PSA was evaluated as reported.[13] Briefly, KYL-TGX (100 µM) was incubated with 10 µg/mL human recombinant PSA at 37 °C. Aliquots of 50 µL were collected at different time intervals for analyzing using a Shimadzu HPLC system (SIL-1QAF auto sampler, LC-2QAT controller pump, SPD-1QA UV detector) on a C-18 reverse-phase column (4.6 × 250 mm, 5 µm).

Cell cytotoxicity IC$_{50}$

C4-2 cells were seeded in a 96-well plate at a density of 1 × 10^4 cells/well and incubated for 24 h before the study. The cells were washed and incubated with KYL-TGX and TGX-D1 at 37 °C for 72 h. Cell viability was measured using MTT assay. The IC$_{50}$ values were calculated by fitting a concentration–response curve using Graphpad Prism 5 (Graphpad Software, Inc.)

Cellular uptake and toxicity study

C4-2 cells (3 × 10^5 cells/well) were seeded in 24-well plates for 24 h and then incubated with KYL-TGX, dipeptide-TGX-D1 and TGX-D1 at 37 °C. After incubation for 1 h, the cells were washed with PBS and then lysed as we described before.[13] Drug molecules were extracted from cell lysate using a liquid–liquid extraction method.[13] Both KYL-TGX and dipeptide-TGX-D1 were cleaved by NaOH to form TGX-D1 during the extraction process. Concentrations of TGX-D1 in the cell lysate were quantitated using LC–MS/MS. BCA protein assay kit (Pierce, Rockford, IL) was used to quantitate the total protein concentration of the cell lysate. Cellular uptake was normalized to the total protein content of the samples.

PSA levels in the cell culture medium were also monitored. Briefly, C4-2 cells were plated at a density of 3 × 10^5 cells/well in 24-well plates for 24 h, followed by replacement with fresh medium. After incubation for 1 h and 24 h, the medium (supernatant) was collected for PSA assay using a human PSA ELISA kit (R&D Systems Inc. Minneapolis).

Cellular cytotoxicity of KYL-TGX and TGX-D1 in C4-2 cells was compared after 1 h and 2 h incubation. Briefly, C4-2 cells (5 × 10^3 cells/well) seeded in 96-well plates were incubated with KYL-TGX and TGX-D1 at 37 °C for 1 h and 2 h, followed by replacement with fresh medium. MTT assay was conducted at 72 h post treatment to determine the cell viability.

Stability study of KYL-TGX in PBS and human serum

Stability of the peptide conjugated drug was studied in PBS and human serum. The peptide drug conjugate KYL-TGX (5 µM) was incubated in PBS or 50% human serum at 37 °C. At various time intervals, aliquots of 20 µL were mixed with 60 µL acetonitrile containing 0.1% formic acid to precipitate serum proteins. After centrifugation at 12,000 ×*g* for 10 min, 50 µL of the supernatant was collected and analyzed using HPLC.
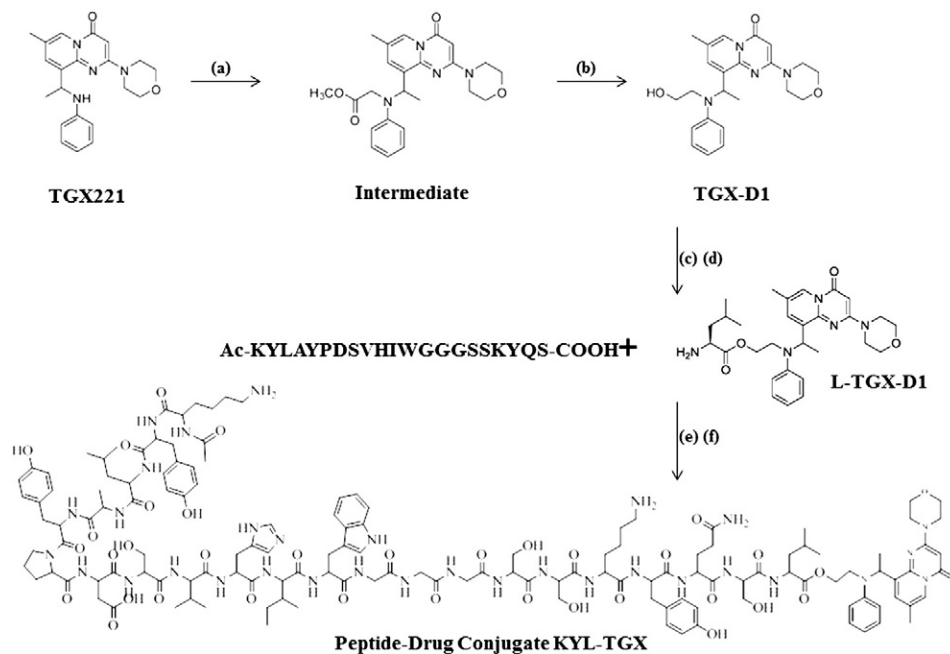
Figure 1. Synthesis scheme of KYL-TGX. Reaction conditions: (**A**) Methylbromoacetate, DIPEA, CH$_3$CN; (**B**) LiCl, NaBH$_4$, THF/EtOH (**C**) Fmoc-Leu, EDCI, DMAP (cat), DCM; (**D**) DCM containing 20% piperidine; (**E**) HATU, NMP, DIPEA; (**F**) TFA/TIPS/DCM (47.5/5/47.5), room temperature.

Biodistribution and pharmacokinetics studies of the peptide drug conjugate

The animal protocol was approved by the University of Missouri-Kansas City, Institutional Animal Care and Use Committee (IACUC). Four- to five-week-old male nude mice were housed in a temperature-controlled room with a 12 h light–dark cycle. The C4-2 xenograft tumor model was developed by subcutaneous injection of 3.5×10^6 cells with Matrigel into the right and left flanks of each mouse. The tumors were allowed to grow to a mean volume of 500 mm^3, and then the mice were randomly assigned into two groups for drug administration via the tail vein. One group of the mice received 2 mg/kg TGX-D1, and the second group received KYL-TGX at the equivalent dose 2 mg/kg TGX-D1. The mice were then sacrificed at various time points ranging from 30 min to 12 h. Blood and major organs such as the tumors, liver, heart, kidneys, lungs and spleen were harvested, washed, blotted dried, weighed, and stored at −80 °C. KYL-TGX and TGX-D1 were extracted from the tissues using a liquid/liquid extraction protocol and then analyzed using LC–MS/MS as we and others reported.[13,22] During the extraction process, KYL-TGX is converted to TGX-D1 by NaOH. As a result, concentrations of TGX-D1 in the samples were quantitated using LC–MS/MS. The detection limit of TGX-D1 is approximately 10 nM. TGX-221 was added as the internal standard before extraction.

Results

Synthesis of KYL-TGX

We previously synthesized a TGX-221 derivative, TGX-D1, which contains a hydroxyl group to conjugate peptides. TGX-D1 exhibits similar activity as TGX-221 in inhibiting androgen-induced gene expression and cell proliferation. We also conjugated a HER2-specific peptide to TGX-D1 and demonstrated increased solubility and cellular uptake in prostate cancer cells.[13] In this study, we used similar approach to attach the LNCaP-specific peptide KYL to TGX-D1 via a PSA-cleavable linker (Figure 1). Fmoc protected Leucine was coupled to the –OH of TGX-D1 through an ester linker to form NH$_2$-L-TGX. Undeprotected peptides Ac-KYLAYPDSVHIWGGGSSKYQS including the LNCaP-specific peptide, Gly-Gly-Gly, and the PSA-cleavable linker were conjugated to NH$_2$-L-TGX through an amide bond. The N-terminal of the peptide was modified with acetyl group to improve its stability. The undeprotected KYL-TGX was deprotected and then purified using HPLC.

Stability and cleavage study of the PSA-cleavable peptide SSKYQSL

We hypothesize that the PSA-cleavable linker SSKYQSL can only be cleaved by enzymatically active PSA in the interstitial space of prostate tumors. It is therefore critical to evaluate its stability in recombinant human PSA and the sera from prostate cancer patients.

We first studied the stability of Ac-SSKYQSL-NH$_2$ in PBS containing recombinant human PSA at various concentrations (0, 0.1, 1, 10, 100 and 250 µg/mL). As Figure 2, *A* shows, the PSA-cleavable substrate is stable up to 6 h when the PSA level is less than 10 µg/mL in the PBS. However, at high PSA levels (10-250 µg/mL), the substrate is steadily cleaved. In a similar study, we evaluated the stability of Ac-SSKYQSL-NH$_2$ in 50% human serum supplemented with recombinant human PSA (Figure 2, *B*). Similarly, the substrate is cleaved by PSA in a dose-dependent manner. In the presence of 100 µg/mL recombinant PSA, the half-life of the PSA substrate in PBS and 50% serum is 2.2 h and
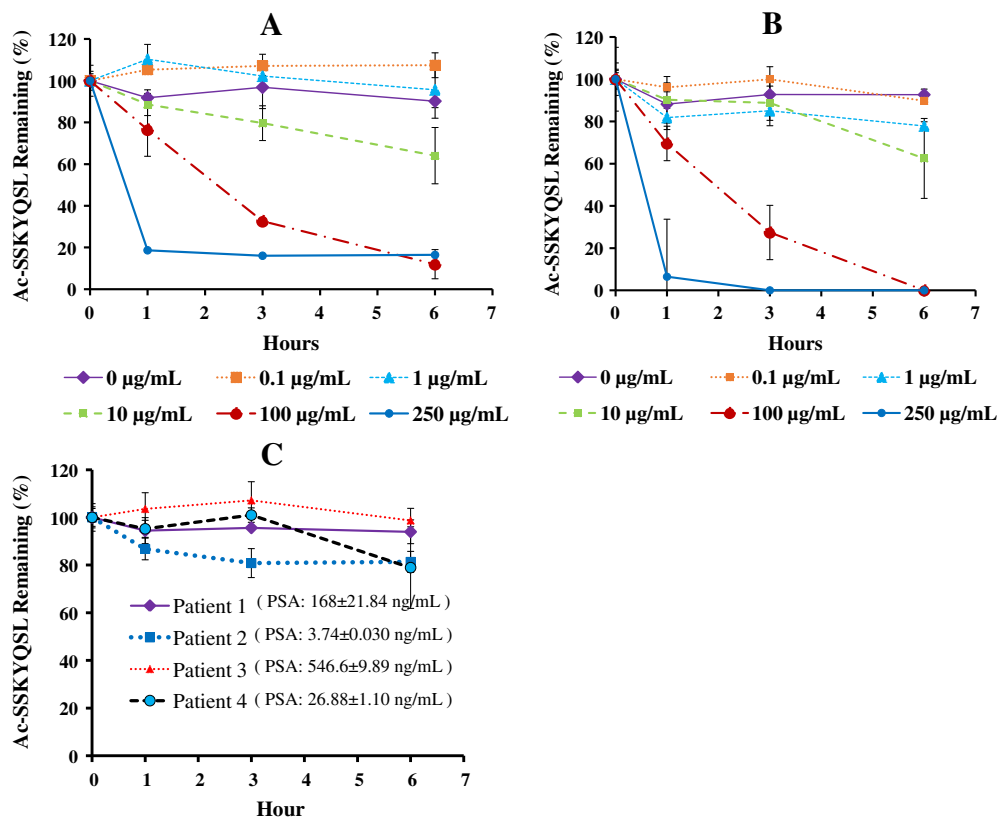
Figure 2. Cleavage study of the PSA-cleavable peptide Ac-SSKYQSL-NH$_2$. (**A**) PBS supplemented with recombinant human PSA. (**B**) 50% human serum supplemented with recombinant human PSA. (**C**) Sera from four unidentified prostate cancer patients. The PSA levels in the serum were measured using a human PSA ELISA kit. The results are presented as mean ± SD (n = 3).
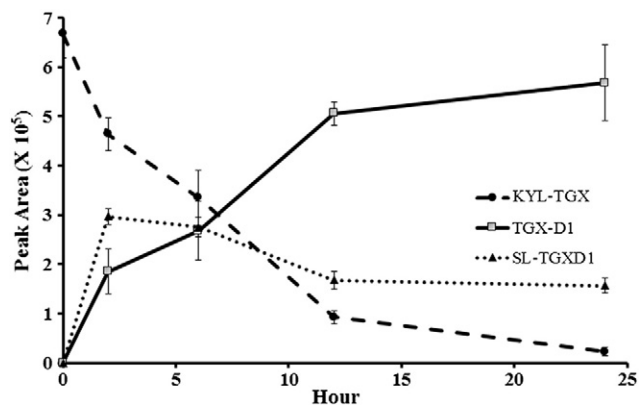

Figure 3. Release of the parent drug from KYL-TGX in the presence of recombinant human PSA. The results are presented as mean ± SD (n = 3).

1.9 h, respectively. The half-life of the PSA substrate in PBS and 50% serum decreases to 0.6 h and 0.5 h, respectively, in the presence of 250 µg/mL recombinant PSA. These results demonstrate that the PSA-cleavable linker can be efficiently cleaved in prostate tumor interstitial space, which contains a substantial level (50-500 µg/mL) of enzymatically active PSA.[17]

We next studied the stability of the PSA substrate Ac-SSKYQSL-NH$_2$ in the sera from four independent prostate cancer patients. The PSA levels in the sera were measured using

a human PSA ELISA kit (R&D Systems Inc. Minneapolis, USA). The serum PSA levels are 168, 3.74, 546.6, and 26.88 ng/mL for the patient 1, 2, 3 and 4 respectively (Figure 2, *C*). As the stability assay shows, the PSA substrate is quite stable in the sera from prostate cancer patients even when the PSA level is higher than 546 ng/mL in the patient 3 (Fig. 2, *C*). These results from Figure 2 (*A, B & C*) clearly suggest the PSA-cleavable substrate SSKYQSL is stable in the blood of prostate cancer patients, but can be readily cleaved in the tumor interstitial space, which contains a high concentration of enzymatically active PSA.

Release of the parent drug from KYL-TGX

We hypothesize that the peptide drug conjugate (KYL-TGX) can be cleaved in the tumor microenvironment by PSA to release the dipeptide drug (SL-TGX), which will be finally cleaved by esterase to release the parent drug TGX-D1 in the cells to exert its activity. The KYL-TGX was therefore incubated with human recombinant PSA (10 µg/mL) at 37 °C for various time intervals. The KYL-TGX, SL-TGX, and TGX-D1 were monitored using HPLC as we reported.[13] As Figure 3 illustrates, KYL-TGX is steadily cleaved by PSA to release SL-TGX with a t$_{1/2}$ of approximately 8 h. We also observed a steady release of the parent drug from SL-TGX, indicating that KYL-TGX can efficiently release the active parent drug in prostate cancers.
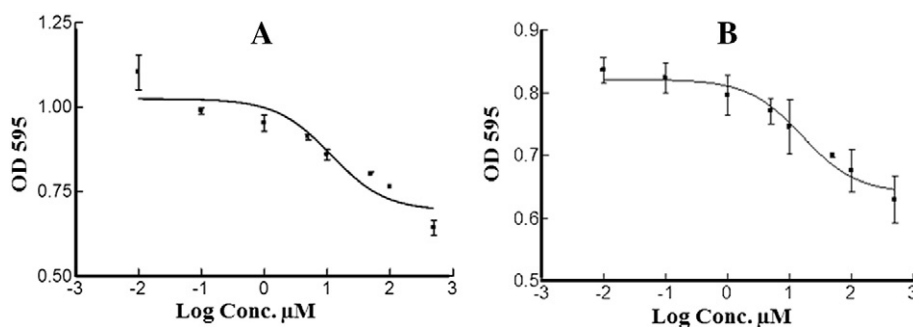
Figure 4. Cell cytotoxicity IC_{50} of TGX-D1 (**A**) and KYL-TGX (**B**) in C4-2 cells. The results are presented as mean ± SD (n = 3).
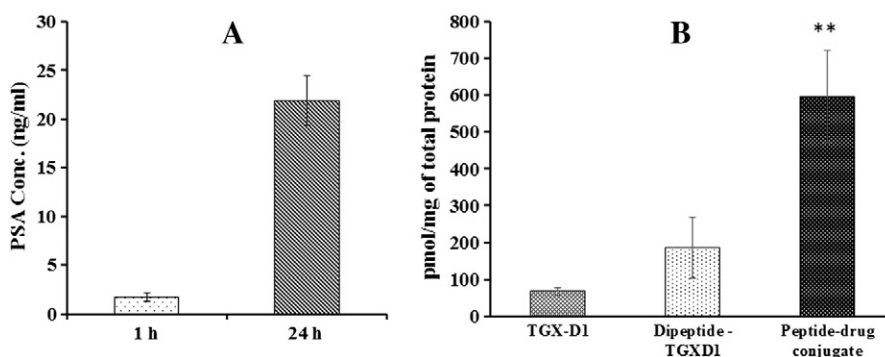

Figure 5. Cellular uptake study of KYL-TGX, dipeptide-TGX-D1, and TGX-D1 in C4-2 cells. (**A**) PSA concentrations in the cell culture medium of C4-2 cells after incubation with fresh medium for 1 and 24 h. (**B**) After incubation with the drug at 1 μM for 1 h, C4-2 cells were washed and lysed, followed by drug quantitation using LC–MS/MS. Cellular uptake was normalized to the total amount of protein. The results are presented as mean ± SD (n = 3). (** $P < 0.01$).

Cell cytotoxicity IC_{50} of KYL-TGX and TGX-D1

We next examined whether peptide modification affects the cellular cytotoxicity IC_{50} of the drug in C4-2 cells. KYL-TGX and TGX-D1 were incubated in C4-2 cells for 72 h, and MTT assay was used to determine cell viability. As shown in Figure 4, TGX-D1 and KYL-TGX induce similar cytotoxicity profile in C4-2 cells. The IC_{50} values for TGX-D1 and KYL-TGX are 11.36 μM and 14.93 μM, respectively. These results suggest that KYL-TGX can efficiently release the parent drug TGX-D1 inside cells to exhibit its activity.

Cellular uptake of KYL-TGX

In order to study whether the LNCaP-specific peptide KYL can enhance the delivery of the KYL-TGX to prostate cancers, we performed cellular uptake study in C4-2, which is a derivative cell line of LNCaP. KYL-TGX, dipeptide-TGX-D1 and TGX-D1 were incubated with C4-2 cells at 1 μM for 1 h. As Figure 5, B shows, compared to TGX-D1, KYL-TGX exhibits nearly nine-fold higher uptake in the cells, demonstrating that coupling of the LNCaP-specific peptide KYL dramatically enhances the delivery of its cargo to prostate cancer cells. Moreover, dipeptide-TGX-D1 shows 2-fold higher uptake compared to TGX-D1, indicating that the dipeptide transporter in prostate cancer cells can be utilized to enhance drug delivery.

The PSA concentration in the cell culture medium was also determined (Figure 5, A). After 1 h incubation of C4-2 cells with fresh medium, the PSA level in the medium is about 2.88 ng/mL.

The PSA level increases to 20.13 ng/mL after 24 h incubation. As shown above in Figure 2, A, the PSA-cleavable linker is stable in the presence of PSA up to 1 μg/ml. Therefore, the PSA in the cell culture medium is not high enough to degrade KYL-TGX during the cellular uptake study.

We further studied whether the enhancement in cellular uptake is correlated with its activity. C4-2 cells were incubated with 1 μM of TGX-D1 and KYL-TGX for 1 h and 2 h, followed by replacement with fresh medium and MTT assay at 72 h post treatment. As Figure 6 illustrates KYL-TGX exhibit higher activity than TGX-D1 at both incubation time intervals, indicating the enhanced cellular uptake and activity of KYL-TGX.

Serum stability of peptide drug conjugate KYL-TGX

Stability of KYL-TGX in 50% human serum and PBS was assayed at 37 °C. As shown in Figure 7, KYL-TGX is generally stable in PBS. After 4 h incubation in PBS, only about 6% of KYL-TGX is degraded. By comparison, KYL-TGX exhibits a time dependent degradation in human serum with a $t_{1/2}$ of 3.5 h, and approximately 58% of KYL-TGX is degraded after 4 h. This result is in consistent with our previous finding from the HER2 peptide modified TGX-D1.[13] The degradation is possible due to the cleavage of the ester linker between the peptide and TGX-D1.

Biodistribution and pharmacokinetic study

Biodistribution and pharmacokinetics of KYL-TGX and TGX-D1 were studied in nude mice bearing human prostate
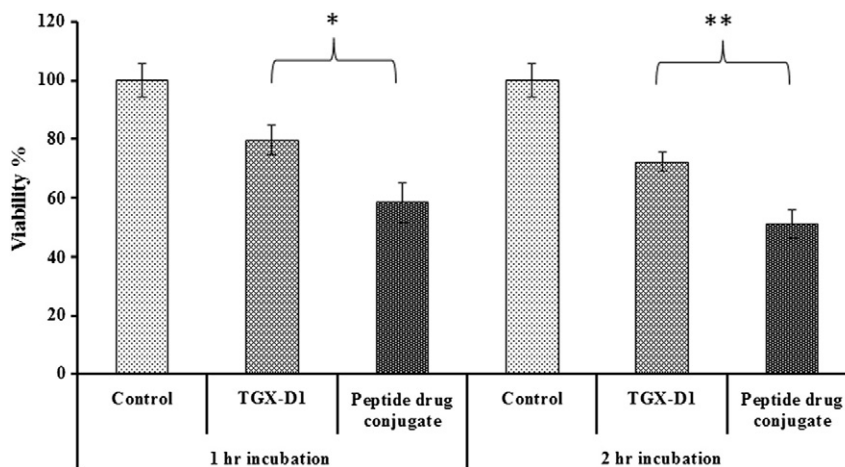
Figure 6. Cytotoxicity of KYL-TGX and TGX-D1 in C4-2 cells after 1 and 2 h incubation. C4-2 cells were incubated with 1 μM TGX-D1 and KYL-TGX for 1 and 2 h, followed by replacement with fresh medium and MTT assay at 72 h post treatment. The results are presented as mean ± SD (n = 3). (*$P < 0.05$; **$P < 0.01$).

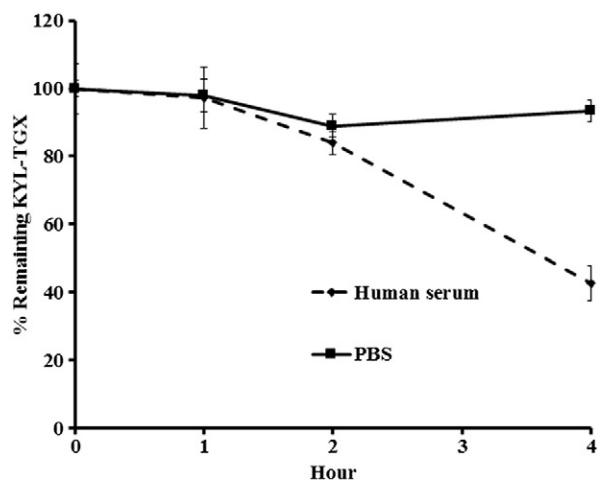Figure 7. Stability of the KYL-TGX in human serum and PBS. KYL-TGX was incubated in PBS (pH 7.5) and 50% human serum at 37 °C for various time intervals. The remaining KYL-TGX was analyzed using HPLC. The results are presented as mean ± SD (n = 3).

Table 1
Pharmacokinetic parameters of KYL-TGX and TGX-D1 in nude mice bearing C4-2 tumor xenografts.

PK Parameter	KYL-TGX	TGX-D1
$t_{1/2\alpha}$ (h)	0.13 ± 0.11	0.03 ± 0.03
$t_{1/2\beta}$ (h)	0.77 ± 0.38	0.64 ± 0.16
V_d (mL)	1.50 ± 001	1.50 ± 001
CL (mL/h)	3.05 ± 0.35	3.14 ± 0.39
AUC 0-inf (μg/mL*h)	16.49 ± 1.78	16.03 ± 1.89
MRT (h)	0.81 ± 0.15	0.89 ± 0.24

drug concentration of KYL-TGX in the tumor is significantly higher than that of TGX-D1 at all the time intervals (Figure 8, *A*). The tumor area under the curve (AUC$_{0-24h}$) of KYL-TGX is approximately 3.2-fold greater than that of TGX-D1 (Figure 8, *B*). These results clearly indicate a high and specific affinity of the KYL-TGX to prostate cancer cells *in vivo*.

Discussions

Despite continuous efforts and advancements, chemotherapy is still the most extensively adopted approach for advanced prostate cancer. Therapeutic effect of chemotherapy is however limited because of nonspecificity, inadequate availability to tumors, and the complicated microenvironment in tumor sites.[1] It is therefore critical to improve the targetability and tumor penetration of chemotherapy agents. A variety of targeting ligands such as antibodies, aptamers, and peptides have been explored to specifically deliver therapeutics to prostate cancer cells via active targeting mechanisms.[2] Compared to other types of ligands, peptide ligands exhibit many advantages including low molecular weight, ease of synthesis, better cellular permeability, low immunogenicity, flexibility in chemical conjugation, and high drug loading capacity.[23] We recently discovered an LNCaP-specific peptide (KYLAYPDSVHIW, KYL), which can specifically deliver its cargo to LNCaP cells.[24]

cancer C4-2 xenografts after intravenous administration. Drug concentrations in the plasma, tumor, liver, kidneys, lungs, and spleen were measured using LC–MS/MS. The plasma concentration data were fitted using a two-compartment model, and pharmacokinetic parameters of KYL-TGX and TGX-D1 were summarized in Table 1. Both KYL-TGX and TGX-D1 exhibit a biphasic plasma elimination profile with similar distribution half-life ($t_{1/2\alpha}$), elimination half-life ($t_{1/2\beta}$), volume of distribution (V_d), clearance, area under the curve (AUC), and mean residence time (MRT).

Biodistribution of KYL-TGX and TGX-D1 in the plasma, tumor, liver, kidneys, lungs, and spleen is presented in Figure 8. Following intravenous administration, both of them exhibit similar and rapid kidney elimination due to their small molecular weight. In addition, KYL-TGX and TGX-D1 exhibit nearly the same distribution profiles in the liver, spleen, and lungs. By contrast,
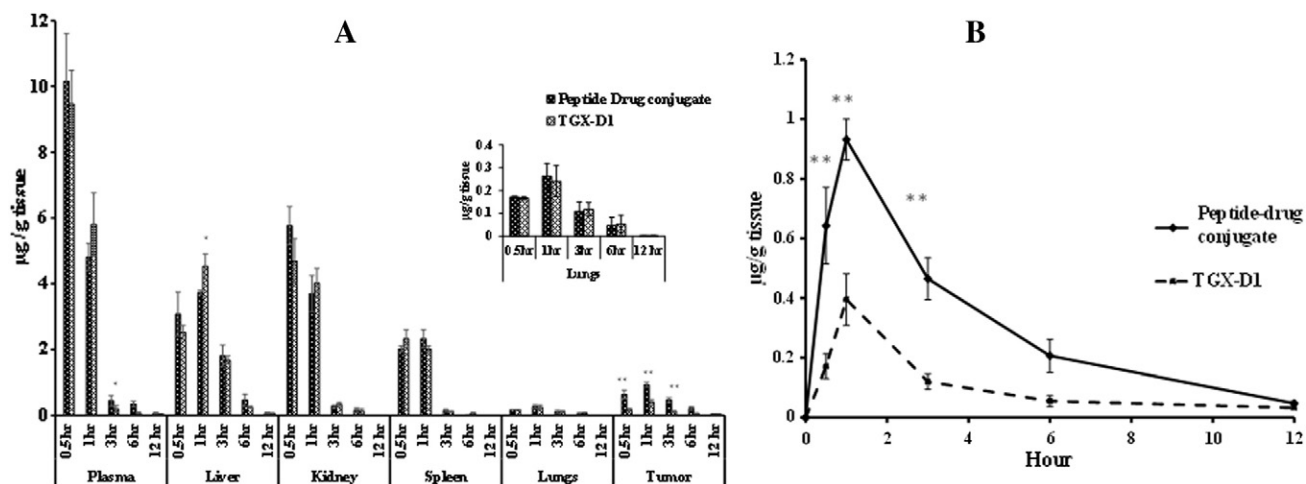
Figure 8. Biodistribution of KYL-TGX and TGX-D1 in nude mice bearing C4-2 tumor xenografts. The drug was administered at 2 mg/kg via the tail vein. At 0.5, 1, 3, 6, and 12 h post administration, the blood and major organs including the tumors, liver, heart, kidneys, lungs and spleen were harvested, washed, blotted dried, and weighed. Drug molecules were extracted from these samples using a liquid/liquid extraction procedure. Drug concentrations were determined using LC–MS/MS and normalized to the total protein content of the samples. (**A**) Biodistribution of KYL-TGX and TGX-D1 in the plasma, liver, kidneys, spleen, lungs, and tumor. (**B**) Tumor area under the curve (AUC) of KYL-TGX and TGX-D1 (*$P < 0.05$; **$P < 0.01$). The results are presented as mean ± SD (n = 3).

Peptide drug conjugate is a simple, promising, and scalable platform to improve the efficacy of chemotherapy agents.[13,23,25] Its small size leads to excellent cell permeability, better tumor penetration, and high drug-loading capability. Moreover, peptide drug conjugate can significantly improve the aqueous solubility of parent drug if the peptide moiety is hydrophilic.[13] We have recently synthesized a TGX-221 derivative, TGX-D1, which contains a hydroxyl group for peptide conjugation and exhibits similar activity and isoform-specificity as TGX-221.[13] In this study, we conjugated the LNCaP-specific peptide KYL to TGX-D1, a PI3K-p110β inhibitor, through a PSA-cleavable linker.

We recently discovered that dipeptide transporters are over-expressed in prostate cancer cells.[26] We also demonstrated that dipeptide linked drug shows higher cellular uptake than unmodified drug in prostate cancer cells, suggesting that peptide transporters in prostate cancers can be utilized to enhance the cellular uptake and tumor penetration of dipeptide linked drug.[13] We therefore include a dipeptide SL between the PSA-cleavage site and the parent drug (Figure 1). We hypothesize that the KYL ligand can specifically deliver the peptide drug conjugate KYL-TGX to prostate cancers. The PSA substrate will be cleaved by PSA in the tumor interstitial space, and the released dipeptide-linked TGX will diffuse into tumor cells via overexpressed dipeptide transporters in prostate cancer cells, thus enhancing tumor penetration.

PSA is a serine protease that is highly expressed in prostate cancers rather than other normal tissues.[2,16] The serum PSA level in prostate cancer patients is much lower than the PSA level in tumor interstitial space (extracellular fluid). For example, one study investigated the serum PSA levels in patients with newly diagnosed prostate cancer. The mean PSA value for 133 African Americans was 14 ng/mL compared with 8.29 ng/mL for 408 white men.[27] In studies where bone scans are positive for prostate cancer metastasis, the serum PSA levels have averaged between 30.0 and 140.0 ng/mL.[28–31] By contrast, the interstitial space of prostate cancer contains a high level of enzymatically active PSA in the range of 50-500 µg/mL,[17] which is about a thousand-fold greater than the serum PSA level in prostate cancer patients. Moreover, PSA is only enzymatically active in the interstitial space of prostate tumors. By contrast, PSA in the blood circulation is not active because of the presence of PSA inhibitors.[18,19,32] Consequently, PSA-cleavable substrate is stable in the blood circulation but can be cleaved in the interstitial space of prostate tumors to release the active drug.

We evaluated the cleavage efficacy of the PSA substrate SSKYQSL in PBS or 50% human serum supplemented with recombinant human PSA. As shown in Figure 2, the PSA substrate can be cleaved by recombinant human PSA when the concentration is higher than 1 µg/mL, suggesting that it can be efficiently cleaved in prostate cancer interstitial space, where the concentration of PSA is in the range of 50-500 µg/mL.

To prove that the PSA substrate is stable in the blood circulation of prostate cancer patients, we performed the stability assay of the substrate in the sera from four unidentified prostate cancer patients. The PSA levels in these patients are in the range of 3.74 to 546.6 ng/mL. The PSA substrate exhibits good stability in all the four serum samples (Figure 2, *C*). This is the first evidence showing that the PSA-cleavable peptide SSKYQSL is stable in the serum from prostate cancer patients regardless of the relatively high PSA levels in the blood. These results clearly demonstrate that the PSA-cleavable linker can be used to specifically release the active drug in prostate cancer interstitial space.

LNCaP-specific peptide (KYL) is adopted as the targeting moiety in the peptide drug conjugate to enhance cellular uptake in prostate cancer cells. LNCaP is an androgen-sensitive human prostate adenocarcinoma cell line and the most extensively used cell line prostate cancer studies.[33–35] Compared to other cell lines, the progression model of LNCaP exhibits more similarities with human prostate cancer. LNCaP cells express many key prostate cancer biomarkers, such as PSMA, PSA, and PAP.[36] It is worth noting that although LNCaP is one of the most widely used prostate

cancer cell lines, it only represents early hormone-responsive prostate cancer. For other types of prostate cancers, we may need to replace the LNCaP-specific ligand. C4-2 is a subline of LNCaP, and the KYL peptide also has a high affinity to C4-2 cells. Cellular uptake study (Figure 5, B) demonstrates that the KYL peptide dramatically increases the uptake of KYL-TGX in C4-2 cells. Accordingly, KYL-TGX exhibits higher activity compared to TGX-D1 in C4-2 cells (Figure 6).

We finally performed biodistribution and pharmacokinetic studies in nude mice bearing C4-2 xenografts (Figure 8). Compared to TGX-D1, KYL-TGX shows approximately 3.2-fold increase in tumor accumulation but similar uptake in other organs including the plasma, liver, kidneys, spleen, and lungs. This result clearly reveals the high specificity and affinity of the KYL-TGX to prostate cancer cells *in vivo*. On the other hand, KYL-TGX and TGX-D1 exhibit very similar distribution and elimination rates. This could be mainly due to the small molecular weight of KYL-TGX (M_w 2909) and TGX-D1 (M_w 409). Another possible reason could be the cleavage of the ester bond between TGX-D1 and the attached peptide. As shown in Figure 7, half-life of the KYL-TGX in human serum is approximately 3.5 h, which is mainly because of the cleavage of the ester bond. This is consistent with our and others' reports.[13,37] Moreover, the esterase activity in mouse serum is about 10 times higher than in human serum.[38] Therefore, some of the administered KYL-TGX could be degraded in the blood circulation by esterase before they can reach prostate cancer cells. This result suggests that replacement of the ester bond with a more stable linker, such as an amide or disulfide bond, may further increase the tumor accumulation of KYL-TGX *in vivo*.

In summary, an enzyme-responsive peptide drug conjugate KYL-TGX consisting of the LNCaP-specific peptide KYL, PSA-cleavable linker, and TGX-D1 was synthesized. We demonstrated for the first time that the PSA-cleavable peptide SSKYQSL is stable in the serum from prostate cancer patients but can be cleaved in prostate cancer interstitial space. Biodistribution study in mouse bearing tumor xenograft reveals high and specific accumulation of KYL-TGX in tumors. These findings indicate that peptide drug conjugate is a relatively simple but promising nanoscale platform for prostate cancer chemotherapy agents.

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