Pharmacokinetically Stabilized Cystine Knot Peptides That Bind Alpha-v-Beta-6 Integrin with Single-Digit Nanomolar Affinities for Detection of Pancreatic Cancer

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Abstract

Purpose: Detection of pancreatic cancer remains a high priority and effective diagnostic tools are needed for clinical applications. Many cancer cells overexpress integrin αvβ6, a cell surface receptor being evaluated as a novel clinical biomarker.

Experimental Design: To validate this molecular target, several highly stable cystine knot peptides were engineered by directed evolution to bind specifically and with high affinity (3–6 nmol/L) to integrin αvβ6. The binders do not cross-react with related integrin αvβ3, integrin αvβ1, or tumor-angiogenesis–associated integrin, α5β3.

Results: Positron emission tomography showed that these disulfide-stabilized peptides rapidly accumulate at tumors expressing integrin αvβ6. Clinically relevant tumor-to-muscle ratios of 7.7 ± 2.4 to 11.3 ± 3.0 were achieved within 1 hour after radiotracer injection. Minimization of off-target dosing was achieved by reformattng αvβ6-binding activities across various natural and pharmacokinetically stabilized cystine knot scaffolds with different amino acid content. We show that the primary sequence of a peptide scaffold directs its pharmacokinetics. Scaffolds with high arginine or glutamic acid content suffered high renal retention of more than 75% injected dose per gram (%ID/g). Substitution of these amino acids with renally cleared amino acids, notably serine, led to significant decreases in renal accumulation of less than 20%ID/g 1 hour postinjection (P < 0.05, n = 3).

Conclusions: We have engineered highly stable cystine knot peptides with potent and specific integrin αvβ6-binding activities for cancer detection. Pharmacokinetic engineering of scaffold primary sequence led to significant decreases in off-target radiotracer accumulation. Optimization of binding affinity, specificity, stability, and pharmacokinetics will facilitate translation of cystine knots for cancer molecular imaging.

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Introduction

Integrins are a family of heterodimeric cell surface receptors that mediate cellular adhesion to extracellular matrix proteins. Integrins also serve as bidirectional signal transducers to regulate differentiation, migration, proliferation, and cell death (1, 2). Integrin αvβ3 promotes tumor neo-vascularization (3, 4). However in certain cancers, other integrins, such as αvβ5, become highly overexpressed on cell surfaces (2, 5, 6). Therefore, this biomarker is being validated for detection of colon, liver, ovarian, pancreatic, and squamous cell cancers (7–9). Molecular imaging of integrin αvβ5 may be used to gauge receptor expression levels to determine prognosis and guide therapy (10–12). Here, we have developed potent integrin αvβ6 binders for clinical translation as radiotracers for early cancer detection.

Previously, several integrin αvβ6 binders were identified from natural and combinatorial sources. Linear αvβ6-binding peptides derived from the coat protein of foot-and-mouth disease viruses (FMDV) generally suffered poor in vivo stability, which raise concerns about their potential immunogenicity. 64Cu-labeled versions of FMDV peptides showed extremely high renal retention, which suggests that these peptides may not be ideal translational candidates. An alternative to radiometals is the use of radiohalogens
Translational Relevance

We describe the engineering and validation of new peptide radiotracers for integrin $\alpha_v\beta_6$, a cell surface biomarker overexpressed in many cancers. Currently, radiotracers that specifically detect pancreatic cancers are clinically unavailable. We validated targeting of integrin $\alpha_v\beta_6$ using pancreatic and epidermoid cancer xenografts or orthotopic models. The new peptides show high affinity and specificity for integrin $\alpha_v\beta_6$ with no cross-reactivity to related integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_1$. Uptake of the radiotracers by integrin $\alpha_v\beta_6$-expressing tumors was rapid and high (~2%–5% injected dose per gram, 1 hour postinjection). Peptide pharmacokinetics has been optimized to minimize off-target background. In contrast to linear peptides, these disulfide-stabilized radiotracers are much more stable in serum, blood, and urine and may therefore be less immunogenic. The cystine knot class of peptides has an extremely long shelf life in dry or solvated forms. The sole N-terminus amine enables site-specific coupling of radiometals or radiohalogens. Together, these characteristics indicate potential for clinical translation.

(7, 9, 13). Phage display systems have identified several linear and disulfide-cyclized peptides that bind $\alpha_v\beta_6$ (14, 15). In one study, a radio-iodinated linear peptide HRP-1 showed rapid degradation in serum (16). One-bead-one-compound libraries have identified many binders, of which 43 $^{18}$F-labeled linear peptides were tested in a high compound libraries have identified many binders, of which 43 $^{18}$F-labeled linear peptides were tested in a high throughput live animal imaging survey (17). Although some of these peptides show promising small animal data, linear peptides or even simple disulfide-bonded peptides with stability problems may discourage translation (17). Peptide fragments can be highly immunogenic thus rendering the parent peptide untranslatable (18). For these reasons, we sought to generate high-affinity binders that are very stable in physiologic media, show low off-target accumulation and effectively detect cancer in living subjects.

Engineered cystine knot peptides (knots) have shown promise for cancer imaging with $\alpha_v\beta_6$ as a target (19–21). The cystine knot is a rigid molecular scaffold of 3 to 4 kDa that owes its exceptionally high stability to 3 interwoven disulfide bonds and a centrally located $\beta$-sheet. Potent receptor-binding activities have been engineered into the scaffold's solvent exposed loops (22). The knotted structure helps to resist degradation/denaturation in hostile biologic, chemical, and physical environments such as strong acids and boiling water (23). Cystine knots have shown exceptional structural stability during prolonged incubation in serum (19). Moreover, their use in humans as uterogenics has not led to reports of adverse side effects, albeit without formal published studies (24, 25). Binding potency remains high for engineered knots that we have subjected to long-term storage (>1 year) in water at 4°C or stored in lyophilized form at room temperature. The N-terminus provides a sole primary amine for site-specific conjugation of imaging labels, bioactive cargo, or pharmacokinetic stabilizers. Collectively, these characteristics bode well for clinical translation.

In this study, we developed new cystine knots that bind integrin $\alpha_v\beta_6$ expressed on pancreatic tumors grown in mice. The binders showed single-digit nanomolar dissociation constants similar to antibodies used clinically for imaging and therapy. These new knots rapidly accumulated in $\alpha_v\beta_6$-positive tumors, which led to excellent tumor-to-normal contrast. The new knots did not accumulate in tumors that do not express integrin $\alpha_v\beta_6$. The peptides were specific for the targeted integrin $\alpha_v\beta_6$ receptors expressed on orthotopic pancreatic tumors and various xenografts used in this study. In addition, pharmacokinetic stabilization strategies endowed knots with rapid renal clearance, which significantly reduced off-target dosing. This study indicates a broadly applicable way to engineer new binders and to stabilize their pharmacokinetics for enhanced clinical performance.

Materials and Methods

The Supplementary Material section provides additional details.

Materials, cell lines, and reagents

BxPC-3 pancreatic cancer cells were obtained from American Type Culture Collection (ATCC) and grown in RPMI-1640 media (ATCC). A431 epidermoid cancer cells, human embryonic kidney 293T cells (293), U87MG (malignant glioma), and MDA-MB-435 (breast cancer) cells were obtained from frozen lab stocks and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and penicillin/streptomycin (Invitrogen). Recombinant human integrins $\alpha_v\beta_6$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_1$ were purchased from R&D Systems. All other chemicals were obtained from Fisher Scientific unless otherwise specified. Yeast media, growth and induction conditions, and integrin-binding buffer (IBB) are previously described (22).

Library synthesis and screening

The open reading frames encoding cystine knot peptides were generated by overlap-extension PCR using yeast-optimized codons. Positions for randomization, denoted by "X" were constructed with NNB degenerate codons (Supplementary materials). PCR products were amplified using primers with overlap to the pCT yeast display plasmid upstream or downstream of the NheI and BamHI restriction sites, respectively. For each library, approximately 40 µg of DNA insert and 4 µg of linearized pCT vector were electroporated into the Saccharomyces cerevisiae strain EBY100 by homologous recombination as previously described (26). For libraries L2 and L3, approximately $5 \times 10^6$ to $7 \times 10^6$ transformants per sub-library were combined for a total diversity of approximately $1 \times 10^7$ clones as estimated by...
serial dilution plating and colony counting. For libraries L2.1 and L3.1, approximately $2 \times 10^6$ transformants per sub-library were combined for a total diversity of approximately $4 \times 10^6$ clones.

For library screening, various concentrations of recombinant $\alpha_\beta_6$ integrin were added to yeast suspended in IBB for various times at room temperature. Next, a 1:250 dilution of chicken anti-cMyc IgY antibody (Invitrogen) was added for 1 hour at 4°C. The cells were washed with ice-cold IBB and incubated with a 1:25 dilution of fluorescein-conjugated anti-$\alpha_6$ integrin antibody (Biolegend) and a 1:100 dilution of Alexa 555–conjugated goat anti-chicken IgG secondary antibody (Invitrogen) for 0.5 hours at 4°C. Cells were washed in IBB and $\alpha_\beta_6$ integrin binders were isolated using a Becton Dickinson FACS Aria III instrument. For the first round of sorting, approximately $2 \times 10^7$ yeast clones were screened with 100 nmol/L $\alpha_\beta_6$ integrin. To increase sort stringency, integrin concentrations were successively decreased to 1 nmol/L in later sort rounds, and a diagonal sort gate was used to isolate yeast cells with enhanced integrin binding (FITC fluorescence) for a given protein expression level (Alexa 555 fluorescence). For the second diversification round, approximately $5 \times 10^6$ yeast clones were screened as described earlier with a final concentration of 300 pmol/L $\alpha_\beta_6$ integrin coupled with 3 days of washing in IBB at 37°C. Plasmid DNA was recovered by Zymoprep (Zymo Research), amplified in Max Efficiency DH5ax Escherichia coli cells (Invitrogen) and sequenced.

**Peptide synthesis/biosynthesis, folding, and radiolabeling**

S2, and R01 were synthesized, folded, and purified as previously described with a modification to the folding buffer, which included the addition of an equal volume of isopropanol and 800 mmol/L guanidium hydrochloride (22). A20 was similarly prepared without folding. R02 and R03 were biosynthesized using Pichia pastoris (Supplementary Materials). Peptides were conjugated through their N-terminal amine to DOTA-NHS and radiolabeled with $^{64}$Cu$^{2+}$ as previously described (19). The radiocchemical purity was determined by high-performance liquid chromatography (HPLC) to be more than 95%. The radiocchemical yield was usually more than 80%. The specific activity of the probe was approximately 500 Ci/mmol. Molecular masses were confirmed by matrix-assisted laser desorption/ionization–mass spectrometry (MALDI-MS; ABI 5800, Supplementary Table S2).

**Binding affinity**

Various concentrations of integrin $\alpha_\beta_6$, $\alpha_\beta_5$, $\alpha_\beta_3$, and $\alpha_\beta_1$ were incubated with $10^7$ yeast cells expressing R01, R02, R03, S02, S03, or 2.5F in the presence of $10^6$ uninduced yeast cells as previously described (22, 27). Prior to flow cytometric analysis, yeast cells were processed, stained, and washed as described earlier in Library Synthesis and Screening.

**Protein and cell capture assays**

Neutavidin-coated wells (Pierce) were saturated with biotinylated peptides A20, R01, S02, or 2.5F. A concentration of 10 nmol/L of recombinant integrins or 10$^3$ cells in IBB were added to wells at room temperature for 2 hours. Wells were washed 3 times with ice-cold IBB. Integrins were detected with mouse anti-$\alpha_6$ or anti-$\alpha_3$ primary antibodies (Biolegend) and anti-mouse Alexa-488 (Cellsignal). Cells were detected with crystal violet (22). Signals were quantified with a plate reader (Tecan). For competition binding assays, integrin $\alpha_\beta_6$, or BxPC-3 cells were preincubated with R01 or S02 for 2 hours prior to introduction into A20-coated wells.

**$^{64}$Cu-DOTA peptide stability**

Aliquots of $^{64}$Cu-DOTA peptides were incubated in an equal volume of mouse or human serum for up to 24 hours. Samples were acidified with trifluoroacetic acid and centrifuged to remove precipitants. In addition, $^{64}$Cu-DOTA peptides were extracted from full mouse bladders 1.5 to 2 hours after injection. Soluble fractions were filtered with a Spin-X 0.2 µm filter (Corning) when necessary. All samples were analyzed by radio-HPLC on a Dionex C$8$ column.

**Tumor models**

Animal procedures were conducted per protocols 11580 and 21637 (Stanford University Administrative Panels on Laboratory Animal Care). Female athymic nude mice, 4 to 6 weeks old (Charles River), were subcutaneously shoulder-injected with $10^6$ cells suspended in 100 µL PBS. Orthotopic tumors were generated with $10^6$ cells/20 µL Matrigel (Supplementary materials). Mice were used for imaging/biodistribution studies when xenografts or orthotopic tumors reached approximately 10 or 5 mm, respectively, in diameter.

**MicroPET imaging**

Tumor-bearing mice ($n = 3$ for each probe) were injected with approximately 50 to $100 \mu$Ci ($\sim 0.15$ nmol) of probe via the tail vein and imaged with a microPET R4 rodent model scanner (Siemens) using 5 minute static scans. Images were reconstructed by a 2-dimensional ordered expectation maximum subset algorithm and calibrated as previously described (28). Region of interests (ROI) were drawn over the tumor on decay-corrected whole body images using ASIPro VM software (Siemens). ROIs were converted to counts/g/min, and %ID/g values were determined assuming a tissue density of 1 g/mL. No attenuation correction was conducted.

**Biodistribution analysis**

Anesthetized nude mice bearing xenograft/orthotopic tumors were injected with approximately 50 to $100 \mu$Ci ($\sim 0.15$ nmol) of $^{64}$Cu-DOTA peptides via tail vein and euthanized after 1 or 24 hours. Tissues were removed, weighed, and measured by scintillation counting (19, 29). Radiotracer uptake in tissues was reported as...
%ID/g and represents the mean ± SD of experiments carried out on 3 mice.

Results

Engineering αvβ6 binders

Nine phage display–derived lead-motif (RTDLXXL) containing sequences were engrafted into loop-1 of an acyclized cystine knot scaffold *Momordica cochinchinensis* Trypsin Inhibitor-II (MCOTI-II) from squash (14, 30, 31). These chimeric binders are referred to as the “R-knots” (R0) as MCOTI-II has high arginine content (Fig. 1). Optimal loop-1 length and motif position were determined, which enabled design of libraries X3RTDLXXLX3 and X4RTDLXXLX3 (L2 and L3 Fig. 1B; Supplementary Fig. S1), which were pooled and sorted by fluorescence-activated cell sorting (FACS). The X4RTDLXXLX3 library dominated sort-round five (1 nmol/L target). Frequent occurrence of arginine or lysine residues at certain loop positions suggested favorable binding interactions. Arginine was fixed at these positions in the second libraries (L2.1 and L3.1) to ensure a sole N-terminus amine for chemical labeling. The remaining loop positions were randomized (Fig. 1, Schematic 1A). The final sort-round was conducted in 300 pmol/L target followed by 3 days of washing at 37°C. The top 3 most-represented binders (1, 2, and 3) were characterized.

Scaffold swapping and its effects on binding affinity and specificity

A novel αvβ6-binding activity, “2,” was grafted into several arginine-rich scaffolds (R0–3), glutamic acid–rich scaffolds (E0–4) and serine-rich scaffolds (S0–3). αvβ6 binding was determined by flow cytometry (Fig. 1B; Supplementary Fig. S2B and Schematic S1) to be comparable across scaffolds; maintenance of high-affinity binding to integrin αvβ6 indicates that engineered loops and knotted scaffolds can function independently and interchangeably. Novel integrin αvβ6-binding activities called “1,” “2,” and “3” were each tested in the context of both the R0 and S0 scaffolds for their ability to bind target. We measured the $K_D$ of R01, R02, and R03 to be $3.6 \pm 0.9$, $3.2 \pm 2.7$, and $3.6 \pm 1.6$ nmol/L, respectively, by flow cytometry using soluble integrin αvβ6 (Fig. 2A). Interestingly, the $K_D$ of the S01 and S02 was only slightly less at $6.5 \pm 0.5$ and $6.0 \pm 0.5$ nmol/L, respectively, whereas the $K_D$ of S03 (3.1 ± 0.5 nmol/L) matched that of its parent (Fig. 2B). These results suggest that the knotted structure is tightly maintained in engineered R0 and S0 binders, so that novel activities can be trans-grafted into other wild-type or rationally designed knots without

Figure 1. Schematic outline of engineering process. A, the primary sequence of the wild-type trypsin inhibitor cystine knot framework (R0) includes 6 cystine residues (white underlined black-back letters, and disulfide connectivities as black lines) and 5 loops. Grafts 1 to 9 (G1–G9) each contain the RTDLXXL motif (gray-back letters). Libraries L2 and L3 were derived from highest affinity grafts, G2 and G3. High-affinity binder R1 emerged after five sort rounds. Libraries, L2.1 and L3.1, fixed arginine residues to prevent the occurrence of lysine at those positions. Binders R2 and R3 emerged from sorting. B, the arginine-rich (R0), glutamic acid-rich (E0), and serine-rich (S0) frameworks show the number and positions of amino acids (underlined gray-back letters) that were substituted. The names of peptides that were fully characterized in vitro and validated in vivo are shown in gray-back letters. C, the structure of MCOTI-II (R0, PDB 2IT8) and its active loop (black) are stabilized by 3 knotted disulfide bonds (black) formed by 6 cysteine residues (I–VI).
notable loss of potency (Supplementary Fig. S2B). We also tested cross-reactivity of peptides used throughout these studies to other integrins. Peptides show very low binding to integrins $\alpha_v \beta_3$, $\alpha_v \beta_5$, and $\alpha_5 \beta_1$ (Fig. 2C). Scrambled peptides did not bind integrin $\alpha_v \beta_6$ (Supplementary Fig. S2A).

**Protein and cell capture assays**

Biotinylated peptides A20, R01, S02, and 2.5F were immobilized onto neutravidin-coated plates. All four peptides captured recombinant integrin $\alpha_v \beta_6$, but only knottin 2.5F captured integrins $\alpha_v \beta_3$, $\alpha_v \beta_5$, and $\alpha_5 \beta_1$.  

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Figure 2. Representative binding curves of A, R01 (circles/solid lines), R02 (squares/dashed lines), and R03 (diamonds/dotted lines). The same activities were reformatted in a serine-rich scaffold to produce B, S01 (circles/solid lines), S02 (squares/dashed lines), and S03 (diamonds/dotted lines). By flow cytometry, all 6 binders showed single-digit nanomolar affinities for integrin $\alpha_v \beta_6$. Data were normalized with respect to saturated fluorescence intensity (plateau) observed at the highest target concentrations. C, indicated peptides were also evaluated for binding to related integrins $\alpha_v \beta_3$, $\alpha_v \beta_5$, and $\alpha_5 \beta_1$, and normalized to binding by 2.5F (19).

Figure 3. Protein and cell capture assays. A, microwells coated with A20, R01, S02, or 2.5F are tested for binding to 10 nmol/L integrins $\alpha_v \beta_6$, $\alpha_v \beta_3$, $\alpha_v \beta_5$, or $\alpha_5 \beta_1$. Binding was normalized to A20. $\alpha_v \beta_6$, $\alpha_v \beta_3$, $\alpha_v \beta_5$, and $\alpha_5 \beta_1$ binding were normalized to 2.5F. B, R01 and S02 inhibit binding of 10 nmol/L $\alpha_v \beta_6$ to A20-coated wells. Data are normalized to noncompetitive binding. C, various cell lines are tested for binding to A20-, R01-, or S02-coated wells. Data are independently normalized to BxPC-3 cell binding. D, R01 and S02 inhibit capture of $10^5$ BxPC-3 cells. Data are normalized to noncompetitive binding.
Engineered binders show different levels of specificity for their targets (Fig. 3A). Biotinylated A20, precoated onto microwell plates, engaged in competitive binding with soluble R01 or S02 for recombinant integrin $\alpha_v\beta_6$. Dose-dependent inhibition indicated competition between peptides for a specific target-binding site (Fig. 3B). A20, R01, and S02 also captured cells that express native integrin $\alpha_v\beta_6$ (Fig. 3C). Flow cytometry showed all tested cell lines (BxPC-3, A431, U87MG, MDA-MB-435) but the 293 cells express integrin $\alpha_v\beta_6$ (Supplementary Fig. S3A and S3B). Peptides R01 and S02 blocked adhesion of BxPC-3 cells onto A20-coated wells confirming specific binding between peptides and functionally active integrins expressed on cellular surfaces (Fig. 3D). $^{64}$Cu-DOTA-labeled peptides also showed binding to target-expressing cells and not to 293 negative controls (Supplementary Fig. S4).

**Scaffold reformatting: serum and metabolic stability of peptides**

Most wild-type and engineered knots resist degradation/denaturation in physiologic media such as serum and urine (19, 23, 29). However, loop engineering can compromise stability. For example, R02 was approximately 80% degraded during 24 hours serum incubation (Fig. 4A). Comparatively, $^{64}$Cu-DOTA-labeled R01 showed much greater stability and approximately 20% degradation occurred during 24 hours serum incubation. These 2 peptides share identical primary sequences in loops 2 through 5 of their structural frames (Supplementary Schematic S1). In contrast, $^{64}$Cu-DOTA-S02 and -E02 showed exceptionally high (>95%) stability during 24 hour serum incubation (Fig. 4B and C). For completeness, the linear control $^{64}$Cu-DOTA-A20 was more than 90% degraded after 24 hours of incubation in serum (Fig. 4D). Urine samples drawn from mice 1.5 to 2

![Image of Figure 4](Figure 4. Radio-HPLC analysis of $^{64}$Cu-DOTA-labeled (A) R02, (B) E02, (C) S02, and (D) A20 prior to (top) and after 24-hour incubation (bottom) in mouse serum at 37°C. *, HPLC retention time of intact radiotracer.)
hours after injection showed approximately 20% degradation of 64Cu-DOTA-R01 and less than 5% degradation of 64Cu-DOTA S02, our current lead translational candidates (Supplementary Fig. S4A and S4B). These results show that nonbinding portions of peptide scaffolds may be used to increase in vivo stability.

MicroPET imaging and biodistribution

Radiolabeled versions of knots were evaluated by positron emission tomography (PET) in mice bearing integrin \( \alpha_v\beta_6 \)-expressing BxPC-3 (pancreatic cancer) or A431 (epidermoid cancer) xenografts and \( \alpha_v\beta_6 \)-negative 293 tumors. Mice were injected with approximately 75 \( \mu Ci \) of 64Cu-DOTA–labeled R02, E02, and S02, and R01, in BxPC-3 xenografts and 293 xenografts (\( n = 3 \) mice; Fig. 5D). Comparatively, tumor uptake of R02 measured 4.3%ID/g ± 0.7%ID/g in BxPC-3 xenografts compared with 1.3%ID/g ± 0.1%ID/g in 293 xenografts. S02 and R01 corroborated these results (Fig. 5D). Collectively, these results show that the \( \alpha_v\beta_6 \)-specific probes selectively bind \( \alpha_v\beta_6 \)-positive (T+) tumors (A431 and BxPC-3) and do not accumulate in the \( \alpha_v\beta_6 \)-negative (T−) 293 tumors (Fig. 5A and D). Unfortunately, conclusive identification of orthotopic BxPC-3 tumors by PET remained elusive.

Biodistribution analysis of R01 and S02 in BxPC-3 xenograft tumors showed 4.13%ID/g ± 1.01%ID/g and 1.80%ID/g ± 0.50%ID/g 1 hour postinjection. These data closely matched corresponding PET data (Table 1 and Fig. 5D). Importantly, S02 effectively accumulated in BxPC-3 orthotopic tumors (1.85%ID/g ± 0.11%ID/g, 1 hour postinjection) compared with normal pancreas (0.2%ID/g–0.3%ID/g, 1 hour postinjection, Tables 1 and 2).

PET studies of BxPC-3 pancreatic xenografts confirmed these results. Importantly, significantly less radiotracer accumulated in \( \alpha_v\beta_6 \)-negative 293 xenografts (\( P < 0.05 \), \( n = 3 \) mice; Fig. 5D). Comparatively, tumor uptake of R02 measured 4.3%ID/g ± 0.7%ID/g in BxPC-3 xenografts compared with 1.3%ID/g ± 0.1%ID/g in 293 xenografts. S02 and R01 corroborated these results (Fig. 5D). Collectively, these results show that the \( \alpha_v\beta_6 \)-specific probes selectively bind \( \alpha_v\beta_6 \)-positive (T+) tumors (A431 and BxPC-3) and do not accumulate in the \( \alpha_v\beta_6 \)-negative (T−) 293 tumors (Fig. 5A and D). Unfortunately, conclusive identification of orthotopic BxPC-3 tumors by PET remained elusive.

**Figure 5.** MicroPET images of (A) A431 xenografts are shown at 1 hour postinjection of 64Cu-DOTA–labeled R02, E02, and S02, and R01. The positive control is indicated by A20. Far right, a BxPC-3 \( \alpha_v\beta_6 \)-positive (T+) and \( \alpha_v\beta_6 \)-negative 293 (T−) double xenograft injected with S02 is shown both as a photograph and microPET image of the same mouse. B and C, quantification of microPET images shown in (A). D, side-by-side comparison of R02, S02, and R01 in BxPC-3 xenografts and 293 xenografts (\( P < 0.05 \)). B–D, four bars indicate 1-, 2-, 4-, and 24-hour postinjection.
Renal retention varied by binding activity and scaffold (Fig. 5C). R01 ranged from approximately 45%ID/g (1 hour) to approximately 20%ID/g (24 hours), whereas R02 ranged from approximately 75%ID/g (1 hour) to approximately 28%ID/g (24 hours). This difference is attributed to the higher arginine content of activity "2." However, transfer of activity "2" to the carboxyl-rich scaffold (E02) did not decrease kidney signal [~72%ID/g (1 hour) and ~16%ID/g (24 hours)]. These results suggest that in addition to arginine residues, the kidneys also actively retain carboxyl-containing residues. Importantly, when the "2" activity was tested in the S0 scaffold (S02), significantly

However, substantial amounts of S02 accumulated in liver, stomach, intestines, and kidneys as measured by biodistribution analysis and seen by PET imaging (Tables 1 and 2 and Fig. 5).

R02 and R01 accumulated to greater levels in A431 tumors than E02 and S02. However, these arginine-rich binders cleared slowest from surrounding muscle tissue. Biodistribution analysis indicated approximately 0.6%ID/g/muscle at 1 hour for the R02, whereas approximately half (~0.3%ID/g) was observed for E02 and S02 (Supplementary Table S1). Therefore, tumor-to-muscle contrast was comparable for each of the engineered knots.

Table 1. Biodistribution of $^{64}$Cu-DOTA-R01 and -S02, in pancreatic cancer models, measured by tissue resection and scintillation counting are reported as mean%ID/g ± SD (n = 3) in various tissues

<table>
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<th>Tissue</th>
<th>$^{64}$Cu-DOTA-R01</th>
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<td>24 h</td>
<td>1 h</td>
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<tr>
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NOTE: Orthotopic tumors are indicated by "*".
Abbreviation: n.d., not determined.

Table 2. The associated tumor-to-normal tissue ratios of $^{64}$Cu-DOTA-R01 and -S02 at 1- and 24-hour postinjection

<table>
<thead>
<tr>
<th>Ratio</th>
<th>$^{64}$Cu-DOTA-R01</th>
<th>$^{64}$Cu-DOTA-S02</th>
<th>$^{64}$Cu-DOTA-S02</th>
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<td></td>
<td>1 h</td>
<td>24 h</td>
<td>1 h</td>
</tr>
<tr>
<td>T/muscle</td>
<td>8.89 ± 2.00</td>
<td>6.52 ± 0.83</td>
<td>6.22 ± 0.93</td>
</tr>
<tr>
<td>T/liver</td>
<td>1.91 ± 0.85</td>
<td>0.72 ± 0.11</td>
<td>0.67 ± 0.32</td>
</tr>
<tr>
<td>T/lung</td>
<td>1.48 ± 0.12</td>
<td>1.54 ± 0.53</td>
<td>1.89 ± 0.81</td>
</tr>
<tr>
<td>T/spleen</td>
<td>8.34 ± 2.14</td>
<td>5.05 ± 0.73</td>
<td>7.22 ± 2.26</td>
</tr>
<tr>
<td>T/pancreas</td>
<td>7.18 ± 0.55</td>
<td>4.96 ± 1.75</td>
<td>10.89 ± 3.95</td>
</tr>
<tr>
<td>T/kidneys</td>
<td>0.06 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>0.07 ± 0.03</td>
</tr>
</tbody>
</table>

NOTE: Orthotopic tumors are indicated by "*".
Abbreviation: n.d., not determined.
lower renal retention was measured (~18%ID/g (1 hour) and ~7%ID/g (24 hours)). The large differences in renal uptake suggest that scaffold pharmacokinetics may be optimized to evade renal reuptake and allow a binder to pass freely into the bladder. Arginine is one of the most versatile amino acids in animal cells, serving as a precursor for the synthesis of proteins, nitric oxide, urea, polyamines, proline, glutamate, creatine, and agmatine (32). Therefore, arginine recycling by the kidneys is beneficial to the organism. By substituting pharmacokinetically favorable amino acids in the diversity-tolerant cystine knot framework, non-specific uptake by off-target tissues may be reduced.

Discussion

Integrin αβ6 is being explored as a clinical target because of its elevated expression in several cancers (2). In this study, yeast surface display and high throughput screening by FACS produced many high-affinity binders that selectively accumulate in αβ6-positive pancreatic and epidermoid cancer models but not in αβ6-negative tumors actively growing in mice. By grafting the loop known as activity "2" (RSIARTDLHDHILRGR) across different cystine knot scaffolds with biased amino acid content (R0, E0, and S0), we achieved significantly lower renal signal without compromising αβ6 binding affinity or specificity. This study suggests that primary sequence composition drives pharmacokinetics, so that it may be possible to optimize tumor uptake, tumor-to-muscle contrast, and off-target accumulation in tissues by fine-tuning the non-target-binding portions of the scaffold in addition to engineering target-binding loops.

Absolute 64Cu-DOTA signal intensity at the tumor was greatest for the 2 R0 binders, R0.1 and R0.2 (~4.5%ID/g at 1 hour postinjection). 64Cu-DOTA-labeled E0.2, S0.2, and A20 generated approximately half the tumor signal intensity compared with the R-rich scaffolds. Given the similar affinities of the engineered knots (Fig. 2A and B), we rationalize that higher similar tumor signal of R0.2 benefited from the inherent "stickiness" of arginine. However, independent of a scaffold’s compositional differences, each of the activity "2" knots (R0.2, E0.2, and S0.2) showed similarly useful tumor-to-normal contrast enabling precise delineation of integrin αβ6-positive tumors (T+) from αβ6-negative tumors (T−) shown in Fig. 5.

For clinical imaging of pancreatic cancer, S0.2 has emerged as our lead candidate. 64Cu-DOTA-S0.2 showed an excellent tumor-to-background ratio (~10 at 1 hour), rapid renal clearance (~25%ID/g 1 hour postinjection to <10%ID/g after 24 hours), and high serum stability (>95% after 24 hours). In contrast, 64Cu-DOTA-R0.2 rapidly degrades in serum (<20% intact 24 hours) and accumulates to a much higher degree in the kidneys (~75%ID/g 1 hour). 64Cu-DOTA-E0.2 also suffers from prolonged and high renal retention (~75%ID/g 1 hour). Therefore, to take advantage of lower renal background of S0.2, we are conducting affinity maturation to achieve higher tumor uptake comparable with R0.2 binders. We are also exploring 111Flabeling strategies as the pharmacokinetics of these binders support a shorter lived isotope for clinical translation.

Biodistribution studies indicated that 64Cu-DOTA-S0.2 also effectively accumulated in orthotopic pancreatic tumors (~1.8%ID/g 1 hour postinjection) compared with normal pancreas (~0.2%ID/g–0.3%ID/g). However, tumor-to-liver/kidney/gut ratios were suboptimal (Tables 1 and 2). The proximity of these PET-avid tissues is approximately 1 to 3 mm from the pancreas and is beyond the spatial resolution of the PET scanner. Therefore, definitive identification of orthotopic tumors by PET was limited by closely packed mouse organs. However, human scanning should not suffer from these limitations.

The engineered knots exhibit specific binding to integrin αβ6. They do not bind related integrins αβ3, αβ5, or ααβ1, and the scrambled (RDTLXXL) versions do not bind αβ6 even at high concentrations (Figs. 2C and 3A; Supplementary Fig. S2A). Low uptake of αβ6-specific radiotracers by 293 xenografts correlated to their limited expression of both the α6 and β6 integrin subunits (Fig. 5, Supplementary Fig. S3A). In a very clear example, 64Cu-DOTA-S0.2 specificity was shown in a mouse bearing both BxPC-3 and 293 xenografts (Fig. 5A, far right). Here, signal intensity for the αβ6-positive tumor (T+, left shoulder) was approximately 2%ID/g, versus approximately 0.5%ID/g for the αβ6-negative tumor (T− right shoulder) at 1 hour postinjection. The "stickier" R0 binders also showed selectivity for integrin αβ6-expressing cells/tumors and recombinant protein (Figs. 2, 3, and 5; Supplementary Fig. S8). However, muscle washout was slower for the R0s, so that contrast was comparable across all binders tested (Fig. 5B and D).

Renal clearance can potentially be controlled and optimized. This study provides insight about the significant differences in renal uptake reported for peptides previously engineered to bind integrin αβ6, a vascular target (19). Knottins 2.5D and 2.5F, based on the cystine knot Ecballium elaterium Trypsin Inhibitor (EETI-II), contain a minimal number of arginines. The renal signals of 64Cu-DOTA-2.5D and -2.5F measured less than 10%ID/g from 1 to 24 hours. In contrast, 64Cu-DOTA-AgrRP-7C, derived from the agouti-related peptide, which contains many complex nitrogen- and oxygen-containing residues, showed significantly higher renal retention of more than 65%ID/g at the same time points (29). Renal uptake of radiometal-labeled affibodies has also been reported to be very high (>100%ID/g) at multiple time points for binders 002, 1907, and 2377 (33–35). Affibody sequence inspection reveals the presence of many K, D/E, and Q/N residues, which can contribute to high renal retention (36–38). The kidneys appear to actively survey for the more complex, charge-rich amino acids (D/E/K/R) while allowing rapid clearance of the simpler noncharged amino acids (e.g., A, G, S). The development of stable, pharmacokinetically optimized scaffolds is highly desirable. Our results suggest one way to gain precise control of binder pharmacokinetics.

In summary, cystine knots are inherently stable scaffolds that are potentially well suited for clinical molecular imaging. Loop-grafting and directed evolution experiments...
generated single-digit nanomolar binders. PET imaging studies produced clinically useful tumor-to-normal contrast within 1 hour. We showed that simple scaffold swapping could enhance knot stability and stabilize pharmacokinetics to evade renal surveillance and approach renal stealth. Importantly, most knots are very well behaved during long-term storage. Collectively, these data suggest that cystine knots warrant further exploration for clinical translation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Pharmacokinetically Stabilized Cystine Knot Peptides That Bind Alpha-v-Beta-6 Integrin with Single-Digit Nanomolar Affinities for Detection of Pancreatic Cancer

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