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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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Abstract

Purpose—Detection of pancreatic cancer remains high priority and effective diagnostic tools are needed for clinical applications. Many cancer cells overexpress integrin $\alpha_v\beta_6$, 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 nM) to integrin $\alpha_v\beta_6$. The binders don't cross-react with related integrin $\alpha_v\beta_5$, integrin $\alpha_5\beta_1$ or tumor-angiogenesis associated integrin, $\alpha_v\beta_3$.

Results—Positron emission tomography showed that these disulfide-stabilized peptides rapidly accumulate at tumors expressing integrin $\alpha_v\beta_6$. Clinically relevant tumor-to-muscle ratios of 7.7 ± 2.4 to 11.3 ± 3.0 were achieved within one hour after radiotracer injection. Minimization of off-target dosing was achieved by reformatting $\alpha_v\beta_6$ -binding activities across various natural and pharmacokinetically-stabilized cystine knot scaffolds with different amino acid content. We demonstrate that a peptide scaffold's primary sequence directs its pharmacokinetics. Scaffolds with high arginine or glutamic acid content suffered high renal retention of > 75 percent 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 < 20 %ID/g 1h post injection ($p < 0.05$, $n=3$).

Conclusions—We have engineered highly stable cystine knot peptides with potent and specific integrin $\alpha_v\beta_6$ 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.

INTRODUCTION

Integrins are a family of heterodimeric cell surface receptors that mediate cellular adhesion to extracellular matrix proteins. Integrins also serve as bi-directional signal transducers to

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regulate differentiation, migration, proliferation, and cell death (1, 2). Integrin $\alpha_v\beta_3$ promotes tumor neovascularization (3, 4). However in certain cancers, other integrins, such as $\alpha_v\beta_6$, 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 $\alpha_v\beta_6$ may be used to gauge receptor expression levels to determine prognosis and guide therapy (10-12). Here, we have developed potent integrin $\alpha_v\beta_6$ binders for clinical translation as radiotracers for early cancer detection.

Previously, several integrin $\alpha_v\beta_6$ binders were identified from natural and combinatorial sources. Linear $\alpha_v\beta_6$ -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. ^{64}Cu -labeled versions of FMDV peptides demonstrated extremely high renal retention, which suggests that these peptides may not be ideal translational candidates. An alternative to radio-metals is the use of radio-halogens (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 HBP-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 throughput live animal imaging survey (17). While 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 physiological media, demonstrate low off target accumulation and effectively detect cancer in living subjects.

Engineered cystine knot peptides (knots) have shown promise for cancer imaging with $\alpha_v\beta_3$ as a target (19-21). The cystine knot is a rigid molecular scaffold of 3-4 kDa that owes its exceptionally-high stability to three 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 biological, 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. Additionally, 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 SUPPLEMENTAL INFORMATION 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 DMEM supplemented with 10% FBS and penicillin/streptomycin (Invitrogen). Recombinant human integrins $\alpha_v\beta_6$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$ 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 (Supplemental). 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, ~40 μ g of DNA insert and 4 μ g of linearized pCT vector were electroporated into the *S. cerevisiae* strain EBY100 by homologous recombination as previously described (26). For libraries L2 and L3, ~5-7 $\times 10^6$ transformants per sub-library were combined for a total diversity of ~1 $\times 10^7$ clones as estimated by serial dilution plating and colony counting. For libraries L2.1 and L3.1, ~2 $\times 10^6$ transformants per sub-library were combined for a total diversity of ~4 $\times 10^6$ clones.

For library screening, various concentrations of recombinant $\alpha_v\beta_6$ 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 1h at 4°C. The cells were washed with ice-cold IBB and incubated with a 1:25 dilution of fluorescein-conjugated anti- α_v integrin antibody (Biolegend) and a 1:100 dilution of Alexa 555-conjugated goat anti-chicken IgG secondary antibody (Invitrogen) for 0.5h at 4°C. Cells were washed in IBB and $\alpha_v\beta_6$ integrin binders were isolated using a Becton Dickinson FACS Aria III instrument. For the first round of sorting, ~2 $\times 10^7$ yeast clones were screened with 100 nM $\alpha_v\beta_6$ integrin. To increase sort stringency, integrin concentrations were successively decreased to 1 nM 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, ~5 $\times 10^6$ yeast clones were screened as described above with a final concentration of 300 pM $\alpha_v\beta_6$ integrin coupled with 3 days of washing in IBB at 37°C. Plasmid DNA was recovered by Zymo prep (Zymo Research), amplified in Max Efficiency DH5 α *E. coli* cells (Invitrogen) and sequenced.

Peptide Synthesis/Biosynthesis, Folding and Radiolabeling

S₀2 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 mM guanidium hydrochloride (22). A20 was similarly prepared without folding. R₀2 and E₀2 were biosynthesized using *Pichia pastoris* (Supplemental). Peptides were conjugated through their N-terminus amine to DOTA-NHS and radiolabeled with ⁶⁴CuCl₂ as previously described (19). The radiochemical purity was determined by HPLC to be > 95%. The radiochemical yield was usually over 80%. The specific activity of the probe was ~500 Ci/mmol. Molecular masses were confirmed by MALDI-MS (ABI 5800, Supplemental Table 2).

Binding Affinity

Various concentrations of integrin $\alpha_v\beta_6$, $\alpha_v\beta_5$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ were incubated with 10^5 yeast cells expressing R₀₁, R₀₂, R₀₃, S₀₁, S₀₂, S₀₃ or 2.5F in the presence of 10^6 un-induced yeast cells as previously described (22, 27). Prior to flow cytometry and analysis, yeast cells were processed, stained, and washed as described above in *Library Synthesis and Screening*.

Protein and Cell Capture Assays

Neuravidin coated wells (Pierce) were saturated with biotinylated peptides A20, R₀₁, S₀₂ or 2.5F. 10nM recombinant integrins or 10^5 cells in IBB were added to wells at room temperature for 2h. Wells were washed 3x with ice-cold IBB. Integrins were detected with mouse anti- α_v or anti- α_5 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 $\alpha_v\beta_6$ or BxPC-3 cells were preincubated with R₀₁ or S₀₂ for 2 hours prior to introduction into A20 coated wells.

⁶⁴Cu-DOTA Peptide Stability

Aliquots of ⁶⁴Cu-DOTA-peptide were incubated in an equal volume of mouse or human serum for up to 24h. Samples were acidified with TFA and centrifuged to remove precipitants. In addition, ⁶⁴Cu-DOTA-peptides were extracted from full mouse bladders 1.5-2 hour after injection. Soluble fractions were filtered with a Spin-X 0.2 μ m filter (Corning) when necessary. All samples were analyzed by radio-HPLC on a Dionex C₄ column.

Tumor Models

Animal procedures were performed per protocol 11580 and 21637 (Stanford University Administrative Panels on Laboratory Animal Care). Female athymic nude mice, 4-6 weeks old (Charles River), were subcutaneously shoulder-injected with 10^7 cells suspended in 100 μ L PBS. Orthotopic tumors were generated with 10^6 cells/20 μ L Matrigel (Supplemental). Mice were used for imaging/biodistribution studies when xenografts or orthotopic tumors reached ~10 mm or ~5 mm, respectively, in diameter.

MicroPET Imaging

Tumor-bearing mice (n=3 for each probe) were injected with ~50-100 μ Ci (~0.15 nmol) of probe via the tail vein and imaged with a microPET R4 rodent model scanner (Siemens) using 5min static scans. Images were reconstructed by a two-dimensional ordered expectation maximum subset algorithm and calibrated as previously described (28). ROIs 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 performed.

Biodistribution Analysis

Anesthetized nude mice bearing xenograft/orthotopic tumors were injected with ~50-100 μ Ci (~0.15 nmol) of ⁶⁴Cu-DOTA-peptides via tail vein, and euthanized after 1h or 24h. Tissues were removed, weighed and measured by scintillation counting (19, 29). Radiotracer uptake in tissues was reported as percent injected dose per gram (%ID/g) and represents the mean $\pm$ standard deviation of experiments performed on three mice.

RESULTS

Engineering $\alpha_v\beta_6$ 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” (R_0) since MCoTI-II has high arginine content (Schematic 1). Optimal loop-1 length and motif position were determined, and enabled design of libraries $X_3RTDLXXLX_3$ and $X_4RTDLXXLX_3$ (L2 and L3 Schematic 1A, Supplemental Figure 1), which were pooled and sorted by FACS. The $X_3RTDLXXLX_3$ library dominated sort-round five (1 nM 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 (Schematic 1A). The final sort-round was conducted in 300 pM target followed by 3 days of washing at 37°C. The top three most-represented binders (1, 2 and 3) were characterized.

Scaffold Swapping and its Effects on Binding Affinity and Specificity

A novel $\alpha_v\beta_6$ -binding activity, “2”, was grafted into several arginine-rich scaffolds (R_{0-3}), glutamic acid-rich scaffolds (E_{0-4}) and serine-rich scaffolds (S_{0-3}). $\alpha_v\beta_6$ -binding was determined by flow cytometry (Schematic 1B, Supplemental Figure 2B and Supplemental Schematic 1) to be comparable across scaffolds; maintenance of high-affinity binding to integrin $\alpha_v\beta_6$ indicates that engineered loops and knotted scaffolds can function independently and interchangeably. Novel integrin $\alpha_v\beta_6$ binding activities called “1”, “2”, and “3”, were each tested in the context of both the R_0 and S_0 scaffolds for their ability to bind target. We measured the K_D of R_{01} , R_{02} , and R_{03} to be 3.6 ± 0.9 nM, 3.2 ± 2.7 nM, 3.6 ± 1.6 nM, respectively, by flow cytometry using soluble integrin $\alpha_v\beta_6$ (Figure 2A). Interestingly, the K_D of the S_{01} , S_{02} was only slightly less at 6.5 ± 2.0 nM and 6.0 ± 0.1 nM, respectively, while the K_D of S_{03} (3.1 ± 0.5 nM) matched that of its parent (Figure 2B). These results suggest that the knotted structure is tightly maintained in engineered R_0 and S_0 binders, so that novel activities can be trans-grafted into other wild type or rationally-designed knots without notable loss of potency (Supplemental Figure 2B). We also tested cross-reactivity of peptides used throughout these studies to other integrins. Peptides demonstrate very low binding to integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ (Figure 2C). Scrambled peptides did not bind integrin $\alpha_v\beta_6$ (Supplemental Figure 2A).

Protein and Cell Capture Assays

Biotinylated peptides A20, R_{01} , S_{02} and 2.5F were immobilized onto neutravidin coated plates. All 4 peptides captured recombinant integrin $\alpha_v\beta_6$, but only knottin 2.5F captured integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$. Engineered binders show different levels of specificity for their targets (Figure 3A). Biotinylated A20, precoated onto microwell plates, engaged in competitive binding with soluble R_{01} or S_{02} for recombinant integrin $\alpha_v\beta_6$. Dose dependent inhibition indicated competition between peptides for a specific target-binding site (Figure 3B). A20, R_{01} and S_{02} also captured cells that express native integrin $\alpha_v\beta_6$ (Figure 3C). Flow cytometry showed all tested cell lines (BxPC-3, A431, U87MG, MDA-MB-435) but the 293 cells express integrin $\alpha_v\beta_6$ (Supplemental Figure 3AB). Peptides R_{01} and S_{02} blocked adhesion of BxPC-3 cells onto A20 coated wells confirming specific binding between peptides and functionally-active integrins expressed on cellular surfaces (Figure 3D). ^{64}Cu -DOTA-labeled peptides also demonstrated binding to target expressing cells and not to 293 negative controls (Supplemental Figure 4).

Scaffold Reformatting: Serum and Metabolic Stability of Peptides

Most wild-type and engineered knots resist degradation/denaturation in physiological media such as serum and urine (19, 23, 29). However, loop engineering can compromise stability. For example, R₀2 was ~80% degraded during 24h serum incubation (Figure 4A). Comparatively, ⁶⁴Cu-DOTA-labeled R₀1 demonstrated much greater stability; approximately 20% degradation occurred during 24h serum incubation. These two peptides share identical primary sequences in loops 2 through 5 of their structural frames (Schematic 1). In contrast, ⁶⁴Cu-DOTA-S₀2 and -E₀2 demonstrated exceptionally high (>95%) stability during 24h serum incubation (Figure 4BC). For completeness, the linear control ⁶⁴Cu-DOTA-A20 was >90% degraded after 24h incubation in serum (Figure 4D). Urine samples drawn from mice 1.5-2h after injection showed ~20% degradation of ⁶⁴Cu-DOTA-R₀1 and <5% degradation of ⁶⁴Cu-DOTA S₀2, our current lead translational candidates (Supplemental Figure 4AB). These results demonstrate that non-binding portions of peptide scaffolds may be used to increase in vivo stability.

MicroPET Imaging and Biodistribution

Radio-labeled versions of knots were evaluated by 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 ~75 uCi of ⁶⁴Cu-DOTA labeled peptides. R₀2, E₀2, S₀2, R₀1 and the positive control A20, rapidly accumulated in $\alpha_v\beta_6$ -positive A431 tumors and generated excellent tumor-to-muscle contrast ratios of 6-11 at 1h post injection (p.i., Figure 5AB). Absolute uptake in A431 tumors was highest for the two R₀-based binders R₀1 (~5 %ID/g) and R₀2 (~4 %ID/g) compared to E₀2 (~1.5 %ID/g), S₀2 (~2 %ID/g) and A20 (~2 %ID/g) at 1h p.i. (Figures 5AB).

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, Figure 5D). Comparatively, tumor uptake of R₀2, measured 4.3 ± 0.7 %ID/g in BxPC-3 xenografts compared to 1.3 ± 0.1 %ID/g in 293 xenografts. S₀2 and R₀1 corroborated these results (Figure 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 (Figure 5AD). 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 ± 1.01 and 1.80 ± 0.50 %ID/g 1h p.i.. These data closely matched corresponding PET data (Table 1A and Figure 5D). Importantly, S₀2 effectively accumulated in BxPC-3 orthotopic tumors (1.85 ± 0.11 %ID/g, 1h p.i.) compared to normal pancreas (~0.2-0.3 %ID/g, 1h p.i., Table 1). However, substantial amounts of S₀2 accumulated in liver, stomach, intestines and kidneys as measured by biodistribution analysis and seen by PET imaging (Table 1, Figure 5).

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

Renal retention varied by binding activity and scaffold (Figure 5C). R₀1 ranged from ~45 %ID/g (1h) to ~20 %ID/g (24h), whereas R₀2 ranged from ~75 %ID/g (1h) to ~28 %ID/g (24h). 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 (1h) and ~16 %ID/g (24h)). 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 S_0 scaffold (S_02), significantly lower renal retention was measured (~18 %ID/g (1h) and ~7 %ID/g (24h)). 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 $\alpha_v\beta_6$ is being explored as a clinical target due to its elevated expression in several cancers (2). In the current study, yeast surface display and high throughput screening by FACS produced many high-affinity binders that selectively accumulate in $\alpha_v\beta_6$ -positive pancreatic and epidermoid cancer models, but not in $\alpha_v\beta_6$ -negative tumors actively growing in mice. By grafting the loop known as activity “2” (RSLARTDLHLRGR) across different cystine knot scaffolds with biased amino acid content (R_0 , E_0 and S_0), we achieved significantly lower renal signal without compromising $\alpha_v\beta_6$ 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 ^{64}Cu -DOTA signal intensity at the tumor was greatest for the two R_0 binders, R_01 and R_02 (~4.5 %ID/g at 1h p.i.). ^{64}Cu -DOTA-labeled E_02 , S_02 and A20 generated approximately half the tumor signal intensity compared to the R-rich scaffolds. Given the similar affinities of the engineered knots (Figure 2AB), we rationalize that R_0 s’ higher tumor signal benefitted from the inherent “stickiness” of arginine. However, independent of a scaffold’s compositional differences, each of the activity “2” knots (R_02 , E_02 , and S_02) demonstrated similarly useful tumor-to-normal contrast enabling precise delineation of integrin $\alpha_v\beta_6$ -positive tumors (T+) from $\alpha_v\beta_6$ -negative tumors (T-) shown in Figure 5.

For clinical imaging of pancreatic cancer, S_02 has emerged as our lead candidate. ^{64}Cu -DOTA- S_02 demonstrated an excellent tumor-to-background ratio (~10 at 1 h), rapid renal clearance (~25 %ID/g 1h p.i. to < 10 %ID/g after 24h), and high serum stability (>95% after 24h). In contrast, ^{64}Cu -DOTA- R_02 rapidly degrades in serum (< 20% intact 24h) and accumulates to a much higher degree in the kidneys (~75 %ID/g 1h). ^{64}Cu -DOTA- E_02 also suffers from prolonged and high renal retention (~75 %ID/g 1h). Therefore, to take advantage of S_0 s’ lower renal background, we are performing affinity maturation to achieve higher tumor uptake comparable to R_0 binders. We are also exploring ^{18}F labeling strategies since the pharmacokinetics of these binders support a shorter lived isotope for clinical translation.

Biodistribution studies indicated that ^{64}Cu -DOTA- S_02 also effectively accumulated in orthotopic pancreatic tumors (~1.8 %ID/g 1h p.i.) compared to normal pancreas (~0.2-0.3 %ID/g). However, tumor-to-liver/kidney/gut ratios were suboptimal (Table 1AB). The proximity of these PET-avid tissues is ~1-3mm 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 $\alpha_v\beta_6$. They do not bind related integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ or $\alpha_5\beta_1$, and the scrambled (*RDTXXL*) versions do not bind $\alpha_v\beta_6$ even at high concentrations (Figures 2C and 3A, Supplemental Figure 2A). Low uptake of $\alpha_v\beta_6$ -specific radiotracers by 293 xenografts correlated to their limited expression of both the α_v and β_6 integrin subunits (Supplemental Figure 3A, Figure 5). In a very clear example, ^{64}Cu -DOTA-S₀2 specificity was demonstrated in a mouse bearing both BxPC-3 and 293 xenografts (far right panel Figure 5A). Here, signal intensity for the $\alpha_v\beta_6$ -positive tumor (T +, left shoulder) was ~2 %ID/g, vs. ~0.5 %ID/g for the $\alpha_v\beta_6$ -negative tumor (T-, right shoulder) at 1h p.i.. The “stickier” R₀ binders also demonstrated selectivity for integrin $\alpha_v\beta_6$ expressing cells/tumors and recombinant protein (Figures 2, 3, 5 and Supplemental Figure 3). However, muscle wash-out was slower for the R₀s, so that contrast was comparable across all binders tested (Figure 5BD).

Renal clearance can potentially be controlled and optimized. Our current study provides insight about the significant differences in renal uptake reported for peptides previously engineered to bind integrin $\alpha_v\beta_3$, 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 ^{64}Cu -DOTA-2.5D and -2.5F measured < 10 %ID/g from 1-24h. In contrast, ^{64}Cu -DOTA-AgRP-7C, derived from the agouti related peptide, which contains many complex nitrogen- and oxygen-containing residues, demonstrated significantly higher renal retention of > 65 %ID/g at the same time points (29). Renal uptake of radio-metal 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 non-charged 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 1h. We demonstrated 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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STATEMENT OF 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 $\alpha_5\beta_1$. Uptake of the radiotracers by integrin $\alpha_v\beta_6$ -expressing tumors was rapid and high (~2-5 percent injected dose per gram, 1 hour post injection). 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.

Figure 1.

Schematic Outline of Engineering Process. (a) The primary sequence of the wild type trypsin inhibitor cystine knot framework (R_0) includes six cysteine residues (white underlined black-back letters, and disulfide connectivities as black lines) and five loops. Grafts one to nine (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 R_{01} emerged after 5 sort rounds. Libraries, L2.1 and L3.1, fixed arginine residues to prevent the occurrence of lysine at those positions. Binders R_{02} and R_{03} emerged from sorting. (b) The arginine-rich (R_0), glutamic acid-rich (E_0) and serine-rich (S_0) 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 (R_0 , PDB 2IT8) and its active loop (black) are stabilized by three knotted disulfide bonds (black) formed by six cysteine residues (I–VI).

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 six binders demonstrated 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 10nM integrins $\alpha_v\beta_6$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ or $\alpha_5\beta_1$. $\alpha_v\beta_6$ binding was normalized to A20. $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ binding were normalized to 2.5F. (b) R₀1 and S₀2 inhibit binding of 10nM $\alpha_v\beta_6$ to A20 coated wells. Data are normalized to non-competitive binding. (c) Various cell lines are tested for binding to A20, R₀1 or S₀2 coated wells. Data are normalized to BxPC-3 cell-binding. (d) R₀1 and S₀2 inhibit capture of 10^5 BxPC-3 cells. Data are normalized to non-competitive binding.

Figure 4. Radio-HPLC analysis of ^{64}Cu -DOTA labeled (a) R_02 , (b) E_02 , (c) S_02 and (d) A_20 prior to (upper panel) and after 24h incubation (lower panel) in mouse serum at 37°C . Asterisk indicates HPLC retention time of intact radiotracer.

Figure 5.

MicroPET images of (a) A431 xenografts are shown at 1h post injection of ^{64}Cu -DOTA labeled R₀₂, E₀₂ and S₀₂ and R₀₁. The positive control is indicated by A20. On the 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,c) Quantification of microPET images shown in panel 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 24h post injection.

Biodistribution of ⁶⁴Cu-DOTA-R₀1, and -S₀2, in pancreatic cancer models, measured by tissue resection and scintillation counting are reported as (a) mean % ID/g ± SD (n=3) in various tissues, and (b) their associated tumor-to-normal tissue ratios at 1 hour and 24 hours post injection are reported as mean ± SD (n=3). Orthotopic tumors are represented by “O*”

Table 1

A. Biodistribution of ⁶⁴Cu-DOTA-labeled peptides in α₁β₆-positive BxPC3 pancreatic tumor models.

Tissue	Data are presented as mean %ID/g ± SD					
	⁶⁴ Cu-DOTA-R ₀ 1		⁶⁴ Cu-DOTA-S ₀ 2		⁶⁴ Cu-DOTA-S ₀ 2 (*)	
	1h	24h	1h	24h	1h	24h
Tumor	4.13±1.01	3.31±0.20	1.80 ±0.50	1.46±0.43	1.85±0.11 (*)	n.d.
Muscle	0.49±0.21	0.33±0.14	0.24±0.14	0.14±0.02	0.21±0.02	n.d.
Blood	0.46±0.18	0.52±0.09	0.29±0.07	0.23±0.03	0.22±0.03	n.d.
Heart	0.39±0.08	0.67±0.12	0.22±0.04	0.47±0.03	0.32±0.01	n.d.
Kidney	68.12±9.69	33.75±1.77	26.53±12.26	10.69±1.63	31.49±2.47	n.d.
Liver	2.28±0.40	4.69±0.75	3.03±1.10	2.79±0.65	2.70±0.53	n.d.
Lung	2.78±0.59	2.34±0.80	1.03±0.29	1.22±0.50	0.95±0.09	n.d.
Spleen	0.52±0.18	0.67±0.11	0.27±0.12	0.36±0.13	0.39±0.09	n.d.
Pancreas	0.57±0.10	0.73±0.28	0.17±0.02	0.25±0.02	orthotopic (*)	n.d.
Stomach	2.31±0.12	2.71±0.42	0.79±0.19	0.98±0.27	1.51±0.11	n.d.
Intestine	1.49±0.2	2.05±0.38	1.280.26	0.92±0.27	1.05±0.11	n.d.
Brain	0.07±0.01	0.15±0.03	0.04±0.01	0.07±0.01	0.06±0.01	n.d.
Bone	0.26±0.08	0.510.24	0.09±0.01	0.19±0.06	0.23±0.01	n.d.
Skin	0.99±0.14	1.04±0.72	0.34±0.09	0.66±0.15	0.46±0.32	n.d.

B. Tumor-to-Normal Tissue Ratios

Ratio	⁶⁴ Cu-DOTA-R ₀ 1		⁶⁴ Cu-DOTA-S ₀ 2		⁶⁴ Cu-DOTA-S ₀ 2 (*)	
	1h	24h	1h	24h	1h	24h
T/Muscle	8.89±2.00	6.52±0.83	6.22±0.93	6.36±1.20	8.54±1.23	n.d.
T/Blood	9.72±3.63	10.81±3.31	9.98±5.95	10.68±3.44	8.86±1.38	n.d.
T/Liver	1.91±0.85	0.72±0.11	0.67±0.32	0.53±0.19	0.70±0.09	n.d.
T/Lung	1.48±0.12	1.54±0.53	1.89±0.81	1.26±0.28	1.95±0.28	n.d.

B. Tumor-to-Normal Tissue Ratios

Ratio	⁶⁴ Cu-DOTA-R ₀ I		⁶⁴ Cu-DOTA-S ₀ 2		⁶⁴ Cu-DOTA-S ₀ 2 (*)	
	1h	24h	1h	24h	1h	24h
T/Spleen	8.34±2.14	5.05±0.73	7.22±2.26	4.23±1.62	6.81±2.54	n.d.
T/Pancreas	7.18±0.55	4.96±1.75	10.89±3.95	5.96±2.20	orthotopic(*)	n.d.
T/Kidneys	0.06±0.02	0.10±0.01	0.07±0.03	0.14±0.06	0.06±0.01	n.d.