SUPPLEMENTARY INFORMATION

Engineered Knottin Peptides: A New Class of Agents for Imaging Integrin Expression in Living Subjects

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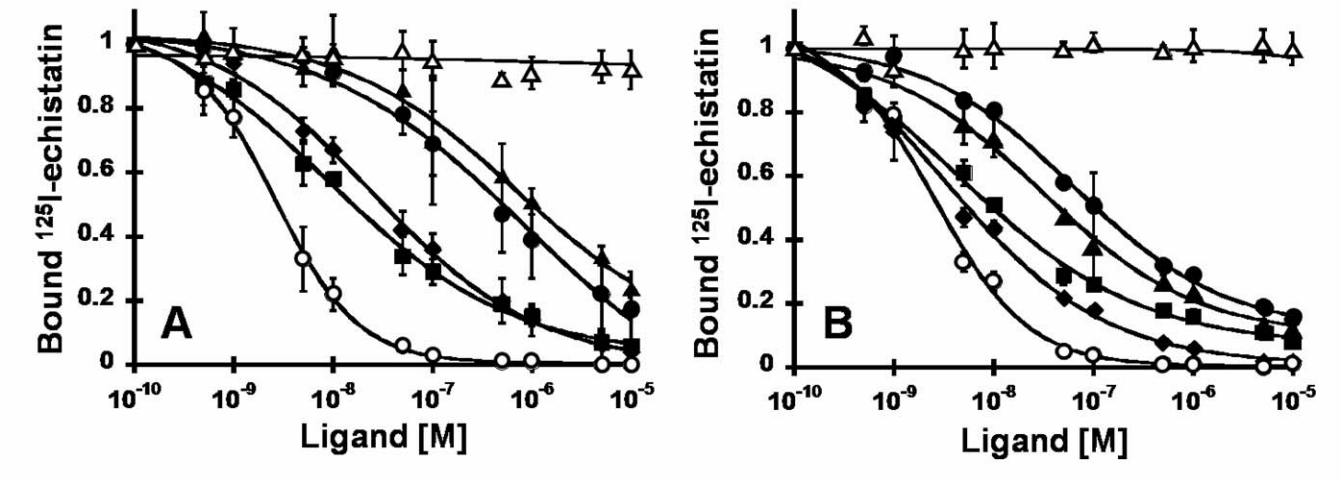
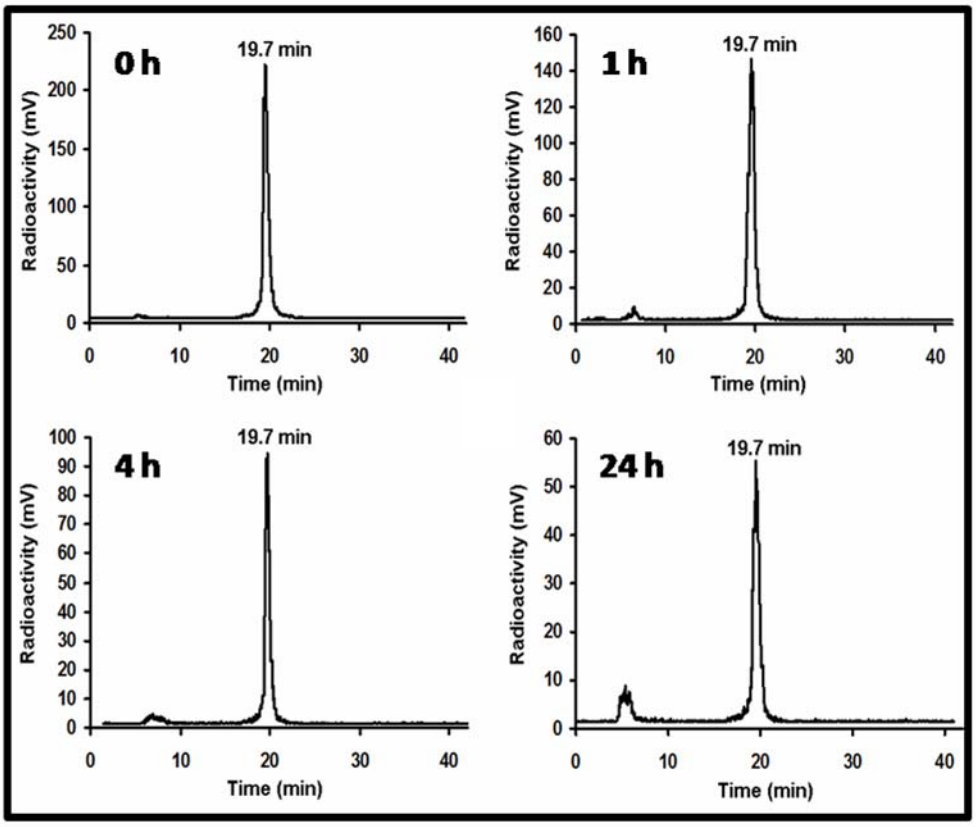
SUPPLEMENTARY METHODS

Peptide synthesis and folding. Knottin peptides were synthesized on a CS Bio CS036 instrument using 9-fluorenylmethylcarbonyl (Fmoc)-based solid phase peptide synthesis. Briefly, rink amide resin (CS Bio Company; loading 0.66 mEq/g) was swollen in *N, N*-dimethylformamide (DMF) for 30 min. Fmoc-protected amino acids were purchased from Novabiochem/EMD Chemicals Inc. Fmoc groups were removed with 20% piperdine in DMF. 1 mmol aliquots of amino acids were activated in a solution containing 1 mmol HOBt and 0.5 M diisopropylcarbodiimide (DIC) in DMF. After synthesis, side-chain deprotection and resin cleavage was achieved by addition of a 94:2.5:2.5:1 (v/v) mixture of trifluoroacetic acid (TFA)/trimethylsilane/ethanedithiol/water for 2 h at room temperature.

The reaction mixtures were precipitated with cold anhydrous ether, and the crude peptides were purified by preparative or semi-preparative reversed-phase HPLC using a Varian Prostar instrument and Vydac C_{18} columns. Linear gradients of 90% acetonitrile in water containing 0.1% (v/v) TFA were used for all peptide purifications, which were monitored at an absorbance of 220 nm. Peptide purity was analyzed by analytical reversed-phase HPLC using a Vydac C_{18} column. Molecular masses were determined by electrospray ionization mass spectrometry (ESI-MS) using a Waters Micromass ZQ and Waters MassLynx v4.0 or Magtran v1.02 (Zhongqi Zhang) analysis software. Large scale folding reactions were performed by incubating peptides with 2.5 mM reduced glutathione and 20% dimethlysulfoxide (v/v) in 0.1 M ammonium bicarbonate, pH 9 with gentle rocking overnight. The final oxidized product was purified by semi-preparative reversed-phase HPLC as described above. Following purification, folded peptides were lyophilized and stored at room temperature until used. Purified peptides were dissolved in water, and concentrations were determined by amino acid analysis (AAA Service Laboratory, Damascus, OR). Peptide purity and molecular mass were confirmed by analytical reversed-phase HPLC and ESI-MS as described above.



Supplementary Figure S1. Competition binding of DOTA- and Cy5.5-labeled peptides to tumor cell surface integrins. Varying concentrations of peptides were incubated with ^{125}I-labeled echistatin and allowed to compete for binding to integrin receptors expressed on the surface of U87MG glioblastoma cells. *A*, DOTA-labeled peptides. *B*, Cy5.5-labeled peptides. The fraction of ^{125}I-echistatin bound to the cell surface is plotted versus the concentration of: c(RGDyK) (●), FN-RGD2 (▲), FN-RDG2 (△), and mutants 2.5D (■) and 2.5F (◆). *A,B* Unlabeled echistatin (○) was used as a positive control to compare binding data from different experiments. Data shown is the average of triplicate values and error bars represent standard deviations. Half-maximal inhibitory concentration (IC_{50}) values obtained from this data are listed in Table 1.



Supplementary Figure S2. Serum stability of ^{64}Cu-DOTA-conjugated knottin 2.5D. To measure serum stability, 50-100 µCi of ^{64}Cu-DOTA-2.5D knottin peptide in 100 µL of PBS, pH 7.4 was added to 1 mL of mouse serum (Sigma Aldrich). After incubation for 1, 4, or 24 h at 37 ºC the solution was filtered through a NanoSep 10K Omega device (Pall Corporation). The filtrate was then analyzed by reversed-phase HPLC under identical conditions used for analyzing the original radiolabeled compound. Radio-HPLC traces of untreated knottin peptide (0 h), or knottin peptide after incubation in mouse serum at 37 ^{o}C for 1, 4, or 24 h.

Supplementary Table S1. Characterization of imaging probes by mass spectrometry. Cy5.5-labeled and DOTA-conjugated peptides were characterized by either: 1) ESI-MS, where we report the neutral mass, or 2) MALDI-TOF-MS where we report the $[M+H]^+$ state (*).

Peptide	Expected Neutral Mass (Da)		Observed Mass (Da)	
	Cy5.5	DOTA	Cy5.5	DOTA
c(RGDyK)	1520.6	1341.5	1519.5	1341.8*
FN-RGD2	4001.3	3490.8	4002.2*	3491.4
2.5D	4139.5	3629.0	4140.3*	3628.6
2.5F	4187.4	3677.1	4188.7*	3678.3*
FN-RDG2	4001.3	3490.8	4002.0	3491.9

Supplementary Table S2. Serum and *in vivo* stability of ^{64}Cu-DOTA-knottin 2.5D. Radio-HPLC was used to analyze the soluble fraction of the tissue homogenate to quantify the intact tracer, which elutes between 19.5 to 20 min, and the primary metabolites, which elute between 4 to 6 min (Fig. 5). The soluble fraction represents the percentage of total activity recovered from a given tissue sample, and was approximately 80-90% for the blood, 70-75% for the tumor, and 40-70% for the kidneys.

	Control	Serum			Blood		Kidney		Tumor	
Time (h)	0	1	4	24	1	4	1	4	1	4
Intact Tracer (%)	97	95	91	82	96	90	88	46	90	79