Electronic Supplementary Material (ESI) for Chemical Communications

Supporting information

New cross-bridged cyclam derivative CB-TE1K1P, an improved bifunctional chelator for copper radionuclides

Dexing Zeng, Ouyang Qin, Zhengxin Cai, Xiang-Qun Xie and Carolyn J. Anderson

Abbreviations. EOB, end of bombardment; TLC, thin-layer chromatography; SEC, size exclusion chromatography; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; TEA, triethylamine; DIEA, N,N-Diisopropylethylamine; TFA, trifluoroacetic acid; EA, ethyl acetate; DCM, dichloromethane; THF, tetrahydrofuran; DMF, Dimethylformamide; DCC, N,N'-Dicyclohexylcarbodiimide; DIC, N,N'-Diisopropylcarbodiimide; EDCI, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, hydroxybenzotriazole; DMAP, 4-Dimethylaminopyridine; NHS, N-Hydroxysuccinimide; EDTA, ethylenediaminetetraacetic acid; DBCO, dibenzo-cyclooctyne, a metal-free click moiety; DPBS, Dulbecco's phosphate-buffered saline; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl.

Reagents. All solvents and reagents were purchased from commercial sources and used without additional purifications, unless otherwise noted. The monoclonal antibody cetuximab (Erbitux, 2.0 mg/mL) was from ImClone Systems Incorporated (New York, NY). Cyclam was purchased from CheMatech (Dijon, France). LC-MS grade acetonitrile and water were obtained from Fisher Scientific (Waltham, MA, USA). Wang resin and

Fmoc protected amino acids were from Chem-Impex International (Wood Dale, IL). Chelex@100 resins were obtained from Bio-Rad (Hercules, CA). Ultrapure Water was produced using a Thermo Scientific Barnstead Nanopure® Ultrapure Water System. Copper-64 chloride (0.1 M HCl) was obtained from Washington University (St. Louis, MO) and University of Wisconsin (Madison, WI). All other chemicals and reagents were from Sigma Aldrich (St. Louis, MO). MultiScreen 96-well microtiter plates for receptor binding assays were counted on a 1450 Microbeta Trilux Liquid Scintillation and Luminescence counter (PerkinElmer Life Sciences).

Instrumentation. A Bio-Scan AR-2000 radio-TLC imaging scanner was used to determine radiochemical purity. A Perkin-Elmer (Packard) Cobra II Gamma counter (Waltham, MA) was used to measure gamma radiation. ^{1}H NMR spectra were recorded on a Bruker DRX 400 MHz spectrometer (Billerica, MA), and ESI-MS were measured on a Waters LCT-Premier XE LC-MS station (Milford, MA). Corning Spin-X® concentrators and Zeba spin desalting columns were from Thermo-Scientific (Waltham, MA). Luna C-18 HPLC columns and Superose™ 12 SEC columns were from Phenomenex (Torrance, CA, USA) and GE (Piscataway, NJ, USA), respectively. A Waters 1525 binary pump chromatography system was used for purification of some synthesized compounds using two elution buffers (0.1 v% TFA in de-ionized water as elution buffer A and 0.1 v% TFA in acetonitrile as elution buffer B). The Waters 1525 binary pump chromatography system was assembled with a SEC Superose FPLC column and used for analysis of the ^{64}Cu labeled cetuximab conjugates using DPBS as the elution buffer. Metal decontamination was done as previously described.[1]

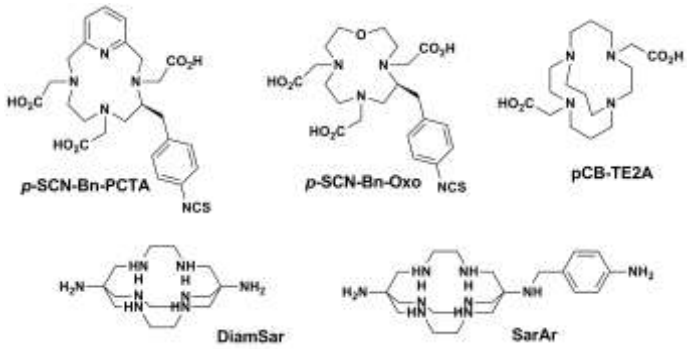
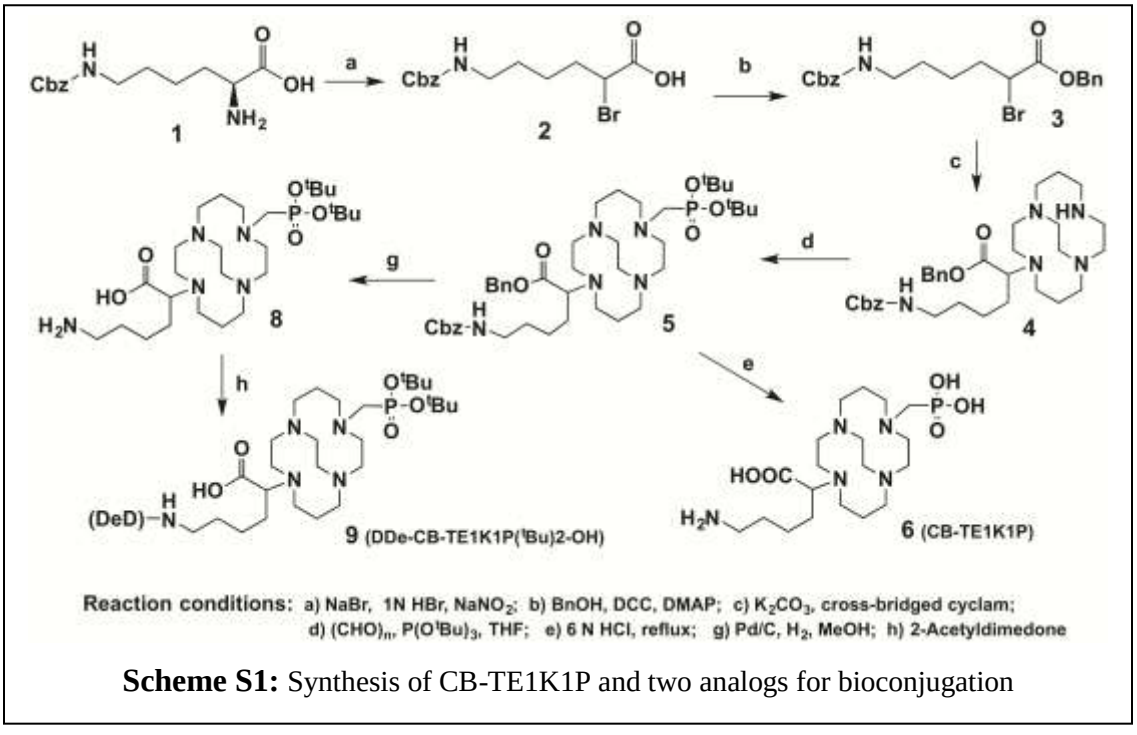


Figure S1: Additional chelators for copper radionuclides

General radio-TLC procedures: ~ 2.0 μCi of ^{64}Cu-labeled conjugate was mixed with 20 μL of 50 mM EDTA buffer (pH = 5.50), and the resulting mixture was vortexed for 10 seconds and incubated for 30 seconds at 37 °C. ~ 2 μL of the mixture was spot onto the TLC plate that was then developed in the mobile phase (0.1 M NH_4OAc : MeOH = 1 : 1). Free ^{64}Cu chelated with EDTA and moved to the solvent front, whereas the ^{64}Cu labeled conjugate remained at the origin.

Synthesis of CB-TE1K1P chelator and its derivatives:



Scheme S1: Synthesis of CB-TE1K1P and two analogs for bioconjugation

All reactions were conducted under N_2, unless otherwise described.

Compound 2. Compound **2** was synthesized using as previously described.[2] After silica gel chromatography purification, 1.49 g compound **2** was obtained in 60.5% yield as a light yellow oil. ESI-MS: observed, *M/Z* $(M+H)^+$ = 343.99, calculated, $(M+H)^+$ = 344.05.

Compound 3. DCC (825 mg, 4.0 mmol) and DMAP (49 mg, 0.4 mmol) were dissolved in 20 mL DCM, and then compound **2** (1.32 g, 3.8 mmol) and benzyl alcohol (450 mg, 4.16 mmol) were added. After the reaction mixture was stirred overnight in the dark, the insoluble solid was filtered out and the filtrate was concentrated. The residue was purified by silica gel chromatography with EA/hexane (5:1) to obtain compound **3** (1.41 g, 85.5%) as a light yellow oil **^{1}H NMR** (400 MHz, $CDCl_3$): δ 1.39-1.35 (m, 2H), 1.51-1.54 (m, 2H), 2.02-2.08 (m, 2H), 3.19 (t, 2H, *J* = 8.0 Hz), 4.26 (t, 1H, *J* = 8.0 Hz), 4.77 (s, br, 1H), 5.11 (s, 2H), 5.22 (s, 2H), 7.37-7.39 (m, 10H); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 24.4, 29.2, 34.4, 40.7, 45.5, 66.8, 67.6, 128.1, 128.2, 128.6, 128.7, 135.1, 155.6, 169.5; **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 434.04, calculated, $(M+H)^+$ = 434.09.

Compound 4. Cross-bridged cyclam[3] (500 mg, 2.2 mmol) and sodium carbonate (235 mg, 2.2 mmol) were suspended in dry CH_3CN (20 mL), and then compound **3** (955 mg, 2.2 mmol) was added in one portion by syringe. The solution was stirred for 2 d at room temperature in the dark, followed by removal of the solvent. The residue was

purified by silica gel chromatography with DCM/MeOH (10:1.5) to obtain compound **4** (688 mg, 53.7%) as a light oil. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 1.20-1.89 (m, 11H), 2.26-3.48 (m, 23H), 4.67 (s, 1H), 5.11 (s, 2H), 5.22 (s, 2H), 7.33-7.35 (m, 10H); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 24.0, 29.5, 33.9, 40.7, 45.0, 45.1, 49.2, 50.3, 51.6, 52.0, 53.0, 53.3, 55.4, 56.8, 63.6, 64.3, 66.5, 128.4, 128.5, 128.6, 135.8, 136.7, 156.5, 173.1; **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 580.45, calculated, $(M+H)^+$ = 580.39.

Compound 5. Tri(tert-butyl)phosphite (500 mg, 2.0 mmol) and paraformaldehyde (65 mg, 2.2 mmol) were added to a solution of compound **4** (580 mg, 1.0 mmol) in dry THF (50 mL) and stirred for 3 d. The solvent was removed, the residue was re-dissolved in H_2O (50 mL), and its pH was adjusted to 10.0 by slow addition of K_2CO_3. The resulting solution was extracted with toluene (3 x 50 mL), the combined organic phases were dried over anhydrous Na_2SO_4. After the solvent was removed, the residue was purified by aluminum oxide chromatography with DCM/MeOH (20:1) to obtain compound **5** (438 mg, 55.7%) as a light yellow oil. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 1.30-1.91 (m, 29H), 2.56-3.59 (m, 24H), 5.02 (s, 1H), 5.11 (s, 2H), 5.21 (m, 2H), 7.32-7.39 (m, 10H); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 24.0, 29.8, 29.9, 30.5, 30.6, 40.5, 50.0, 51.4, 51.8, 52.7, 53.4, 53.5, 56.1, 56.5, 63.1, 66.5, 66.8, 81.9, 82.0, 127.9, 128.0, 128.1, 128.8, 128.9, 129.0, 135.3, 156.6, 172.2; **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 786.56, calculated, $(M+H)^+$ = 786.49.

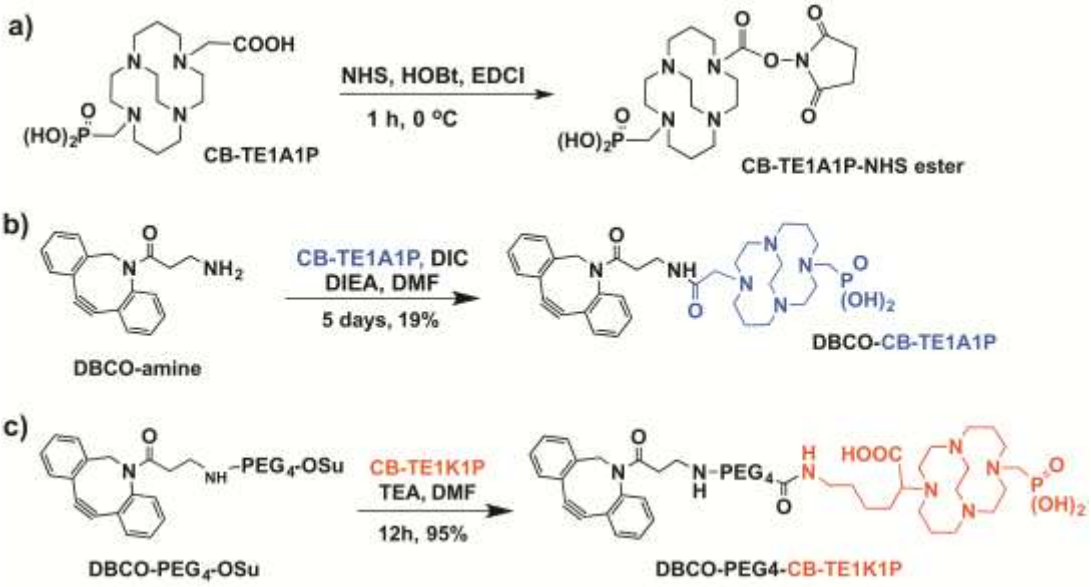
Compound 6. **5** (200 mg, 0.25 mmol) was dissolved in 25 mL of 6 M HCl and refluxed overnight, and the solvent was removed under reduced pressure. The residue

was re-dissolved in 0.5 mL H_2O, and then its pH was adjusted to 3.0 by slow addition of 1.0 M NH_4OH. The resulting solution was purified by preparative HPLC, and **6** (52 mg, 46%) was obtained as colorless solid. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 1.50-1.53 (m, 2H), 1.65-1.88 (m, 8H), 2.25-2.38 (m, 2H), 2.46 (d, 1H, *J* = 12.4), 2.71-3.77 (m, 21H), 3.92-3.96 (m, 1H), 4.27 (d, 1H, *J* = 9.6); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 18.7, 19.36, 22.24, 23.1, 24.5, 26.6, 39.1, 45.2, 47.1, 47.6, 50.1, 51.3, 52.6, 53.1, 53.5, 57.9, 58.1, 62.3, 173.9; **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 450.21, calculated, $(M+H)^+$ = 450.28.

Compound 9. **5** (200 mg, 0.25 mmol) was dissolved in MeOH (10 mL). Pd/C (20 mg) was added to the solution to afford a slurry, and the resulting mixture was stirred overnight under H_2 (balloon, 1 atm). The Pd/C was filtered off and the filtrate was concentrated to yield compound **8** as a colorless oil, which was used without further purification in the subsequent reaction step. **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 562.28, calculated, $(M+H)^+$ = 562.41. Crude compound **8** was dissolved in 10 mL of THF, and then 2-acetyldimedone (137 mg, 0.30 mmol) and TEA (76 mg, 0.75 mmol) was added. After the reaction mixture was stirred overnight, the solvent was removed under reduced pressure. The residue was purified using preparative HPLC, and compound **9** (96 mg, 50.2%) was obtained as a white solid. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 1.00 (s, 6H), 1.49 (s, 18H), 1.52-1.91 (m, 10H), 2.33 (S, 4H), 2.52 (s, 3H), 2.67-3.39 (m, 24H), 4.25 (s, br, 1H), 10.62 (s, br, 1H), 13.38 (s, 1H); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 18.0, 24.0, 24.7, 25.3, 28.35, 29.6, 30.2, 30.3, 30.6, 30.7, 43.6, 50.4, 50.8, 52.4, 53.0, 53.6, 54.4, 54.7, 54.8, 55.3, 56.0, 56.1, 56.7, 59.6, 59.8, 69.7, 83.1,

83.0, 82.9, 82.8, 107.9, 173,5, 175.2, 197.7; **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 726.43, calculated, $(M+H)^+$ = 726.49.

Chelator activation and functionalization:



Scheme S2: Chelator activation and/or functionalization

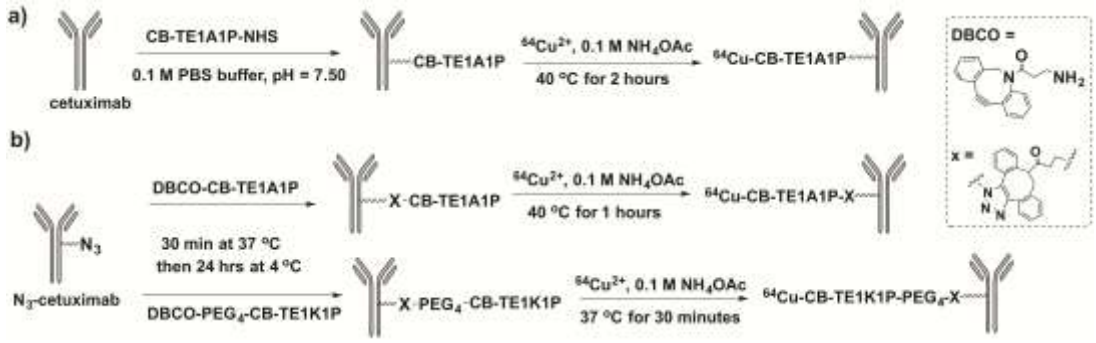
Synthesis of CB-TE1A1P-NHS ester: To the mixture of CB-TE1A1P (9.6 mg, 25 µmol), NHS (2.5 mg, 22 µmol) and HOBt (3.0 mg, 22 µmol) in 0.5 mL anhydrous DMF, EDCI (3.9 mg, 20 µmol) was added. After it was stirred 1 h at 0 °C, the solvent was removed under reduced pressure to yield the title compound as a colorless solid that was used without further purification for conjugation to cetuximab.

Synthesis of DBCO-CB-TE1A1P: DBCO-amine (24 mg, 87 µmol) and CB-TE1A1P (33 mg, 87 µmol) were dissolved in 1.0 mL of anhydous DMF, and then DIC (11 mg,

87 µmol) and DIEA (23 mg, 178 µmol) were added. After the resulting mixture was stirred for 5 d at room temperature, the reaction yield was 19% based on HPLC analysis. The solvent was removed, and the residue was purified using preparative HPLC. Pure DBCO-TE1A1P was then obtained as a colorless oil. **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 637.15, calculated, $(M+H)^+$ = 637.23.

Compound 7 (DBCO-PEG_4-CB-TE1K1P**):** TEA (20 mg, 200 µmol) and CB-TE1K1P (compound **6**, 20 mg, 45 µmol) were dissolved in 1.0 mL anhydous DMF, and then 10 mg of DBCO-PEG_4-OSu was added. After the resulting mixture was stirred overnight, over 95% yield was obtained based on HPLC analysis. The solvent was removed, and the residue was purified using preparative HPLC. Pure compound **7** was then obtained as a colorless oil. **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 1028.47, calculated, $(M+H)^+$ = 1028.55.

Preparation of chelator-cetuximab conjugates and radiolabelling with ^{64}Cu



Scheme S3: Preparation of the chelator-cetuximab conjugates and radiolabelling with ^{64}Cu

Conjugation of CB-TE1A1P to Cetuximab and radiolabelling with ^{64}Cu

Cetuximab (2.9 mL, 40 nmol) and Na_2HPO_4 buffer (0.1 M, pH = 8.2, 2.1 mL) was added to the above NHS/EDCI activated CB-TE1A1P (20 µmol, calculated on the basis of EDCI), and the resulting mixture was incubated for 2 d at 4 °C with gentle rotation. The resulting non-click cetuximab-CB-TE1A1P conjugate was purified by Corning Spin-X® concentrator and a Zeba desalting column to a concentration of 2.0 mg/mL as determined by FPLC using a GE Superose™ 12 column with isocratic elution (0.08 mL/min flow rate; 1 X DPBS).

The ^{64}Cu-labelling of non-click CB-TE1A1P-cetuximab was performed in NH_4OAc buffer as follows: ~70 µL of the non-click CB-TE1A1P-cetuximab (2.0 mg/mL) solution was added to 250 µL of 0.1 M NH_4OAc (pH = 8.2) in a 1.5 mL tube, and then 500 µCi (EOB) of $^{64}Cu^{2+}$ in 50 µL of 0.1 M NH_4OAc solution (pH = 8.0) was added. The mixture was vortexed for 10 sec, and incubated in a thermomixer at 40 °C for 2 h. The radiolabelling yield was determined by FPLC.

Conjugation of CB-TE1A1P and CB-TE1K1P to N_3-cetuximab via SPAAC and radiolabelling with ^{64}Cu

3-azidopropionic acid succinimidyl ester was prepared from 3-bromopropanoic acid as previously described.[4] The 3-azidopropionic acid succinimidyl ester (4.5 mg, 20.0 µmol) was added to the mixture of the cetuximab solution (14.5 mL, 0.2 µmol) and Na_2HPO_4 buffer (0.1 M, pH = 8.2, 5.4 mL), and then the resulting mixture was incubated overnight at 4 °C with gentle rotation. The resulting azide-cetuximab conjugate was purified by Corning Spin-X® concentrator and a Zeba desalting column column to a concentration of 2.0 mg/mL as previously described.

The above purified azide-cetuximab was mixed with 100-fold CB-TE1K1P-PEG_4-DBCO (or CB-TE1A1P-DBCO), and the resulting mixture was incubated for 30 min at 37 °C, followed by incubation for 2 d at 4 °C with gentle rotation. The CB-TE1K1P-PEG_4-click-cetuximab (or CB-TE1A1P-click-cetuximab) conjugate was then purified by Corning Spin-X® concentrator and a Zeba desalting column to a concentration of 2.0 mg/mL as previously described.

The two chelator-click-cetuximab conjugates were radiolabelled with Cu-64 using the following protocol: ~ 30 μL of the CB-TE1A1P-click-cetuximab (or ~ 10 μL of CB-TE1K1P-PEG_4-click-cetuximab) solution (2.0 mg/mL) was added to 250 μL of 0.1 M NH_4OAc (pH = 8.2) in a 1.5 mL tube, and then 500 μCi of $^{64}Cu^{2+}$ in 50 μL of 0.1 M NH_4OAc solution (pH = 8.0) was added. The mixture was vortexed for 10 sec, and incubated in a thermomixer at 40 °C for 2 h (or at 37 C for 30 min for the CB-TE1K1P clicked cetuximab conjugate). The radiolabelling yield was determined by FPLC.

Determination of chelator to antibody ratio:

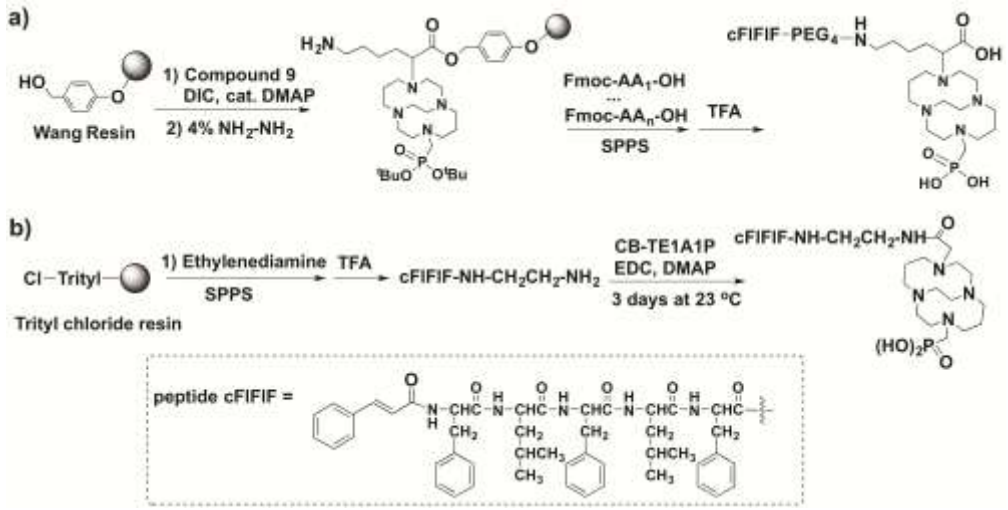
A carrier-added copper solution (10 μM), prepared by mixing 100 μL $^{64}Cu^{2+}$ solution (1.0 μCi/μL) with 10 μL of 1.0 mM non-radioactive copper solution and 890 μL of 0.1 M NH_4OAc (pH=8.2), was used to determine the number of CB-TE1A1P (or CB-TE1K1P) moieties on each cetuximab protein by titration experiments using a published method.[5]

Serum stability measurement: ^{64}Cu-labeled cetuximab-chelator conjugate (~100 μCi) was mixed with 0.2 mL fetal bovine serum, and the resulting mixture was incubated at 37 °C for 1, 2 and 3 days. After incubation, the radiochemical purity of the ^{64}Cu labeled cetuximab-chelator conjugate was determined by radio-FPLC.

***In vitro* Binding Affinity**

The HCT116 cell line was provided by Dr. Bert Vogelstein (Johns Hopkins University). HCT116 cells were cultured in DMEM medium supplemented with 10% FBS and 0.1% gentamicin in a 37ºC humidified 95% air, 5% CO2 incubator. The affinity of ^{64}Cu-labeled cetuximab conjugates for EGFR was determined by a saturation binding assay based on previously published methods.[6] HCT116 cell membrane preparations were diluted in binding buffer [0.1% bovine serum albumin, 50 mM Tris-HCl (pH 7.4), 5.0 mmol/L $MgCl_2$, 0.5 μg/mL aprotinin, 200 μg/mL bacitracin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin A] and 15 μg of membrane was added to each well of a 96-well filtration plate (Multiscreen Durapore; Millipore; Billerica, MA). Membranes were incubated with increasing concentrations of ^{64}Cu-CB-TE1K1P-click-cetuximab for 2 h at room temperature. Non-specific binding was determined by saturating receptors with excess cetuximab. When reaching equilibrium, unbound radioactivity was filtered out and the membranes were washed twice with 200 μL binding buffer. Bound radioactivity was measured with a liquid scintillation and luminescence plate reader (1450 Microbeta; Perkin Elmer; Waltham, MA). Total binding sites (B_{max}) and binding affinity (K_d) were determined by a non-linear regression fit of bound peptides per mg of protein versus concentration of radio-ligand using GraphPad Prism v 5.0 (San Diego, CA).

Preparation of cFlFlF-chelator conjugates and radiolabelling with ^{64}Cu



Scheme S4: Preparation of the cFlFlF-chelator conjugates and radiolabelling with ^{64}Cu

cFlFlF-PEG$_4$-CB-TE1K1P (**Scheme S4a)**: p-Benzyloxybenzyl alcohol (Wang) resin (20 μmol) was swelled in DMF (1.0 mL), and then Dde-CB-TE1K1P(^{t}Bu$_2$)-OH (40 μmol) was coupled to the resin by mixing with HATU (40 μmol) and DIEA (100 μmol) in DMF (1.0 mL) for 2 h at room temperature, followed by thorough washing with DMF. The resulting resin was subsequently capped by incubating with acetic anhydride (100 μmol) and DIEA (300 μmol) in DMF (1.0 mL) for 30 min. The Dde protection group was removed by mixing with 4% hydrazine in DMF (3 x 1.0 mL). The title peptide conjugate was prepared by coupling the above resin containing a primary amine with the amino acids and special residues in the following order: Fmoc-PEG$_4$-OH, Fmoc-Phe, Fmoc-(D)Leu, Fmoc-Phe, Fmoc-(D)Leu, Fmoc-Phe, and Trans-cinnamic acid, using standard Fmoc-SPPS. The resulting cFLFLF-PEG$_4$-CB-TE1K1P conjugate was cleaved from the

resin support using a 95% TFA, and purified by HPLC. **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 1494. 68, calculated, $(M+H)^+$ = 1494.84

cFlFlF-PEG$_4$-NH-CH$_2$CH$_2$-NH-CB-TE1A1P (Scheme S4b): 4-Methyltrityl chloride resin (20 μmol) was swelled in 2.0 mL DMF, and incubated with ethylenediamine (100 μmol) and TEA (200 μmol) in 1.0 mL DMF for 2 h, followed by thorough washing with DMF. The title peptide was prepared by coupling the above resin containing a primary amine with the amino acids and special residues in the following order: Fmoc-PEG$_4$-OH, Fmoc-Phe, Fmoc-(D)Leu, Fmoc-Phe, Fmoc-(D)Leu, Fmoc-Phe, and Trans-cinnamic acid, using standard Fmoc-SPPS. The resulting cFlFlF-PEG$_4$-NH-CH$_2$CH$_2$-NH$_2$ peptide was cleaved from the resin support using 95% TFA, and purified by HPLC. **ESI-MS:** observed, *M/Z* $(M+H)^+$ = 1105.60, calculated, $(M+H)^+$ = 1105.63. The CB-TE1A1P was then conjugated to the purified cFlFlF-PEG$_4$-NH-CH$_2$CH$_2$-NH$_2$ using the same method used for DBCO-CB-TE1A1P preparation, and the resulting cFlFlF-PEG$_4$-NH-CH$_2$CH$_2$-NH-CB-TE1A1P conjugate was purified by HPLC. **ESI-MS**: observed, *M/Z* $(M+H)^+$ = 1465.69, calculated, $(M+H)^+$ = 1465.82

Conventional radiolabelling of the cFlFlF-chelator conjugates with $^{64}Cu^{2+}$

5 μL of 100 μM the cFlFlF-chelator solution was firstly added to 145 μL of 0.1 M NH_4OAc (pH = 8.2) in a 1.5 mL tube, and 500 μCi of $^{64}Cu^{2+}$ in 50 μL of 0.1 M NH_4OAc solution (pH = 8.0) was added. The mixture was vortexed for 10 sec, and incubated in a thermomixer at 22 or 37 °C for 5, 10, 20, 30 or 60 min. The radiolabelling yield was determined by radio-TLC (Figure **S2a)**.

Serum stability measurement: ^{64}Cu-labeled cFLFLF-chelator conjugate (~50 μCi) was mixed with 0.2 mL fetal bovine serum, and the resulting mixture was incubated at 37 °C for 1 and 2 d. After incubation, the radiochemical purity of the ^{64}Cu labeled cFLFLF-chelator conjugate was determined by radio-TLC (Figure **S2b)**.

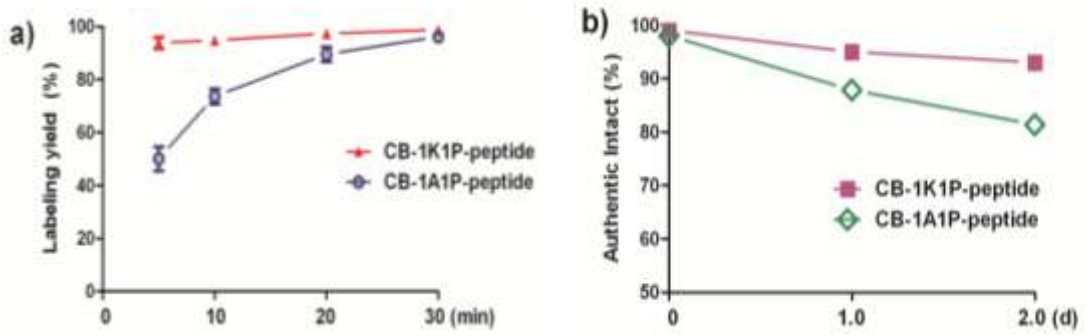


Figure S2: a) ^{64}Cu-labeling of two cFlFlF-chelate conjugates; b) serum stability of these two ^{64}Cu-labelled conjugates

Log P measurement: ^{64}Cu-labeled cFLFLF-chelator conjugate (~5 μCi) was added to a 1.5 mL tube containing 500 μL of water and 500 μL of octanol. The resulting mixture was shaken vigorously for 20 min at room temperature, and then centrifuged at 5000 rpm for 10 min. Triplicate aliquots (200 μL) of octanol and aqueous layers were carefully separated and radioactivity of samples was counted on a Cobra II gamma counter. Log P was measured as log (radioactivity in octanol / radioactivity in water).

Density Functional Theory (DFT) Calculations: All calculations were conducted using density functional theory (DFT) as implemented in the Gaussian03 suite of ab initio quantum chemistry programs.[7] Geometry optimizations and vibrational frequency calculations were performed by using the unrestricted B3LYP exchange and correlation

functionals and the double-ζ 6-31+G(d,p) basis set for all atoms.[8-11] Normal self consistent field (SCF) and geometry convergence criteria were employed and the geometry of complex Cu-CB-TE1K1P and Cu-CB-TE1A1P was optimized in the gas phase without the use of symmetry constraints. The effect of stereochemistry was not investigated in the calculation and the program selected the stereoisomers of both Cu complexes randomly.

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