Supporting Information

Thiol-reactive bifunctional chelators for the creation of site-selectively modified radioimmunoconjugates with improved stability

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I. General information

1. *Reagents*

All chemicals, unless otherwise noted, were acquired from Sigma-Aldrich and Fisher Scientific and were used as received without further purification. All water used was ultra-pure (>18.2 MΩcm-1), and dimethylsulfoxide was of molecular biology grade (>99.9%). All the bifunctional chelators were purchased from Macrocyclics. ^{89}Zr was produced at Memorial Sloan-Kettering Cancer Center on a TR19/9 cyclotron (Ebco Industries, Inc.) via the ^{89}Y(*p*,*n*)^{89}Zr reaction and purified to yield ^{89}Zr with a specific activity of 196-496 MBq/μg. ^{177}Lu was obtained from Perkin-Elmer.

2. Instrumentation

All instruments were calibrated and maintained in accordance with standard quality-control procedures. UV-Vis measurements were taken on a Shimadzu BioSpec-Nano spectrophotometer. Activity measurements were made using a CRC-15R Dose Calibrator (Capintec). For the quantification of activity, experimental samples were counted for 1 min on an Automatic Wizard γ–counter (Perkin-Elmer). Radiolabeling experiments with ^{89}Zr and ^{177}Lu were monitored using silica-impregnated instant thin-layer chromatography (iTLC) paper (Pall Corp.) eluted with an aqueous solution of EDTA 50 mM at pH 5.0 and analyzed on an AR-2000 iTLC plate reader (Bioscan Inc.). PET images were recorded on a MicroPET Focus 120 (Concorde MicroSystem, Inc.). Electrospray ionization mass spectrometry (ESI-MS) spectra were recorded using a Waters Acquity UPLC (Milford, CA) with an electrospray ionization SQ detector. High-resolution mass spectrometry (HRMS) spectra were recorded with a Waters LCT Premier system (ESI).

3. HPLC

HPLC purifications were performed using a Shimadzu HPLC equipped with a C_{18} reversed-phase column (XTerra® Preparative MS OBDTM; 10 μm, 19×250 mm), a SPD-M20A photodiode array detector, two LC-20AP pumps, a CBM-20A communication BUS module, and a FRC-10A fraction collector, and using a flow rate of 6 mL/min and a gradient of MeCN:H_2O (both with 0.1% TFA). The quality control analysis of purified compounds was performed using a C_{18} reversed-phase column (Phenomenex Jupiter analytical; 5 μm, 4.6×250 mm) with a flow-rate of 1 mL/min and a gradient of MeCN:H_2O (both with 0.1% TFA).

4. Cell culture, animal protocols, and xenograft procedures

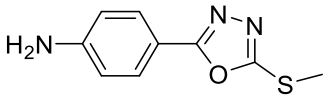
Human colorectal cancer cell line SW1222 was obtained from the Ludwig Institute of Cancer Research and maintained in Iscove's Modified Dulbecco's Medium, supplemented with 10% heat-inactivated fetal bovine serum, 2.0 mM glutamine, 100 units/mL penicillin, and 100

units/mL streptomycin in a 37°C environment containing 5% CO_2. Cell lines were harvested and passaged weekly using a formulation of 0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium.

All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committees of Weill Cornell Medical Center, Hunter College, and Memorial Sloan Kettering Cancer Center, and the experiments followed institutional guidelines for the proper and humane use of animals in research. Six to eight week-old athymic nude female (Hsd: Athymic Nude-nu) mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in ventilated cages, given food and water *ad libitum*, and allowed to acclimatize for approximately 1 week prior to inoculation. SW1222 tumors were induced on the left shoulder by a subcutaneous injection of 5×10^6 cells in suspension in 150 µL of a 1:1 mixture of fresh media:BD Matrigel (BD Biosciences, Bedford, MA). The xenografts reached ideal size for imaging and biodistribution (~100-150 mm^3) in approximately 12-14 days.

II. Synthesis

4-(5-(Methylthio)-1,3,4-oxadiazol-2-yl)aniline **(1)**



In a glass vessel protected from light with aluminium foil, 5-(4-aminophenyl)-1,3,4-oxadiazole-2-thiol (100 mg; 0.517 mmol; 1 eq.) was dissolved in 3 mL of methanol, and diisopropylethylamine (DIPEA; 360 µL; 2.07 mmol; 4 eq.) was added to the solution. The mixture was stirred at room temperature for 10 minutes before slowly adding iodomethane (32 µL; 0.517 mmol; 1 eq.). After 45 minutes, the solvent was removed under reduced pressure. The white solid was dissolved in 3 mL of ethyl acetate and washed with a 0.1 M solution of sodium carbonate and then washed with water until reaching pH 7. The organic phase was dried over $MgSO_4$ before the evaporation of the volatiles under reduced pressure, ultimately affording white needles (107 mg; yield: 100%). Because of its slight light-sensitiveness, this compound was kept in foil-covered glass vials.

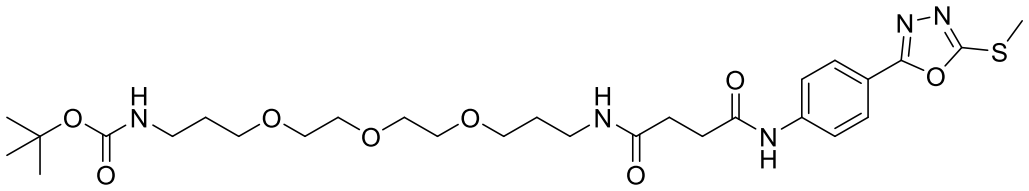
TLC (Ethyl acetate:triethylamine, 9:1): Rf 0.65

^{1}H NMR (500 MHz, $CDCl_3$) 7.79 (2H, d, J = 8.5 Hz), 6.72 (2H, d, J = 8.5 Hz), 4.04 (2H, br s), 2.75 (3H, s)

^{13}C NMR (125 MHz, $CDCl_3$)166.3, 163.7, 149.7, 128.5, 114.8, 113.5, 14.8

HRMS-ESI *m/z* Calcd for $[C_9H_9N_3OS+H]^+$: 208.0539; found: 208.0539; Δ: -0.80 ppm.

***tert-Butyl (18-((4-(5-(methylthio)-1,3,4-oxadiazol-2-yl)phenyl)amino)-15,18-dioxo-4,7,10-trioxa-14-azaoctadecyl)carbamate* (2)**



To a solution of *N*-Boc-*N'*-succinyl-4,7,10-trioxa-1,13-tridecanediamine (386.5 mg; 0.919 mmol; 1 eq) in dichloromethane (3 mL) in a glass vessel protected from light with aluminium foil was added diisopropylethylamine (480 µL; 3 eq) and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI; MW = 191.7 g/mol; 1.379 mmol; 1.5 eq.). To this solution was added 200 mg of **1** (0.965 mmol; 1.05 eq). The reaction mixture was stirred at room temperature for 5 days. The mixture was then washed three times with 5 mL of an aqueous solution of 1 M hydrochloric acid. The organic phase was washed twice with 5 mL of an aqueous solution of 1 M Na_2CO_3 and then with water until pH neutral. The organic phase was dried on $MgSO_4$ and evaporated. The off-white solid residue was dissolved in 10 mL of ethyl acetate, and the target compound was precipitated by slow addition of 30 mL of cyclohexane. After filtration, the final product was obtained as a white powder (310 mg; yield: 55%). Because of its slight light-sensitiveness, this compound was kept in foil-covered glass vials.

This yield can certainly be improved by the addition of a recrystallization step after complete precipitation of the final product in pure cyclohexane, but our aim was to keep the experimental procedure as simple as possible.

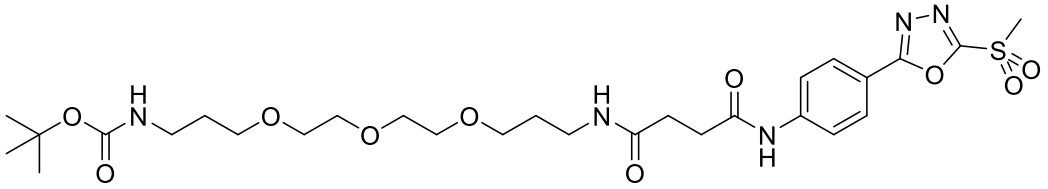
TLC (acetonitrile:water, 3:1): Rf 0.73

^{1}H NMR (500 MHz, $CDCl_3$) 9.68 (1H, s), 7.91 (2H, d, *J*= 9.0 Hz), 7.71 (2H, d, *J*= 8.5 Hz), 6.82 (1H, s), 4.99 (1H, s), 3.70-3.45 (12H, m), 3.41 (2H, q, *J*= 6.0 Hz), 3.20 (2H, q, *J*= 6.5 Hz), 2.76 (3H, s), 2.71 (2H, m), 2.63 (2H, m), 1.80-1.70 (4H, m), 1.42 (9H, s).

^{13}C NMR (125 MHz, $CDCl_3$) 172.6, 171.3, 165.8, 164.6, 156.2, 141.8, 127.7, 119.6, 118.6, 79.2, 70.6, 70.5, 70.3, 70.1, 69.6, 38.8, 38.5, 33.5, 31.6, 29.9, 28.6, 14.8.

HRMS-ESI *m/z* Calcd for $[C_{28}H_{43}N_5O_8S+Na]^+$: 632.2725; found: 632.2722; Δ: 0.35 ppm.

***tert-Butyl (18-((4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenyl)amino)-15,18-dioxo-4,7,10-trioxa-14-azaoctadecyl)carbamate* (3)**



In a glass vessel protected from light with aluminium foil, 30 mg of **2** (0.049 mmol, 1 eq.) was dissolved in 4 mL of dichloromethane before the slow addition of 48.5 mg of *m*-chloroperbenzoic acid (70%, 0.197 mmol, 4 eq.). The reaction mixture was stirred at room temperature overnight. The resulting yellow mixture was washed three times with 8 mL of an aqueous 0.1 M solution of NaOH and then with water until the pH of the solution reached 7. The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure to give a pale solid (28.5 mg, yield: 90%).

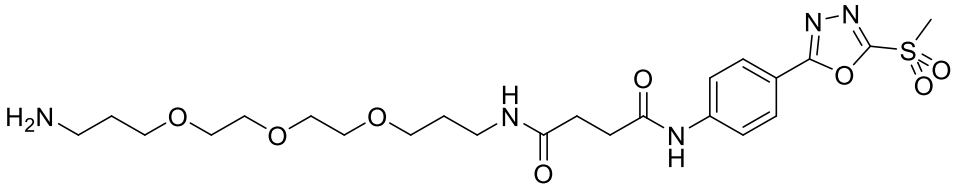
TLC (acetonitrile): Rf 0.51

^{1}H NMR (500 MHz, $CDCl_3$) 9.99 (1H, s), 7.98 (2H, d, *J*= 9.0 Hz), 7.75 (2H, d, *J*= 8.5 Hz), 6.88 (1H, s), 4.99 (1H, s), 3.66-3.50 (15H, m), 3.41 (2H, q, *J*= 6.0 Hz), 3.20 (2H, q, *J*= 6.5 Hz), 2.71 (2H, m), 2.65 (2H, m), 1.80-1.70 (4H, m), 1.43 (9H, s).

^{13}C NMR (125 MHz, $CDCl_3$) 172.6, 171.5, 166.5, 161.6, 156.1, 143.4, 128.7, 119.6, 116.4, 79.1, 70.5, 70.4, 70.2, 70.0, 69.4, 43.0, 38.8, 38.4, 33.2, 31.3, 29.7, 28.4.

HRMS-ESI *m/z* Calcd for $[C_{28}H_{43}N_5O_{10}S+H]^+$: 642.2803; found: 642.2797; Δ: 1.06 ppm.

***N*^{1}*-(3-(2-(2-(3-Aminopropoxy)ethoxy)ethoxy)propyl)-*N*^{4}*-(4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenyl)succinamide* (PODS)**



To a solution of **3** in dichloromethane (30.0 mg, 46.8 µmol; in 1.6 mL) was added trifluoroacetic acid (400 µL). The reaction mixture was stirred at room temperature for 3 hours, and the volatiles were then removed by evaporation under reduced pressure. The oily residue was dissolved in 7 mL of water and 4 mL of ethyl acetate. The aqueous phase was then washed twice with 4 mL of ethyl acetate. The aqueous layer was lyophilized to afford the final product as a white powder (25.0 mg, yield: 98%).

TLC (acetonitrile:water, 3:1): Rf 0.38

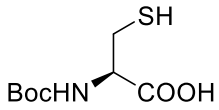
^{1}H NMR (500 MHz, D_2O) 7.85 (2H, d, *J* = 9.0 Hz), 7.55 (2H, d, *J* = 8.5 Hz), 3.60-3.45 (15H, m), 3.45 (2H, t, *J*= 6.5 Hz), 3.20 (2H, t, *J*= 6.5 Hz), 3.04 (2H, t, *J*= 7.0 Hz),

2.67 (2H, t, *J*= 6.5 Hz), 2.54 (2H, t, *J*= 6.5 Hz), 1.87 (2H, qt, *J*= 6.5 Hz), 1.70 (2H, qt, *J*= 6.5 Hz).

^{13}C NMR (125 MHz, D_2O) 174.5, 173.2, 166.8, 161.4, 142.2, 128.6, 120.3, 116.6, 69.4, 69.4, 69.3, 69.2, 68.2, 68.2, 42.5, 37.6, 36.2, 31.9, 30.7, 28.2, 26.4.

HRMS-ESI *m/z* Calcd for $[C_{23}H_{35}N_5O_8S+H]^+$: 542.2279; found: 542.2281; Δ: -0.36 ppm.

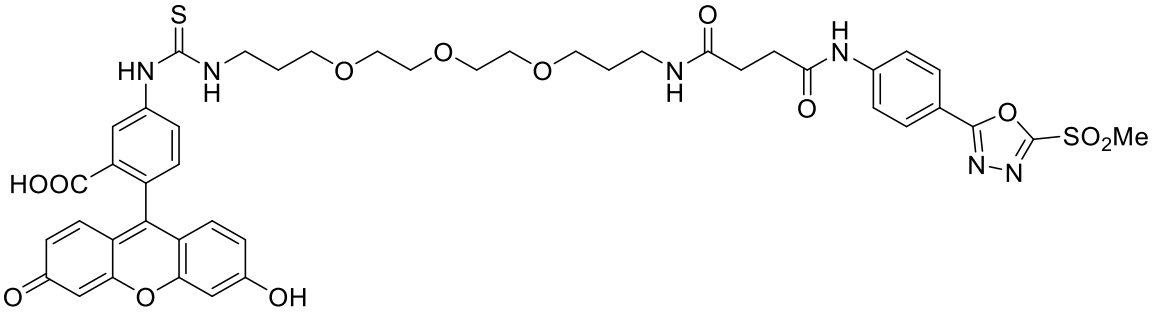
Boc-L-Cysteine



Cysteine (1.0 g, 8.25 mmol, 1 eq.) was suspended in 5 mL of methanol. This suspension was cooled on ice and trimethylamine (2.8 mL, 19.8 mmol, 2.4 eq.) was added, followed by the slow addition of Boc_2O (2.16 g, 9.9 mmol, 1 eq,) over 2 min. The clear solution was left at room temperature overnight, and the volatiles were then removed under reduced pressure. The resulting clear oil was dissolved in 10 mL of dichloromethane, and this solution was extracted with a 1 M Na_2CO_3 solution. The aqueous phase was then acidified to pH 2 with 6 N HCl and extracted with dichloromethane. The organic phase was dried over $MgSO_4$ and evaporated to yield the final product as a thick colorless oil (1.13 g, yield 63%).

^{1}H NMR (500 MHz, $CDCl_3$) 5.44 (1H, m), 4.5 (1H, m), 3.05 (2H, m), 1.46 (9H, s).
Analytical data identical to those reported in the literature.[1]

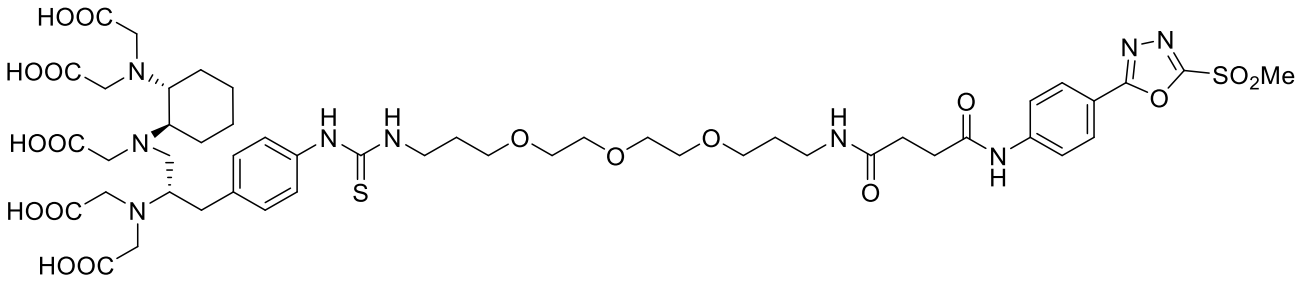
PODS-FL



To a solution of 6.0 mg of **PODS** in 600 uL of DMF (11.1 µmol, 1 eq.) was added 3.9 uL of DIPEA (22.2 µmol, 2 eq.) and 122 µL of a 0.1 M solution of fluorescein-isothyocyanate in DMSO (12.2 µmol, 1.1 eq.). The mixture was protected from light and let to react overnight at room temperature. The product was then purified by HPLC (gradient MeCN/H_2O + 0.1% TFA, 0% MeCN to 100% in 30 min, R_t = 22.5 min) to afford 3.8 mg of an orange powder (yield: 37%)

HRMS-ESI *m/z* Calcd for $[C_{44}H_{46}N_6O_{13}S_2+H]^+$: 931.2637; found: 931.2634; Δ: 0.27 ppm.

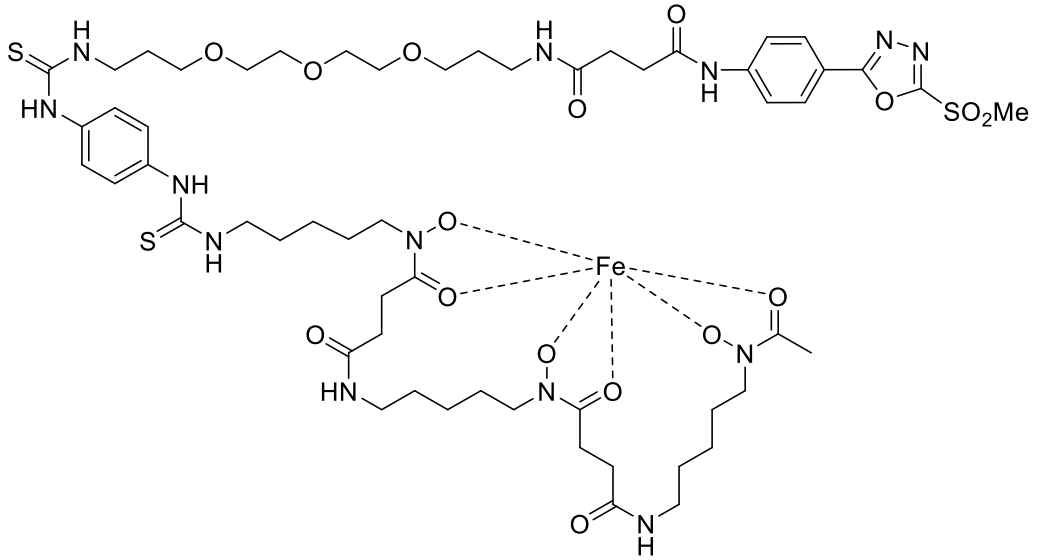
PODS-CHX-A″-DTPA



To a solution of **PODS** (2.1 mg, 3.84 µmol, 1 eq.) in 200 µL of DMSO was added DIPEA (6.7 µL, 10 eq.). To this solution was added 3.0 mg of p-SCN-Bn-CHX-A″-DTPA•3HCl (4.25 µmol, 1.1 eq.; Macrocyclics, Inc.), and the mixture was stirred at room temperature overnight. The product was then purified by HPLC (gradient MeCN/H_2O + 0.1% TFA, 0% MeCN to 100% in 25 min, R_t = 19.5 min) to afford 3.0 mg of a white powder (yield: 69%).

HRMS-ESI *m/z* Calcd for $[C_{49}H_{69}N_9O_{18}S_2+H]^+$: 1136.4275; found: 1136.4273; Δ: 0.18 ppm.

PODS-DFO-Fe

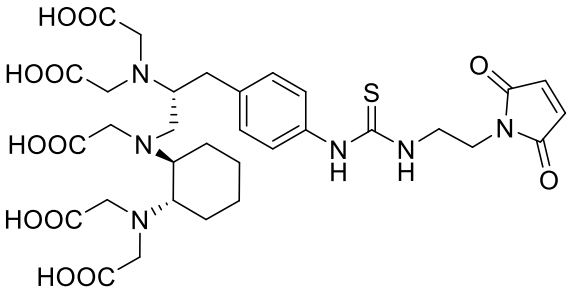


To a solution of *p*-SCN-Bn-DFO (5.0 mg, 6.65 µmol, 1.2 eq.; Macrocyclics, Inc.) in 100 uL of DMSO was added 1.8 mg of $FeCl_3$ hexahydrate (6.65 µmol, 1.2 eq.). The resulting dark red solution was added to a solution of **PODS** (3.0 mg, 5.54 µmol, 1 eq.) in 100 µL of DMSO

with DIPEA (4.83 µL, 5 eq.). The mixture was stirred at room temperature overnight. The product was then purified by HPLC (gradient MeCN/H_2O +0.1% TFA, 30% MeCN to 100% in 30 min, R_t = 16.5 min) to afford 5.2 mg of a dark red solid (yield: 72%).

HRMS-ESI *m/z* Calcd for $[C_{56}H_{84}FeN_{13}O_{16}S_3+H]^+$: 1347.4745; found: 1347.4735; Δ: 0.74 ppm. °

Mal-CHX-A"-DTPA



p-SCN-Bn-Chx-A″-DTPA.3HCl (5.0 mg, 7.1 mmol, 1 eq.) was dissolved in 100 uL of DMSO. To this solution was added 12.4 uL of DIPEA (10 eq.) and a solution of *N*-(2-aminoethyl)maleimide trifluoroacetate salt (1.99 mg in 150 uL of DMSO, 7.8 mmol, 1.1 eq.). The mixture was stirred at room temperature overnight. The product was then purified by HPLC preparative (H_2O/MeCN + 0.1% TFA, 0% MeCN to 85% at 30 min, R_t = 26 min) to give a white solid (4.9 mg, yield: 93%)

HRMS-ESI *m/z* Calcd for $[C_{32}H_{42}N_6O_{12}S+H]^+$: 735.2654; found: 735.2654; Δ: 0.09 ppm.

III. PODS reactivity assays

1. General procedure for the kinetic studies

All kinetics experiments were conducted with the same initial concentrations: 0.925 mM of PODS, 9.25 mM of thiol (10 eq.), and 4.625 mM of TCEP (5 eq.). The samples were analyzed on an Agilent 6340 Ion Trap with Electron Transfer Dissociation (ETD) LC/MS coupled with an Agilent 1200 Series HPLC system. The elution solvents (water and acetonitrile) were both complemented with 0.1% of formic acid. The samples were kept at 25°C and analyzed repeatedly for 1 h or until completion using a 5 to 60% gradient of MeCN over 5 min. The divert valve was switched to waste from 0 to 1.5 min to avoid salts in the nebulizer and then switched to source for the rest of the run. The acquisition was started immediately after the addition of PODS to the mixture (t = 0). After the first injection, the nature of each peak was confirmed by mass

spectrometry. The ratios of starting material and conjugation products were calculated from integration of the corresponding peaks at 252-256 nm.

2. Preparation of the samples

Boc-L-cysteine pH 4.5

To 374 µL of PBS was added 10 µL of cysteine (82 g/L in water, 10 eq.) and 10 µL of fresh TCEP (53 g/L in water, 5 eq.). The pH of the solution of adjusted to pH 4.5 with Na_2CO_3 0.1 M. To this mixture was added 10 µL of PODS (20 g/L in DMSO, 1 eq.).

Boc-L-cysteine pH 7.5

To 374 µL of PBS at pH 7.5 was added 10 µL of cysteine (82 g/L in Na_2CO_3 0.1 M, pH 7.5, 10 eq.) and 10 µL of fresh TCEP (53 g/L in in Na_2CO_3 0.3 M, pH 7.5, 5 eq.). To this mixture was added 10 µL of PODS (20 g/L in DMSO, 1 eq.).

Glutathione pH 7.5

To 352 µL of PBS at pH 7.5 was added 28.4 µL of glutathione (40 g/L in Na_2CO_3 0.1 M, pH 7.5, 10 eq.) and 10 µL of fresh TCEP (53 g/L in in Na_2CO_3 0.3 M, pH 7.5, 5 eq.). To this mixture was added 10 µL of PODS (20 g/L in DMSO, 1 eq.).

IV. Preparation of antibody-PODS-FL conjugates

To a suspension of 200 µg of antibody in PBS pH 7.4 (1 mg/mL) was added 1.33 µL of a fresh TCEP solution (10 mM in water, 10 eq) and the appropriate volume of a solution of PODS-FL (1 mM in DMSO). The reaction mixture was stirred on a thermomixer (25°C or 37°C) for 30 min, 2 h, or 24 h. The conjugate was then purified on a size exclusion column (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2 mL of PBS, pH 7.4) and concentrated using centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp. Billerica, MA). The fluorescein:mAb ratio was determined via UV-Vis spectrophotometry of the conjugate at 280 nm and 495 nm followed by calculation using the following equation:

$$A_{mAb} = A_{280} - (A_{495} * CF)$$

$$DOL = [A_{max} * MW_{mAb}] / [[mAb] * \varepsilon_{Dye495}]$$

in which the correction factor (CF) for PODS-FL was 0.60 based on the absorbance spectrum of PODS-FL in PBS, MW_{mAb} = 150,000, ε_{Dye495} =75,000, and $\varepsilon_{280,\ mAb}$= 210,000.

For the re-oxidation experiment, 200 µg of trastuzumab in PBS pH 7.4 (1 mg/mL) were first reduced with 1.33 µL of a fresh TCEP solution (10 mM in water, 10 eq). After 30 min of

reaction, the reduced antibody was purified on a size exclusion column (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2 mL of PBS, pH 7.4) and concentrated using centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp. Billerica, MA). This solution was then allowed to re-oxidize in open air for 10 min before the addition of 13.3 µL of a solution of PODS-FL (1 mM in DMSO). The reaction mixture was incubated for 2 h at 37°C and then processed as described above.

V. Transchelation of iron from PODS-DFO-Fe with EDTA

100 µL of a solution of PODS-DFO-Fe (2 mM in DMSO) was diluted to 2 mL with PBS at pH 7.4. This solution was split in 100 uL aliquots, and these aliquots were supplemented with various volumes of EDTA 25 g/L (10 or 20 µL), adjusted at various pH with 0.25 M H_2SO_4, and incubated at different temperatures (25°C or 37°C). The transchelation of iron was monitored at different timepoints by measuring the absorbance of these solutions at 290 nm and 420 nm (DFO-Fe complex has a broad absorption peak centred at 420 nm). As the extinction coefficient of PODS-DFO at 290 nm does not change upon transchelation, the ratio Abs_{420}/Abs_{290} was used as a surrogate to assess the transchelation of Fe(III) from DFO to EDTA and was compared to the ratio of a reference solution of PODS-DFO-Fe in PBS.

VI. Preparation of mAb-DFO conjugates

1. Preparation of mAb-mal-DFO conjugates

To a suspension of 1 mg of antibody in PBS pH 7.4 (1 mg/mL) was added 6.7 µL of a fresh TCEP solution (10 mM in water, 10 eq) and 4.76 µL of DFO-maleimide (Macrocyclics, Inc.; 10 mg/mL in DMSO). The reaction mixture was stirred on a thermomixer at 25 °C for 2 hours. The conjugate was then purified on a size exclusion column (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2 mL of PBS, pH 7.4) and concentrated using centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp. Billerica, MA).

2. Preparation of mAb-PODS-DFO conjugates

To a suspension of 1 mg of antibody in PBS pH 7.4 (1 mg/mL) was added 6.7 µL of a fresh TCEP solution (10 mM in water, 10 eq) and 33 µL of a solution of PODS-DFO-Fe (2 mM in DMSO). The reaction mixture was stirred on a thermomixer at 2 5°C for 2 hours. To this yellow solution was then added 100 µL of EDTA (tetrasodic salt, 25 g/L in water), and the pH was adjusted to 4.5 with 0.25 M H_2SO_4. The mixture was stirred at 25 °C for 30 min to yield a colorless solution, indicating the transchelation of the Fe(III) from the DFO. The conjugate was

then purified on a size exclusion column (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2 mL of PBS, pH 7.4) and concentrated using centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp. Billerica, MA).

VII. Preparation of trast-CHX-A″-DTPA conjugates

1. Preparation of CHX-A"-DTPA-mal-trast conjugate

To a suspension of 500 µg of trastuzumab in PBS pH 7.4 (1 mg/mL) was added3.35 µL of a fresh TCEP solution (10 mM in water, 10 eq) and 6.7 µL of a solution of Mal-CHX-A″-DTPA (5 mM in DMSO). The reaction mixture was stirred on a thermomixer at 25°C for 2 hours. The conjugate was then purified on a size exclusion column (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2 mL of PBS, pH 7.4) and concentrated using centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp. Billerica, MA).

2. Preparation of CHX-A"-DTPA-PODS-trast conjugate

To a suspension of 500 µg of trastuzumab in PBS pH 7.4 (1 mg/mL) was added a 3.35 µL of a fresh TCEP solution (10 mM in water, 10eq) and 6.7 µL of a solution of PODS-CHX-A″-DTPA (5 mM in DMSO). The reaction mixture was stirred on a thermomixer at 25°C for 2 hours. The conjugate was then purified on a size exclusion column (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2 mL of PBS, pH 7.4) and concentrated using centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp. Billerica, MA).

VIII. Radiolabeling

1. Radiolabeling of the immunoconjugates with ^{89}Zr

For each DFO-bearing conjugate, 500 µg of immunoconjugate was diluted to 400 µL with PBS, pH 7.4. [^{89}Zr]Zr-oxalate (1500 µCi) in 150 µL of 1.0 M oxalic acid was adjusted to pH 7.0-7.5 with 1.0 M Na_2CO_3. After the bubbling of CO_2 stopped, the ^{89}Zr solution was added to the antibody solution, and the resulting mixture was incubated at room temperature for 1 h. The reaction progress was then assayed using iTLC. Subsequently, the reaction was quenched with 13 µL of 50 mM of EDTA (pH = 5). After 10 min, the radiolabeled construct was purified using size exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 500 µL fractions of PBS, pH 7.4) and, if necessary, concentrated via centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 4

Centrifugal Filtration Units, Millipore Corp. Billerica, MA). The radiochemical purity of the final radiolabeled bioconjugates was assayed by iTLC again. In the iTLC experiments, free $^{89}Zr^{4+}$ cations and [^{89}Zr]-EDTA elute with solvent front, while radiolabeled antibody constructs remain at the baseline.

2. Radiolabeling of the immunoconjugates with ^{177}Lu

300 μg of CHX-A″-DTPA-bearing conjugate were dissolved in 300 μL of 0.25 M ammonium acetate buffer supplemented with 6 g/L of ascorbic acid, pH 5.5. To this solution was added 500 μCi of [^{177}Lu]$LuCl_3$ in solution in 0.05 M HCl, and the resulting mixture was stirred at 37 °C for 1 h. The progress of the reaction was then assayed using iTLC. Subsequently, the radiolabeled construct was purified using size exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 500 μL fractions of PBS supplemented with 6 g/L of ascorbic acid, pH 7.4) and, if necessary, concentrated via centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp. Billerica, MA). The radiochemical purity of the final radiolabeled bioconjugates was assayed by iTLC again. In the iTLC experiments, free $^{177}Lu^{3+}$ cations and [^{177}Lu]-EDTA elute with solvent front, while radiolabeled antibody constructs remain at the baseline.

IX. *In vitro* stability assays

The stability of the radioimmunoconjugates with respect to radiochemical purity and loss of radioactivity from the antibody was investigated via incubation of 30-50 μCi of the radiolabeled antibodies in human serum for 7 days at 37 °C (n = 3). Every 24 hours, the radiochemical purity of the antibodies was determined via iTLC with an eluent of 50 mM EDTA, pH 5.0.

X. Immunoreactivity assays

The immunoreactivity of the huA33-DFO conjugates was determined using an antigen saturation assay. Briefly, suspensions of 2.0×10^7 SW1222 colorectal cancer cells in 100 μL of media were prepared in microcentrifuge tubes. In parallel, a solution of 0.4 ng/μL radioimmunoconjugate was prepared in PBS supplemented with 1% bovine serum albumin. 2 ng (5 μL) of the radioimmunoconjugate solution was then added to the cell suspension, and the resulting mixture was agitated via pipetting and subsequently incubated on ice for 1 h. After 1 h, the cells were pelleted via centrifugation (600×g for 5 min). After centrifugation, the media was carefully removed and placed in a separate tube, and 1 mL of ice-cold PBS was added to the original tube containing the cells. The cells were then pelleted again via centrifugation (600×g for 5 min). Following centrifugation, the PBS was removed and placed in a separate tube. These

PBS washing steps were repeated two more times. After washing, the amount of radioactivity in all five tubes — cell pellet, media, wash 1, wash 2, and wash 3 — was assayed using a gamma counter. The counting data was then background corrected, and the immunoreactive fraction was calculated by dividing the counts in the cell pellet by the sum of the counts in the cell pellet, media, and three washes. No weighing was applied to the data, and the data were obtained in triplicate.

XI. PET imaging

PET imaging was performed on a micro-PET Focus 120 scanner (Concorde Microsystems). Athymic nude mice bearing subcutaneous SW1222 xenografts (left shoulder, ~100-150 mm^3, 12-14 days after inoculation) were administrated with the ^{89}Zr-labeled conjugates (140 μCi, ~60 μg, in solution in 200 μL of saline) via tail vein injection. Approximately 5 min before imaging, the mice were anesthetized by inhalation of a 2% isoflurane (Baxter Healthcare):oxygen gas mixture and placed on the scanner bed. Anesthesia was maintained using a 1% isoflurane mixture. PET data for each mouse were recorded via static scans at 24 h, 72 h, and 120 h p.i.

The imaging data was normalized to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay to the time of injection but no attenuation, scatter, or partial-volume averaging correction was applied. Activity concentrations (percentage of injected dose per gram of tissue; %ID/g) were determined by conversion of the counting rates from the reconstructed images. All of the resulting PET images were analyzed using ASIPro VM™ software.

XII. Biodistribution

Athymic nude mice bearing subcutaneous SW1222 xenografts (left shoulder; ~100-150 mm^3) were randomized before the study and were administrated with the radioimmunoconjugates (30 μCi, ~15 μg, in solution in 200 μL of saline) via tail vein injection. Subsequently, the animals (n = 4 per group) were euthanized by CO_2(g) asphyxiation at 24 h, 72 h, and 120 h post-injection, and 13 tissues (including tumor) were removed, washed, dried, weighed, and counted in a gamma-counter. The number of counts in each tissue were background- and decay-corrected to the time of injection and converted to activity units (μCi) using a calibration curve generated from known standards. The %ID/g for each tissue sample was then calculated by normalization to the total activity injected and the mass of each tissue.

XIII. Statistical analysis

Data were analyzed by the unpaired, two-tailed Student's t-test. Differences at the 95% confidence level ($P < 0.05$) were considered to be statistically significant.

XIV. Supplementary figures

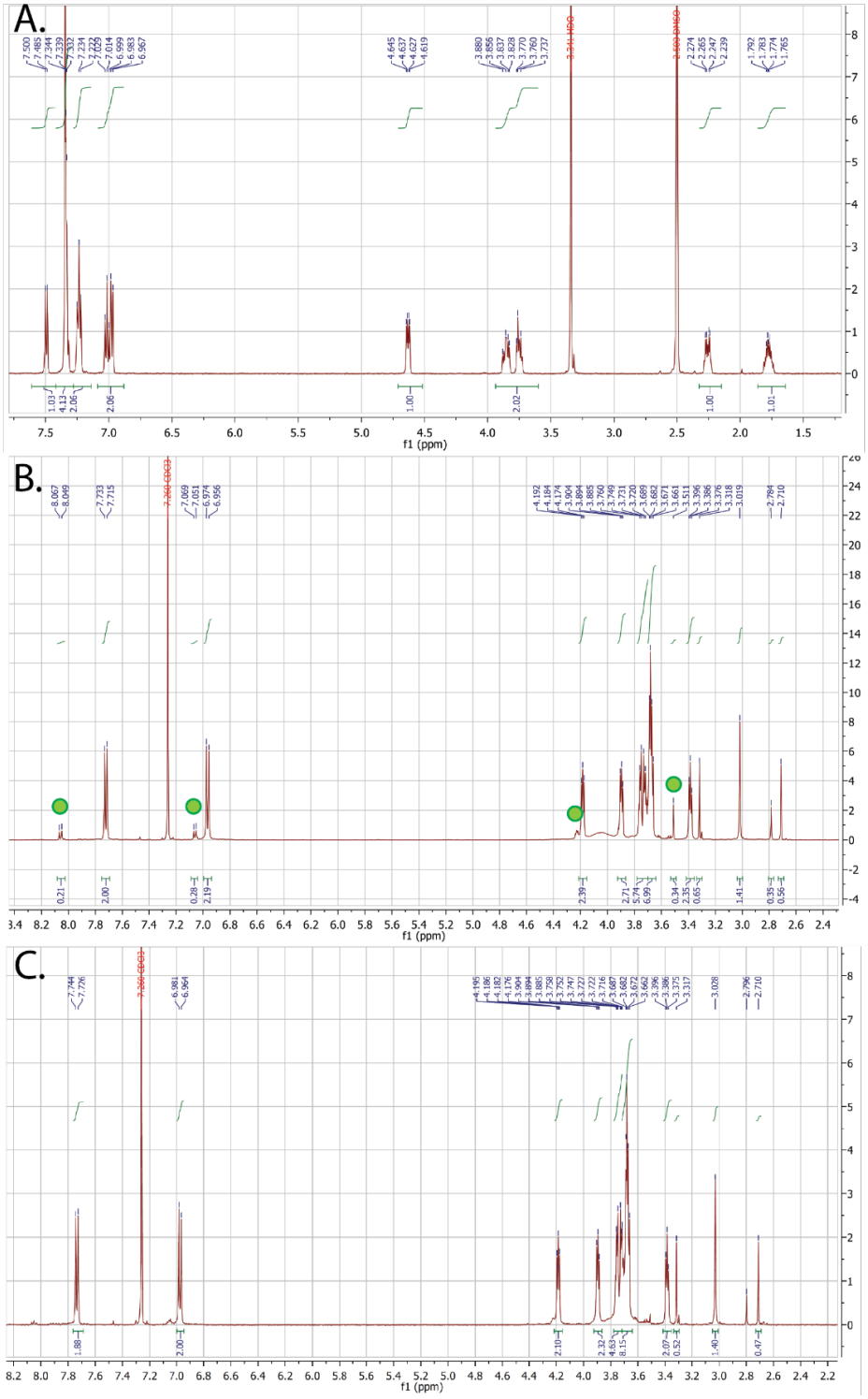


Figure S1. [1]H NMR spectra of the content of three different vials of the "azido-PEG_4-phenyloxadiazole methylsulfone" ordered from Sigma Aldrich, Inc.. Lot numbers: B00237343 (A) and MKBX9525V (B and C). According to the previously published chemical shifts,[2] the vial B contained less than 15% of the reagent (the green dots mark the characteristic signals of the compound). The two other vials contained only trace amount of the the reagent (C) or none at all (A).

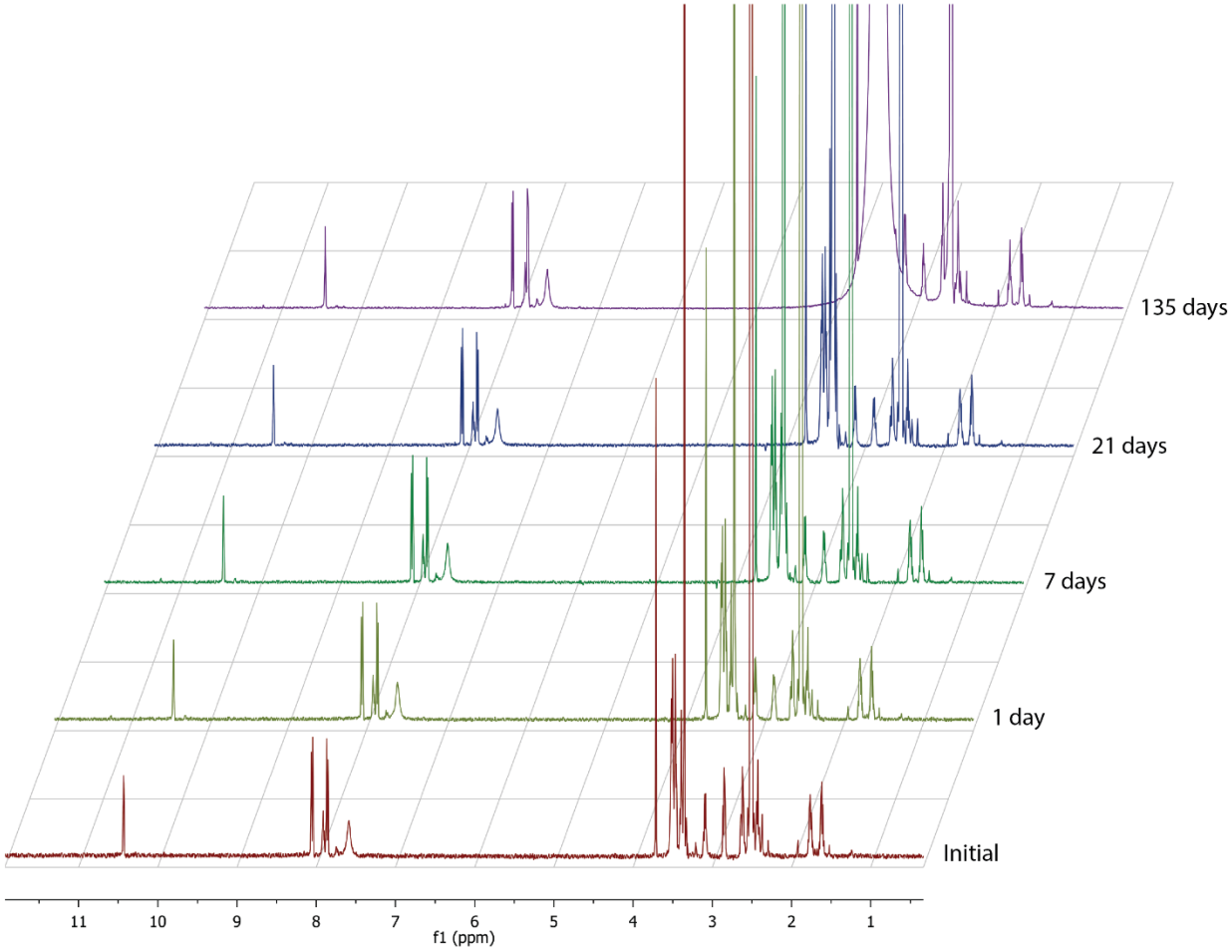


Figure S2. Stacked ^{1}H NMR spectra of a solution of PODS in DMSO-d_6, kept at room temperature for 135 days. No degradation of the compound was observed over this timeframe. The increase of the H_2O signal at 3.33 ppm over time was due to absorption of water by the DMSO, although the solution was kept in a closed container.

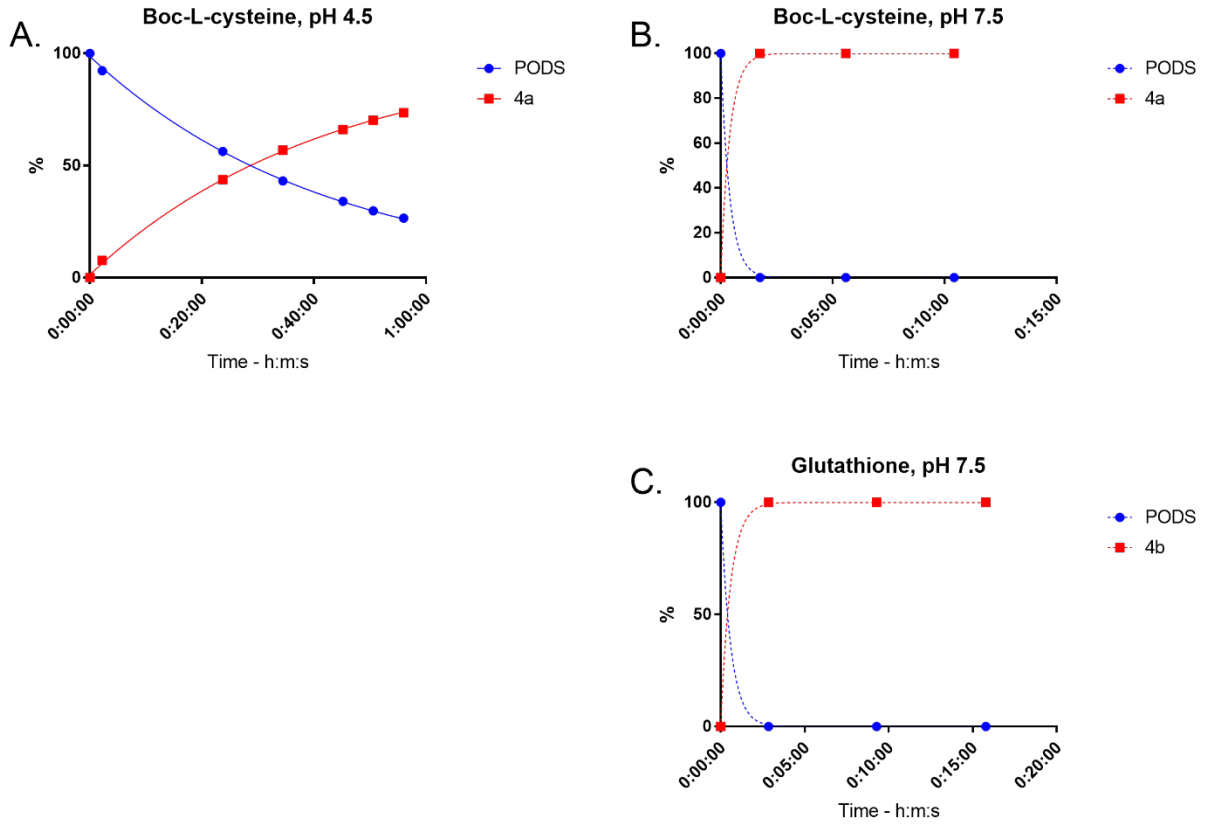


Figure S3. Evolution of the reaction between PODS and model thiol-bearing biomolecules at various pH: (A) coupling of PODS and Boc-L-cysteine at pH 4.5; (B) coupling of PODS and Boc-L-cysteine at pH 7.5; (C) Coupling of PODS and glutathione at pH 7.5.

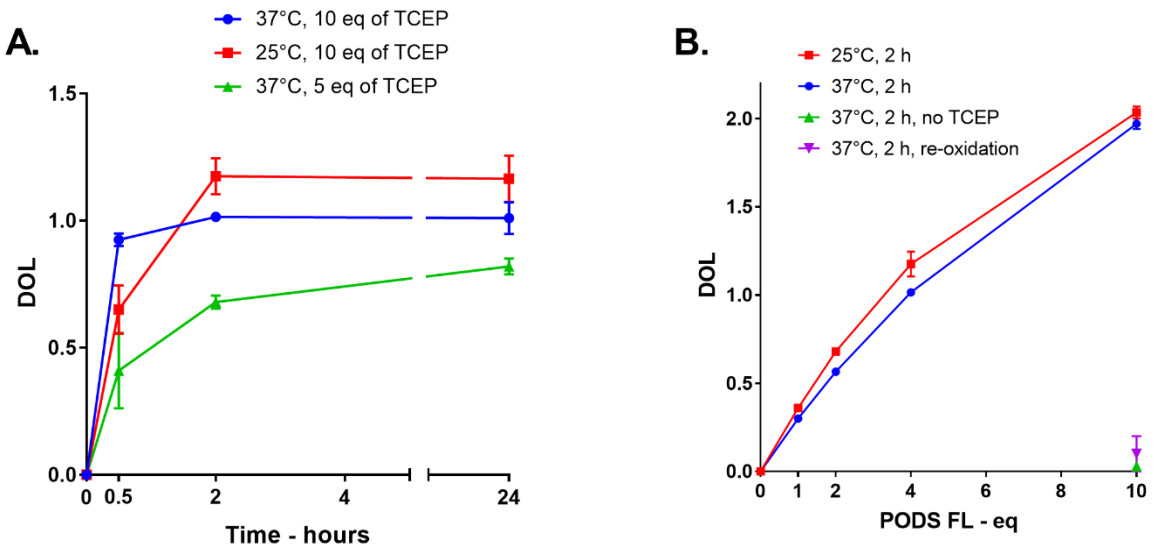


Figure S4. Conjugation of trastuzumab with PODS-FL. (A) DOL of the trastuzumab-FL conjugates obtained with 4 equivalents of PODS-FL at different temperatures and with different amounts of reducing agent after reaction times of 30 min, 2 h and 24 h (n=2). (B) DOL of the trastuzumab-FL conjugates obtained after 2 h of reaction with different amounts of PODS-FL at 37 °C and 25 °C with 10 equivalents of TCEP (n = 2).

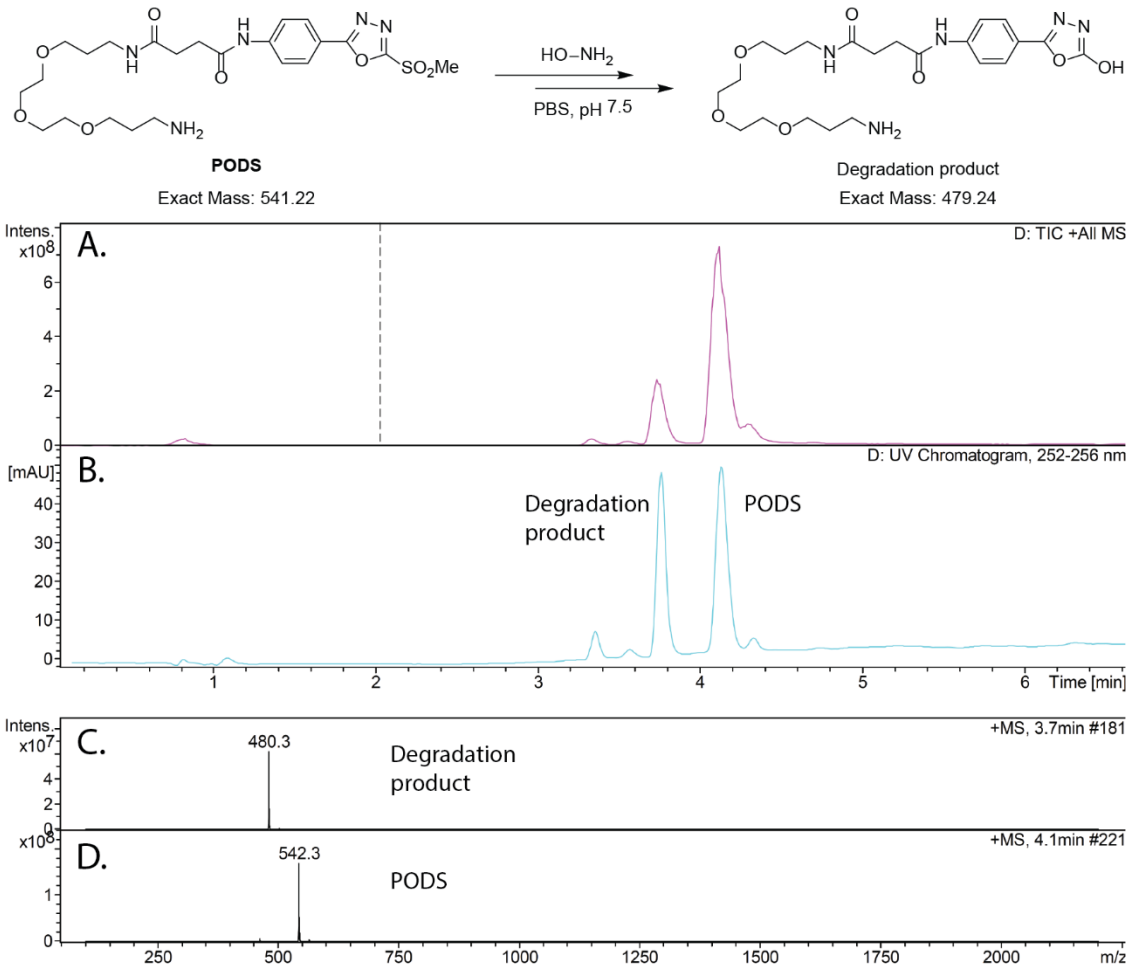


Figure S5. LC-MS chromatogram of a mixture of PODS (10 mM) with 5 equivalents of hydroxylamine in PBS pH 7.5 after 1 hour at room temperature. (A) Total ion count chromatogram and (B) chromatogram at 254 nm, mass spectra of the 3.7 min peak (C) and 4.1 min peak (D). The peak at 4.1 min corresponds to PODS, and the peak at 3.7 min corresponds to the hydroxyl-substitute, likely formed by the degradation of the substitution product.

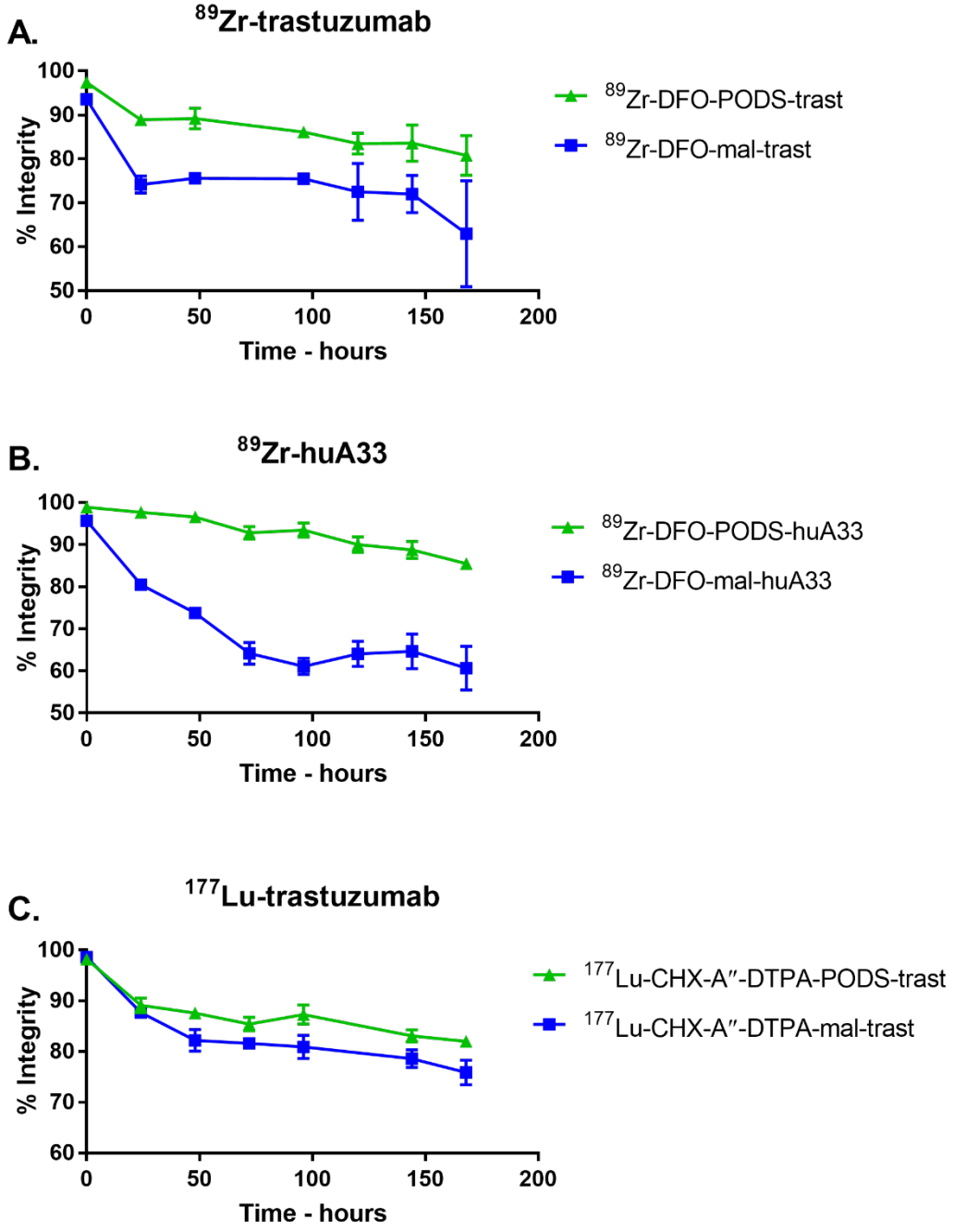


Figure S6. Stability of the radiolabeled conjugates in human serum at 37°C. (A) Integrity of ^{89}Zr-DFO-PODS-trast and ^{89}Zr-DFO-mal-trast; (B) integrity of ^{89}Zr-DFO-PODS-huA33 and ^{89}Zr-DFO-mal-huA33; (C) integrity of ^{177}Lu-CHX-A″-DTPA-PODS-trast and ^{177}Lu-CHX-A″-DTPA-mal-trast.

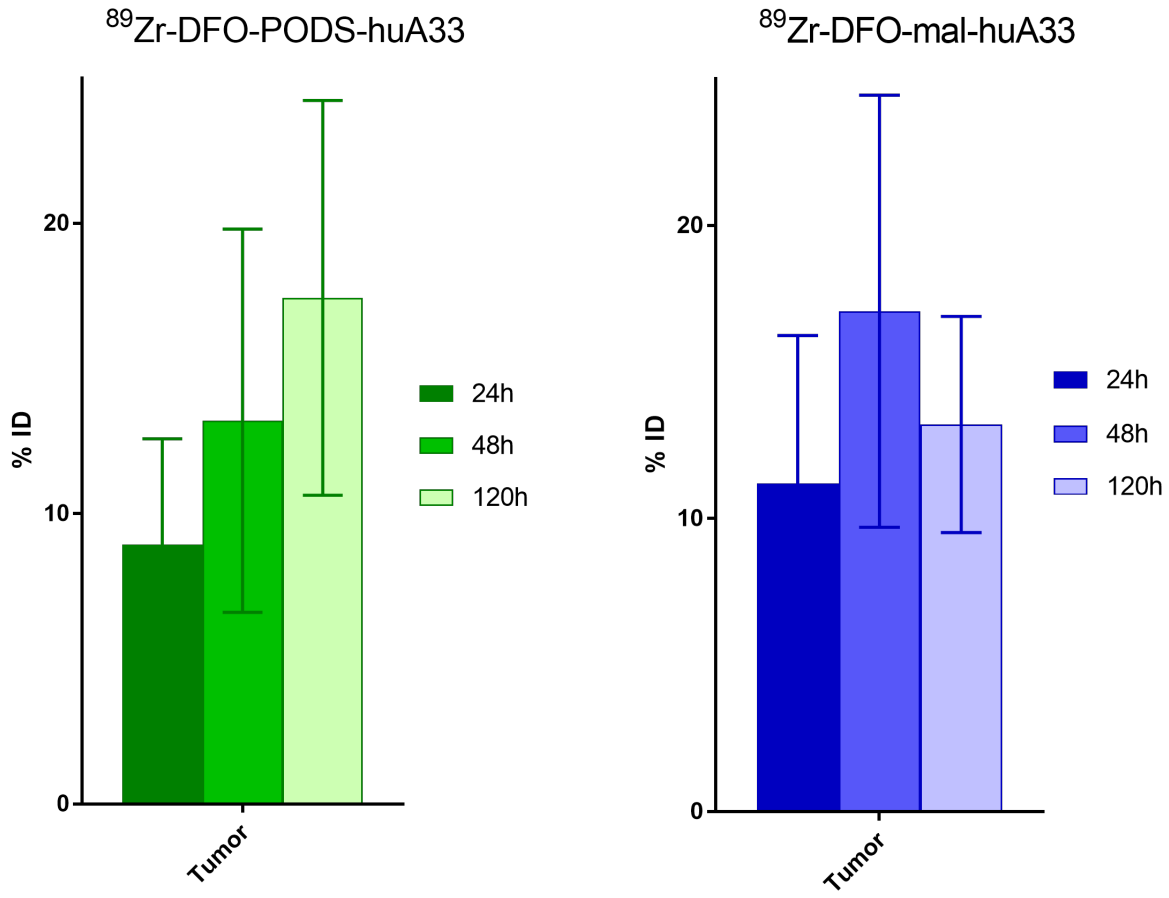


Figure S7. Activity uptake in the tumor (in %ID) after the administration of ^{89}Zr-DFO-PODS-huA33 and ^{89}Zr-DFO-mal-huA33 (30 µCi, 15-18 µg) to athymic nude mice bearing A33-expressing subcutaneous SW1222 human colorectal cancer xenografts.

XV. Supplementary tables

Table S1. De-complexation of Fe(III) from PODS-DFO-Fe for various conditions of pH, temperature and scavenger chelator (EDTA).

Reaction time	Temperature	EDTA g/L	pH					
			4.0	4.5	5.0	5.5	6.5	7.5
30 min	25 °C	2.2	7.6%	4.8%	87.9%	94.1%		
	25 °C	4.4	6.2%	63.7%	85.0%	100%		
	37 °C	2.2	4.5%	3.5%	54.0%	69.5%	97.1%	92.2%
2 h	25 °C	2.2	6.3%	0.8%	69.4%	70.5%		
	25 °C	4.4	6.7%	17.9%	52.3%	84.7%		
	37 °C	2.2	8.9%	8.7%	38.6%	57.0%		
15 h	25 °C	2.2	0.0%	6.0%	8.5%	35.5%		
	25 °C	4.4	0.0%	5.8%	13.1%	34.2%		
	37 °C	2.2	5.7%	3.5%	7.5%	18.5%	58.5%	77.0%

% Intact DFO-Fe
100-80%
80-50%
50-10%
< 10%

Table S2. Results of the radiolabeling of CHX-A″-DTPA-PODS-trast and CHX-A″-DTPA-mal-trast with ^{177}Lu, and of the ^{89}Zr-labeling of DFO-PODS-trast, DFO-mal-trast, DFO-PODS-huA33 and DFO-mal-huA33.

Radiotracers	Specific activity - Ci/g	RCY - %	RCP - %
^{177}Lu-CHX-A″-DTPA-PODS-trast	1.3 ± 0.1	97%	> 99 %
^{177}Lu-CHX-A″-DTPA-mal-trast	1.3 ± 0.1	97%	> 99 %
^{89}Zr-DFO-PODS-trast	2.5 ± 0.2	88%	> 99 %
^{89}Zr-DFO-mal-trast	2.4 ± 0.4	85%	> 99 %
^{89}Zr-DFO-PODS-huA33	2.8 ± 0.4	95%	> 99 %
^{89}Zr-DFO-mal-huA33	2.8 ± 0.2	88%	> 99 %

Table S3. Biodistribution data for the ^{89}Zr-DFO-mal-huA33 and ^{89}Zr-DFO-PODS-huA33 at 24 h, 48 h, and 120 h after injection in nude mice bearing SW1222 tumors.

	^{89}Zr-DFO-mal-huA33			^{89}Zr-DFO-PODS-huA33		
	24h	48h	120h	24h	48h	120h
Blood	7.1 ± 2.2 [a]	3.8 ± 1.2	1.5 ± 0.3	8.0 ± 2.5	4.8 ± 0.4	0.6 ± 0.3
Tumor	30.9 ± 5.4	49.6 ± 9.3	36.8 ± 3.3	42.0 ± 18.8	56.4 ± 6.9	28.1 ± 7.3
Heart	3.1 ± 0.6	1.9 ± 0.3	1.1 ± 0.1	3.4 ± 0.8	2.2 ± 0.3	0.5 ± 0.1
Lung	3.6± 0.8	2.3 ± 0.5	1.3 ± 0.2	4.3 ± 0.9	2.8 ± 0.4	0.6± 0.3
Liver	3.3 ± 0.5	2.9 ± 0.6	2.7 ± 0.4	3.3 ± 0.4	2.0 ± 0.2	1.2 ± 0.3
Spleen	3.6 ± 2.0	2.4 ± 0.6	2.5 ± 0.1	2.7 ± 0.3	1.8 ± 0.1	1.2 ± 0.6
Stomach	0.8 ± 0.4	0.7 ± 0.1	0.4 ± 0.1	1.9 ± 0.3	1.2 ± 0.1	0.21 ± 0.1
S. Intestine	1.5 ± 0.7	1.1 ± 0.3	0.5 ± 0.1	1.9 ± 0.3	1.3 ± 0.1	0.3 ± 0.1
L. Intestine	0.6 ± 0.2	0.5 ± 0.1	0.3 ± 0.1	1.7 ± 0.6	1.2 ± 0.2	0.4 ± 0.1
Kidney	5.5 ± 0.7	4.6 ± 0.8	3.1 ± 0.5	3.6 ± 0.7	2.6 ± 0.1	1.4 ± 0.1
Muscle	1.0 ± 0.3	0.7 ± 0.1	0.7 ± 0.4	1.1 ± 0.2	0.8 ± 0.1	0.3 ± 0.1
Bone	6.5 ± 1.2	11.7 ± 4.3	12.2 ± 2.5	2.8 ± 0.2	3.7 ± 0.4	4.3 ± 0.6

[a]Values are %ID/g ± SD. Nude mice (n = 4) bearing subcutaneous BT474 xenografts were administered the ^{89}Zr-labeled constructs (30 μCi, ~15 μg) via tail vein injection. Stomach, small intestine, and large intestine values include contents.

Table S4. Tumor-to-organ activity uptake ratios for ^{89}Zr-DFO-mal-huA33 and ^{89}Zr-DFO-PODS-huA33 at 24h, 48 h, and 120 h after injection in nude mice bearing SW1222 tumors.

	^{89}Zr-DFO-mal-huA33			^{89}Zr-DFO-PODS-huA33		
	24h	48h	120h	24h	48h	120h
Tumor : Blood	4.4 ± 1.5	13.1 ± 4.9	25.1 ± 5.0	5.2 ± 2.8	11.9 ± 1.7	46.9 ± 29.6
Tumor : Heart	10.1 ± 2.6	26.8 ± 6.8	33.3 ± 4.3	12.4 ± 6.3	25.9 ± 4.7	54.4 ± 18.5
Tumor : Lung	8.5 ± 2.3	21.6 ± 6.5	28.9 ± 5.8	9.9 ± 4.9	20.0 ± 4.0	50 ± 26.4
Tumor : Liver	9.3 ± 2.2	17.4 ± 4.9	13.6 ± 2.4	12.8 ± 5.9	28.8 ± 4.2	24.4 ± 8.5
Tumor : Spleen	8.5 ± 4.9	20.9 ± 6.8	14.9 ± 1.6	15.6 ± 7.2	31.3 ± 4	23.2 ± 13.4
Tumor : Stomach	37.7 ± 19.8	67.1 ± 17.5	90.8 ± 20.5	21.8 ± 10.4	45.4 ± 6.4	119.5 ± 39.6
Tumor : S. Intestine	20.3 ± 10.3	45.0 ± 15.2	75.9 ± 9.3	22.1 ± 10.3	43.6 ± 7	86.6 ± 39.5
Tumor : L. Intestine	53.9 ± 20.0	103.6 ± 32.3	131.6 ± 29.8	24.8 ± 14.2	46.5 ± 9.4	67.3 ± 17.9
Tumor : Kidney	5.6 ± 1.2	10.8 ± 2.7	12.0 ± 2.3	11.7 ± 5.7	21.5 ± 2.7	19.4 ± 5.2
Tumor : Muscle	30.0 ± 9.2	67.3 ± 14.3	51.8 ± 21.8	38.2 ± 18.9	70 ± 14.4	106.7 ± 37.4
Tumor : Bone	4.8 ± 1.2	4.2 ± 1.7	3.0 ± 0.7	15.2 ± 6.9	15.2 ± 2.4	6.5 ± 1.9

XVI. References

1. Bai, C.-G., Xu, Y.-T., Li, N.-N., Wang, J.-H., Yang, C., Chen, Y. & Zhou, H.-G. (2015) Cysteine and Its Derivatives as New Delhi Metallo-beta-lactamase-1 Inhibitors. *Curr. Enzyme Inhib. 11*, 46–57
2. Toda, N., Asano, S. & Barbas, C. F. (2013), Rapid, Stable, Chemoselective Labeling of Thiols with Julia–Kocieński-like Reagents: A Serum-Stable Alternative to Maleimide-Based Protein Conjugation. *Angew. Chem. Int. Ed. 52*, 12592–12596