Supporting information for

Development of a Novel Pretargeted Imaging Strategy Based on HaloTag technology

James C. Knight,[a] Michael Mosley,[a] Michael R. Stratford,[a] H. Tetsuo Uyeda,[b] Hélène A. Benink,[b] Mei Cong,[b] Frank Fan,[b] Stephen Faulkner,[c] and Bart Cornelissen*[a]

[a] *CR-UK/MRC Oxford Institute for Radiation Oncology, University of Oxford, Oxford, OX3 7DQ, UK.*
[b] *Promega Corporation, Madison, WI, USA.*
[c] *Chemistry Research Laboratory, University of Oxford, 12 Mansfield Road, Oxford, OX1 3TA, UK.*

bart.cornelissen@oncology.ox.ac.uk

Table of Contents

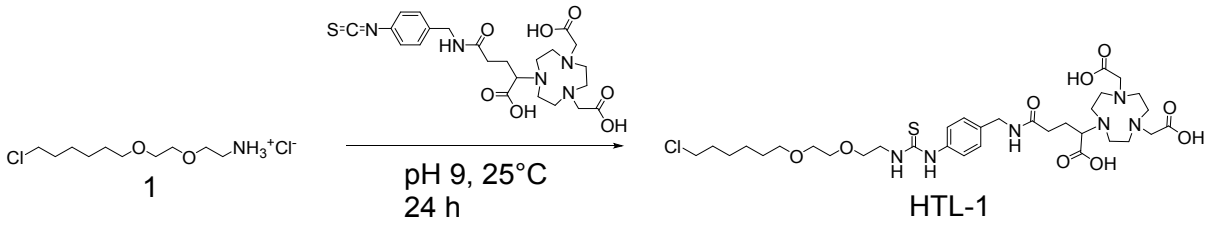
Supplemental Methods

Supplemental Figures

Chemical syntheses

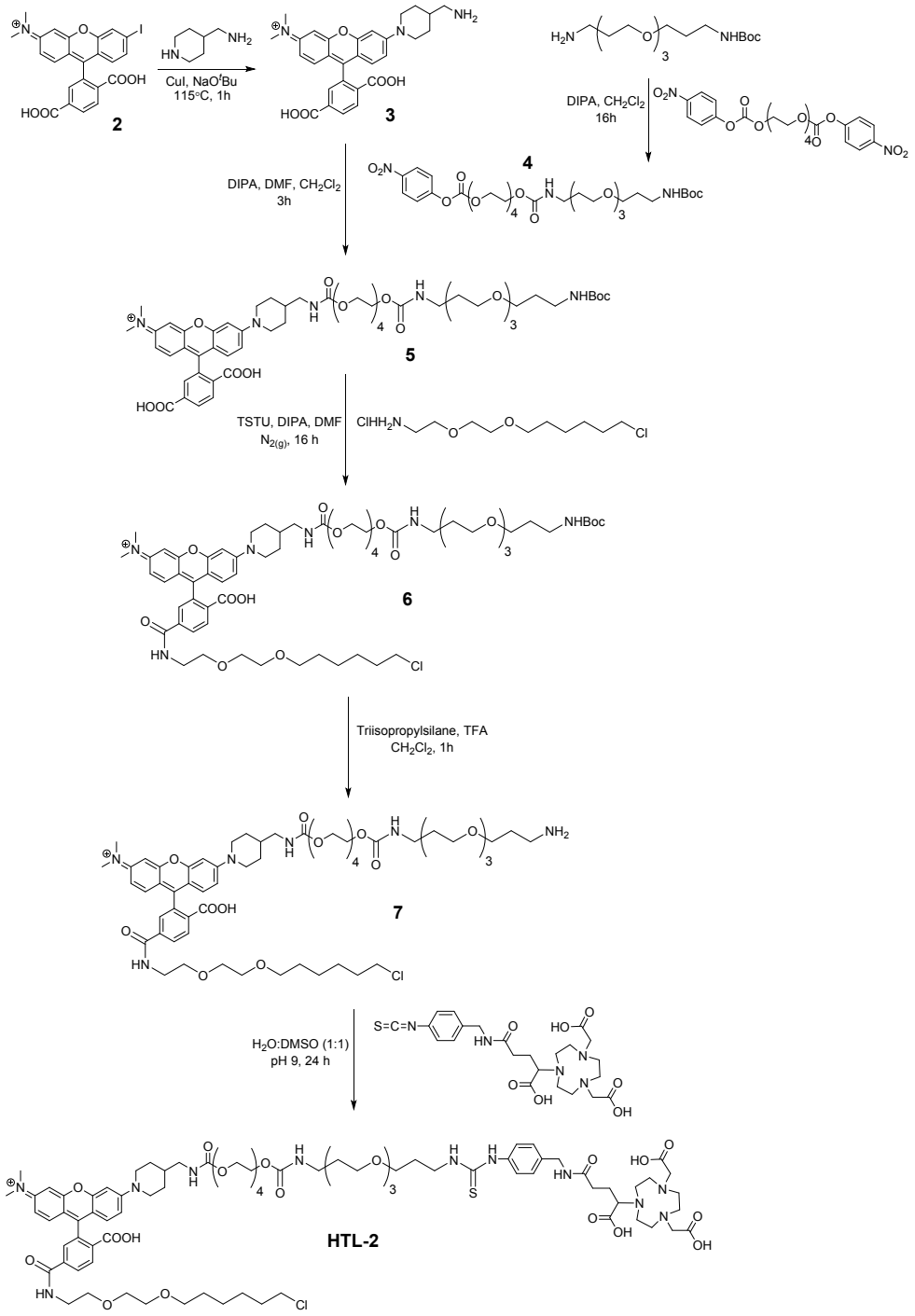
General methods

All solvents and reagents were obtained from commercial suppliers unless otherwise stated. H_2O was deionised using a Purite Neptune purification system. ^{1}H NMR was performed on a Bruker AVII spectrometer. Accurate masses were determined to four decimal places using a Bruker μTOF spectrometer at the Chemistry Research Laboratory of the University of Oxford. UV/vis spectra were acquired using a Jenway 6505 spectrophotometer. The commercially available HaloTag ligand building block **1** was obtained from Promega Corporation. The bifunctional chelating agent p-NCS-benzyl-NODA-GA was obtained from CheMatech. The precursor compounds bis(4-nitrophenyl)(((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl))bis(carbonate) and compound **2** were prepared according to known procedures.[1,2]



Scheme 1: Synthesis of HaloTag ligand, HTL-1

Synthesis of HTL-1: To p-NCS-benzyl-NODA-GA (14 mg, 19.2 μmol) in deionised H_2O (500 μL) was added 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-aminium chloride **1** (5 mg, 19.2 μmol) in DMSO (500 μL). The reaction mixture was adjusted to pH 9 with 1 M NaOH and then stirred for 24 h. The crude reaction mixture was then purified by HPLC-LCMS (Chromolith RP18e column, 100 x 4.6mm, with 10mM HCOOH and MeOH, running a linear gradient from 40-95% over 12 min, flow rate 1.3 mL min^{-1}, t_R = 5.9 min). The HPLC solvent was removed by lyophilisation to yield the product as a white solid (4 mg, 5.4 μmol, 28% yield). ^{1}H-NMR δ_H (500 MHz; D_2O): 8.19 (s, 1H); 7.29 (d, 2H, J = 10.0 Hz); 7.19 (d, 2H, J = 10.0 Hz); 4.31 (m, 1H); 3.68-2.94 (m, 30H); 2.42 (t, 2H, J = 7.5 Hz); 2.08 (m, 1Hz); 1.96 (m, 1H); 1.64 (q, 2H, J = 8.8 Hz); 1.44 (m, 2H); 1.31 (m, 2H); 1.22 (m, 2H); HRMS (ES +ve) m/z: 743.3215 $(M-H)^+$, $C_{33}H_{52}ClN_6O_9S$ requires 743.3210.



Scheme 2: Synthesis of HaloTag ligand, HTL-2

N-(6-(4-(aminomethyl)piperidin-1-yl)-9-(2,5-dicarboxyphenyl)-3H-xanthen-3-ylidene)-N-methylmethanaminium (3): Compound **2** (5.0 g, 9.72 mmol), 4-(aminomethyl)piperidine (3.33 g, 29.2 mmol), CuI (926 mg, 4.86 mmol) and NaO^{t}Bu (934 mg, 9.72 mmol) were added to trifluoroethanol (50 mL) and heated to 115°C in a pressure reactor with stirring. After 1 h, the reaction was cooled, filtered and rinsed with water. The solution was concentrated under vacuum and the crude material was purified by RP-HPLC (linear gradient of 5-100% CH_3CN with H_2O+0.1% TFA over 30 minutes). The HPLC solvent was removed under vacuum to yield a red solid (3.02 g, 62%). ^{1}H-NMR δ_H (300 MHz; MeOD): 8.41 (s, 1H); 8.40 (s, 1H); 7.99 (s, 1H); 7.19 (m, 5H); 7.00 (s, 1H); 4.40 (d, 2H, J = 12 Hz); 3.22 (s, 6H); 3.20 (d, 2H, J = 12 Hz); 2.84 (d, 2H, J = 9.8 Hz); 2.10 (s, 1H); 1.97 (m, 2H); 1.40 (m, 2H); ESI-MS m/z: 500.4 $(M)^+$, $C_{29}H_{30}N_3O_5$ requires 500.57.

1-(4-nitrophenoxy)-1-oxo-2,5,8,11-tetraoxatridecan-13-yl(2,2-dimethyl-4-oxo-3,9,12,15-tetraoxa-5-azaoctadecan-18-yl)carbamate (4): To a stirring solution of bis(4-nitrophenyl)(((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl)) bis(carbonate) (21.58 g, 41.15 mmol) in dichloromethane (200 mL) was added a solution of tert-butyl(3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate (8.14 g, 25.4 mmol) and diisopropylamine (4.54 mL, 25.4 mmol) in a dropwise manner. The resulting solution was stirred at room temperature for 16 h. The solvent was evaporated and the resulting yellow oil was purified by column chromatography (5% MeOH in CH_2Cl_2) to give a clear yellow oil (10.23 g, 57%). ^{1}H-NMR δ_H (300 MHz; CD_2Cl_2): 8.28 (d, 2H, J = 7.8 Hz); 7.41 (d, 2H, J = 7.8 Hz); 4.41 (m, 2H), 4.19 (m, 2H); 3.80 (m, 2H); 3.59 (m, 18H); 3.20 (m, 6H); 1.78 (m, 6H); 1.40 (s, 9H). ESI-MS m/z: 706.4 $(MH)^+$, $C_{31}H_{51}N_3O_{15}$ requires 706.75.

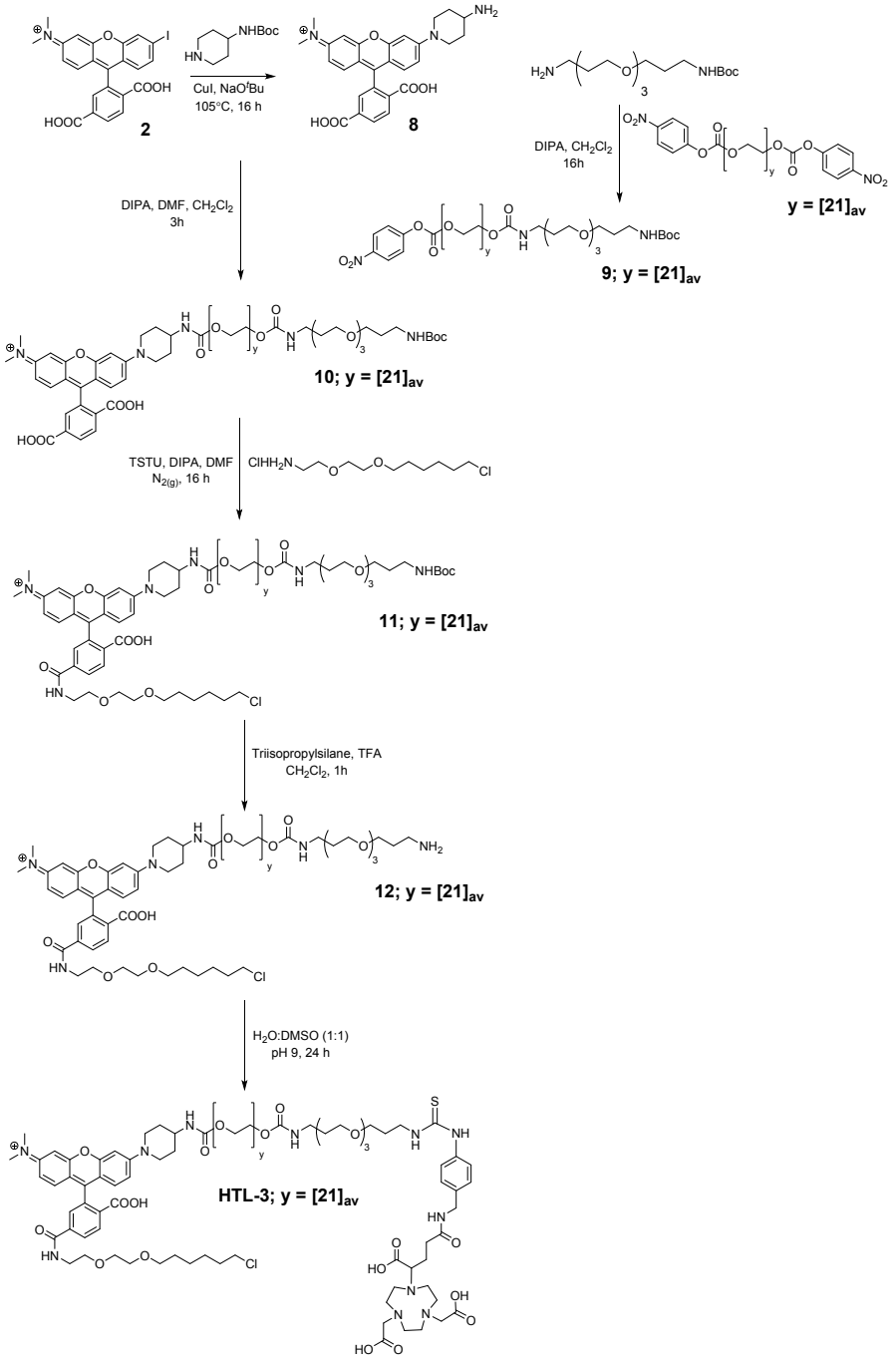
N-(9-(2,5-dicarboxyphenyl)-6-(4-(35,35-dimethyl-3,17,33-trioxo-4,7,10,13,16,22,25,28,34-nonaoxa-2,18,32-triazahexatriacontyl)piperidin-1-yl)-3H-xanthen-3-ylidene)-N-methylmethanaminium (5): Compound **3** (423 mg, 0.85 mmol) was dissolved in DMF (50 mL) and a solution of compound **4** (1.10 g, 1.56 mmol), diisopropylamine (2.0 mL, 11.1 mmol) and dichloromethane (5 mL) was added dropwise. After 3 h, the solvent was removed and the product was purified by RP-HPLC (linear gradient of 5-100% CH_3CN with H_2O+0.1%TFA over 30 minutes) to give a red oil (280 mg, 31%). ^{1}H-NMR δ_H (300 MHz; CD_3CN): 8.33 (s, 1H); 8.35 (s, 1H); 7.94 (s, 1H); 7.05 (m, 5H); 6.80 (d, 1H J = 2.3 Hz); 4.1 (m, 8H); 3.55 (m, 26 H); 3.21 (s, 6H); 3.08 (m, 10H); 1.92 (m, 4H); 1.68 (m, 4H); 1.40 (s, 9H). ESI-MS m/z: 1067.0 $(M)^+$, $C_{54}H_{76}N_5O_{17}^+$ requires 1067.2.

N-(9-(2-carboxy-5-((2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)phenyl)-6-(4-(35,35-dimethyl-3,17,33-trioxo-4,7,10,13,16,22,25,28,34-nonaoxa-2,18,32-triazahexatriacontyl)piperidin-1-yl)-3H-xanthen-3-ylidene)-N-methylmethanaminium (6): To a solution of compound **5** (720 mg, 0.67 mmol), TSTU (345 mg, 1.15 mmol) and DMF (50 mL) was added diisopropylamine (2.0 mL, 11.1 mmol) dropwise under nitrogen. After 1 h, a solution of 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine hydrochloride (275 mg, 1.06 mmol) in DMF (2 mL) was added and the reaction was stirred for a further 16 h at room temperature. The solvent was removed and the product was isolated by RP-HPLC (linear gradient of 5-100% CH_3CN with H_2O+0.1%TFA over 30 minutes) as a red oil (480 mg, 56%). ^{1}H-NMR δ_H (300 MHz; CD_3CN): 8.38 (d, 1H, J = 7.8 Hz); 8.12 (d, 1H, J 7.9 Hz); 7.79 (s, 1H); 7.50 (bs, NH); 7.05 (m, 5H); 6.81 (s, 1H); 4.21 (m, 2H); 4.10 (m, 4H); 3.60 (m, 45H); 3.21 (s, 6H); 3.05 (m, 10H); 1.86 (m, 4H); 1.70 (m, 6H); 1.39 (s, 9H). ESI-MS m/z: 1272 $(M)^+$, $C_{64}H_{96}ClN_6O_{18}^+$ requires 1272.93.

N-(6-(4-(31-amino-3,17-dioxo-4,7,10,13,16,22,25,28-octaoxa-2,18-diazahentriacontyl)piperidin-1-yl)-9-(2-carboxy-5-((2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)phenyl)-3H-xanthen-3-ylidene)-N-methylmethanaminium (7): Compound **6** (760 mg, 0.59 mmol) was dissolved in dichloromethane (50 mL) with triisopropylsilane (1 mL). Trifluoroacetic acid (7.0 mL, 91 mmol) was added and the solution was stirred at room temperature for 1 h. The solvent was removed and the product was isolated by RP-HPLC (linear gradient of 5-100% CH_3CN with H_2O+0.1%TFA over 30 minutes) as the TFA salt (610 mg, 88%). ^{1}H-NMR δ_H (300 MHz; CD_3CN): 8.39 (d, 1H, J = 7.9 Hz); 8.12 (d, 1H, J = 8.0 Hz); 7.79 (s, 1H); 7.49 (bs, NH); 7.0 (m, 6H); 6.81 (s, 1H); 4.21 (m, 2H); 4.15 (m, 4H); 3.60 (m, 45H);3.23 (s, 6H); 3.04 (m, 10H); 1.90 (m, 4H); 1.72 (m, 6H); 1.5 (m, 10H). ESI-MS m/z: 586.5 $(M)^{2+}$, $(C_{64}H_{96}ClN_6O_{18})/2^+$ requires 586.41.

Synthesis of HTL-2: To p-NCS-benzyl-NODA-GA (7 mg, 13 µmol) in deionised H_2O (500 µL) was added compound **7** (8 mg, 6.7 µmol) in DMSO (500 µL). The reaction mixture was adjusted to pH 9 with 1 M NaOH and then stirred for 24 h. The crude reaction mixture was then purified by HPLC-LCMS (Chromolith RP18e column, 100 x 4.6 mm, with 10 mM HCOOH and MeCN, running a linear gradient from 10-90% over 10 min, flow rate 1.3 mL min^{-1}, t_R = 6.5 min). The HPLC solvent was removed under vacuum to yield the product as a red oil (2 mg, 1.2 µmol, 18% yield). ^{1}H NMR δ_H (500 MHz; D_2O): 8.40 (s, 1H, NH); 8.05 (d, 1H, J = 5.0 Hz); 7.99 (d, 1H, J = 10.0 Hz); 7.70 (s, 1H); 7.23 (d, 2H, J = 5.0 Hz); 7.18 (d, 1H, J = 10.0 Hz); 7.14-7.10 (m, 2H); 6.97 (d, 1H, J = 10.0 Hz); 6.89-6.85 (m, 2H); 6.62 (s, 1H); 4.35–4.22 (m, 1H); 4.19-4.07 (m, 5H); 3.74-3.41 (m, 44H); 3.39-3.28

(m, 8H); 3.24-3.01 (m, 18H); 2.69 (s, 1H); 2.42 (t, 2H, J = 7.5 Hz); 2.19 (s, 1H); 2.11-2.06 (m, 1H); 2.03 (s, 1H, NH); 2.01-1.62 (m, 9H); 1.56-1.51 (m, 2H); 1.38-1.28 (m, 3H); 1.22-1.08 (m, 6H); 0.85 (m, 1H); HRMS (ES +ve) m/z: 1648.8004 $(M\text{-}COOH)^+$, $C_{81}H_{119}ClN_{11}O_{21}S$ requires 1648.7986; UV/Vis [λ_{max}, nm (εM, $M^{-1}cm^{-1}$)]: 555 (67,500) in H_2O.



Scheme 3: Synthesis of HaloTag ligand, HTL-3

N-(6-(4-aminopiperidin-1-yl)-9-(2,5-dicarboxyphenyl)-3H-xanthen-3-ylidene)-N-methylmethanaminium (8): Compound **2** (797 mg, 1.55 mmol), 4-(*N*-Boc-amino)piperidine (624 mg, 3.12 mmol), CuI (205 mg, 1.08 mmol) and NaO^{t}Bu (155 mg, 1.61 mmol) were added to trifluoroethanol (20 mL) and heated to 105°C in a pressure reactor with stirring. After 16 h, the reaction was cooled, filtered and rinsed with water. The solution was concentrated and purified by

RP-HPLC (linear gradient of 5-100% CH_3CN with H_2O+0.1%TFA over 30 minutes) to give a red solid (300 mg, 40%) ^{1}H-NMR δ_H (300 MHz; CD_3CN): 8.35 (m, 2H); 7.81 (m, 1H); 7.40 (m, 1H); 7.19 (m, 2H); 7.01 (m, 2H) 6.81 (s, 1H); 3.50 (m, 5H); 3.23 (s, 6H); 3.02 (m, 4H). ESI-MS *m/z*: 486.4 $(M)^+$, $C_{28}H_{28}N_3O_5^-$ requires 486.2.

Compound 9: Compound **9** was prepared in a similar manner to compound **4** using the bis-nitrophenylcarbonate prepared from a PEG1000 sample (52%). ^{1}H-NMR δ_H (300 MHz; CD_2Cl_2): 8.24 (d, 2H, *J* = 8.2 Hz); 7.41 (d, 2H, *J*= 8.2 Hz); 4.41 (m, 2H); 4.17 (bs, NH); 3.78 (m, 2H); 3.6 (m, 100H); 3.20 (m, 4H); 1.71 (m, 2H); 1.40 (s, 9H). ESI-MS *m/z*: 1455.8 ± 44 $(M)^-$, $C_{65}H_{119}N_3O_{32}^-$ requires 1454.64.

Compound 10: Compound **10** was prepared and purified in a similar manner to compound **5** (72%). ^{1}H-NMR δ_H (300 MHz; CD_3CN): 8.39 (s, 1H); 8.38 (s, 1H); 7.98 (s, 1H); 7.02 (m, 5H); 6.83 (d, 1H, *J* = 2.2 Hz); 5.70 (bs, NH); 4.23 (m, 2H); 4.11 (m, 4H); 3.6 (m, 98H); 3.23 (s, 6H); 3.12 (m, 10H); 1.90 (m, 3H); 1.68 (m, 4H); 1.41 (s, 9H).

Compound 11: Compound **11** was prepared and purified in a similar manner to compound **6** (59%). ^{1}H-NMR δ_H (300 MHz; CD_3CN): 8.39 (d, 1H, *J* = 8.0 Hz); 8.19 (d, 1H, *J* = 7.9 Hz); 7.80 (s, 1H); 7.59 (bs, NH); 7.03 (m, 5H); 6.84 (d, 1H, *J* = 2.2 Hz); 5.75 (bs, NH); 4.23 (m, 2H); 4.10 (m, 4H); 3.6 (m, 104H); 3.25 (s, 6H); 3.05 (m, 10H); 1.90 (m, 3H); 1.72 (m, 6H); 1.49 (m, 4H); 1.40 (s, 9H); 1.35 (m, 4H). ESI-MS *m/z*: 1012 ± 22 $(M)^{2+}$, $(C_{98}H_{164}ClN_6O_{35})/2^+$ requires 1010.9.

Compound 12: Compound **12** was prepared and purified in a similar manner to compound **7** (91%). ^{1}H-NMR δ_H (300 MHz; CD_3CN): 8.39 (d, 1H, *J* = 7.9 Hz); 8.15 (d, 1H, *J* = 8.0 Hz); 7.80 (s, 1H); 7.55 (bs, NH); 7.04 (m, 7H); 6.82 (s, 1H); 5.90 (bs, NH); 5.71 (bs, NH); 4.2 (m, 2H); 3.6 (m, 116H); 3.23 (s, 6H); 3.05 (m, 8H); 1.90 (m, 4H); 1.74 (m, 4H); 1.40 (m, 10H). ESI-MS *m/z*: 1930.1 $(M+H+Na)^+$, $(C_{92}H_{155}ClN_6O_{33}Na)^+$ requires 1930.02.

Synthesis of HTL-3: To p-NCS-benzyl-NODA-GA (4 mg, 5.2 µmol) in deionised H_2O (500 µL) was added compound **12** (10 mg, 5.2 µmol) in DMSO (500 µL). The reaction mixture was adjusted to pH 9 with 1 M NaOH and then stirred for 24 h. The crude reaction mixture was then purified by HPLC-LCMS (Chromolith RP18e column, 100 x 4.6mm, with 10mM HCOOH and MeCN, running a linear gradient from 30-70% over 12 min, flow rate 1.3 mL min^{-1}, t_R = 6.1 min). The HPLC solvent was removed under vacuum to yield the product as a red oil (5 mg, 2.1 µmol, 40% yield); ESI-MS *m/z*: 811 ± 14.7 $(M)^{3+}$, $(C_{115}H_{188}ClN_{11}O_{40}S)/3^+$ requires 810.42. UV/Vis [λ_{max}, nm (εM, $M^{-1}cm^{-1}$)]: 555 (71,500) in H_2O.

Radiosynthesis of ^{111}In-HTL-1, 2, and 3:

General methods

$^{111}InCl_3$ was purchased from either Perkin Elmer (provided in 0.05 M HCl) or Mallinckrodt (provided in 0.02 M HCl). HPLC was performed on a Waters Alliance 2695 separation module using a Waters XBridge BEH C_{18} column (130 Å, 3.5 µm, 4.6 × 150 mm). UV/Vis and radioactivity measurements on the HPLC were acquired using a Waters 2489 UV/Vis detector and a Ludlum gamma detector (NaI, Model 44.2) in conjunction with a Ludlum scalar ratemeter (Model 2000), respectively.

Radiosynthesis of ^{111}In-HTL-1: 15 MBq of $^{111}InCl_3$ in 0.1 M citrate buffer (10 µL, pH 5.5, Chelex-treated) was added to HTL-1 (10 µg) dissolved in 0.1 M citrate buffer (100 µL, pH 5.5, Chelex-treated). The reaction mixture was incubated at 37°C for 30 minutes and then ^{111}In-HTL-1 was separated and collected by HPLC [30:70 MeCN/H_2O (containing 0.1 % TFA) to 70:30 MeCN/H_2O over 15 minutes, t_R = 6 min]. Non-decay-corrected radiochemical yields of 46±12% were obtained. Post-purification analysis by HPLC routinely indicated >95% radiochemical purity and yielded a specific activity of 949 MBq/µmol (Fig. S2). For subsequent cell-based *in vitro* or *in vivo*

experiments, the HPLC solvent was removed under a flow of $N_{2(g)}$ and ^{111}In-HTL-1 was reconstituted in either cell culture medium or saline solution (0.9% NaCl) + 5% ethanol, respectively.

Radiosynthesis of ^{111}In-HTL-2: 20 MBq of $^{111}InCl_3$ in 0.1 M citrate buffer (10 µL, pH 5.5, Chelex-treated) was added to HTL-2 (100 µg) dissolved in 0.1 M citrate buffer (90 µL, pH 5.5, Chelex-treated). The reaction mixture was incubated at 37°C for 30 minutes and then ^{111}In-HTL-2 was separated and collected by HPLC [30:70 MeCN/H_2O (both containing 0.1 % TFA) to 70:30 MeCN/H_2O over 30 minutes, t_R = 11 min]. Non-decay-corrected radiochemical yields of 23±4% were obtained. Post-purification analysis by HPLC routinely indicated >95% radiochemical purity and yielded a specific activity of 1160 MBq/µmol (Fig. S2). For subsequent cell-based *in vitro* or *in vivo* experiments, the HPLC solvent was removed under a flow of $N_{2(g)}$ and ^{111}In-HTL-2 was reconstituted in either cell culture medium or saline solution (0.9% NaCl) + 5% ethanol, respectively.

Radiosynthesis of ^{111}In-HTL-3: 13 MBq of $^{111}InCl_3$ in 0.1 M citrate buffer (10 µL, pH 5.5, Chelex-treated) was added to HTL-2 (10 µg) dissolved in 0.1 M citrate buffer (90 µL, pH 5.5, Chelex-treated). The reaction mixture was incubated at 37°C for 30 minutes and then ^{111}In-HTL-3 was purified by size-exclusion chromatography (Sephadex G-25, PD-10 column, eluted with 500 µL fractions of PBS, pH 7.4). Non-decay-corrected radiochemical yields of 22±3% were obtained, and post-purification analysis was performed by radio-iTLC-SA (Agilent, catalogue no. A120B12) using an eluting solvent of 0.1 M citrate buffer (pH 5.5). Upon completion, the iTLC-SA strips were analysed on a Cyclone Plus Storage Phosphor System (Perkin Elmer). In these conditions, ^{111}In-HTL-3 was found to remain on the baseline, and $^{111}InCl_3$ and ^{111}In-citrate travelled with the solvent front (Fig. S2). Radiochemical purities of >95% were routinely achieved and a specific activity of 723 MBq/µmol was determined.

Log P determination

To a biphasic mixture of octan-1-ol (2 mL) and deionised water (1.995 mL) was added the relevant ^{111}In-labelled HaloTag ligand (5 µL, *ca.* 10 kBq). The mixture was vortexed thoroughly and then centrifuged (1,200 rpm for 5 min). From each layer, 100 µL was transferred to a microcentrifuge tube and the radioactivity was measured using a 2480 WIZARD^2 gamma counter (Perkin Elmer). All measurements were performed in triplicate. Mean Log P values for ^{111}In-HTL-1, ^{111}In-HTL-2, and ^{111}In-HTL-3 were -1.14±0.00, -1.05±0.02, and -1.74±0.04, respectively.

Cell culture

The human osteosarcoma cell line, U2OS, the human mammary carcinoma cell line, MDA-MB-231, and the murine mammary carcinoma cell line, 4T1, were acquired from ATCC. The HER2-tranfected cell line, MDA-MB-231/H2N, was a gift from Dr. Robert Kerbel (Sunnybrook Health Sciences Centre, Toronto, ON).[3] Each of these cell lines was maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. The HaloTag-transfected cell lines, U2OS-ECS and 4T1-ECS, were obtained from Promega Corporation and maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 0.4 mg/mL G418 (Promega). All cell lines were cultured in a 37°C environment containing 5% CO_2. Cells were harvested and passaged as required using Trypsin-EDTA solution (Sigma). Cells were tested and authenticated by the providers. The cumulative length of culture was less than 6 months following retrieval from liquid nitrogen storage.

Cell binding on U2OS-ECS and 4T1-ECS cells: Radioactivity assay

Aliquots of 2×10^5 cells (U2OS, U2OS-ECS, 4T1, or 4T1-ECS) were seeded in 24-well plates in cell culture medium (500 µL). The cells were allowed to adhere overnight and the supernatant medium was replaced with fresh cell culture medium (400 µL). The relevant ^{111}In-labelled HaloTag ligand (25 kBq in 100 µL of cell culture medium) was then added to each well and incubated at 37°C in 5% CO_2. At 0.25, 1, 4, or 24 h, the cell culture medium was removed and combined with a single wash of

phosphate buffered saline (PBS, pH 7.4, 500 μL). The remaining monolayer of cells was then incubated with 0.1 M glycine (pH 2.5, 500 μL) for 6 minutes at 4°C to remove non-covalently bound ^{111}In-labelled HaloTag ligand from the cell surface. The glycine was then removed and combined with a single wash of cold 0.1 M glycine (pH 2.5, 500 μL). Lastly, the cells were lysed with 0.1 M sodium hydroxide for 20 minutes at room temperature. The amount of radioactivity contained within the cell culture medium, the glycine wash, and the cell lysate fractions were measured using a gamma counter. For blocking studies, the cells were pre-incubated with a commercially available HaloTag TMR ligand (5 μM; Promega, catalogue no. G8251) in cell culture medium (500 μL) for 24 h prior to incubation with the ^{111}In-labelled HaloTag ligands. These experiments were performed in triplicate on at least three separate occasions.

Cell binding on U2OS-ECS and 4T1-ECS cells: Confocal Microscopy

Aliquots of 5×10^4 cells (U2OS, U2OS-ECS, 4T1, or 4T1-ECS) were seeded in 0.5 mL of cell culture medium in an 8-well Falcon CultureSlide (BD Bioscience). The cells were allowed to adhere overnight and the supernatant medium was replaced with fresh cell culture medium (400 μL). The relevant HaloTag ligand (25 μM in 100 μL of cell culture medium) was then added to each well to give a final HaloTag ligand concentration of 5 μM. The cells were incubated at 37°C in 5% CO_2. At 0.25, 1, 4, or 24 h, the cell culture medium was removed, the remaining monolayer of cells was washed with (PBS, pH 7.4, 500 μL) and the cells were then fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. The cells were then mounted using Vectashield mounting medium with DAPI (Vector labs, Peterborough, UK). Confocal microscopy images were acquired using a Zeiss 530 microscope (Zeiss, Welwyn Garden City, UK).

Non-specific protein binding of ^{111}In-HTL-1, 2, and 3

In vitro protein binding in (i) cell culture medium supplemented with 10% fetal bovine serum (FBS), and (ii) FBS:

In triplicate, 5 μL of the relevant ^{111}In-labelled HaloTag ligand (60 kBq) in 0.1 M sodium citrate buffer (pH 5.5) was added to 495 μL of either (*i*) cell culture medium supplemented with 10% FBS, or (*ii*) FBS, and the samples were incubated at 37°C. At 0.25, 1, or 24 h, ice-cold methanol (1 mL) or CH_3CN+0.1% TFA (1 mL) was added to the FBS and cell culture medium samples, respectively. The samples were vortexed thoroughly then placed at -20°C for 2 h. The precipitated proteins were pelleted by centrifugation (13,000 rpm for 5 min) and the supernatant liquid was transferred to a separate microcentrifuge tube. The amount of radioactivity in both the precipitated protein fraction and the supernatant fraction was measured using a gamma counter.

In vivo protein binding in mouse blood:

BALB/c mice were injected with 0.44-1.22 MBq of the relevant ^{111}In-labelled HaloTag ligand *via* tail vein. At 1 h p.i., the mice were euthanized by intraperitoneal injection of sodium pentobarbital (Euthatal; Merial Animal Health Ltd.). A blood sample (*ca.* 0.25 mL) was collected from the heart using a needle and syringe which had been pre-rinsed with a 1xEDTA solution and then transferred to a heparin-coated vial. The blood sample was centrifuged (13,000 rpm for 5 min) to pellet the blood cells, and the supernatant serum was transferred to a separate microcentrifuge tube. To the serum was added ice-cold methanol (1 mL) and the mixture was briefly vortexed. The precipitated proteins were pelleted by centrifugation (13,000 rpm for 5 min) and the supernatant serum was then transferred to a separate microcentrifuge tube. The amount of radioactivity in each fraction was measured using a gamma counter.

Animal model

All animal procedures were carried out in accordance with the UK animals (Scientific Procedures) Act 1986 and with local ethical committee approval. 4T1 or 4T1-ECS tumours were established in

female BALB/c mice (Charles River, UK) by subcutaneous injection of 10^6 cells in DMEM into the right flank. Subsequent SPECT/CT imaging and *ex vivo* biodistribution experiments were performed when the tumours had reached a diameter of approximately 10 mm.

Small animal SPECT/CT images and analysis

For both the 4T1 and 4T1-ECS tumour models, groups of 3-4 mice received approximately 2 MBq of either ^{111}In-HTL-1, ^{111}In-HTL-2, or ^{111}In-HTL-3. In some cases, a 100-fold molar excess of cold, unlabelled HaloTag ligand (HTL-1, HTL-2, or HTL-3, respectively) was co-injected as a blocking agent. Static SPECT-CT images were acquired at 3 h and 24 h p.i. with a nanoSPECT/CT scanner (Bioscan). Throughout the scanning procedure, mice were kept under anaesthesia by inhalation of 2% isofluorane. SPECT images were analysed using Inveon Research Workplace software (Siemens).

***Ex vivo* biodistribution data**

At 24 h p.i., mice were euthanized by cervical dislocation and selected organs, tissues and blood were removed. The samples were immediately rinsed with water, dried, and transferred into a pre-weighed counting tube. After weighing the filled counting tubes, the amount of radioactivity in each was measured using a 1470 WIZARD gamma counter (Perkin Elmer). Counts per minute were converted into radioactivity units (MBq) using a calibration curve generated from known standards. These values were decay-corrected to the time of injection, and the percentage of the injected dose per gram (%ID/g) of each sample was calculated.

HaloTag protein expression

The 6HisHalo_Control plasmid (Promega), encodes for the His_6HaloTag fusion protein, and was used to transform BL21 (DE3) cells (New England Biolabs), and generate an over-night mini-culture from a single colony. Terrific Broth (1000 mL) supplemented with 50 μg/mL ampicillin was seeded with a Halo-Tag mini-culture (1 mL) and allowed to grow at 37°C to an OD_{600} of between 1.0 - 2.0, at which point peptide expression was induced with 1 mM IPTG. The culture was continued over-night at 25°C, was centrifuged at 4,000xg for 15 minutes at 4°C, and the pellet re-suspended in 180 mL lysis buffer (100mM HEPES, pH 7.5, 500 mM NaCl, 0.1 mg/mL lysozyme, 50 units RQ1 DNase). Lysis was continued by incubation at room temperature for 45 minutes followed by sonication on ice using a Sonics Vibracell sonicator equipped with a 6 mm sonicating probe (3 x 5 second pulses at 50% amplitude, repeated for 6 minutes). Insoluble debris was pelleted by centrifugation at 9,000xg for 20 minutes, and the soluble fraction filtered through a 0.8 μm filter.

His_6HaloTag protein was isolated on a 15 mL bedded-volume HisLink column (Promega). The HisLink matrix was re-suspended and incubated for 10 minutes at 4°C in 10 mL buffer (100 mM HEPES, pH 7.5, 100 mM NaCl, 500 mM imidazole) after sealing the column. The eluent was then collected and the elution process repeated with a further 20 mL of buffer (100mM HEPES, pH 7.5, 100 mM NaCl, 500 mM imidazole). The eluent was filtered through a 0.8 μm filter and dialyzed in a 10 kDa MWCO Slide-A-Lyzer Dialysis Cassette against several large volumes of PBS buffer at 4°C. The isolated His_6HaloTag was validated by UV spectrophotometry and 12% Bis-Tris PAGE (Novex).

Trastuzumab-HaloTag conjugation

Trastuzumab-HaloTag conjugate protein was prepared using a Click&Go™ Protein-Protein Conjugation Kit (Click Chemistry Tools, catalogue no. 1008) using the manufacturer's instructions.

IC_{50} determination

The 50% inhibitory concentration (IC_{50}) of the Trastuzumab-HaloTag conjugate protein for binding the HER2 receptor was determined on MDA-MB-231/H2N cells. Cells were grown to confluence in 12-well plates. Cells were washed with PBS and then incubated at room temperature for 10 minutes in binding buffer (DMEM supplemented with 0.5% *w/v* FBS). A trace amount of 1 kBq of ^{111}In-DTPA-

Trastuzumab was added, followed by increasing concentrations of Trastuzumab-HaloTag conjugate protein (0.001 – 100 nM). After incubation for 2 h at 4°C, binding buffer was removed, cells were washed twice with PBS and cell-associated radioactivity was determined using a gamma counter. IC_{50} values were calculated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The trastuzumab-HaloTag conjugate protein yielded an IC_{50} value of 56 nM.

Pretargeting experiments: Radioactivity assay

Aliquots of 2×10^5 cells (MDA-MB-231 or MDA-MB-231/H2N) were seeded in 24-well plates in warm cell culture medium (500 µL). The cells were allowed to adhere overnight, and the old medium was replaced with fresh cell culture medium (270 µL). Either unmodified HaloTag protein, unmodified Trastuzumab, Trastuzumab-HaloTag conjugate, or non-specific rabbit IgG-HaloTag conjugate (2 µM, 30 µL) were added to yield a final concentration of 200 nM. The cells were incubated at 37°C in 5% CO_2 for 30 minutes at room temperature. The cell culture medium was removed and the cells were washed twice with PBS (pH 7.4, 500 µL). Fresh cell culture medium (400 µL) was added to each well and the relevant ^{111}In-labelled HaloTag ligand (25 kBq) in cell culture medium (100 µL) was then added. The cells were incubated for a further 30 minutes at room temperature. The cell culture medium was removed and combined with two washes of PBS (pH 7.4, 500 µL). The remaining monolayer of cells was then lysed with 0.1 M sodium hydroxide for 20 minutes at room temperature. The amount of radioactivity contained within the cell culture medium and the cell lysate fractions were measured using a gamma counter. All experiments were performed in triplicate on at least three separate occasions.

Pretargeting experiments: Confocal Microscopy

Aliquots of 5×10^4 cells (U2OS, U2OS-ECS, 4T1, or 4T1-ECS) were seeded in 0.5 mL of cell culture medium in an 8-well Falcon CultureSlide (BD Bioscience). The cells were allowed to adhere overnight and the medium was replaced with fresh cell culture medium (270 µL). Unmodified HaloTag protein, unmodified Trastuzumab, or Trastuzumab-HaloTag conjugate (2 µM in 30 µL of cell culture medium) was then added to yield a final concentration of 200 nM. The cells were incubated at 37°C in 5% CO_2 for 30 minutes. The cell culture medium was removed and the remaining monolayer of cells was washed twice with PBS (pH 7.4, 500 µL). Fresh cell culture medium (400 µL) was added to each well. The relevant HaloTag ligand (25 µM in 100 µL of cell culture medium) was added to each well to give a final HaloTag ligand concentration of 5 µM. The cells were incubated at 37°C in 5% CO_2. The cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. The cells were mounted using Vectashield mounting medium with DAPI. Confocal microscopy images were acquired using a Zeiss 530 microscope.

Statistical analyses

All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). 1-way ANOVA with Tukey post-tests was used for multiple comparisons.

Supplemental Figures

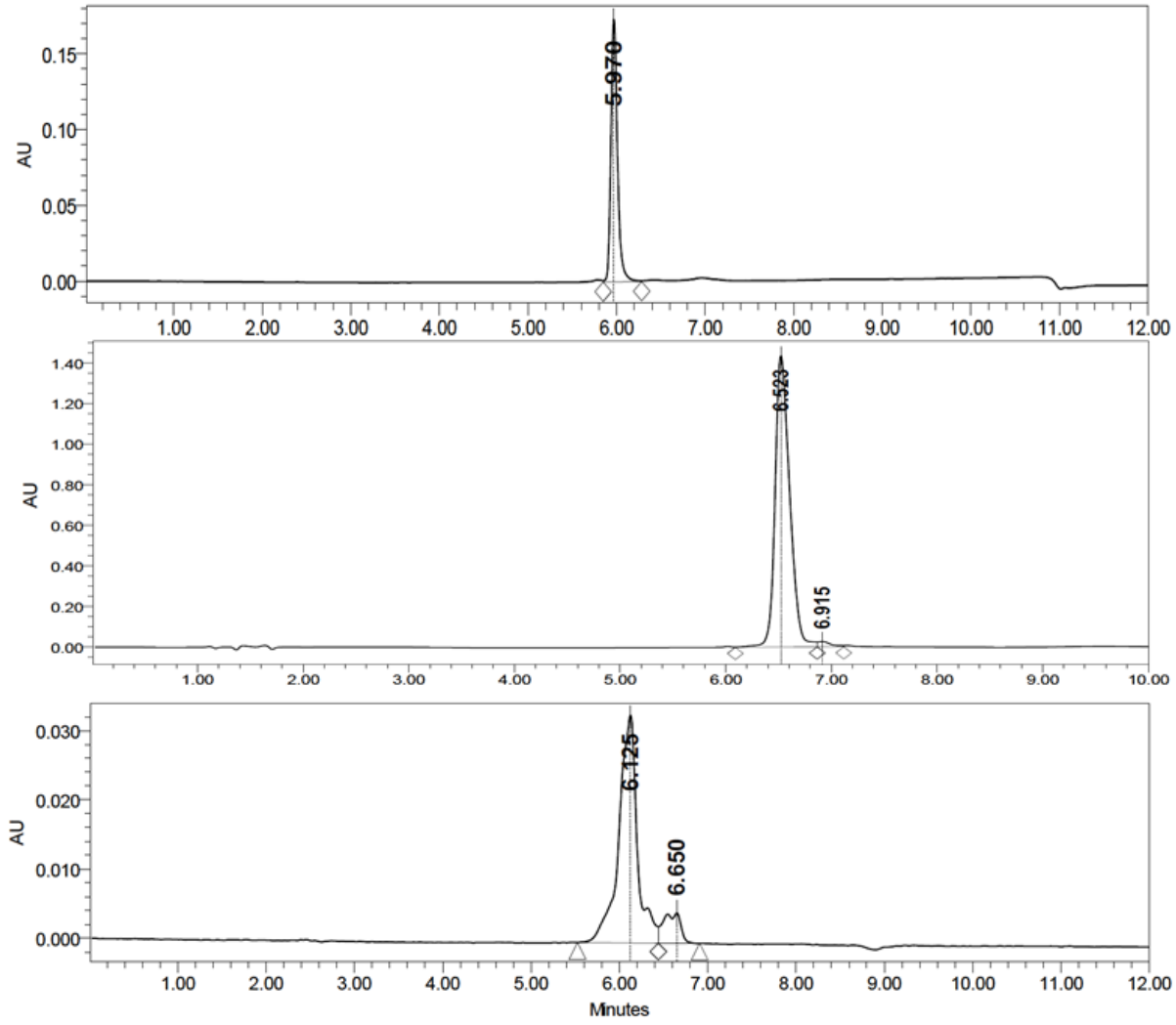


Figure S1. HPLC chromatograms of HTL-1 (top), HTL-2 (middle), and HTL-3 (bottom)

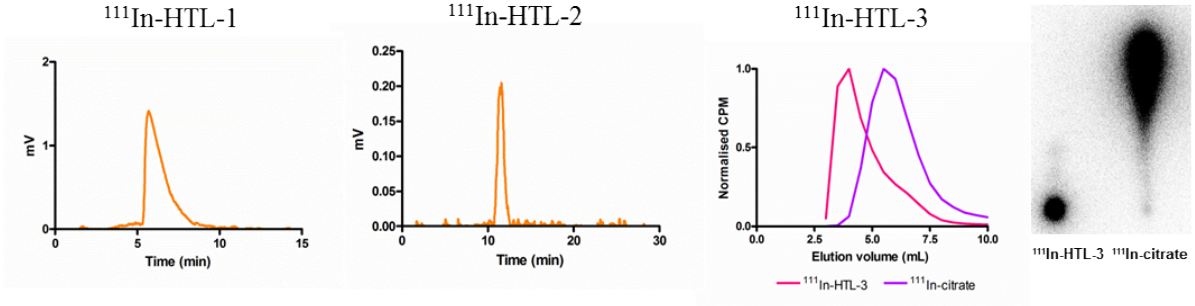


Figure S2. Radio-HPLC chromatograms of ^{111}In-HTL-1 (left) and ^{111}In-HTL-2 (middle), and size exclusion chromatography (Sephadex G25) of ^{111}In-HTL-3 alongside a radio-TLC of the purified product (right).

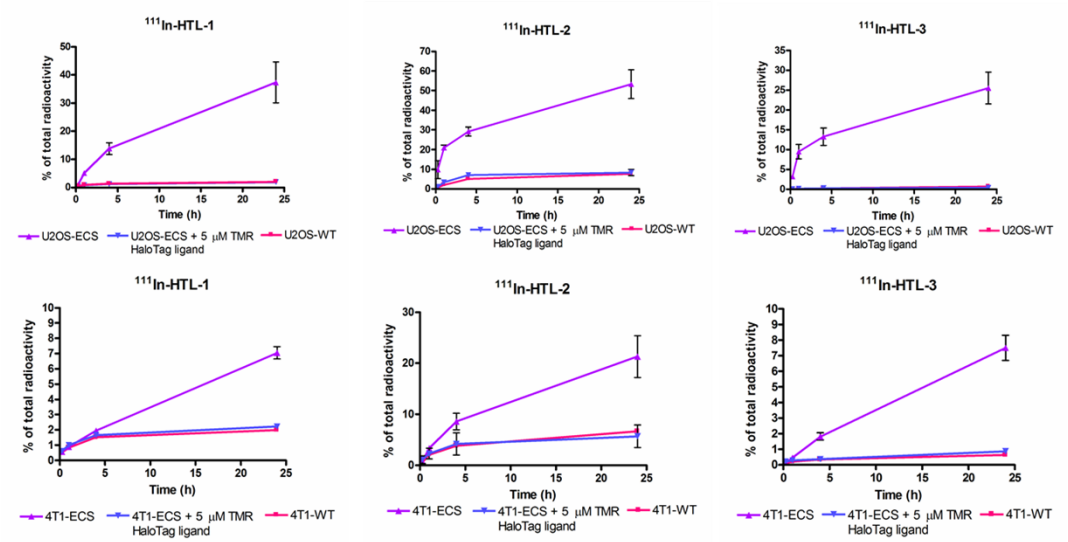


Figure S3. The ability of these novel ^{111}In-labelled HaloTag ligands to specifically bind HaloTag protein expressed on the surface of both U2OS-ECS (top row) and 4T1-ECS (bottom row) cancer cells was confirmed by assaying cell-bound radioactivity. High specific binding to cells expressing HaloTag was observed which increased over time.

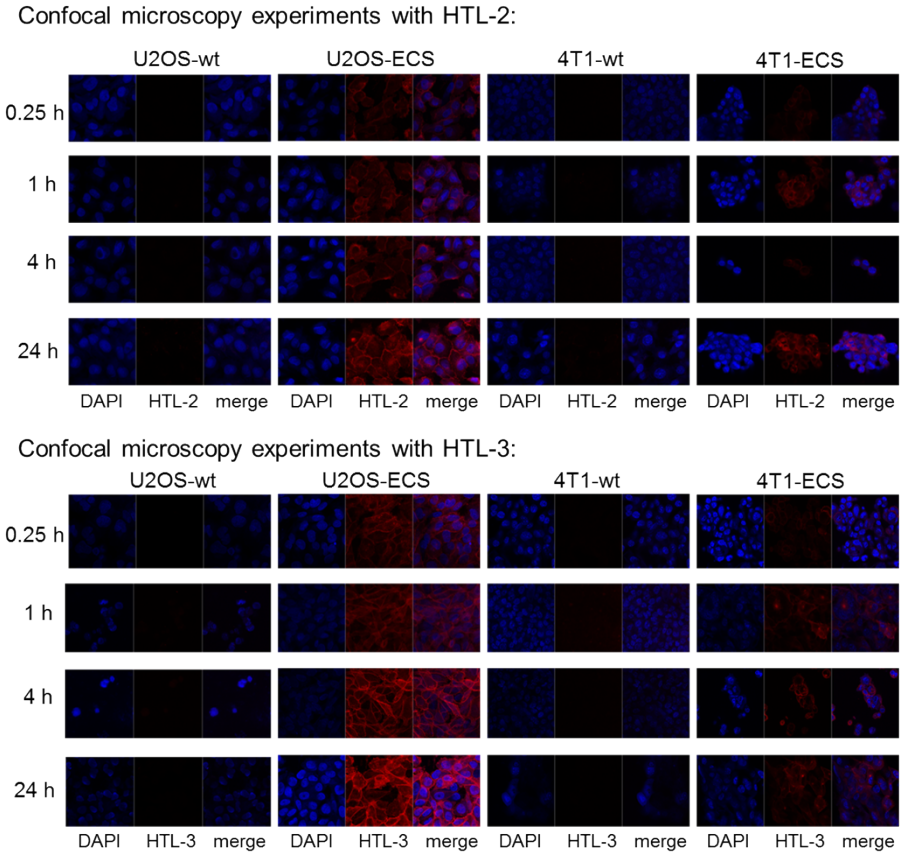


Figure S4. The ability of the fluorescent TMR-containing HaloTag ligands (HTL-2 and HTL-3; red) to bind to cells transfected with HaloTag on the extracellular surface (ECS) was confirmed by confocal microscopy. Higher HaloTag expression on U2OS-ECS versus 4T1-ECS cells was reflected by higher fluorescence intensity. For both HTL-2 and HTL-3, the fluorescence intensity increased with longer incubation times on both of the HaloTag transfected cell lines.

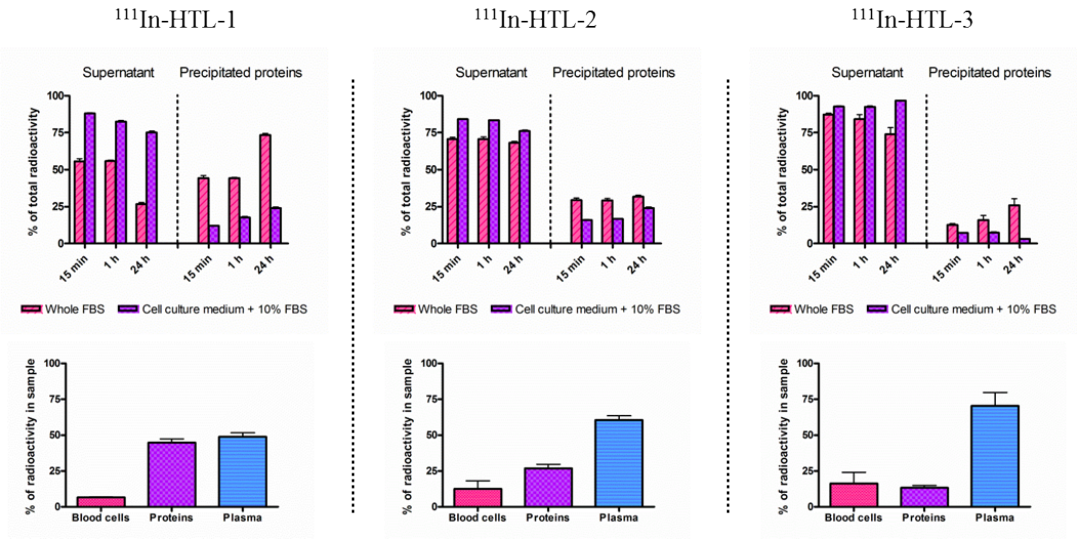


Figure S5. Non-specific protein binding of ^{111}In-HTL-1, 2, and 3. Top row: *In vitro* protein binding in whole FBS and cell culture medium supplemented with 10% FBS. Bottom row: Distribution of radioactivity in mouse blood at 1 h post-injection.

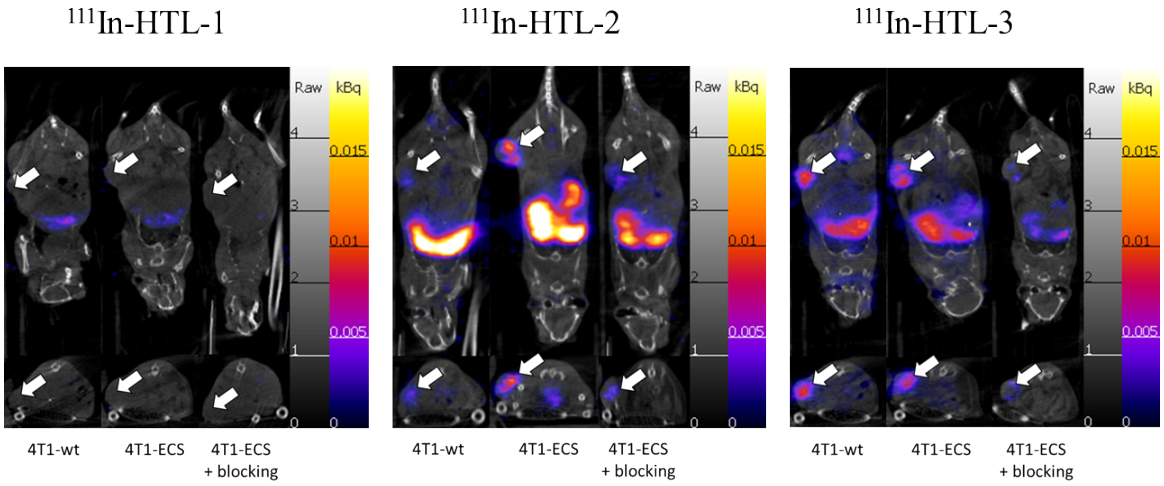


Figure S6. Small animal SPECT/CT imaging experiments were performed using BALB/c mice bearing either wild-type 4T1 or 4T1-ECS (HaloTag positive) subcutaneous tumours (white arrows). Images were acquired at 24 h p.i. of the ^{111}In-labelled HaloTag ligand. Coronal (top) and transverse (bottom) planar images intersect the centre of the tumour.

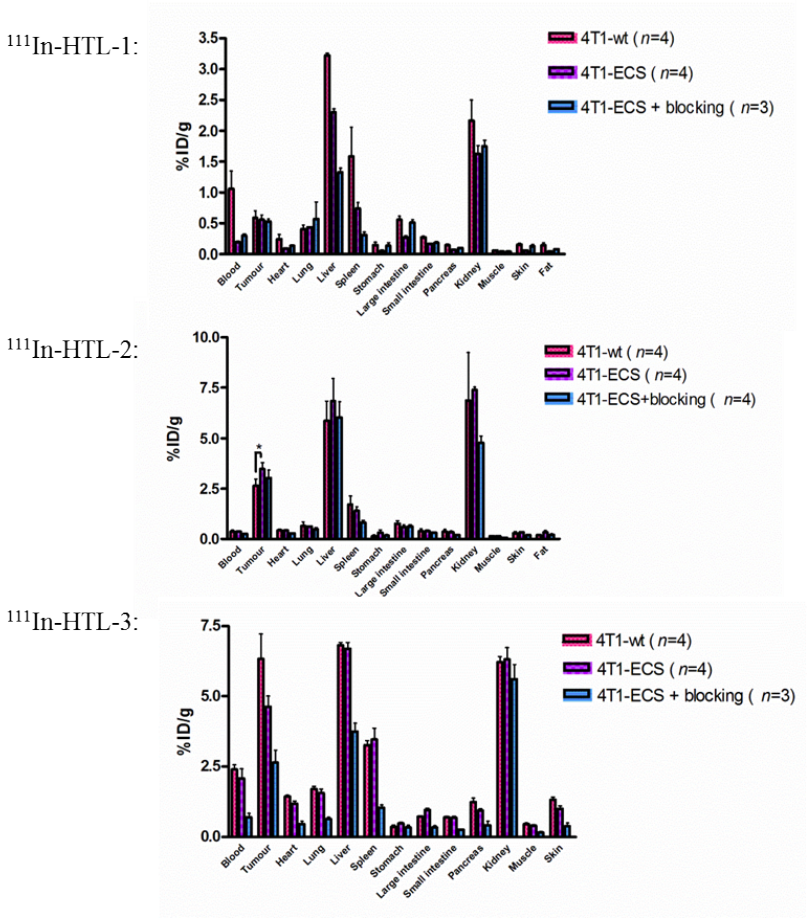


Figure S7. *Ex vivo* biodistribution experiments were performed at 24 h post-injection. In the case of ^{111}In-HTL-2, significantly higher uptake was observed for the HaloTag transfected tumour compared with the wild-type tumour (3.49±0.58 and 2.64±0.34 %ID/g, respectively; $P<0.05$).

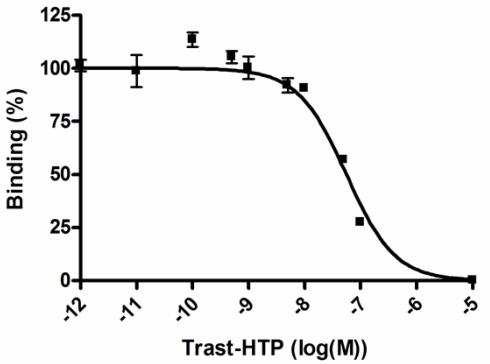


Figure S8. IC_{50} determination of Trastuzumab-HaloTag conjugate protein on MDA-MB-231/H2N cells. ^{111}In-Bn-DTPA-Trastuzumab was used as radioactive tracer.

References

1. A. R. Yeager, N. S. Finney, J. Org. Chem., 2004, **69**, 613-618.
2. WO pat., 2013134686 A1, 2013.
3. J. M. du Manoir, G. Francia, S. Man, M. Mossoba, J. A. Medin, A. Viloria-Petit, D. J. Hicklin, U. Emmenegger, R. S. Kerbel, Clin. Cancer Res., 2006, **12**, 904-916.