**Electronic Supplementary Information**

# Contents

General Experimental ..... 2
Procedures & Characterisation ..... 3
$H_3$DFOSqOEt ..... 3
$H_3$DFOSqTaur ..... 4
$^{nat}$ZrDFOSqTaur ..... 5
$H_3$DFO-p-Ph-$SO_3H$ ..... 6
$H_3$DFOSq-trastuzumab ..... 6
$^{89}$Zr-DFOSq-trastuzumab ..... 6
DFO-p-Ph-NCS-trastuzumab & $^{89}$Zr-DFO-p-Ph-NCS-trastuzumab ..... 9
Competition study by radio-iTLC (EDTA challenges) ..... 10
MicroPET Imaging ..... 11
$^{89}$ZrDFOSq-trastuzumab in SKOV3 xenograft-bearing mice ..... 11
Mouse 1/3 ..... 11
Mouse 2/3 ..... 12
Mouse 3/3 ..... 12
$^{89}$ZrDFO-p-Ph-NCS-trastuzumab in SKOV3 xenograft-bearing mice ..... 13
Mouse 1/3 ..... 13
Mouse 2/3 ..... 13
Mouse 3/3 ..... 13
$^{89}$ZrDFOSq-trastuzumab in BT474 xenograft-bearing mice ..... 15
Mouse 1/2 ..... 15
Mouse 2/2 ..... 16
$^{89}$ZrDFO-p-Ph-NCS-trastuzumab in BT474 xenograft-bearing mice ..... 17
Mouse 1/4 ..... 17
Mouse 2/4 ..... 17
Mouse 3/4 ..... 18
Mouse 4/4 ..... 18
$^{89}$ZrDFOSq-trastuzumab in LS174T xenograft-bearing mice ..... 19
Mouse 1/3 ..... 19
Mouse 2/3 ..... 19
Mouse 3/3 ..... 20

## General Experimental

All reagents and solvents were obtained from standard commercial sources and unless otherwise stated were used as received. $^{1}H$ and $^{13}C$ spectra were recorded with a Varian FT-NMR 500, Varian FT-NMR 600, or Bruker Avance III HD 700 spectrometer. $^{1}H$-NMR spectra were acquired at 500, 600 or 700 MHz and $^{13}C$-NMR spectra were acquired at 125 or 175 MHz. All NMR spectra were recorded at 25°C unless otherwise stated. The reported chemical shifts (in parts per million) are referenced relative to residual solvent signal, except for $D_2O$ solutions which were spiked with acetone and referenced accordingly. ESI-MS were recorded on an Agilent 6510 ESI-TOF LC/MS Mass Spectrometer (Agilent, California USA). Analytical reverse phase HPLC were performed on an Agilent 1100 Series. Protein samples were analysed using an Agilent 6220 ESI-TOF LC/MS Mass Spectrometer coupled to an Agilent 1200 LC system (Agilent, Palo Alto, CA). All data were acquired and reference mass corrected via a dual-spray electrospray ionisation (ESI) source. Acquisition was performed using the Agilent Mass Hunter Acquisition software version B.02.01 (B2116.30). Ionisation mode: Electrospray Ionisation; Drying gas flow: 7 L/min; Nebuliser: 35 psi; Drying gas temperature: 325°C; Capillary Voltage (Vcap): 4000 V; Fragmentor: 300 V; Skimmer: 65 V; OCT RFV: 250 V; Scan range acquired: 300–3200 m/z Internal Reference ions: Positive Ion Mode = m/z = 121.050873 & 922.009798. Protein desalting and chromatographic separation was performed using an Agilent Poroshell C18 2.1 x 75 mm, 5μm column using 5% (v/v) acetonitrile ported to waste (0-5min). Upon desalting of sample the flow was ported back into the ESI source for subsequent gradient elution with (5% (v/v) to 100% (v/v)) acetonitrile / 0.1% formic acid over 8 min at 0.25 mL/min. Analysis was performed using Mass Hunter version B.06.00 with BioConfirm software using the maximum entropy protein

deconvolution algorithm; mass step 1 Da; Baseline factor 3.00; peak width set to uncertainty. For analysis of radioactive samples, size exclusion HPLC was performed on a Shimadzu SCL-10A VP/LC-10 AT VP system with a Shimadzu SPD-10A VP UV detector followed by a radiation detector (Ortec model 276 photomultiplier base with preamplifier, Ortec 925-SCINT ACE mate preamplifier, BIAS supply and SCA, Bicron 1M 11/2 photomultiplier tube). A Biosuite 125 HR SEC 5 μm 7.8 x 300 mm column was used with a flow rate of 0.6 mL/min and Dulbecco's PBS with 5% isopropanol as eluent. Radio-iTLC were analysed using a Raytest Rita-Star TLC scanner.

For HPLC purification and analysis of non-radioactive samples, solvent A = 0.5 % TFA in MilliQ, solvent B = 0.5% TFA in $CH_3CN$.

SKOV-3, BT-474 and LS174T tumour bearing mice were injected intravenously via a tail vein with $^{89}$Zr-labelled trastuzumab. At 24 h, 48 h, 96 h and/or 8 days post-injection the animals were anaesthetised in 2.5% isoflurane and 50% O2 in air and placed on the bed of a Philips Mosaic small animal PET scanner. The images were reconstructed using a 3D RAMLA algorithm as described previously. (D. S. Dorow, C. Cullinane, N. Conus, P. Roselt, D. Binns, T. J. McCarthy, G. A. McArthur and R. J. Hicks, Eur. J. Nucl. Med. Mol. Imaging, 2006, 33, 441–452) Quantification was performed using software developed in-house (MARVn). (M. E. Trinkaus, R. Blum, D. Rischin, J. Callahan, M. Bressel, T. Segard, P. Roselt, P. Eu, D. Binns, M. P. MacManus, D. Ball and R. J. Hicks, J. Med. Imaging Radiat. Oncol., 2013, 57, 475–481) All data are presented as mean ± standard error.

## Procedures & Characterisation

### *$H_3$DFOSqOEt*

A mixture of Desferrioxamine B mesylate (0.20 g, 0.31 mmol) and N,N-diisopropylethylamine (0.05 mL, 0.3 mmol) was stirred in ethanol (6 mL) at 50°C. After 1 h, 3,4-diethoxy-3-cyclobutene-1,2-dione (0.1 mL, 0.7 mmol) in ethanol (9 mL) was added.

After a further 30 mins of stirring at 50°C the solvent was removed under reduced pressure, and the residue triturated with ethanol (3 x 10 mL). The product was dried *in vacuo* to give $H_3$DFOSqOEt as a white powder (0.17 g, 83%). $^1$H NMR ($d_6$-DMSO, 500 MHz) δ 9.61 (s, 6H), 8.77 (t, J = 5.8 Hz, 1H), 8.58 (t, J = 5.7 Hz, 1H), 7.77 (d, J = 4.7 Hz, 5H), 4.64 (p, J = 6.9 Hz, 5H), 3.45 (t, J = 7.0 Hz, 3H), 3.38 (s, 5H), 3.26 (dd, J = 13.0, 6.6 Hz, 1H), 3.00 (dd, J = 12.7, 6.4 Hz, 10H), 2.56 (d, J = 6.5 Hz, 1H), 2.26 (t, J = 7.2 Hz, 1H), 1.96 (s, 7H), 1.50 (d, J = 6.6 Hz, 3H), 1.42 – 1.31 (m, 3H), 1.24 (ddd, J = 20.2, 14.7, 8.0 Hz, 2H); $^{13}$C NMR ($d_6$-DMSO, 101 MHz) δ 189.4, 189.3, 182.0, 181.8, 176.9, 176.5, 172.6, 172.2, 172.00, 171.3, 170.1, 70.2, 68.8, 68.7, 47.1, 47.0, 46.8, 43.7, 43.4, 39.5, 38.4, 30.1, 29.9, 29.6, 28.8, 27.6, 26.0, 25.8, 23.5, 22.9, 20.3, 15.6; ESI MS [M+H$^+$]: 685.3768, calculated for $(C_{31}H_{53}N_6O_{11})^+$: 685.3767, [M+Na$^+$]: 707.3589, calculated for $(C_{31}H_{52}NaN_6O_{11})^+$: 707.3586.

### *$H_3$DFOSqTaur*

$H_3$DFOSqOEt (124 mg, 0.181 mmol) and a large excess of taurine (227 mg, 1.81 mmol) were stirred in a mixture of ethanol/H2O (1:1), and N,N-diisopropylethylamine (204 μL, 1.81 mmol) was added. The mixture was stirred for 6 hours at 60°C, the ethanol removed in vacuo and the aqueous solution filtered. $H_3$DFOSqTaur was then purified by semi-preperative HPLC (Phenomenex Synergi Max RP 4.6 x mm column, 5-25 % solvent B over 25 minutes) to give a white powder (12.4 mg, 10 %). ESI MS [M+H+]: 764.3411, calculated for $(C_{31}H_{54}N_7O_{13}S)^+$: 764.3495. $^1$H NMR (500 MHz, dmso) δ 10.99 (br s, 1H), 7.83 – 7.74 (m, 1H), 3.78 (br s, 1H), 3.51 – 3.39 (m, J = 14.7, 7.6 Hz, 1H), 3.36 – 3.27 (m, J = 11.8, 4.6 Hz, 1H), 3.13 – 3.04 (m, J = 16.0, 8.7 Hz, 1H), 3.04 – 2.93 (m, J = 12.4, 6.7 Hz, 1H), 2.73 (dd, J = 12.4, 6.1 Hz, 1H), 2.60 (s, 1H), 2.59 – 2.51 (m, J = 6.4 Hz, 1H), 2.41 (s, 1H), 2.31 – 2.22 (m, 1H), 1.65 – 1.55 (m, J = 13.7, 7.6 Hz, 1H), 1.55 – 1.42 (m, 1H), 1.37 (dt, J = 14.7, 7.3 Hz, 1H), 1.33 – 1.12 (m, 1H). $^{13}$C NMR (176 MHz, DMSO) δ 182.53, 182.28, 173.91, 173.63,

172.06, 172.02, 171.43, 170.83, 170.21, 167.82, 167.52, 158.77, 158.55, 158.33, 158.12, 117.63, 115.99, 114.35, 112.71, 51.78, 50.26, 50.20, 47.13, 47.07, 46.84, 43.21, 42.95, 40.33, 38.48, 38.15, 30.73, 30.44, 30.38, 30.16, 30.06, 29.98, 29.25, 28.84, 28.62, 28.59, 27.64, 26.07, 26.00, 23.54, 23.17, 23.15, 23.06, 22.99, 22.76, 20.38.

### *ZrDFOSqTaur*

ZrDFOSqTaur for NMR analysis was produced by direct addition of 1 eq $[ZrCl_4(THF)_2]$ in $d_6$-DMSO to the NMR sample of the ligand, and confirmed by ESI MS $[M^+]$: 850.2231, calculated for $(C_{31}H_{50}N_7O_{13}SZr)^+$: 850.2229. $^1$H NMR (700 MHz, DMSO) δ 11.55 – 11.44 (m, *J* = 23.5 Hz, 2H), 11.42 (s, 2H), 8.40 – 8.21 (m, 2H), 8.23 – 8.02 (m, 4H), 7.96 – 7.81 (m, 3H), 4.84 (s, 42H), 3.74 (s, 5H), 3.67 – 3.60 (m, 5H), 3.58 (t, *J* = 5.5 Hz, 13H), 3.49 – 3.44 (m, *J* = 6.0 Hz, 3H), 3.34 – 3.27 (m, 1H), 3.08 (s, 2H), 3.06 – 2.96 (m, 9H), 2.77 – 2.72 (m, 6H), 2.68 – 2.61 (m, 2H), 2.60 (s, 2H), 2.58 (s, 1H), 2.51 – 2.49 (m, *J* = 1.4 Hz, 4H), 2.47 – 2.42 (m, *J* = 5.4 Hz, 1H), 2.42 – 2.36 (m, *J* = 9.7 Hz, 4H), 2.32 – 2.21 (m, *J* = 19.1, 12.5 Hz, 3H), 2.17 – 2.09 (m, 3H), 2.06 (s, 3H), 1.97 – 1.93 (m, 1H), 1.75 – 1.71 (m, 14H), 1.68 – 1.58 (m, *J* = 30.7, 15.4 Hz, 9H), 1.56 – 1.47 (m, 7H), 1.41 – 1.34 (m, 9H), 1.34 – 1.23 (m, 8H). (Note: all $^1$H signals are broad). $^{13}$C NMR (151 MHz, DMSO) δ 182.43, 182.17, 182.03, 177.86, 173.85, 173.57, 172.06, 171.66, 171.00, 170.62, 170.57, 170.19, 168.07, 167.89, 167.80, 165.11, 164.21, 163.12, 158.67, 158.42, 158.16, 157.91, 116.08, 114.17, 67.11, 52.05, 50.72, 50.42, 50.16, 50.10, 50.04, 43.16, 43.04, 40.34, 38.64, 38.45, 38.27, 37.73, 37.62, 37.47, 30.75, 30.45, 30.19, 29.40, 28.94, 28.89, 28.81, 28.59, 28.56, 28.37, 28.11, 26.75, 26.48, 26.42, 26.35, 25.20, 24.69, 24.64, 24.34, 24.23, 23.47, 23.35, 23.26, 23.23, 23.12, 22.92, 22.87, 22.72, 22.67, 16.40.

### *$H_3$DFO-p-Ph-$SO_3$H*

DFO-*p*-Ph-$SO_3$H was prepared from $H_3$DFO mesylate and 4-isothiocyanatobenzenesulfonic acid using analogous procedures to the synthesis of $H_3$DFOSqTaur. ESI MS [M+$H^+$]: 776.3318, calculated for $(C_{32}H_{54}N_7O_{11}S_2)^+$: 776.3317

### *$H_3$DFOSq-trastuzumab*

A solution of $H_3$DFOSqOEt (0.455 mg, 20 eq) in DMSO/$H_2O$ was added to a solution of trastuzumab (5 mg) in pH 9 borate buffer (0.5 M) to give a total volume of 1 mL. The reaction mixture was allowed to stand at room temperature overnight, and was then filtered using Amicon 50 kDa centrifuge filters. The crude product was washed on the filter with NaCl solution (0.9% w/v, 4 × 400 μL) and the concentrate collected to give $H_3$DFOSq-trastuzumab (56 mg/mL). The product was analysed by LC-MS, which indicated a mixture of trastuzumab with 2-7 chelators, and an average of 4.5 chelators/mAb.

### *$^{89}$Zr-DFOSq-trastuzumab*

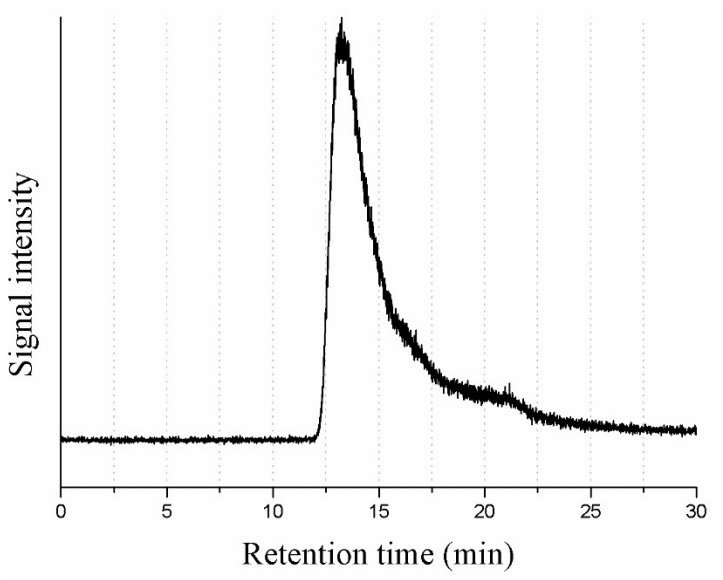
Aqueous $Na_2CO_3$ (25 uL, 2 M) was added to a solution of $^{89}$Zr in 1 M oxalic acid (75 μL, ca. 56 MBq) until the pH was slightly basic. HEPES buffer (100 μL, 0.5 M, pH 7.0) was then added and the solution allowed to stand for 5 min. Neutral pH was confirmed, and then a solution of $H_3$DFOSq-trastuzumab (4 μL, 225 ug in saline) was added. Reaction progress was monitored by radio-iTLC (Silica infused glass fiber plate, 20 mM pH 5 citrate buffer, product $R_f$ = 0).

After 25 mins reaction time, the crude $^{89}$ZrDFOSq-trastuzumab was purified on a PD-10 size exclusion column using pH 7 Dulbecco's PBS (20 mM, with 5% sodium gentisate) as eluent. After column loading, flow through was discarded and three fractions were collected (Fraction 1: 1.5 mL, 45 MBq, Fraction 2: 1.0 mL, 11 MBq, Fraction 3: 1.5 mL, 1 MBq).

Fraction 1 was analysed by radio-iTLC and radio-SEHPLC (Figure 1a, product retention time 12.55 min).

Two doses (200 μL, 6.0 MBq each) of $^{89}$ZrDFOSq-trastuzumab in PBS with 5% sodium gentisate (prefiltered for injection using a MF Millipore membrane MCE Cathivex GS 0.22 μm filter unit) were prepared and administered to BT474 tumour-bearing mice via tail vein injection. PET images were taken at intervals of approximately 24 h, 48 h 96 h and 8 days post administration.

(a)


Signal intensity
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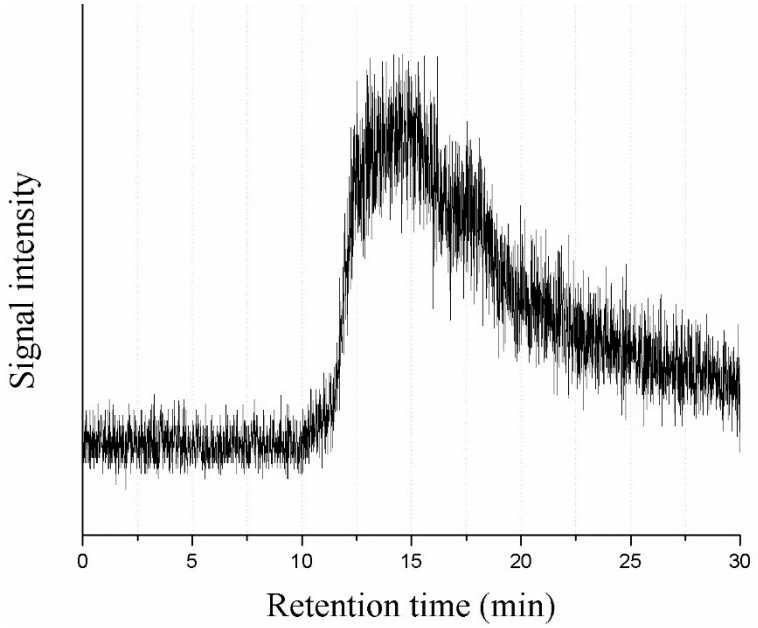
5
10
15
20
25
30
Retention time (min)

(b)


Signal intensity
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20
25
30
Retention time (min)

( c)

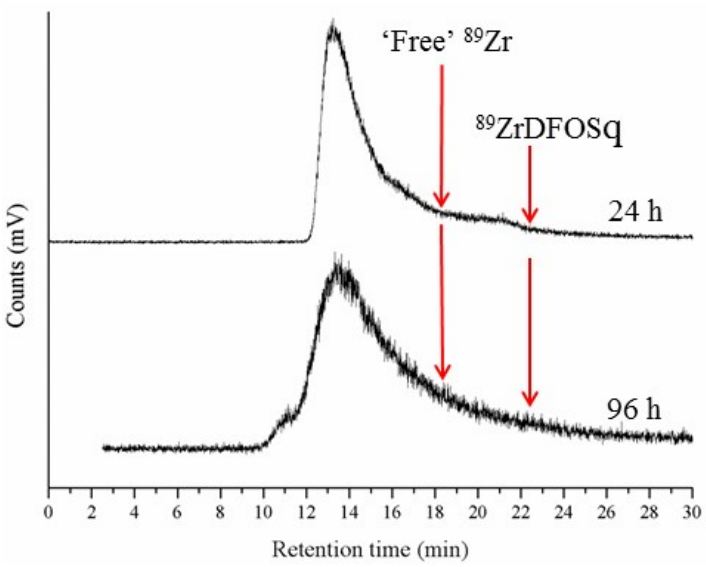



**Figure S1.** Radio-SEHPLC traces of (a) $H_3$DFOSq-trastuzumab following PD10 purification. (b) $H_3$DFO-*p*-Ph-trastuzumab following PD10 purification. The failure of the signal to return to baseline indicates potential radiolysis of the conjugate in the PBS buffer during elution. (c) $H_3$DFOSq-trastuzumab after 24 and 96 hours incubation in PBS/gentisate buffer at ambient temperature, with expected retention times for breakdown products indicated.

## *DFO-p-Ph-NCS-trastuzumab & $^{89}$Zr-DFO-p-Ph-NCS-trastuzumab*

$H_3$DFO-*p*-PhNCS was purchased from Macrocyclics (Cat. No. B-705). Trastuzumab conjugations and radiolabelling were carried out following reported procedures (M. J. W. D. Vosjan, L. R. Perk, G. W. M. Visser, M. Budde, P. Jurek, G. E. Kiefer and G. A. M. S. van Dongen, *Nature Protocols*, 2010, **5**, 739-743)

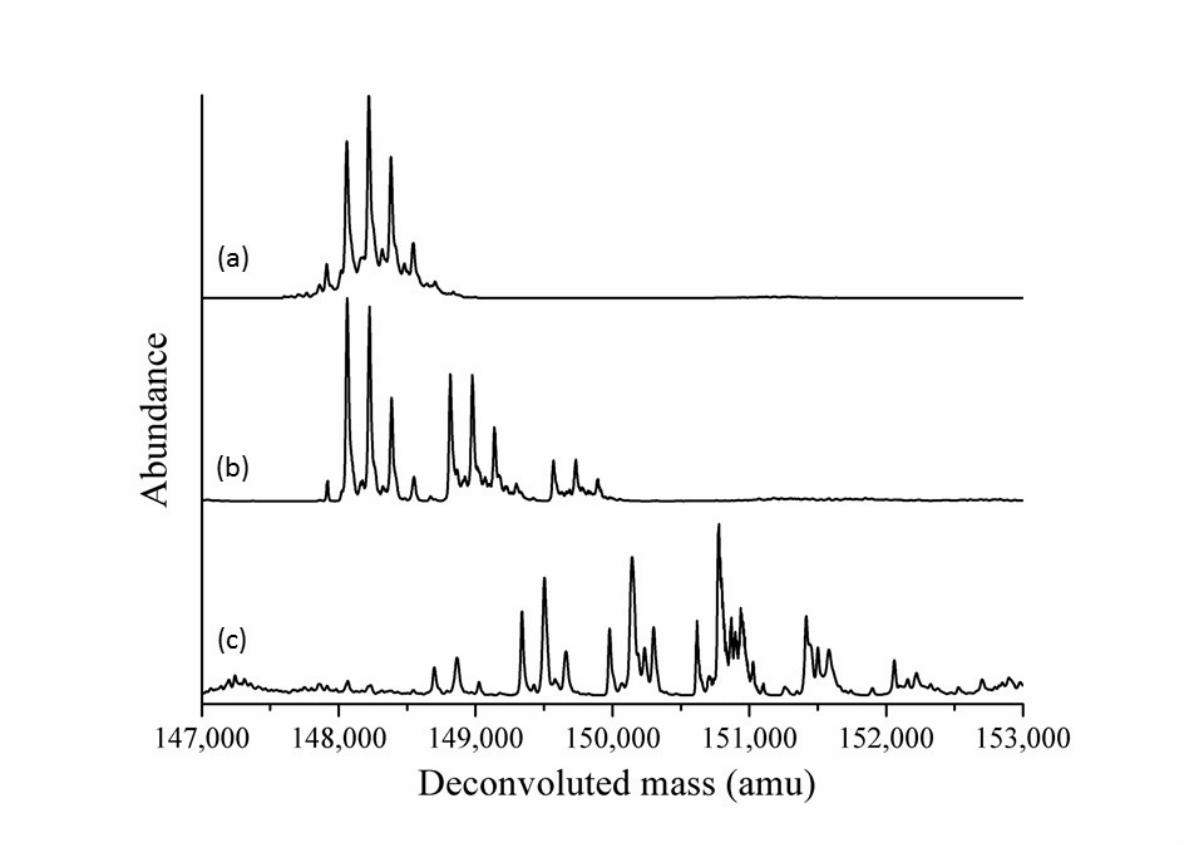



**Figure S2.** Deconvoluted ESI mass spectra of (a) unconjugated trastuzumab, (b) (DFO-p-Ph)$_x$-trastuzumab conjugate where x=0-2 (average 0.6 chelators/mAb) and (c) (DFOSq)$_x$-trastuzumab conjugate where x=1-6 (average 3.4 chelators/mAb)

## Competition study by radio-iTLC (EDTA challenges)

All ligand challenge experiments were carried out at pH 7.0. Analysis of the mixtures was performed by iTLC using 20 mM citrate buffer (pH 5) as eluent. In the forward direction ($^{89}$ZrDFO derivatives challenged with EDTA), the concentration of the DFO derivative in each reaction mixture was 0.096 mM. 24 hours reaction time was required in these DFO challenges to form a reasonable amount of $^{89}$ZrEDTA for reliable TLC analysis. In the reverse direction, the two DFO derivatives were able to transchelate the $^{89}$Zr in reasonable amounts within 20 minutes at DFO and EDTA concentrations of 3.2 mM and 0.27 mM, respectively.

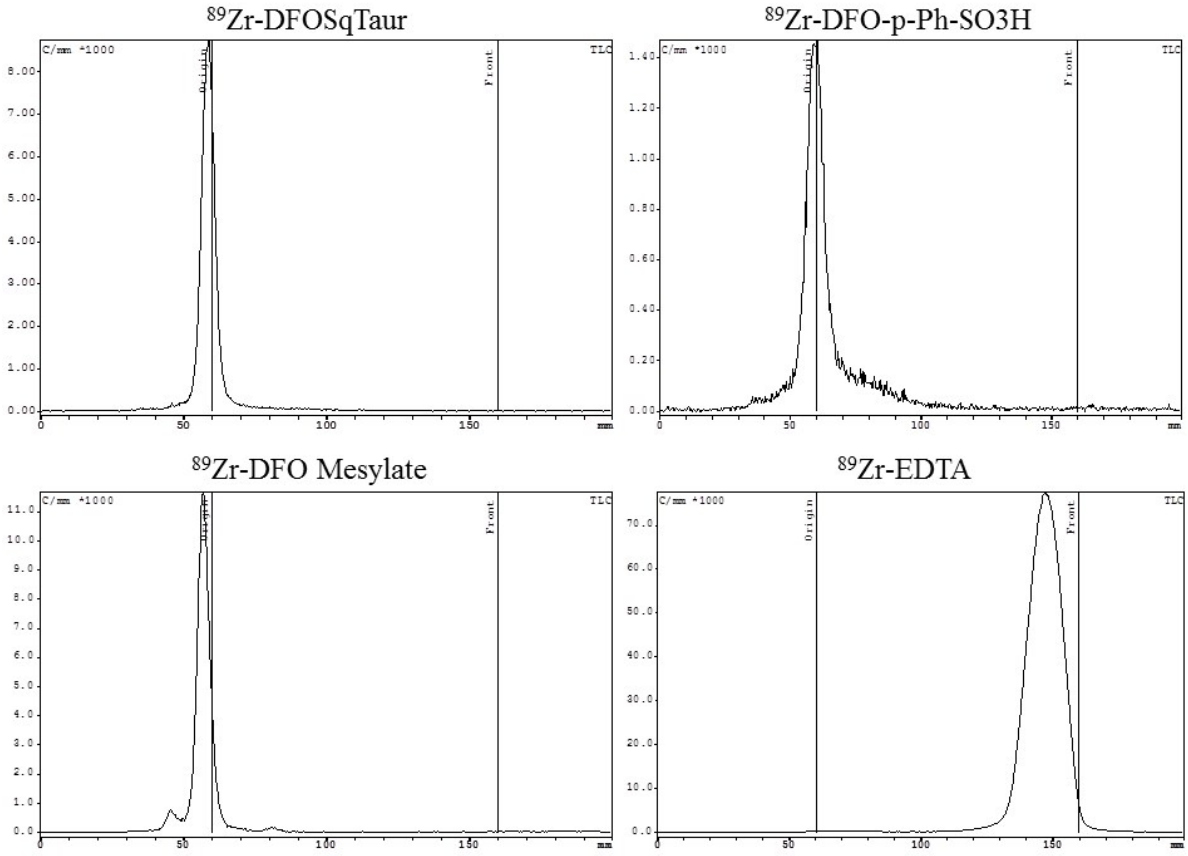


**Figure S3.** radio-iTLC of the four zirconium-89 complexes used in the competition studies. $^{89}$ZrDFOSqTaur, $^{89}$ZrDFO-*p*-Ph-$SO_3H$ and $^{89}$Zr-DFO mesylate remain at the baseline under these conditions, while $^{89}$ZrEDTA moves with the solvent front.

**Table 1.** Amount of $^{89}$ZrEDTA formed after 50mM EDTA challenge, 24 hours, given as % total $^{89}$Zr.

| | $^{89}$Zr-DFOSq-Taur | $^{89}$Zr-DFO-p-Ph-$SO_3$H |
|---|---|---|
| Run 1 | 18 | 41 |
| Run 2 | 9 | 33 |
| Run 3 | 8 | 17 |
| Average | 12 | 30 |
| SEM | 3.2 | 7.1 |

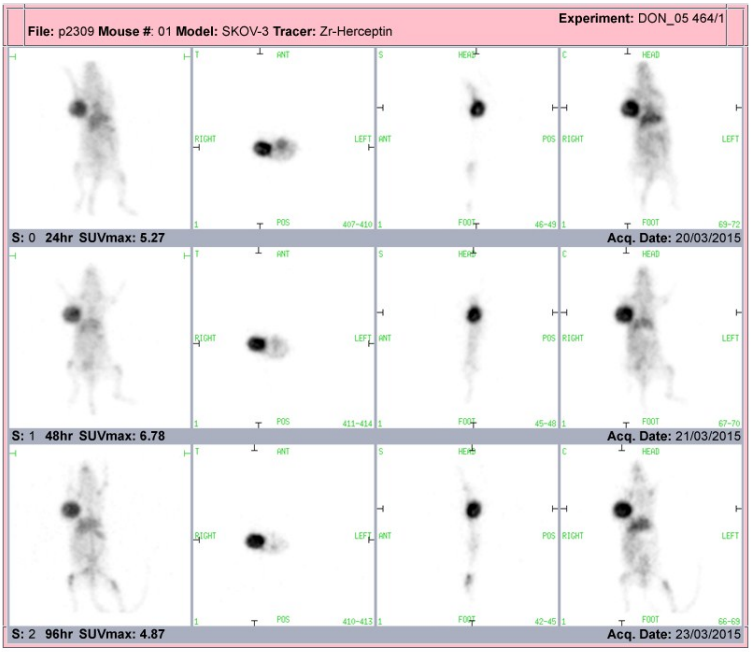
**Table 2**. Amount of $^{89}$ZrEDTA remaining after challenging a $^{89}$ZrEDTA solution with each ligand for 20 min.

| | $^{89}$Zr-DFOSq-Taur | $^{89}$Zr-DFO-p-Ph-$SO_3$H |
|---|---|---|
| % $^{89}$Zr bound by EDTA | 12 | 63 |

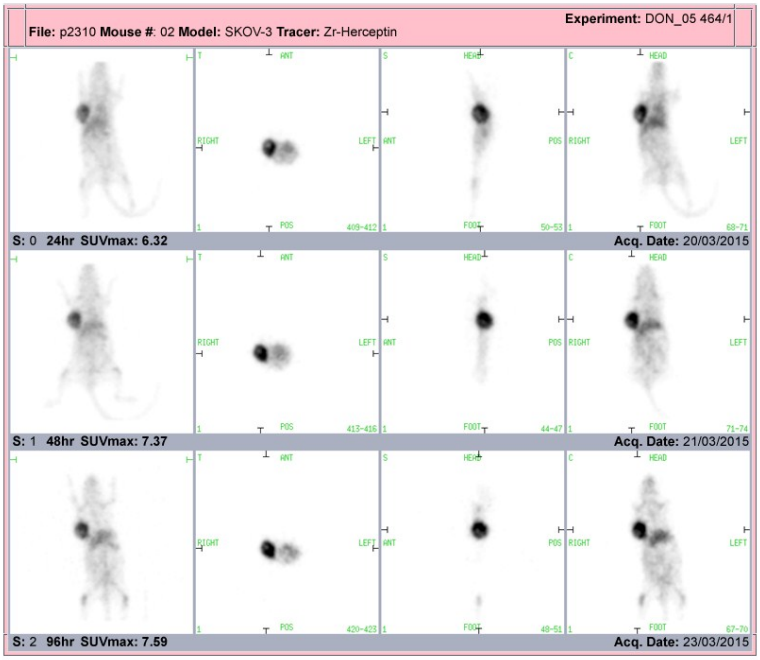
# MicroPET Imaging

## *$^{89}$ZrDFOSq-trastuzumab in SKOV3 xenograft-bearing mice*

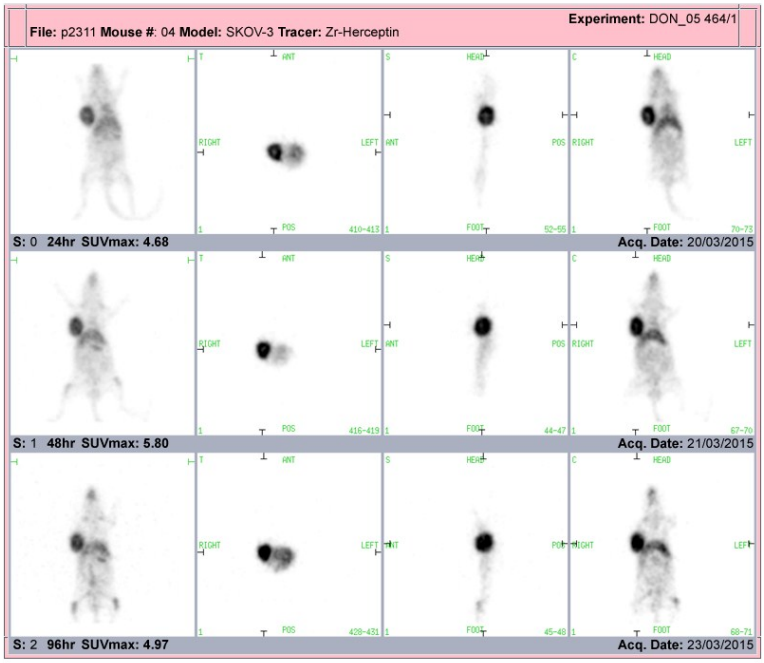
### Mouse 1/3




### Mouse 2/3

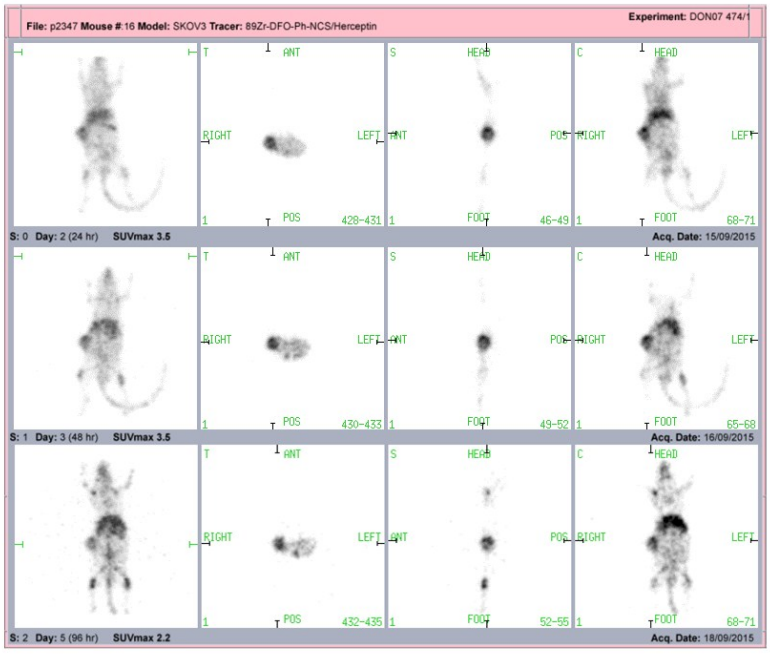


**Mouse 3/3**

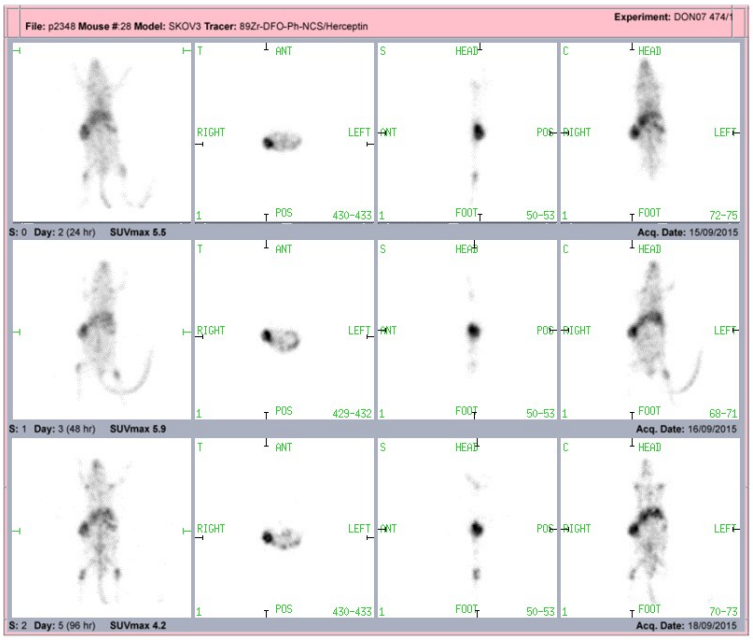



## *$^{89}$ZrDFO-p-Ph-NCS-trastuzumab in SKOV3 xenograft-bearing mice*

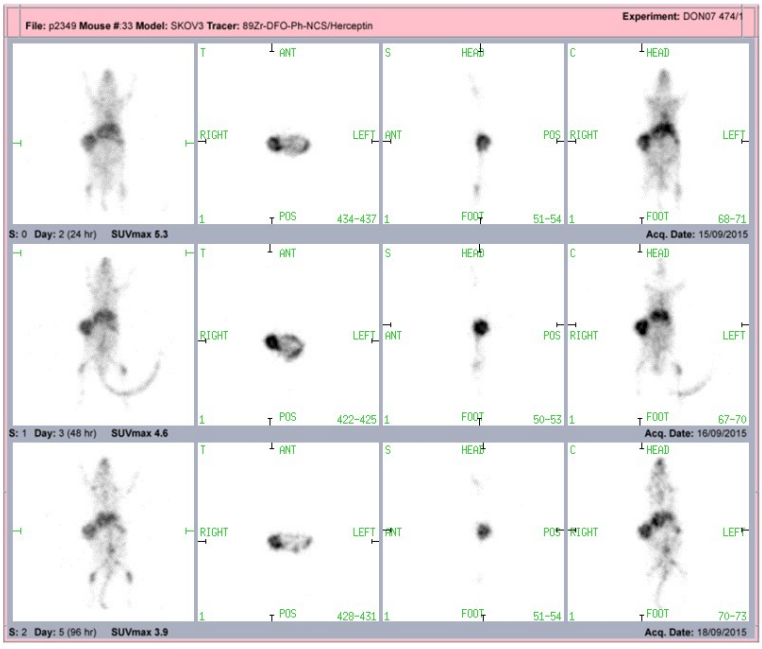
**Mouse 1/3**



## Mouse 2/3

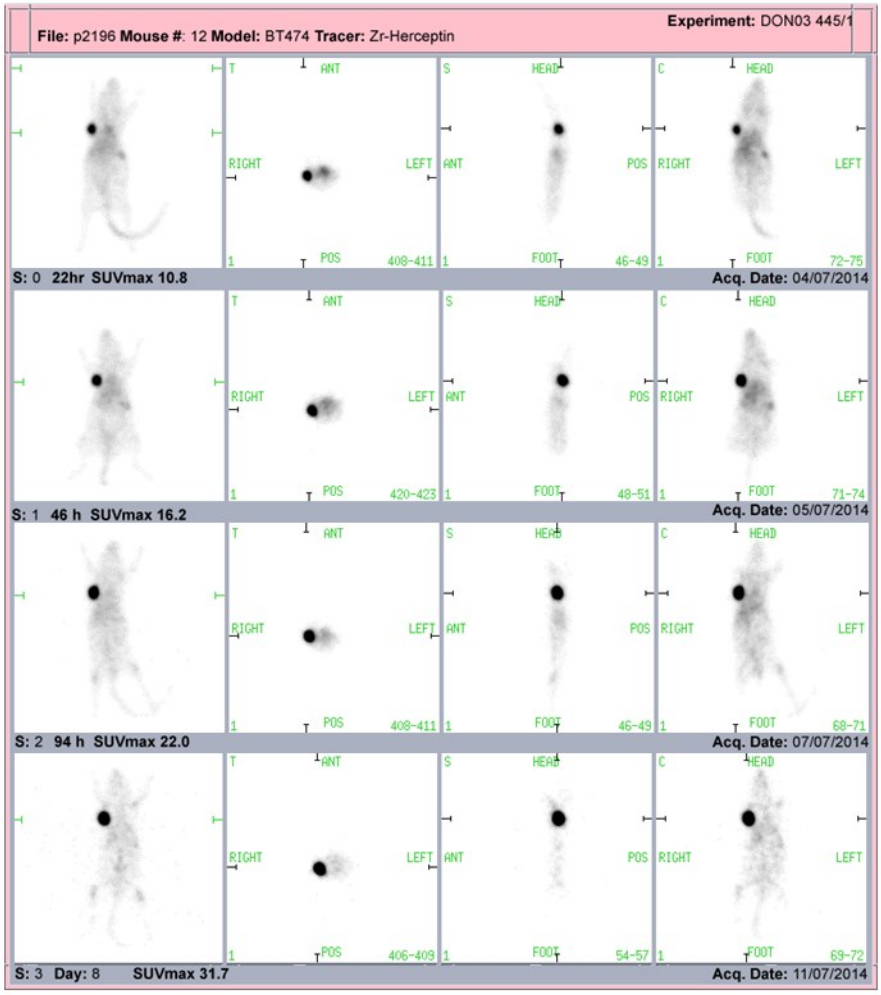



## Mouse 3/3

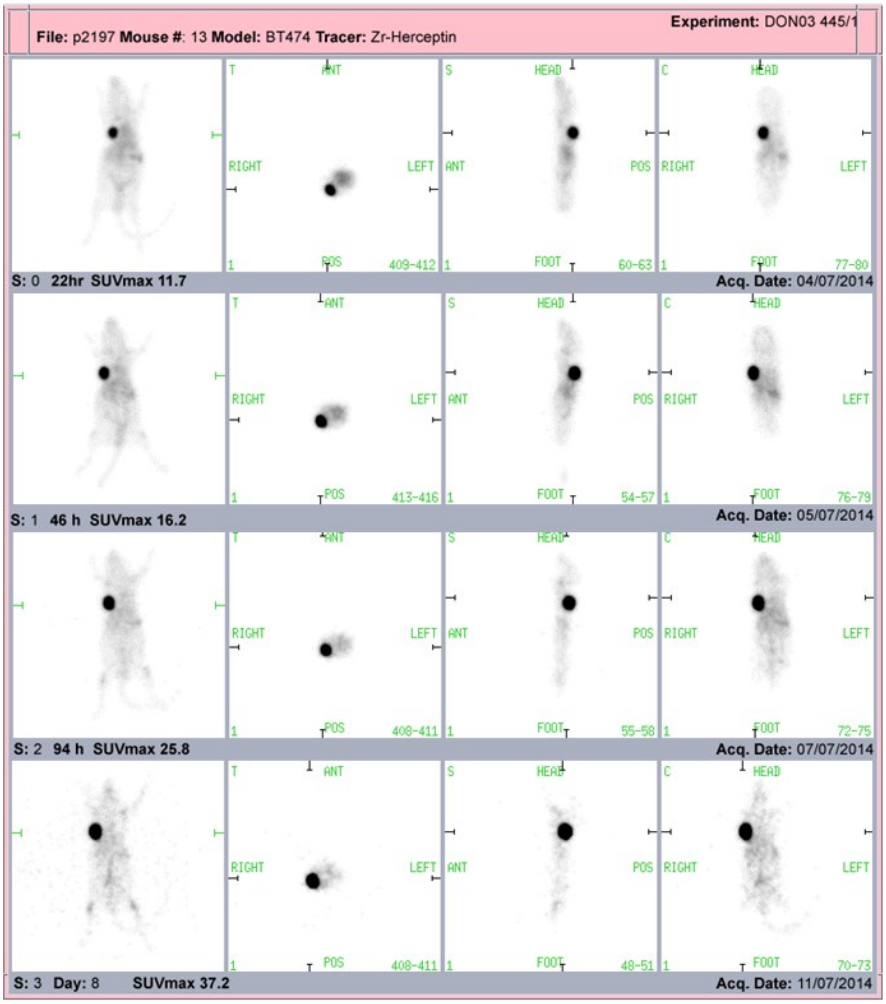


## *$^{89}$ZrDFOSq-trastuzumab in BT474 xenograft-bearing mice*

**Mouse 1/2**



**Mouse 2/2**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Experiment: DON03 445/1
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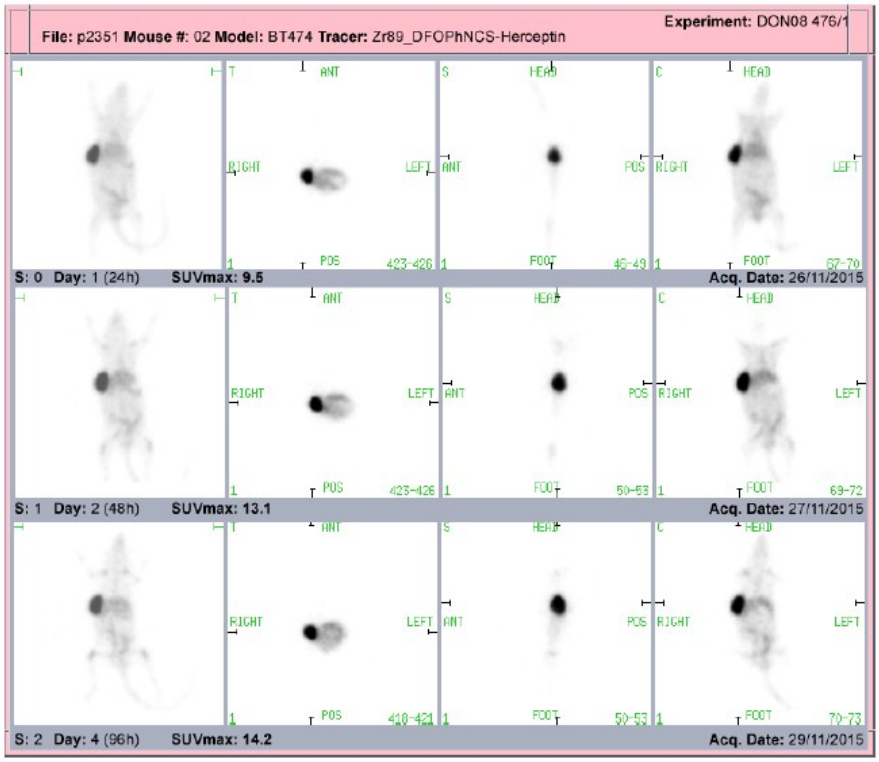
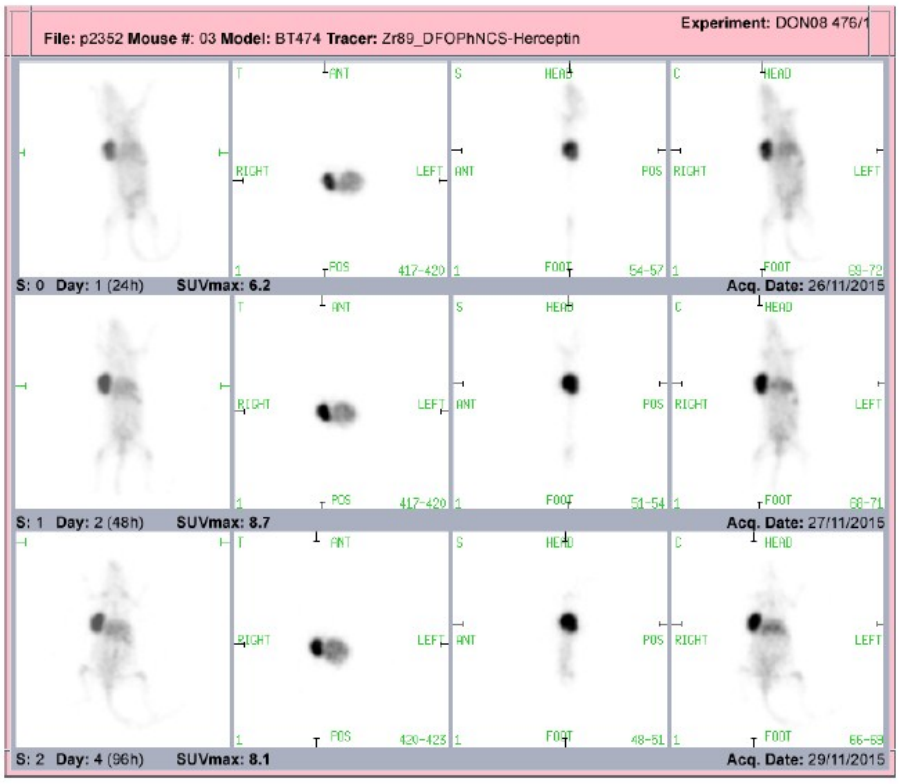
Acq. Date: 04/07/2014
S: 1 46 h SUVmax 16.2
Acq. Date: 05/07/2014
S: 2 94 h SUVmax 25.8
Acq. Date: 07/07/2014
S: 3 Day: 8 SUVmax 37.2
Acq. Date: 11/07/2014

## *$^{89}$ZrDFO-p-Ph-NCS-trastuzumab in BT474 xenograft-bearing mice*

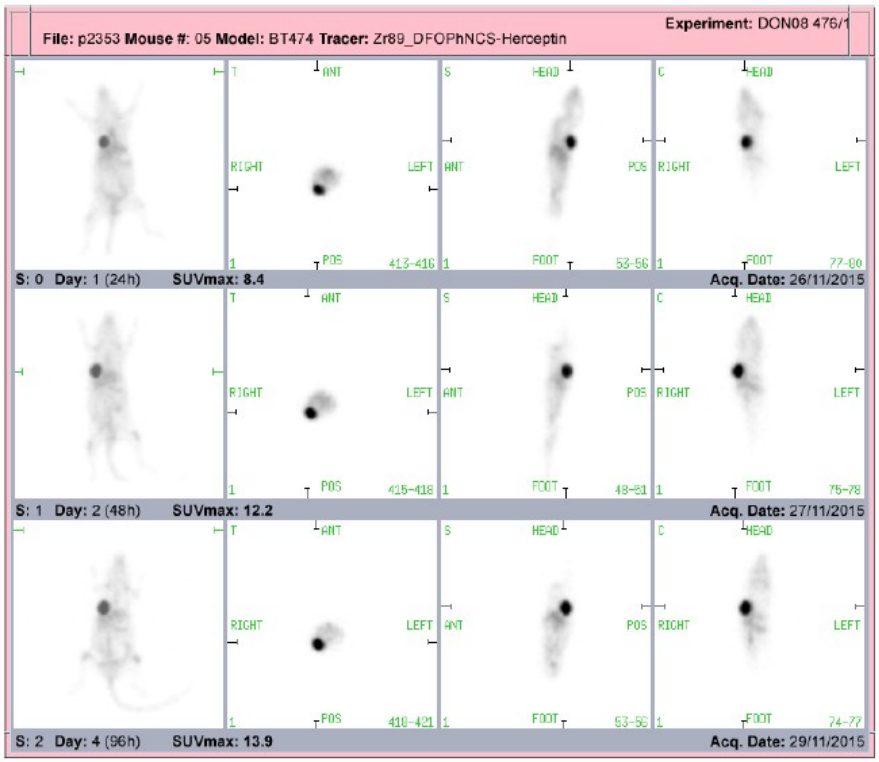
### Mouse 1/4




### Mouse 2/4



**Mouse 3/4**

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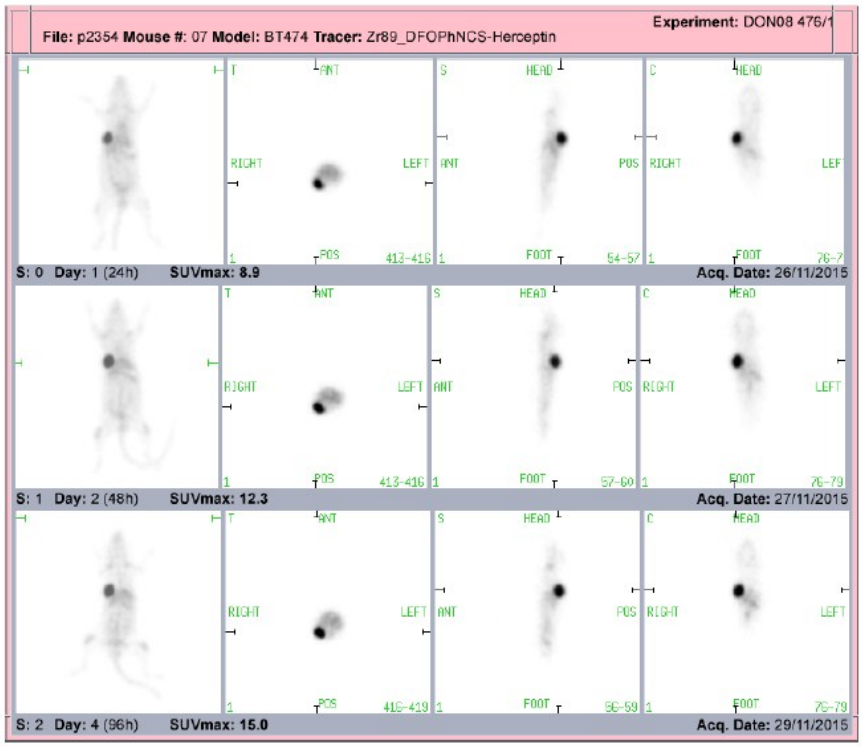
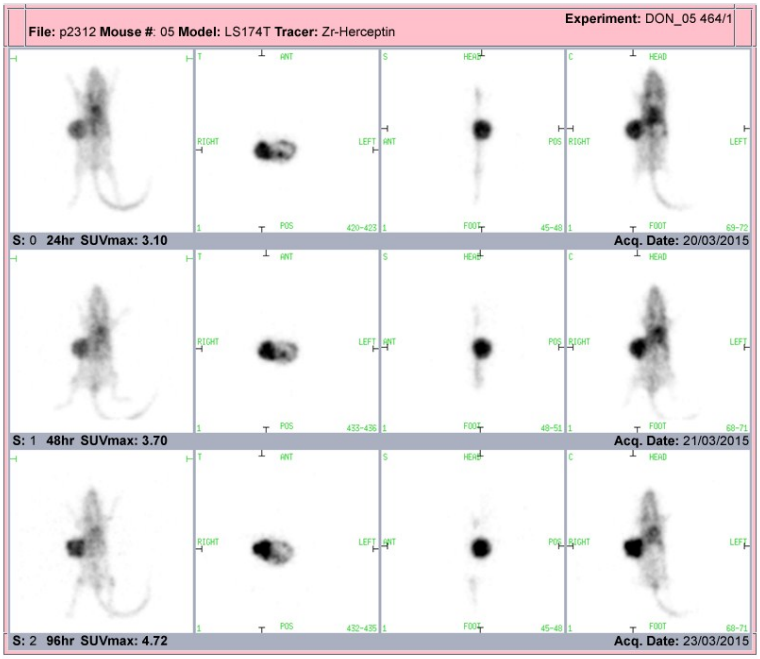
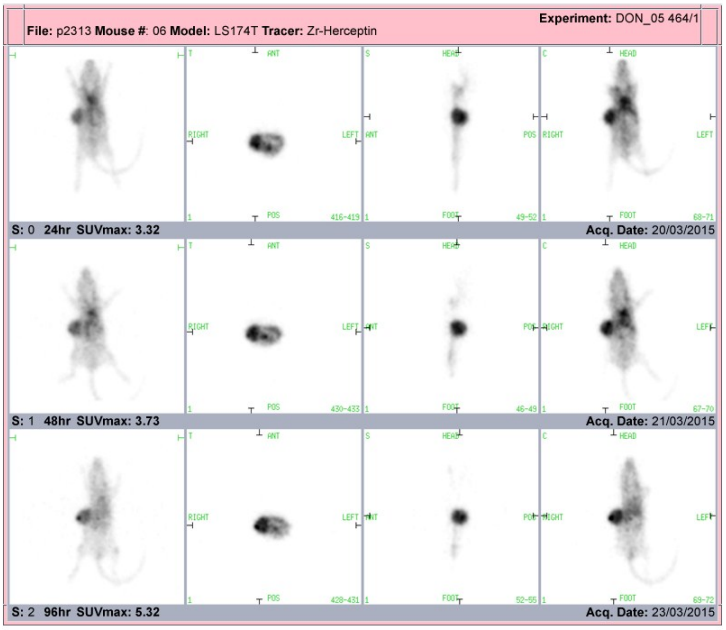


## *$^{89}$ZrDFOSq-trastuzumab in LS174T xenograft-bearing mice*

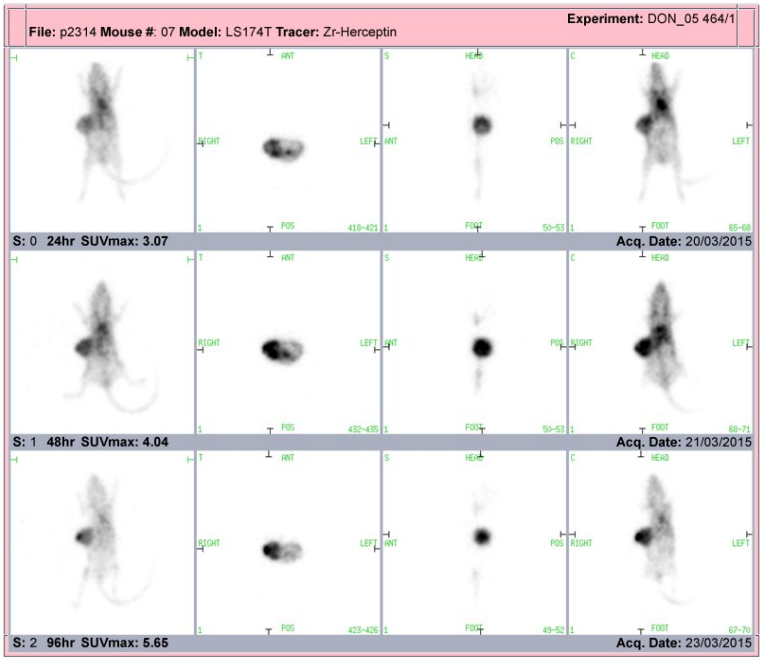
### Mouse 1/3




### Mouse 2/3



## Mouse 3/3


File: p2314 Mouse #: 07 Model: LS174T Tracer: Zr-Herceptin
Experiment: DON_05 464/1
S: 0 24hr SUVmax: 3.07
Acq. Date: 20/03/2015
S: 1 48hr SUVmax: 4.04
Acq. Date: 21/03/2015
S: 2 96hr SUVmax: 5.65
Acq. Date: 23/03/2015