Supporting Information

Bioorthogonal masking of circulating antibody-TCO groups using tetrazine-functionalized dextran polymers

Jan-Philip Meyer[1], Kathryn M. Tully[1,2], James Jackson[1], Thomas R. Dilling[1], Thomas Reiner[1,3*], and Jason S. Lewis[1,2,3,4*]

[1]Department of Radiology, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, New York 10065, United States

[2]Department of Pharmacology, Weill Cornell Medical College, 1300 York Avenue, New York, New York 10065, United States

[3]Department of Radiology, Weill Cornell Medical College, 1300 York Avenue, New York, New York 10065, United States

[4]Program in Molecular Pharmacology, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, New York 10065, United States

Corresponding Authors

*(J.S.L) E-Mail: lewisj2@mskcc.org

*(T.R.) E-Mail: reinert@mskcc.org

1 | General information

1.1 | Reagent and instrument specifications

NOTA-Bn-p-NCS was purchased from Macrocyclics. All other starting materials were purchased from Sigma-Aldrich (synthetic-grade). All starting materials were used without further purification. All solvents used for HPLC analysis and purification were purchased from Fisher Scientific (HPLC grade). Metal-free DMSO (≥99.99995%) and MeCN (≥99.999%) were purchased from Sigma-Aldrich. Water (>18.2 MΩ cm^{-1} at 25 °C) was obtained from an Alpha-Q Ultrapure water system from Millipore (Bedford, MA). C18 light Sep-Pak® cartridges were obtained from Waters (Milford, MA). $^{68}Ga^{3+}$ was received from received from an Eckert & Ziegler ^{68}Ga-generator (Model IGG 100). C18 cartridges were equilibrated using absolute ethanol (10 mL) followed by deionized water (5 mL). QMA cartridges used a Chromafix 30-PS-HCO_3-resin for ion-exchange and were equilibrated using $KHCO_3$-solution (0.4 M, 5 mL) followed by deionized water (10 mL). High performance liquid chromatography (HPLC) purification and analysis was performed on a Shimadzu UFLC HPLC system equipped with a DGU-20A degasser, a SPD- M20A UV detector, a RF-20Axs fluorescence detector, a LC-20AB pump system, and a CBM-20A communication BUS module. A LabLogic Scan-RAM radio-TLC/HPLC-detector was used for purifications while a PosiRAM Model 4 was used for analysis. HPLC solvents for analytical HPLC (Buffer A: 0.1% TFA in water, Buffer B: 0.1% TFA in MeCN) and size-exclusion HPLC (1x PBS pH 7.4) were filtered before use. HPLC analysis of radioactive and non-radioactive compounds was performed on a reversed phase Atlantis T3 column (C18, 5 µm, 4.6 mm × 250 mm). Preparative HPLC purification was carried out on a reversed phase Waters XTerra Prep C18 OBD (C18, 10 µm, 19 mm × 250 mm). Size-exclusion chromatography was conducted using a Superdex® 200 10/300 GL column (1x PBS, 1mL/min flow rate). For radioactive thin-layer chromatography (TLC) analysis throughout this work, Merck pre-coated TLC plates (C18, reversed-phase) were used. Radio-TLC was performed using a Bioscan AR-2000 (Eckert & Ziegler, Berlin, Germany) instrument. Radioactivity was determined using a calibrated ion chamber (Capintec CRC-15R). All PET imaging experiments were conducted on a Focus 120 MicroPET camera (Siemens, Knoxville, TN).

1.2 | Animal protocol, cell culture and xenograft procedures

All animal experiments within this study were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of MSKCC and followed National Institutes of Health

guidelines for animal welfare. Female athymic nude CrTac:NCr-Foxn1^{nu} mice at age 6-8 weeks were purchased from Charles River Laboratories.

BxPC3 cells were purchased from ATCC (Manassas, VA) and grown in RPMI modified to contain 4.5g/L glucose and 1.5g/L sodium bicarbonate and supplemented with 10% (v/v) fetal calf serum, 10mM HEPES, 1mM sodium pyruvate, 2mM L-glutamine, 10cc/L non-essential amino acids, 100IU penicillin and 100ug streptomycin. The human colorectal cancer cell line SW1222 was obtained from Sigma Aldrich 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. For subcutaneous injections, mice were anesthetized with 2% isoflurane (Baxter Healthcare) (2 L/min medical air) before BxPC3 cells were implanted subcutaneously (5×10^6 cells in 150 µL 1:1 growth media/Matrigel® (BD Biosciences, San Jose, CA) in the right shoulder and allowed to grow for approximately 3-4 weeks until the tumors reached 5–10 mm in size. For all intravenous injections, mice were gently warmed with a heat lamp and placed on a restrainer. The tails were sterilized with alcohol pads, and injection took place via the lateral tail vein. Healthy mice were given a two-week recovery period before reuse in another experiment.

2 | Synthesis and radiolabeling data

2.1. DP-NH_2 modification with Tz-NHS

DP–Tz constructs were synthesized by incubating amino-dextran (NH_2–DP, MW = 0.5, 2 MDa, 1–36.9 mg, 2–18.5 nmol) with an excess of Tz-NHS ester (2-8 mg, 6.5-25 µmol), resulting in Tz/DP ratios of 62-654, depending on utilized dextran and reaction conditions (*Scheme 1, Supplementary Information*). NH_2-DP was dissolved in PBS (pH = 7.4) before the pH was adjusted to 8.5–9 using 0.1 M Na_2CO_3 (40 µL). Tz-NHS ester was dissolved in DMF (20–40 µL) and added to the aqueous solution containing NH_2-DP. The mixture was incubated at room temperature with agitation (700 rpm) for 2 h. Subsequently, Tz–DP conjugates were purified using PD–10 size-exclusion columns using metal-free water and subsequently lyophilized. The purity of Tz–DP was determined using size–exclusion HPLC (0.9% saline, 0.75 mL/min). The dried construct was weighed and dissolved in PBS, and Tz/DP ratios were then calculated using UV/VIS spectroscopy. Constructs dissolved in PBS were stored at -80 °C.

Run #	MW (DP-amine, [kDa])	n (DP-amine [nmol])	n (Tz-NHS [µmol])	Conjugate Tz/DP ratio
1	500	2	6	50
2	2000	3	9	371
3	2000	3	8	99
4	2000	3	8	137
5	500	12	39	109
6	2000	3	9	480
7	500	114	363	83
8	2000	17	56	510
9	2000	19	61	600
10	2000	18	59	600

Table S1. Summary of obtained tetrazine-dextran (Tz/DP) ratios using DP-amine with molecular weights of 500 and 2,000 kDa and a Tz/DP ration in the reaction mixture of ≈3190.

1. QC run (size-exclusion) of DP-NH_2 (500 kDa, 90 amine groups/DP) prior to conjugation:

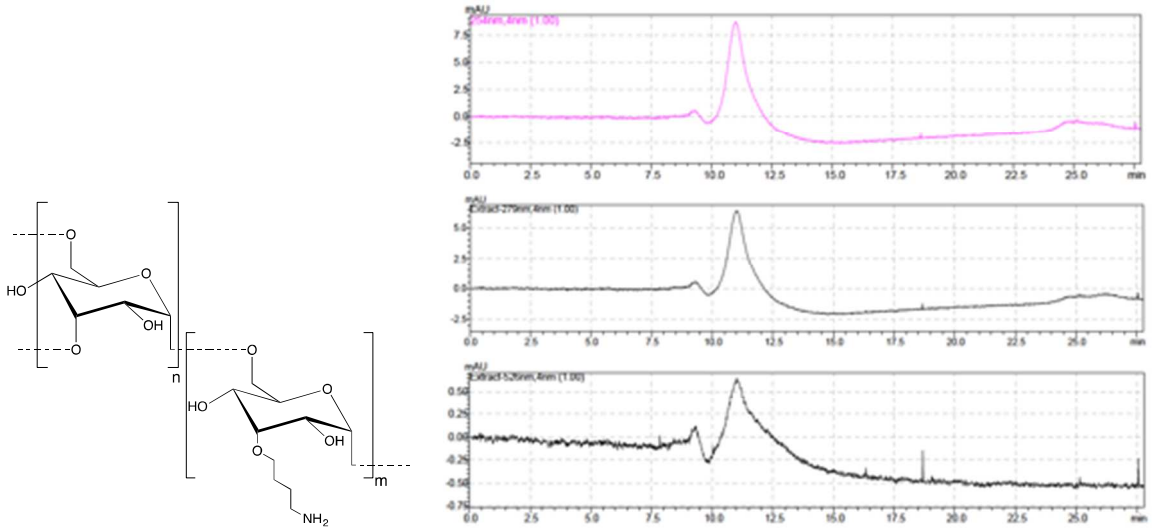


Figure S1. Structure (left) and size-exclusion chromatograms (right) for DP–NH_2.

2. QC run (size-exclusion) of DP-Tz (run #1, 500 kDa, 62 Tz moieties/DP):

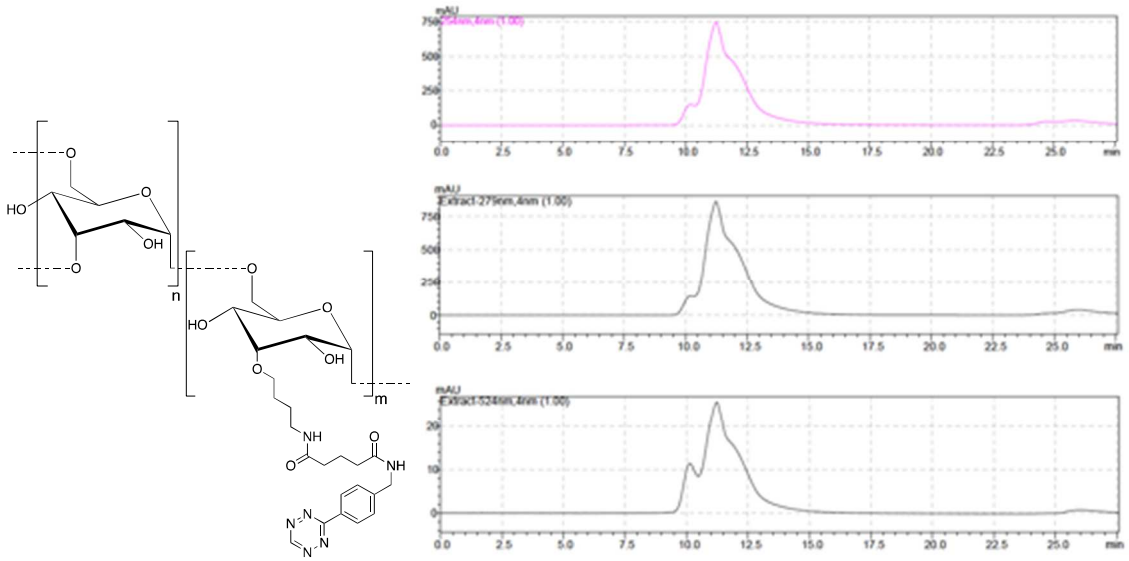


Figure S2. Structure (left) and size-exclusion chromatograms (right) for DP–Tz (2).

3. QC run (size-exclusion) of DP-Tz-5B1-TCO adduct:

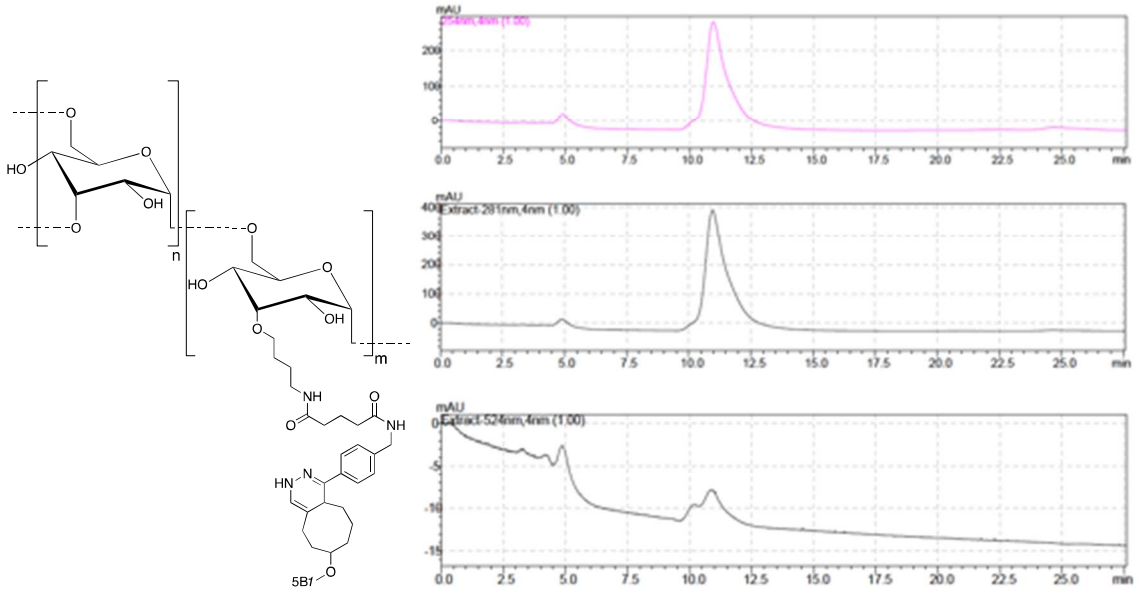
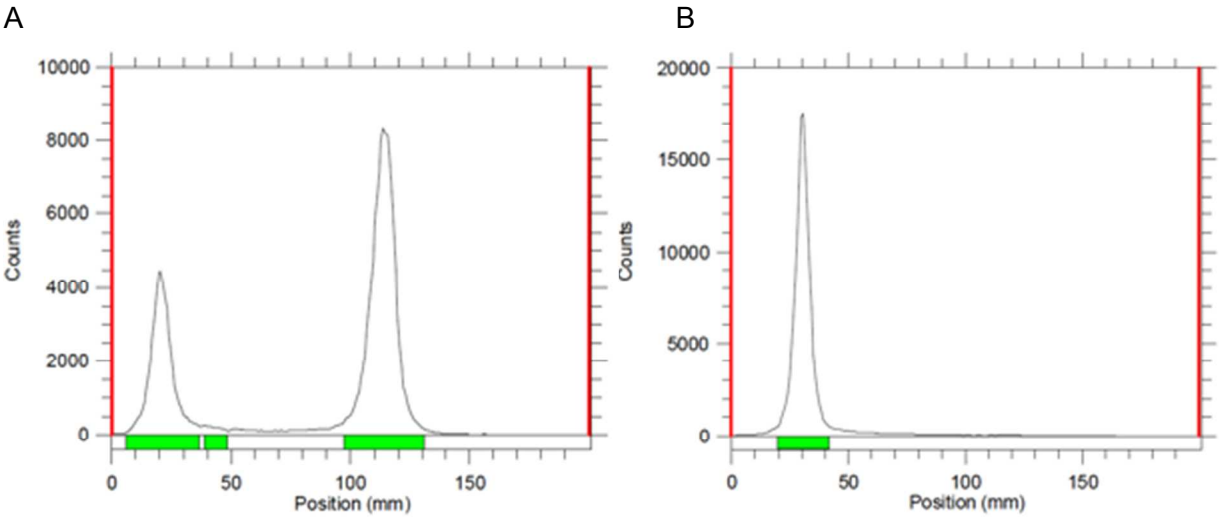


Figure S3. Structure (left) and size-exclusion chromatograms (right) for DP–Tz-TCO-5B1 (3).

2.2. DP-NH_2 modification with Tz-NHS, DFO-NCS, and subsequent ^{89}Zr-radiolabeling

DP–Tz–DFO constructs were synthesized by incubating amino-dextran (2,000 kDa, 5 mg, 2.5 nmol) first by incubation with an excess of Tz-NHS ester (2-8 mg, 6.5-25 µmol), resulting in Tz/DP ratios of 110 ± 22 (n = 3) after purification as described above. The DP–Tz constructs were subsequently incubated with a 25-fold molar excess of DFO-NCS ester at pH = 8.5 in carbonate buffer. DP–Tz–DFO conjugates were finally purified using PD-10 desalting columns. DP–Tz–DFO constructs were then labeled with ^{89}Zr using the standard labeling protocol (pH = 6.8, 37 °C, 1 h with 500 rpm agitation). Conversion and radiochemical purity was determined via iTLC (mobile phase: 50 mM EDTA, pH = 5). Labeling yields of approx. 30% were achieved. Longer incubation times did not lead to higher radiolabeling yields. The radiolabeled construct was purified using PD-10 desalting columns.



Reg	(mm) Start	(mm) Stop	(mm) Centroid	RF	Region Counts	Region CPM	% of Total	% of ROI
Rgn 1	5.9	36.7	21.0	0.105	52151.0	125162.4	28.82	30.58
Rgn 2	38.4	48.7	42.6	0.213	2413.0	5791.2	1.33	1.41
Rgn 3	97.5	131.0	113.6	0.568	115977.0	278344.8	64.09	68.01
3 Peaks					170541.0	409298.4	94.24	100.00

Reg	(mm) Start	(mm) Stop	(mm) Centroid	RF	Region Counts	Region CPM	% of Total	% of ROI
Rgn 1	19.6	41.9	30.3	0.152	148362.0	684747.7	91.43	100.00
1 Peaks					148362.0	684747.7	91.43	100.00

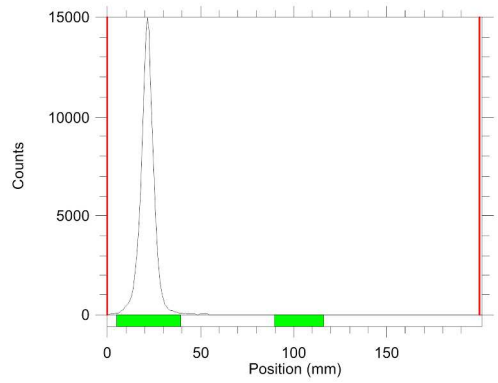
Figure S4. iTLC chromatograms for the reaction mixture (A) of ^{89}Zr-labeled DP-Tz-DFO and the purified polymer (B) after PD-10 purification.

2.3. 5B1 and huA33 conjugation with TCO-NHS

TCO-conjugation of 5B1 and huA33 was performed as previously described.[1, 2]

2.3. Antibody–DFO–TCO conjugation and ^{89}Zr–labeling

TCO-conjugation of 5B1 was performed as described above, except that 25 eq. of TCO–NHS were used instead of 30–35 eq. as for the regular TCO-modification. Purified 5B1-TCO (2–3 mg) was subsequently incubated with a 20-fold molar excess of DFO-NCS at pH = 8.5 in carbonate buffer. The resulting 5B1–DFO–TCO conjugates were purified using PD-10 desalting columns, and the number of TCO-groups per mAb was determined as described above. 5B1–DFO–TCO constructs were radiolabeled with ^{89}Zr using an aqueous buffer (pH = 6.8) at 37 °C for 1 h with 500 rpm agitation. Conversion and radiochemical purity was determined via iTLC (mobile phase: 50 mM EDTA, pH = 5). Quantitative labeling yields were achieved using this labeling protocol, despite the modification with two different functionalities.



Reg	(mm) Start	(mm) Stop	(mm) Centroid	RF	Region Counts	Region CPM	% of Total	% of ROI
Rgn 1	5.0	39.3	21.4	0.107	135948.0	370767.3	98.71	99.77
Rgn 2	89.8	116.4	103.7	0.518	314.0	856.4	0.23	0.23
2 Peaks					136262.0	371623.6	98.94	100.00

Figure S5. iTCL of the purified ^{89}Zr-labeled 5B1-DFO-TCO immunoconjugate.

3 | In *vivo* biodistribution and pretargeting experiments

3.1 Biodistribution of ^{89}Zr-labeled DP-Tz-DFO constructs

^{89}Zr-labeled DP–Tz–DFO conjugates (0.2 nmol) were administered into BxPC3-tumor bearing mice (n = 3) 2 h after the injection of 5B1-TCO (1.2 nmol) via the lateral tail vein. Control animals (n = 3) did not receive 5B1-TCO. PET Imaging was performed 2, 24, 48, and 144 h p.i. of ^{89}Zr-DP–Tz–DFO.

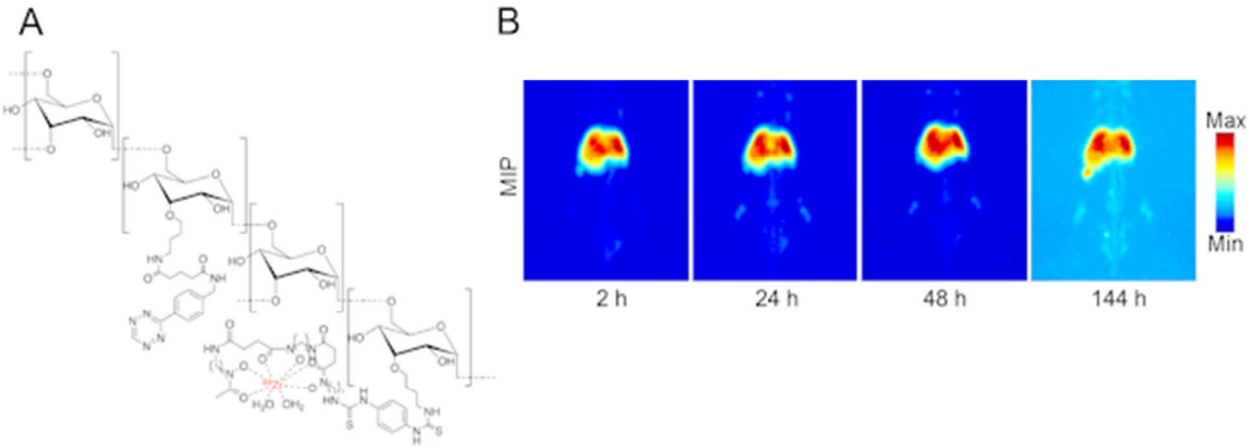


Figure S6. (A) Structure of the ^{89}Zr-labeled DP-Tz-DFO constructs utilized in preliminary biodistribution studies. (B) PET images obtained through injection of ^{89}Zr-DP-Tz-DFO (200 µCi, 0.5 nmol) into BxPC3-tumor bearing mice.

3.2 Investigation of in vivo click chemistry between DP–Tz and 5B1-DFO-TCO

We labeled 5B1–DFO–TCO with ^{89}Zr and determined how the biodistribution of the mAb is influenced upon injection of DP–Tz. For that, BxPC3-tumor bearing mice were injected with ^{89}Zr–5B1–DFO–TCO. After 20 min, mice of the experimental cohort received DP–Tz (Tz = 200 nmol/mouse), whereas the control group did not receive DP–Tz. PET imaging of both cohorts was performed 1 and 24 h p.i. of ^{89}Zr-5B1–DFO–TCO. Ex *vivo* biodistribution data were obtained for the last time point (24 h) for both cohorts (n = 5).

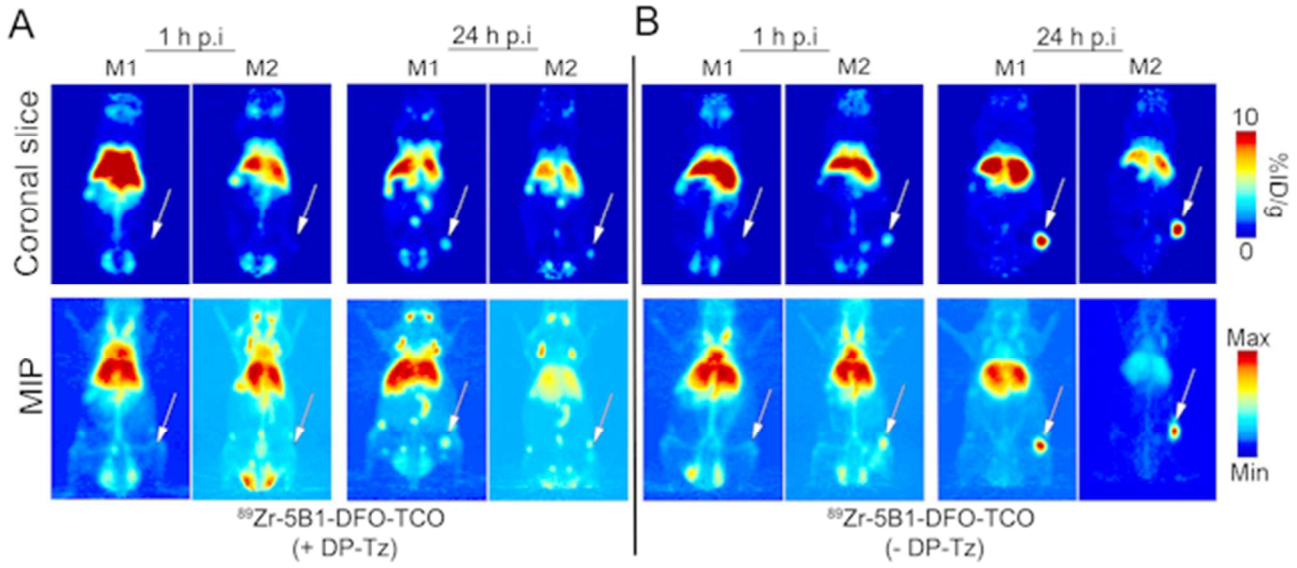


Figure S7. PET imaging results demonstrating the ability of DP–Tz to effectively bind circulating antibody-TCO.

3.3. Proof-of-concept Pretargeting study evaluating DP-Tz constructs in the SW1222 model

The *in vivo* performance of the newly developed DP–Tz constructs was measured by comparing TTB and tumor-to-organ (TTO) ratios as well as absolute tumoral tracer uptake in experiments using Tz–DP compared to standard pretargeting experiments. PET imaging studies and *ex vivo* organ biodistribution experiments were performed 2 h post [^{68}Ga]**1** injection. For pretargeting studies (n = 4) huA33-TCO (150 µg, 0.7 nmol/mouse) was injected 48 h prior to [^{68}Ga]**1** (50-500 µCi, 1.8-2.5 nmol, in 200 µL saline).

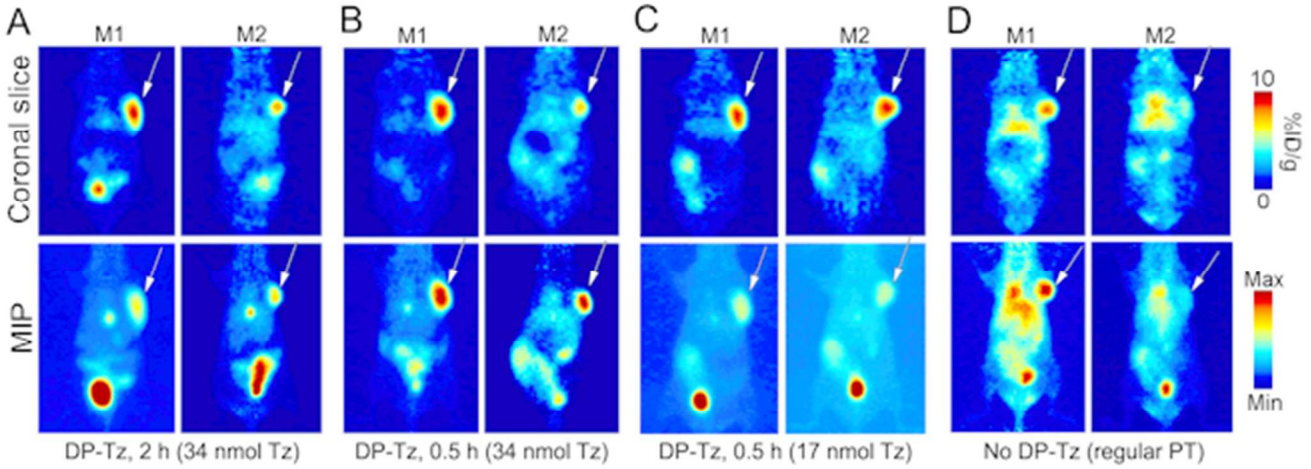


Figure S8. Pretargeted PET imaging results of additional mice of the 3 experimental (A-C) and the control (D) cohort.

3.4. Pretargeting study evaluating DP-Tz constructs in the BxPC3 model

The *in vivo* performance of the newly developed DP–Tz constructs was further investigated by comparing TTB and tumor-to-organ (TTO) ratios as well as absolute tumoral tracer uptake in experiments using Tz–DP compared to standard pretargeting experiments in the BxPC3 PDAC model. PET imaging studies and *ex vivo* organ biodistribution experiments were performed 2 h post [^{68}Ga]**1** injection. For pretargeting studies (n = 4) 5B1-TCO (200 µg, 1.2 nmol/mouse) was injected 48 h prior to [^{68}Ga]**1** (50-500 µCi, 1.8-2.5 nmol, in 200 µL saline).

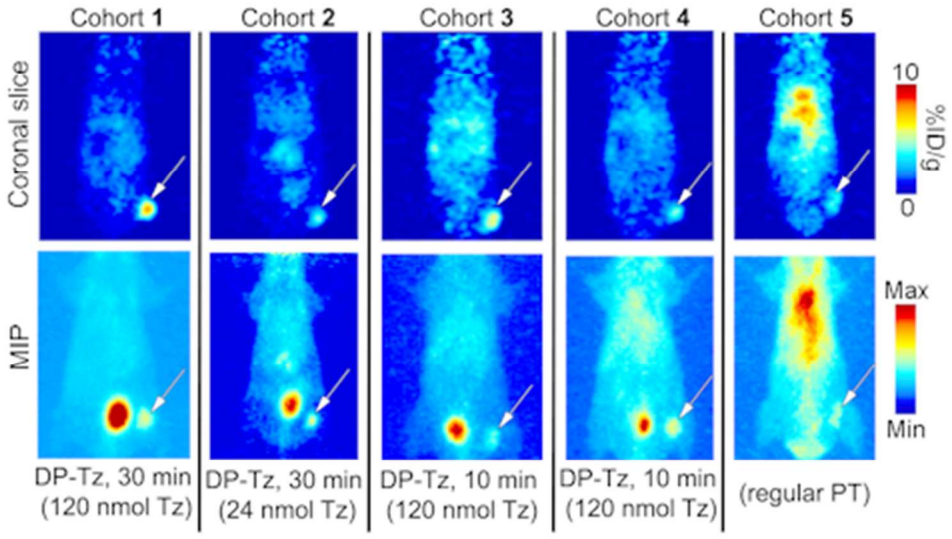


Figure S9. PET imaging results demonstrating the ability of DP–Tz to significantly enhance TTB and TTO ratios.

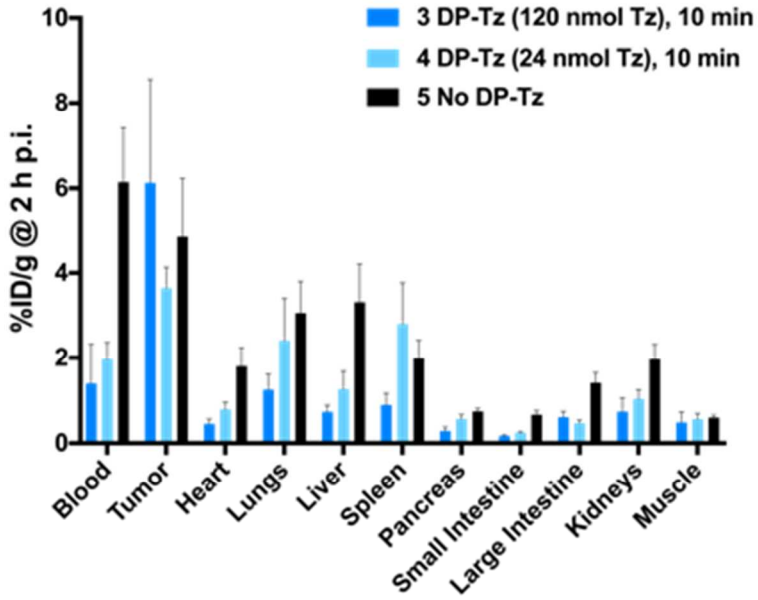


Figure S10. Ex *vivo* biodistribution data obtained for high and low concentrated DP-Tz injected 10 min prior to radioligand [^{68}Ga]**1.**

References

(1) Meyer, J. P., Houghton, J. L., Kozlowski, P., Abdel-Atti, D., Reiner, T., Pillarsetty, N. V., Scholz, W. W., Zeglis, B. M., and Lewis, J. S. (2016) (18)F-Based Pretargeted PET Imaging Based on Bioorthogonal Diels-Alder Click Chemistry. *Bioconjug Chem 27*, 298-301.

(2) Zeglis, B. M., Sevak, K. K., Reiner, T., Mohindra, P., Carlin, S. D., Zanzonico, P., Weissleder, R., and Lewis, J. S. (2013) A pretargeted PET imaging strategy based on bioorthogonal Diels-Alder click chemistry. *J Nucl Med 54*, 1389-1396.