### Supplemental data

### MATERIALS AND METHODS

#### Synthesis and Quality Control of Conjugated and Radiolabeled Anticalins

PRS-110 (57 kDa; 3.8 mg/mL; Pieris-AG), the MET specific Anticalin containing a 40 kDa (2 x 20 kDa PEG, NOF) branched PEG moiety (1), was allowed to react with deferoxamine-*p*-SCN (Macrocyclics) and subsequently loaded with <sup>89</sup>Zr in analogy to the protocol described for antibodies by Vosjan *et al.* (2). In brief, the pH of a 1 mg/mL solution of Anticalin (Pieris AG) was set at pH 8.9 - 9.1 with 0.1 M Na<sub>2</sub>CO<sub>3</sub>. Different molar equivalents (2.5, 5 and 10) of the chelator were added from a stock solution of 2.5 mg/mL in DMSO. The mixture — containing the Anticalin at a concentration of 0.96–0.97 mg/mL — was allowed to react for 60 min at 20 °C. Excess deferoxamine-*p*-SCN was removed and the buffer was exchanged with highly purified water (water for injections, B. Braun) with repeated ultracentrifugation (5x) using a 30 kDa polyethersulfone (PES) 2 mL Vivaspin-2 filter (Sartorius). The deferoxamine-conjugated Anticalin was stored at -20 °C until use.

To radiolabel the conjugate, <sup>89</sup>Zr-oxalate solution (740–1850 MBq/mL, IBA Molecular) was set at pH 4.0 - 4.5 with 2 M  $Na_2CO_3$  and incubated for 3 min at room temperature (2). The pH of the solution was brought to 6.8 - 7.2 with an excess of 0.5 M HEPES (pH 7.2) and the chelated Anticalin was added and incubated for 60 min and used as such.

The radiochemical purity of the radiolabeled <sup>89</sup>Zr-PRS-110 Anticalin was assessed by size exclusion high performance liquid chromatography (SE-HPLC) coupled to UV-Vis and radiodetection, which also provided an assessment of potential aggregation by the conjugation and labeling procedure. Radiochemical purity was further assessed by trichloroacetic acid (TCA) precipitation (*3, 4*). Here, protein was precipitated by addition of an equal volume of phosphate-buffered saline (PBS) containing 0.5% human serum albumin (Sanquin) and 30% TCA, leaving free, non-protein bound <sup>89</sup>Zr in solution. The radioactivity of the precipitate was determined with a calibrated well-type γ-counter (LKB Wallac 1282). The relative radioactivity of the precipitate compared to the radioactivity of the non-precipitated solution provided the radiochemical purity.

Analysis of MET binding capacity of PRS-110 after conjugation (not loaded with zirconium-89) was performed by a direct binding ELISA analysis. A 12-point dilution series of conjugated and unconjugated PRS-110 was prepared in assay buffer: PBS (Life technologies) containing 0.1% Tween 20 (Roth) and 2 % bovine serum albumin (Roth). Recombinant human MET extracellular domain, consisting of HGF R  $\alpha$  E25-R307 and HGF R  $\beta$  S308-T932 fused to Fc (Recombinant Human HGF R/c-MET Fc Chimera) (R&D Systems) in PBS (3 µg/mL), was added to each well of a 384 -microtiter plate and incubated overnight at 4 °C. The plates were blocked for 1 h with assay buffer before the Anticalin dilution was added and incubated for 1 h at room temperature. Anti-Anticalin-horseradish peroxidase (Pieris AG) was added and incubated at room temperature for 1 h. In between each step the plate was washed with PBS containing 0.05% Tween 20 using a Biotek ELx405 Select CW washer. Fluorescence signals in RFU (relative fluorescence units) were measured 5 min after addition of QuantaBlu (Thermo Scientific) using the Safire2 microplate reader (Tecan) at an excitation wavelength of 320 nm and emission wavelength of 430 nm. The half maximal effective concentration (EC50) values were calculated using a one site - specific binding model according to RFU = RFU<sub>max</sub> × c(PRS-110) / (EC50 + c(PRS-110)), with c(PRS-110) being the concentration of the conjugated and unconjugated PRS-110 molecules, respectively.

The stable attachment of the radiolabel to the Anticalins was monitored up to 96 h for all conjugates in an excess of 0.9% NaCl at 4  $^{\circ}$ C and in human serum at 37  $^{\circ}$ C by the TCA precipitation assay as described above.

The generation and quality control of a negative control radiotracer, <sup>89</sup>Zr-Tlc-PEG, was carried out in an identical fashion. The negative control is based on the wild-type tear lipocalin (Tlc), which is identical to PRS-110 with the exception of specific mutations, which were introduced by protein evolution and engineering to facilitate MET binding (1). It therefore exhibits a very similar molecular weight and is expected to possess a nearly identical overall structure. Tlc-PEG is a PEGylated version of Tlc containing the same 40kD-PEG moiety as PRS110, and has been described previously (1). Tlc-PEG was conjugated to deferoxamine-p-SCN at a ratio of 1:5, the optimal ratio as determined for PRS-110.

For fluorescence imaging experiments, PRS-110 was labeled with the infrared-fluorescent dye IRDye 800CW (Licor Biosciences) according to Licor's protocol. In short, the pH of a 1 mg/mL solution of PRS-110 was set at 8.5 with 0.1 M Na<sub>2</sub>CO<sub>3</sub>. The NHS ester of the IRDye 800CW was added at a molar ratio of 1:5 and incubated for 2 h at 20 °C. The mixture was purified by PD-10 desalting column filtration and analyzed by SE-HPLC as described above.

### RESULTS

## Synthesis and Quality Control of Conjugated and Radiolabeled <sup>89</sup>Zr-PRS-110

PRS-110 was conjugated with deferoxamine-*p*-SCN at three different ratios of Anticalin to deferoxamine-*p*-SCN (1:2.5, 1:5, 1:10). In all cases, there was no impact of conjugation on target binding according to an ELISA assay (Supplemental Fig. 2A).

The conjugated Anticalins were subsequently labeled with <sup>89</sup>Zr and the aggregation tendency and radiochemical purity of the products was analyzed by SE-HPLC and a TCA precipitation assay. The latter assay was also used to assess the stable attachment of the radiolabel to the Anticalins over time in 0.9% NaCl at 4 <sup>o</sup>C and in human serum at 37 <sup>o</sup>C. For all labeling ratios, we found a stable product in both conditions, with only minor release of <sup>89</sup>Zr from PRS-110 up to 96 h after radiolabeling (Supplemental Fig. 2B). PRS-110 labeled at a ratio of 1:5 or 1:10 allowed for a specific activity of 100 MBq/mg at a radiochemical purity >95% without purification, while this was not obtainable with the 1:2.5 labeled PRS-110. According to SE-HPLC, the products at a labeling ratio of 1:2.5 and 1:5 remained monomeric and free of aggregates and fragments or radioactive impurities (Supplemental Fig. 2C). At a ratio of 1:10, however, aggregates became visible presumably due to the increased hydrophobicity of the molecule after conjugation (data not shown). Based on the purity, stability and high attainable specific activity, we selected the 1:5 conjugated PRS-110 molecule for

the experiments described herein. A negative control molecule, <sup>89</sup>Zr-Tlc-PEG, was generated with the procedure found to be optimal for PRS-110. Conjugation was performed at a Tlc-PEG to deferoxamine-*p*-SCN ratio of 1:5, and the molecule was purified and assessed for radiochemical purity and stability as described for <sup>89</sup>Zr-PRS-110, with identical results (data not shown).



## Supplemental Figure 1.

H441, U87-MG and A2780 tumor cells were analyzed for MET expression by flow cytometry analysis. The results are presented in mean fluorescence intensity (MFI); data was obtained in 3 independent experiments.



### Supplemental Figure 2.

Quality control of <sup>89</sup>Zr-PRS-110. Binding of <sup>89</sup>Zr-PRS-110 at different chelation ratios was tested in a binding ELISA (A), fluorescence signals in RFU (relative fluorescence units). Panel B shows the stability of <sup>89</sup>Zr-PRS-110 in 0.9% NaCl at 4 °C and in human serum at 37 °C expressed as radiochemical purity (RCP). Panel C shows a typical SE-HPLC chromatogram of <sup>89</sup>Zr-PRS-110 with detection at 280 nm for the protein signal and co-registration of radioactive <sup>89</sup>Zr signal. Data was obtained in 3 independent experiments.



## Supplemental Figure 3.

<sup>89</sup>Zr-PRS-110 microPET imaging of H441 (A), U87-MG (B) and A2780 (C) bearing mice. Representative maximum intensity projection microPET images are shown at 96 h after tracer injection of <sup>89</sup>Zr-PRS-110.

# Supplemental Table 1:

%ID/g values of biodistribution results 96h after injection of 10, 50, 100 and 500  $\mu g$   $^{89}$ Zr-PRS-110 to H441 human tumor bearing mice.

%ID/g	<sup>89</sup> Zr-PRS-110											
_	1	)	5	0 μο	)	1	00 µ	g	500 µg			
Heart	1.19	±	0.12	1.26	±	0.19	1.12	±	0.14	1.27	±	0.14
Blood	1.73	±	0.18	1.95	±	0.09	1.31	±	0.09	2.03	±	0.08
Lung	1.21	±	0.11	5.83	±	1.56	4.11	±	1.82	2.01	±	0.18
Liver	3.01	±	0.39	4.87	±	0.46	3.81	±	0.59	3.51	±	0.23
Kidney	2.06	±	0.15	3.90	±	0.28	2.00	±	0.17	2.60	±	0.22
Urine	0.58	±	0.11	0.76	±	0.26	0.73	±	0.08	0.47	±	0.08
Bladder	0.89	±	0.04	1.23	±	0.14	0.98	±	0.07	0.98	±	0.09
Stomach	0.39	±	0.02	0.50	±	0.14	0.38	±	0.03	0.46	±	0.14
Pancreas	0.40	±	0.02	0.55	±	0.07	0.42	±	0.05	0.39	±	0.03
Spleen	2.67	±	0.58	8.13	±	2.00	5.94	±	0.94	5.99	±	0.38
Small intestine	0.32	±	0.07	0.46	±	0.10	0.32	±	0.02	0.31	±	0.02
Colon	0.41	±	0.16	0.54	±	0.06	0.36	±	0.05	0.32	±	0.03
Muscle	0.26	±	0.03	0.31	±	0.06	0.33	±	0.06	0.25	±	0.07
Bone	0.58	±	0.14	1.38	±	0.29	0.77	±	0.12	0.82	±	0.13
Brain	0.06	±	0.01	0.08	±	0.01	0.09	±	0.04	0.08	±	0.01
Tumor	7.53	±	3.38	5.92	±	1.14	4.87	±	0.46	2.93	±	0.89

# Supplemental Table 2:

%ID/g values of biodistribution results 96h after injection of 50 μg <sup>89</sup>Zr-PRS-110 H441, U87-MG and A2780 bearing mice.

%ID/g	H	441	U87	-MG	A2780					
	<sup>89</sup> Zr-PRS-110	<sup>89</sup> Zr-Tlc-PEG	<sup>89</sup> Zr-PRS-110	<sup>89</sup> Zr-Tlc-PEG	<sup>89</sup> Zr-PRS-110	<sup>89</sup> Zr-Tlc-PEG				
Heart	1.26 ± 0.19	1.31 ± 0.17	1.03 ± 0.30	1.42 ± 0.21	0.90 ± 0.12	1.80 ± 0.21				
Blood	1.95 ± 0.09	1.96 ± 0.33	1.72 ± 1.52	1.96 ± 0.39	1.23 ± 0.48	2.53 ± 0.33				
Lung	5.83 ± 1.56	2.01 ± 0.59	4.00 ± 1.26	1.67 ± 0.19	1.41 ± 0.53	1.87 ± 0.36				
Liver	4.87 ± 0.46	4.85 ± 0.80	6.88 ± 1.84	7.96 ± 2.12	5.84 ± 2.12	6.51 ± 0.79				
Kidney	3.90 ± 0.28	3.88 ± 0.70	1.39 ± 1.16	5.50 ± 0.88	2.92 ± 0.56	5.55 ± 1.22				
Urine	0.76 ± 0.26	$0.66 \pm 0.08$	1.70 ± 1.36	0.96 ± 0.68	0.74 ± 0.31	0.65 ± 0.22				
Bladder	1.23 ± 0.14	1.30 ± 0.17	0.94 ± 0.39	1.06 ± 0.41	1.01 ± 0.26	1.74 ± 0.20				
Stomach	0.50 ± 0.14	$0.48 \pm 0.05$	0.35 ± 0.10	0.52 ± 0.10	0.39 ± 0.09	0.83 ± 0.35				
Pancreas	0.55 ± 0.07	$0.61 \pm 0.04$	0.51 ± 0.11	0.52 ± 0.11	0.45 ± 0.09	0.91 ± 0.24				
Spleen	8.13 ± 2.00	9.43 ± 1.15	5.72 ± 1.65	$6.00 \pm 0.60$	4.14 ± 1.22	5.49 ± 1.52				
Small intestine	0.46 ± 0.10	$0.95 \pm 0.36$	$0.90 \pm 0.86$	0.49 ± 0.13	0.42 ± 0.18	1.18 ± 0.25				
Colon	0.54 ± 0.06	0.84 ± 0.16	0.56 ± 0.18	0.51 ± 0.08	0.44 ± 0.22	0.78 ± 0.19				
Muscle	0.31 ± 0.06	$0.44 \pm 0.08$	0.32 ± 0.12	0.39 ± 0.06	0.34 ± 0.18	0.59 ± 0.20				
Bone	1.38 ± 0.29	2.08 ± 0.37	1.30 ± 0.38	1.65 ± 0.44	1.22 ± 0.15	3.23 ± 0.52				
Brain	0.08 ± 0.01	$0.07 \pm 0.02$	$0.06 \pm 0.02$	0.07 ± 0.01	0.06 ± 0.01	0.10 ± 0.02				
Tumor	5.92 ± 1.14	$3.90 \pm 0.84$	1.78 ± 0.25	1.19 ± 0.29	1.71 ± 0.52	2.53 ± 0.90				

# Supplemental Table 3:

Organ-to-blood values of biodistribution results 96h after injection of 50 μg<sup>89</sup>Zr-PRS-110 H441, U87-MG and A2780 bearing mice.

		H4	41		U87	-MG		A2780										
	<sup>89</sup> Zr-PRS-110			<sup>89</sup> Zr-Tlc-PEG			<sup>89</sup> Zr-PRS-110			<sup>89</sup> Zr-Tlc-PEG			<sup>89</sup> Zr-PRS-110			<sup>89</sup> Zr-Tlc-PEG		
Heart	0,68	±	0,11	0,67	±	0,07	1,07	±	0,81	0,73	±	0,09	0,80	±	0,22	0,71	±	0,02
Blood	1,05	±	0,06	1,00	±	0,00	1,00	±	0,00	1,00	±	0,00	1,00	±	0,00	1,00	±	0,00
Lung	3,15	±	0,91	1,02	±	0,15	4,46	±	3,74	0,87	±	0,15	1,16	±	0,10	0,74	±	0,10
Liver	2,61	±	0,20	2,50	±	0,33	9,22	±	11,23	4,43	±	2,45	5,69	±	3,83	2,59	±	0,28
Kidney	2,10	±	0,22	1,99	±	0,24	2,57	±	3,31	2,88	±	0,64	2,53	±	0,57	2,23	±	0,55
Urine	0,41	±	0,14	0,34	±	0,03	1,14	±	0,31	0,56	±	0,52	0,60	±	0,12	0,20	±	0,12
Bladder	0,66	±	0,08	0,68	±	0,17	0,90	±	0,77	0,53	±	0,13	0,88	±	0,26	0,69	±	0,06
Stomach	0,27	±	0,08	0,25	±	0,02	0,55	±	0,68	0,28	±	0,10	0,33	±	0,06	0,32	±	0,11
Pancreas	0,30	±	0,04	0,32	±	0,05	0,57	±	0,53	0,27	±	0,04	0,39	±	0,11	0,36	±	0,06
Spleen	4,37	±	1,12	6,47	±	1,04	7,82	±	9,42	3,14	±	0,49	3,51	±	0,60	6,13	±	1,05
Small intestine	0,25	±	0,05	0,52	±	0,30	2,24	±	3,92	0,25	±	0,03	0,35	±	0,11	0,48	±	0,17
Colon	0,29	±	0,04	0,44	±	0,15	0,98	±	1,40	0,27	±	0,08	0,36	±	0,10	0,31	±	0,06
Muscle	0,17	±	0,03	0,24	±	0,09	0,32	±	0,22	0,20	±	0,02	0,34	±	0,28	0,19	±	0,12
Bone	0,74	±	0,16	1,11	±	0,40	1,71	±	2,08	0,91	±	0,50	1,13	±	0,47	0,99	±	0,61
Brain	0,04	±	0,00	0,03	±	0,00	0,06	±	0,04	0,04	±	0,00	0,05	±	0,02	0,04	±	0,01
Tumor	3,17	±	0,55	2,09	±	0,75	2,41	±	2,86	0,64	±	0,25	1,51	±	0,57	1,00	±	0,33

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