Protein Ligand Production

The Affibody molecules were fused with the C-terminal Sel-tag utilizing the method described by Cheng et al. (*1*). The gene sequences of $Z_{EGFR:2377}$ (*2*) and $Z_{HER2:342}$ (*3*) were PCR amplified in two separate reactions using a forward primer (5'-acgagccatcctgcaggtagataacaaattcaacaaag) introducing a *PstI* restriction site and a reverse primer introducing the Sel-tag-encoding sequence, the bacterial SECIS element and a *Hin*dIII restriction site (5'-agtgataagcttggccgcataggctaacgattggtgcagacctgcaaccgattattagcctcagcagcctttcggcgcctgagcatcatttagc) (Eurofins mwg operon). The PCR products were purified using QIAquick PCR purification kit (QIAGEN) before restriction with *Pst*I and *Hin*dIII (New England Biolabs) according to the manufacturer's recommendations. The restricted gene fragments were ligated into the pET-derived expression vectors pAY457 and pAY430, both containing a multiple cloning site, a gene for kanamycin (Km) resistance and the T7 promoter (*4*). In addition, pAY430 carries a gene fragment for an N-terminal His_6-tag. The ligation products were transformed into the *E. coli* strain RR1ΔM15 (*5*) and confirmed by DNA sequencing. To improve the efficiency of Sec insertion, BL21(DE3) cells (Novagen) already harboring the pSUABC plasmid (*6,7*) were transformed with the plasmids and double transformants were selected on agar plates containing Km and chloramphenicol (Cm). Protein expression was subsequently performed with selected clones, whereby Sec insertion was verified in pilot experiments using ^{75}Se labeling (*1*).

Large-scale recombinant production of the His_6-tagged proteins, His_6-$Z_{HER2:342}$-ST (-ST denoting the Sel-tag) and His_6-$Z_{EGFR:2377}$-ST, was performed by shake flask cultivations. 1.5 L LB medium (10 g/L pepton, 10 g/L yeast extract, 10 g/L NaCl) with 50 μg/mL Km and 34 μg/mL Cm was inoculated with 1.5 mL overnight cultures and grown in 37°C, 200 rpm. When the cultivations reached late exponential phase, gene expression was induced by adding IPTG to a final concentration of 0.5 mM. L-cysteine and sodium selenite were added to final

concentrations of 100 μg/L and 5 μM, respectively. Cultivations were then continued over night in 25°C, 200 rpm, before harvesting by centrifugation (5 000 x *g*, 15 min, 3°C), followed by disruption of the cells by incubation with lysozyme, 0.5 mg/mL, for 1 h on ice. The two His_6-tagged proteins were purified under native conditions in 50 mM Tris-HCl, 500 mM NaCl, pH 7.5 using immobilized metal ion affinity chromatography (IMAC) on an ÄKTA explorer system (GE Healthcare) with a 5 mL HisTrap FF column (GE Healthcare). The proteins were eluted with 50 mM Tris-HCl, 500 mM NaCl, pH 7.5, containing 250 mM imidazole and further purified by gel filtration on a HiLoad 16/60 Superdex 200 pg size exclusion column eluted with phosphate buffered saline (PBS), pH 7.4.

The Sel-tagged HER2-binding Affibody variant without the N-terminal His_6-tag, $Z_{HER2:342}$-ST, was produced in a 20 L batch fermenter using TSB + YE medium (30 g/L tryptic soy broth, 5 g/L yeast extract). Upon induction with IPTG, the cultures were supplemented with L-cysteine and selenite as described above and cultivation was continued over night. Immediately after harvest, the cells were centrifuged (15 900 x *g*, 15 min), the pellet resuspended in TST buffer (25 mM Tris-HCl, 1 mM EDTA, 0.2 M NaCl and 0.05% Tween 20) and then heated (90°C, 3 min) to disrupt the cells, precipitate cell debris material and as an initial purification step. After cooling on ice, cell debris was removed by centrifugation (25 000 x *g*, 20 min, 4°C). The total yield of $Z_{HER2:342}$-ST from this 20 L cultivation was approximately 7 g. The protein was further purified by affinity chromatography, using an anti-idiotypic Affibody molecule binding to the $Z_{HER2:342}$ as previously described (*8*), with a final, un-optimized yield of approximately 15 mg of protein per liter cultivation. Identity and purity of all purified proteins were assessed by SDS-PAGE analysis and LC-MS using an Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS system. The purified protein was lyophilized using a Thermo Savant Mudulyo D (Techtum Lab).

Fluorescent Labeling of Sel-Tagged Proteins

After initial reduction using 2 mM dithiothreitol (DTT) for 30 min at room temperature (RT), 5-IAF dissolved in DMSO and urea were added to the proteins to final concentrations of 5 mM and 2.2 M, respectively. The proteins were incubated in RT for 20 min followed by purification on a NAP-5 column (GE Healthcare) to remove DTT, urea and unconjugated 5-IAF. The fluorescence of the proteins was verified by SDS-PAGE and UV-light inspection.

Cell Lines, Culture Conditions and FACS analysis

The HER2-expressing cell lines SKOV-3 (ovarian carcinoma) and MDA-MB-453 (breast carcinoma) were cultured in McCoy´s 5A media and Dulbecco´s Modified Eagle´s Medium (DMEM), respectively, both supplemented with 10 % fetal bovine serum (FBS) and 100 units penicillin/mL, 100 µg streptomycin/mL (PEST). The EGFR-expressing cell line A431 (epidermal carcinoma) was cultured in DMEM with L-glutamine and 4500 mg/L D-glucose supplemented with 1 mM sodium pyruvate, PEST and 10 % FBS. All cell lines were purchased from ATCC-LGC, their integrity independently verified with short tandem repeat profiling cell authentication analysis (LGC Standards) in December 2010 and cultured at 37°C and 5 % CO_2 in a humidified environment.

For cell binding analysis by FACS, cells were washed with ice-cold PBS containing 10 mg/mL albumin (PBS/albumin), resuspended in 100 µL PBS/albumin containing 100 nM IAF-labeled protein and incubated for 1 h in RT. To a subset of the cells, a tenfold excess of non-labeled Affibody molecule was also added, in order to investigate the binding specificity. The cells were washed with PBS/albumin and the cell-associated fluorescence of the blocked and non-blocked cells was evaluated using a FACSsort Calibur flow cytometer (Becton-Dickinson) and analyzed using FlowJo 7.6 flow cytometry analysis software (Becton-Dickinson).

References

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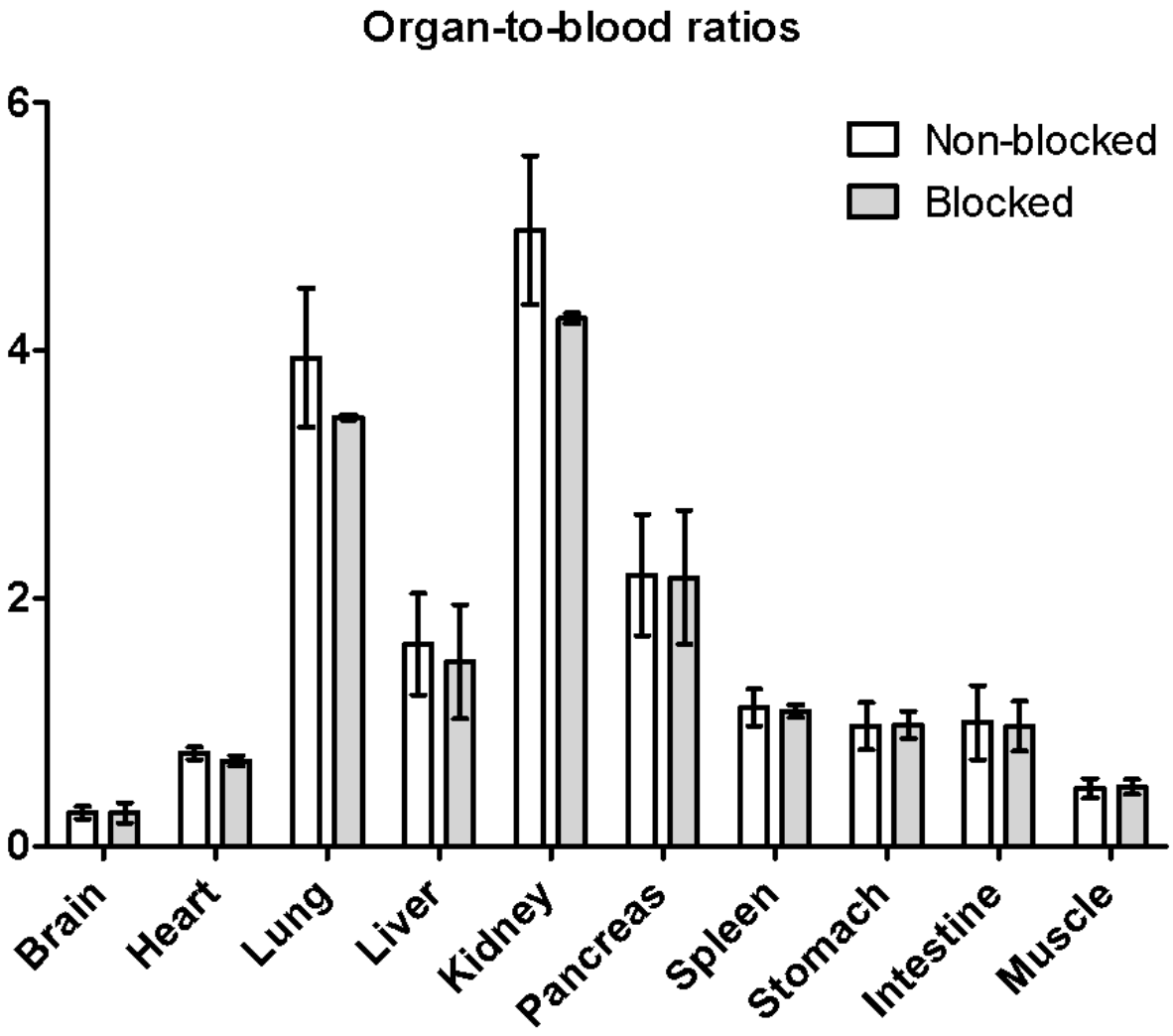

SUPPLEMENTAL FIGURE 1: Comparative biodistribution of ^{11}C-$Z_{HER2:342}$-ST in mice before and after blocking of receptors with an excess of non-labeled tracer. The overall distribution profile was very similar before and after blocking.