

# High contrast tumor imaging with radio-labeled antibody Fab fragments tailored for optimized pharmacokinetics via PASylation

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**Abbreviations:** ABD, albumin binding domain; CDC, complement-dependent cytotoxicity; CDR, complementarity-determining region; CLL, chronic lymphocytic leukemia; DMEM, Dulbecco's modified Eagle medium; EPR, enhanced permeability and retention effect; Fab, antigen-binding fragment; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FcRn, neonatal Fc receptor; HER2, human epidermal growth factor receptor 2; ID, injected dose; IDA, iminodiacetic acid; Ig, immunoglobulin; mAb, monoclonal antibody; MIP, maximum intensity projection; NHL, non-Hodgkin lymphoma; PET, positron emission tomography; p.i., post injection; PK, pharmacokinetics; RIT, radioimmuno therapy; scFv, single-chain variable antibody fragment; SEC, size exclusion chromatography; SPECT, single photon emission computed tomography; TLC, thin layer chromatography

Although antigen-binding fragments (Fabs) of antibodies constitute established tracers for in vivo radiodiagnostics, their functionality is hampered by a very short circulation half-life. PASylation, the genetic fusion with a long, conformationally disordered amino acid chain comprising Pro, Ala and Ser, provides a convenient way to expand protein size and, consequently, retard renal filtration. Humanized  $\alpha$ HER2 and  $\alpha$ CD20 Fabs were systematically fused with 100 to 600 PAS residues and produced in *E. coli*. Cytofluorimetric titration analysis on tumor cell lines confirmed that antigen-binding activities of the parental antibodies were retained. The radio-iodinated PASylated Fabs were studied by positron emission tomography (PET) imaging and biodistribution analysis in mouse tumor xenograft models. While the unmodified  $\alpha$ HER2 and  $\alpha$ CD20 Fabs showed weak tumor uptake (0.8% and 0.2% ID/g, respectively; 24 h p.i.) tumor-associated radioactivity was boosted with increasing PAS length (up to 9 and 26-fold, respectively), approaching an optimum for Fab-PAS<sub>400</sub>. Remarkably, 6- and 5-fold higher tumor-to-blood ratios compared with the unmodified Fabs were measured in the biodistribution analysis (48 h p.i.) for  $\alpha$ HER2 Fab-PAS<sub>100</sub> and Fab-PAS<sub>200</sub>, respectively. These findings were confirmed by PET studies, showing high imaging contrast in line with tumor-to-blood ratios of 12.2 and 5.7 (24 h p.i.) for  $\alpha$ HER2 Fab-PAS<sub>100</sub> and Fab-PAS<sub>200</sub>. Even stronger tumor signals were obtained with the corresponding  $\alpha$ CD20 Fabs, both in PET imaging and biodistribution analysis, with an uptake of 2.8% ID/g for Fab-PAS<sub>100</sub> vs. 0.24% ID/g for the unmodified Fab. Hence, by engineering Fabs via PASylation, plasma half-life can be tailored to significantly improve tracer uptake and tumor contrast, thus optimally matching reagent/target interactions.

## Introduction

Monoclonal antibodies (mAbs), including their antigen-binding fragments (Fabs), constitute the fastest growing class of biotherapeutic agents today.<sup>1,2</sup> Among those, in particular the “blockbuster” antibodies trastuzumab (Herceptin<sup>®</sup>) and rituximab (Rituxan<sup>®</sup>), which are directed against human epidermal growth factor receptor 2 (HER2) and the B-cell antigen CD20, respectively, are successfully applied for the treatment of tumors in patients with metastatic breast

cancer and non-Hodgkin lymphoma (NHL).<sup>3</sup> Apart from such naked IgGs, radio-immunoconjugates like <sup>90</sup>Y ibritumomab tiuxetan (Zevalin<sup>®</sup>), also directed against CD20, have shown promise for tumor targeting in clinical therapy.<sup>4</sup>

HER2 is overexpressed in 20–30% of primary breast cancers,<sup>5</sup> but also in other tumor types, including ovarian, bladder, pancreatic and lung cancer, which is usually associated with poor prognosis.<sup>6</sup> In 1998 the US Food and Drug Administration (FDA) approved the humanized IgG1/ $\kappa$  trastuzumab – initially obtained by

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complementarity-determining region (CDR) grafting from the mouse mAb 4D5 and subsequently engineered toward improved affinity<sup>7</sup> – for the treatment of patients with HER2-positive metastatic breast cancer alone or in combination with chemotherapy.<sup>8</sup> Recently, the antibody-drug conjugate ado-trastuzumab emtansine (T-DM1, Kadcyla<sup>®</sup>), developed by chemical coupling of trastuzumab with the cytotoxin mertansine, was approved.<sup>9</sup>

CD20 is expressed on the pre-B to mature B-cell lineage and occurs in >90% of patients with B-cell lymphoma, whereas most plasma immune cells are CD20 negative.<sup>10,11</sup> The human IgG1/ $\kappa$  ofatumumab (2F2, Arzerra<sup>®</sup>), generated in human Ig-transgenic mice, was approved by the FDA in 2009 for patients with chronic lymphocytic leukemia (CLL).<sup>12</sup> The reduced immunogenicity of ofatumumab compared to the first CD20-targeting mAb to be approved, rituximab (Rituxan<sup>®</sup>/MabThera<sup>®</sup>), led to reduced side effects in clinical studies.<sup>13</sup> Its lower off-rate further contributed to more efficient complement-dependent cytotoxicity (CDC) on target cells compared to rituximab.<sup>14</sup>

Although several radio-labelled mAbs have been developed for cancer radioimmunotherapy (RIT) and evaluated in clinical trials during the last decades,<sup>15</sup> radioimmuno-imaging has attracted even greater attention for target evaluation and dosimetry. Recently, immuno-positron emission tomography (Immuno-PET) has gained particular interest since long-lived positron emitters such as <sup>124</sup>I and <sup>89</sup>Zr have become commercially available.<sup>16</sup> For example, <sup>89</sup>Zr-trastuzumab was applied to non-invasively assess the HER2 status in patients with metastatic breast cancer.<sup>17,18</sup> In another study, the chimeric IgG1/ $\kappa$  antibody <sup>124</sup>I-cG250 directed against carbonic anhydrase IX was used to diagnose clear-cell renal carcinoma in 26 patients.<sup>19</sup>

Due to their intrinsically long plasma half-life, typically in the range of 2–3 weeks,<sup>20</sup> which allows extended contact time with cell surface antigens, and also owing to the enhanced permeability and retention effect (EPR),<sup>21,22</sup> full-size antibodies can reach high uptake levels in tumor tissue. On the other hand, their poor tissue penetration and often inhomogeneous distribution, which leaves areas of the tumor untargeted, is a general problem for tumor therapy<sup>23</sup> as well as high-precision imaging. Furthermore, the prolonged circulation time leads to high background in the blood, especially at shorter time points, as well as exposure of healthy organs to the radionuclide conjugate, which often causes side effects and appears dose limiting in RIT.<sup>24</sup>

Especially for in vivo diagnostic applications, where high tumor imaging contrast (i.e., signal-to-background ratio) is desired shortly after tracer administration, antibody fragments with their better tissue penetration, much faster blood clearance and lack of FcRn binding, which is responsible for Fc-mediated endosomal recycling of full-size immunoglobulins (Igs), are preferred.<sup>25,26</sup> Conversely, their smaller size and very fast kidney clearance (from minutes to a few hours in humans) often prevents sufficient accumulation of the tracer at the targeted tumor tissue, and is responsible for the rapid wash-out at later time points when plasma concentrations are decreased.<sup>25,27,28</sup> Consequently, improving the targeting properties of antibody fragments by manipulating their plasma half-life via protein

engineering may provide even more useful radioimaging reagents.

Up to now, several attempts were made to prolong the circulation and control the in vivo biodistribution of protein-based tracers. For example, fusion with a phage-derived albumin binding peptide has been employed to extend plasma half-life of the HER2-specific Fab 4D5, which indeed led to higher tumor accumulation.<sup>27</sup> This approach took advantage of the high abundance and intrinsically long life-time of serum albumin, which like IgGs possesses a size above the threshold for renal filtration and moreover gets recycled via FcRn.<sup>29,30</sup> However, as albumin is catabolized by many tumors,<sup>31,32</sup> which generally promotes tumor uptake of an associated tracer, it is difficult to ascribe the observed effects to the increased plasma half-life alone.

An alternative, clinically-approved method to retard renal clearance of bioactive proteins is the chemical conjugation with polyethylene glycol (PEG), which enlarges the hydrodynamic molecular volume beyond the glomerular pore size.<sup>33</sup> Previous studies have shown that PEGylation of a single-chain variable antibody fragment (scFv), and also of a designed ankyrin repeat protein (DARPin), both directed against HER2, led to enhanced tumor uptake.<sup>34,35</sup> However, the longer persistence in plasma due to the fusion with a 20 kDa PEG moiety also resulted in lower tumor-to-blood ratios.

Recently, PASylation technology was developed as a biological alternative to PEGylation.<sup>36</sup> In this case, a long biosynthetic polypeptide composed of the 3 small amino acids Pro, Ala and Ser (PAS), was designed to adopt a highly solvated random coil structure under physiological conditions with very similar biophysical properties as PEG, thus drastically increasing the hydrodynamic volume of the attached protein reagent. In this manner, much prolonged plasma half-life, depending on the length of the PAS sequence, can be achieved while, in contrast to PEG, PAS constitutes a biodegradable and non-immunogenic polymer. Another major advantage of PASylation is that the amino acid polymer can be genetically encoded and directly produced as a fusion protein, thus avoiding the laborious optimization of chemical coupling conditions.<sup>37</sup> Using pre-made gene cassettes, the precise tuning of the length of the PAS tag attached to a functional binding protein is easily achieved. Hence, the loss of binding activity, which often accompanies PEGylation of proteins, as well as the formation of a heterogeneous bioconjugate, is prevented.

In the present study, we have employed PASylation of recombinant Fab fragments with specific tumor-targeting activities to tailor their pharmacokinetic (PK) properties and investigated the effect on both tumor uptake and tumor-to-normal tissue or tumor-to-blood ratio. To this end, we utilized the  $\alpha$ HER2 Fab derived from trastuzumab (hu4D5-v8)<sup>7</sup> and the  $\alpha$ CD20 Fab derived from ofatumumab (2F2)<sup>38</sup> as model tracers with clinical relevance because both their antigens represent established tumor markers.<sup>39</sup> The two Fab fragments were fused to PAS sequences of 100 up to 600 residues and functionally produced in *E. coli*, followed by radio-iodination and PET imaging, as well as biodistribution analysis in CD1-Foxn1<sup>tm</sup> mice bearing HER2- and CD20-positive tumor xenografts, respectively.

## Results

### Preparation of PASylated $\alpha$ HER2 and $\alpha$ CD20 Fabs with extended plasma half-life

For in vivo imaging of HER2-positive xenograft tumors, an affinity-matured version of the  $\alpha$ HER2 Fab fragment based on trastuzumab was used. First, a couple of conservative amino acid exchanges at the N- and C-termini of the V-gene regions that had been inserted to facilitate cloning during construction of the previously described *E. coli* expression vector pASK88-4D5<sup>40</sup> were reversed by site-directed mutagenesis to fully match the hu4D5-v8 sequence.<sup>7</sup> Second, a single point mutation, D<sup>H198</sup>W, which was described to result in threefold improved antigen affinity,<sup>41</sup> was introduced into CDR-H3.

For in vivo imaging of CD20-positive xenograft tumors, the recombinant Fab fragment 2F2 derived from ofatumumab<sup>14</sup> was cloned after gene synthesis of the V-gene regions by insertion into pASK88 in the same format as the  $\alpha$ HER2 Fab. pASK88 provides for human IgG1/ $\kappa$  constant gene regions, with a His<sub>6</sub>-tag attached to the C-terminus of the heavy chain as well as *E. coli* signal sequences to direct periplasmic secretion.<sup>42</sup>

To dissect the influence of circulation half-life on tumor uptake in mouse xenograft models, PAS sequences comprising 100, 200, 400 and 600 amino acid residues were fused to the C-terminus of the light chain according to a previously published strategy<sup>36</sup> (Fig. 1). For comparison, analogous fusion proteins of both Fabs with the *Streptococcal* albumin binding domain (ABD),<sup>43</sup> thus utilizing complex formation with serum albumin,<sup>27</sup> were constructed. The Fab of the murine mAb IN1 directed against the unrelated neuronal surface protein Nogo-A<sup>44</sup> served as a negative control.

All Fabs were produced as functional proteins in the periplasm of *E. coli*, where disulfide bond formation and light/heavy chain association can readily occur, and purified to homogeneity by means of His<sub>6</sub> and *Strep*-tag II affinity chromatography, size exclusion chromatography (SEC) and ion exchange chromatography. A protein yield of 0.4 mg l<sup>-1</sup> OD<sup>-1</sup> from shake flask culture was obtained for the  $\alpha$ CD20 Fab, comparable to the 4D5 Fab,<sup>40</sup> while the affinity-matured version of the latter Fab showed

slightly decreased yield (0.2 mg l<sup>-1</sup> OD<sup>-1</sup>). Notably, PASylation did not significantly affect protein yield.

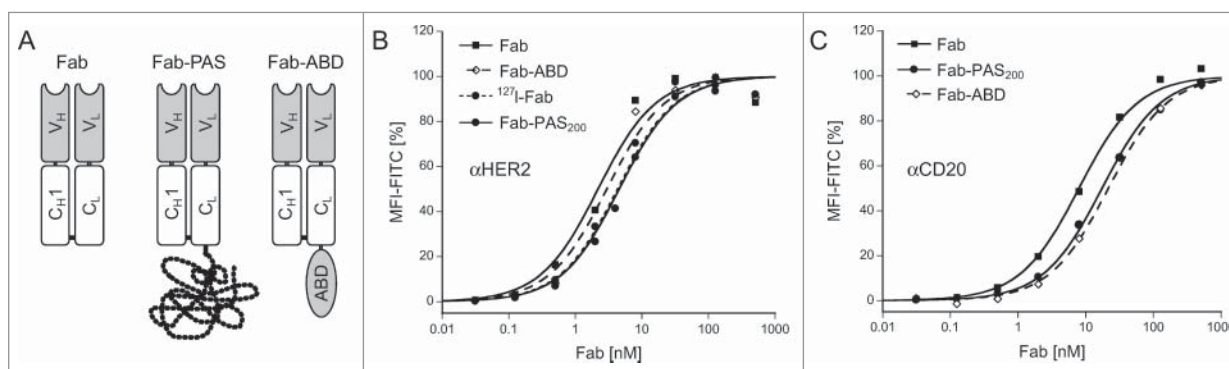
Proper disulfide bridge formation, with  $\geq 95\%$  covalent linkage between light and heavy chains as well as a reduced electrophoretic mobility depending on the length of the attached PAS tag, was detected by SDS-PAGE analysis. These results were consistent with a previous report on protein PASylation.<sup>36</sup> Likewise, SEC analysis revealed a homogeneous peak with decreasing elution volume upon increasing PAS length, indicating an expanded molecular size and a much enlarged hydrodynamic volume of the PASylated Fabs, as expected.

To assess binding activity towards the corresponding tumor antigens, fluorescence-activated cell sorting (FACS) titration analyses were performed on the HER2- and CD20-positive human tumor cell lines SK-BR-3<sup>45</sup> and Granta,<sup>46</sup> respectively. While the affinity-matured  $\alpha$ HER2 Fab showed a dissociation constant of 2.2 nM, a K<sub>D</sub> value of 8.0 nM was measured for the  $\alpha$ CD20 Fab (Fig. 1). Neither PASylation nor fusion with the ABD had a strong influence on this functional binding activity, which was even less than the small reduction in affinity observed during surface plasmon resonance measurements before.<sup>36</sup> The same was the case for non-radioactive iodination of the  $\alpha$ HER2 Fab. Overall, the measured affinities were comparable to those of the parental antibodies trastuzumab and ofatumumab, for which K<sub>D</sub> values of around 5 nM had been reported on the basis of in vitro cell assays.<sup>47,48</sup>

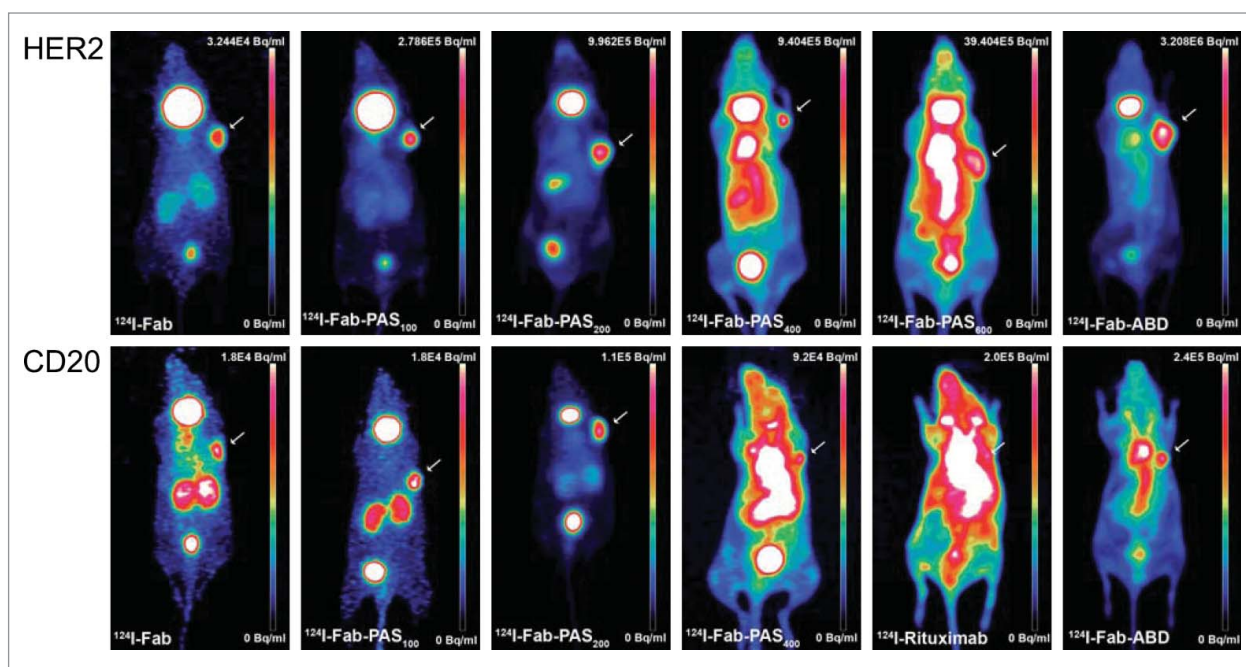
For subsequent in vivo tumor imaging, the  $\alpha$ HER2 and  $\alpha$ CD20 Fabs as well as their fusion proteins were radiolabeled via the Iodogen coupling procedure<sup>49</sup> with the  $\beta^+/\gamma$  emitter <sup>124</sup>I, whereas the  $\gamma$  emitter <sup>125</sup>I was chosen for in vivo biodistribution analysis. In addition, <sup>125</sup>I was used in combination with <sup>124</sup>I or with the  $\gamma$  emitter <sup>123</sup>I for dual tracer experiments.

### In vivo PET tumor imaging

To evaluate the PK and biodistribution properties of the radio-iodinated  $\alpha$ HER2 and  $\alpha$ CD20 Fabs in their various PASylated formats, in vivo PET imaging was performed using CD1-*Foxn1*<sup>tmu</sup> mice bearing HER2- or CD20-positive human tumor



**Figure 1.** In vitro characterization of purified recombinant Fabs. **(A)** Schematic representation of the bacterially produced Fab format and its fusions with a PAS polypeptide or an albumin binding domain (ABD). **(B, C)** FACS titration analyses of  $\alpha$ HER2 and  $\alpha$ CD20 Fabs in different formats versus corresponding tumor cell lines. Fabs were incubated with SK-BR-3 (HER2) or Granta (CD20) cells for 1 h at 4°C and, after washing, bound Fab was detected with a fluorescein-conjugated  $\alpha$ hu-kappa-light chain antibody. Hyperbolic curve fit (see Materials and Methods) revealed K<sub>D</sub> values of 2.2 ± 0.6 nM for  $\alpha$ HER2 Fab, 4.6 ± 0.9 nM for  $\alpha$ HER2 Fab-PAS<sub>200</sub>, 3.0 ± 0.7 nM for  $\alpha$ HER2 Fab-ABD, 4.3 ± 0.6 nM for the (non-radioactively) iodinated  $\alpha$ HER2 Fab as well as 8.0 ± 1.1 nM for  $\alpha$ CD20 Fab, 15.6 ± 5.7 nM for  $\alpha$ CD20 Fab-ABD and 17.5 ± 1.0 nM for  $\alpha$ CD20 Fab-PAS<sub>200</sub>.



**Figure 2.** MIP-PET images of xenograft tumors in mice using  $^{124}\text{I}$ -labeled  $\alpha\text{HER2}$  and  $\alpha\text{CD20}$  Fab fragments, respectively. CD1-*Foxn1*<sup>nu</sup> mice bearing s.c. HER2-positive (SK-BR-3) or CD20-positive (Granta) xenografts at the right shoulder (see arrows) were injected with  $^{124}\text{I}$ -labeled recombinant Fabs in different molecular formats: Fab, Fab-PAS<sub>100</sub>, Fab-PAS<sub>200</sub>, Fab-PAS<sub>400</sub>, Fab-PAS<sub>600</sub>, Fab-ABD as well as, for comparison, the full-size IgG rituximab. PET scans were performed 24 h p.i. for HER2 (top) and CD20 (bottom). Regardless of the reagent used, the thyroid gave rise to elevated background signals in all mice because this organ was not blocked for iodine uptake in these experiments.

xenografts. For comparison of the different constructs, PET images corresponding to representative static maximum intensity projections (MIP) were collected 24 h p.i. (Fig. 2), when a high tumor-to-background ratio was reached. At this time point, both unmodified Fabs allowed visualization of the tumor.

Notably, the  $\alpha\text{HER2}$  Fab fragment led to a better tumor-to-background ratio and lower kidney accumulation, possibly due to its higher target affinity. A moderate radioactive signal in the bladder indicated renal excretion of both  $^{124}\text{I}$ -Fabs, as expected for proteins smaller than the glomerular pore size. The observed considerable thyroid accumulation was likely due to dehalogenation of the tracer, as iodide uptake by this organ was not specifically blocked.<sup>50</sup> Iodide is also transported from the bloodstream into the gastric lumen,<sup>51</sup> as was detectable via stomach uptake of radioactivity for some of the constructs.

Apart from this general pattern, which is in line with previous *in vivo* imaging and biodistribution studies of radio-iodinated Fabs,<sup>52</sup> there was a strong effect on the signal distribution upon applying PASylation, corresponding to an extension of (murine) plasma half-life in the range from approximately 1.3 (for the unfused Fab) to 28 h (in the case of the PAS<sub>600</sub> tag).<sup>36</sup> In particular, PASylation led to a much enhanced imaging contrast 24 h p.i. for Fab-PAS<sub>100</sub> and Fab-PAS<sub>200</sub> compared with the unmodified Fabs, both for the HER2 and CD20 target (Fig. 2). This effect was particularly evident for the  $\alpha\text{CD20}$  Fab, where the fusion with 200 PAS residues led to comparable high tumor-to-background ratio as for the  $\alpha\text{HER2}$  Fab-PAS<sub>200</sub> in spite of its lower target affinity, as noted above.

In contrast, investigation of the Fabs carrying longer PAS sequences of 400 or 600 residues revealed an increasing background signal 24 h p.i. which was mainly detectable for the highly perfused organs, eventually leading to a lower tumor-to-normal tissue ratio. In fact, for the long PAS tags the biodistribution pattern approached that of full-length antibodies, such as  $^{124}\text{I}$ -rituximab (Fig. 2), with a typically poor tumor-to-blood ratio of 0.5 at 24 h p.i., which is in agreement with published data.<sup>53</sup>

Notably, fusion of the  $\alpha\text{CD20}$  and  $\alpha\text{HER2}$  Fabs with the ABD, as an alternative strategy to prolong circulation via association with albumin,<sup>27</sup> also resulted in good imaging contrast 24 h p.i. Compared with the structurally more expanded Fab-PAS<sub>600</sub> construct, which has similar plasma half-life,<sup>36</sup> the tumor-to-normal tissue ratio was actually better. However, albumin binding resulted in unfavorable accumulation of radioactivity in the blood, especially for the  $\alpha\text{CD20}$  Fab-ABD construct (cf. Fig. 2 and Table 2). Thus, the highly perfused organs like heart and lung became strongly visible, resulting in much poorer imaging contrast for the ABD fusion than for the PAS<sub>100/200</sub> versions described above.

#### Biodistribution of radiolabeled $\alpha\text{HER2}$ and $\alpha\text{CD20}$ Fabs

*In vivo* biodistribution studies were performed using the same subcutaneous human tumor xenograft models. Mice were injected with 5 pmol (specific activity 80–100 MBq/nmol) of the radio-iodinated Fabs. At 24 h p.i., both unmodified Fabs showed specific but rather low tumor accumulation of 0.80% ID/g for HER2-positive and 0.24% ID/g for CD20-positive xenografts (Tables 1 and 2). Nevertheless, a respectable tumor-to-blood

**Table 1.** Biodistribution of radio-iodinated  $\alpha$ HER2 Fabs (for tumor-to-organ ratios see Fig. S1)

[%ID/g]	<sup>125</sup> I-Fab			<sup>125</sup> I-Fab-PAS <sub>100</sub> *			<sup>125</sup> I-Fab-PAS <sub>200</sub> *		
	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Tumor	2.67 ± 0.33	0.80 ± 0.32	0.33 ± 0.15	7.41 ± 1.02	2.69 ± 1.05	1.20 ± 0.43	3.40 ± 0.70	2.15 ± 0.95	2.77 ± 0.79
Blood	2.46 ± 0.47	0.15 ± 0.02	0.13 ± 0.03	4.10 ± 0.39	0.22 ± 0.10	0.08 ± 0.04	4.16 ± 0.82	0.38 ± 0.11	0.24 ± 0.03
Heart	0.91 ± 0.17	0.06 ± 0.01	0.06 ± 0.02	1.49 ± 0.12	0.10 ± 0.05	0.04 ± 0.01	1.24 ± 0.13	0.11 ± 0.04	0.08 ± 0.01
Lung	1.76 ± 0.34	0.16 ± 0.07	0.14 ± 0.08	2.90 ± 0.34	0.25 ± 0.09	0.10 ± 0.03	2.18 ± 0.33	0.25 ± 0.08	0.17 ± 0.02
Liver	0.96 ± 0.19	0.09 ± 0.02	0.12 ± 0.04	1.14 ± 0.13	0.11 ± 0.04	0.07 ± 0.03	1.04 ± 0.22	0.10 ± 0.02	0.08 ± 0.01
Spleen	1.73 ± 0.52	0.06 ± 0.01	0.05 ± 0.01	1.96 ± 0.41	0.17 ± 0.09	0.05 ± 0.01	1.18 ± 0.18	0.10 ± 0.03	0.11 ± 0.03
Kidney	2.34 ± 0.36	0.21 ± 0.03	0.16 ± 0.03	8.39 ± 1.19	0.35 ± 0.08	0.12 ± 0.02	3.09 ± 0.49	0.27 ± 0.07	0.23 ± 0.02
A. gland	0.83 ± 0.21	0.09 ± 0.03	0.14 ± 0.07	1.62 ± 0.35	0.29 ± 0.12	0.07 ± 0.02	1.63 ± 0.39	0.10 ± 0.02	0.16 ± 0.02
Pancreas	1.56 ± 0.26	0.04 ± 0.01	0.04 ± 0.01	2.11 ± 0.70	0.13 ± 0.07	0.03 ± 0.02	0.93 ± 0.14	0.07 ± 0.02	0.06 ± 0.01
Stomach	10.03 ± 1.70	0.21 ± 0.07	0.13 ± 0.04	10.67 ± 3.89	0.59 ± 0.31	0.10 ± 0.05	3.25 ± 0.80	0.21 ± 0.08	0.16 ± 0.07
S. int.	1.93 ± 0.36	0.09 ± 0.02	0.11 ± 0.07	2.12 ± 0.33	0.20 ± 0.09	0.05 ± 0.03	0.83 ± 0.14	0.07 ± 0.03	0.07 ± 0.02
L. int.	2.13 ± 0.54	0.11 ± 0.05	0.12 ± 0.04	2.51 ± 0.64	0.26 ± 0.20	0.06 ± 0.04	0.55 ± 0.11	0.06 ± 0.01	0.07 ± 0.01
Muscle	0.46 ± 0.09	0.02 ± 0.00	0.02 ± 0.01	0.75 ± 0.21	0.05 ± 0.02	0.03 ± 0.04	0.35 ± 0.03	0.04 ± 0.01	0.03 ± 0.01
Bone	0.76 ± 0.16	0.03 ± 0.01	0.03 ± 0.01	0.88 ± 0.13	0.08 ± 0.03	0.03 ± 0.01	0.57 ± 0.09	0.05 ± 0.01	0.04 ± 0.01
Skin	1.86 ± 0.45	0.14 ± 0.03	0.10 ± 0.04	2.69 ± 0.81	0.25 ± 0.09	0.06 ± 0.01	1.30 ± 0.18	0.15 ± 0.05	0.12 ± 0.02
Brain	0.13 ± 0.02	0.01 ± 0.00	0.02 ± 0.01	0.15 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	0.13 ± 0.02	0.01 ± 0.00	0.01 ± 0.00
Thyroid	5.28 ± 3.06	8.47 ± 4.33	3.34 ± 1.40	6.72 ± 3.11	6.30 ± 2.71	8.53 ± 1.35	0.74 ± 0.22	2.56 ± 0.81	4.26 ± 1.49
[%ID/g]	<sup>123</sup> I-Fab-PAS <sub>400</sub> *			<sup>124</sup> I-Fab-PAS <sub>600</sub> *			<sup>125</sup> I-Fab-ABD		
	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Tumor	6.64 ± 0.59	7.19 ± 2.22	5.30 ± 1.01	4.61 ± 1.31	5.77 ± 0.94	3.72 ± 0.37	10.79 ± 2.07	15.42 ± 5.63	12.41 ± 3.19
Blood	18.46 ± 1.63	7.31 ± 0.94	2.06 ± 0.66	17.39 ± 4.35	8.22 ± 0.56	4.16 ± 0.63	21.17 ± 4.17	9.86 ± 2.85	5.75 ± 1.34
Heart	5.56 ± 0.89	1.98 ± 0.39	0.52 ± 0.15	4.24 ± 1.08	1.87 ± 0.36	0.98 ± 0.10	6.97 ± 1.73	3.16 ± 1.06	1.79 ± 0.34
Lung	7.32 ± 1.04	2.90 ± 0.45	1.05 ± 0.21	7.10 ± 2.16	2.90 ± 0.33	1.53 ± 0.23	10.33 ± 2.28	4.90 ± 1.96	2.74 ± 0.73
Liver	4.45 ± 0.67	1.64 ± 0.09	0.61 ± 0.10	3.55 ± 1.09	1.51 ± 0.14	0.99 ± 0.13	4.53 ± 0.60	2.01 ± 0.62	1.14 ± 0.24
Spleen	2.72 ± 0.20	1.46 ± 0.28	0.62 ± 0.13	2.60 ± 0.79	1.18 ± 0.30	1.19 ± 0.21	3.64 ± 0.86	1.55 ± 0.54	0.95 ± 0.17
Kidney	7.53 ± 1.03	3.13 ± 0.29	0.85 ± 0.20	4.24 ± 1.26	1.91 ± 0.13	1.15 ± 0.25	4.76 ± 0.99	2.33 ± 0.71	1.32 ± 0.26
A. gland	3.73 ± 0.99	2.13 ± 0.34	0.42 ± 0.07	5.20 ± 1.76	2.19 ± 0.93	1.14 ± 0.32	4.10 ± 1.82	2.46 ± 0.81	1.11 ± 0.15
Pancreas	1.85 ± 0.47	1.16 ± 0.16	0.29 ± 0.09	1.62 ± 0.35	0.63 ± 0.10	0.41 ± 0.07	1.77 ± 0.54	1.14 ± 0.28	0.65 ± 0.12
Stomach	3.82 ± 0.82	2.30 ± 0.64	0.65 ± 0.19	3.07 ± 0.60	1.14 ± 0.13	0.79 ± 0.13	3.79 ± 0.75	1.57 ± 0.32	0.91 ± 0.14
S. int.	1.84 ± 0.21	1.03 ± 0.23	0.28 ± 0.07	1.48 ± 0.28	0.48 ± 0.06	0.40 ± 0.03	2.45 ± 0.36	1.04 ± 0.33	0.62 ± 0.13
L. int.	1.67 ± 0.36	1.05 ± 0.25	0.31 ± 0.06	0.91 ± 0.22	0.37 ± 0.08	0.34 ± 0.04	1.44 ± 0.33	0.67 ± 0.16	0.47 ± 0.11
Muscle	0.66 ± 0.20	0.48 ± 0.04	0.16 ± 0.07	0.55 ± 0.07	0.37 ± 0.08	0.23 ± 0.10	0.90 ± 0.25	0.74 ± 0.28	0.50 ± 0.09
Bone	1.39 ± 0.36	0.98 ± 0.18	0.20 ± 0.05	1.48 ± 0.30	0.66 ± 0.16	0.34 ± 0.06	1.82 ± 0.17	0.98 ± 0.38	0.54 ± 0.11
Skin	2.56 ± 0.97	1.64 ± 0.28	0.44 ± 0.08	2.28 ± 0.54	1.39 ± 0.08	0.81 ± 0.07	3.62 ± 0.87	2.48 ± 0.73	1.64 ± 0.26
Brain	0.52 ± 0.06	0.22 ± 0.05	0.06 ± 0.02	0.47 ± 0.11	0.18 ± 0.04	0.12 ± 0.02	0.53 ± 0.07	0.21 ± 0.07	0.14 ± 0.02
Thyroid	1.16 ± 0.58	2.89 ± 1.49	6.08 ± 1.40	0.54 ± 0.22	2.07 ± 0.32	2.63 ± 0.82	0.19 ± 0.04	2.41 ± 0.97	2.20 ± 1.32

\*Biodistribution was quantified in dual tracer experiments in the combinations <sup>125</sup>I-Fab-PAS<sub>100</sub> / <sup>123</sup>I-Fab-PAS<sub>400</sub> and <sup>125</sup>I-Fab-PAS<sub>200</sub> / <sup>124</sup>I-Fab-PAS<sub>600</sub>.

ratio of 5.3 and 4.0 was detected for the  $\alpha$ HER2 and  $\alpha$ CD20 Fabs, respectively. As expected, no accumulation of the negative control Fab IN1 was observed for the SK-BR-3 xenograft (0.07% ID/g 24 h p.i.) (cf. Fig. 3).

Compared to the unmodified protein reagents, the series of different PASylated  $\alpha$ HER2 and  $\alpha$ CD20 Fabs with their successively extended plasma half-lives exhibited increasing tumor uptake with a maximum for 400 PAS residues: 7.2% ID/g for  $\alpha$ HER2 and 6.3% ID/g for  $\alpha$ CD20 24 h p.i. (Tables 1 and 2; Fig. 3A). Notably, the highest tumor accumulation with 15.4% ID/g for  $\alpha$ HER2 and 12.3% ID/g for  $\alpha$ CD20 was observed for the <sup>125</sup>I-Fab-ABD version 24 h p.i., yet at a relatively low tumor-to-blood ratio of 1.6, likely due to the association with albumin in the blood plasma. Again, dehalogenation of the radiotracer seemed

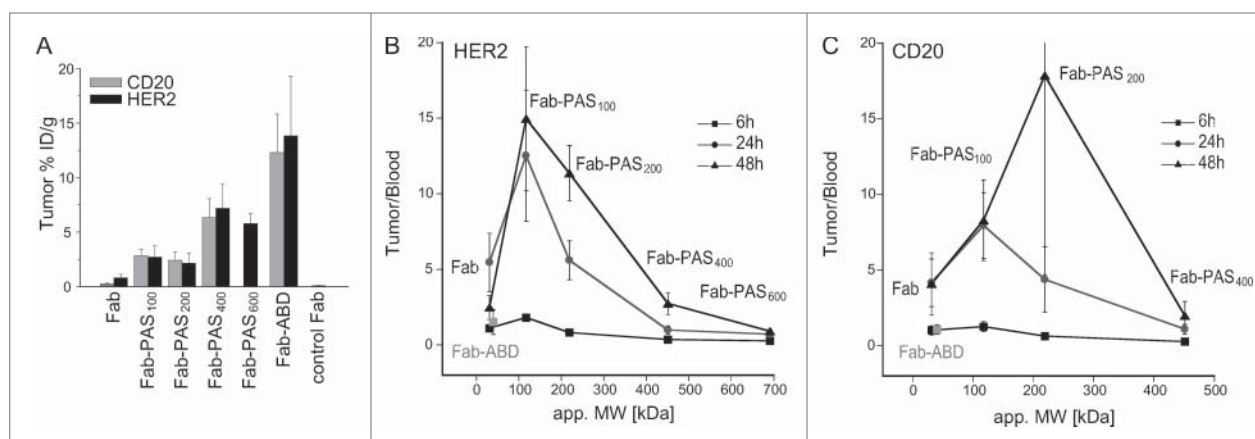
responsible for generally high levels of radioactivity in thyroid and stomach, as evident in the biodistribution for Fab and Fab-PAS<sub>100</sub> 6 h p.i. (Tables 1 and 2). Possibly, PASylation with 200 or more amino acid residues as well as fusion with an ABD improved the in vivo stability of the Fab fragments, since, for these tracers, less of the iodine isotope could be detected in those organs.

In control experiments, CD1-*Foxn1*<sup>tm</sup> mice bearing HER2-positive xenografts were treated with a 1,000-fold excess of trastuzumab before measuring target-specific accumulation of radio-iodinated Fab-ABD, Fab-PAS<sub>200</sub> and Fab-PAS<sub>600</sub> (Fig. S3). Under these conditions, biodistribution studies 24 h p.i. showed clearly reduced tumor uptake for <sup>125</sup>I-Fab-ABD, from 15.4% ID/g to 6.2% ID/g ( $p < 0.05$ ). Likewise, the  $\alpha$ HER2 Fab-PAS<sub>200</sub> and Fab-PAS<sub>600</sub> also yielded much lower tumor uptake after blocking with the antibody

**Table 2.** Biodistribution of radio-iodinated  $\alpha$ CD20 Fabs (for tumor-to-organ ratios see Fig. S2)

[%ID/g]	$^{125}\text{I}$ -Fab*				$^{125}\text{I}$ -Fab-PAS <sub>100</sub> *				$^{125}\text{I}$ -Fab-PAS <sub>200</sub> *				$^{125}\text{I}$ -Fab-PAS <sub>400</sub> *				$^{125}\text{I}$ -Fab-PAS <sub>200</sub>		$^{125}\text{I}$ -Fab-ABD	
	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	Block 24 h	24 h	
Tumor	1.33 ± 0.14	0.24 ± 0.09	0.17 ± 0.07	8.45 ± 1.38	2.82 ± 0.58	2.18 ± 1.15	4.15 ± 1.21	2.41 ± 0.76	1.47 ± 0.67	4.03 ± 0.71	6.33 ± 1.73	2.92 ± 1.55	0.68 ± 0.38	12.29 ± 3.53				0.68 ± 0.38	12.29 ± 3.53	
Blood	1.39 ± 0.37	0.06 ± 0.01	0.04 ± 0.01	6.98 ± 1.77	0.37 ± 0.08	0.26 ± 0.08	6.71 ± 0.65	0.59 ± 0.24	0.09 ± 0.02	15.63 ± 2.89	5.86 ± 0.52	1.58 ± 0.66	0.63 ± 0.11	11.52 ± 0.17				0.63 ± 0.11	11.52 ± 0.17	
Heart	0.52 ± 0.18	0.03 ± 0.00	0.02 ± 0.00	2.58 ± 0.56	0.17 ± 0.05	0.12 ± 0.04	2.42 ± 0.49	0.19 ± 0.09	0.04 ± 0.00	4.98 ± 0.76	1.77 ± 0.20	0.50 ± 0.22	0.25 ± 0.09	3.54 ± 0.45				0.25 ± 0.09	3.54 ± 0.45	
Lung	1.16 ± 0.43	0.08 ± 0.04	0.06 ± 0.02	5.11 ± 0.94	0.44 ± 0.13	0.35 ± 0.12	3.82 ± 0.71	0.43 ± 0.18	0.14 ± 0.09	6.98 ± 0.46	2.43 ± 0.08	0.71 ± 0.28	0.47 ± 0.16	5.36 ± 0.41				0.47 ± 0.16	5.36 ± 0.41	
Liver	0.52 ± 0.12	0.04 ± 0.01	0.04 ± 0.01	2.44 ± 0.56	0.21 ± 0.06	0.26 ± 0.09	1.62 ± 0.16	0.18 ± 0.06	0.06 ± 0.01	3.18 ± 0.48	1.10 ± 0.11	0.35 ± 0.10	0.21 ± 0.03	2.03 ± 0.08				0.21 ± 0.03	2.03 ± 0.08	
Spleen	0.97 ± 0.54	0.04 ± 0.01	0.03 ± 0.01	3.77 ± 0.36	0.22 ± 0.06	0.20 ± 0.08	2.02 ± 0.51	0.25 ± 0.06	0.08 ± 0.02	2.63 ± 0.46	1.00 ± 0.15	0.42 ± 0.15	0.26 ± 0.08	1.65 ± 0.13				0.26 ± 0.08	1.65 ± 0.13	
Kidney	2.75 ± 0.51	0.41 ± 0.07	0.23 ± 0.04	19.39 ± 1.78	0.86 ± 0.23	0.56 ± 0.19	13.40 ± 2.33	0.92 ± 0.33	0.17 ± 0.03	11.15 ± 2.44	2.62 ± 0.25	0.83 ± 0.30	1.10 ± 0.33	2.63 ± 0.32				1.10 ± 0.33	2.63 ± 0.32	
A. gland	0.73 ± 0.42	0.04 ± 0.02	0.04 ± 0.01	4.38 ± 1.09	0.45 ± 0.15	0.27 ± 0.16	3.46 ± 1.20	0.37 ± 0.12	0.11 ± 0.03	5.78 ± 1.97	2.67 ± 0.63	0.58 ± 0.18	0.98 ± 1.37	2.75 ± 0.47				0.98 ± 1.37	2.75 ± 0.47	
Pancreas	1.10 ± 0.44	0.02 ± 0.01	0.01 ± 0.00	4.92 ± 0.68	0.17 ± 0.05	0.09 ± 0.04	1.89 ± 0.20	0.19 ± 0.07	0.03 ± 0.01	2.02 ± 0.25	0.76 ± 0.09	0.18 ± 0.11	0.14 ± 0.02	1.31 ± 0.17				0.14 ± 0.02	1.31 ± 0.17	
Stomach	6.02 ± 3.11	0.10 ± 0.04	0.04 ± 0.01	15.47 ± 6.09	0.62 ± 0.10	0.27 ± 0.17	6.53 ± 2.87	0.59 ± 0.31	0.08 ± 0.03	3.36 ± 1.06	1.64 ± 0.47	0.33 ± 0.18	0.29 ± 0.05	2.31 ± 0.24				0.29 ± 0.05	2.31 ± 0.24	
S. int.	1.13 ± 0.63	0.04 ± 0.01	0.03 ± 0.01	3.95 ± 0.73	0.24 ± 0.04	0.18 ± 0.07	1.47 ± 0.46	0.18 ± 0.08	0.04 ± 0.01	1.75 ± 0.16	0.66 ± 0.12	0.16 ± 0.07	0.18 ± 0.02	1.29 ± 0.05				0.18 ± 0.02	1.29 ± 0.05	
L. int.	1.06 ± 0.19	0.04 ± 0.01	0.03 ± 0.01	3.18 ± 0.31	0.28 ± 0.05	0.22 ± 0.07	1.11 ± 0.14	0.15 ± 0.08	0.04 ± 0.01	1.23 ± 0.17	0.44 ± 0.07	0.11 ± 0.04	0.17 ± 0.03	0.95 ± 0.13				0.17 ± 0.03	0.95 ± 0.13	
Muscle	0.27 ± 0.08	0.01 ± 0.00	0.01 ± 0.00	1.60 ± 0.20	0.08 ± 0.02	0.04 ± 0.01	0.66 ± 0.07	0.11 ± 0.04	0.02 ± 0.00	0.68 ± 0.14	0.40 ± 0.07	0.11 ± 0.03	0.08 ± 0.02	0.64 ± 0.06				0.08 ± 0.02	0.64 ± 0.06	
Bone	0.46 ± 0.10	0.02 ± 0.01	0.02 ± 0.01	2.18 ± 0.71	0.10 ± 0.02	0.06 ± 0.02	1.11 ± 0.14	0.14 ± 0.03	0.05 ± 0.02	1.63 ± 0.50	0.66 ± 0.14	0.15 ± 0.05	0.09 ± 0.01	0.85 ± 0.19				0.09 ± 0.01	0.85 ± 0.19	
Skin	1.03 ± 0.28	0.04 ± 0.01	0.03 ± 0.01	5.19 ± 1.10	0.33 ± 0.09	0.19 ± 0.06	2.41 ± 0.21	0.31 ± 0.09	0.05 ± 0.01	2.89 ± 0.52	1.61 ± 0.40	0.44 ± 0.35	0.32 ± 0.04	3.02 ± 0.13				0.32 ± 0.04	3.02 ± 0.13	
Brain	0.07 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.37 ± 0.09	0.02 ± 0.01	0.03 ± 0.01	0.23 ± 0.02	0.02 ± 0.01	0.01 ± 0.00	0.45 ± 0.13	0.15 ± 0.02	0.04 ± 0.01	0.02 ± 0.00	0.28 ± 0.03				0.02 ± 0.00	0.28 ± 0.03	
Thyroid	7.2 ± 1.1	4.6 ± 1.2	3.7 ± 1.5	13.6 ± 2.74	13.1 ± 8.0	23.58 ± 6.0	4.2 ± 0.5	3.1 ± 0.3	2.9 ± 1.0	1.5 ± 0.3	3.4 ± 1.7	2.4 ± 0.8	5.0 ± 2.2	2.4 ± 0.5				5.0 ± 2.2	2.4 ± 0.5	

\*Biodistribution was quantified in dual tracer experiments in the combinations  $^{125}\text{I}$ -Fab /  $^{125}\text{I}$ -Fab-PAS<sub>100</sub> and  $^{125}\text{I}$ -Fab-PAS<sub>200</sub> and  $^{125}\text{I}$ -Fab-PAS<sub>100</sub> /  $^{125}\text{I}$ -Fab-PAS<sub>400</sub>.



**Figure 3.** Correlation of tumor uptake and tumor-to-blood ratio with plasma half-life. CD1-*Foxn1*<sup>tm</sup> mice bearing s.c. SK-BR-3 or Granta xenografts in the right shoulder were injected with <sup>125</sup>I-labeled Fabs. **(A)** Tracer uptake for HER2- and CD20-positive tumors 24 h p.i. quantified in a  $\gamma$ -counter for various PASylated Fabs (mean values  $\pm$  standard deviation;  $n = 5$ ). The IN-1 Fab served as negative control. **(B, C)** Tumor-to-blood ratio plotted against the apparent molecular size (MW) – correlating both with the length of the attached PAS tag and with plasma half-life<sup>36</sup> – of the different PASylated  $\alpha$ HER2 **(B)** and  $\alpha$ CD20 **(C)** Fabs 6 h, 24 h and 48 h p.i. ( $n = 5$ ). Data points for Fab-ABD are shown for comparison in gray.

( $p < 0.05$  and  $p < 0.001$ ). Similarly, in analogous control experiments with CD20-positive xenografts, tumor uptake of radio-iodinated  $\alpha$ CD20 Fab-PAS<sub>200</sub> was effectively blocked with a 1,000-fold excess of rituximab (0.68% ID/g *vs.* 2.41% ID/g; 24 h p.i.;  $p < 0.05$ ; cf. Table 2). It should be noted that rituximab recognizes loop regions of CD20 in close proximity to the ofatumumab epitope,<sup>38</sup> such that binding of the corresponding Fab is prevented in a competitive manner.

#### Relationship between tumor-to-blood ratio and pharmacokinetics of PASylated Fabs

The tumor-to-blood ratios determined in the biodistribution studies for the  $\alpha$ HER2 and  $\alpha$ CD20 Fabs with PAS tags of different lengths described above were plotted against their apparent molecular sizes (Fig. 3), which were previously determined by analytical SEC and are known to correlate with plasma half-life in mice.<sup>36</sup> For both  $\alpha$ HER2 and  $\alpha$ CD20 Fab versions the resulting graphs revealed a bell-shaped curve, both at 24 h and at 48 h p.i., starting at a low level for the unmodified Fabs, followed by an initial increase due to PASylation, with a pronounced optimum occurring in the range of 100 to 200 PAS residues.

In fact, excellent tumor-to-blood and tumor-to-normal tissue ratios, with 12.2 in the case of HER2 and 7.5 in the case of CD20, were observed for Fab-PAS<sub>100</sub> 24 h p.i. While tumor-to-blood ratios for the  $\alpha$ HER2 Fab-PAS<sub>200</sub> were comparable to the unmodified Fab at this time point, the Fab-PAS<sub>200</sub> format revealed similar or even better tumor-to-blood ratio than the 100 residue PAS tag at 48 h p.i., especially in the case of the corresponding  $\alpha$ CD20 Fab version (cf. Fig. 3B and 3C). This behavior was most likely the result of a longer contact and accumulation time due to the slower plasma clearance and possibly also to a reduced wash-out from tumor tissue. Taken together, based on these data both Fab-PAS<sub>100</sub> and Fab-PAS<sub>200</sub> constructs appear clearly superior for *in vivo* imaging purposes in comparison with the unmodified Fabs.

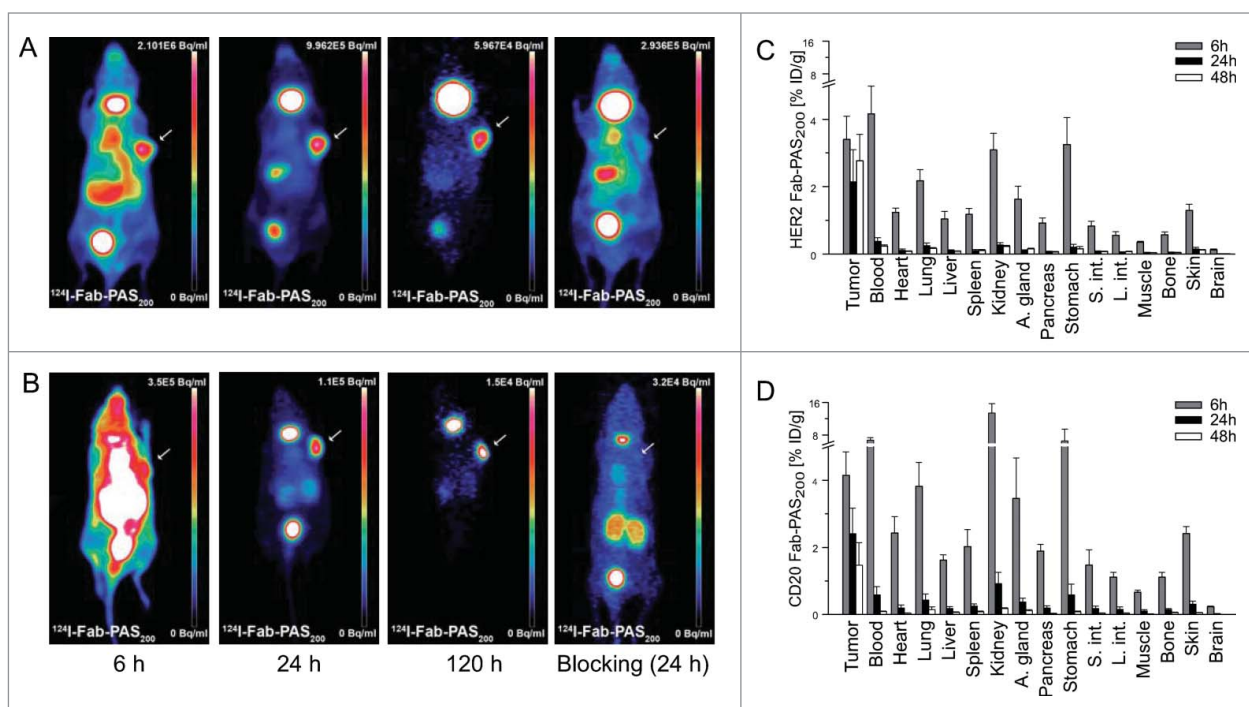
When further increasing the molecular size of the Fabs via fusion with PAS sequences of greater length, i.e., with 400 or 600 residues, the circulation time in blood was extended to a similar level as with the ABD.<sup>36</sup> Interestingly, under these conditions the high blood radioactivity 24 h p.i. led to decreased tumor-to-blood ratios as well as poor imaging contrast, thus approaching the unfavorable imaging properties of full-size antibodies.

#### Time-dependent biodistribution and PET imaging of $\alpha$ HER2 and $\alpha$ CD20 Fab-PAS<sub>200</sub>

Since the Fab-PAS<sub>200</sub> format had essentially shown optimal tumor accumulation in the preceding experiments, sequential static PET scans were performed 6 h, 24 h and 120 h p.i. to investigate the distribution of both  $\alpha$ HER2 and  $\alpha$ CD20 Fab-PAS<sub>200</sub> over time (Fig. 4). The best imaging contrast was seen 24 h p.i. for both tracers, while even after 120 h signals were still detectable in the tumor. Upon antigen blocking with a 1,000-fold excess of trastuzumab or rituximab, representative PET-images 24 h p.i. showed strong reduction in tumor uptake, confirming the previously observed high specificity of these protein tracers. Detailed biodistribution analyses 6 h, 24 h and 48 h p.i. further revealed that already 24 h p.i. both  $\alpha$ HER2 and  $\alpha$ CD20 Fab-PAS<sub>200</sub> versions were almost entirely cleared from blood and normal tissue (Fig. 4C, 4D), thus resulting in the high tumor contrast.

## Discussion

For clinical tumor imaging the use of smaller functional fragments instead of full size mAbs has shown promise, in particular with regard to their faster blood clearance and better tumor penetration.<sup>25,26</sup> While scFvs, which constitute the smallest kind of functional Ig fragments, are somewhat easier to prepare in form of recombinant proteins, they often show an oligomerization



**Figure 4.** Evaluation of the Fab-PAS<sub>200</sub> format as radioactive imaging tracer. (A, B) PET scans were recorded at varying time points on CD1-Foxn1<sup>fl/fl</sup> mice carrying s.c. HER2-positive (SK-BR-3) or CD20-positive (Granta) tumors (see arrows) after administration of <sup>124</sup>I-Fab-PAS<sub>200</sub>. Sequential MIP images were collected 6 h, 24 h, and 120 h p.i. for HER2 (A) and CD20 (B). To investigate tumor target blocking in a control experiment, 0.56 mg trastuzumab or rituximab, respectively, was injected both 24 h before and together with the radiolabeled Fab, followed by scanning 24 h p.i. as above. For each tumor type, Fab-PAS<sub>200</sub> shows increasing contrast over time and stable tumor uptake till 120 h after injection. Note that thyroid was not blocked for iodine uptake (as in Fig. 2). (C, D) Biodistribution analysis of radio-iodinated αHER2 (C) and αCD20 (D) Fab-PAS<sub>200</sub>, illustrating the percentage of injected dose per gram (% ID/g) 6 h, 24 h and 48 h p.i. (mean values ± standard deviation; n = 5).

tendency, thus resulting in inhomogeneous preparations with variations in molecular size and target avidity. In contrast, Fabs offer clear benefits with regard to much higher protein stability, which also has led to several biopharmaceutical products.<sup>1</sup> In fact, several Fabs have been approved by the FDA or the European Medicines Agency as imaging reagents for single photon emission computed tomography (SPECT): <sup>99m</sup>Tc-arcitumomab (CEA-Scan),<sup>54</sup> <sup>99m</sup>Tc-sulesomab (LeukoScan)<sup>55</sup> and <sup>99m</sup>Tc-beatumomab (LymphoScan).<sup>56</sup>

Compared with full-size mAbs, Fabs have a much shorter plasma half-life (approximately by a factor 30 – in humans)<sup>57</sup> due to their lack of FcRn-mediated recycling and their smaller size, which boosts renal elimination. The shorter circulation is generally considered beneficial for in vivo imaging because it (i) allows better tumor/tissue penetration, and (ii) leads to quick clearance of background radioactivity in the blood. However, the small monovalent Fabs, which cannot take advantage of an avidity effect such as bivalent IgGs, also tend to diffuse out of the tumor when their plasma concentration drops, thus lowering the effective tumor uptake. Consequently, apart from antigen affinity, there is also an interdependent relationship between molecular size and circulation half-life of a protein tracer with regard to tumor targeting efficacy, which was previously noted by others, too.<sup>27,35,58</sup>

Recent analyses of literature data<sup>58,59</sup> from applications of various radiotracers for tumor imaging or cancer therapy indicated how molecular size of the radioconjugate in relation to antigen affinity can influence selective tumor targeting. While the affinity for the cell-surface receptor directly correlates with tumor accumulation, the molecular diameter has mixed effects on several parameters like capillary extravasation from blood, diffusion across the tumor interstitium, available volume fraction in the tumor and, in particular, circulation time. Astonishingly, a theoretical model of tumor targeting<sup>58</sup> predicted lowest tumor uptake for agents of intermediate size with a molecular weight around 25 kDa. Whereas smaller compounds accumulate more rapidly in the tumor, they require higher target affinity in order to achieve similar tumor retention as larger molecules. On the other hand, larger molecules, including mAbs, take advantage of the enhanced permeability and retention effect (EPR). According to these considerations, tumor-targeting properties of mid-size protein tracers may be improved by increasing their molecular dimensions, in line with prolonged plasma half-life.

In this study, we optimized the PK properties of αHER2 and αCD20 Fabs by applying PASylation technology. PASylation offers an ideal tool for such an endeavor as the increase in molecular size, and the resulting retarding effect on kidney filtration, can be smoothly tuned on the genetic level by



varying the length of the encoded PAS polypeptide as part of the recombinant Fab fusion protein.<sup>36</sup> The possibility to directly adjust the parameter plasma half-life of a protein tracer, here Fab, using PAS tags of different lengths without affecting its target-binding activity provides the opportunity to investigate the relation between circulation time, molecular size and tumor-to-blood ratio in an unbiased manner. Remarkably, PET imaging and biodistribution studies of all PASylated Fabs did not show unfavorable accumulation in any normal tissue, thus confirming the biologically inert behavior of the PAS polypeptides and demonstrating that PASylation is compatible with *in vivo* imaging in general.

As a result, especially the Fab-PAS<sub>100</sub> and Fab-PAS<sub>200</sub> formats which carry PAS tags of moderate lengths exhibited excellent tumor-to-blood and tumor-to-normal tissue ratios and, thus, offer promising tracers for clinical applications. Specific binding to HER2 and CD20 tumor antigens was verified by competitive blocking experiments with the cognate antibodies trastuzumab or rituximab. Absence of non-specific tissue staining was confirmed by applying the human NogoA-specific Fab IN1, whose central nervous system antigen is not accessible for circulating antibodies, as a negative control.

Both unmodified  $\alpha$ HER2 and  $\alpha$ CD20 Fabs showed rapid blood clearance and, consequently, were washed out from tissues and tumor over time, as expected for these small proteins with short plasma half-lives of around 1.3 h in mice, as previously determined for the 4D5 Fab.<sup>36</sup> However, by fusing the recombinant Fabs with PAS sequences of increasing lengths, it was possible to successively prolong their circulation time resulting in increasing tumor accumulation, similar to the effect previously described for PEGylated antibody fragments.<sup>34,60</sup> In our study, maximal tumor uptake 24 h p.i. was seen for Fab-PAS<sub>400</sub>, which has a plasma half-life of around 14 h in mice and a hydrodynamic volume of 451 kDa as previously determined by analytical SEC.<sup>36</sup> The larger fusion protein Fab-PAS<sub>600</sub> with its further prolonged circulation time, which was investigated in the case of the  $\alpha$ HER2 Fab, showed slightly lower tumor accumulation at this time point, but may not yet have reached its maximum.

Whereas Fab-ABD showed good tumor-to-normal tissue ratios, albumin binding was obviously accompanied by a high level of radioactivity in the blood and, consequently, led to a poor tumor-to-blood ratio, almost like for a full-length mAb. Compared with Fab-PAS<sub>600</sub>, the similarly extended plasma half-life of 28 h for Fab-ABD with its intrinsically much smaller molecular size (in the absence of albumin) seemed to result in even higher tumor accumulation, in line with a previous study that utilized an albumin-binding peptide fused to the trastuzumab Fab.<sup>27</sup> This may be due to the well-known phenomenon that albumin itself is actively taken up by the tumor, probably as a nutrient supply.<sup>31,32</sup>

Interestingly, in a study using the  $\alpha$ HER2 affibody <sup>114m</sup>In-CHX-A"-DTPA-ABD-(Z<sub>HER2:342</sub>)<sub>2</sub>, a similar biodistribution pattern was observed in nude mice bearing LS174T xenografts, revealing 14.5% ID/g in the tumor and 12.6% ID/g in the blood 18 h p.i.<sup>61</sup> Taken together, this indicates that the tumor targeting behavior of albumin-binding agents is only partially dependent

on the properties of the antigen-specific module of the protein tracer, at least for HER2-positive xenograft tumors and at earlier time points. In fact, an albumin-mediated tumor accumulation of Fab-ABD might also be responsible for the incomplete blocking with trastuzumab as observed here in the control experiments.

Previous experiments with a PEGylated  $\alpha$ HER2 scFv (4D5-PEG20) in an SK-OV-3 tumor xenograft model<sup>35</sup> showed that tumor accumulation can also be boosted by extending the plasma half-life using an albumin-independent biophysical principle. However, this resulted in a much lower tumor-to-blood ratio 24 h p.i. compared to the unmodified protein tracer. These findings are in agreement with our observations for those Fab constructs having a circulation time  $\geq 14$  h, i.e., Fab-PAS<sub>400</sub> and Fab-PAS<sub>600</sub>.

Conversely, much improved tumor-to-blood ratios were observed for Fab-PAS<sub>100</sub> (24 h p.i.) and Fab-PAS<sub>200</sub> (48 h p.i.) compared to the unmodified  $\alpha$ HER2 and  $\alpha$ CD20 Fabs. Thus, it seems that a plasma half-life in mice of 2.7 h or 5.2 h, respectively, and an effective molecular size of 117 kDa or 219 kDa for Fab-PAS<sub>100</sub> and Fab-PAS<sub>200</sub><sup>36</sup> provide a favorable combination between tracer size and circulation time for tumor targeting in a diagnostic setting. In fact, the higher apparent molecular weight and the moderately prolonged circulation led to a 3-fold (HER2) and 12-fold (CD20) increased tumor uptake compared to the unmodified Fabs. Furthermore, a 2-fold improved tumor-to-blood ratio for Fab-PAS<sub>100</sub> 24 h p.i. and a 2-4-fold improvement for Fab-PAS<sub>200</sub> 48 h p.i. were observed in the biodistribution analyses for HER2- and CD20-positive tumors.

Interestingly, the hydrodynamic volume of the larger fusion proteins is actually in the range of full-size IgGs, but does not seem to hinder efficient tumor accumulation. In this regard, it has to be kept in mind that PASylated proteins do not quite behave like solid globular proteins (such as albumin or an antibody), rather the dynamically disordered random coil nature of the slim, linear PAS polypeptide confers enhanced diffusional properties in a crowded molecular environment, similar to PEG.<sup>36</sup> Consequently, at least for the PAS fusions with moderate lengths, the effect of prolonged plasma half-life and, hence, extended tumor contact time, which allows enduring tumor accumulation, comes fully into play. Indeed,  $\alpha$ HER2 Fab-PAS<sub>200</sub> showed ongoing tumor uptake between 24 h and 48 h p.i., which probably results from maintaining a concentration gradient between blood plasma and tumor over a longer period of time, as previously discussed by others.<sup>27,35</sup> Therefore, both the Fab-PAS<sub>100</sub> and the Fab-PAS<sub>200</sub> format should be suitable candidates for clinical application, depending on the optimal time point for read out, offering enhanced tumor-to-normal tissue ratios and also improved tumor uptake.

Our findings are in agreement with several previous studies on radiolabelled mAbs or antibody fragments that target the tumor antigen HER2. For example, biodistribution of <sup>111</sup>In-DOTA-trastuzumab and <sup>111</sup>In-DOTA-F(ab')<sub>2</sub>-trastuzumab showed specific tumor uptake (31 and 20% ID/g, respectively, 24 h p.i.) in mice bearing HER2 positive BT-474 tumor xenografts.<sup>62</sup> In spite of the generally higher tumor accumulation, which might be due

to the larger size of these protein tracers and the use of a residualizing radiometal ion, better tumor-to-blood ratios of 5.3 for the unmodified Fab and even of 12.2 for Fab-PAS<sub>100</sub> were reached in our study, compared to 3.4 for trastuzumab and 10 for the corresponding F(ab')<sub>2</sub>.

The CD20-targeting mAb <sup>99m</sup>Tc/<sup>188</sup>Re(CO)<sub>3</sub>-RTXred (rituximab) showed rather low tumor uptake of 0.8-2.7% ID/g at 24 h p.i. in mice bearing a Ramos lymphoma xenograft, depending on the radioisotope, and a poor tumor-to-blood ratio of 0.5. In contrast, an engineered dimeric scFv fusion protein derived from rituximab, the <sup>124</sup>I-minibody, revealed clearly higher tumor uptake (13% ID/g) and tumor-to-blood ratio (4.8) 21 h p.i. using the murine B-cell lymphoma cell line 38C13 transduced with human CD20 in a xenograft model.<sup>63,64</sup> For comparison, the monomeric αCD20 Fab-PAS<sub>100</sub> and Fab-PAS<sub>200</sub> constructs analyzed in our study showed somewhat lower total tumor accumulation of 2.8 and 2.4% ID/g 24 h p.i., but also considerably lower blood and normal tissue burden, thus yielding better imaging contrast 24 h p.i., with tumor-to-blood ratios of 4-8.

In a previous study on the role of PK for tumor targeting, two versions of a <sup>99m</sup>Tc(CO)<sub>3</sub>-labeled scFv derived from trastuzumab were investigated: the small monomeric antibody fragment itself and the corresponding scFv chemically conjugated to 20 kDa linear PEG.<sup>35</sup> Although PEGylation led to a marked decrease in antigen affinity in this study, contrasting with PASylation, these two molecules appear comparable to the αHER2 Fab and Fab-PAS<sub>400</sub> constructs, respectively, with regard to plasma half-life.<sup>36</sup> PEGylation of the αHER2 scFv led to a 5-fold increase in tumor accumulation from 1.7% ID/g (monomer) to 9.3% ID/g (monomer-PEG20k) at 24 h p.i. while PASylation with 400 residues of the αHER2 Fab resulted in a 9-fold (for HER2) to 26-fold (for CD20) increase in tumor uptake in our study. Interestingly, in both cases the tumor-to-blood ratio was concomitantly decreased, 4-fold for the scFv coupled to PEG20k and 4-5-fold for the Fab fused to 400 PAS residues.

A similar effect of molecular size increase by PEGylation was observed for HER2-specific DARPins, an engineered non-Ig protein scaffold, in a SK-OV-3 mouse xenograft model.<sup>34</sup> In this report, conjugation to PEG20k led to a 1.6-5-fold improved tumor uptake, depending on the affinity of the investigated DARPIn, but also to lower tumor-to-blood ratio. Thus, if compared according to molecular size, both PASylation and PEGylation show similar effects on tumor uptake of the tracers used for in vivo imaging, which is in agreement with the analogous biophysical properties of both polymers and their prolonging effect on circulation.<sup>36</sup>

Nevertheless, a more modest extension of plasma half-life in the range of 2-14 h has not been described up to now. In fact, our study demonstrates for the first time that prolongation of circulation just by a factor 2-3 compared with the unmodified recombinant Fab considerably improves tumor-to-blood ratio, as was observed here for Fab-PAS<sub>100</sub> (24 h p.i.) and Fab-PAS<sub>200</sub> (48 h p.i.). Apparently, such positive effects on imaging contrast were missed by the previous PEGylation studies, since only rather large PEG polymers (20, 40 or 60 kDa) were investigated.<sup>34,35</sup> Instead, PAS polypeptides with 100-200 residues would

correspond to 5-10 kDa PEG chains with regard to molecular size as well as retardation of kidney clearance. Consequently, for effective tumor targeting of the protein tracer and, in particular, high imaging contrast, a moderate increase in the hydrodynamic molecular volume is crucial to achieve an optimum between plasma concentration / circulation time and tissue penetration / tumor accumulation.

Apart from that, compared to chemical conjugation with PEG, which is a synthetic polymer with inherent size polydispersity, PASylation yields a truly uniform protein preparation, avoids laborious in vitro coupling procedures, and does not interfere with the biological activity or affinity of the protein, as was specifically demonstrated for the αHER2 Fab before.<sup>36</sup> This offers a convenient route to quickly generate superior Fab reagents from cloned humanized antibodies to also address other disease-relevant antigens for in vivo imaging applications, taking advantage of an already clinically-approved class of tracer molecules. Furthermore, it even seems that PASylation may improve the in vivo stability of the radiolabeled Fab fragments since Fab-PAS<sub>200</sub>, Fab-PAS<sub>400</sub> and Fab-PAS<sub>600</sub> showed less accumulation of free iodide in the thyroid, otherwise an indication for proteolysis, dehalogenation and metabolism.<sup>65</sup> Finally, PASylation also appeared to prevent overt accumulation in the kidneys.

Neglecting effects of allometric scaling when transferring pre-clinical data from mice to man, the two PASylated αHER2 Fab versions with superior tumor imaging properties, Fab-PAS<sub>100</sub> and Fab-PAS<sub>200</sub>, may also offer promising tracer reagents for in vivo imaging of pathological HER2 expression in patients. Thus, they could be useful for the diagnosis of HER2-positive tumor recurrence, dose optimization for HER2-targeted therapy and for monitoring of tumor treatment efficiency in a clinical setting. Likewise, the αCD20 Fab-PAS<sub>200</sub> version appears attractive for noninvasive in vivo monitoring of B-cell lymphoma growth and metastasis, both in animal models during preclinical research and in human therapy.

In conclusion, a few attempts to specifically tailor the plasma half-life of radiolabelled monovalent antibody fragments (Fab or scFv) for imaging and/or tumor targeting have been reported before, which made use of (i) PEGylation<sup>35</sup> and (ii) albumin association.<sup>27</sup> In both cases, significantly higher tumor accumulation in comparison with the unmodified antibody fragment was described. However, this effect, which should be beneficial for radiotherapy, came at the expense of a diminished tumor-to-blood ratio, a parameter crucial for in vivo imaging, obviously because of the much longer persistence of the modified proteins in circulation. Hence, there are two major opposing effects of PK extension: longer perfusion of the tumor leads to higher accumulation of the tracer – owing to the slow dissociation rate once the antibody fragment has bound to its antigen, which is often followed by receptor endocytosis – while at the same time there is a stronger background of radioactivity in the blood and an associated exposure of highly vascularized organs.

In these previous investigations, the tumor-to-blood ratio was eventually improved by performing imaging or biodistribution analyses at later time points, i.e., 48 h p.i. and beyond. In the present study, we followed a different approach: taking advantage

of the easily applicable PASylation technology that offers a purely biophysical and biologically inert way to retard kidney filtration, the plasma half-life was systematically varied about a wide range employing the same Fab fragment as targeting module, thereby avoiding influences on affinity, valency or albumin/FcRn-binding effects upon changing molecular formats. Thus, we found that there is an optimum for plasma half-life / molecular size, which lies around 100-200 PAS residues, roughly equivalent to a single PEG chain of 5-10 kDa. Notably, the same trend was observed for two different Fabs, targeting HER2 and CD20, respectively, which also differed in their antigen affinities by a factor 3.6. This clearly indicates a general mechanism and strongly suggests that PK should be worth optimizing for biomolecular imaging reagents. Beyond that, the beneficial effect of PASylation on specific tumor accumulation demonstrated here should also be applicable to drug conjugates of antibody fragments (or alternative binding proteins) in a therapeutic setting.

## Materials and Methods

### Preparation of recombinant $\alpha$ HER2 and $\alpha$ CD20 Fabs

The following amino acid exchanges were introduced via QuikChange mutagenesis (Stratagene, La Jolla, CA) into a version of the trastuzumab Fab that was previously cloned on the bacterial expression vector pASK88:<sup>40</sup> K<sup>H3</sup>Q, Q<sup>H5</sup>V, E<sup>L3</sup>Q, L<sup>L4</sup>M and L<sup>L104</sup>V. In addition, the amino acid substitution D<sup>H98</sup>W (Kabat numbering) was introduced to improve the affinity for the HER2 tumor antigen.<sup>41</sup> Also, the mutated V-genes were subcloned via *Xba*I and *Sac*II on the vector pASK106-4D5<sup>66</sup> to produce the Fab as fusion protein with the ABD from *Streptococcal* protein G at the C-terminus of its light chain.<sup>40</sup> Corresponding Fabs fused with PAS polypeptides of different lengths were prepared using a recently published series of compatible expression plasmids.<sup>36</sup>

The V-genes corresponding to the amino acid sequence of ofatumumab<sup>14</sup> were obtained by gene synthesis (Mr. Gene, Regensburg, Germany) and subcloned via standardized restriction sites on pASK88, providing human C<sub>H</sub>1 $\gamma$ <sub>1</sub> and C $\kappa$  constant domain regions, to yield the  $\alpha$ CD20 Fab.<sup>42</sup> After that, appropriate derivative plasmids were constructed by subcloning as above to express analogous fusion proteins as for the  $\alpha$ HER2 Fab.

The different Fab versions were produced in *E. coli* JM83 at 22°C in shake flasks using LB medium containing 100 mg/l ampicillin according to a published procedure.<sup>40</sup> In case of the PASylated Fabs, 6 g/l glucose and 1 g/l proline were added. Three hours after induction at OD<sub>550</sub> = 0.5 with 200  $\mu$ g/l anhydrotetracycline (Acros Organics, Geel, Belgium) bacterial cells were harvested by centrifugation and the periplasmic extract was prepared.<sup>67</sup> The dialyzed protein fraction was applied to a Zn(II)-charged IDA Sepharose column and eluted with an imidazole/HCl concentration gradient.<sup>67, 68</sup> Fabs fused to PAS<sub>400</sub> and PAS<sub>600</sub> sequences were further purified via *Strep*-Tactin affinity chromatography utilizing the *Strep*-tag II<sup>69</sup> at the C-terminus of the PAS tag that was appended to the light chain. To isolate monodisperse protein and deplete bacterial endotoxin,

SEC on a Superdex 200 HR 10/300 GL column (GE Healthcare, Munich, Germany), as well as ion exchange chromatography on Q-Sepharose (GE Healthcare), were performed using phosphate-buffered saline (PBS; 4 mM KH<sub>2</sub>PO<sub>4</sub>, 16 mM Na<sub>2</sub>HPO<sub>4</sub>, 115 mM NaCl) as running buffer.

The purified recombinant Fabs were concentrated by ultrafiltration (Amicon Ultra centrifugal filter devices 30,000 MWCO; Millipore, Billerica, MA). Purity and disulfide bond formation was analyzed by SDS-PAGE and analytical SEC. Protein concentration was determined according to absorption at 280 nm using calculated extinction coefficients<sup>70</sup> of 77405 M<sup>-1</sup>cm<sup>-1</sup> for  $\alpha$ HER2 Fab and its PASylated versions, 81875 M<sup>-1</sup>cm<sup>-1</sup> for  $\alpha$ HER2 Fab-ABD, 75915 M<sup>-1</sup>cm<sup>-1</sup> for  $\alpha$ CD20 Fab and its PASylated versions and 80385 M<sup>-1</sup>cm<sup>-1</sup> for  $\alpha$ CD20 Fab-ABD.

### *In vitro* binding studies of $\alpha$ HER2 and $\alpha$ CD20 Fabs

The HER2-positive human breast adenocarcinoma cell line SK-BR-3<sup>45</sup> was cultured to 80–90% confluence in Dulbecco's Modified Eagle Medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 10% v/v Fetal Bovine Serum (FBS) (Biochrom) and 1% w/v penicillin/streptomycin at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were detached with Accutase (Sigma-Aldrich, St. Louis, MO), counted with a Neubauer counting chamber and washed with 10% v/v FBS, 2 mM EDTA, 0.002% w/v sodium azide (FACS buffer). The CD20-positive human mantle cell lymphoma line Granta-519<sup>46</sup> was cultured in DMEM supplemented with 20% v/v FBS.

In each case, 10<sup>5</sup> cells were incubated with 100  $\mu$ l of the Fab preparation (0.031-512 nM), diluted in FACS buffer, for 1 h at 4°C in a 96-well microtiter plate. After two washing steps with FACS buffer, the cells were incubated with 50  $\mu$ l of a fluorescein-conjugated  $\alpha$ hu-kappa-light chain antibody (Invitrogen / Life Technologies, Darmstadt, Germany) for 20 min at 4°C. Cells were finally centrifuged and resuspended in 500  $\mu$ l FACS buffer. Then, propidium iodide was added to a final concentration of 1  $\mu$ g/ml to label (and exclude during counting) dead cells, and antibody-stained cells were counted with a FACSAria Cell-Sorting system (BD Biosciences, Heidelberg, Germany). Data were analyzed using FlowJo software (Tree Star, Ashland, OR) and the mean fluorescence intensity (MFI; normalized against the isotype control) was plotted against the applied concentration of the Fab. The apparent K<sub>D</sub> value was fitted using the formula  $MFI = \Delta MFI_{max} \cdot [Fab] / (K_D + [Fab])$  and plotted using Kaleidagraph Software (Synergy, Reading, PA) with  $\Delta MFI_{max}$  set to 100%.

### Radio-iodination of Fabs

The purified Fab versions were labeled with various radioactive isotopes of iodine using the Iodogen method,<sup>49</sup> aiming at a molar ratio between iodine and Fab of 1:1 (as specifically adjusted for <sup>125</sup>I). Typically, 50  $\mu$ g Iodogen (Pierce, Rockford, IL) was coated at the bottom of a plastic vial by drying a 1 mg/ml solution in dichloromethane with a gentle argon stream and stored at -20°C. For radiolabeling, 0.44 nmol purified Fab or mAb diluted in 200  $\mu$ l PBS (1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 137 mM NaCl, 2.6 mM KCl) was mixed with

37 MBq of either Na<sup>125</sup>I with a specific activity of 74 MBq/nmol (Hartman Analytic, Braunschweig, Germany), Na<sup>123</sup>I with a specific activity of 185 MBq/nmol (GE Healthcare, Buckinghamshire, UK), Na<sup>124</sup>I with a specific activity of ~1.1 MBq/pmol (IBA Molecular, VU Amsterdam, The Netherlands) or 0.5 nmol sodium salt of the stable isotope <sup>127</sup>I, each dissolved in 100 µl 1 mM NaOH, and added to the vial. After 15 min incubation at room temperature, the reaction was stopped by addition of 10 µl 25 mg/ml Na-ascorbate/HCl pH 4–5 and the mixture was immediately pipetted out of the plastic vial and, hence, separated from the insoluble oxidizing agent.

Coupling yields of 80–90%, depending on the iodine isotope, were achieved as determined by thin layer chromatography (TLC) on glass micro fiber paper (Agilent Technologies, Santa Clara, CA) using 0.9% w/v NaCl as mobile phase, leading to retention factors (Rf) of 0.0 and 1.0 for the radiolabeled Fab and free iodide, respectively. The radio-iodinated Fabs were separated from excess reagents by gel filtration on a PD-10 column (GE Healthcare) using 5.2 mM histidine/HCl pH 6, 2% w/v trehalose, 0.9% w/v NaCl, 0.009% v/v Tween20 as running buffer, thus preventing loss by adsorption. Radiochemical purity, analyzed by TLC, was >98% for the initial 1 ml elution fraction. A specific activity of approximately 80 MBq/nmol was calculated from the radioactivities of the reagents and the reaction yields. Protein concentration was verified with a micro-Bradford assay (Bio-Rad Protein Assay Kit II; Bio-Rad Laboratories, Hercules, CA). Iodinated Fabs were further analyzed by SDS-PAGE and the <sup>127</sup>I-labeled HER2 Fab was tested for in vitro antigen binding activity via FACS titration of tumor cells as described above.

### *In vivo* studies

For biodistribution analyses and PET imaging, female CD1-*Foxn1<sup>tm</sup>* mice of age 6–8 weeks (Charles River Laboratories, Sulzfeld, Germany) were injected subcutaneously with 5 × 10<sup>6</sup> SK-BR-3 cells suspended in 100 µl DMEM into the right shoulder. After approximately two weeks, tumors had reached a size of 300–500 mm<sup>3</sup>. Alternatively, 5 × 10<sup>6</sup> Granta-519 cells were mixed with Matrigel Basement Membrane Matrix (BD Biosciences) for subcutaneous injection to achieve more uniform tumor growth over 4–5 weeks, eventually achieving sizes of 300–500 mm<sup>3</sup>.

Micro-PET studies were performed after tail-vein injection of 6–12 MBq <sup>124</sup>I-labeled Fab having a specific activity of 80 MBq/nmol (n = 2). 60-min static PET images were acquired 6 h, 24 h and 120 h post injection (p.i.) under isoflurane anesthesia on an Inveon PET/CT small animal scanner (Siemens Medical Solutions, Knoxville, TN). Images were reconstructed using a 3-dimensional ordered-subsets expectation maximum algorithm (3D-OSEM) and analyzed using Inveon Research Workplace software (Siemens Medical Solutions). All images are shown as maximum intensity projections (MIP) after scaling to achieve a similar intensity of each tumor, thus allowing better comparison of tumor-to-background ratios.

For biodistribution studies, mice were injected intravenously with 0.37–0.74 MBq of <sup>123</sup>I-Fab, <sup>124</sup>I-Fab or <sup>125</sup>I-Fab (specific activity 80 MBq/nmol). At 6 h, 24 h and 48 h p.i., mice (n = 5–6) were sacrificed in a CO<sub>2</sub> atmosphere, the stomach was emptied, blood and organs of interest were dissected, weighed and analyzed in a Wallac Wizard γ-counter (PerkinElmer, Turku, Finland). The total applied radioactivity was determined by measuring the syringes before and after injection with a CRC-15R Dose Calibrator (Capintec, Ramsey NJ). The percentage of injected dose (% ID), corrected for the dose remaining in the tail, per gram of each organ (% ID/g) was calculated using a standard containing 1% of the injected activity diluted in water.

<sup>125</sup>I/<sup>124</sup>I or <sup>125</sup>I/<sup>123</sup>I dual tracer experiments for different PASylated Fab combinations directed against the same tumor target were performed as indicated in Tables 1 and 2. The different iodine activities were individually quantified in the γ-counter with appropriate calibration. In case of the combination with <sup>124</sup>I, which emits high energy γ-radiation, <sup>125</sup>I was measured after decay for 10 half-life periods of the former.

For antigen blocking experiments (n = 3–4), 0.56 mg trastuzumab or rituximab from the hospital pharmacy or 0.2 mg Fab-ABD was injected into the tail vein once 24 h before and once together with the radiolabeled Fab.

All animal experiments were approved by local authorities (Regierung von Oberbayern, Germany; license no. 55.2–1–54–2532–46–12) and were in compliance with the local regulatory and institutional guidelines.

### Disclosure of Potential Conflicts of Interest

AS and MS are cofounders of XL-protein GmbH, Germany.

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### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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