ADAPT, a Novel Scaffold Protein-Based Probe for Radionuclide Imaging of Molecular Targets That Are Expressed in Disseminated Cancers

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Abstract

Small engineered scaffold proteins have attracted attention as probes for radionuclide-based molecular imaging. One class of these imaging probes, termed ABD-Derived Affinity Proteins (ADAPT), has been created using the albumin-binding domain (ABD) of streptococcal protein G as a stable protein scaffold. In this study, we report the development of a clinical lead probe termed ADAPT6 that binds HER2, an oncoprotein overexpressed in many breast cancers that serves as a theranostic biomarker for several approved targeting therapies. Surface-exposed amino acids of ABD were randomized to create a combinatorial library enabling selection of high-affinity binders to various proteins. Furthermore, ABD was engineered to enable rapid purification, to eradicate its binding to albumin, and to enable rapid blood clearance. Incorporation of a unique cysteine allowed site-specific conjugation to a maleimido derivative of a DOTA chelator, enabling radionuclide labeling. 111In for SPECT imaging and 68Ga for PET imaging. Pharmacologic studies in mice demonstrated that the fully engineered molecule 111In/68Ga-DOTA-(HE)3-ADAPT6 was specifically bound and taken up by HER2-expressing tumors, with a high tumor-to-normal tissue ratio in xenograft models of human cancer. Unbound tracer underwent rapid renal clearance followed by high renal reabsorption. HER2-expressing xenografts were visualized by gamma-camera or PET at 1 hour after infusion. PET experiments demonstrated feasibility for discrimination of xenografts with high or low HER2 expression. Our results offer a preclinical proof of concept for the use of ADAPT probes for noninvasive in vivo imaging. Cancer Res; 75(20); 4364-71. ©2015 AACR.

Introduction

Specific radionuclide imaging of therapeutic molecular targets in vivo may provide a noninvasive tool for repeatable determination of their expression levels in disseminated cancer to stratify patients for a targeted therapy (1).

Radiolabeled therapeutic monoclonal antibodies have demonstrated utility in visualization of molecular targets (2–4). However, poor extravasation and penetration of intact antibodies into tumor masses in combination with slow clearance from blood and tissues (5) cause modest imaging contrast. Besides, antibodies as well as other proteins with molecular weights over 45 kDa accumulate in tumors unspecifically (6), which may cause false-positive findings. The use of smaller antibody fragments improves tumor-to-organ radioactivity concentration ratios, and therefore imaging contrast (1). This creates preconditions for better sensitivity. Mathematical modeling suggests that the smaller the imaging agent is, the better the targeting would be if the affinity is high enough (7). The smallest immunoglobulin-based imaging agents utilize camelid VHH fragments (15 kDa) and demonstrate very good imaging properties (8). Still, further size reduction is desirable.

A possible way to generate high-affinity targeting proteins is to combine molecular display techniques (e.g., phage, ribosomal, yeast, or bacterial display) with the use of engineered scaffold proteins (9). Scaffold proteins contain a robust structurally defined framework, which provides rigidity, and a variable surface area, where amino acids are randomized to create libraries for selection of binders. The use of scaffold proteins has enabled the generation of binders with small size (4–20 kDa), well-defined specificity and low nanomolar or subnanomolar affinity. The first scaffold protein for molecular imaging was an affibody molecule targeting HER2, a receptor tyrosine kinase that is overexpressed in cancer and serves as a target for several mAbs and tyrosine kinase inhibitors (10). In preclinical models, this scaffold protein has demonstrated tumor-to-organ ratios exceeding those of monoclonal antibodies by one-two orders of magnitude (11). Clinical studies have confirmed the feasibility of imaging of HER2-expressing metastases using affibody molecules within hours after injection (12). Affibody molecules have also demonstrated successful preclinical imaging of other molecular targets, including...
Materials and Methods

All animal experiments were performed in accordance with national legislation on laboratory animal's protection and have been approved by the Ethics Committee for Animal Research in Uppsala. Detailed descriptions of materials, equipments, and methods used in this study are given in the Supplementary Data.

Cell culture

For in vitro studies and for in vivo models with high HER2 expression, SKOV-3 human ovarian cancer cells (3.16 × 10^6 receptors/cell; ref. 29) were used. To model tumors with low target expression, colorectal carcinoma LS174T cells (~3.9 × 10^6 receptors/cell; ref. 29) were used. HER2-negative Ramos lymphoma cells were used for implantation of HER2-negative control xenografts. All cell lines were from the ATCC. Immediately before experiment, HER2 expression in the cell lines was confirmed by measurement of specific binding of radiolabeled anti-HER2 antibody molecule. Cells were cultured in RPMI medium (Flow Irvine), supplemented with 10% fetal calf serum (Sigma), 2 mmol/L l-glutamine, and PEST (penicillin, 100 IU/mL, and streptomycin, 100 mg/mL; Biokrom Kg).

Tracer design, production, and labeling

The binding of the selected molecule, ADAPT6, to albumin was deleted by complementing the substitution L37R at the albumin-binding surface with two alanine substitutions (S18Y20). The novel molecule is denoted ADAPT6. A CXXXHEXEDAVDANS-sequence was introduced at the N-terminus both as a tag for immobilized metal ion affinity chromatography (IMAC) purification and as a biodistribution modifier (30). The variant was designated as C-(HE)3-ADAPT6. In addition, an ADAPT6 molecule with an N-terminal GXSSHEHHEAVDANS-sequence was also produced for blocking experiments, H6-ADAPT. For site-specific labeling, a maleimido-derivative of the versatile DOTA-chelator was conjugated to the unique cysteine introduced at the N-terminus of the (HE)3-ADAPT6. The conjugate was designated as DOTA-C-(HE)3-ADAPT6. The identity of the conjugate was confirmed by liquid chromatography-electrospray ionization mass spectrometry (LC/ESI-MS). Purity of the conjugate was determined by reversed phase high-performance liquid chromatography (RP-HPLC). The thermal stability and secondary structure of DOTA-C-(HE)3-ADAPT6 and H6-ADAPT6 were determined using a JASCO J-810 spectropolarimeter (Jasco). Binding of DOTA-C-(HE)3-ADAPT6 and H6-ADAPT6 to HER2 and human serum albumin (HSA) was evaluated on a ProteOn XPR36 Protein interaction array system (Bio-Rad). For experimental details, see Supplementary Data.

For labeling of DOTA-C-(HE)3-ADAPT6 with 111In, the lyophilized protein (50 µg) was reconstituted in 80 µL of 0.2 mol/L ammonium acetate, pH 5.5, mixed with 67 µL of 111In stock solution (30–60 MBq), and incubated at 90°C for 35 minutes.

For labeling with 68Ga, lyophilized DOTA-C-(HE)3-ADAPT6 (50 µg) was reconstituted in 100 µL 1.25 mol/L sodium acetate buffer, pH 3.6. 68Ga, up to 3 MBq per microgram of DOTA-C-(HE)3-ADAPT6, was added to the mixture. The mixture was incubated at 95°C for 30 minutes. After labeling, the conjugate was purified using a NAP-5 size-exclusion column equilibrated with PBS.

Measurements of labeling yield and radiochemical purity of conjugates were performed by radioisotopic thin layer chromatography (ITLC) cross-validated by radio–SDS-PAGE, as described earlier (31).

To evaluate the stability of the label, the conjugates were incubated at room temperature in a 500-fold molar excess of EDTA for 1 hour and analyzed using radio–ITLC. The experiments were performed in duplicate. In control experiments, the conjugates were incubated with PBS only.

In vitro cell binding and processing of radiolabeled ADAPT6

Affinity of binding of radiolabeled 111In-DOTA-C-(HE)3-ADAPT6 to HER2-expressing cells was measured using LigandTracer...
In vivo evaluation of radiolabeled DOTA-C(HE)_3-ADAPT6

Euthanasia was performed under Rompun/ketalar anesthesia. Animals were purchased from Taconic M&B.

A group of four mice was used for each data point. The mice were euthanized at predetermined time points post-injection (p.i.) by overdosing of anesthesia followed by heart puncture and exsanguination. Blood and organ samples were collected and their weight and radioactivity were measured. Organ uptake values were calculated as the percentage of injected activity per gram of tissue (%IA/g).

A study in normal NMRI mice was performed to evaluate the biodistribution pattern of ^111^In-DOTA-C(HE)_3-ADAPT6, particularly the blood clearance rate. Female NMRI mice (average weight 24 ± 2 g) were injected with 30 kBq (1 μg) of ^111^In-DOTA-C(HE)_3-ADAPT6 in 100 μL PBS and biodistribution was measured at 1, 4, and 24 hours p.i.

Targeting properties and in vivo specificity of ^111^In-DOTA-C(HE)_3-ADAPT6 were evaluated in female BALB/C nu/nu mice bearing SKOV-3 xenografts. For establishing HER2-positive xenografts, 10^6^ SKOV-3 cells were s.c. implanted in the right hind leg. For HER2-negative controls, 5 × 10^6^ Ramos cells were s.c. implanted. At the time of experiments, the average animal weight was 19 ± 1 g. The average weight of xenografts was 0.31 ± 0.18 g and 0.11 ± 0.09 g for SKOV-3 and Ramos, respectively. ^111^In-DOTA-C(HE)_3-ADAPT6 (30 kBq, 3 μg in 100 μL PBS) was injected i.v. (tail vein) in three groups of mice with SKOV-3 xenografts and one control group with Ramos xenografts. To saturate HER2 receptors in tumors, another control group of mice was injected with ^111^In-DOTA-C(HE)_3-ADAPT6 (30 kBq) at the protein dose of 300 μg. Biodistribution was measured at 1, 4, and 24 hours p.i. Biodistribution in control groups was measured at 1 hour p.i.

Targeting of human tumor xenografts with high (SKOV-3) and low (LS174T) expression of HER2 in mice using ^68^Ga-DOTA-C(HE)_3-ADAPT6 was compared at 1 hour p.i. The goal of this experiment was to confirm that DOTA-C(HE)_3-ADAPT6 labeled with positron-emitting nuclide ^68^Ga can specifically target HER2-expressing xenografts, and that the discrimination between tumors with high and low HER2 expression is better at a higher injected protein dose. At the time of experiments, the average animal weight was 19 ± 1 g in both groups. The average weight of SKOV-3 xenografts was 0.21 ± 0.09 g and 0.3 ± 0.2 g for LS174T xenografts. All animals were injected with 300 kBq ^68^Ga-DOTA-C(HE)_3-ADAPT6 in 100 μL PBS. For each type of xenograft, the injected protein dose was adjusted with unlabeled DOTA-C(HE)_3-ADAPT6 to 1 μg for one group and to 15 μg for the other group.

In vitro cell binding and processing of radiolabeled ADAPT6

According to Interaction Map analysis (Supplementary Figs. S6 and S7), binding of the ^111^In-DOTA-C(HE)_3-ADAPT6 to living HER2-expressing SKOV-3 cells had an affinity (K_d) of 1.13 ± 0.05 nM/L.

In vitro saturation experiments (Fig. 1C) demonstrated that addition of a 100-fold molar excess of the nonlabeled ADAPT6 significantly (P < 0.05) reduced the binding of ^111^In-DOTA-C(HE)_3-ADAPT6 to HER2-expressing SKOV-3 cells. This demonstrated a saturable character of the binding, proving its specificity. Moreover, the binding of ^111^In-DOTA-C(HE)_3-ADAPT6 was prevented by addition of an excess of trastuzumab. However, adding an excess amount of an anti-HER2 ^22^ZnHER2:342 affibody molecule did not affect the binding of ^111^In-DOTA-C(HE)_3-ADAPT6. This shows that ADAPT6 competes for the binding site with trastuzumab but not with the ^22^ZnHER2:342 affibody molecule, which is in agreement with previous results (28). Binding of ^111^In-DOTA-C(HE)_3-ADAPT6 to LS174T had the same saturability.
pattern, but the binding to nonsaturated cells was much lower than in case of SKOV3 cells (0.39% vs. 11.0% of added radioactivity), reflecting the low HER2 expression in this cell line.

The binding and internalization of $^{111}$In-DOTA-C-{(HE)}$_3$-ADAPT6 by SKOV-3 cells is shown in Fig. 1D. The internalization of the ADAPT6 protein was slow: Only 5% and 26% of the total bound radioactive molecules were internalized after one and 24 hours of incubation, respectively.

In vivo evaluation of radiolabeled DOTA-C-{(HE)}$_3$-ADAPT6

A first evaluation of $^{111}$In-DOTA-C-{(HE)}$_3$-ADAPT6 was performed in normal NMRI mice (Fig. 2). The rapid clearance of the tracer from blood (0.36 ± 0.09 %IA/g at 1 hour p.i.) confirmed that there was no residual binding to albumin. $^{111}$In-DOTA-C-{(HE)}$_3$-ADAPT6 also cleared rapidly from other organs and tissues (uptake of less than 1 %IA/g at 1 hour p.i.) except from kidneys. Low radioactivity in the gastrointestinal tract including content (0.44% ± 0.08% of injected activity at 1 hour p.i.) suggested that hepatobiliary excretion played a minor role in the clearance. The renal uptake was high, 259 ± 30 %IA/g at 1 hour p.i.

Biodistribution of $^{111}$In-DOTA-C-{(HE)}$_3$-ADAPT6 in BALB/C nu/nu mice bearing SKOV-3 xenografts is shown in Figs. 3 and 4. The specificity of HER2 targeting in vivo was demonstrated by saturation of HER2 receptors and by the use of HER2-negative Ramos xenografts (Fig. 3). The use of a saturating amount (300 μg) of nonlabeled H$_6$-ADAPT6 resulted in a reduction of tumor uptake of $^{111}$In-DOTA-C-{(HE)}$_3$-ADAPT6 from 19 to 3.3 %IA/g ($P < 0.005$). Some reduction of the uptake was observed also in kidneys ($P < 0.05$). Interestingly, the uptake in spleen was significantly ($P < 0.05$) higher in mice injected with 300 μg...
H$_4$-ADAPT6. Uptake in Ramos xenografts (0.112 ± 0.002 %IA/g) was significantly ($P < 0.05$) lower than uptake in SKOV-3 xenografts, on the same level as in muscles.

The biodistribution in normal organs (Fig. 4A) was characterized by rapid clearance from normal tissues except from kidneys and was in an excellent agreement with the data for NMRI mice. The tumor uptake was 18.7 ± 6.1, 13.9 ± 5.2, and 10.8 ± 2.5 %IA/g at 1, 4, and 24 hours p.i., respectively. Although the time points do not differ significantly ($P > 0.05$), there is a clear trend that tumor uptake decreases over time. Such biodistribution pattern provided high tumor-to-organ ratios already at 1 hour p.i. (Fig. 4B). For example, the tumor-to-blood ratio was as high as 43 ± 11 at this time point. At later time points, there was a significant ($P < 0.05$) increase only in tumor-to-blood and tumor-to-lung ratios.

Biodistribution and tumor-to-organ ratios of $^{68}$Ga-DOTA-C-(HE)$_3$-ADAPT6 in BALB/C nu/nu mice bearing SKOV-3 xenografts (high HER2 expression) and LS174T (low HER2 expression) xenografts at injected dose of 1 and 15 μg, the uptake in SKOV-3 xenografts was lower at 15 μg, the tumor-to-organ ratios were not lower (Table 2).

**Imaging**

Experimental imaging (Fig. 5) confirmed the biodistribution results. The only normal organ with high uptake of radioactivity was kidney. No other organs were visualized. SKOV-3 xenografts with high HER2 expression were clearly visualized with a high contrast using 3 μg $^{111}$In-DOTA-C-(HE)$_3$-ADAPT6. Saturation of receptors with DOTA-C-(HE)$_3$-ADAPT6 caused dramatic decrease of tumor uptake (Fig. 5A). PET imaging (Fig. 5B) demonstrated that xenografts with high (SKOV-3) and low (LS174T) HER2 expression could be easily distinguished using 15 μg $^{68}$Ga-DOTA-C-(HE)$_3$-ADAPT6.

**Discussion**

Both theoretical calculations and experimental data (5, 7, 34) suggest that size reduction is the most promising way to increase imaging contrast provided by proteinaceous imaging agents.

### Table 1. Biodistribution of $^{68}$Ga-DOTA-C-(HE)$_3$-ADAPT6 in BALB/C nu/nu mice bearing SKOV-3 xenografts (high HER2 expression) and LS174T (low HER2 expression) at 1 hour p.i.

<table>
<thead>
<tr>
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<th>1 μg</th>
<th>15 μg</th>
<th>1 μg</th>
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<tbody>
<tr>
<td><strong>Blood</strong></td>
<td>0.59 ± 0.07</td>
<td>0.50 ± 0.06</td>
<td>0.60 ± 0.09</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td><strong>Lung</strong></td>
<td>0.70 ± 0.02</td>
<td>0.53 ± 0.06</td>
<td>0.67 ± 0.09</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>1.7 ± 0.1</td>
<td>1.21 ± 0.05</td>
<td>1.8 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>0.62 ± 0.05</td>
<td>0.50 ± 0.07</td>
<td>0.74 ± 0.07</td>
<td>0.56 ± 0.34</td>
</tr>
<tr>
<td><strong>Stomach</strong></td>
<td>0.65 ± 0.11</td>
<td>0.35 ± 0.07</td>
<td>0.54 ± 0.06</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td>5.08 ± 24</td>
<td>277 ± 5</td>
<td>332 ± 27</td>
<td>311 ± 31</td>
</tr>
<tr>
<td><strong>Tumor</strong></td>
<td>10.3 ± 10$^{-6}$</td>
<td>8.4 ± 0.6$^a$</td>
<td>2.9 ± 0.5$^a$</td>
<td>0.9 ± 0.6</td>
</tr>
<tr>
<td><strong>Muscle</strong></td>
<td>0.18 ± 0.01</td>
<td>0.12 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td><strong>Bone</strong></td>
<td>0.5 ± 0.1</td>
<td>0.34 ± 0.07</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
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</table>

**NOTE:** Data are presented as an average %IA/g and SD for four mice.

$^a$Injected dose of 1 and 15 μg in LS174T xenografts.

$^b$Difference was significant ($P < 0.05$) between.

$^c$Injected dose of 1 and 15 μg in SKOV3 xenografts.

$^d$SKOV3 and LS174T xenografts at injected dose of 1 μg.

$^e$SKOV3 and LS174T xenografts at injected dose of 15 μg.
ADAPT (scaffold size 5.1 kDa without linkers and tags) has a size advantage in comparison with many other scaffold proteins, such as affibody molecules (7 kDa), designed ankyrin repeat proteins (14 kDa), anticalins (20 kDa), fibronectin domains (adnectins) (10 kDa), and VHHs (15 kDa). Knottins (4 kDa) are smaller, but they and many of the other scaffold proteins contain cysteines. The cysteine-free structure of ADAPT enables the use of oxidizing and reducing conditions during conjugation and labeling without affecting the structure of the domain. Moreover, the possibility to introduce a unique cysteine, as in this study, provides easy site-specific conjugation of chelators and other prosthetic groups that gives homogenous ADAPT conjugates. However, these potential advantages of ABD-based imaging agents could be realized only with three preconditions:

1. Generation of ABD derivatives with affinity in the range of 10 pmol/L to 10 nmol/L (35) to therapeutic targets should be possible;
2. Binding to albumin should be reduced to a level that permits rapid clearance from blood;
3. Off-target interaction of the scaffold should not cause any noticeable uptake in normal tissues.

An optimal affinity ($K_D$) for imaging agents should be in the range between 10 pmol/L and 10 nmol/L. If $K_D$ is lower than 10 pmol/L (very high affinity), pharmacokinetics of the agent would depend heavily on blood flow or vascular permeability. Furthermore, if $K_D$ is greater than 10 nmol/L, the off-rate will be too fast, and the retention at specific binding sites might be insufficient (35). This and previous studies (21, 23, 24) have demonstrated that selection of ADAPT binders with an optimal affinity to several therapeutic targets (HER2, HER3, and TNFα) is possible. This study has demonstrated that reduction of the affinity to albumin to a nonmeasurable level is also possible. The data from the first animal study (Fig. 2) demonstrated rapid clearance from blood. Furthermore, these data showed very low uptake in normal tissues (except kidneys), which suggests absence of noticeable off-target interactions. The high renal uptake is a general feature of all radiometal-labeled scaffold proteins, including nanobodies (36), affibody molecules (13–15), fibronectin domains (19), and designed ankyrin repeats (20). This uptake is caused by reabsorption from primary urine in proximal tubuli. After rapid internalization and proteolytic degradation, bulky hydrophilic radiometabolites get trapped inside proximal tubuli cells. However, renal metastases are rather rare, and the well-defined shape of kidneys excludes false-positive findings. Moreover, our clinical study with affibody molecules suggests that high renal uptake does not prevent visualization of adrenal metastases and absorbed doses to kidneys permit multiple imaging procedures (12). Considerations concerning dosimetry aspects of high renal uptake are presented in Supporting Information. In addition, earlier we have shown for affibody molecules that the use of radiohalogen labels yields lipophilic radiometabolites, which “leak” rapidly from kidneys (11, 37). In the case of slow internalization by cancer cells, the use of radiohalogen labels has very little influence on tumor uptake of affibody molecules (11, 37). As internalization of ADAPT is also slow (Fig. 1D), the use of nonresidualizing radiohalogen labels may provide ADAPT-based

Table 2. Tumor-to-organ ratios of $^{68}$Ga-DOTA-C-(HE)$_3$-ADAPT$_6$ in BALB/C nu/nu mice bearing SKOV-3 xenografts (high HER2 expression) and LS174T (low HER2 expression) at 1 hour p.i.

<table>
<thead>
<tr>
<th></th>
<th>SKOV3 xenografts</th>
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<th>LS174T xenografts</th>
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<tbody>
<tr>
<td></td>
<td>1 μg</td>
<td>15 μg</td>
<td>1 μg</td>
</tr>
<tr>
<td>Blood</td>
<td>17.5 ± 0.3</td>
<td>17 ± 2</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>15 ± 1</td>
<td>16 ± 1</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Liver</td>
<td>6.0 ± 0.1</td>
<td>7.0 ± 0.4</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>17 ± 2</td>
<td>17 ± 1</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Stomach</td>
<td>16 ± 1</td>
<td>24 ± 4</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.034 ± 0.005</td>
<td>0.030 ± 0.003</td>
<td>0.009 ± 0.002</td>
</tr>
<tr>
<td>Muscle</td>
<td>56 ± 9</td>
<td>70 ± 6</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>Bone</td>
<td>20 ± 4</td>
<td>25 ± 4</td>
<td>7 ± 3</td>
</tr>
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NOTE: Data are presented as an average value and SD for four mice.

Figure 5.
Imaging of HER2 expression in xenografts using radiolabeled DOTA-C-(HE)$_3$-ADAPT$_6$. A, gamma-camera imaging of SKOV-3 xenografts using $^{111}$In-DOTA-C-(HE)$_3$-ADAPT$_6$ (5 μg). Contours were derived from a digital photograph and superimposed over images to facilitate interpretation. A control animal (right) was injected with a saturating amount of nonlabeled ADAPT$_6$. B, PET/CT imaging of SKOV-3 (high expression) and LS174T (low expression) xenografts using $^{68}$Ga-DOTA-C-(HE)$_3$-ADAPT$_6$. Injected protein dose was 15 μg. Arrows are pointing at tumors (T) and kidneys (K).
Both in vivo specificity tests (saturation and the use of HER2-negative xenografts) confirmed highly specific targeting of HER2-positive xenografts using 111In-DOTA-C-(HE)_3-ADAPT6 (Fig. 3). Interestingly, the spleen uptake was increased in a saturation experiment. This might be due to weak cross-reactivity with other molecular targets expressed in the spleen or with a target that is taken up by the spleen. Such weak cross-reactivity is primarily manifested when a large amount of conjugate is injected (For more detailed consideration see Supplementary Information).

Considerations concerning splenic uptake at high injected dose of 111In-DOTA-C-(HE)_3-ADAPT6. Importantly, the uptake in HER2-positive xenografts exceeded uptake in negative ones by more than 160-fold. This demonstrated absence of an enhanced permeability and retention effect for ADAPTs, and excludes false-positive findings due to unspecific uptake in tumors. The uptake of the ADAPT in SKOV-3 xenografts (19 ± 6 %IA/g at 1 hour) was at the same level as for radiometal-labeled HER2-targeting affbody molecules (17 ± 2 %IA/g; ref. 38), and exceeded reported uptake for DARPin (8.3 ± 2.7 %IA/g; ref. 20) and nanobodies (4.7 ± 0.7 %IA/g; ref. 36) at the same point in time in the same xenografts. The data concerning higher tumor uptake of smaller ADAPTs and affbody molecules in comparison with larger DARPin and nanobodies are consistent with a previous comparison with dimeric affbody molecules. It has been shown that the smaller monomers have higher tumor uptake than larger dimers (37). Together, these data suggest that the use of smaller scaffold proteins improves tumor targeting, presumably due to better extravasation. Importantly, clearance of 111In-DOTA-C-(HE)_3-ADAPT6 was more rapid than clearance of, for example, affbody molecules. As a result, the highest tumor-to-organ ratios were reached very early (Fig. 4B), which enabled high-contrast imaging already at 1 hour p.i. (Fig. 5A). Ability to provide high-contrast images shortly after injection offers clear clinical and logistical advantages. Importantly, it permits the use of short-lived positron emitting radionuclides for labeling and therefore PET for imaging is feasible (Fig. 5B).

A rapid blood clearance in combination with high affinity might cause equal uptake of an imaging agent in tumors with high and low target expression. However, this limitation can be circumvented by increasing injected protein dose. We have shown earlier for affbody molecules in preclinical models (39). In this study, an increase of the injected dose from 1 to 15 μg increased the difference in uptake in xenografts with low and high expression from 3-fold to more than 9-fold. This indicates that during possible clinical translation a dose-finding study should be performed to determine an optimal injected dose permitting clear discrimination between tumors with high and low target expression.

In conclusion, ADAPTs can be used as imaging agents that provide high-contrast PET imaging of HER2 expression in cancer shortly after injection. This shows that ADAPT is a very promising novel scaffold for development of imaging agents for personalized cancer treatment.

Disclosure of Potential Conflicts of Interest

J. Buijs is a CTO for Ridgeview Instruments AB. A. Orlova has ownership interest (including patents) and is a consultant/advisory board member for AbbVie AB, Sweden. V. Tolmachev has ownership interest (including patents) and is a consultant/advisory board member for AbbVie AB, Solna, Sweden. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Garousi, J. Nilvebrant, M. Sandstrom, H. Honarvar, A. Orlova, V. Tolmachev

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Garousi, S. Lindbo, J. Nilvebrant, M. Åstrand, J. Buijs, M. Sandstrom, H. Honarvar, A. Orlova, V. Tolmachev

Writing, review, and/or revision of the manuscript: J. Garousi, S. Lindbo, J. Nilvebrant, M. Åstrand, J. Buijs, M. Sandstrom, H. Honarvar, A. Orlova, V. Tolmachev, S. Hober

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Tolmachev

Study supervision: M. Sandstrom, V. Tolmachev, S. Hober

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