# Reduction of renal activity retention of radiolabeled albumin binding domain-derived affinity proteins using a non-residualizing label strategy compared with a cleavable glycine-leucine-glycine-lysine-linker

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**Abstract.** The feasibility of targeted imaging and therapy using radiolabeled albumin-binding domain-derived affinity proteins (ADAPTs) has been demonstrated. However, high renal uptake of radioactivity limits the maximum tolerated dose. Successful reduction of renal retention of radiolabeled Fab fragments has been demonstrated by incorporating a cleavable linker between the targeting agent and the radiometal chelator. The present study investigated if the introduction of a glycine-leucine-glycine-lysine (GLGK)-linker would reduce the kidney uptake of radiolabeled ADAPT6 and also compared it with the non-residualizing [$^{125}$I]I-[(4-hydroxyphenyl)ethyl] maleimide ([$^{125}$I]I-HPEM) labeling strategy. GLGK was site-specifically coupled to human epidermal growth factor receptor 2 (HER2)-targeting ADAPT6. Conjugates without the cleavable linker were used as controls and all constructs were labeled with lutetium-177 ($^{177}$Lu). [$^{125}$I]I-HPEM was coupled to ADAPT6 at the C-terminus. Biodistribution of all constructs was evaluated in NMRI mice 4 h after injection. Specific binding to HER2-expressing cells *in vitro* was demonstrated for all constructs. No significant difference in kidney uptake was observed between the [$^{177}$Lu]Lu-2,2',2",2"'-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetic acid-GLGK-conjugates and the controls. The renal activity of [$^{125}$I]I-HPEM-ADAPT6 was significantly lower compared with all other constructs. In conclusion, the incorporation of the cleavable GLGK-linker did not result in lower renal retention. Therefore, the present study emphasized that, in order to achieve a reduction of renal retention, alternative molecular design strategies may be required for different targeting agents.

## Introduction

Targeted therapies have gained interest as a way for safe and selective delivery of cytotoxic agents, such as radionuclides, toxins, and drugs, to malignant cells (1). The use of high affinity small engineered scaffold proteins (ESPs) as targeting agents enables the efficient delivery of payloads to targets such as cancer-specific markers on tumor cells. Compared with antibodies, ESPs have a more rapid clearance from blood and non-targeted tissues (2-4). Radiolabeled affibody molecules and Albumin binding domain (ABD)-Derived Affinity ProTeins (ADAPTs) are ESPs that can provide high contrast visualization of human epidermal growth factor receptor 2 (HER2)-expressing tumors in breast cancer patients (5-7). During the past years, several other promising ESPs, such as anticalins, cystine-knot peptides, and designed ankyrin repeat proteins (DARPins), have been developed and investigated in preclinical or early clinical studies (8-12).

ADAPTs are small (5 kDa) engineered non-immunoglobulin proteins, with the selected variant against HER2 named ADAPT6 (13). In previous studies, we have investigated different aspects of the molecular design of radiolabeled ADAPTs to increase the sensitivity for imaging of disseminated cancers (14-19). For stratification of cancer patients for HER2 targeting therapy, [$^{99m}$Tc]Tc-ADAPT6 has demonstrated

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*Abbreviations:* ADAPT, albumin-binding domain-derived affinity protein; ABD, albumin binding domain; DARPin, designed ankyrin repeat protein; DOTA, 2,2',2",2"'-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; ESP, engineered scaffold protein; HER2, human epidermal growth factor receptor 2; HPEM, [(4-hydroxyphenyl)ethyl]maleimide; SPECT, single-photon emission computed tomography

*Key words:* kidney retention, cleavable linker, renal brush border enzyme, non-residualizing label, radionuclide

excellent sensitivity and specificity as an imaging agent using single-photon emission computed tomography (SPECT). Moreover, no signs of acute toxicity in patients at doses up to 1 mg were observed (6). ADAPTs have also shown promising potential as therapeutic agents. ABD-fused ADAPT6 labeled with $^{177}$Lu has shown promising results in radionuclide therapy of HER2-expressing SKOV-3 xenografts in mice. It was demonstrated that the median survival in mice was increased more than two-fold after a single injection of 18 MBq of [$^{177}$Lu]Lu-2,2',2",2"'-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetic acid (DOTA)-ADAPT6-ABD035 (20).

The kidneys are the main excretion pathway of peptides and proteins below 60 kDa and therefore, a high renal uptake is common for ESP-based radiopharmaceuticals. High renal uptake leads to reduced sensitivity for detection of abdominal lesions close to the kidneys and it also decreases the maximum tolerated therapeutic dose. Radiopharmaceuticals excreted via the glomeruli undergo reabsorption and internalization in proximal tubular cells. Internalized radiopharmaceuticals are degraded by lysosomal enzymes leading to the formation of radiometabolites. The main reason for the high retention of radioactivity in kidneys is due to the degradation of proteins labeled with residualizing radiometals (e.g. $^{177}$Lu) leading to the formation of hydrophilic radiometabolites that cannot cross the cellular membrane and will therefore be trapped inside the cells. High renal retention has been observed for ADAPTs labeled with radiometals regardless of the label position, at both N- and C-terminus (13,16,17).

Several strategies have been developed to reduce the renal retention of ESP-based radiopharmaceuticals, and one strategy is to prevent glomerular filtration by fusing the radiopharmaceutical to an ABD. The ABD will bind to albumin in the blood which will increase the total size of the construct above the kidney filtration barrier (60 kDa). This strategy has been successfully applied to both $^{177}$Lu-labeled anti-HER2 affibody molecules and [$^{177}$Lu]Lu-ADAPT6, where the kidney radioactivity retention was reduced 25- and 14-fold compared with the native constructs, respectively (20,21). However, the increased circulatory half-life by ABD-fusion will also increase the risk of undesired bone marrow exposure. Therefore, exploring alternative strategies to reduce the kidney accumulation of ADAPTs is of high interest for radionuclide therapy. Pretargeting is a possible approach to reduce the renal activity uptake using a non-labeled primary agent coupled to a recognition tag injected prior to the radiolabeled probe (22-24). A study using affibody-based peptide nucleic acid (PNA)-mediated pretargeting in mice demonstrated a 20-fold lower uptake of radioactivity in kidneys compared with the regular targeting strategy (24).

Another method that does not require modification of the ESP is the use of a pharmacological agent as a competitor or inhibitor of the reabsorption process in kidneys. These agents may act on transporters (e.g. saturation by lysine, arginine, gelofusine, or blocking by probenecid) or by decreasing the level of energy-mediated endocytosis (e.g. pre-injection of sodium maleate and fructose) (25). We have recently investigated these pharmacological approaches for renal uptake reduction of DARPins, affibodies, and ADAPT6. It was shown that sodium maleate and fructose could reduce the accumulation of activity in kidneys, however, both agents are toxic at the required doses (26-28).

An alternative approach for reducing the activity uptake in the kidneys is the use of a non-residualizing label (e.g. radioiodine). Radiometabolites of a non-residualizing label are lipophilic which enables diffusion through the cell membrane out into the extracellular space. In that way, the radiometabolites can return to the blood circulation and be excreted from the body. Reduction of the renal activity retention using a non-residualizing label has been demonstrated for [$^{125}$I]I-HPEM-ADAPT6 in mice bearing SKOV-3 xenografts (17). This approach is particularly useful for radionuclide therapy with the β-emitter $^{131}$I. $^{131}$I has suitable physicochemical properties and the emitted 365 keV gamma-rays (81% abundance) could be used for monitoring therapy. On the other hand, it has been demonstrated that ADAPT6 labeled with radiometals provided higher radioactivity retention in the tumor com-pared to a non-residualizing radiohalogen label (14). Longer retention of radioactivity at the target location reduces the frequency of injections for radionuclide therapy. Therefore, it is of high interest to find a strategy to reduce the reabsorption in the kidneys of ADAPTs labeled with radiometals (e.g. beta-emitting $^{177}$Lu).

The introduction of an enzyme-cleavable peptide linker between the radiometal-chelator complex and the scaffold protein could be another method for reducing the renal reabsorption of radiopharmaceuticals. Once the peptide linker has been cleaved by the enzyme located in the kidneys, the radiometal-chelator complex will be separated from the scaffold protein and excreted with the urine. It is crucial that the peptide linker has high selectivity to the selected enzyme and not to other enzymes (e.g. in the blood). The proximal tubular brush border enzyme is a potential target enzyme for this approach. Previous studies have shown that the glycine-lysine (GK) sequence is a substrate for carboxypeptidase M located at the renal brush border membrane (29-31). Uehara *et al* also demonstrated that the construct $^{188}$Re-tricarbonyl-(cyclopenta dienyl)-glycyl-lysine-Fab was recognized and cleaved by the brush border enzymes, resulting in a significant decrease of renal activity uptake 6 h after injection without influencing the tumor uptake (32). Another study demonstrated the same activity uptake in the tumor but a lower renal activity uptake due to an efficient cleavage of $^{66/67}$Ga-NOTA-Met from a $^{66/67}$Ga-labeled Fab fragment (33,34).

In this study, we investigated if the introduction of a cleavable glycine-leucine-glycine-lysine (GLGK) peptide linker between the [$^{177}$Lu]Lu-DOTA-complex and ADAPT6 would reduce the renal activity retention due to cleavage by the brush border enzymes. This strategy was also compared to the use of a non-residualizing [$^{125}$I]I-HPEM label.

## Materials and methods

*Preparation of radiolabeled ADAPT6 constructs.* Three constructs (1-3) of ADAPT6 (Fig. 1A), containing cysteine either at the N- or C-terminus, were recombinantly produced in E. coli BL21*(DE3) cells and purified as described earlier (13,14,17). The molecular mass was confirmed by MALDI. DOTA-GLGK(maleimide)-COOH was synthesized in two steps (Data S1). First, DOTA-GLGK-COOH (4)

Table I. Radiochemical yields of radiolabeled ADAPT6 constructs.

| ADAPT6 construct | Radiochemical yield (%) | Radiochemical purity (%) | Radiochemical purity after challenge (%) |
|---|---|---|---|
| [$^{177}$Lu]Lu-DOTA-GLGK-1 | 82±2 | >99 | >99[a] |
| [$^{177}$Lu]Lu-DOTA-1 | 90±4 | >99 | >99[a] |
| [$^{177}$Lu]Lu-DOTA-GLGK-2 | 87±3 | >99 | >99[a] |
| [$^{177}$Lu]Lu-DOTA-2 | 95±2 | >99 | >99[a] |
| [$^{125}$I]I-HPEM-3 | 60±6 | >99 | >99[b] |

[a]Incubation with a 1000-fold molar excess of potassium iodide; [b]Incubation with a 5000-fold molar excess of EDTA. As shown in Fig. 1A, construct 1: ADAPT6 with a cysteine at the N-terminus; 2: ADAPT6 with a cysteine at the C-terminus; 3: ADAPT6 with HPEM at C-terminus. ADAPT, albumin-binding domain-derived affinity proteins; $^{177}$Lu, lutetium-177; DOTA, 2,2',2",2"'-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetic acid; [$^{125}$I]I-HPEM, [$^{125}$I]I-[(4-hydroxyphenyl)ethyl]maleimide.

(Fig. 1B) was synthesized by Fmoc solid phase peptide synthesis on 2-CTC resin using Oxyma Pure and N,N'-diisopropylcarbodiimide (DIC) as coupling reagents and the cleaved peptide was purified by RP-HPLC. In a second step, N-(methoxycarbonyl) maleimide was coupled to the free amine in 4 to generate DOTA-GLGK(maleimide)-COOH (5) (Fig. 1B) which was purified by RP-HPLC. The identity and purity of the compound were determined by LC-MS. Through a Michael addition, constructs 1 and 2 were conjugated with compound 5 and DOTA-maleimide separately followed by purification by RP-HPLC. $^{177}$Lu-labeling was performed according to a method optimized by Altai *et al* (35). Construct 3 was labeled with [$^{125}$I]I-HPEM according to the protocol optimized by Tolmachev *et al* for indirect iodination of affibody molecules (36).

*The specificity of in vitro cell binding of radiolabeled ADAPT6.* The specificity of binding of ADAPT6 constructs labeled with $^{177}$Lu or $^{125}$I was tested using the HER2-expressing SKOV3 ovarian cancer cell line (1.6x10$^6$ receptors/cell) as described by Wållberg and Orlova (37). Briefly, 50 nM of the labeled compound was added to two sets of Petri dishes in triplicates (1 million cells/dish). HER2 receptors in one set of the dishes were saturated by the addition of non-labeled ADAPT6 construct (1000-fold molar excess) 15 min prior to the addition of the labeled compound. The dishes were incubated at 37˚C for 1 h in a humidified incubator. The media were aspirated, the cells were harvested by trypsinisation and radioactivity content in the samples was measured. The percent of cell-associated radioactivity was calculated and the data were normalized to the highest value of average cell-associated radioactivity.

*In vivo studies.* The animal experiments were planned in agreement with the Swedish laws on laboratory animal welfare and were approved by the Ethics Committee for Animal Research in Uppsala (Permit Number C4/2016). The biodistribution properties of ADAPTs labeled with $^{177}$Lu or $^{125}$I were evaluated in non-tumor bearing NMRI mice, randomized into five groups with four animals per group. Mice were injected with a total dose of 3 $\mu$g per mouse (40 kBq/15 kBq of $^{177}$Lu/$^{125}$I, correspondingly, and the dose was adjusted by the addition of non-labeled compound) in 100 $\mu$l PBS. At 4 h after injection, animals were euthanized by intraperitoneal injection of xylazine and ketamine. The dose of xylazine was 20 mg/kg, the dose of ketamine was 200 mg/kg. After the injection of anesthesia, its effectiveness was ensured by the absence of a paw withdrawal reflex in mice. The sedated mice were euthanized by exsanguination via cardiac puncture and collection of blood. Tissue samples and organs of interest were collected, weighed, and the uptake of activity was measured using an automated γ-spectrometer. The data were corrected for dead time, spillover, background, and decay.

*Statistical analysis.* GraphPad Prism (version 8.00 for Windows; GraphPad Software, San Diego, CA, USA) was used for statistical analysis. To determine significant differences ($P<0.05$) between two groups the obtained data were analyzed by an unpaired two-tailed t-test. Data analysis for more than two groups was performed using one-way ANOVA with Bonferroni's multiple comparisons test.

## Results

*Synthesis of DOTA-GLGK(maleimide)-COOH (5).* DOTA-GLGK(maleimide)-COOH was successfully synthesized and purified. The purity was >98 % (Fig. S1) and the identity of the product was confirmed (calculated m/z 840.4, found m/z 840.1) by LC-MS (Fig. S2).

*Preparation of radiolabeled ADAPT6 constructs.* ADAPT6 constructs were successfully produced and purified. MALDI-TOF analysis confirmed the expected mass of the constructs (6447 Da), which was in good agreement with the theoretical value (6445.2 Da). The difference was within the accuracy of the method. The constructs intended for labeling with $^{177}$Lu were successfully conjugated with construct 5 or DOTA-maleimide, and the purity and identity of the constructs were confirmed by mass spectrometry (Fig. S3). All ADAPT6 conjugates were successfully labeled with $^{177}$Lu or [$^{125}$I]I-HPEM, and the radiochemical yields are presented in Table I. The purity after size-exclusion chromatography was over 99% for all constructs. No release of the radiolabels could be detected after incubation with an excess of EDTA or KI over 3 h (Table I). The radiolabeling of construct 3 (Fig. 1A) with [$^{125}$I]I-HPEM was in good agreement with the previously reported data for indirect radioiodination of ADAPT6 and

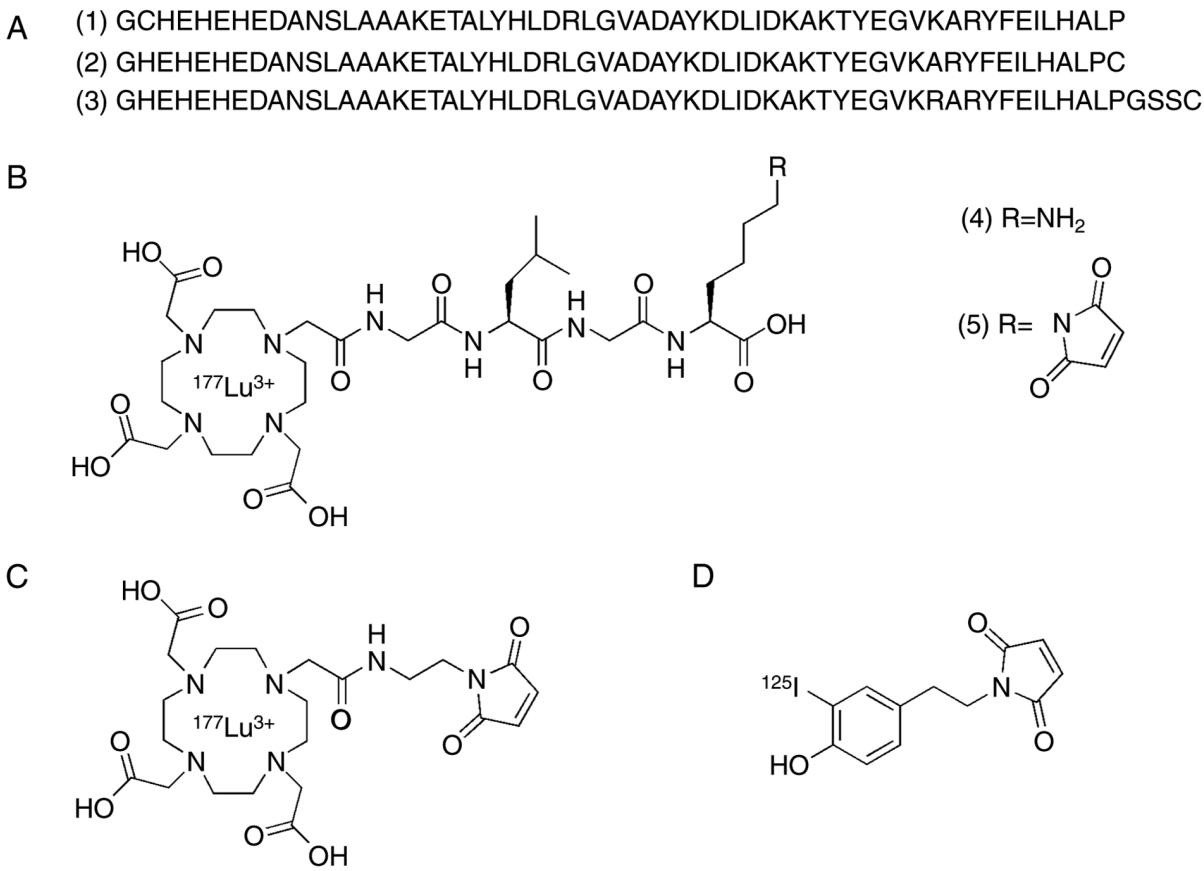


Figure 1. Schematic overview of the structures of ADAPT6 and radiolabeling methods used in the study. (A) Amino acid sequences of ADAPT6 constructs (1-3) containing cysteine (red) either at the N- or C-terminus. (B) Structure of the cleavable peptide linker (4) [$^{177}$Lu]Lu-DOTA-GLGK-COOH and (5) [$^{177}$Lu]Lu-DOTA-GLGK-(maleimide)-COOH. (C) Structure of [$^{177}$Lu]Lu-DOTA-maleimide. (D) Structure of [$^{125}$I]I-HPEM. ADAPT, albumin-binding domain-derived affinity protein; $^{177}$Lu, lutetium-177; DOTA, 2,2',2",2"'-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid; GLGK, glycine-leucine-glycine-lysine; [$^{125}$I]I-HPEM, [$^{125}$I]I-[(4-hydroxyphenyl)ethyl]maleimide.

affibody molecules (17,37). The maximum achieved effective specific radioactivity was 1 MBq/$\mu$g (7 GBq/$\mu$mol) and 0.3 MBq/$\mu$g (1.96 GBq/$\mu$mol) for $^{177}$Lu and $^{125}$I, respectively.

*Specificity of in vitro cell binding of radiolabeled ADAPT6 constructs*. The in vitro specificity test demonstrated HER2-mediated binding to HER2-expressing SKOV-3 cells for all radiolabeled ADAPT6 constructs (Fig. 2). Presaturation of HER2 receptors by non-labeled ADAPT6 resulted in a significant decrease of cell-associated radioactivity ($P<0.001$).

*In vivo studies*. The biodistribution study in NMRI mice 4 h after injection demonstrated a fast excretion and an activity in blood below 0.2% ID/g for all radiolabeled ADAPT6 constructs (Fig. 3). Constructs labeled with $^{177}$Lu at the C-terminus had a significantly ($P<0.001$, one-way ANOVA test) lower activity uptake in blood compared to the N-terminus constructs. The liver uptake of [$^{125}$I]I-HPEM-ADAPT6 was significantly higher ($P<0.001$, one-way ANOVA test) compared with the $^{177}$Lu-labeled constructs. For all constructs, the uptake of activity in non-targeted organs and tissues was lower than 1% ID/g (except in the kidneys for the $^{177}$Lu-labeled constructs). There was no significant difference ($P>0.05$, one-way ANOVA test) in the radioactivity uptake in the kidneys between the $^{177}$Lu-labeled ADAPT6 constructs containing the cleavable peptide linker and the controls, regardless of the label position. The renal radioactivity uptake of [$^{125}$I]I-HPEM-ADAPT6 was significantly lower than that of all other constructs. For example, the renal activity of [$^{125}$I]-HPEM-ADAPT6 was 507-fold lower than the activity of [$^{177}$Lu]Lu-DOTA-GLGK-2 (0.59±0.21% ID/g vs. 299±21% ID/g, respectively). Numerical biodistribution data is presented in Table SI.

## Discussion

ESPs, such as ADAPTs, have a high affinity to their targets and possess suitable pharmacokinetics for selective delivery of radioactivity for imaging and therapy. However, due to their small size (<60 kDa), their biodistribution is associated with high renal uptake as a result of reabsorption in proximal tubular cells (5-7,13). After reabsorption, degradation of the radiopharmaceuticals occurs leading to the formation of radiometabolites. Radiometabolites of proteins labeled with radiometals ($^{177}$Lu, $^{111}$In) usually have better residualizing properties than proteins labeled with radiohalogens ($^{125}$I, $^{131}$I). The two most common ways to lower renal radioactivity exposure are to re-duce the uptake of the radiopharmaceutical in the kidneys or to reduce the renal retention of the radiometabolites. We have already demonstrated two different methods for the reduction of kidney uptake of radiolabeled ADAPTs. However, these methods are associated with toxicity in some organs and tissues such as the bone marrow (20-28).

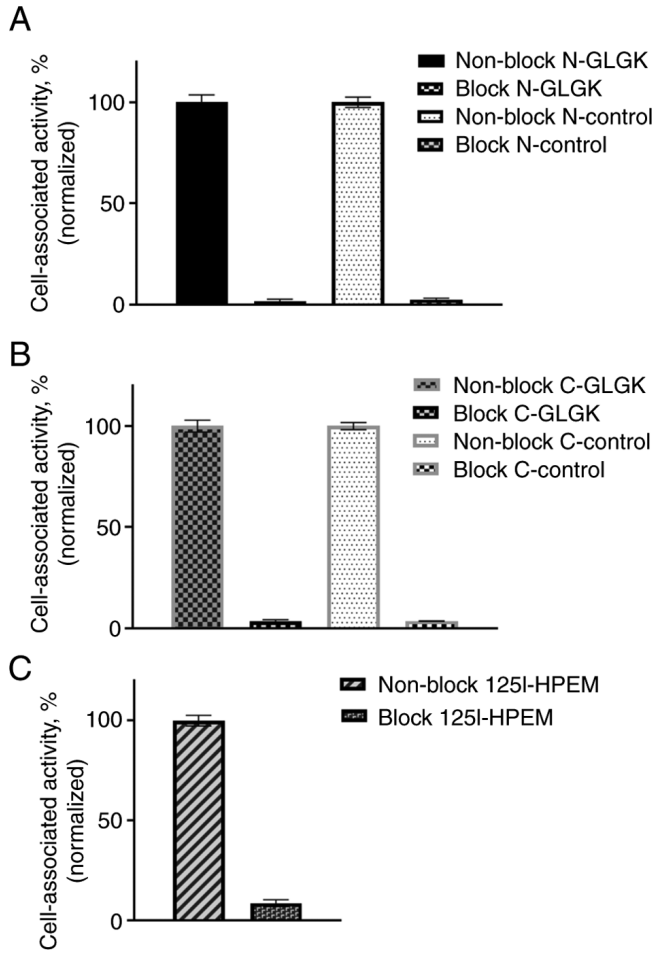


Figure 2. Binding specificity of radiolabeled ADAPT6 constructs to HER2-expressing SKOV3 cells. (A) [$^{177}$Lu]Lu-DOTA-GLGK-1 and [$^{177}$Lu]Lu-DOTA-1; (B) [$^{177}$Lu]Lu-DOTA-GLGK-2 and [$^{177}$Lu]Lu-DOTA-2; (C) [$^{125}$I]I-HPEM-3. In the blocked groups, HER2 receptors were presaturated by an excess of non-labeled ADAPT6. ADAPT, albumin-binding domain-derived affinity protein; $^{177}$Lu, lutetium-177; DOTA, 2,2',2",2"'-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetic acid; GLGK, glycine-leucine-glycine-lysine; [$^{125}$I]I-HPEM, [$^{125}$I]I-[(4-hydroxyphenyl)ethyl]maleimide.

Therefore, it is desirable to investigate other approaches for this purpose.

In this study, we have investigated if the strategy of incorporating a cleavable linker between the radiometal-chelator complex and the targeting agent would result in a reduction of renal activity retention, which has been demonstrated for Fab-fragments earlier (32-34). The cleavable linker used in this study was GLGK, a substrate for the brush border enzymes in kidneys, which upon cleavage is suggested to lead to excretion of the radiometal-chelator complex with the urine. We also compared the use of a cleavable linker with another strategy based on the use of a non-residualizing label via indirect radiohalogenation. $^{177}$Lu and $^{125}$I (a surrogate of $^{131}$I used for therapy) were used as radionuclides to evaluate and compare the two strategies. Their preferable characteristics, such as a physical half-life between 6 and 7 days, stable daughter products, well-developed chemistry for radiolabeling with high stability, and association of an additional low energy gamma emission for diagnostic purposes make them suitable for radionuclide therapy in clinics (38).

After successful preparation of all ADAPT6 constructs, the two labeling approaches provided stable labels. All radiolabeled ADAPT6 constructs demonstrated specific binding to the HER2-expressing cells *in vitro*.

Surprisingly, no significant difference in renal activity retention could be demonstrated between the constructs containing the cleavable linkers and the controls. Despite the same structure of the cleavable linker as in the construct [$^{111}$In]In-DOTA-GLGK-Cys-diabody used by Li and co-workers, there was no reduction in renal activity retention in our study (39). An explanation for this could be the differences in the type and size of the targeting agent, which could have negatively affected the cleavage of the linker. The different types of radionuclides used ($^{177}$Lu vs. $^{111}$In) is also a factor that can influence the results. Arano *et al* demonstrated that the efficiency of this approach is influenced in different ways by the size and composition of the linker for different radionuclides and chelators (40). Based on this, the application of in vitro system using brush border membrane enzymes could be helpful for the selection of a suitable peptide linker for future studies (34,41).

While the presence and position of the cleavable linker did not affect the kidney uptake, results from the biodistribution study demonstrated that the uptake of radioactivity in blood was significantly lower for the C-terminus constructs than for the N-terminus constructs. An explanation for this could be that the placement of DOTA-GLGK(maleimide)-COOH at the C-terminus increases the local hydrophilicity at this position, which would decrease the interactions with blood proteins and thereby result in a lower blood uptake. In that way, these constructs would have a local hydrophilicity at both termini: the HEHEHE-tag at the N-terminus and the cleavable peptide linker at the C-terminus. The N-terminus construct will only have a local hydrophilicity at one terminus due to the placement of the HEHEHE-tag and the cleavable peptide linker at the same position. Higher local hydrophobicity at the C-terminus of these constructs could also be a possible explanation for the slightly, but not significantly, higher uptake in the liver.

When comparing the cleavable linker strategy with the use of a non-residualizing label, the renal retention of [$^{125}$I]I-HPEM-ADAPT6 was significantly lower than for all the $^{177}$Lu-labeled constructs. This was an expected possibility as a non-residualizing iodine label is not trapped inside the cell after internalization to the same extent as a residualizing radiometal (17). Amongst the constructs studied herein, [$^{125}$I]I-HPEM-ADAPT6 was deemed optimal due to its lower uptake in the kidneys. The use of a non-residualizing label resulted in significantly lower renal activity retention of radiolabeled ADAPT6 compared with the use of a residualizing radiometal in combination with the cleavable GLGK-linker.

It was previously observed that an elevated lipophilicity was associated with elevated hepatic uptake for affibody molecules and DARPins (42-44). The addition of hydrophilic chelators such as DOTA increases the hydrophilicity of radiolabeled ADAPTs, while the addition of the HPEM group increases their lipophilicity. This might explain the observed phenomenon that the liver uptake of [$^{125}$I]I-HPEM-ADAPT6 (0.70±0.19% ID/g) was ca. two to

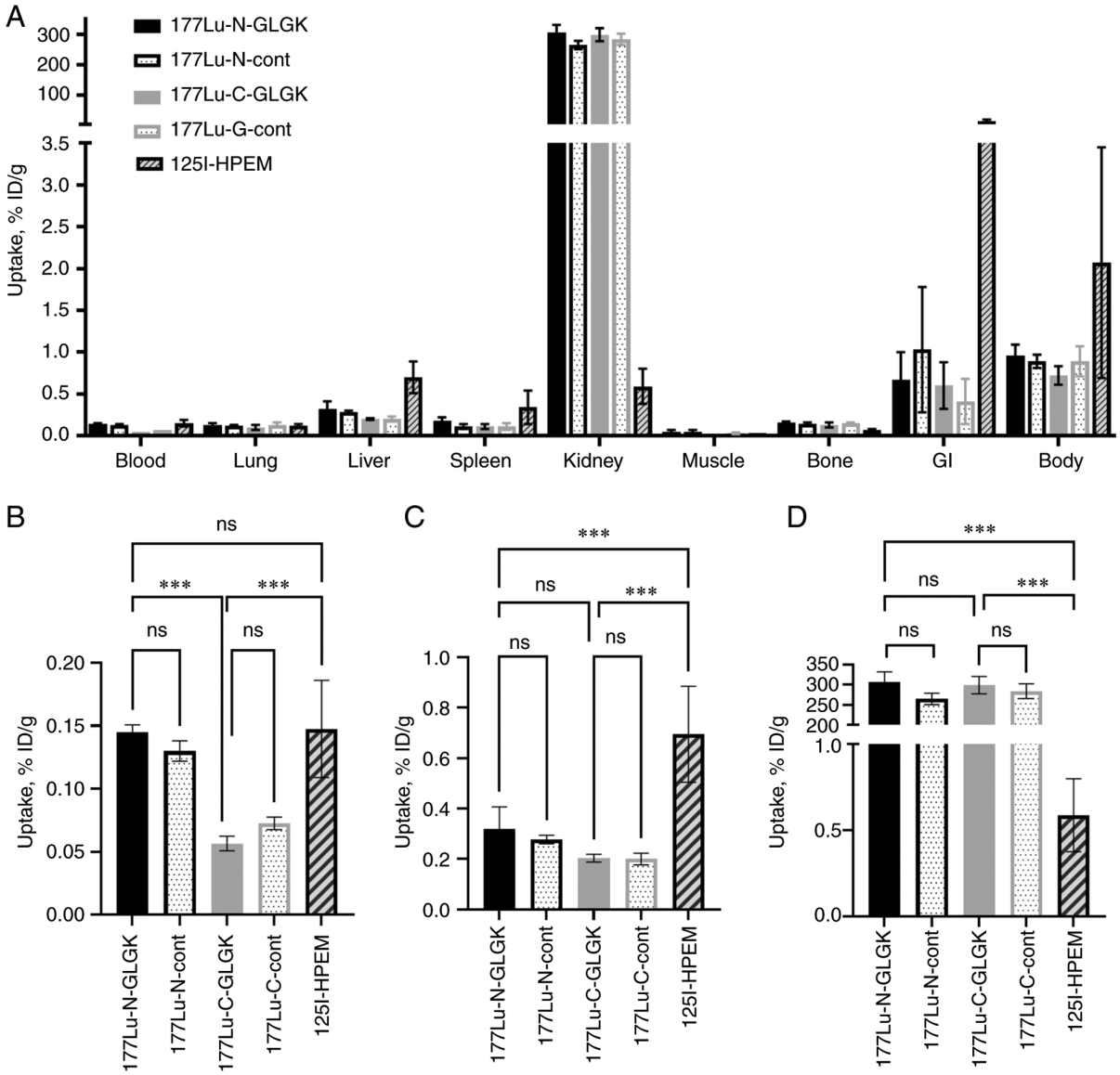


Figure 3. Biodistribution profile of ADAPT6 constructs labeled with $^{177}$Lu or [$^{125}$I]I-HPEM in NMRI mice at 4 h after injection: (A) Whole biodistribution; (B) blood uptake; (C) liver uptake; and (D) kidney uptake. Results are presented as an average % ID/g and SD of four animals. ***P<0.001. ns, not significant; ADAPT, albumin-binding domain-derived affinity protein; $^{177}$Lu, lutetium-177; GI, gastrointestinal; DOTA, 2,2',2",2"'-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid; GLGK, glycine-leucine-glycine-lysine; [$^{125}$I]I-HPEM, [$^{125}$I]I-[(4-hydroxyphenyl)ethyl]maleimide.

three times higher than of the $^{177}$Lu-labeled constructs (ca. 0.2-0.4% ID/g). It should be noted that the tumor uptake of ADAPT6 is typically much higher (ca. 10-20% ID/g; 17) and an elevated hepatic uptake would not be a limitation for radionuclide therapy

In conclusion, to prevent the kidneys from high radiation exposure in the course of targeted therapy, molecular design can be used to reduce renal activity retention. Earlier studies showed that the incorporation of a cleavable linker between the targeting agent and the radiometal chelator reduced renal retention of radiolabeled Fab fragments (32-34). In the present study, we investigated if the same strategy could be applied to reduce the renal retention of radiolabeled ADATPs. Surprisingly, incorporation of the cleavable GLGK-linker did not result in lower renal retention of $^{177}$Lu-labeled ADAPT6. This study emphasizes that when aiming to reduce the renal retention of ESPs labeled with radiometals, the molecular design of each construct is crucial. However, further investigations into suitable methods for the reduction of renal activity uptake of radiolabeled ADAPTs during radionuclide therapy are needed.

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## Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

JG designed, coordinated and supervised the study. FL, AV, YL, SL, TX, MO, SSR and JG performed the experiments. JG analyzed the data. FL, SL and JG performed production, purification and analysis of compounds. UR participated in the molecular design and supervised the production, purification and analysis. FL, AV and JG wrote the first version of the manuscript. All authors read and approved the manuscript. FL and JG confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

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## Competing interests

The authors declare that they have no competing interests.

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