# Advantage of Lutetium-177 *versus* Radioiodine Immunoconjugate in Targeted Radionuclide Therapy of B-cell Tumors

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**Abstract.** *Background: We herein report a comparison of the radiolabels $^{177}$Lu and $^{125}$I bound to the monoclonal antibody HH1 that targets the CD37 antigen expressed on non-Hodgkin B-cell lymphomas. Materials and Methods: Mixtures of $^{177}$Lu and $^{125}$I-labeled HH1 antibody were co-injected into nude mice carrying Ramos xenografts and the biodistribution using the paired label format allowing tracer comparisons in each individual mouse. Results: Products of the two radionuclides had very similar immunoractivity in vitro but showed different properties in vivo. Both products had relevant stability in blood and most normal tissues in nude mice carrying subcutaneous Ramos xenografts. However, both the tumor uptake and retention were significantly higher for $^{177}$Lu vs. $^{125}$I labeled HH1. The tumor to normal tissue ratios were several-fold improved for $^{177}$Lu compared to radioiodine labeled antibodies. Conclusion: The data presented herein support the evaluation of CD37 as a target for clinical $^{177}$Lu-based radioimmunotherapy against b-cell malignancies.*

In therapeutic nuclear medicine there is a substantial effort currently underway to develop radiolabeled peptides and monoclonal antibodies (1). In recent years there has been a shift from radioiodine-based conjugates to radioimmunoconjugates (RICs) based on metal radionuclides (2). The latter are conjugated to proteins *via* chelators. These are considered as residualizing radiolabels compared to conventional non-residualizing radioiodine label, which is obtained with the reaction of oxidized iodine towards tyrosine groups on proteins.

Radioimmunotherapy (RIT), *i.e.* therapy with radiolabeled monoclonal antibodies, has obtained approval in B-cell Non-Hodgkin lymphoma (NHL) (3). Iodine-131 and $^{90}$Y-labeled antibodies targeting the CD20 antigen have so far been the only licensed products in NHL (4, 5). External-beam radiotherapy is highly effective in localized disease from NHL (6). Thus, NHL is *per se* a radio-responsive disease (7) and RIT should be a relevant strategy from that perspective. Another aspect supporting the usefulness of RIT is the relatively rapid and efficient antibody targeting achieved in NHL (8).

The CD20 antigen is the target for an effective therapy with the non-radioactive and humanized monoclonal antibody, rituximab (9). A combination of immunotherapy and RIT against the same antigen may not be optimal, since both clinical and experimental studies in mice have shown that in some circumstances even quite low rituximab concentrations in the blood can reduce tumor cell targeting and thus impair the clinical efficacy of CD20-directed RIT (10). Furthermore, the majority of patients selected for CD20-based RIT have received several cycles of "cold" rituximab, which may result in selection of tumor cells with low CD20 expression and thus could lower the effect of subsequent anti-CD20 treatments (11-13). An alternative would be to target a different antigen than CD20 in RIT against NHL. Several studies have been performed against other NHL-associated antigens including CD19, CD22, and CD37 (8, 14-17).

Twenty-five years ago $^{131}$I-labeled CD20- and CD37-targeting RICs were compared (18, 19) and both produced clinical responses. By combinations with chemotherapy and autologous bone marrow transplantation, complete responses of long duration were reported (19). High-dose therapy using $^{131}$I-MB-1 against CD37 was later abandoned. The emphasis was focused on CD20 targeting since it was possible to give



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higher dosages of $^{131}$I-anti-CD20 RICs than for $^{131}$I-anti-CD37 RICs and since higher dosages of antibody was necessary for anti-CD37 antibodies than for anti-CD20 antibodies to get a good bio-distribution (18, 19). Later work has indicated that the use of $^{131}$I-labeled antibodies in RIT against internalizing antigens like CD22 would be a suboptimal strategy since $^{131}$I labeled onto tyrosine would constitute a non-residualizing label which upon internalization could release free $^{131}$I or other proteolytic products diffusing out of the cell membrane (20).

Lutetium-177 labeled HH1 is a CD37-targeting RIC which in preclinical testing has shown promising antitumor activity in NHL models (14). Using the residualizing $^{177}$Lu labeled *via* p-SCN-Bn-DOTA to the HH1 (also named tetulomab), good tumor targeting in xenografts was demonstrated (21).

In this study we report the comparison of $^{125}$I- and $^{177}$Lu-labeled HH1 anti-CD37, in targeting of Ramos human lymphoma xenografts in nude mice. Our aim was to explore whether possible therapeutic gain could be achieved by switching from a non-residualizing, covalently bound, halogen radionuclide to a complex-conjugated metallic radionuclide in RIT against CD37-expressing malignancies.

## Materials and Methods

*Preparation of DOTA-labeled antibody*. The procedures were performed according to previously published methods (21) but with a few modifications. Using centrifuge microconsentrators (Vivaspin 20, Sartorius Stedim Biotech, Göttingen Germany) HH1 was subjected to buffer exchanges and concentration adjustment to a final solution of 10-20 mg/ml in disodium carbonate/sodium bicarbonate (0.25 M) with a pH of approximately 9.0. Using a chelator to antibody ratio of 5-7, 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (p-SCN-Bn-DOTA) (Macrocyclics, TX, USA) dissolved at 10 mg/ml in 5 mM HCl was added to the antibody solutions and incubated with gentle shaking at room temperature for 2 hours before the reaction was terminated by adding 0.3 M glycine in carbonate buffer followed by 15 min shaking. Using centrifuge micro-concentrator, the buffer was exchanged to 0.9% sodium chloride and the concentration adjusted to 20-35 mg/ml as measured by spectrophotometry at 280 nm.

*Radiolabeling of antibody*. Lutetium-177 in 10 mM HCl (ITG, Garching, Germany) was added to approximately 0.5 mg of DOTA-labeled HH1 in ammonium acetate buffer pH 5.0-5.5 and gently shaken at 37˚C for 25 min. Thereafter the product was purified by elution through a Sephadex G-25 PD-10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using Dulbecco's PBS as eluant. The antibody fraction was sterile filtered and used for further work.

*Radioiodination*. Iodine-125 as sodium iodide (Hartmann Analytic, Braunschweig, Germany) was added to an iodogen tube (Pierce, Rockford, IL, USA) with 100 μl 0.025 M TRIS/0.4 M NaCl, pH 7.5, and incubated for 7 min before transfer to a reaction vial with approximately 0.2 mg of HH1 (Nordic Nanovector AS, Oslo, Norway) and incubated for 7 more min then added 100 μl of 0.1 M L-tyrosine in TRIS and after 5 min incubation eluted on a Sephadex G-25 PD-10 column.

*Quality control*. $^{177}$Lu-labeled antibody was evaluated on thin layer chromatography using chromatography strips for $^{111}$In and $^{90}$Y supplied by Biodex.com. This chromatography system had been verified for $^{177}$Lu previously. As eluant 0.9% NaCl was used. The samples were mixed in a 1:9 ratio with a buffer containing 7% human serum albumin (HSA) and 10 mM diethylene triamine pentaacetic acid (DTPA). Free $^{177}$Lu would be complexed by the DTPA and follow the solvent front while the radiolabeled antibody would be retained at the base line.

*Measurements of immunoreactive fraction*. A one point assay was used. Approximately 8 ng of radiolabeled antibody was added to 0.2 ml cell suspension (50 million Ramos cells per ml) and incubated for 1 hour, counted on an LKB Multigamma Counter to determine applied activity and washed 3 times with 0.5 ml DPBS/0.5% BSA. Finally, cell pellets were counted on the LKB Multigamma counter to determine cell bound activity. As a control, cells pretreated with 50-100 μg of antibody to block the antigens were used in the procedure. Two parallels were used for unblocked and pre-blocked cells respectively.

*In vitro cell binding assay*. Ramos cells were cultured as single cell suspensions in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine and 1% pen-strep (all from Gibco, Life Technologies) in a humidified atmosphere of 95% air and 5% $CO_2$. Cells were counted using a Countess cell counter (Invitrogen, Life Technologies, Carlsbad, CA, USA). One ml of cells suspension of 10 millions cells per ml in growth medium were transferred to 5 ml tubes and added a mixture of $^{125}$I-labeled and $^{177}$Lu-labeled monoclonal antibodies. After 1 hour of incubation the medium were replaced to remove unconjugated antibody and the tubes placed in a cell incubator at 37˚C and 5% $CO_2$.

After 1 day and 5 days 100 or 200 μl samples were withdrawn and counted on a Cobra II Auto Gamma counter (Packard Instruments, Downers Grove IL, USA) using a dual window setting for $^{125}$I and $^{177}$Lu. Due to spillover for $^{177}$Lu into the $^{125}$I-window the output from the counter had to be corrected accordingly. Control samples with either pure $^{177}$Lu or $^{125}$I were run through the counter to determine spillover in each series of countings. The samples were counted before and after 3 times centrifuging-washing of cells to determine cell bound activity. By comparing the cell bound fraction for $^{125}$I and $^{177}$Lu the retention properties of the two radiolabeled products were studied.

*Biodistribution*. Institutionally bred female athymic nude mice, Foxn1$^{nu}$, were used in the experiments. The animals were maintained under pathogen-free conditions, and food and water were supplied *ad libitum*. All procedures and experiments involving animals in this study were approved by the National Animal Research Authority and carried out according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes. Nude mice were injected subcutaneously with a solution of $10^6$ cells/ml of Ramos lymphoma cells in both flanks. Initial tumor diameters at the beginning of the experiments ranged from 7 to 12 mm. The Radioimmunoconjugate was sterile filtered and diluted in DPBS to final concentrations of 2 to 10 MBq/ml. Samples of the injectates were used as references in the measurement procedures. For the paired label biodistributions $^{177}$Lu-HH1 and $^{125}$I-HH1 of concentrations 3 MBq/ml were mixed at 1:1. Standard solutions from each individual RIC were kept to assess possible

spillovers during sample counting. Specific activities of the RICs ranged between 60-200 MBq/mg and 110-350 MBq/mg for $^{125}$I labeled and $^{177}$Lu-labeled products respectively. Two to five animals were used per time point. Autopsies were performed after cervical dislocation at various time points after injection. The weight of each tissue sample was determined, and the amount of $^{177}$Lu and $^{125}$I was measured by a calibrated LKB Multigamma Counter. The decay corrected percentages of the injected dose per gram tissue (%ID/g) and the non-decay corrected activity per gram tissue (specific activity) were calculated for each time point. The paired label biodistribution samples were counted using a protocol with two energy windows, one for each radionuclide. Standards from each separate RIC were counted at each time point in order to evaluate possible spillover effects from the two energy windows. There was no significant spillover from $^{125}$I into the $^{177}$Lu window but there was an average of 2.8% spillover from $^{177}$Lu into the $^{125}$I window. Thus, each sample was counted with the dual energy window protocol and data was corrected for spillover by subtracting 2.8% of the $^{177}$Lu counts from the $^{125}$I counts to determine net amount of $^{125}$I counts.

*Calculation of absorbed radiation dose*. The absorbed radiation doses of $^{177}$Lu-HH1 and $^{131}$I-HH1 to blood and tumor were calculated assuming dose contributions from the main β-particle emissions of each RIC (mean energy: $E_\beta$=0.1342 MeV for $^{177}$Lu and $E_\beta$=0.1920 MeV for $^{131}$I) for simplicity. It was assumed that the radionuclides were distributed homogenously and that there was 100% absorption of the β-particles in the tissues. The half-lives ($T_{1/2}$) of $^{177}$Lu and $^{131}$I used in the calculations were 6.7 and 8.02 days respectively. The biodistribution data was normalized to an injection of 1 MBq/kg body weight. The activity at t=0 was estimated to be zero in tumor and 100 % of the total injected activity in blood. The biodistribution of $^{125}$I-HH1 was used to determine the absorbed doses of the $^{131}$I-HH1 treatment. The activities of $^{125}$I-HH1 in each tissue for each time point ($t$) were divided by $e^{-Ln(2)/T1/2(125I)t}$ where $T_{1/2(125I)}$ is the half-life of $^{125}$I considered to be 59.4 days and then multiplied by $e^{-Ln(2)/T1/2(131I)t}$ where $T_{1/2(131I)}$ is the half-life of $^{131}$I (8.02 days). The total number of disintegrations from the time of injection of the RICs until no activity was left in the body was calculated by estimating the area under the curve (AUC) as described by Yuan (22). The extrapolation of the data from the last time point (14 days) to infinite time after injection was performed by assuming there was no more biological decay but only radioactive decay taking place after 14 days. The extrapolated contribution to the area under the curve represented 2 to 25% of the total area under the curve. The absorbed dose was then calculated as the multiplication of AUC by $E_\beta$.

## Results

*Preparation of DOTA-conjugates*. The DOTA-labeling gave a product with typically 1-2 DOTA per antibody molecule. This was determined by using an assay including the reaction of a mixture of stable and radioactive lutetium chloride with the DOTA-labeled protein and measuring % protein binding using thin layer chromatography (data not shown). The radiolabeling of the DOTA-antibody conjugate gave a yield of 80% or higher and the purity after elution through the Sephadex G-25 PD-10 gel filtration column resulted in a product with radiochemical purity of at least 97% as determined by thin-layer chromatography.

Table I. *Relative retention of $^{177}$Lu- vs. $^{125}$I- labeled HH1 on Ramos cells in vitro measured in a paired label binding assay.*

| Relative retention[1] | |
|---|---|
| 1 day | 5 days |
| 0.90±0.03 | 0.92±0.07 |

[1]Relative retention was calculated by dividing the percentage of cell bound activity of $^{125}$I-HH1 by the percentage cell bound activity of the $^{177}$Lu-HH1. Values are mean±SD.

*$^{125}$I-labeling*. Iodination of the antibody was performed using the iodogen tube method (www.fishersci.com) and gave a radiochemical yield of typically 55%-80% as measured by the Sephadex G-25 PD10 eluate fractions.

*Immunoreactive fraction*. Using approximately $50\times10^6$ cells per ml in 0.2 ml and approximately 8 ng total RIC's, the net immunoreactive fractions (unblocked minus blocked samples) in the one point assay were 85.6% (range 85.2% to 86.0%) for $^{177}$Lu-HH1 and 83.7% (range 81.9% to 85.4%) for $^{125}$I-HH1.

*In vitro cell retention*. Table I presents the relative retention data for Ramos cells of HH1 labeled with $^{125}$I and $^{177}$Lu. The retention was similar for $^{125}$I and $^{177}$Lu versions of the antibody. The data indicate that both RICs work well in terms of antigen binding and retention *in vitro*.

*Paired label biodistribution experiment*. The data show that iodine-labeled HH1 had a good stability in circulation with blood levels similar to that of lutetium-labeled HH1 (Figure 1). In the Ramos tumors, however, there were significantly higher levels of Lu-labeled antibody (Figure 2). Also in liver, and to some degree in the spleen, the accumulation of lutetium was higher than that of iodine (Figure 1). This may be due to the retention of degradation products from RIC metabolism. A significant uptake of iodine in the thyroid was observed at all time points (Table II). This uptake indicates release of free iodine from the product probably occurring in the tumor, spleen and liver. Overall, the tumor-to-blood and tumor-to-tissue ratios were favorable for lutetium labeled HH1 and unfavorable for iodine labeled HH1 (Figure 3). The data indicate that lutetium-labeled HH1 shows promising properties in targeted therapy against CD37 antigen expressing malignancies. The absorbed radiation dose deposition in tumor and blood (Table III) suggests significant therapeutic potential for $^{177}$Lu-HH1.

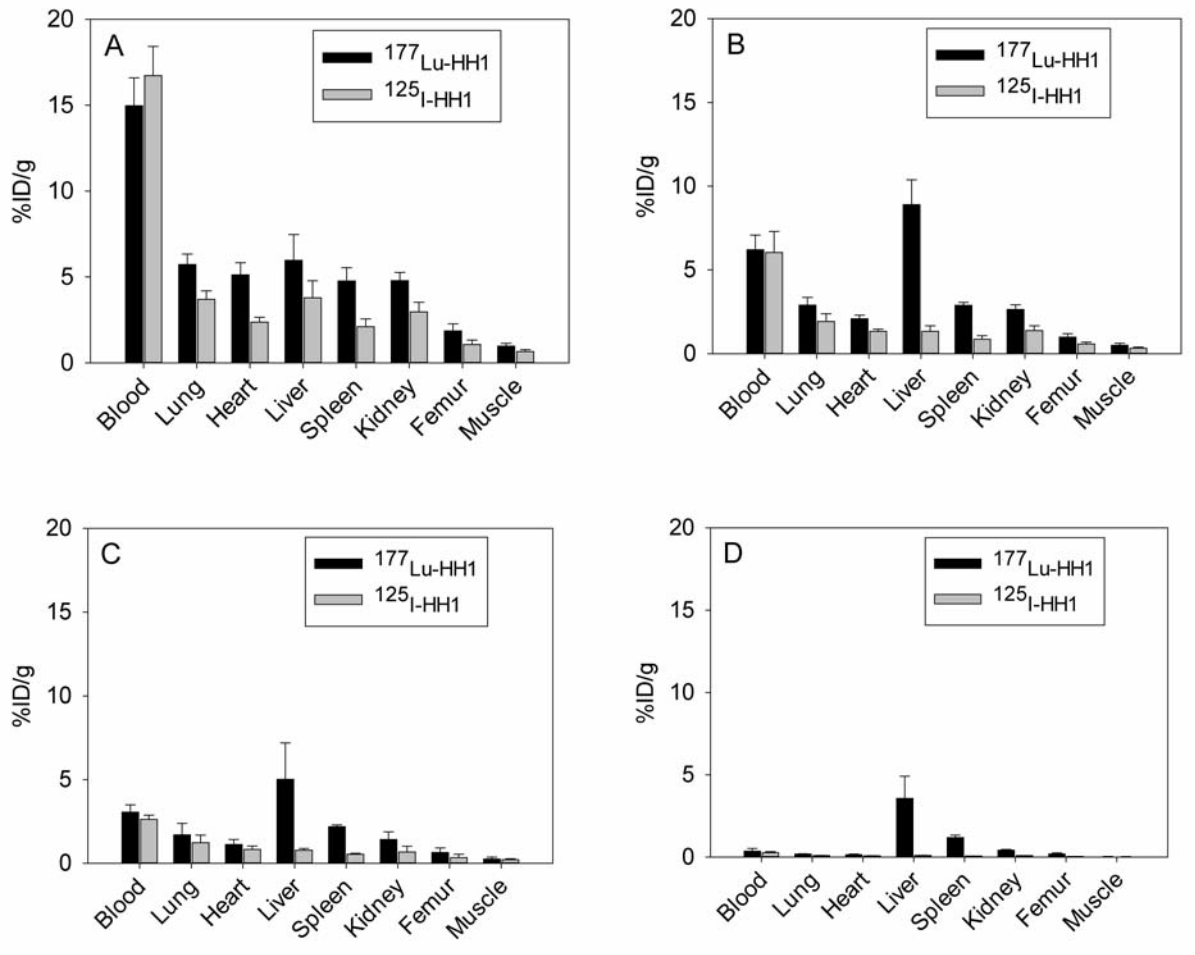


Figure 1. *Biodistribution of $^{177}$Lu-HH1 and $^{125}$I-HH1 in normal tissues of nude mice with Ramos human lymphoma xenografts. Products were injected in a paired-label fashion, i.e., the two RIC's were mixed and co-injected for direct comparison in each animal. Mean±SE. A) 1 day B) 3 days C) 7 days D) 14 days after treatment injection.*

## Discussion

Based on the radio-responsiveness of NHL and the disease being targetable with monoclonal antibodies, two RICs; Zevalin and Bexxar, both targeting the CD20 antigen, were developed and approved for treatment of NHL. Although strong anti-tumor activity has been demonstrated, foremost in indolent lymphoma, these two products have so far reached a modest sale, probably partly because of competing anti-CD20 rituximab and issues related to preparation and handling of the products. Lately, more evidence for the efficacy of RIT for B-cell lymphoma has appeared (23). In order to further improve on RIT in NHL other antigens have been proposed as targets. The current study shows that products whereby *in vitro* data indicate relevant stability may behave quite differently *in vivo*. When $^{125}$I-HH1 and $^{177}$Lu-HH1 were incubated in cell suspensions of Ramos cells in culture medium, the two products had similar retention properties in terms of fraction

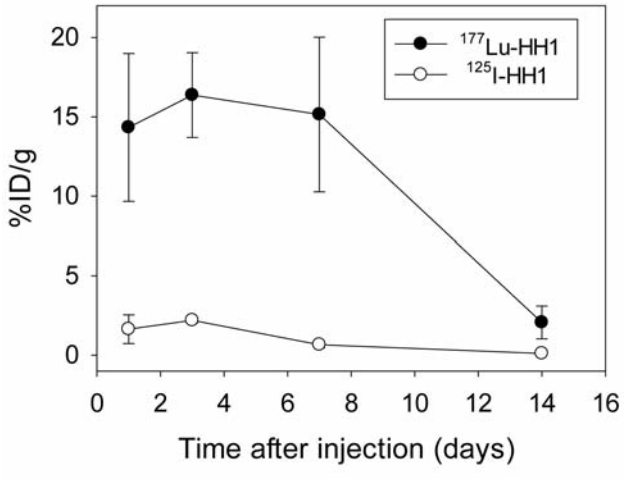


Figure 2. *Uptake in tumors of $^{177}$Lu-HH1 and $^{125}$I-HH1 injected in a paired-label format in nude mice with Ramos xenografts. Mean±SE.*

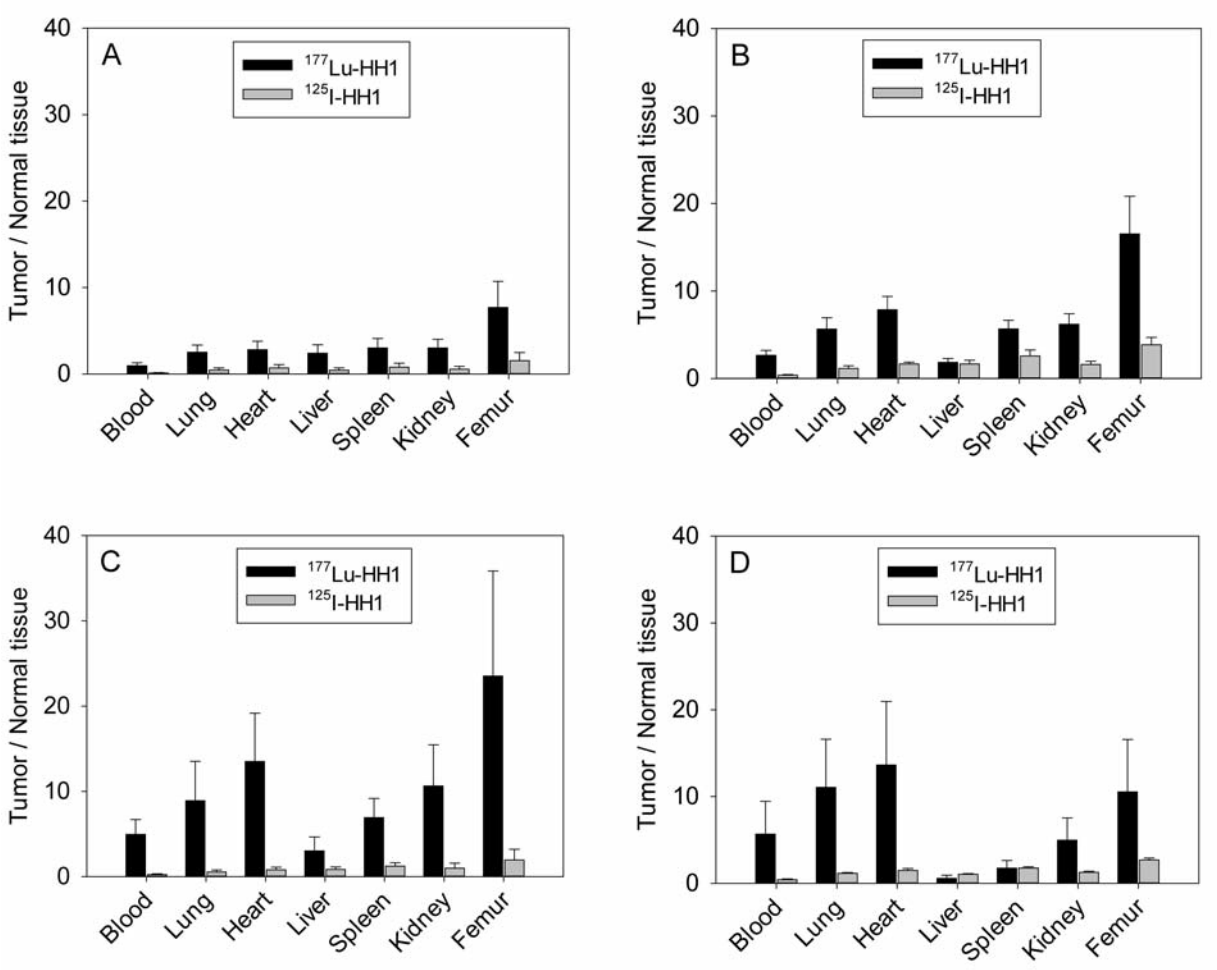


Figure 3. *Tumor to normal tissue ratio for nude mice with Ramos xenografts injected in paired label fashion with $^{177}$Lu-HH1 and $^{125}$I-HH1. Mean±SE. A) 1 day B) 3 days C) 7 days D) 14 days after treatment injection.*

Table II. *Retention of radionuclides in the neck (including thyroid) after co-injection of $^{177}$Lu- and $^{125}$I-HH1 in animals with Ramos xenografts.*

| Time points (days) | $^{177}$Lu (% I.D*) | $^{125}$I(% I.D) |
|---|---|---|
| 1 | 0.3±0.1 | 0.9±0.2 |
| 3 | 0.1±0.02 | 3.2±1.2 |
| 7 | 0.1±0.05 | 3.4±1.8 |
| 14 | 0.01±0.003 | 2.7±0.4 |

*Percent of injected dose per gram, mean±SD.

Table III. *Radiation doses of $^{177}$Lu-HH1 and $^{131}$I-HH1 to blood and tumor.*

| | Dose to Tissues (Gy) | |
|---|---|---|
| | $^{177}$Lu-HH1 | $^{131}$I-HH1 |
| Blood | 2.4±0.2 | 2.0±0.1 |
| Tumor | 7.0±1.6 | 0.29±0.04 |

Values are mean±SD.

of radionuclide bound to the cells. In contrast, it was shown that, *in vivo*, there was a significant tumor targeting advantage of using $^{177}$Lu *vs*. radioiodine-labeled anti-CD37 RIC. Older clinical data related to RIT with anti-CD37 RIC labeled with $^{131}$I may therefore have underestimated the clinical potential of this antigen as a target for RIT. Assuming $^{125}$I behaves as $^{131}$I, the switching from $^{131}$I to $^{177}$Lu could improve radionuclide retention in tumor with as much as a factor of 20. Another advantage is the low thyroid uptake with $^{177}$Lu since thyroid is the main target organ for released radioiodine.

There may be large individual variations in tumor processing of iodine-labeled antibodies in the clinic and a xenograft model has significant limitations in predicting the clinical tumor properties. At least one study, however, has reported lack of tumor targeting with $^{131}$I-labeled antibody in NHL despite confirmed antigen expression (24). Therefore, this may have important implications to the clinical use of RIT against B-cell malignancies. We predict that $^{177}$Lu-labeled anti-CD37 antibody may deliver a more tumor-specific radiation compared to a historical $^{131}$I-labeled anti-CD37 version due to chemical properties more suitable for cell internalization and more favorable radiation properties with a higher component of beta emission *vs*. gamma. Ongoing and future clinical studies will hopefully answer whether $^{177}$Lu-HH1 could be an effective therapeutic tool against B-cell malignancies, exploiting the B-cells inherent radiation sensitivity and their broad expression of the CD37 antigen.

In summary, we have shown that the residualizing radiometal $^{177}$Lu had considerably higher tumor retention than the non-residualizing radiohalogen $^{125}$I as label for the therapeutic monoclonal antibody HH1 in a CD37 expressing xenograft/nude mouse model. This difference could be important and supports further evaluation of CD37 as a target in clinical $^{177}$Lu-based radioimmunotherapy against non-Hodgkin lymphoma.

## Disclosure

Ada Repetto-Llamazares, Øyvind S. Bruland, Jostein Dahle and Roy H. Larsen are stock owners in Nordic Nanovector AS.

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