Effect of Modulating FcRn Binding on Direct and Pretargeted Tumor Uptake of Full-Length Antibodies

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Abstract

Full-length antibodies lack ideal pharmacokinetic properties for rapid targeted imaging, prompting the pursuit of smaller peptides and fragments. Nevertheless, studying the disposition properties of antibody-based imaging agents can provide critical insight into the pharmacology of their therapeutic counterparts, particularly for those coupled with potent payloads. Here, we evaluate modulation of binding to the neonatal Fc receptor (FcRn) as a protein engineering-based pharmacological strategy to minimize the overall blood pool background with directly labeled antibodies and undesirable systemic click reaction of radiolabeled tetrazine with circulating pretargeted trans-cyclooctene (TCO)-modified antibodies. Noninvasive SPECT imaging of mice bearing HER2-expressing xenografts was performed both directly (\(^{111}\)In-labeled antibody) and indirectly (pretargeted TCO-modified antibody followed by \(^{111}\)In-labeled tetrazine). Pharmacokinetic modulation of antibodies was achieved by two distinct methods: Fc engineering to reduce binding affinity to FcRn, and delayed administration of an antibody that competes with binding to FcRn. Tumor imaging with directly labeled antibodies was feasible in the absence of FcRn binding, rapidly attaining high tumor-to-blood ratios, but accompanied by moderate liver and spleen uptake. Pretargeted imaging of tumors with non-FcRn-binding antibody was also feasible, but systemic click reaction still occurred, albeit at lower levels than with parental antibody. Our findings demonstrate that FcRn binding impairment of full-length IgG antibodies moderately lowers tumor accumulation of radioactivity, and shifts background activity from blood pool to liver and spleen. Furthermore, reduction of FcRn binding did not eliminate systemic click reaction, but yielded greater improvements in tumor-to-blood ratio when imaging with directly labeled antibodies than with pretargeting.
INTRODUCTION

The prolonged serum persistence of target-specific antibodies has provided numerous therapeutic agents with favorable pharmacological properties, so that weekly to near monthly dosing is common (1,2). However, the slow systemic clearance of antibodies is a double-edged sword since wait times of days are needed until target-to-blood ratios become optimal (3,4). For diagnostic imaging, this limitation has been addressed by pursuing smaller, faster clearing antibody fragments such as Fabs (5-7), minibodies (8), or diabodies (9), all lacking the Fc region of the antibody responsible for neonatal Fc receptor (FcRn)-mediated recycling. These same-day imaging agents have overcome the slow pharmacokinetics of immunoPET/SPECT imaging agents comprised of full-length antibodies, thus benefiting patient convenience, compliance, diagnosis time, and radiation dosimetry.

While this “smaller/faster” strategy decreases serum persistence, it also shifts radioactivity away from target receptors and towards clearance organs, particularly kidneys, which can result in dose-limiting radiotoxicity (10). Using non-residualizing radiolabels (e.g. $^{124}$I, $^{18}$F) and/or metabolically labile linkers can overcome high renal dosimetry (11), but at the expense of losing the residualization advantage within tumor/target tissues for internalizing receptors. Furthermore, these liabilities raise similar concerns in the pharmacological context of the corresponding biotherapeutic agents, particularly those coupled with potent payloads, so that both systemic exposure and normal tissue uptake/catabolism are intimately linked to therapeutic index.

Recently, pretargeted imaging via in vivo click chemistry has been investigated as an alternative approach to address the undesirably slow pharmacokinetics of antibody-based imaging agents (12,13). The implications of this technology on therapeutic drug delivery have
also been explored (14), but several limitations remain. Tumor-to-background ratios in pretargeted imaging are typically better for slow/non-internalizing targets than for internalizing targets. For instance, we previously demonstrated that same-day imaging of pretargeted anti-HER2 (internalizing) antibodies was inferior to imaging with directly labeled antibodies, as substantial levels of systemic click reaction limited delineation of tumors from blood pool background (15). Furthermore, antibody pretargeting often benefits from clearing/blocking agents to reduce systemic reaction/labeling (16), but this adds another step that could be disadvantageous from an operational viewpoint.

Here, we further pursue a protein engineering strategy to modulate antibody pharmacokinetics by lowering FcRn binding affinity to achieve rapid target delineation. Previously, $^{124}$I-labeled scFv-Fc variants (~105 kDa) were employed in slow-internalizing receptor models in an imaging context (17-19). We have instead chosen to evaluate a full-length IgG (~150 kDa) with a residualizing label ($^{111}$In) in an internalizing target (HER2) expressing tumor model using immunoSPECT as a readout but with intended therapeutic implications.

To directly assess the impact of FcRn-mediated IgG recycling and the resulting impact on serum persistence and tumor-to-background ratio, we evaluated the presence and absence of FcRn binding activity using two methods: Fc engineering of intact IgG to ablate FcRn binding (20) and administration of a competitive FcRn binding antibody, effectively acting as a clearing agent, with engineered Fc mutations (YTE-KF) to enhance FcRn binding as described in a previous report of the ‘ABDEG™’ antibody (21,22). We investigated these two approaches in the context of both direct and pretargeted imaging, taking advantage of the click reaction between trans-cyclooctane (TCO)-modified antibodies and radiolabeled tetrazines.
MATERIALS AND METHODS

All animal studies were conducted in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the Genentech Institutional Animal Care and Use Committee (IACUC). Details regarding all reagents and general procedures may be found in the Supplementary data (available at http://mct.aacrjournals.org/).

Antibody engineering and conjugation

Anti-HER2 THIOMAB™ antibodies (clone 7C2, which binds a different epitope than trastuzumab or pertuzumab; see Supplementary Table 1 for 7C2 sequence as previously reported in (23)) were engineered to introduce cysteine residues at three positions (24) for conjugation to the click-reactive TCO-PEG₃-maleimide (PEG = polyethylene glycol) linker (25,26), the metal chelator DOTA-maleimide (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7-triy1)triacetic acid) for imaging via ¹¹¹In-DOTA₆-mAbs, and the capping reagent N-ethyl maleimide (NEM) as previously described (15) to give final conjugates with ~6 TCO, DOTA, or NEM groups per antibody (Supplementary Table 2). These cysteine mutations were engineered alone or in combination with additional mutations – H310A/H435Q – to ablate binding to FcRn (20). Selected groups of mice received an anti-gD YTE-KF hIgG1 antibody, a competitive binder to FcRn, with the same mutations as the ‘Abdeg’ antibody previously reported (21,22) and featuring single-digit nanomolar affinity to mouse FcRn at both pH 6.0 and 7.2 (27).

Radiochemistry and chromatography

7C2-NEM₆, 7C2-HAHQ-NEM₆, 7C2-TCO₆, and 7C2-HAHQ-TCO₆ antibodies were radioiodinated (¹²⁵I) for invasive biodistribution assessment through tyrosine residues, (Supplement S1) (15,28). Radiosynthesis of ¹¹¹In-DOTA₆-labeled 7C2 and 7C2-HAHQ for
direct imaging was achieved via engineered thiols (Supplement S2) (15); additionally, $^{111}$In-DOTA-Tz was synthesized (Supplement S3) for pretargeted imaging (15,29).

**SPECT-CT imaging**

The KPL-4 tumor-bearing C.B-17 SCID.bg mouse model (mean tumor volume at least 250 mm$^3$) was utilized as previously described (15). An overview of the SPECT-CT imaging study design is presented in Fig. 1. All dosing was single intravenous (tail vein) bolus in sterile saline vehicle (100 µL), with all antibody tracers adjusted with unlabeled protein to 5 mg/kg total dose. Directly labeled groups received 185 kBq of $^{125}$I-7C2-NEM$_6$ plus 11.1 MBq $^{111}$In-DOTA$_6$-7C2 (group 1) or 185 kBq of $^{125}$I-7C2-HAHQ-NEM$_6$ plus 11.1 MBq $^{111}$In-DOTA$_6$-7C2-HAHQ (group 2), each followed 24 h later by vehicle. In vivo click reactions were performed by administering 185 kBq of $^{125}$I-7C2-TCO$_6$ (group 4) or $^{125}$I-7C2-HAHQ-TCO$_6$ (group 5) followed 24 h later by 11.1 MBq $^{111}$In-DOTA-Tz. Selected groups received $^{111}$In-DOTA$_6$-7C2 (group 3) or $^{125}$I-7C2-TCO$_6$ (group 6) followed 24 h later by IgG-YTE-KF (50 mg/kg), thereafter by vehicle or $^{111}$In-DOTA-Tz at 48 h post initial injection.

SPECT images were acquired in two 20% windows centered at the 173- and 247-keV photopeaks of $^{111}$In using an ultra-high sensitivity mouse 2.0-mm-pinhole collimator (MILabs, Utrecht, Netherlands). For each animal, two frames (15 min each) were acquired. Image reconstruction and co-registration (MILabs software), SPECT quantitation (VivaQuant software), and visualization (Amira software) were performed. Regions of interest were drawn around heart (surrogate for blood exposure) and tumor, based on CT anatomical images.

**Pharmacokinetics and biodistribution**

Blood samples from all mice in the SPECT imaging study were collected at 0.25, 24.25, and 48 h (0.25, 48, and 72 h for the IgG-YTE-KF groups) post initial antibody injection via
retroorbital bleed, with terminal tissue harvest performed at 2 days (3 days for the IgG-YTE-KF
groups) post initial antibody injection. Tissue samples collected from the terminal harvests
included tumor, kidney, liver, spleen, muscle, fat pad, small intestine, skin, heart, lungs, and
brain. Tissues collection, analysis, and calculations were performed as previously described (15).
Sparse blood pharmacokinetic data were fit to a standard linear two-compartment
pharmacokinetic model (Supplement S4) (30).

RESULTS

Antibody engineering and conjugation

DOTA-maleimide, TCO-PEG3-maleimide, and NEM were conjugated through the
engineered thiols of 7C2 (LC: K149C, HC: L177C, HC: Y376C), with >95% efficiency by mass
spectrometry. Conjugation ratios are reported in Supplementary Table 2.

Radiochemistry and chromatography

Radioiodination of the antibodies with 37 MBq input gave ~50% yields for 125I-7C2-
TCO₆ (~340.4 kBq/µg, 99% pure) and 125I-7C2-HAHQ-TCO₆ (~314.5 kBq/µg, 99% pure), 64%
for 125I-7C2-NEM₆ (~510.6 kBq/µg, 98% pure), and 74% for 125I-7C2-HAHQ-NEM₆ (~418.1
kBq/µg, 97% pure). Radiometal labeling of 7C2-DOTA₆ and 7C2-HAHQ-DOTA₆ antibodies
gave 77% yield for 111In-DOTA₆-7C2 (~210.9 kBq/µg, 99% pure) and 38% for 111In-DOTA₆-
7C2-HAHQ (~173.9 kBq/µg, 96% pure); that of DOTA-Tz provided ~71% yield of 111In-
DOTA-Tz (8.11 MBq/µg based on amount of tetrazine prior to Sep-Pak, 95% pure)
(Supplementary Fig. 1).

SPECT-CT imaging
All data in this manuscript were collected in a single comprehensive imaging study (Fig. 2), with blood concentrations (Fig. 3) derived by sparse sampling, and tissue harvest (Fig. 4-6) performed after the last image acquisition.

In the direct imaging study arm, good tumor delineation was observed at 28 h for both parental (7C2) and FcRn non-binding (7C2-HAHQ) tracers (Fig. 2 and Supplementary Table 3, groups 1-2). The background signal in animals injected with $^{111}$In-7C2 was primarily in heart/blood pool, that with $^{111}$In-7C2-HAHQ in liver and spleen. Tumor-to-blood ratios were ~2-fold higher for the HAHQ variant relative to the parental molecule, and two-fold higher at the 48 h (than 24 h) post antibody injection.

In the pretargeted study arm, the majority of signal was observed in circulation at 4 h post $^{111}$In-DOTA-Tz administration, consistent with tumor-to-blood ratios <1, although with distinguishable tumor delineation (Fig. 2 and Supplementary Table 3, groups 4-5). However, a next-day SPECT scan revealed ~2-fold increase in tumor-to-blood ratio for both variants relative to same-day imaging.

Mice receiving directly labeled $^{111}$In-7C2 followed by anti-gD-YTE-KF demonstrated good tumor delineation at 52 h, with ~2-fold increase in tumor-to-blood ratio as compared to $^{111}$In-DOTA-7C2-HAHQ (Fig. 2 and Supplementary Table 3, group 3). However, significant blood pool signal was observed for the pretargeted group with anti-gD-YTE-KF, resulting in a similar tumor-to-blood ratio at 52 h to that of 7C2-HAHQ-TCO$_6$ + $^{111}$In-DOTA-Tz group (Fig. 2 and Supplementary Table 3, group 6). The 72-h SPECT images for direct labeling followed by anti-gD-YTE-KF showed a slight improvement of already well-resolved image (Fig. 2 and Supplementary Table 3, group 3). The pretargeted group followed by anti-gD-YTE-KF also demonstrated better tumor delineation (Fig. 2 and Supplementary Table 3, group 6).
Pharmacokinetics and biodistribution

In the direct labeling study arm, the FcRn-binding impaired variant $^{111}$In-DOTA$_6$-7C2-HAHQ exhibited faster systemic clearance than parental $^{111}$In-DOTA$_6$-7C2 with the blood concentration being 2-fold lower for the HAHQ variant at 24 h post antibody injection (8.9 ± 1.4 and 19.5 ± 3.4 %ID/mL, respectively) (black vs blue, Fig. 3). By 48 h post-antibody injection, the difference in pharmacokinetics between the HAHQ variant and parental antibody was even more dramatic with >4-fold difference in whole blood of 3.6 ± 0.6 and 15.2 ± 3.0 %ID/mL, respectively. See Supplementary Table 4 for fitted pharmacokinetic parameter values.

A similar discrepancy between HAHQ variants and parental molecules was observed for the co-administered $^{125}$I-labeled mAb-NEM$_6$ conjugates in the direct labeling study arm and for the mAb-TCO$_6$ conjugates in the pretargeted arm (black vs blue, Supplementary Fig. 3). In addition, the clearance of all parental 7C2 antibodies (independent of labeling method or conjugation) increased from the time at which 50 mg/kg anti-gD-YTE-KF was administered due to effective blocking of FcRn by the clearing agent (red, Fig. 3; Supplementary Fig. 3).

Pretargeted groups demonstrated rapid blood clearance of $^{111}$In label due to the pharmacokinetic properties of the small molecule tracer, $^{111}$In-DOTA-Tz (Supplementary Fig. 4). Only 15 min after the $^{111}$In-DOTA-Tz administration, pretargeted groups showed ~40% less blood-circulating $^{111}$In tracer (on a dose-normalized basis) as compared to the intact antibody (13.6 ± 1.4 vs 21.8 ± 1.3 %ID/mL, respectively) (Supplementary Fig. 4 vs Supplementary Fig. 3). While there was no dramatic difference in blood levels of $^{111}$In-DOTA-Tz between the parental 7C2-TCO$_6$ (blue, Supplementary Fig. 4 A) and 7C2-HAHQ-TCO$_6$ (black, Supplementary Fig. 4 B) at 15 min post tetrazine, the blood level at 24 h was three times higher in mice receiving parental 7C2-TCO$_6$ than for 7C2-HAHQ-TCO$_6$. 
Biodistribution data for $^{111}$In-DOTA$_6$-7C2 showed high tumor uptake of the $^{111}$In label whether alone (blue, Fig. 4 A; 48.5 %ID/g at 48 h) or followed by 50 mg/kg anti-gD-YTE-KF (72 h uptake) (red, Fig. 4 B; 45.9 %ID/g at 72 h). In contrast, $^{111}$In-DOTA$_6$-7C2-HAHQ showed signal accumulation not only in the tumor (28.2 %ID/g) but also significant amounts in the liver (15.1 %ID/g) and spleen (28.3 %ID/g) (black, Fig. 4 A; 48 h). Mice receiving $^{111}$In-DOTA$_6$-7C2 followed by anti-gD-YTE-KF had 72-h hepatic and splenic uptake intermediate between the 48-h levels observed in $^{111}$In-DOTA$_6$-7C2 and $^{111}$In-DOTA$_6$-7C2-HAHQ groups (Fig. 4). Tumor-to-blood ratios by gamma counting largely mirrored those obtained by SPECT during the last image acquisition (Supplementary Table 3).

Similar to the directly labeled groups (Fig. 4), 7C2-TCO$_6$ followed by the tetrazine tracer (blue, Fig. 5 A) demonstrated higher $^{111}$In accumulation in the tumor than 7C2-HAHQ-TCO$_6$ (black, Fig. 5 A) (10.4 ± 1.0 and 6.3 ± 1.0 %ID/g, respectively at 48 h, ~1.5-fold) with ~2-fold higher tumor-to-blood ratio for the latter (Supplementary Table 3); but only modest accumulation in liver and spleen. Results for click reaction of $^{111}$In-DOTA-Tz after anti-gD-YTE-KF administration at 72 h (red, Fig. 5 B) were similar to the corresponding 7C2-HAHQ-TCO$_6$ group at 48 h, but with lower tumor, liver, and spleen uptake.

Biodistribution data for $^{125}$I-7C2-NEM$_6$ and $^{125}$I-7C2-HAHQ-NEM$_6$ (solid blue and black, respectively, Supplementary Fig. 5 A) at 48 h post administration revealed that FcRn binding impairment by point mutations yielded ~3-fold signal reduction in tumor and most tissues, largely mirroring exposure. Consistent with previous work, far less tumor uptake was observed with the non-residualizing $^{125}$I label than with $^{111}$In-DOTA (31). Distribution of $^{125}$I-7C2-TCO$_6$ and $^{125}$I-7C2-HAHQ-TCO$_6$ (hatched blue and black, respectively, Supplementary Fig. 5 A) were similar to that of the $^{125}$I-labeled mAb-NEM$_6$ variants in the direct study arm.
Data for $^{125}\text{I-7C2-NEM}_6$ followed 24 h later by 50 mg/kg anti-gD-YTE-KF and harvested at 72 h post administration (red, Supplementary Fig. 5 B) was similar to that of $^{125}\text{I-7C2-HAHQ-NEM}_6$ at 48 h.

DISCUSSION

Our primary goal was to compare two methods, direct FcRn binding attenuation via site mutagenesis and use of a competitive FcRn binding antibody, for reducing systemic exposure of a directly-labeled immunoSPECT antibody to provide pharmacological insight on both imaging and biotherapeutic dosing strategies. Assessment of tumor-to-blood ratios is relevant to diagnostic imaging agents and certain biotherapeutic applications, but additional considerations are required for intracellular drug delivery of toxic payloads. For instance, target-independent uptake and catabolism of conventional IgG-based antibody-drug conjugates by non-specific pinocytosis likely contributes to normal tissue toxicity (32). Exploiting the accelerated systemic clearance of fragments that undergo renal filtration may reduce pinocytosis in most tissues by reducing plasma exposure, but at the cost of high renal uptake and low efficiency of payload delivery to tumor. Moreover, FcRn mutation of full-length IgG is not an ideal platform for optimizing toxin delivery either because the mechanism for increased clearance relies on blocking the recycling ability of FcRn after internalization to achieve widespread elimination via endothelial cells throughout the body, most evident by the increased uptake and catabolism in liver and spleen. Nevertheless, the use of rapidly clearing antibody-based vehicles for drug delivery of potent payloads may be relevant to therapeutic index by limiting the degree of non-specific pinocytosis in normal tissues, assuming that clearance organ uptake – whether kidney, liver or otherwise – is tolerated.
While both methods were successful in reducing exposure, engineering of HAHQ mutations into our antibody was more efficient than adding anti-gD-YTE-KF in terms of the reduction in systemic blood pool (Fig. 3), even when considering the deliberate 24-h time delay. We could have co-administered the anti-gD-YTE-KF simultaneously with our labeled anti-HER2 antibodies to compare the FcRn binding attenuation and the competitive FcRn binding blockade methods at matched time points. However, we opted to investigate a staggered dosing interval to allow more time for efficient tumor uptake before modulating exposure, and reduce the amount of hepatic/splenic accumulation expected based on our previous results with HAHQ mutant antibodies (20).

Our secondary goal was to reduce the undesirable click reaction of $^{111}$In-labeled tetrazine that we previously encountered in pretargeted imaging with circulating TCO-modified antibodies (15), while still considering potential therapeutic implications (14). We considered that FcRn binding attenuation might induce undesirable accumulation in liver and spleen, the prominent tissue sites of IgG degradation in the absence of FcRn protection (20). However, based on prior dual isotope biodistribution studies (20), we hypothesized that rapid intracellular degradation in these sites would limit the amount of extracellular mAb-TCO$_6$ below the levels necessary for appreciable click reaction.

Our results with parental 7C2-TCO$_6$ using this approach were consistent with our previous work (15), reinforcing that the slow internalization of HER2 (33) and constant cell surface receptor pool allow efficient tumor click reaction. However, pretargeted imaging with 7C2-HAHQ-TCO$_6$ resulted in greater hepatic and splenic uptake than anticipated (Fig. 2, group 4 and Fig. 5). Even though the systemic levels of 7C2-HAHQ-TCO$_6$ were more than twice lower than those of 7C2-TCO$_6$ at 24 h (Fig. 3), it led to sufficient levels of systemic click reaction,
some of which distributed to these tissues. Accordingly, only a modest reduction in systemic blood pool activity was observed at 15 min post $^{111}$In-labeled tetrazine with the HAHQ group relative to parental (10.1 ± 0.9 and 13.6 ± 1.4 %ID/mL, respectively) (Supplementary Fig. 4).

Administering $^{111}$In-labeled tetrazine 48 h (rather than 24 h) after mAb-TCO$_6$ (when levels of 7C2-HAHQ-TCO$_6$ are 4-fold lower relative to 7C2-TCO$_6$) may have reduced systemic click reaction, but lower tumor cell surface mAb-TCO$_6$ may have negated the potential benefits. Furthermore, mice receiving the anti-gD-YTE-KF clearing agent did experience a 48 h time delay between mAb-TCO$_6$ and tetrazine; results showed similar levels of $^{111}$In in whole blood, lower in tumor (Fig. 5, group 6), and lower in liver and spleen, albeit giving good tumor imaging on the day after tetrazine (Fig. 2, group 6).

Our study adds to the relatively small literature on examining the tumor uptake of full IgG with attenuated FcRn binding affinity (18,19,22,34,35). Notably, Burvenich et al reported moderate to high tumor uptake, depending on the engineered mutations, of $^{111}$In-labeled antibodies, but with very limited SPECT imaging data (34). Olafsen et al (35) demonstrated lower tumor uptake using minibodies (~80 kDa) and scFv-Fc variants (~105 kDa, with HAHQ mutation); their renal uptake was inversely proportional to tumor uptake (~28 and 13 % ID/g for minibody and scFv-Fc, respectively). It is possible that our use of an even larger molecule (~150 kDa) promoted higher tumor uptake by reducing renal clearance.

Consistent with the findings of Swiercz et al (22), we observed that administering an antibody containing the YTE-KF mutation increased contrast. These effects on pharmacokinetics may be explained by this mutation yielding an IgG variant with single-digit nanomolar binding affinity to mouse FcRn at both extracellular and lysosomal pH (27). An antibody fragment (efgartigimod) engineered with ‘ABDEG™’ technology to treat autoimmune disorders has
demonstrated similar effects in cynomolgus monkeys and in healthy volunteers (36), suggesting that clinical feasibility for imaging applications is within grasp. Our IgG-based clearing agent with the same mutations accelerated antibody clearance from the point of administration (Fig. 3) and preserved tumor uptake in direct imaging (Fig. 4 B; Fig. 2, group 3). However, these benefits came at the expense of waiting an additional day until image acquisition, and despite producing a favorable next-day image (Fig. 2, group 6), the clearing agent was less useful in the pretargeting study arm as it worsened tumor uptake (Fig. 5 B). We postulate that the additional 24 h delay allowed depletion of cell surface HER2 receptors available for click reaction, which decreased tumor uptake. However, this delay also improved contrast relative to images without clearing agent by increasing the time between mAb-TCO₆ and tetrazine, further demonstrating the importance of timing in pretargeted imaging.

CONCLUSION

Herein, we have applied pharmacokinetic modulation via FcRn binding mutations or a competitive clearing agent to both direct and pretargeted SPECT imaging with a full-length antibody to gain insight into pharmacological implications for the corresponding biotherapeutic and/or potent immunoconjugates thereof. Tumor delineation against background in an internalizing, high HER2 expressing model could be achieved using directly labeled antibodies irrespective of FcRn recycling. However, results from imaging with directly labeled antibodies benefited more from reducing exposure via Fc point mutation or using an FcRn competitive clearing agent than with pretargeted imaging. Overall, both the HAHQ mutations and YTE-KF clearing agent approaches provided improved tumor-to-blood ratios, but each improvement came
at a cost: HAHQ mutations with higher spleen and liver uptake, and YTE-KF clearing agent with
the additional delay in image acquisition.

ACKNOWLEDGEMENTS

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REFERENCES


FIGURES LEGENDS

FIGURE 1. Schematic overview of imaging study design. The numbering of Groups 1-6 is maintained throughout all subsequent figures for clarity and cross-referencing.

FIGURE 2. Representative SPECT-CT images across both direct (\(^{111}\)In-DOTA-mAb) and pretargeted (mAb-TCO\(_6\) followed by \(^{111}\)In-DOTA-tetrazine) arms. The color scales apply only to the SPECT image, not the CT skeleton, which is presented in beige for anatomic reference. Note the differences in timing between panels. KPL-4 tumor bearing mice were injected with either directly labeled \(^{111}\)In-DOTA-labeled mAb (co-injected with 5 mg/kg unlabeled mAb) (direct labeling groups 1-3) or 5 mg/kg of 7C2-TCO\(_6\) antibodies, followed 24 (or 48) h later by \(^{111}\)In-DOTA-Tz (pretargeting groups 4-6). Imaging was performed at 28 and 48 h post-\(^{111}\)In-DOTA-mAb or mAb-TCO (4 h post \(^{111}\)In-DOTA-Tz for pretargeted groups) for all groups except those whose time points are shifted 1 day later to accommodate injection by 5B6-YTE-KF (groups 3 and 6). (All): Total body radioactivity at time of imaging is indicated for each image. Animals from each group were imaged for the same amount of time and images rendered with the same voxel range to indicate relative differences within each group. Vehicle = Phosphate-buffered saline; TCO= trans-cyclooctene; Tz= tetrazine.

FIGURE 3. Sparse pharmacokinetics of \(^{111}\)In-labeled antibodies in the direct labeling arm of the imaging study. Dashed lines are model fits, and symbols are observed data (n=3). (Parental and HAHQ / groups 1-2): KPL-4 tumor bearing mice received 11.1 MBq of \(^{111}\)In-DOTA\(_6\)-mAb (blue/black). Whole blood was collected at 0.25, 24.25 h (15 min post tetrazine injection) and 48
h post antibody injection. (YTE-KF / group 3): KPL-4 tumor bearing mice received 11.1 MBq of $^{111}$In-DOTA$_6$-mAb (red) followed 24 h later by 50 mg/kg dose of anti-gD-YTE-KF. Whole blood was collected at 0.25, 48.25 h (15 min post tetrazine injection), and 72 h post antibody injection. For model fit, data at 24 h was assumed to be same as for parental group. (All): Samples were subjected to gamma counting and values were plotted as %ID/mL of blood. ID= injected dose.

FIGURE 4. Tissue distribution (intact plus residualized) for directly ($^{111}$In-DOTA) labeled antibodies assessed by tissue harvest after the SPECT imaging study. A. (Parental and HAHQ / groups 1-2): Data included for parental 7C2 antibody (blue), the FcRn binding impaired 7C2-HAHQ (black) for tissue harvest at 48 h. B. (YTE-KF / group 3): Data included for parental 7C2 followed 24 h later by 50 mg/kg anti-gD-YTE-KF (red) and tissue harvest at 72 h. (All): KPL-4 tumor-bearing mice were injected with $^{111}$In-mAb (11.1 MBq) mixed with 5 mg/kg total mAb. Tissues were harvested, rinsed with saline, weighed, and gamma counted to produce data plotted as %ID/g of tissue. ID= injected dose.

FIGURE 5. Tissue distribution of intact plus residualized ($^{111}$In-labeled) tetrazine for the pretargeted (TCO) arm of the imaging study. Tissue distribution data for antibody-TCO conjugates followed by $^{111}$In-labeled tetrazine. A. (Parental and HAHQ / groups 4-5): KPL-4 tumor-bearing mice were injected with 5 mg/kg total of mAb-TCO$_6$ followed by $^{111}$In-Tz (11.1 MBq) at 24 h and subsequent tissue harvest at 48 h. Data is included for the TCO conjugates of parental 7C2 antibody (blue), the FcRn binding impaired 7C2-HAHQ (black). B. (YTE-KF /
**group 6:** KPL-4 tumor-bearing mice were injected with 5 mg/kg total of parental 7C2-TCO₆ followed 24 h later by 50 mg/kg anti-gD-YTE-KF, ¹¹¹In-Tz (11.1 MBq) at 48 h (red). Tissue harvest was at 72 h. **(All):** Tissues were harvested, rinsed with saline, weighed, and gamma counted to produce data plotted as %ID/g of tissue. Data is tracking ¹¹¹In-DOTA-Tz (residualizing), a portion of which has click reacted with mAb-TCO₆.

ID= injected dose; TCO= *trans*-cyclooctene.

**FIGURE 6.** Tumor-to-background ratios of %ID/g values achieved at last imaging time point (48 h, 72 h for groups receiving YTE-KF) across all treatment groups.

TCO= *trans*-cyclooctene.
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<td><img src="image4" alt="ⁱ¹ⁱIn-DOTA" /></td>
<td><img src="image5" alt="trans-cyclooctene" /></td>
<td><img src="image6" alt="tetrazine" /></td>
<td><img src="image7" alt="saline" /></td>
<td><img src="image8" alt="SPECT-CT" /></td>
<td><img src="image9" alt="Euthanasia / tissue harvest" /></td>
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* time (h): 0 24 28 48 52 72

†
Figure 2

1. $^{111}$In-7C2
   - 0 h: $^{111}$In-7C2
   - 24 h: Vehicle
   - 48 h: Vehicle
   - 28 h: 3.9 MBq
   - 48 h: 3.9 MBq

2. $^{111}$In-7C2-HAQ
   - 0 h: $^{111}$In-7C2-HAQ
   - 24 h: Vehicle
   - 48 h: Vehicle
   - 28 h: 3.9 MBq
   - 48 h: 3.9 MBq

3. $^{111}$In-7C2
   - 0 h: $^{111}$In-7C2
   - 24 h: 5B6-YTE-KF
   - 48 h: Vehicle
   - 28 h: 2.8 MBq
   - 72 h: 2.8 MBq

4. 7C2-TCO₆
   - 0 h: 7C2-TCO₆
   - 24 h: $^{111}$In-DOTA-Tz
   - 28 h: 3.4 MBq
   - 24 h: 3.4 MBq
   - 48 h: 2.4 MBq
   - 72 h: 2.4 MBq

5. 7C2-TCO₆-HAQ
   - 0 h: 7C2-TCO₆-HAQ
   - 24 h: $^{111}$In-DOTA-Tz
   - 28 h: 2.1 MBq
   - 24 h: 1.3 MBq
   - 48 h: 1.3 MBq

6. 7C2-TCO₆
   - 0 h: 7C2-TCO₆
   - 24 h: 5B6-YTE-KF
   - 48 h: $^{111}$In-DOTA-Tz
   - 28 h: 1.9 MBq
   - 52 h: 1.9 MBq
   - 72 h: 1.9 MBq
Figure 3

Time post Antibody injection (h)

%ID/mL

5B6-YTE-KF (selected groups)*

- $^{111}$In-DOTA$_6$-7C2 (group 1)
- $^{111}$In-DOTA$_6$-7C2; 5B6-YTE-KF* (group 3)
- $^{111}$In-DOTA$_6$-7C2-HAHQ (group 2)
Figure 4

A

48 h

%ID/g

Blood
Tumor
Spleen
Liver
Kidney
Muscle

$^{111}\text{In-DOTA}_6\text{-7C2 (group 1)}$

$^{111}\text{In-DOTA}_6\text{-7C2-HAHAQ (group 2)}$

B

72 h

%ID/g

Blood
Tumor
Spleen
Liver
Kidney
Muscle

$^{111}\text{In-DOTA}_6\text{-7C2; gD-YTE-KF (group 3)}$
Figure 5

A

$^{111}$In-DOTA-Tetrazine (group 4)
(predose: 7C2-TCO$_6$)

$^{111}$In-DOTA-Tetrazine (group 5)
(predose: 7C2-HAHQ-TCO$_6$)

48 h

%ID/g

B

$^{111}$In-DOTA-Tetrazine (group 6)
(predose: 7C2-TCO$_6$; gD-YTE-KF)

72 h

%ID/g
Figure 6

![Bar chart showing tumor-to-normal ratio by different groups.

- **111**In-DOTA₆-7C2 (group 1)
- **111**In-DOTA-Tetrazine (group 4) (predose: 7C2-TCO₆)
- **111**In-DOTA₆-7C2-HAHQ (group 2)
- **111**In-DOTA-Tetrazine (group 5) (predose: 7C2-HAHQ-TCO₆)
- **111**In-DOTA₆-7C2 + gD-YTE-KF (group 3)
- **111**In-DOTA-Tetrazine (group 6) (predose: 7C2-TCO₆; gD-YTE-KF)
Molecular Cancer Therapeutics

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Lidia Nazarova, Hanine Rafidi, Danielle Mandikian, et al.

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