

Phase I trial of ^{99m}Tc -(HE)₃-G3, a DARPIn-based probe for imaging of HER2 expression in breast cancer

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ABSTRACT

Radionuclide molecular imaging of human epidermal growth factor type 2 (HER2) expression may enable a non-invasive discrimination between HER2-positive and HER2-negative breast cancers for stratification of patients for HER2-targeted treatments. DARPin G3 is a small (molecular weight 14 kDa) scaffold protein with picomolar affinity to HER2. The aim of this first-in-human study was to evaluate the safety, biodistribution and dosimetry of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$.

Methods. Three cohorts of patients with primary breast cancer (each including at least 4 patients with HER2-negative and 5 patients with HER2-positive tumors) were injected with either 1000, 2000 or 3000 μg of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ (287 ± 170 MBq). Whole-body planar imaging followed by SPECT was performed at 2, 4, 6 and 24 h after injection. Vital signs and possible side effects were monitored during imaging and up to 7 days after injection.

Results. All injections were well tolerated. No side effects were observed. The results of blood and urine analyses did not differ before and after studies. $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ cleared rapidly from the blood. The highest uptake was detected in the kidneys and liver followed by the lungs, breasts and small intestinal content. The hepatic uptake after injecting with 2000 or 3000 μg was significantly ($p < 0.05$) lower than the uptake after injecting with 1000 μg . Effective doses did not differ significantly between cohorts (average 0.011 ± 0.004 mSv/MBq). Tumor-to-contralateral site ratios for HER-positive tumors were significantly ($p < 0.05$) higher than for HER2-negative at 2 and 4 h after injection.

Conclusions. Imaging of HER2 expression using $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ is safe, well-tolerated and provides a low absorbed dose burden on patients. This imaging enables discerning HER2-positive and HER2-negative breast cancer. Phase I study data justifies further clinical development of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$.

KEYWORDS: HER2; DARPin G3, ^{99m}Tc , SPECT, Phase I

INTRODUCTION

Breast cancer with high levels of human epidermal growth factor receptor type 2 (HER2) expression (3+ immunohistochemistry status) or *HER2* gene amplification (six or more copies found using in-situ hybridization measurements) is clinically defined as HER2-positive (1). HER2-positive tumors (15-20% of total cases) respond to HER2-targeting therapeutics, such as the antibodies trastuzumab, and pertuzumab, the tyrosine kinase inhibitor lapatinib or trastuzumab-based antibody-drug conjugates. Information concerning HER2 expression levels is required for every invasive primary or recurrent breast cancer (2) as it is critical for making a decision to use HER2-targeting therapies. To determine HER2-expression in breast cancer, the American Society of Clinical Oncology and College of American Pathologists has recommended biopsy sampling, followed by testing using immunohistochemistry and in situ hybridization in the case of equivocal results from immunohistochemistry tests (2).

Although biopsy-based methodology is instrumental in clinical decision-making, it is associated with a number of issues, such as HER2-expression heterogeneity, changing of HER2 expression levels with time and poor accessibility of some metastases for sampling (3, 4). Since radionuclide molecular imaging provides global information about HER2 expression and is non-invasive, and therefore might be used repeatedly, there is an appreciable interest to develop radiolabeled probes for HER2 visualization (5-7).

A common approach to imaging HER2 is through the use of therapeutic monoclonal antibodies labeled with long-lived positron emitters, e.g. ^{89}Zr or ^{64}Cu , for PET imaging (8). However, the clearance of antibodies from the blood is slow, which is associated with a high background even 4-7 days after injection. Furthermore, harnessing the high sensitivity of PET is essential in this case. This approach is viable in Western Europe and North America, where PET infrastructure is routinely available. However, access to PET is limited in the majority of the

world's populations living in Africa, Latin America and Asia. For these regions, a ^{99m}Tc -labeled imaging probe would be desirable as SPECT cameras are much more common there.

The use of ^{99m}Tc -labeled engineered scaffold proteins for SPECT imaging is a feasible approach for HER2 imaging because these types of imaging probes provide a high contrast only several hours after injection, according to preclinical studies (9). Designed ankyrin repeat proteins (DARPin) are one such promising class of engineered scaffold proteins, having small molecular weights (14-18 kDa), high affinity and specificity to selected targets, high chemical and thermal stability, and potentially low production costs (10). DARPin G3 binds to HER2 with an affinity of 90 pM and exquisite selectivity (11). Goldstein and co-workers demonstrated that ^{111}In -labeled G3 provides specific high-contrast imaging of HER2 in human xenografts in mice 4 h after injection (12). For site-specific labeling of G3 with ^{99m}Tc , we evaluated the use of $^{99m}\text{Tc}(\text{CO})_3^+$ in combination with histidine-containing tags at different positions in the DARPin (13). For clinical translation we selected DARPin (HE)₃-G3 with the HEHEHE-tag placed at the N-terminus. This variant demonstrated significantly ($p < 0.05$) higher tumor-to-liver, tumor-to-muscle and tumor-to-bone uptake ratios compared to the other tested variants. This indicated that the imaging contrast would be higher in the main metastatic sites of breast cancer.

The aim in this first-in-human study was to evaluate the safety and distribution of ^{99m}Tc -(HE)₃-G3 in patients with primary HER2-positive and HER2-negative breast cancer.

The primary objectives of this study (ClinicalTrials.gov ID: NCT04277338) were: to obtain initial information concerning the safety and tolerability of ^{99m}Tc -(HE)₃-G3 after a single intravenous injection; to assess the distribution of ^{99m}Tc -(HE)₃-G3 in normal tissues and in tumors over time; to evaluate the dosimetry of ^{99m}Tc -(HE)₃-G3.

The secondary objective was to compare the tumor imaging data with HER2 expression data obtained by immunohistochemistry and fluorescent in situ hybridization (FISH) analysis of biopsy samples.

Since clinical data for other scaffold proteins (*14,15*) had demonstrated that the mass of injected protein has a strong influence on its biodistribution and targeting properties, three levels of the injected protein mass (1000, 2000 and 3000 μg) were tested.

MATERIALS AND METHODS

Patients

This was a prospective, open-label, non-randomized Phase I diagnostic study in patients with untreated primary breast cancer (ClinicalTrials.gov Identifier: NCT04277338). Both the initial protocol and the further extension of patient cohorts has been approved by Scientific Council of the Cancer Research Institute and Board of Medical Ethics, Tomsk National Research Medical Center of the Russian Academy of Sciences, and all subjects provided written informed consent. Female patients (18-80 years) with HER2 status previously determined using biopsy material from the primary tumor according to the guidelines of the American Society of Clinical Oncology (2) were eligible.

According to protocol, patients were divided into three cohorts (injected with 1000, 2000 or 3000 μg $^{99\text{m}}\text{Tc}-(\text{HE})_3\text{-G3}$), each including at least 5 patients with HER2-positive and at least 4 patients with HER2-negative tumors. Enrollment into cohort with higher injected dose was initiated after accomplished safety evaluation for preceding cohort with lower injected dose. In each cohort, consecutive patients were enrolled until required numbers for each category (HER2-positive and HER2-negative) were obtained.

The inclusion criteria were: diagnosis of primary breast cancer with possible lymph node metastases; at least one lesion > 1.0 cm in greatest diameter; hematological, liver and renal function test results within the normal limits; a negative pregnancy test for patients of childbearing potential and capability to undergo the diagnostic investigations planned in the study.

The exclusion criteria were: other concurrent malignancies; autoimmune disease or history of autoimmune disease; active infection or history of severe infection. One patient had a previous nephrectomy because of a car accident injury. Although such situation was not listed in the exclusion criteria, the patient was excluded from the study because this might influence the renal elimination rate and result in a non-representative biodistribution pattern.

Twenty eight patients were enrolled in the trial (Table 1; Fig. 1).

Mammograms (Giotto Image), biopsy samples, breast, regional lymph nodes and liver ultrasound images (GE LOGIQ E9), bone scans (Siemens E.Cam 180) using ^{99m}Tc -pyrophosphate, and chest CT scans (Siemens Somatom Emotions 16 ECO) were obtained for all patients according to the local standard of care.

The level of HER2 expression in biopsy samples was determined by immunohistochemistry using the Herceptest (DAKO). In tumors with a score of 2+ or in cases of questionable results, HER2 amplification was assessed by FISH using KBI-10701 probe (Kreatech) . To confirm imaging results, FISH analysis was performed after imaging for all patients. The tumors were classified as HER2-positive (HercepTest score 3+ or HercepTest score 2+ and FISH-positive) and HER2-negative (HercepTest score 0 or 1+, or score 2+ but FISH-negative).

Imaging Protocol

(HE)₃-G3 was labeled with ^{99m}Tc using the protocol reported earlier (13). The yield was 83±9 % and the radiochemical purity was over 98 %.

^{99m}Tc-(HE)₃-G3 was injected as an intravenous bolus. The injected protein dose was 1000 µg (HE)₃-G3 for patients 1-9, 2000 µg (HE)₃-G3 for patients 10-18, and 3000 µg (HE)₃-G3 for patients 19-29. The average injected activity was 287±170 MBq. A Siemens E.Cam 180 scanner equipped with a high-resolution low-energy collimator was used for imaging. Anterior and posterior whole-body planar imaging (at a scan speed of 12 cm/min, 1024x256 pixel matrix) and SPECT scans (32 projections, 30 seconds each, 128x128 pixel matrix) were performed at 2, 4, 6 and 24 h. The SPECT data was reconstructed by iterative reconstruction with a Gaussian filter applying scatter correction using the E.Soft computer system for scintigraphic data processing. Patient 27 was imaged using a Siemens Symbia Intevo Bold scanner equipped with a high-resolution low-energy collimator. Whole-body imaging (at 2, 4, 6 and 24 h) and SPECT scans (at 4, 6 and 24 h) were performed in the same mode as above. After 2 h, two SPECT/CT bed scans were performed (60 projections, 20 seconds each, 256x256 pixel matrix, 130 kV, effective 36 mAs).

Vital signs and possible side effects were monitored during the investigation (0-24 h after injection) and 3-7 days after the injection. Blood and urine analyses were performed 1 and 3 days after the injection.

Evaluation of Distribution and Dosimetry

Regions of interest (ROI) were drawn over organs of interest and the body contour on the anterior and posterior whole-body images and a geometric mean of counts at 2, 4, 6 and 24 h was calculated for each ROI. For quantification, a known activity of ^{99m}Tc in a water-filled phantom

in combination with Chang's correction was used. A ROI was placed over the heart to assess the activity in the blood. Data were fitted to single exponential functions and residence times were calculated as areas under fitted curves using Prism 8 (GraphPad Software, LLC). Absorbed doses were calculated by OLINDA/EXM 1.1 using an Adult Female phantom.

To calculate tumor-to-contralateral breast and tumor-to-liver ratios, a 3.5-cm³ volume of interest (VOI) was drawn on tomograms centered on the highest tumor uptake, and counts were recorded. Thereafter, this VOI was copied to the contralateral breast to obtain reference counts. The tumor-to-contralateral breast ratio for each primary tumor was calculated and matched with the biopsy-based data concerning HER2-expression in the same tumor.

Statistics

Values are reported as mean \pm standard deviation. Differences between uptakes in organs at different time points were analyzed using 1-way ANOVA. The nonparametric Mann-Whitney U-test was used to determine if the differences between tumor-to-contralateral breast ratios for HER2-positive and HER2-negative tumors were significant. A two-sided P value of less than 0.05 was considered significant.

RESULTS

Safety and Tolerability

The intravenous bolus administration of ^{99m}Tc-(HE)₃-G3 was well tolerated by all 29 patients, independent of the injected protein mass. Changes in vital signs or adverse reactions were not registered during imaging or the follow-up period. No relevant changes in blood or urine samples were found after their analyses.

Evaluation of Distribution and Dosimetry

The kinetics of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ elimination from blood is shown in Fig. 2. The elimination half-lives were comparable for all injected protein doses. These elimination half-lives were 3.5 h (95% confidence interval (CI) 2.3 - 6.0 h), 3.8 h (95% CI 3.4 - 4.3 h), and 3.4 h (95% CI 2.6 - 4.6 h), for 1000 μg , 2000 μg and 3000 μg , respectively.

The kidneys and liver were the organs with the highest uptake of activity (Table 2; Fig. 3). Noticeable activity was also observed in the lungs, small intestines, lacrimal and salivary glands. Injections with 2000 and 3000 μg resulted in a significant ($p < 0.05$) reduction in hepatic uptake compared with 1000 μg injections. The difference between hepatic uptakes after injection with 2000 and 3000 μg was not significant, although there was a strong tendency toward reduced uptake with an increase in injected mass (R^2 in the range between 0.86 and 0.95) (Supplemental Figure S1). The decay-corrected uptake in the liver and kidneys did not differ significantly between 2 and 24 h. There was a significant gradual decrease in uptake within the small intestines and lungs between the first and last time points.

The evaluation of the absorbed doses is shown in Table 3. The highest absorbed dose was in the kidneys, followed by the adrenals, urinary bladder wall, liver, gall bladder wall and ovaries. The absorbed dose to the liver was significantly ($p < 0.05$) higher after injection with 1000 μg compared to the doses after injecting with 2000 and 3000 μg . The effective doses were 0.011 ± 0.001 , 0.012 ± 0.006 and 0.012 ± 0.003 mSv/MBq for 1000, 2000 and 3000 μg , respectively. An effective dose of 3.2-3.4 mSv would result from the typical injected activity of 250 MBq used in this study.

Discrimination between Tumors with High and Low HER2 Expression

Imaging using $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ enabled the visualization of all known tumors (including HER2-negative tumors) already 2 h after injection (Fig. 3 and 4). HER2-positive tumors remained visible at all time points, but HER2-negative tumors could not be visualized 24 h after injection. Involved lymph nodes were also visualized in nine patients. The involvement of lymph nodes was confirmed by histological analysis after core biopsies (n = 2) or cytological analysis after fine-needle biopsies (n = 7).

Tumor-to-contralateral site ratios were significantly higher ($p < 0.05$, Mann-Whitney test) for HER2-positive tumors than for HER2-negative ones 2 and 4 h after injection with 1000 and 2000 μg , but the difference was not significant 6 h after injection (Fig. 5). Tumor-to-contralateral ratios after injection with 3000 μg were significantly ($p < 0.05$, Mann-Whitney test) higher for HER2-positive tumors 2, 4, and 6 h after injection (Fig. 5).

Patient 26 was enrolled in this study as a patient with a HER2-negative tumor based on a 1+ immunohistochemistry score from her core biopsy sample (Fig. 6A). However, the tumor-to-contralateral breast ratio was unusually high (12.5 at 2 h, 3000 μg dose) for a patient with a HER2-negative lesion (Fig. 6B). Upon our request, her surgery samples were evaluated and 35% of the tumor was found to display elevated HER2 expression from immunohistochemistry (Fig. 6C) while the rest of the tumor was HER2-negative (Fig. 6D). Based on this finding, trastuzumab treatment was added to her adjuvant therapy. Similarly, a high tumor-to-contralateral site ratio (14.4 at 2 h, 2000 μg dose) for patient 16 prompted the re-evaluation of her biopsy samples using a FISH-analysis which actually suggested a HER2-positive tumor. Further postimaging FISH analysis demonstrated agreement between ICH and FISH data for all other patients except from patient 6, who had HER2-overexpression without gene amplification.

Patient 27 was enrolled in the study with a large HER2-positive inflammatory breast cancer with axillary node involvement, as well as suspected metastatic sites in the liver. ^{99m}Tc -(HE)₃-G3 imaging demonstrated multiple sites of abnormal accumulation of activity. Presence of metastases in the sites of abnormal accumulation in e.g. the liver (Fig. 7A, tumor-to-reference zone ratio 2.97) and in iliac bone (Fig. 7C, tumor-to-reference zone ratio 9.5) was confirmed by diagnostic CT images (Fig. 7B and 7D).

DISCUSSION

Numerous preclinical studies have demonstrated that small scaffold proteins are promising types of probes for molecular imaging (16). Still, only a limited number of such probes have been tested in clinical trials: affibody molecules (17), ADAPTs (15), and adnectins (18). At the same time, scaffold proteins represents a large variety of molecular forms with different structures, charges and lipophilicity of solvent-exposed amino acids (19). These features can substantially modify off-target interactions, impact the biodistribution of these probes and influence their imaging contrast. Such considerations necessitate the clinical evaluation of different scaffolds in order to select the most promising ones.

This study demonstrated that the injection of DARPins (up to 3 mg) is well-tolerated and not associated with any adverse effects. A combination of rapid systemic clearance and the favorable dosimetry properties of ^{99m}Tc ensured a moderate effective dose. In the current study, typical equivalent doses were 3.2-3.4 mSv. However, since the optimal imaging time is between 4 and 6 h, the injected activity could still be reduced at least twice and accordingly further reduce the dose burden to patients. This aspect offers advantages compared to immunoPET, where the radiation burden is higher by an order of magnitude.

The important features of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ are rapid localization in tumors and quick clearance from the blood (Fig. 3). Both of these features might be explained by the small size of this targeting probe and they enabled the clear visualization of tumors as early as 2 h after injection (Fig. 3). It should also be noted that cells in clinically defined HER2-negative tumors (immunohistochemistry score of 2+ and ISH negative) might still express hundreds of thousands of HER2 receptors per cell (20). Accordingly, HER2-negative tumors were also visualized (Fig. 4). However, tumor-to-contralateral breast ratios were significantly ($p < 0.05$, Mann-Whitney test) higher for HER2-positive lesions 2 and 4 h in 1000- μg - and 2000 μg -cohorts, and at 2, 4 and 6 h in the 3000- μg cohort (Fig. 5). Such simple approach is feasible even in developing countries as it does not require regular exact calibration of SPECT cameras. Unsuspectingly, HER2 imaging using $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ has already proved its worth in this study. High tumor-to-contralateral breast ratios triggered re-evaluating the HER2 status for patients 16 and 26, who were enrolled in this trial as having HER2-negative tumors. In both cases, a HER2-positive status has been confirmed. This indicates that radionuclide molecular imaging can overcome the limitations of biopsy-based methods caused by heterogeneous target expression and associated sampling errors.

The aspect of injected mass was found essential in earlier studies with affibody molecules (14,17) and ADAPT6 (15). When a low protein mass is injected (100 μg for affibody molecules or 250 μg for ADAPT6 in the case of HER2 imaging), the binding to HER2 expressed on hepatocytes results in a high liver uptake and sequestration of the probe from circulation. This prevents delivery of the radionuclide to tumors and increases the background during the imaging of liver metastases, which are common in breast cancer. The current study demonstrated that optimization of the injected mass is essential for DARPIn-based HER2-imaging probes as well. An increase of the injected mass from 1000 to 3000 μg decreased hepatic uptake by more than half (Table 2, Supplemental Fig. S1). This created a precondition for the clear visualization of

HER2-positive liver metastases in patient 27 (Figure 7). The example of patient 27 indicates that $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ has the capability to enable visualization of metastases in multiple locations, including the bone and liver. Ultimately, the tracer should be used for detection of HER2 expression in metastatic breast cancer. Such findings during the Phase I trial motivate its further development.

CONCLUSION

Injections of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ are safe and result in low absorbed and effective doses. Preliminary data suggests that SPECT/CT using $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ could distinguish HER2-positive from HER2-negative primary breast cancer. An injected protein mass between 2000 and 3000 μg is desirable in order to suppress hepatic uptake. Further clinical development of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ for imaging of HER2-expression in cancers is warranted.

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KEY POINTS

- **QUESTION:** Is the use of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ for HER2 imaging tolerable, **safe and informative?**
- **PERTINENT FINDINGS:** $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ is safe, associated with low dose burdens and enables distinguishing HER2-positive and HER2-negative primary breast cancer lesions 2-4 h after injection.
- **IMPLICATION FOR PATIENT CARE:** Results from this study suggest that further clinical development of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$ might provide a probe for the SPECT imaging of HER2 expression.

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TABLES

Table 1. Patient Characteristics

Patient no	Age (y)	HER2 status in primary tumor before imaging (IHC ^a /FISH)	<i>HER2</i> gene amplification (determined after imaging)	Primary tumor status (ER/PgR)	Clinical stage before imaging
1000 µg; mean tumor size 28±11 mm					
1	68	1+ (IHC)	FISH-	ER +/PgR -	IIA (T2N0M0)
2	62	1+ (IHC)	FISH-	ER +/ PgR +	I (T1N0M0)
3	66	1+ (IHC)	FISH-	ER +/PgR +	IIA (T2N0M0)
4	48	0 (IHC)	FISH-	ER -;/PgR -	IIA (T2N0M0)
5	50	3+ (IHC)	FISH+	ER +/ PgR +	IIA (T2N0M0)
6	70	3+ (IHC)	FISH-	ER +/ PgR +	IIA (T2N0M0)
7	30	3+ (IHC)	FISH+	ER +/PgR +	IIB (T2N1M0)
8	59	2+ (IHC)/ FISH +		ER -/PgR -	IIA (T2N0M0)
9	45	(IHC)3+	FISH-	ER +/ PgR -	IIB (T2N1M0)
2000 µg; mean tumor size 25±6 mm					
10	50	1+ (IHC)	FISH-	ER +/ PgR +	IIA (T2N0M0)
11	57	1+ (IHC)	FISH-	ER +/ PgR +	I (T1N0M0)
12	43	1+ (IHC)	FISH-	ER +/ PgR +	IIA (T2N0M0)
13	51	1+ (ICH)	FISH-	ER -/ PgR -	IIA (T2N0M0)
14	65	3+ (IHC)	FISH+	ER +/ PgR +	IIA (T2N0M0)
15	35	3+ (IHC)	FISH+	ER +/ PgR +	I (T1N0M0)
16	50	1+ (IHC) *	FISH+	ER +/ PgR +	IIA (T2N0M0)
17	68	2+ (IHC) /FISH +		ER +/ PgR -	IIA (T2N0M0)
18	68	3+ (IHC)	FISH+	ER +/ PgR +	IIA (T2N0M0)
3000 µg; mean tumor size 22±7 mm					
19	37	1+ (IHC)	FISH-	ER -/ PgR -	IIA (T2N0M0)
20	45	1+ (IHC)	FISH-	ER +/ PgR +	IIA (T2N0M0)
21	56	1+ (IHC)	FISH-	ER +/ PgR +	IIA (T2N0M0)
22	45	1+ (IHC)	FISH-	ER +/ PgR +	IIA (T2N0M0)
23	36	1+ (ICH)	FISH-	ER +/ PgR +	IIA (T2N0M0)
24	48	3+ (IHC)	FISH+	ER +/ PgR +	IIB (T2N1M0)
25	58	3+ (IHC)	FISH+	ER +/ PgR +	IIA (T2N0M0)
26	47	1+ (IHC) *	FISH+	ER +/ PgR +	IIA (T2N0M0)
27	61	3+ (ICH)	FISH+	ER +/ PgR -	IV (T4N3M1)
28	49	3+ (IHC)	FISH+	ER +/ PgR +	I (T1N0M0)

^a IHC: immunohistochemistry

* Postimaging FISH evaluation demonstrated *HER2* gene amplification

Table 2. Uptake of ^{99m}Tc in Tumor-Free Areas of Organs with Highest Uptake on SPECT Images After Injection of $^{99m}\text{Tc-HE}_3\text{-G3}$ (decay corrected).

	2 h			4 h			6 h			24 h		
	1000 μg	2000 μg	3000 μg	1000 μg	2000 μg	3000 μg	1000 μg	2000 μg	3000 μg	1000 μg	2000 μg	3000 μg
Breast	2.1 \pm 0.6	2.4 \pm 0.6	2.4 \pm 0.6	2.1 \pm 0.4	2.0 \pm 0.5	2.1 \pm 0.5	2.3 \pm 1.1	1.8 \pm 0.5	1.9 \pm 0.3	1.7 \pm 0.2	1.7 \pm 0.5	1.6 \pm 0.4
Small intestines	2.5 \pm 0.6	2.4 \pm 0.5	2.9 \pm 0.6	2.1 \pm 0.4	2.2 \pm 0.4	2.5 \pm 0.6	2.1 \pm 0.8	1.9 \pm 0.5	2.0 \pm 0.4	1.7 \pm 0.4	1.7 \pm 0.5	1.8 \pm 0.6
Kidney	24 \pm 5	28 \pm 6	29 \pm 11	22 \pm 5	28 \pm 6	29 \pm 11	24 \pm 5	28 \pm 6	29 \pm 11	21 \pm 5	21 \pm 5	26 \pm 12
Liver	11 \pm 2	6 \pm 2 ^a	5 \pm 2 ^a	12 \pm 4	6 \pm 2 ^a	5 \pm 2 ^a	10 \pm 3	6 \pm 2 ^a	4 \pm 1 ^a	9 \pm 3	5 \pm 2 ^a	4 \pm 2 ^a
Lungs	2.4 \pm 0.6	2.4 \pm 0.7	2.7 \pm 0.9	2.2 \pm 0.5	2.2 \pm 0.7	2.2 \pm 0.6	1.9 \pm 0.6	2.0 \pm 0.6	2.0 \pm 0.4	1.9 \pm 0.5	1.8 \pm 0.6	1.6 \pm 0.4

^a Significant difference in uptake compared to the injection of 1000 μg $^{99m}\text{Tc-HE}_3\text{-G3}$

Table 3. Absorbed Doses after Injection of 1000, 2000 and 3000 μg $^{99\text{m}}\text{Tc}-(\text{HE})_3\text{-G3}$.

	Absorbed dose, mGy/MBq		
	1000 μg	2000 μg	3000 μg
Adrenals	0.031 \pm 0.007	0.031 \pm 0.007	0.032 \pm 0.002
Brain	0.0010 \pm 0.0004	0.0011 \pm 0.0002	0.0012 \pm 0.0001
Breasts	0.008 \pm 0.002	0.007 \pm 0.001	0.008 \pm 0.001
Gallbladder Wall	0.017 \pm 0.003	0.015 \pm 0.004	0.014 \pm 0.002
LLI Wall	0.005 \pm 0.001	0.006 \pm 0.003	0.006 \pm 0.001
Small Intestine	0.0076 \pm 0.0010	0.009 \pm 0.004	0.009 \pm 0.002
Stomach Wall	0.0060 \pm 0.0008	0.006 \pm 0.001	0.007 \pm 0.002
ULI Wall	0.007 \pm 0.001	0.008 \pm 0.003	0.009 \pm 0.002
Heart Wall	0.004 \pm 0.001	0.004 \pm 0.001	0.0042 \pm 0.0007
Kidneys	0.10 \pm 0.02	0.10 \pm 0.03	0.13 \pm 0.05
Liver	0.016 \pm 0.003	0.011 \pm 0.003*	0.0100 \pm 0.0008*
Lungs	0.005 \pm 0.001	0.005 \pm 0.001	0.006 \pm 0.001
Muscle	0.0024 \pm 0.0005	0.003 \pm 0.001	0.0028 \pm 0.0007
Ovaries	0.014 \pm 0.005	0.014 \pm 0.008	0.013 \pm 0.003
Pancreas	0.012 \pm 0.001	0.013 \pm 0.003	0.016 \pm 0.004
Red Marrow	0.0033 \pm 0.0007	0.004 \pm 0.001	0.004 \pm 0.001
Osteogenic Cells	0.006 \pm 0.002	0.006 \pm 0.002	0.007 \pm 0.001
Skin	0.0014 \pm 0.0004	0.0015 \pm 0.0003	0.0017 \pm 0.0003
Spleen	0.010 \pm 0.001	0.010 \pm 0.003	0.012 \pm 0.004
Thymus	0.006 \pm 0.001	0.007 \pm 0.003	0.0068 \pm 0.0002
Thyroid	0.017 \pm 0.003	0.018 \pm 0.005	0.022 \pm 0.003
Urinary Bladder Wall	0.013 \pm 0.007	0.014 \pm 0.009	0.019 \pm 0.007
Uterus	0.008 \pm 0.002	0.055 \pm 0.01	0.009 \pm 0.003
Total Body	0.004 \pm 0.001	0.004 \pm 0.001	0.004 \pm 0.001
Effective Dose Equivalent (mSv/MBq)	0.017 \pm 0.002	0.020 \pm 0.012	0.019 \pm 0.005
Effective Dose (mSv/MBq)	0.011 \pm 0.001	0.012 \pm 0.006	0.012 \pm 0.003

* Significant ($p < 0.05$) difference with absorbed dose after the injection of 1000 μg $^{99\text{m}}\text{Tc-HE3-G3}$

FIGURES CAPTIONS

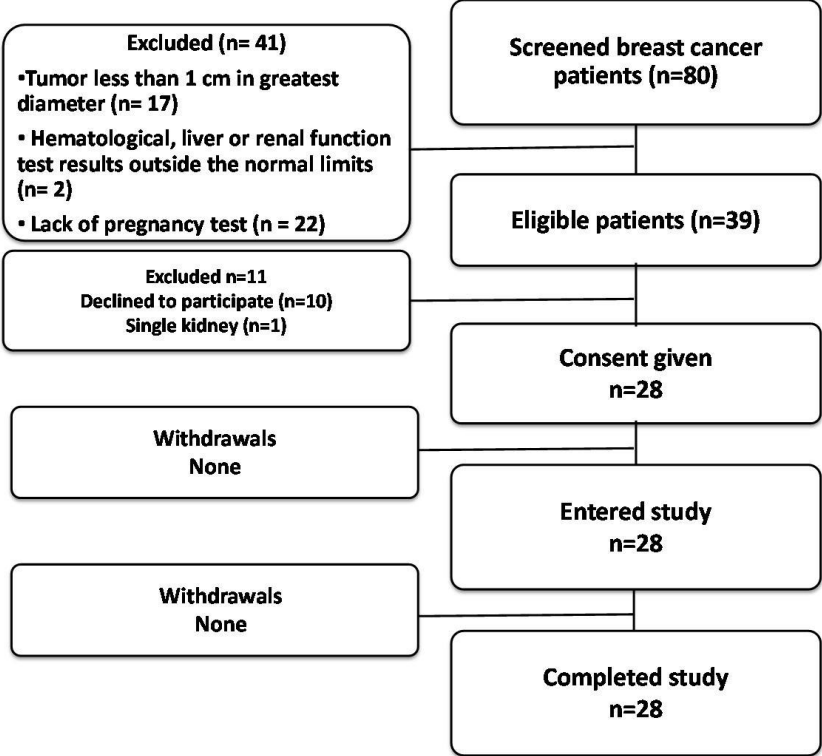


Figure 1. Standards for Reporting of Diagnostic Accuracy Studies (STARD) flow diagram.

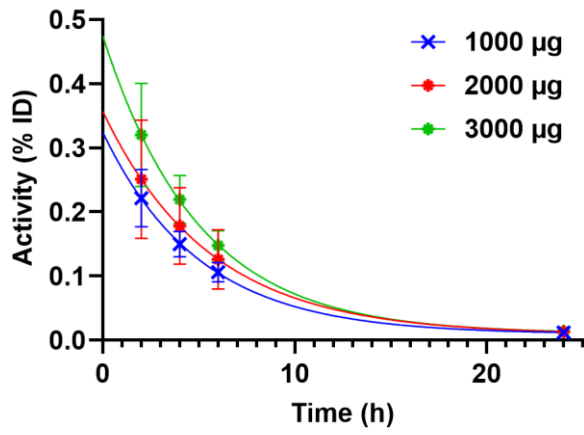


Figure 2. Kinetics of elimination of $^{99m}\text{Tc-HE}_3\text{-G3}$ from blood. Data were calculated based on count rates in ROIs placed over hearts.

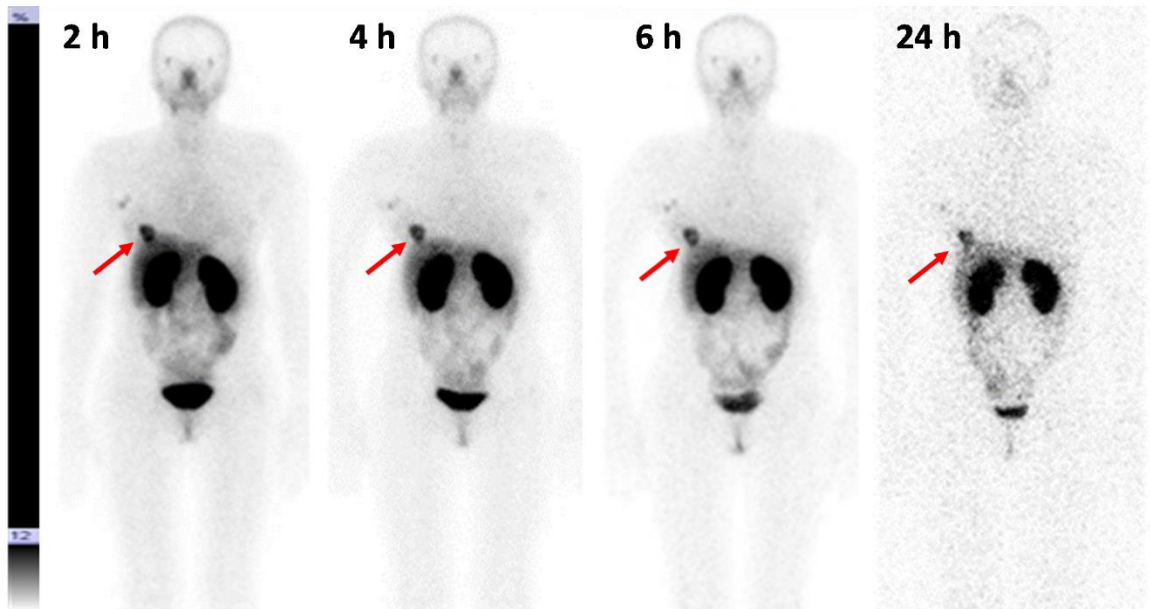


FIGURE 3. Anterior images at 2, 4, 6 and 24 h after the injection of 3000 μg $^{99\text{m}}\text{Tc-HE}_3\text{-G3}$ (Patient 24). The upper setting of the scale window is **12 %** of the maximum counts.

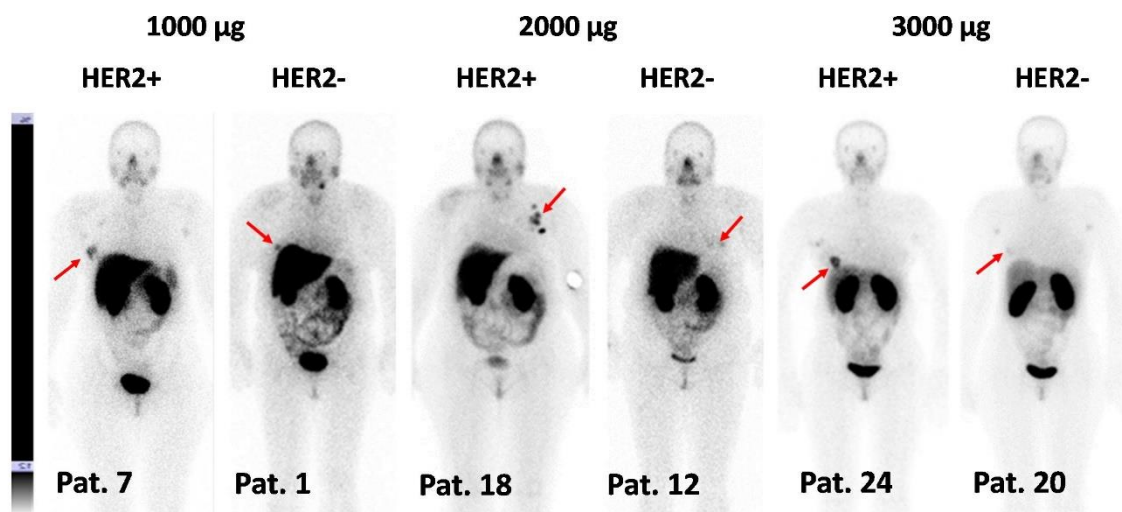
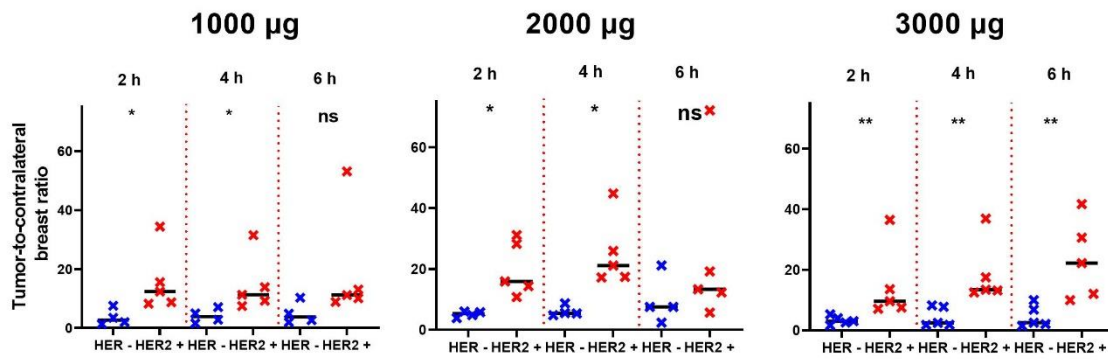


FIGURE 4. Representative anterior images of patients with HER2-positive and HER2-negative tumors 4 h after the injections of 1000, 2000 or 3000 μg $^{99\text{m}}$ Tc-HE3-G3. The upper setting of the scale window is the same for all images, 12 % of the maximum count rate.



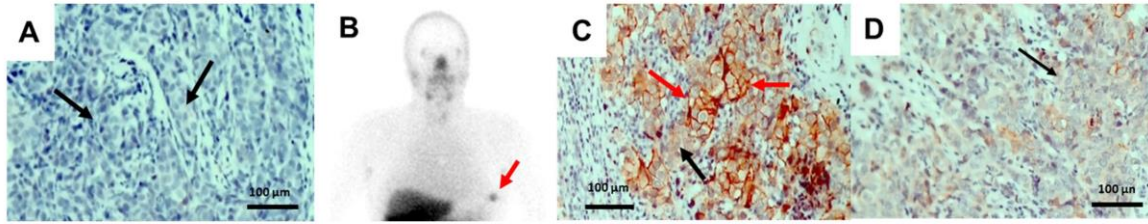


FIGURE 6. Patient 26. (A) Immunohistochemistry analysis shows very low HER2 expression in biopsy material (A). Anterior image 4 h after injection, the upper setting of the scale window is 12% of the maximum counts, showing a tumor-to-contralateral site ratio typical for HER2-positive tumors (B); Immunohistochemistry analysis of surgery material shows areas with high (B) and low (C) HER2 expression. Magnification 400x. Black arrows show cells with low and red cells with high HER2 expression.

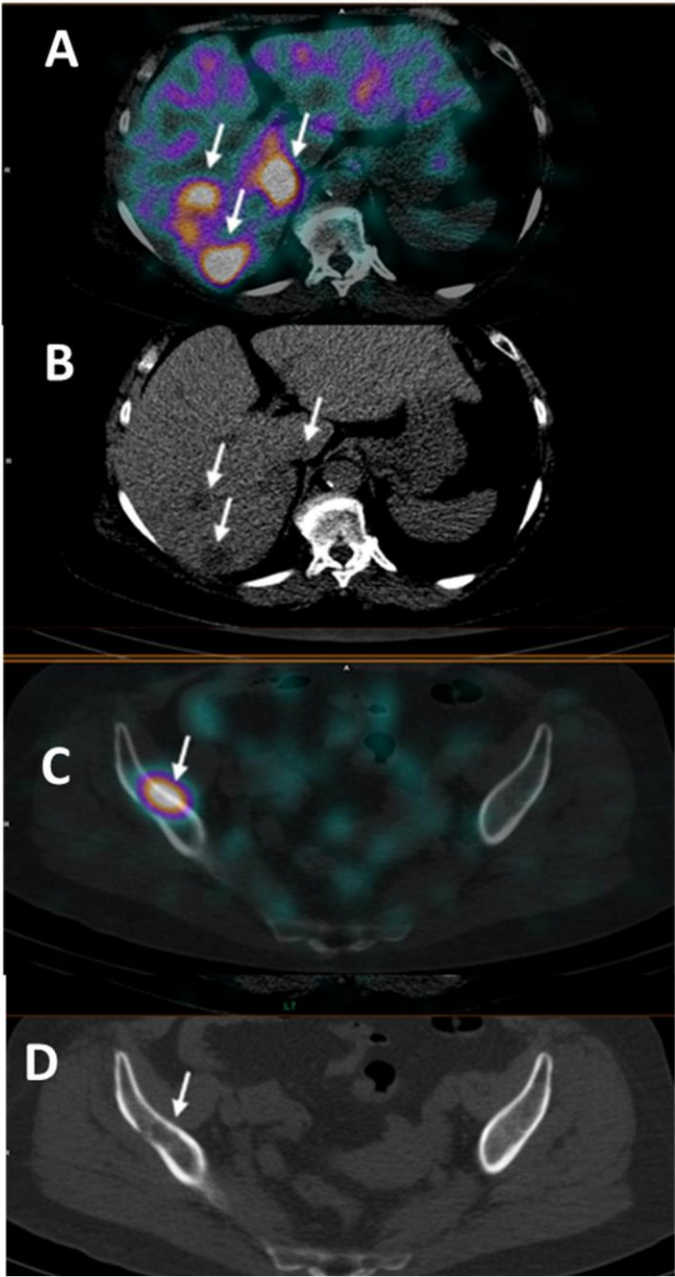
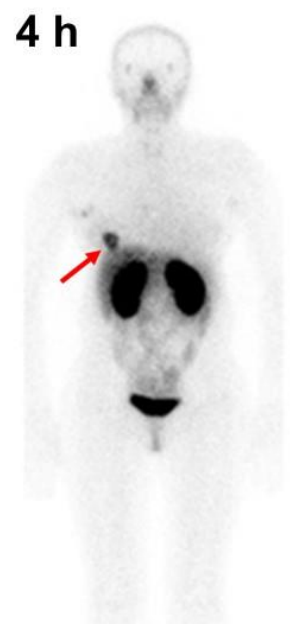
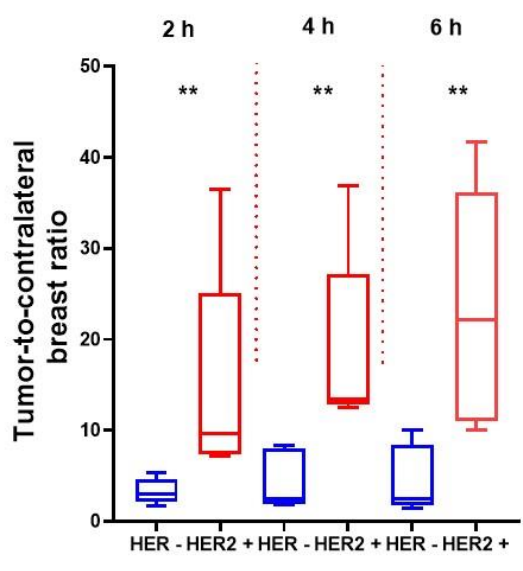
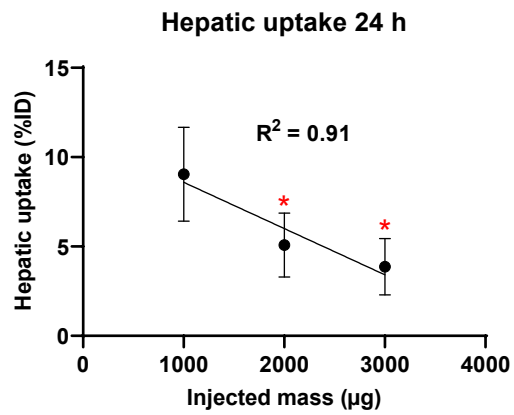
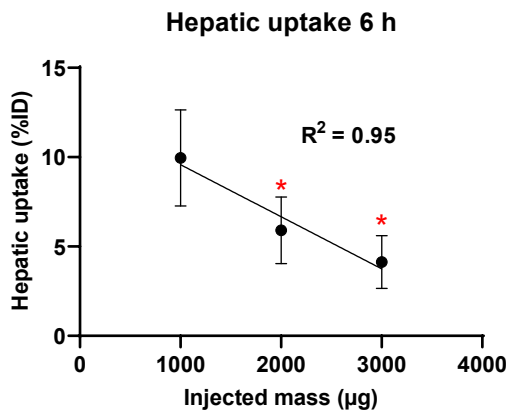
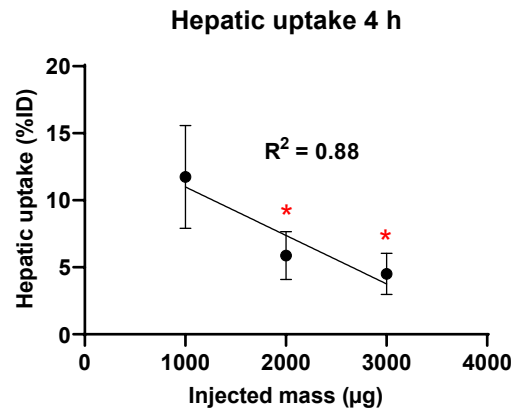
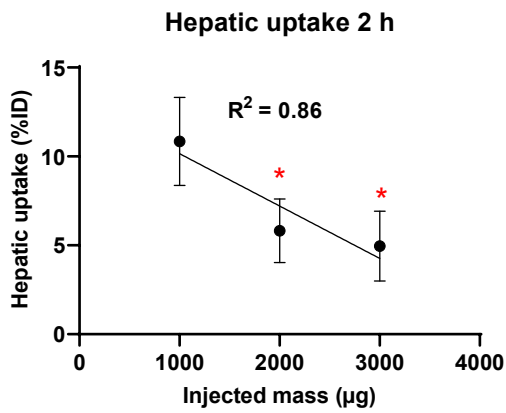


FIGURE 7. Patient 27. (A) SPECT/CT and (B) CT images of hepatic lesions; (C) SPECT/CT and (D) CT images of iliac bone lesions.



Graphical Abstract



Supplemental Figure S1. Influence of injected mass on hepatic uptake of $^{99m}\text{Tc}-(\text{HE})_3\text{-G3}$.

*Significant difference ($p < 0.005$) with uptake after injection of 1000 μg (ANOVA test).