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Synthesis and biodistribution studies of ^{177}Lu-trastuzumab as a therapeutic agent in the breast cancer mice model

Samira Rasaneh,[a] Hossein Rajabi,[a]* Mohammad Hossein Babaei,[b] and Fariba Johari Daha[b]

Trastuzumab is a humanized monoclonal antibody against the HER2 that has the potential to be used as radioimmunotherapy (RIT) agent in treatment of breast cancer. Lutetium-177 has beta energy suitable for therapy and gamma photons for imaging. We labeled trastuzumab with lutetium-177 via DOTA as chelator and performed some necessary tests for the first stage in using complex as a RIT agent. Radiochemical purity, immunoreactivity and stability of complex were determined. The biodistribution and imaging studies were determined in mice bearing breast tumor. The radiochemical purity was 94 ± 0.9%. Lutetium-Trastuzumab showed a good stability at biological condition. The tumor to blood ratio was calculated 3.29(± 0.09) after 7 days. The good tumor uptake in biodistribution studies was agreed with gamma camera images after 7 days. The results showed that the new complex could be considered for further evaluation in animals and possibly in humans as a new radiopharmaceutical for use in RIT against breast cancer.

Keywords: radioimmunotherapy; trastuzumab; lutetium-177; DOTA; breast cancer

Introduction

Recently, targeted therapy of radiopharmaceuticals has shown promising effects for delivering higher radiation doses to tumor cells or metastases. Monoclonal antibodies have attracted much attention due to their targeting capabilities and served as selective carriers of radioisotopes to specific antigens in radioimmunotherapy (RIT) for tumor treatment.[1,2]

Trastuzumab, trade-name Herceptin, is the second monoclonal antibody approved by food and drug administration (FDA, USA) for the treatment of metastatic breast cancer.[3] Trastuzumab continuously suppresses HER2 activity that may lead to tumor proliferation and provides constant inhibition of the HER2 receptor.[4–6] Trastuzumab leads to cell stasis and death.[6–7] Trastuzumab has recently been used in RIT as a tumor specific carrier.[8–11]

^{177}Lu is a radiolanthanide with a β-emission similar to ^{131}I but with two advantages over it. The main γ-photons of ^{177}Lu (208 keV, 11% abundance) are more suitable for imaging with a gamma camera than those of ^{131}I (364 keV, 82% abundance), and ^{177}Lu does not need special design of residualizing for labeling with internalizing monoclonal antibodies. The half-life of ^{177}Lu is 6.65 days. This simplifies radiation protection and patient-handling issues and should improve the quality of dosimetric images, particularly by quantitative SPECT. So, ^{177}Lu is being increasingly used in therapeutic research studies.[12]

Targeted radiopharmaceuticals, whether designed for diagnostics or therapy, often involve the use of a radiometal. The DOTA analogues have generally resulted in more stable radiometal bioconjugates. DOTA-NHS(one of the DOTA analogues) was investigated in some of researches. The radiolabeled cG250 with ^{177}Lu via DOTA-NHS, cDTPA and SCN-Bz-DTPA exhibited that ^{177}Lu-DOTA-cG250 had a higher labeling efficiency, immunoreativity, invitro and invivo stability than the DTPA analogues.[13]

The pharmacokinetics and biodistribution studies of ^{177}Lu-labeled J591 by DOTA-NHS in prostate cancer patients showed ^{177}Lu formed strong complex with antibody via this chelator.[14]

However, there were also reports showing that NHS-DOTA was not showed good stability. For example, Back-DOTA-B72.3 and Arm-DOTA-B72.3 were anticipated to be more stable in vitro and in vivo than NHS-DOTA-B72.3 radiolabeled with ^{111}In, ^{90}Y and ^{177}Lu.[15]

It was found that minimizing normal organ accumulation of ^{177}Lu activity after injection of ^{177}Lu-labeled mAb–chelate conjugates depends on both the characteristics of the mAb as well as the properties of the chelate.[16]

We have already been labeled Trastuzumab with ^{177}Lu and performed the necessary in vitro quality control procedures and investigated its cytotoxicity on MCF7 breast cancer cell line.[17] In the present study, we modified the labeling technique and considered the biodistribution and imaging study of complex in mice bearing tumor.

[a]*Department of Medical Physics, Tarbiat Modares University, Tehran, Iran*

[b]*Department of Radioisotope, Nuclear Science and Technology Research Institute, Tehran, Iran*

**Correspondence to: Hossein Rajabi, Department of Medical Physics, Tarbiat Modares University, P. O. Box 14115-331, Tehran, Iran.*
E-mail: hrajabi@modares.ac.ir

Results

Quality control tests

On the average 5.7–6 DOTA analogues were conjugated to trastuzumab and the labeling yield and radiochemical purity of the complex was 68 ± 1.7 and 94 ± 0.9%, respectively. The complex stability in human blood serum and phosphate buffer at 24, 48, 72 and 96 h after incubation is presented in Figure 2. The complex showed a good stability up to 96 h in both of the media. On the average 86 ± 2.3 and 81 ± 2.7% of the complex was stable in phosphate buffer and in human blood serum up to 96 h, respectively. The complex stablity in phosphate buffer was higher than that in human blood serum because the protease enzymes in blood serum could affect and hydrolyze the antibody.[18]

The result of immunoreactivity test showed that the binding of ^{177}Lu to trastuzumab did not affect the immunoreactivity of the antibody. The percentage of immunoreactivity was calculated 85.3 ± 2.5%.

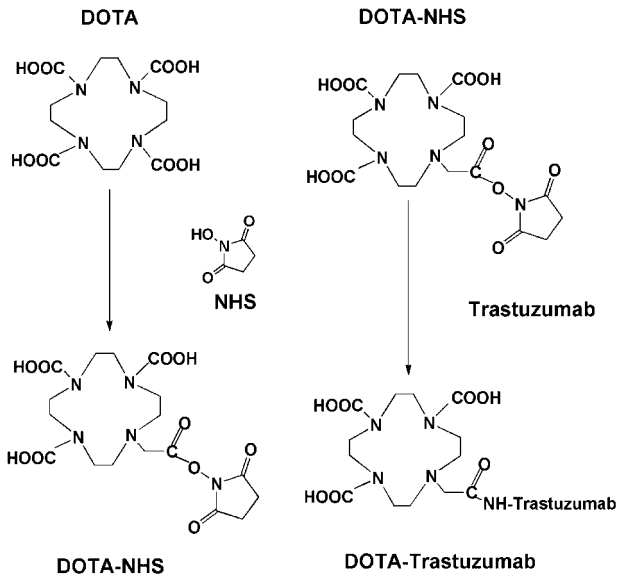

Figure 1. Synthesis strategies of trastuzumab–DOTA conjugation.

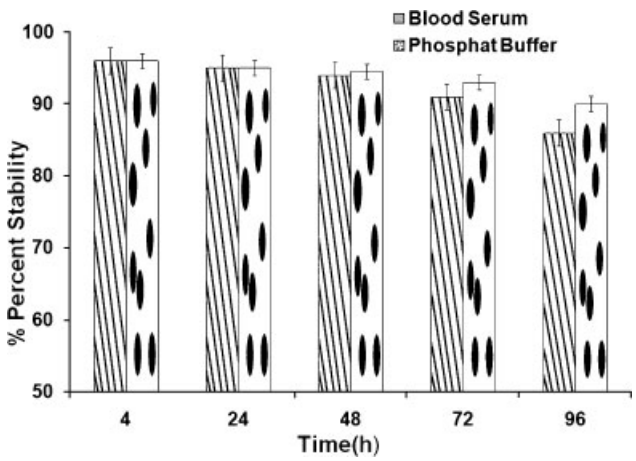

Figure 2. The complex stability in phosphate buffer and human blood serum up to 96 h after labeling.

Biodistribution studies

The immunohistochemistry results showed the spontaneous breast tumor that we used in this study expressed medium levels of HER2. The initial set of ^{177}Lu-trastuzumab biodistribution was performed in normal mice and the results are presented in Table 1. One factor likely to contribute to differences in ^{177}Lu tissue uptake is the *in vivo* stability of the metalchelate complex. The tissue distribution of [^{177}Lu]lutetium chloride was determined for comparative purposes. As the table shows, after 24 h postinjection the activity of ionic lutetium [^{177}Lu] is high in bone, liver, kidney and low in blood but ^{177}Lu-trastuzumab has very high blood uptake and low accumulation in other organs. As expected for ^{177}Lu-trastuzumab, slow clearance from the blood pool was observed. The retention of ^{177}Lu from Lutetium chloride was significantly higher than ^{177}Lu-trastuzumab in bone, liver and spleen (p-value < 0.05).

The second set of tissue distribution experiments were performed in inbred female BALB/c mice bearing breast tumor. Figure 3 illustrates the distribution of ^{177}Lu-trastuzumab 1–7 days after injection of ^{177}Lu-trastuzumab. As can be seen, the

Table 1. Biodistribution of ^{177}Lu chloride and ^{177}Lu-trastuzumab in normal mice 24 h after injection

Tissue	^{177}Lu chloride	177Lu-trastuzumab
Blood	0.97 (±0.07)	24.76 (±4.31)
Liver	9.05 (±1.73)	6.02 (±0.92)
Spleen	4.19 (±0.80)	2.70 (±0. 1)
Kidney	4.53 (±1.08)	2.90 (±0.72)
Bone	13.62 (±3.15)	2.14 (±0. 6)
Stomach	1.91 (±0.06)	1.25 (±0.04)
Intestine	1.95 (±0.67)	1.36 (±0.2)
Colon	1.89 (±0.50)	0.89 (±0.07)
Muscle	0.18 (±0.06)	0.23 (±0.02)
Lung	2.36 (±0.9)	2.09 (±0.4)
Heart	0.95 (±0.04)	2.81 (±0.89)

Values are presented as percent injected dose per gram tissue (mean ± standard deviation, $n = 5$).

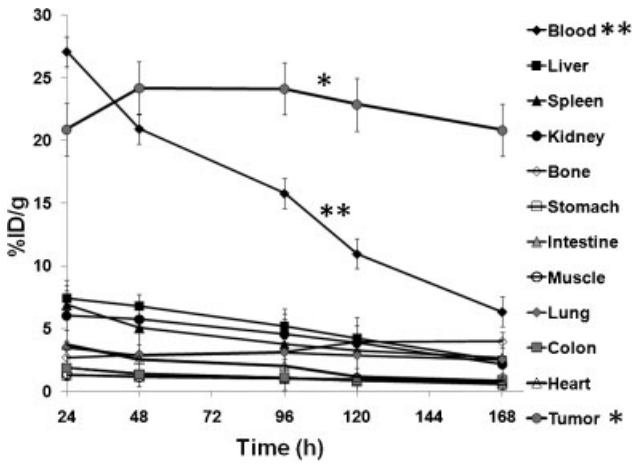

Figure 3. Tissue distribution of ^{177}Lu activity in mice bearing tumor after injection of ^{177}Lu-trastuzumab. Values are presented as percent injected dose per gram of tissue (%ID/g, $n = 5$), the tumor uptake(*) is increased with time and blood activity(**) decreased.

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Accumulation of radioactivity in the tumor after 48 h was reached to a maximum of 24.14 ± 3.96 %ID/g and remained about constant (24.11 ± 3.61 %ID/g) up to 96 h. This concentration decreased with time in all organs, excluding the tumor, which continued to accumulate radioactivity until 96 h after injection. The radioactivity concentration was always below 8% for all normal organs. The tumor uptake values were at all times higher than the values in normal organs.

The tumor to organ ratio is important to evaluate the potential future use of the labeled antibody for HER2 receptor visualization. In Table 2 the tumor to organ ratio of all organs are presented. Table 2 showed that tumor to blood ratio was 0.77 at 24 h after injection and continually increased to 3.29 after 168 h.

Gamma camera imaging

The gamma camera images at 24 and 168 h after injection of ^{177}Lu-trastuzumab are shown in Figure 4. The tumors accumulated large amounts of ^{177}Lu. Small amounts of radioactivity could also be found in the liver area. The tumor uptake was even high up to 168 h after injection. A region of interest analysis of the tumor and corresponding lateral region gave a tumor to background ratio of 20 ± 3.5 for 24 h and 30 ± 1.2 for 168 h.

Discussion

Breast cancer patients are usually tested for the levels of HER2 in their tumors. In a normal cell, HER2 controls aspect of cell growth and division. When activated in cancer cells, HER2 accelerates tumor growth.[19] HER2 is overexpressed in about 20–30% of breast cancers.[4] These patients may be candidate for trastuzumab treatment in the postsurgical or metastatic setting. Trastuzumab potentially may be used as a tumor-specific carrier in RIT.[8–10] Owing to the cardiotoxicity of trastuzumab and its complication in cardiac patients this new application can be helpful at least for the patients who are unable to tolerate trastuzumab treatment because of pre-existing heart problems.[20] Moreover trastuzumab is expensive and the cost of cancer treatment is very high.[21] In RIT less amount of trastuzumab is required therefore the side effects and the cost of treatment will be less. There are many radioisotopes that could be used to label with trastuzumab.[11] Lutetium, radionuclide emitting short-range β-particles (0.04–1.8 mm) with low energy(0.497 MeV, 78.7%), is suitable for therapeutic purposes because of its low tissue penetration range which may render it more effective for small tumors.[11]

In this study we modified labeling method of ^{177}Lu to trastuzumab and performed the quality control tests, biodistribution study and gamma camera imaging in mice bearing tumor.

In previous study the DOTA/Antibody ratio was calculated 2.5–3.[17] With some modifications in present study this ratio improved to 5.7–6. We were able to raise the labeling yield to 68 ± 1.7% and radiochemical purity to 94 ± 0.7%. The immunoreactivity of the complex was 85.3 ± 2.5%, which did not show much reduction in binding ability of the antibody. The stability in phosphate buffer and blood serum was 86 ± 2.3 and 81 ± 2.7% up to 96 h, respectively.

Table 2. Tumor/Organ ratios for 177Lu-trastuzumab in mice bearing tumor

Tissue	24 h	48 h	96 h	120 h	168 h
Blood	0.77 (±0.04)	1.15 (±0.08)	1.52 (±0.07)	2.08 (±0.10)	3.29 (±0.09)
Liver	2.82 (±0.97)	3.58 (±0.52)	4.65 (±1.04)	5.45 (±1.28)	8.59 (±1.57)
Spleen	3.03 (±1.03)	4.78 (±1.0)	6.42 (±1.09)	7.11 (±1.43)	7.79 (±0.97)
Kidney	3.47 (±0.97)	4.22 (±1.11)	5.29 (±1.08)	5.91 (±0.99)	9.81 (±1.96)
Bone	7.78 (±0.97)	8.26 (±1.52)	7.67 (±1.08)	5.9 (±0.88)	5.26 (±0.97)
Stomach	16.17 (±2.61)	20.99 (±3.53)	24.35 (±4. 10)	26.88 (±4.98)	37.83 (±5.17)
Intestine	11.16 (±2.34)	17.49 (±2.68)	22.74 (±3.74)	28.92 (±3.39)	32.51 (±5.20)
Colon	11.21 (±2.10)	18.15 (±2.06)	22.32 (±3.39)	26.56 (±3.54)	24.48 (±2.81)
Muscle	15.81 (±1.88)	21.74 (±1.95)	23.63 (±2.63)	23.55 (±2.49)	25.07 (±2.67)
Lung	5.99 (±1.05)	8.41 (±1.29)	7.8 (±1.47)	8.01 (±1.35)	8.324 (±1.43)
Heart	5.62 (±1.28)	9.54 (±1.56)	11.87 (±1.64)	19.83 (±2.55)	25.69 (±3.12)

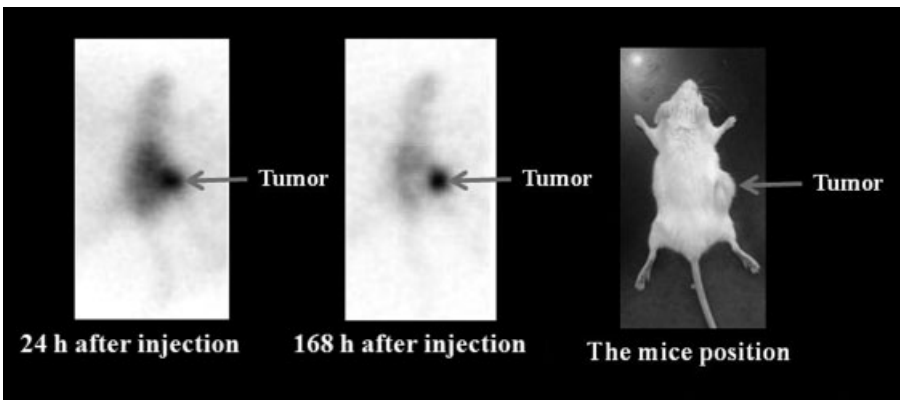

Figure 4. The gamma camera images were acquired at 24 and 168 h after injection of ^{177}Lu-trastuzumab.

In selecting a labeling method for RIT, minimizing normal organ activity levels is most important because dose limiting normal tissue toxicity is perhaps the greatest impediment to successful tumor treatment. For this reason, we evaluated the tissue distribution of the ^{177}Lu-labeled trastuzumab. A comparison between ^{177}Lutetium chloride and ^{177}Lu-labeled trastuzumab in normal mice showed ionic ^{177}Lu was significantly (p-value < 0.05) higher in bone, liver and kidney after administration of ^{177}Lutetium chloride. The biodistribution and imaging studies in mice bearing tumor showed a good tumor uptake up to 7 days and low acumulations in other organs. There was no non-specific uptake controls performed in this study, but the high tumor uptake and tumor to blood ratios greater than 3:1 after 168 h suggest that this uptake is specific.

It was reported that the tumor to blood ratio with ^{188}Re-trastuzumab was 1.86 at 48 h after administration in nude mice bearing NPC cells (overexpress HER2).[9]

The biodistribution study of ^{177}Lu-DTPA-pertuzumab in BALB/c (nu/nu) mice carrying SKOV-3 (overexpress HER2) showed the tumor to blood ratio was 1.6 at 24 h.[22] In another study with ^{177}Lu-mu81C6 the tumor to blood ratio in athymic mice bearing subcutaneous human glioma D-54 MG xenografts was calculated 0.73, 1.15, 1.32 and 1.34 at 24, 48, 120 and 168 h, respectively.[16]

In this study, the tumor to blood ratio was increased gently (0.77, 1.15, 2.08 and 3.29 at 24, 48, 120 and 168 h, respectively) because the tumor that we used expressed medium levels of HER2 (from immunohistochemistry results), so its uptake from ^{177}Lu-trastuzumab was lower than the tumor that overexpress HER2.

It was reported that antibody labeled with ^{177}Lu via DOTA-NHS had high activity accumulation in normal organs.[16] In that study antibody was labeled to lutetium via DOTA-NHS with chelate/antibody ratio of 4. The antibody-NHS-DOTA demonstrated 29% incorporation of ^{177}Lu and a high accumulation of ^{177}Lu in the bone, liver and spleen at 24 h, while ^{177}Lu-DOTA-trastuzumab demonstrated good tumor uptake and low activity in normal tissues. Most probably the normal organ activity of ^{177}Lu depends on both the characteristics of the mAb as well as the properties of the chelate being used.

Materials and methods

Materials

Trastuzumab was purchased in 140 mg vials (Genentech, South San Francisco). ^{177}Lu was produced by bombarding $^{176}Lu_2O_3$ (CAMPRO scientific, Germany, 74%) dissolved in 0.05 M HCl in the Tehran research reactor (at 2.6×10^{13} n Cm^{-2} S^{-1} flux for 14 days). All chemical agents were purchased from Fluka.

SKBr3(Pasteur institute, Tehran, Iran) were grown and routinely maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated in a humidified atmosphere (95% air and 5% CO_2) at 37°C. SKBr3 is a hormone-independent cell line originally derived from a breast adenocarcinoma expressing high level of HER2.[23]

Buffers

All buffers utilized for radiometal ion labeling must be prepared as metal-free solutions. For preparing a 10× conjugation carbonate buffer, $NaHCO_3$ (40.22 g), Na_2CO_3 (2.25 g) and NaCl (87.66 g) were dissolved in 1 l deionized water. Before using trustuzumab in conjugation, it must be dialyzed against carbonate buffer combined with 0.5 M EDTA at pH 8.6 for 24 h then againts carbonate buffer without EDTA for 48 h. The Cu(II)–Arsenazo(III) complex was supplied by dissolving Arsenazo(2 mg), $CuCl_2$ (21.3 mg) and NH_4OAc (2.9 g) in 250 ml deionized water (pH = 7) and storing in dark at 4°C.

Preparation of trastuzumab–DOTA

DOTA-NHS ester prepared before[17](1 mg) was dissolved in 2.5 ml carbonate buffer (pH 8.6). An aliquot of trastuzumab (5 mg, 2 ml) in carbonate buffer was slowly added to DOTA-NHS solution and gently mixed for 24 h at room temperature. Trastuzumab–DOTA conjugation was then separated from unreacted chelate by dialysis against ammonium acetate buffer (0.25 M, pH = 7) combined with 0.5 M EDTA for 24 h and without EDTA for 48 h. The synthesis strategies of trastuzumab–DOTA conjugation are shown in Figure 1.

The number of chelate conjugated to trastuzumab was determined by spectrophotometric assay based on the titration of the Cu(II)–Arsenazo(III) complex.[24] Breifly, A UV/V spectrometer was zeroed against a cuvette filled with 1 ml of Cu-AA reagent. Then 50 μl of trastuzumab–DOTA was added to the cuvette after removing 50 μl of Cu-AA reagent and incubated for 20 min in dark. The absorbance values were read at 590 nm. The final protein concentration was also determined by Lowry method.[25]

Labeling of trastuzumab–DOTA with ^{177}Lu

5–8 mCi of $^{177}LuCl_3$ was added to ammonium acetate buffer (0.10 ml, 0.25 M, pH = 7.0), after addition of trastuzumab–DOTA conjugate (100–200 μg), the pH was adjusted to 5 with ammonia. The complex was incubated at 37°C for 3 h then an aliquot of EDTA (10 mM, 1/9 volume of the samples) was added to the sample and allowed to bind with free lutetium (15 min at room temperature). Trastuzumab–DOTA-^{177}Lu complex were purified by gel filtration on Sephadex G-25 column and eluted with PBS supplemented with 0.5% bovine serum albumin.

Quality control of the trastuzumab–DOTA-^{177}Lu complex

Labeling yield and radiochemical purity of the complex was determined as described previously.[17] The complex stability in buffer and human blood serum was analyzed by ITLC at 24, 48, 72 and 96 h after incubation. The immunoreactivity of the complex on SKBr3 cells was also checked based on the method described by Lindmo *et al.*[26]

Biodistribution studies in mice bearing tumor

The tumors were originally established from a spontaneous breast tumor (a murine mammary carcinoma) in an inbred female BALB/c mouse. The breast tumor was established by subcutaneous implantation of the tumor fragments ($\approx 1\ mm^3$) in the right flank region of normal inbred female BALB/c mice (20–25 g, 8–10 weeks old). The tissue distribution experiments were performed when the tumor volumes reached 7–8 mm^3. The reaction between trastuzumab and the tumor cells was demonstrated by immunohistochemistry using the rabbit antimouse peroxidase technique.[27]

The first set of tissue biodistribution experiments were performed in inbred femal normal BALB/c mice weighing 20–25 g. Ten mice were injected (250 μCi/0.1 ml) intravenously with ^{177}Lu-trastuzumab. The Groups of five animals were killed by

CO_2 gas at 4 and 24 h after injection. The animals were dissected, tissues were removed, weighed and counted for ^{177} Lu using a dual-channel automated gamma counter. The harvested tissues (blood, liver, spleen, kidney, stomach, intestine, colon, heart, lung and bone) were compared with the percentage of injected activity per gram of tissues (%ID/g). In order to facilitate analyses of these biodistribution results, an additional experiment was performed in a group of five animals which received 250 μCi/0.1 ml of ^{177}Lu (lutetium chloride) were killed 4 and 24 h after injection.

The second set of tissue distribution experiments were performed in inbred female BALB/c mice bearing breast tumor. In seven separate studies, mice ($n = 5$) were injected intravenously with ^{177}Lu-trastuzumab(250 μCi/0.1 ml). Groups of five animals were killed at 24, 48, 96, 120 and 168 h postinjection and analyzed as described above. Animal experiments were performed in compliance with the regulation of our institution and with generally accepted guidelines governing such work.

Imaging studies

The biological behavior of ^{177}Lu-trastuzumab was also ascertained by carrying out simultaneous scintigraphic imaging studies in mice bearing breast tumor ($n = 5$). ^{177}Lu-trastuzumab(250 μCi/0.1 ml) was injected intravenously via the tail vein. Serial scintigraphic images were recorded at 24, 48, 96 and 168 h after injection using a low-energy, high-resolution collimator. Prior to the acquisition of images, the animals were anesthetized using a combination of xylazine hydrochloride and ketamine hydrochloride. All the images were recorded by acquiring 300 K counts per pixel in 512 × 512 matrix size.

Statistical analysis

The tissue uptakes were compared among the groups by one-way analysis of variance using statistical software SPSS 13.0. For all the tests, p-values < 0.05 were considered to indicate statistical significance. The results are presented as mean ± SD.

Conclusion

From these results we can conclude that the combination of trastuzumab and ^{177}Lu has some good promising properties on mice bearing tumor including the good *in vivo* stability and tumor uptake. So, the complex has the potential for further investigation as a RIT agent in the treatment of the breast cancer.

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