**¹¹¹In-Labeled Trastuzumab-Modified Gold Nanoparticles are Cytotoxic
In Vitro to HER2-Positive Breast Cancer Cells and Arrest Tumor
Growth *In Vivo* in Athymic Mice After Intratumoral Injection**

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ABSTRACT

Introduction: Gold nanoparticles (AuNP; 30 nm) were modified with polyethylene glycol (PEG) chains linked to trastuzumab for binding to HER2-positive breast cancer (BC) cells and diethylenetriaminepentaacetic acid (DTPA) for complexing the Auger electron-emitter, ^{111}In (trastuzumab-AuNP- ^{111}In). Our objective was to determine the cytotoxicity of trastuzumab-AuNP- ^{111}In on HER2-positive BC cells *in vitro* and evaluate its tumor growth-inhibition properties and normal tissue toxicity *in vivo* following intratumoral (i.t.) injection in mice with s.c. HER2-overexpressing BC xenografts.

Methods: Binding and internalization of trastuzumab-AuNP- ^{111}In or non-targeted AuNP- ^{111}In in SK-BR-3 ($1-2 \times 10^6$ HER2/cell) and MDA-MB-361 (5×10^5 HER2/cell) human BC cells were studied. The surviving fraction (SF) of SK-BR-3 or MDA-MB-361 cells exposed to trastuzumab-AuNP- ^{111}In or AuNP- ^{111}In was determined. DNA double-strand breaks (DSBs) were assayed by probing for $\gamma\text{-H2AX}$. Tumor growth was monitored over 70 days in CD1 athymic mice with s.c. MDA-MB-361 xenografts after i.t. injection of 10 MBq (0.7 mg; 2.6×10^{12} AuNP) of trastuzumab-AuNP- ^{111}In and normal tissue toxicity was assessed by monitoring body weight, complete blood cell (CBC) counts and serum alanine aminotransferase (ALT) and creatinine (Cr).

Results: Trastuzumab-AuNP- ^{111}In was specifically bound by SK-BR-3 and MDA-MB-361 cells. Trastuzumab-AuNP- ^{111}In was more efficiently internalized than AuNP- ^{111}In and localized to a peri-nuclear region. The SF fraction of SK-BR-3 cells was reduced by 1.8-fold by treatment with 3 nM (7 MBq/mL) of trastuzumab-AuNP- ^{111}In . The SF of MDA-MB-361 cells was reduced by 3.7-fold at 14.4 nM (33.6 MBq/mL). In comparison, non-targeted AuNP- ^{111}In at these concentrations reduced the SF of SK-BR-3 or MDA-MB-361

cells by 1.2-fold ($P=0.03$) and 1.7-fold ($P<0.0001$), respectively. DNA DSBs were greater in SK-BR-3 and MDA-MB-361 cells exposed to trastuzumab-AuNP- ^{111}In compared to AuNP- ^{111}In , but unlabeled trastuzumab-AuNP did not increase DNA DSBs. Local i.t. injection of trastuzumab-AuNP- ^{111}In in CD1 athymic mice with s.c. MDA-MB-361 tumors arrested tumor growth for 70 days. There was no apparent normal tissue toxicity. The radiation absorbed dose deposited in the tumor by trastuzumab-AuNP- ^{111}In was 60.5 Gy, while normal organs received <0.9 Gy.

Conclusion: These results are promising for further development of trastuzumab-AuNP- ^{111}In as a novel Auger electron-emitting radiation nanomedicine for local treatment of HER2-positive BC.

Advances in Knowledge and Implications for Patient Care: A local radiation treatment for HER2-positive BC based on AuNP modified with trastuzumab and labeled with the Auger electron-emitter, ^{111}In was developed and shown to arrest tumor growth with no normal tissue toxicity.

Keywords: : indium-111; gold nanoparticles; radiotherapy; breast cancer; HER2; Auger electrons; antibody immunoconjugates

Abbreviated title

^{111}In -labeled and trastuzumab-modified AuNP

1. Introduction

Gold nanoparticles (AuNP) have been studied as radiosensitizers for X-radiation treatment of cancer due to their ability to convert low linear energy transfer (LET) X-rays interacting with the gold surface to high LET and more radiobiologically damaging Auger and photoelectrons [1]. AuNP may passively localize in tumors following systemic intravenous (i.v.) injection via the enhanced permeability and retention (EPR) effect due to leaky tumor vasculature and poor lymphatic drainage [2]. AuNP may also be surface modified with monoclonal antibodies (mAbs) or peptide ligands to bind to tumor-associated antigens or receptors for active tumor targeting [3]. A major limitation of i.v. administration of AuNP is sequestration by the liver and spleen, causing rapid elimination from the blood and low tumor uptake [4]. We previously modified AuNP (30 nm) with polyethylene glycol (PEG) chains linked to trastuzumab to target HER2-positive breast cancer (BC) cells and to 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid (Bn-DTPA) to complex ^{111}In [5, 6]. These trastuzumab-AuNP- ^{111}In were further surface coated with PEG to stabilize the particles to aggregation and minimize liver and spleen uptake [4]. Despite PEG modification, tumor uptake in athymic mice with s.c. HER2-overexpressing MDA-MB-361 human BC xenografts at 48 h post i.v. injection (p.i.) was low [1.2 percent injected dose/g (% i.d./g)] [6]. Liver uptake of trastuzumab-AuNP- ^{111}In was modest (<3% i.d/g) and spleen uptake was high (20% i.d./g). To avoid liver and spleen uptake, and maximize tumor localization of trastuzumab-AuNP for X-ray radiosensitization, we studied local intratumoral (i.t.) injection of these AuNP. This route of administration yielded 25-fold higher tumor concentrations of trastuzumab-AuNP- ^{111}In

at 48 h p.i. (30% i.d./g) and spleen uptake was decreased by 10-fold (<2% i.d./g) [6]. Liver uptake was modestly reduced (1.6% i.d./g). Thus, i.t. injection of trastuzumab-AuNP was employed to study their radiosensitizing effects on MDA-MB-361 tumors **treated with X-radiation** [7]. Treatment of MDA-MB-361 tumors *in vivo* with trastuzumab-AuNP combined with 11 Gy of X-radiation decreased tumor size by 2-fold, whereas tumors treated with X-radiation alone increased slightly in size. Untreated tumors grew exponentially.

Although trastuzumab-AuNP enhanced the effectiveness of X-radiation treatment of MDA-MB-361 tumors, the radiosensitizing properties were **quite** modest [7]. A relatively unexplored strategy is the use of AuNP to deliver radionuclides to cancer cells for radiation treatment. We recently reported that AuNP (30 nm) modified with PEG chains linked to panitumumab for binding epidermal growth factor receptors (EGFR) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelators for complexing the β -particle emitter, ^{177}Lu were bound and internalized by EGFR-positive human BC cells [8]. Exposure of MDA-MB-468 cells with high EGFR density (1.3×10^6 receptors/cell) *in vitro* to panitumumab-AuNP- ^{177}Lu decreased their survival to <0.001%. Subsequently, we found that i.t. injected panitumumab-AuNP- ^{177}Lu arrested the growth of MDA-MB-468 tumor xenografts in athymic mice, with no normal tissue toxicity at the amounts administered (4.5 MBq; 6×10^{11} AuNP) [9]. These results are highly promising for a novel “radiation nanomedicine” strategy for local treatment of BC. This strategy has potential clinical application as a form of neoadjuvant brachytherapy in which the seeds are nanometer in size and are **bound and internalized** into tumor cells to increase the

pathology complete response (pCR) rate, correlated with improved post-surgical outcome in BC, particularly for locally-advanced tumors [10].

In addition to β -particles, Auger electrons have potential for cancer treatment [11].

^{111}In emits 15 very low energy (<25 keV) Auger electrons, in addition to its γ -emissions [$E_{\gamma}=171$ keV (90%) and 245 keV (94%)]. These electrons have nanometer-micrometer range, but inflict lethal DNA damage in cells, especially if released near the cell nucleus, due to their high LET (4-26 keV/ μm) [12]. We previously reported that trastuzumab labeled with ^{111}In and modified with nuclear translocation sequence (NLS) peptides to route the internalized radioimmunoconjugates to the cell nucleus, caused multiple DNA double-strand breaks (DSBs) decreasing the surviving fraction of SK-BR-3 human BC cells by $>90\%$ [13]. Furthermore, a single dose of ^{111}In -NLS-trastuzumab (9.25 MBq; 4 mg/kg) strongly inhibited the growth of s.c. HER2-overexpressing MDA-MB-361 human BC xenografts in athymic mice with no normal tissue toxicity [14]. Since we previously constructed trastuzumab-AuNP- ^{111}In and showed that they provided high concentrations of radioactivity in tumors after i.t. injection which were retained, we hypothesized that these AuNP may be effective for local treatment of HER2-positive BC due to the Auger electron emissions of ^{111}In . In this report, we now describe for the first time the cytotoxicity *in vitro* of trastuzumab-AuNP- ^{111}In on HER2-positive BC cells and their tumor growth-inhibitory properties and normal tissue toxicity *in vivo* after i.t. injection in athymic mice with s.c. HER2-overexpressing MDA-MB-361 human BC xenografts.

2. Materials and methods

2.1 ¹¹¹In-labeled gold nanoparticles

Trastuzumab-modified **HER2-targeted** gold nanoparticles (AuNP) labeled with ¹¹¹In (trastuzumab-AuNP-¹¹¹In-), **non-targeted** ¹¹¹In-AuNP and unlabeled trastuzumab-modified AuNP (trastuzumab-AuNP) were constructed as previously reported [6]. Briefly, 500 µg of trastuzumab (Herceptin, Roche Pharmaceuticals, Mississauga, ON, Canada) in 50 µL of 100 mM NaHCO₃, pH 9.3 was reacted with a 25-fold molar excess of orthopyridyldisulfide-polyethyleneglycol-*N*-hydroxysuccinimide (OPSS-PEG_{5k}-SVA, MW = 5 kDa; Laysan Bio, Arab, AL) overnight at 4°C. PEGylated trastuzumab (trastuzumab-PEG-OPSS) was purified and buffer exchanged into phosphate buffered saline (PBS), pH 7.5, by ultrafiltration on a Vivaspın device (MW cut-off = 30 kDa; Sartorius, Bohemia, NY). Thiolated PEG (MW = 2 kDa) derivatized with benzylisothiocyanate diethylenetriaminepentaacetic acid (SH-PEG_{2k}-Bn-DTPA; 10 µg) was then **complexed** with ¹¹¹InCl₃ (Nordion, Kanata, ON, Canada) in 1 M sodium acetate buffer, pH 6.0 at room temperature (RT) for 30 min. The final radiochemical purity of SH-PEG_{2k}-Bn-DTPA-¹¹¹In was >95% measured by instant thin layer-silica gel chromatography (ITLC-SG; Pall Life Sciences, Mississauga, ON, Canada) developed in 100 mM sodium citrate, pH 5.0. Finally, trastuzumab-AuNP-¹¹¹In were constructed by reacting 1 mL of 30 nm AuNP (2 × 10¹¹ AuNP; Ted Pella, Redding, CA) with 0.4 µg of SH-PEG_{3k}-Bn-DTPA-¹¹¹In (0.7-0.8 MBq) at 4°C for 30 min, then with 10 µg trastuzumab-PEG-OPSS for 5 min. Trastuzumab-AuNP-¹¹¹In were finally surface-coated with an excess (7.6 µg) of PEG_{2k}-SH for 30 min to

prevent aggregation *in vitro* and minimize liver and spleen uptake *in vivo*. Trastuzumab-AuNP-¹¹¹In were purified from excess reagents by centrifugation at $2,500 \times g$ for 30 min at 4°C followed by $15,000 \times g$ for 30 min at 4°C. The supernatant was carefully removed and trastuzumab-AuNP-¹¹¹In were re-suspended in 1 mL of PBS, pH 7.5. This process was repeated once. It was determined that there were 37 SH-PEG_{2k}-Bn-DTPA and 14 trastuzumab-PEG-OPSS per AuNP using a **previously reported** radiometric assay [6]. Non-HER2 targeted AuNP-¹¹¹In not modified with trastuzumab and unlabeled trastuzumab-AuNP were similarly prepared without reaction with trastuzumab-PEG-OPSS or with SH-PEG_{2k}-Bn-DTPA-¹¹¹In, respectively.

2.2 Breast cancer (BC) cells and tumor xenograft mouse model

SK-BR-3 and MDA-MB-361 human breast cancer (BC) cells overexpressing HER2 ($1-2 \times 10^6$ or 5×10^5 HER2 per cell, respectively) [15] were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SK-BR-3 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) under a 5% CO₂ atmosphere at 37°, while MDA-MB-361 cells were cultured in Leibovitz L15 medium (Sigma-Aldrich) supplemented with 20% FBS under 100% air at 37°C. A BC xenograft mouse model was established by first implanting a 0.72 mg, 60 **day** sustained release 17β-estradiol pellet (Innovative Research of America, Sarasota, FL) subcutaneously (s.c.) into female CD1-athymic mice (Charles River, Wilmington, MA) followed 2 **days** later by s.c. inoculation of 1×10^7 MDA-MB-361 cells in 250 μL of a 1:1 mixture of Matrigel (BD Biosciences, Franklin Lakes, NJ) and serum-free media. Tumor xenografts were permitted to grow to a

diameter of 5-8 mm before i.t. injection with trastuzumab-AuNP-¹¹¹In or normal saline. All animal experiments were carried out in compliance with Canadian Council on Animal Care (CCAC) guidelines and under a protocol approved by the Animal Care Committee of the University Health Network (Protocol 2780.4).

2.3 HER2 immunoreactivity

The immunoreactivity of trastuzumab-AuNP-¹¹¹In with HER2-overexpressing SK-BR-3 cells was determined in a direct (saturation) binding assay. Briefly, 1.5×10^4 SK-BR-3 cells per well were seeded into 96-well plates and cultured overnight at 37 °C. The cells were incubated at 4 °C for 30 min with PBS, pH 7.5 with or without 1 mM trastuzumab. The buffer solution was removed, and the cells were incubated for 3 h at 4°C with trastuzumab-AuNP-¹¹¹In (0 to 15 nM in PBS, pH 7.5) with or without 1 mM trastuzumab to block HER2 to measure non-specific binding (NSB) or total binding (TB), respectively. Specific binding was determined by subtracting NSB from TB. Following the incubation period, non-cell bound radioactivity was removed. The cells were then dissolved by incubation in 0.1 M NaOH at RT for 5 min and the cell lysates transferred to γ -counting tubes. This process was repeated 3 times and the radioactivity in the combined cell lysates was measured in a γ -counter (Wizard Model 1480, PerkinElmer, Turku, Finland). The number of cells in each well was estimated by counting 3 untreated control wells seeded overnight with 1.5×10^4 SK-BR-3 cells using a Scepter automatic cell counter (EMD Millipore, Billerica, MA). TB, NSB and SB were plotted vs. the concentration (nM) of trastuzumab-AuNP-¹¹¹In. The SB curve was fitted to a 1-site saturation receptor binding model using Prism Ver. 4.0 software (GraphPad, San Diego,

CA) and the dissociation constant (K_D) and maximum receptors per cell (B_{max}) were calculated.

2.4 Internalization studies

The internalization of trastuzumab-AuNP- ^{111}In or AuNP- ^{111}In by SK-BR-3 or MDA-MB-361 cells was determined by subcellular fractionation. Briefly, 1×10^5 cells per well were seeded overnight into 24-well plates, then incubated for 24 h with trastuzumab-AuNP- ^{111}In or AuNP- ^{111}In (200 μL , 3.0 nM, 1.4 MBq). The cells were washed with normal saline twice after removal of the medium, then harvested by trypsinization, counted and transferred to 1.5 mL Eppendorf tubes. The cell pellets were separated from the supernatant, which contained some cell surface (CS) bound radioactivity, by centrifugation at $500 \times g$ for 5 min. The remaining cell-surface bound radioactivity was then displaced from the cells by resuspending for 5 min on ice with 500 μL of 200 mM sodium acetate/0.5 M NaCl, pH 2.5. The cell suspensions were centrifuged again at $1,020 \times g$ for 5 min to separate the radioactivity internalized into the cytoplasm (Cy) in the cell pellet from radioactivity on the CS in the supernatant, and each was measured in a γ -counter. Images of the same slides used for evaluation of DNA double-strand breaks (DSBs) in SK-BR-3 and MDA-MB-361 cells exposed to trastuzumab-AuNP- ^{111}In and AuNP- ^{111}In (see section 2.5) were taken with an Olympus BX50 upright microscope (Olympus, Tokyo, Japan) in dark field mode using a 20 $\times$ /0.55 lens and wide field fluorescence mode with excitation at 364 nm for visualization of DAPI using 385-470 nm emission filters. Images were merged using ImageJ (U.S. National Institutes of Health, Bethesda, MD) and stored as tiff files.

These images permitted qualitative assessment of the binding and internalization of the AuNP into these cells.

2.5 Clonogenic assays and DNA double-strand breaks (DSBs)

The effect of exposure *in vitro* to trastuzumab-AuNP-¹¹¹In or AuNP-¹¹¹In on the surviving fraction (SF) of SK-BR-3 and MDA-MB-361 cells was measured in clonogenic assays. Sub-confluent cells were harvested from culture by trypsinization and seeded into 24-well tissue culture plates (1×10^5 cells/well) and cultured overnight. The culture medium was then replaced with 200 μ L of fresh medium with or without 3 nM (7.0 MBq/mL) or 14.4 nM (33.6 MBq/mL MBq/mL) of trastuzumab-AuNP-¹¹¹In or AuNP-¹¹¹In. The cells were then incubated for 18 h. Cells were harvested by trypsinization, counted using a Scepter automatic cell counter and 500 cells per well were seeded in triplicate into 6-well tissue culture plates containing 3 mL of fresh medium per well. Following culturing at 37°C and 5% CO₂ for 12 days, surviving colonies were stained with methylene blue (1% in a 1:1 mixture of ethanol and water). Three images of each stained dish were scanned using a FluorChem gel documentation system (Bio-Rad, Mississauga, ON, Canada) and analyzed by ImageJ software with customized macros to count surviving colonies, following a protocol **reported** previously [16]. Only colonies containing >50 cells were counted. The plating efficiency (PE) was defined as the number of surviving colonies divided by the number of cells seeded. The SF was calculated by dividing the PE of treated cells by the PE of untreated cells.

The effect of trastuzumab-AuNP-¹¹¹In, AuNP-¹¹¹In or unlabeled AuNP exposure on induction of DNA DSBs in SK-BR-3 and MDA-MB-361 cells was studied by confocal

immunofluorescence microscopy probing for phosphorylated histone-2A (γ -H2AX) which accumulate at sites of unrepaired DSBs [17]. Approximately 1×10^5 cells were seeded onto a sterilized glass coverslip (10 mm diameter, 0.13-0.17 mm thickness, Ted Pella, Redding, CA) in wells in a 24-well tissue culture plate and cultured overnight. The culture medium was then replaced with 200 μ L of fresh medium with or without 3.0 nM (7.0 MBq/mL) of trastuzumab-AuNP- ^{111}In or AuNP- ^{111}In and the cells incubated for an additional 18 h as per the clonogenic assay procedure. The medium was removed, the cells were rinsed with PBS and then immunostained for γ -H2AX as previously reported [18]. Briefly, cells were fixed, permeabilized, blocked with bovine serum albumin (BSA)-donkey serum (Sigma-Aldrich), then incubated with anti-phospho-histone H2AX (Ser139, clone JBW301) mouse monoclonal IgG₁ (Upstate Biotechnology, Billerica, MA) followed by Alexa Fluor® 488 donkey anti-mouse IgG (H+L) (Invitrogen Molecular Probes, Carlsbad, CA). Cover glasses were mounted on microscope slides (25 $\times$ 75 $\times$ 1 mm, Fisher Scientific, Toronto, ON, Canada) using Vectashield® Mounting Medium with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories, Burlington, ON Canada) to counterstain the cell nucleus. Cover glasses were wrapped in aluminum foil and stored at 4 °C for image acquisition. Images of γ -H2AX foci and nuclei were acquired with a confocal microscope (LSM 700, Carl Zeiss MicroImaging, Thornwood, NY) using acquisition software LSM Zen 2009, a Plan-Apochromat 40x/1.4 Oil DIC M27 objective (Carl Zeiss), and laser scanning mode set as plane with two solid state laser lines (405 and 488 nm) chosen. An optical section ($\sim$ 1.0 μ m) through the center of the cell nucleus was imaged. The laser intensity (2% for both lines), signal amplification and offset, and image resolution (0.156 μ m/pixel) were kept constant for all image acquisitions. At least 50 cells were imaged per

slide. Images were processed to derive the mean integrated density of γ -H2AX foci per unit nucleus area using ImageJ software with customized macros as reported [18].

2.6 Radiation dose estimates *to tumors and normal organs*

Radiation absorbed doses to normal organs **in CD1 athymic mice with s.c. MDA-MB-361 tumors** were estimated using the Medical Internal Radiation Dose (MIRD) formalism: $D = \tilde{A} \times S$, where D is the dose to a target organ from cumulative radioactivity ($\tilde{A}$) in a source organ, and S is the Snyder Factor [19]. The $\tilde{A}$ in selected source organs was quantified from microSPECT/CT images obtained at 2, 24 and 48 h post i.t. injection of trastuzumab-AuNP- ^{111}In or AuNP- ^{111}In (10 MBq, 0.7 mg; 2.6×10^{12} AuNP). The microSPECT/CT imaging protocol and representative images were previously reported by our group [6]. Since the liver and tumor were the only two major organs visualized on the images, and the S -values for mouse organs reported by Bitar et al. [20] did not include tumor as a source organ, the $\tilde{A}$ in the liver and the remainder of the body (excluding the liver) source organs were used to estimate the absorbed dose to target normal organs. The dose to the tumor was estimated based on the self-dose from radioactivity **accumulated** in the tumor. Cumulative radioactivity from 0-48 h ($\tilde{A}_{0-48\text{h}}$) was estimated from the area under the curve [$\text{AUC}_{0-48\text{h}}$; $\text{Bq} \times \text{second (s)}$] for the tumor, liver and remainder of the body using Prism Ver. 4.0 software (GraphPad). The cumulative radioactivity from 48 h to infinity ($\tilde{A}_{48\text{h}-\infty}$) was obtained by dividing the radioactivity at 48 h by the physical decay constant of ^{111}In ($2.86 \times 10^{-6} \text{ s}^{-1}$), thus assuming elimination only by radioactive decay after 48 h. The combined $\tilde{A}_{0-\infty}$ values for each source organ were multiplied by the S -value ($\text{Gy} \times \text{Bq}^{-1} \times \text{s}^{-1}$) for ^{111}In to estimate the radiation absorbed doses (Gy). The S -values for mouse

organs were taken from Bitar et. al. [20], while the S-values ($3.47 \times 10^{-11} \text{ Gy} \times \text{Bq}^{-1} \times \text{s}^{-1}$) for the tumor were obtained from Olinda/EXM Radiation Dose Assessment software (Vanderbilt University, Nashville, TN, USA) [21], assuming that ^{111}In was homogenously distributed in a 6.4 mm diameter sphere.

2.7 Tumor growth inhibition and normal tissue toxicity studies

Tumor growth inhibition and normal tissue toxicity were assessed in groups of 6 female CD1 athymic mice with s.c. MDA-MB-361 human BC xenografts injected i.t. with trastuzumab-AuNP- ^{111}In (10 MBq, 0.7 mg; 2.6×10^{12} AuNP) in 100 μL of normal saline. Control mice received an i.t. injection of normal saline only. The injected volume (100 μL) was administered in multiple fractions at different sites in the tumor to allow more homogeneous distribution of AuNP. Since trastuzumab-AuNP- ^{111}In exhibited greater cell-binding and internalization into MDA-MB-361 cells, and caused more DNA DSBs that reduced the clonogenic survival compared to AuNP- ^{111}In (see Results, Figs. 2-5), and radiation absorbed dose estimates showed significantly greater dose deposition in tumors by trastuzumab-AuNP- ^{111}In than AuNP- ^{111}In (see Results, Table 1), only trastuzumab-AuNP- ^{111}In were studied for treatment in comparison to control tumor-bearing mice treated with normal saline. Our previous studies showed that unlabeled trastuzumab-AuNP (~0.8 mg) injected i.t. in CD1 female CD1 athymic mice with s.c. MDA-MB-361 tumor xenografts exhibited no tumor growth inhibition or normal tissue toxicity [7]. Tumor length and width were measured using digital Vernier calipers by two observers (NC and ZC) prior to treatment, then at 20 days after i.t. injection of trastuzumab-AuNP- ^{111}In or normal saline, then every 7 days until 70 days. The average of the two independent

measurements were used to calculate the tumor volume as $V = (\text{length} \times \text{width}^2) \times 0.5$. The tumor growth index (TGI) was calculated by dividing the tumor volume at each time point by the initial tumor volume prior to treatment.

The body weight of the mice was monitored to evaluate generalized normal tissue toxicity and a body weight index (BWI) was calculated by dividing the body weight at each time point by the pre-treatment body weight. At 70 **days** post-treatment, samples of blood were collected by cardiac puncture under anaesthesia into a Microvette 200 K3 EDTA (Sarstedt, Nümbrecht, Germany) capillary blood collection tube for hematology analysis, and into a Microvette 200 Serum (Sarstedt) tube for measurement of serum alanine aminotransferase (ALT) and creatinine (Cr). Hematology analysis was performed on a Hemavet 950FS analyzer (Drew Scientific, Oxford, CT) and included a complete blood cell count (CBC) for white blood cells (WBC), red blood cells (RBC), platelets (PLT), as well as hemoglobin (Hb) and hematocrit (Hct). Blood collected in Microvette 200 Serum was centrifuged at $500 \times g$ for 5 min to isolate serum. Serum creatinine and ALT were measured using Infinity Creatinine and Infinity ALT/GPT clinical chemistry kits (Fisher Diagnostics, Middletown, VA).

2.8 Statistical analysis

Data were expressed as mean $\pm$ SD and analyzed for statistical significance using Student's t-test ($P < 0.05$). All statistical analyses were performed using Prism Ver. 4.0 software (GraphPad).

3. Results

3.1 HER2 immunoreactivity

Increasing concentrations (0 to 15 nM) of trastuzumab-AuNP-¹¹¹In were incubated with HER2-overexpressing SK-BR-3 cells in the presence or absence of a 67-fold molar excess (1 μ M) of trastuzumab (Fig. 1). The decreased binding observed in the presence of trastuzumab indicated specific HER2-mediated binding (SB) to SK-BR-3 cells, but the SB binding curve did not reach saturation over the range of concentrations examined. Higher concentrations could not be evaluated due to greater adherence of trastuzumab-AuNP-¹¹¹In to the walls of the assay tubes. Since the $B_{\max}$ value previously reported for binding of ¹¹¹In-labeled trastuzumab to SK-BR-3 cells was 1.34×10^6 HER2/cell [15], and 1.7×10^4 cells were used to assess the binding of trastuzumab-AuNP-¹¹¹In to SK-BR-3 cells, this corresponded to an estimated $B_{\max}$ at saturation of 3.7×10^{-5} nmol. Fitting of the SB curve to a 1-site direct (saturation) receptor-binding model setting a constraint of $B_{\max}$ equal to 3.7×10^{-5} nmol, revealed a $K_D = 7.8 \pm 1.0 \times 10^{-8}$ mols/L.

3.2 Internalization studies

The internalization of trastuzumab-AuNP-¹¹¹In or AuNP-¹¹¹In by SK-BR-3 and MDA-MB-361 cells with high or intermediate HER2 density, respectively, was assessed by subcellular fractionation. When SK-BR-3 cells were incubated overnight with 3.0 nM of AuNP, radioactivity on the CS and internalized into the Cy was 3.4-fold and 8.4-fold significantly greater for trastuzumab-AuNP-¹¹¹In than AuNP-¹¹¹In ($P=0.0034$ and $P=0.0015$, respectively; Fig. 2). For MDA-MB-361 cells incubated with 3 nM of AuNP,

radioactivity bound to the CS was 4.6-fold and 1.9-fold lower for trastuzumab-AuNP-¹¹¹In and AuNP-¹¹¹In than for SK-BR-3 cells, but there was no significant difference in CS radioactivity between these two AuNP forms. However, at this concentration of AuNP, there was 2.0-fold significantly greater internalization into the Cy for trastuzumab-AuNP-¹¹¹In than AuNP-¹¹¹In ($P=0.02$). In order to determine if there may be greater differences between trastuzumab-AuNP-¹¹¹In and AuNP-¹¹¹In at a higher concentration, MDA-MB-361 cells were incubated with these AuNP at 14.4 nM. At this concentration, the CS radioactivity was 1.9-fold significantly greater for trastuzumab-AuNP-¹¹¹In than AuNP-¹¹¹In ($P=0.0075$; Fig. 2). Radioactivity internalized into the Cy was 5.8-fold significantly higher for trastuzumab-AuNP-¹¹¹In than AuNP-¹¹¹In ($P=0.0004$) at 14.4 nM. Confocal fluorescence microscopy in dark field mode of SK-BR-3 and MDA-MB-361 cells exposed to trastuzumab-AuNP-¹¹¹In or AuNP-¹¹¹In showed peri-nuclear localization of trastuzumab-AuNP-¹¹¹In while no uptake was visualized for AuNP-¹¹¹In (Fig. 3). The uptake of trastuzumab-AuNP-¹¹¹In visualized in SK-BR-3 cells with high HER2 density was greater than for MDA-MB-361 cells with intermediate HER2 expression.

3.3 Clonogenic assays and DNA double-strand breaks (DSBs)

The SF of SK-BR-3 cells exposed overnight to trastuzumab-AuNP-¹¹¹In (3.0 nM, 7.0 MBq/mL) was reduced by 1.8-fold, whereas AuNP-¹¹¹In decreased the SF by 1.2-fold ($P=0.03$; Fig. 4). However, only minor decreases in SF were noted for MDA-MB-361 cells exposed to this low concentration of trastuzumab-AuNP-¹¹¹In or AuNP-¹¹¹In, and there was no significant difference in SF between these two forms of AuNP ($P=0.146$). When the concentration of trastuzumab-AuNP-¹¹¹In was increased to 14.4 nM (33.6 MBq/mL),

the SF of MDA-MB-361 cells was decreased by 3.7-fold compared to 1.7-fold with AuNP-¹¹¹In ($P<0.0001$; Fig. 4). The number of unrepaired DNA DSBs in SK-BR-3 and MDA-MB-361 cells exposed to 3.0 nM (7.0 MBq/mL) of trastuzumab-AuNP-¹¹¹In or AuNP-¹¹¹In was assessed by immunofluorescence for γ -H2AX and compared to cells exposed to unlabeled trastuzumab-AuNP or untreated cells (Fig. 5A). Trastuzumab-AuNP-¹¹¹In caused 1.8-fold and 2.5-fold significantly more unrepaired DNA DSBs than AuNP-¹¹¹In in SK-BR-3 and MDA-MB-361 cells, respectively ($P=0.0026$ and $P=0.023$, respectively; Fig. 5B). Trastuzumab-AuNP-¹¹¹In and AuNP-¹¹¹In caused 4.8-fold and 2.7-fold significantly more DNA DSBs in SK-BR-3 cells than unlabeled trastuzumab-AuNP ($P=0.0001$ and $P=0.0002$, respectively). In MDA-MB-361 cells, trastuzumab-AuNP-¹¹¹In and AuNP-¹¹¹In caused 15.2-fold and 6.2-fold significantly more γ -H2AX foci than unlabeled trastuzumab-AuNP ($P=0.0025$ and $P=0.0006$, respectively). There were no significant differences in γ -H2AX foci in SK-BR-3 or MDA-MB-361 cells exposed to unlabeled trastuzumab-AuNP compared to untreated cells ($P=0.489$ and $P=0.245$, respectively).

3.4 Radiation dose estimates to tumors and normal organs

Radiation absorbed doses to the tumor and normal organs in CD1 athymic mice with s.c. MDA-MB-361 xenografts injected i.t. with trastuzumab-AuNP-¹¹¹In or AuNP-¹¹¹In (10 MBq, 0.7 mg; 2.6×10^{12} AuNP) are shown in Table 1. The tumor doses from trastuzumab-AuNP-¹¹¹In were 2.1 times significantly greater than for AuNP-¹¹¹In (60.5 ± 7.8 Gy vs 28.4 ± 12.0 Gy, $P<0.001$). Most normal organs except for the liver and total body received <0.9 Gy. The highest normal organ dose was the liver for both trastuzumab-AuNP-¹¹¹In and AuNP-¹¹¹In (0.88 ± 0.62 Gy and 0.76 ± 0.35 Gy, respectively).

3.5 Tumor growth inhibition studies and normal tissue toxicity

Treatment of CD1 athymic mice with s.c. HER2-positive MDA-MB-361 xenografts with 10 MBq (0.7 mg; 2.6×10^{12} AuNP) of trastuzumab-AuNP- ^{111}In injected i.t. arrested tumor growth over a 70 days observation period (Fig. 6A). In contrast, untreated mice receiving only normal saline injections exhibited rapidly increasing tumor growth reaching 8-times their initial size over 70 days. There was no loss of body weight in mice treated with trastuzumab-AuNP- ^{111}In or in control mice receiving normal saline injections (Fig. 6B), indicating no generalized toxicity caused by trastuzumab-AuNP- ^{111}In over a 70 days period. There were also no significant differences in serum ALT or Cr or CBC between mice injected with trastuzumab-AuNP- ^{111}In and mice receiving normal saline injections (Table 2). These results indicated no apparent toxicity towards the liver, kidneys or hematopoietic system at the 70 days end-point of the study.

4. Discussion

In this study, we describe for the first time the cytotoxicity *in vitro* against HER2-positive human BC cells of AuNP (30 nm) modified with PEG chains (5 kDa) linked to trastuzumab and PEG chains (2 kDa) linked to DTPA for complexing ^{111}In (trastuzumab-AuNP- ^{111}In) and the tumor growth inhibitory properties and normal tissue toxicity *in vivo* of these AuNP [6]. Trastuzumab-AuNP- ^{111}In were bound and internalized by HER2-positive SK-BR-3 and MDA-MB-361 human BC cells (Fig. 1 and 2). The estimated K_D for binding of trastuzumab-AuNP- ^{111}In to HER2 on SK-BR-3 cells was $7.8 \pm 1.0 \times 10^{-8}$ mols/L, which is 46-fold lower than previously reported for ^{111}In -labeled trastuzumab (K_D

$= 1.7 \pm 0.8 \times 10^{-9}$ nmols/L) [15]. This lower binding affinity for trastuzumab-AuNP- ^{111}In may be due to steric hindrance of HER2 binding caused by the AuNP as well as by the multiple (14) trastuzumab molecules and PEG polymers (37) bound to each AuNP which further contribute to steric inhibition of receptor binding. In a competition assay, we previously found a 2-fold increase in the K_D value for binding of panitumumab-AuNP- ^{177}Lu to EGFR compared to ^{123}I -labeled panitumumab [8]. This lower effect on the K_D for panitumumab-AuNP- ^{177}Lu may be due to a lower density of panitumumab molecules (8 per AuNP) or lower PEG polymer modification (not measured). However, conjugation of multiple trastuzumab molecules per AuNP may provide greater more avid multivalent binding to BC cells for trastuzumab-AuNP- ^{111}In compared to ^{111}In -labeled trastuzumab. Improvements in the binding affinity may possibly be achieved for trastuzumab-AuNP- ^{111}In by decreasing the number of trastuzumab conjugated per AuNP or the number of PEG polymers to minimize steric hindrance in HER2 binding. Nonetheless, binding was correlated with the HER2 density of SK-BR-3 and MDA-MB-361 cells ($1-2 \times 10^6$ and 5×10^5 HER2/cell, respectively) and was significantly greater for trastuzumab-AuNP- ^{111}In than non-targeted AuNP- ^{111}In (Fig. 2). Internalization was also more efficient for trastuzumab-AuNP- ^{111}In than AuNP- ^{111}In . HER2 is only slowly internalized [22] but trastuzumab binding promotes HER2 internalization [23, 24]. Widefield microscopy in fluorescence and dark field mode revealed that trastuzumab-AuNP- ^{111}In were internalized and deposited near the nucleus in SK-BR-3 and MDA-MB-361 cells, whereas cell binding and internalization were not visible for AuNP- ^{111}In (Fig. 3). The much greater visualization of trastuzumab-AuNP- ^{111}In compared to AuNP- ^{111}In bound to BC cells in dark field mode may be due to clustering of AuNP bound to HER2, since these receptors have been found

to cluster on lipid rafts on the cell surface [25]. In a previous study, we determined by transmission electron microscopy of explanted tumor cells that trastuzumab-AuNP injected i.t. in MDA-MB-361 tumor xenografts in athymic mice were located in lysosomes [6]. The proximity to the nucleus of lysosomes containing trastuzumab-AuNP-¹¹¹In will affect the potency of the emitted Auger electrons for causing lethal DNA DSBs due to their short range, but our studies reported here reveal that these AuNP were deposited in a peri-nuclear location. Moreover, Lee et al. reported that gold nanostars modified with anti-HER2 aptamers were internalized via HER2-mediated endocytosis in BC cells and were found in lysosomes [26]. Lysosomal uptake promoted degradation of HER2 and caused cell death. Thus, it is possible that trastuzumab-AuNP-¹¹¹In may inhibit tumor growth by decreasing HER2 as well as by causing lethal DNA damage in BC cells due to the Auger electron emissions. Previously, we reported that ¹¹¹In-labeled trastuzumab modified with NLS peptides was internalized and translocated to the nucleus in SK-BR-3 and MDA-MB-361 cells [13]. In the current study, trastuzumab-AuNP-¹¹¹In were not modified with NLS peptides, but HER2 nonetheless harbors a putative NLS [27] which may mediate routing of internalized AuNP to a peri-nuclear location in BC cells. We similarly found that ¹¹¹In-labeled and trastuzumab-modified G4 dendrimers with or without NLS peptide modification were internalized and transported to the nucleus in SK-BR-3 cells [28]. Deposition of trastuzumab-AuNP-¹¹¹In near the nucleus will maximize the lethal DNA-damaging effects of the Auger electrons emitted by ¹¹¹In [11], but it has also been proposed that Auger electrons may damage the cell membrane, leading to cell death [29].

At a low concentration (3 nM; 7.0 MBq/mL), trastuzumab-AuNP-¹¹¹In reduced the SF of SK-BR-3 cells ($1-2 \times 10^6$ HER2/cell) *in vitro* by 1.8-fold and was more effective

than AuNP-¹¹¹In (Fig. 4). At this low concentration of trastuzumab-AuNP-¹¹¹In or AuNP-¹¹¹In, only a small decrease in SF was found for MDA-MB-361 cells which have lower HER2 density (5×10^5 HER2/cell). MDA-MB-361 cells are more radioresistant to γ -rays or X-rays than SK-BR-3 cells [7, 16], which may additionally contribute to the lower cytotoxic potency of trastuzumab-AuNP-¹¹¹In on these cells. However, by increasing the concentration of trastuzumab-AuNP-¹¹¹In to 14.4 nM (33.6 MBq/mL), the SF of MDA-MB-361 cells was reduced by 3.7-fold, which was significantly more effective than for AuNP-¹¹¹In. These results agree with our previous report which noted a 3-4 fold lower sensitivity of MDA-MB-361 than SK-BR-3 cells to the cytotoxic effects of ¹¹¹In-NLS-trastuzumab [13]. The cytotoxicity of AuNP-¹¹¹In on SK-BR-3 and MDA-MB-361 cells may be due to non-specific endocytosis of AuNP (Fig. 2). Others have reported binding and internalization of non-targeted AuNP in HER2-positive BT-474 and SK-BR-3 human BC cells [30]. The cytotoxicity of trastuzumab-AuNP-¹¹¹In was directly correlated with the induction of DNA DSBs in SK-BR-3 and MDA-MB-361 cells caused by the Auger electrons emitted by ¹¹¹In (Fig. 5). Trastuzumab-AuNP-¹¹¹In caused significantly more γ -H2AX foci in the nucleus of SK-BR-3 and MDA-MB-361 cells than AuNP-¹¹¹In. The density of γ -H2AX foci in the nucleus of cells exposed to unlabeled trastuzumab-AuNP was not significantly different than for untreated cells. Our previous studies also revealed multiple γ -H2AX foci in the nucleus of SK-BR-3 and MDA-MB-361 cells caused by Auger electrons emitted by ¹¹¹In-NLS-trastuzumab [13] or trastuzumab-modified G4 dendrimers labeled with ¹¹¹In [28]. Only one other study has described the cytotoxicity of ¹¹¹In-labeled AuNP on BC cells [31]. In this study, AuNP (14 nm) were directly linked to ¹¹¹In-DTPA-EGF to bind epidermal growth factor receptors (EGFR) on BC cells. These

¹¹¹In-labeled AuNP were bound and internalized by EGFR-overexpressing MDA-MB-468 human BC cells, reducing their SF by >80%. No assessment of DNA DSBs caused by the Auger electrons was reported.

Local i.t. injection of trastuzumab-AuNP-¹¹¹In in CD1 athymic mice with s.c. MDA-MB-361 xenografts arrested tumor growth over a 70 days period while tumors grew rapidly in untreated mice reaching more than 8-times their initial size (Fig. 6A). The radiation dose delivered to the tumor by trastuzumab-AuNP-¹¹¹In was 60.5 ± 7.8 Gy (Table 1). There was no effect of trastuzumab-AuNP-¹¹¹In injection on body weight compared to normal saline treated control mice over this 70 days period (Fig. 6B), suggesting no generalized normal tissue toxicity. Measurement of serum ALT and Cr, CBC, Hb and Hct confirmed that there was no liver or renal toxicity or hematopoietic toxicity at 70 days. These results were in good agreement with the low radiation doses (<0.9 Gy) delivered to normal organs (Table 1). However, the estimated total body dose was 0.66 ± 0.07 Gy (Table 1), which is higher than previously associated with hematopoietic toxicity (0.2-0.3 Gy) in humans for systemically administered radiotherapeutic agents [32]. Hematopoietic toxicity was not manifested in our study. These relatively high total body dose estimates are due to the small mass of the mouse (25 g). By scaling to a human adult female mass of 60 kg, we project a total body dose in humans of 1 mGy. In addition, since the radioactivity is localized to the tumor, irradiation of the hematopoietic bone marrow would be minimal due to the short range of the Auger electrons. Normal tissue toxicity was assessed at 70 days, and it is possible that toxicity may be manifested at an earlier time point. However, our results are similar to those found for i.t. injected panitumumab-AuNP-¹⁷⁷Lu, in which the growth of s.c. EGFR-overexpressing MDA-MB-468 human BC

xenografts in athymic mice was arrested with no normal tissue toxicity [9]. The tumor dose from panitumumab-AuNP-¹⁷⁷Lu was 30.4 Gy and the whole body dose was 0.35 Gy. The strong tumor growth inhibition properties of trastuzumab-AuNP-¹¹¹In and panitumumab-AuNP-¹⁷⁷Lu are due to local i.t. injection which provides high concentrations of radioactivity that are mostly retained in the tumor and do not redistribute readily to normal tissues, resulting in high tumor radiation doses [6]. Moreover, binding and internalization of trastuzumab-AuNP-¹¹¹In are required to cause lethal DNA DSBs, due to the subcellular range of the Auger electrons and most normal tissues express low levels of HER2 [33]. The described radiation nanomedicine approach for local treatment of tumors provides several advantages, since the AuNP may be modified with multiple copies of antibodies that may multivalently bind to tumor cells, and multiple copies of PEG polymers derivatized with radiometal chelators may be conjugated to the AuNP to amplify delivery of radionuclides to tumors. In addition, the AuNP provide versatility to be modified with different antibodies to tailor the treatment to a particular tumor phenotype, as well as a variety of radiometal chelators to allow complexation of therapeutic radionuclides that emit different forms of radiation (e.g. β -particles or Auger electrons).

5. Conclusion

In conclusion, trastuzumab-AuNP-¹¹¹In were specifically bound, internalized and routed to a peri-nuclear location in HER2-positive BC cells. Emission of subcellular range Auger electrons caused lethal DNA DSBs which decreased the SF of these cells. Trastuzumab-AuNP-¹¹¹In exhibited greater cytotoxic potency than non-targeted AuNP-¹¹¹In. Local i.t.

injection of trastuzumab-AuNP-¹¹¹In arrested tumor growth in mice with s.c. HER2-positive BC xenografts, with no **apparent** normal tissue toxicity. These results are promising for further development of trastuzumab-AuNP-¹¹¹In as a novel Auger electron-emitting radiation nanomedicine for local treatment of HER2-positive BC.

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LEGENDS FOR FIGURES

Fig. 1. Binding of increasing concentrations of ^{111}In -labeled and trastuzumab-modified gold nanoparticles (trastuzumab-AuNP- ^{111}In) to HER2-positive SK-BR-3 human breast cancer cells in the absence (total binding; TB) or presence (non-specific binding (NSB) of an excess (1 mM) of trastuzumab. Specific binding (SB) was obtained by subtraction of NSB from TB.

Fig. 2. Internalization of 3 nM (7.0 MBq/mL) or 14.4 nM (33.6 MBq/mL) of ^{111}In -labeled and trastuzumab-modified gold nanoparticles (trastuzumab-AuNP- ^{111}In) or unmodified ^{111}In -labeled gold nanoparticles (AuNP- ^{111}In) into HER2-positive SK-BR-3 ($1-2 \times 10^6$ receptors/cell) or MDA-MB-361 (5×10^5 receptors/cell) human breast cancer cells. CS: cell surface radioactivity. Cy: Cytoplasmic radioactivity. Significant differences ($P < 0.05$) are indicated by asterisks.

Fig. 3. Dark field and fluorescence microscopy of SK-BR-3 ($1-2 \times 10^6$ receptors/cell) or MDA-MB-361 (5×10^5 receptors/cell) human breast cancer cells incubated with ^{111}In -labeled and trastuzumab-modified gold nanoparticles (trastuzumab-AuNP- ^{111}In) or unmodified ^{111}In -labeled gold nanoparticles (AuNP- ^{111}In). The nucleus was counterstained blue with DAPI. Light scattering of AuNP was shown by the yellow pseudocolor.

Fig. 4. Surviving fraction of HER2-positive SK-BR-3 ($1-2 \times 10^6$ receptors/cell) or MDA-MB-361 (5×10^5 receptors/cell) human breast cancer cells incubated with 3 nM (7

MBq/mL) or 14.4 nM (33.6 MBq/mL) of ^{111}In -labeled and trastuzumab-modified gold nanoparticles (trastuzumab-AuNP- ^{111}In) or unmodified ^{111}In -labeled gold nanoparticles (AuNP- ^{111}In). Significant differences ($P < 0.05$) are indicated by asterisks.

Fig. 5. A. Confocal immunofluorescence microscopy probing for $\gamma\text{-H2AX}$ (green foci) in the nucleus (counterstained blue with DAPI) of HER2-positive SK-BR-3 ($1\text{-}2 \times 10^6$ receptors/cell) or MDA-MB-361 (5×10^5 receptors/cell) human breast cancer cells. Cells were exposed to unlabeled trastuzumab-modified gold nanoparticles (trastuzumab-AuNP) or ^{111}In -labeled unmodified gold nanoparticles (AuNP- ^{111}In) or ^{111}In -labeled and trastuzumab modified gold nanoparticles (trastuzumab-AuNP- ^{111}In) or untreated. **B.** Quantification of $\gamma\text{-H2AX}$ foci in the nucleus of SK-BR-3 or MDA-MB-361 cells following exposure to these treatments. Significant differences ($P < 0.05$) are indicated by asterisks.

Fig. 6. A. Tumor growth index in CD1 athymic mice with s.c. MDA-MB-361 human breast cancer xenografts injected intratumorally with ^{111}In -labeled and trastuzumab-modified gold nanoparticles (trastuzumab-AuNP- ^{111}In ; 10 MBq, 0.7 mg, 2.6×10^{12} AuNP) or receiving no treatment. **B.** Body weight index for trastuzumab-AuNP- ^{111}In treated or untreated mice.

Table 1

Radiation absorbed doses to the tumor and normal organs in CD1 athymic mice with s.c. MDA-MB-361 human breast cancer xenografts injected intratumorally (i.t.) with trastuzumab-AuNP-¹¹¹In or AuNP-¹¹¹In ^a

Organ	Radiation absorbed dose (Gy) ^{b, c}	
	Trastuzumab-AuNP- ¹¹¹ In	AuNP- ¹¹¹ In
Heart	0.24 ± 0.01	0.14 ± 0.02
Lungs	0.33 ± 0.05	0.20 ± 0.01
Liver	0.88 ± 0.62	0.76 ± 0.35
Kidneys	0.19 ± 0.02	0.11 ± 0.01
Spleen	0.19 ± 0.01	0.11 ± 0.02
Pancreas	0.19 ± 0.01	0.11 ± 0.01
Stomach	0.23 ± 0.06	0.15 ± 0.02
Small Intestine	0.20 ± 0.01	0.11 ± 0.02
Colon	0.18 ± 0.01	0.10 ± 0.03
Tumor	60.50 ± 7.80	28.4 ± 12.0
Total body	0.66 ± 0.07 ^d	0.35 ± 0.12 ^d

^a The injected amount was 10 MBq (0.7 mg; 2.6×10^{12} AuNP)

^a Radiation absorbed doses to target organs were estimated based on the cumulative radioactivity in the liver and remainder of the body (including the tumor) source organs measured by quantitative microSPECT/CT imaging. These organs were the only source

organs visualized [6]. Mouse S-values were obtained from Bitar et al. [20].

OLINDA/EXM was used to estimate the dose to the tumor [21].

^c Dose estimates are the mean $\pm$ SD of duplicate values.

^d Significantly different ($P < 0.001$).

Table 2

Results of biochemistry and hematology analyses at 70 days after i.t. injection of ^{111}In -Au-T or normal saline

Parameter ^b	Mean $\pm$ SD ^c	
	Trastuzumab-AuNP ^{111}In	Normal saline
ALT (U/L)	43 $\pm$ 9	55 $\pm$ 20
Cr ($\mu\text{mol/L}$)	29 $\pm$ 5	18 $\pm$ 14
WBC ($10^9/\text{L}$)	1.3 $\pm$ 0.6	1.6 $\pm$ 0.8
RBC ($10^{12}/\text{L}$)	6.0 $\pm$ 1.2	5.6 $\pm$ 0.7
PLT ($10^9/\text{L}$)	382 $\pm$ 334	223 $\pm$ 148
Hb (g/L)	99 $\pm$ 22	91 $\pm$ 9
Hct (%)	33 $\pm$ 9	30 $\pm$ 3

^a The injected amount was 10 MBq (0.7 mg; 2.6×10^{12} AuNP)

^b Parameters: ALT: serum alanine aminotransferase; Cr: serum creatinine; WBC: white blood cells; RBC: red blood cells; PLT: platelets; Hb: hemoglobin; Hct: hematocrit.

^c There were no significant differences in the mean value for any parameters for trastuzumab-AuNP- ^{111}In and normal saline controls.

Fig. 1

Fig. 2

SK-BR-3

MDA-MB-361

Trastuzumab-
AuNP-¹¹¹In

AuNP-¹¹¹In

Fig. 3

Fig. 4

Fig. 5

Fig. 6