Combination of 131I-trastuzumab and lanatoside C enhanced therapeutic efficacy in HER2 positive tumor model

Nagarajan Vinod
Korea Institute of Radiological and Medical Sciences
Jae Kim
Korea Institute of Radiological and Medical Sciences
Seungbum Choi
Korea Institute of Radiological and Medical Sciences
Ilhan Lim (ilhan@kirams.re.kr)
Korea Institute of Radiological and Medical Sciences

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Combination of ^{131}I-trastuzumab and lanatoside C enhanced therapeutic efficacy in HER2 positive tumor model

Nagarajan Vinod[1+], Jae Hyung Kim[2+], Seungbum Choi[2], and Ilhan Lim[1,2,3*]

[1]Department of Nuclear Medicine, Korea Institute of Radiological and Medical Sciences, (KIRAMS), Seoul, 01812, Republic of Korea

[2]Division of RI-convergence Research, Korea Institute of Radiological and Medical Sciences, (KIRAMS), Seoul, 01812, Republic of Korea

[3]Department of Radiological & Medico-Oncological Sciences, University of Science and Technology, Korea Institute of Radiological and Medical Sciences (KIRAMS), Seoul, 01812, Republic of Korea

*corresponding author email: ilhan@kirams.re.kr

[+]these authors contributed equally to this work

Abstract

Lanatoside C has a promising anti-tumor activity and is a potential candidate for radiosensitizers. In this study, we have investigated the therapeutic efficacy of the combination of ^{131}I-trastuzumab and lanatoside C for inhibition of human epidermal growth factor receptor 2 (HER2) positive tumor progression in NCI-N87 xenograft model. The combination treatment (^{131}I-trastuzumab and lanatoside C) showed highest cytotoxicity when compared to non-treated control or trastuzumab alone or ^{131}I alone or ^{131}I-trastuzumab alone *in vitro*. Biodistribution studies using ^{131}I-trastuzumab or combination of ^{131}I-trastuzumab and lanatoside C showed tumor uptake in BALB/c nude mice bearing HER2 positive NCI-N87 tumor xenograft model. The higher tumor uptake was observed in ^{131}I-trastuzumab (19.40±0.04% ID/g) than in the combination of ^{131}I-trastuzumab and lanatoside C (14.02±0.02% ID/g) at 24 hours post-injection. Most importantly, an antitumor effect was observed in mice that received the combination of ^{131}I-trastuzumab and lanatoside C ($p=0.009$) when compared to control. In addition, mice received lanatoside C alone ($p=0.085$) or ^{131}I-trastuzumab alone ($p=0.160$) did not significantly inhibit tumor progression compared with control. Taken together, our data suggest that combination of ^{131}I-trastuzumab and lanatoside C might be a potential synergistic treatment for radioimmunotherapy to control the HER2 positive tumor.

Introduction

Radioimmunotherapy (RIT) represents an attractive approach that combines the advantage of radiation therapy and immunotherapy using monoclonal antibodies (mAbs) [1,2]. Currently, the targeted radiation delivered by mAbs kills explicitly cancer cells or the tumor microenvironment [3]. RIT have been used primarily for the treatment of lymphoma, mostly with radiolabeled mAbs against CD20 [4-7]. Recent research trends found the treatment of tumors using isotope-releasing beta-emitters such as ^{90}Y, ^{177}Lu, ^{131}I and ^{124}I [8-10]. The radioactive isotope is selected considering the physical properties such as path length, emission energy, and half-life [10,11]. This is to establish a therapeutic strategy to effectively reduce the size of tumors [10,12]. Among the many radioisotopes used for RIT, ^{131}I has advantages of being easy to use. The 8-day half-life of ^{131}I increases the efficiency of the treatment, consistent with the biological half-life of the antibody [11,13]. In addition, the path length of the beta-particle of ^{131}I is relatively short and effectively treats small tumors. It is also easy to discharge outside the body. However, RIT has the problem of producing radio-resistance tumors in solid tumors and bone marrow toxicity is a problem [12,14]. Therefore, RIT processing capacity is limited and needs to be improved for these problems. Recently several studies have tried to improve therapeutic efficacy of RIT through Radiosensitizer [14,15].

Radiosensitizers are agents that sensitize the tumor cells to radiation [15]. Many drugs and chemicals have been reported as radiosensitizers. Recently, it has been reported that lanatoside C can be used as a radiosensitizer in radiotherapy [16]. Previous studies have shown the effect of lanatoside C as a radiosensitizer at radiotherapy, but RIT in HER2 positive tumor is not yet known. Therefore, we hypothesis that lanatoside C has an effect of radiosensitizer at ^{131}I-trastuzumab RIT in HER2 positive tumor.

In the present study, we investigated the effect of cell proliferation of lanatoside C on two cancer cells (NCI-N87 and MDA-MB231). In addition, the cytotoxicity and therapeutic effects of combined treatment with ^{131}I-trastuzumab and lanatoside C were evaluated in HER2 positive cancer cells *in vitro* and *in vivo*.

Results

Effect of lanatoside C on cell proliferation of cancer cells

Before investigating the ^{131}I-trastuzumab in combination with lanatoside C, we determined the cytotoxic effects of lanatoside C in NCI-N87 and MDA-MB231 cancer cells. Both the cells were treated with various concentrations of lanatoside C and assesses for cell viability using Ez-Cytox cell viability assay. All the doses of lanatoside C shows strong decreases of cell proliferation in both cancer cells when compared to non-treated control cells, suggesting efficient cellular uptake of those lanatoside C concentrations (Fig. 1). Significant decrease of cell viability relative to untreated control was apparent, and most evident following treatment of NCI-N87 with 0.125 nM/well lanatoside C. A notable difference in cell viability was observed between 0.125 µM and 1 µM of lanatoside C. However, no significant difference was found between 0.25 µM and 0.5 µM in both cancer cells.

Lanatoside C increases the sensitivity of NCI-N87 cells to ^{131}I-trastuzumab radioimmunotherapy *in-vitro*

The cytotoxic effects of lanatoside C on treatment of ^{131}I-trastuzumab in NCI-N87 cells was determined using the Ez-Cytox cell viability assay following 96 h incubation. NCI-N87 cells were treated with lanatoside C in combination of ^{131}I-trastuzumab RIT. The maximum cell death was found in NCI-N87 cells treated with combination of ^{131}I-

trastuzumab RIT and lanatoside C (~99%) compared to ^{131}I-trastuzumab RIT alone (~77%) or ^{131}I alone (~44%) or trastuzumab alone (~58%) (Fig. 2). However, no significant differences were observed in the case of 50, 100, 200 μCi of ^{131}I-trastuzumab RIT in combination of lanatoside C.

Lanatoside C increases the sensitivity of NCI-N87 xenografts to ^{131}I-trastuzumab radioimmunotherapy *in-vivo*

The maximum dose of ^{131}I for therapeutic efficacy is well known through several studies. Therefore, we performed experiments at a concentration of 400 μCi based on the results of previous studies. We observed that tumor growth rapidly occurred in the control group administered 0.01% DMSO in saline. Most importantly, it was confirmed that the growth of the tumor was significantly decreased in the group treated with combination of ^{131}I-trastuzumab and lanatoside C compared to the control group ($p=0.009$; Fig. 3). Moreover, there is no significant differences were found in lanatoside C group ($p=0.085$) and ^{131}I-trastuzumab group ($p=0.160$) when compared to the control group. These results showed that tumor growth was significantly suppressed when ^{131}I-trastuzumab and lanatoside C were treated together.

Biodistribution of ^{131}I-trastuzumab or combination of ^{131}I-trastuzumab and lanatoside C *in vivo*

The results of the biodistribution data of ^{131}I-trastuzumab or combination of ^{131}I-trastuzumab and lanatoside C in NCI-N87 xenografted BALB/c nude mice obtained at 4, 24, 48 h.p.i are shown in Fig. 4. Significant uptake of ^{131}I-trastuzumab was clearly observed in blood, spleen and NCI-N87 tumor. The data reveal that NCI-N87 tumor uptake was shown 4

h.p.i in ^{131}I-trastuzumab (11.1±0.01% ID/g) with a steady increase through 24 h (19.4±0.04% ID/g) and 48 h (16.8±0.04% ID/g) (Fig. 4A). This high accumulation of ^{131}I-trastuzumab is consistent with extraction of the activity from the blood (4 h: 24.9±0.09% ID/g, 24 h: 16.5±0.02% ID/g and 48 h: 11.4±0.04% ID/g). However, combination of ^{131}I-trastuzumab and lanatoside C uptake in NCI-N87 tumors at 4 h (8.5±0.06% ID/g, P=0.0025), 24 h (14.2±0.02% ID/g, P=0.0017) and 48 h (10.4±0.05% ID/g, P=0.0017) showed statistically decrease in ^{131}I-trastuzumab accumulation compared with uptake in ^{131}I-trastuzumab solely (Fig. 4B). This result shows that treatment of lanatoside C with ^{131}I-trastuzumab was decreased uptake of ^{131}I-trastuzumab in tumor. However, previous results in this article shows that treatment of lanatoside C with ^{131}I-trastuzumab has significantly suppressed tumor growth. Accordingly, lanatoside C was decreased uptake of ^{131}I-trastuzumab in tumor. However, lanatoside C was increased inhibition of tumor growth. Hence, we concluded that lanatoside C may be sufficient for use with radioactive sensitizers in HER2 positive tumors.

Discussion

The present study demonstrated that the combination of ^{131}I-trastuzumab RIT and lanatoside C can improve the therapeutic effects in HER2 positive tumor. We demonstrated that lanatoside C increases the sensitivity of NCI-N87 cells and xenograft models to ^{131}I-trastuzumab RIT *in vitro* and *in vivo* (Fig. 2 & 3). The results of the present study are consistent with those of earlier studies, in which treatment with lanatoside C led to dose-dependent cytostatic or cytotoxic responses of radiosensitization in two colorectal cancer cell line [17]. Previous studies have shown the effect of lanatoside C as a radiosensitizer at external radiotherapy. However, no study has been conducted in case of RIT. We demonstrated that effect of lanatoside C as a radiosensitizer at ^{131}I-trastuzumab RIT in HER2 positive cells (Fig.

2). According to literature, tumor cells are most radiosensitive in the M and G2 phases. Moreover, lanatoside C induces cell cycle arrest in the G2/M phase could be responsible for the difference in radiosensitization. Another study also shown that lanatoside C increased cell sensitivity to radiation by inhibiting DNA damage repair [17].

Lanatoside C is known to inhibit cell proliferation and induces cell apoposis in tumor cells involving various cellular signaling pathways [18-20]. Additional killing of NCI-N87 cells by ^{131}I-trastuzumab will be due to associated high energy beta radiation (0.2 MeV of ^{131}I). The enhanced magnitudes of damage are due to the localization of radioisotope very close to cellular targets at membrane and cytoplasm level. The present study also showed that the higher level of cell death measured by Ez-Cytox cell viability, proliferation, and cytotoxicity assay kit was observed after treatment of lanatoside C at various concentrations. Moreover, lanatoside C enhanced ^{131}I-trastuzumab RIT *in vitro*. Combination with ^{131} I-trastuzumab and lanatoside C showed highest cell apoptosis when compared to other groups such as ^{131}I - trastuzumab RIT alone, trastuzumab alone and ^{131}I alone in NCI-N87 cells. The combinatorial treatment of lanatoside C would result in higher apoptosis and thus, there is an increase in the number of cell lysis at the advanced stage of cell death.

Radionuclide ^{131}I emits both β-emission and γ-emission which could be used for radiotherapy. In this work, BALB/c nude mice bearing NCI-N87 tumors were intravenously injected with 400 μCi ^{131}I-trastuzumab. It was found that ^{131}I-trastuzumab after intravenous injection exhibited obvious tumor accumulation. Moreover, the biodistribution of ^{131}I-trastuzumab in mice bearing HER2 positive tumors showed higher tumor uptake than the combination of ^{131}I-trastuzumab and lanatoside C, indicating that ^{131}I-trastuzumab showed greater tumor accumulation and retention effect. Biodistribution studies in nude mice showed that ^{131}I-trastuzumab targeted the tumors overexpressing the Human HER2 receptor *in vivo*. ^{131}I-trastuzumab accumulated to a significant extent in tumors with % ID/g of 19.4±0.04 in

the tumor tissues at 24 h.p.i. which decreased to 16.8 ± 0.045 at 48 h.p.i. Combination of ^{131}I-trastuzumab and lanatoside C also showed similar pattern of tumor uptake in mice bearing HER2 positive tumors. Steady blood clearance of ^{131}I-trastuzumab and ^{131}I-trastuzumab combined with lanatoside C demonstrated the stability of the complex under *in vivo* conditions. The high uptake of ^{131}I-trastuzumab by the liver, lungs and spleen may be due to the rich blood flow and its effective metabolism in the reticuloendothelial system of these organs. The tumor uptake and biodistribution ratio of ^{131}I-trastuzumab was found to be higher than combination of ^{131}I-trastuzumab and lanatoside C at 4, 24, and 48 h.p.i. The expectation of high tumor uptake of the radiolabeled ^{131}I-trastuzumab in HER2 positive tumors was confirmed by the present biodistribution study.

We have performed the clinical trial of RIT for NHL patients using ^{131}I-rituximab for 16 years [21-25]. RIT demonstrated excellent outcomes, but there are some refractory patients who revealed resistance to RIT. We expected that it is necessary to treat these refractory patients using more enhanced RIT protocols. We hope that ^{131}I-rituximab with lanatoside C can be applied for refractory NHL patients, because we found out that addition of lanatoside C can enhance the RIT of ^{131}I-rituximab.

Conclusions

In conclusion, our findings suggest that lanatoside C has the potential to sensitize ^{131}I-trastuzumab induced cytotoxicity in NCI-N87 cells *in vitro* and enhanced strong antitumor effect in a HER2 positive xenograft model. As the results, the combined therapy with ^{131}I-trastuzumab and lanatoside C achieved excellent synergistic *in vivo* therapeutic effects in HER2 positive tumor bearing mice. Therefore, our *in vitro* and *in vivo* results

provide potentially important and promising therapeutic strategies for future clinical translations in radioimmunotherapy.

Materials and methods

Cells and reagents

NCI-N87 and MDA-MB231 cell lines were purchased from the American Type Culture Collection (New York, USA). All these cells were cultured in RPMI-1640 (WELGENE Inc., Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Omega Scientific, Inc., Tarzana, CA, USA), 2 mmol/L L-glutamine, 5% Penicillin/Streptomycin in a humidified atmosphere of 5% CO_2 at 37° C. Lanatoside C was purchased from Sigma-Aldrich (St. Louis, MO, USA). Herceptin (Trastuzumab), A therapeutic agent that targets HER2 (Human Epidermal growth factor Receptor 2) was purchased from Roche. ^{131}I was purchased in New Korea Industrial Co., Ltd. Ez-Cytox cell viability, proliferation, and cytotoxicity assay kit was purchased from DoGenBio (Seoul, Korea).

Radiolabeling

Radiolabeling of trastuzumab with ^{131}I was achieved using the Pierce Pre-coated Iodination Tubes (Thermo scientific, U.S.A.) and carried out in accordance with the protocol provided by Thermo scientific[26]. Briefly, the pierce pre-coated iodination tube was wetted with 1 ml of Tris iodination buffer and decanted. 500 μCi of ^{131}I was added to the Pierce pre-coated iodination tube and activated for 5 min at room temperature. Subsequently, 100 μg of trastuzumab was added to the tubes and the reaction mixture was incubated for 10 min at room temperature. Radiolabeling purity was determined by instant thin-layer chromatography

(Agilent Technologies) using saline. Incorporation purity was always exceeded 95%.

Determination of the effect of lanatoside C on cancer cells

For the *in vitro* cell viability assay was carried out according to protocol described by Ez-Cytox cell viability, proliferation, and cytotoxicity assay kit[27]. Briefly, 100 µl of NCI-N87 or MDA-MB231 cells were firstly seeded into 96-well plate for 24 h and then incubated with various concentration of lanatoside C (0.125, 0.250, 0.500 and 1.000 µM). After 96 h incubation, 10 µl of Ez-Cytox solution were added into each well and incubated at 37° C for 0.5-4 h. The absorbance was quantified at 450 nm using a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA). All experiments were repeated three times with at least triplicate readings for each concentration. Percent cell viability was calculated as the percentage of the ratio of optical density (OD) of treated and non-treated samples.

Estimation of cell death in NCI-N87 cells treated with ^{131}I-trastuzumab and lanatoside C *in vitro* study

Cells were seeded at a density of $2x10^4$ cells per well in a 96-well plate and incubated for 24 h at 37° C. After incubation, cells were treated with trastuzumab or ^{131}I alone or ^{131}I-trastuzumab alone or ^{131}I-trastuzumab combined with lanatoside C. After 96 h of incubation, cell viability was determined using the Ez-Cytox (cell viability, proliferation and cytotoxicity assay kit) following the manufacturer's instructions. The absorbance was measured at 450 nm using a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA). All experiments were repeated three times with at least triplicate readings for each concentration.

Experimental animals

Pathogen-free BALB/c nude mice were obtained from Dooyeol Biotech, Korea. All animal experiments were approved by the Committee for the Handling and Use of Animals and performed in accordance with institutional guidelines at Korea Institute of Radiological and Medical Sciences in compliance with the ARRIVE guidelines.

Xenograft model

To make a xenograft mouse model, NCI-N87 cells (5×10^6/mouse/0.1 ml) were injected subcutaneously into the dorsal right flank of 6-week-old BALB/c nude mice. When the tumor volume reached approximately 150 mm^3, the mice were randomly assigned to four groups (7 mice/group): (1) control group (non-treated control: NCI-N87 tumor), (2) vehicle group (0.01% DMSO in saline, 100 μl, the first 3 days), (3) lanatoside C (6 mg/kg body weight, the first 3 days intraperitoneal injection), (4) ^{131}I-trastuzumab group (400 μCi, once tail vein injection), and (5) ^{131}I-trastuzumab and lanatoside C combination (lanatoside C, 6 mg/kg body weight, the first 3 days intraperitoneal injection, and ^{131}I-trastuzumab, 400 μCi, once tail vein injection). Tumor size and body weight were measured once a week, and the tumor volume (V) was calculated using the following formula: $V = L \times W^2/2$ (L, long diameter of the tumor; W, short diameter of the tumor).

Biodistribution Study

The biodistribution of the ^{131}I radiolabeled trastuzumab was assessed in BALB/c nude mice bearing established NCI-N87 xenografts. Mice were injected with ^{131}I-trastuzumab (400 μCi) or combination of ^{131}I-trastuzumab and lanatoside C by tail vein injection. At 4, 24 and 48 hours after post-injection (h.p.i), groups of 4 mice were euthanized by isofluorane

anesthesia and then immediately bled via cardiac puncture. Tumors and normal tissues (muscle, bone, lipid, spleen, pancreas, intestine, liver, heart, lung, kidney and tail) were then resected and placed in individual γ-counter tubes. The activity of all samples were then counted on a gamma counter (2480 Wizard^2, PerkinElmer, Waltham, MA, USA), and the percent injected dose per gram (% ID/g) calculated. Results were expressed as Mean ± SD for each time point.

Statistical analysis

All data are expressed as the mean ± SD and are representative of at least triplicate experiments. The significance was determined using Kruskal-Wallis test and Mann-Whitney test. A value of $p < 0.05$ was considered to be significant.

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Author Contributions

N.V. and J.H.K. designed and performed most of the *in vitro* and *in vivo* experiments and wrote the manuscript. S.C. performed the *in vitro* and the *in vivo* experiments and analyzed

the data. I.L. designed, supervised the experiments, review and approved the final version of the manuscript.

Conflict of interest

The authors declare no competing interests.

Figure legends

Figure 1. Lanatoside C suppressed growth of cancel cell lines. Inhibitory effect of lanatoside C on cell viability of NCI-N87 (A) and MDA-MB231 (B) cells. Data are presented as percentage of cell viability in which the untreated control sample is set 100%. The average of experimental triplicates ± standard deviation is shown.

Figure 2. Lanatoside C enhanced ^{131}I-trastuzumab RIT *in vitro*. NCI-N87 cells were treated with various activity of ^{131}I-trastuzumab RIT (A), ^{131}I-trastuzumab RIT combined with lanatoside C (B), ^{131}I alone (C) and trastuzumab alone (D), and cell viability was determined by Ez-Cytox assay. Data are presented as percentage of cell viability in which the untreated control sample is set 100%. The average of experimental triplicates ± standard deviation is shown.

Figure 3. Lanatoside C enhanced ^{131}I-trastuzumab RIT in xenograft model. When the NCI-N87 tumor volume reached approximately 150 mm^3, the BALB/c nude mice were treated with control group (0.01% DMSO in saline, 100 μl, the first 3 days), vehicle group (non-treated control), lanatoside C (6 mg/kg body weight, the first 3days intraperitoneal injection), ^{131}I trastuzumab group (400 μCi, once tail vein injection), and combination of ^{131}I-

387 trastuzumab and lanatoside C (lanatoside C, 6 mg/kg body weight, the first 3 days
388 intraperitoneal injection, and ^{131}I-trastuzumab, 400 μCi, once tail vein injection).
389
390 **Figure 4.** Biodistribution pattern (% ID/g) of ^{131}I-trastuzumab (A) or combination of ^{131}I-
391 trastuzumab and lanatoside C (B) in tumor (tumor xenograft NCI-N87) bearing BALB/c nude
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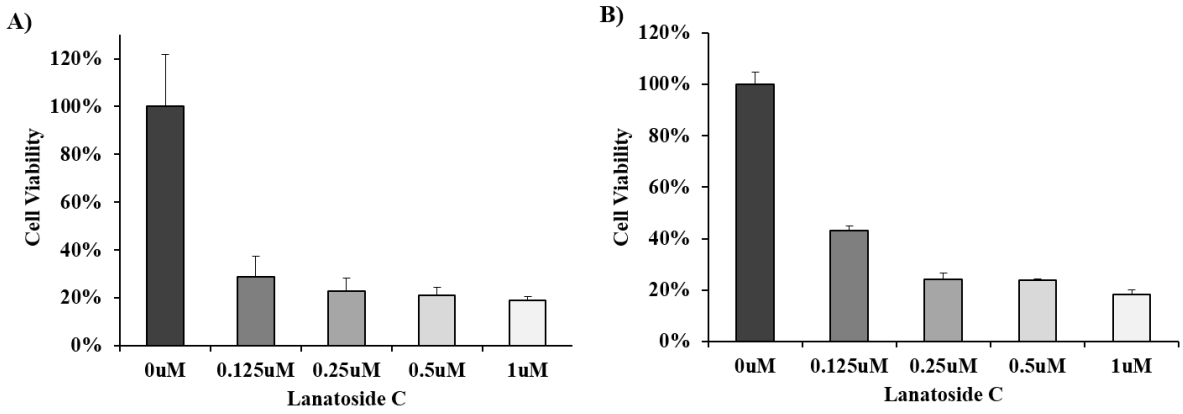
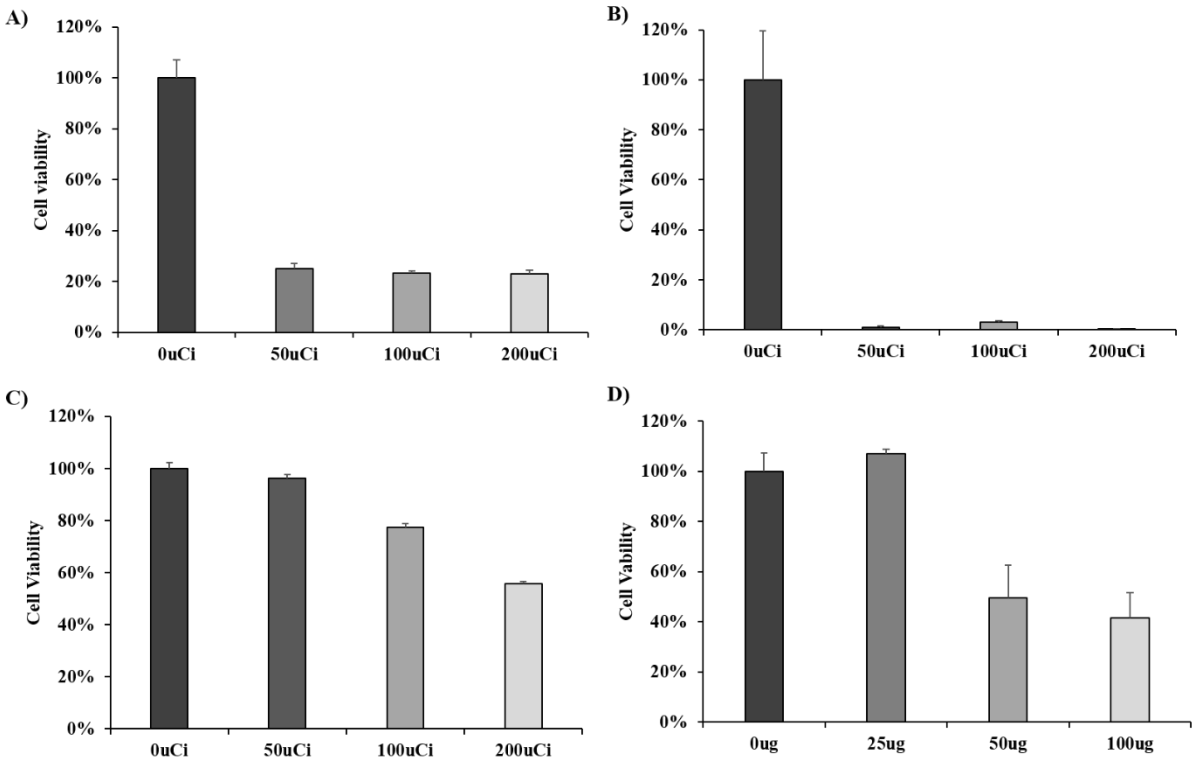

A)
120%
100%
80%
60%
40%
20%
0%
Cell Viability
0uM
0.125uM
0.25uM
0.5uM
1uM
Lanatoside C
B)
120%
100%
80%
60%
40%
20%
0%
Cell Viability
0uM
0.125uM
0.25uM
0.5uM
1uM
Lanatoside C

Figure 1.


A)
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100%
80%
60%
40%
20%
0%
0ug
25ug
50ug
100ug

Figure 2.

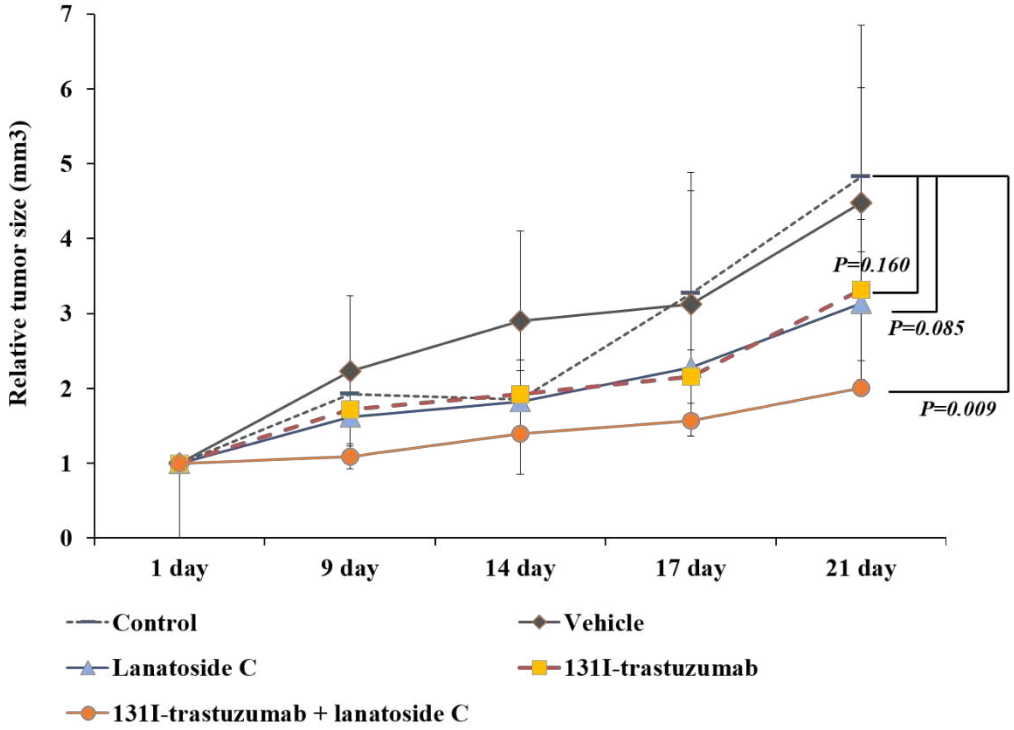
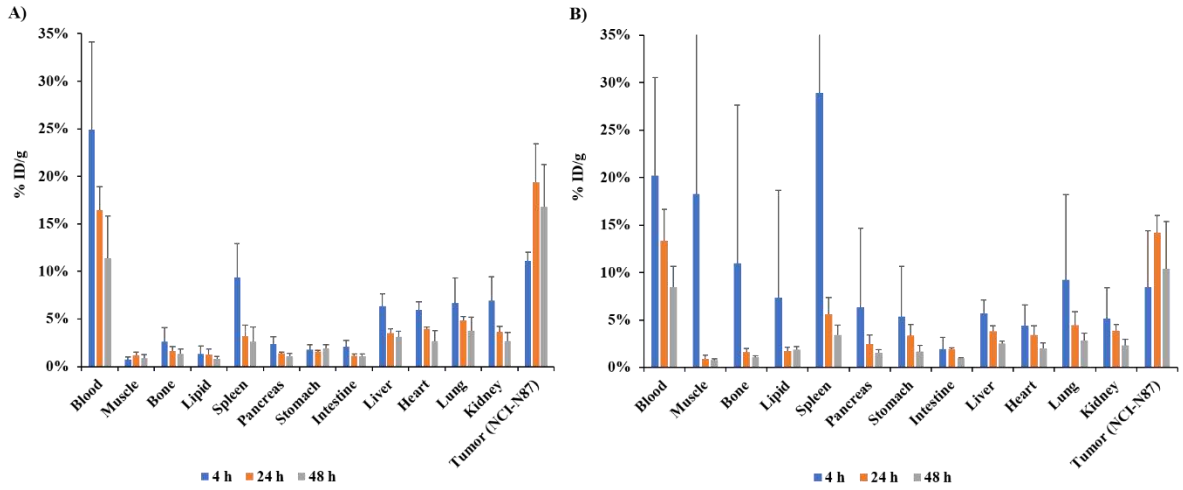

Relative tumor size (mm3)
7
6
5
4
3
2
1
0
1 day
9 day
14 day
17 day
21 day
P=0.160
P=0.085
P=0.009
Control
Vehicle
Lanatoside C
131I-trastuzumab
131I-trastuzumab + lanatoside C

Figure 3.



460

461 **Figure 4.**

Figures

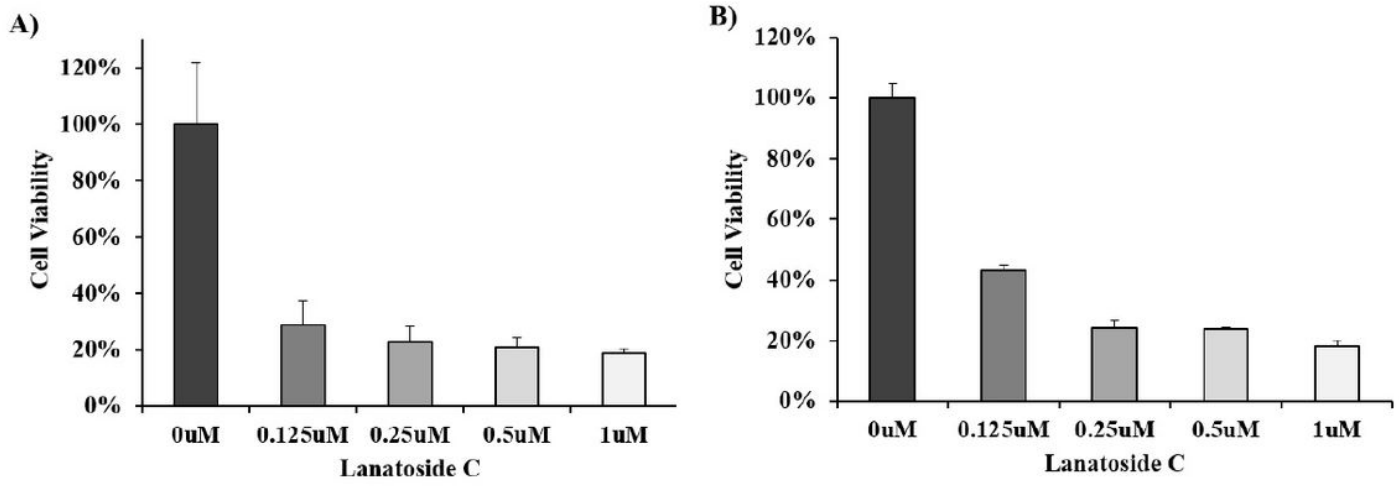


Figure 1

Lanatoside C suppressed growth of cancel cell lines. Inhibitory effect of lanatoside C on cell viability of NCI N87 (A) and MDA MB231 (B) cells. Data are presented as percentage of cell viability in which the untreated control sample is set 100%. The average of experimental triplicates ± standard deviation is shown.

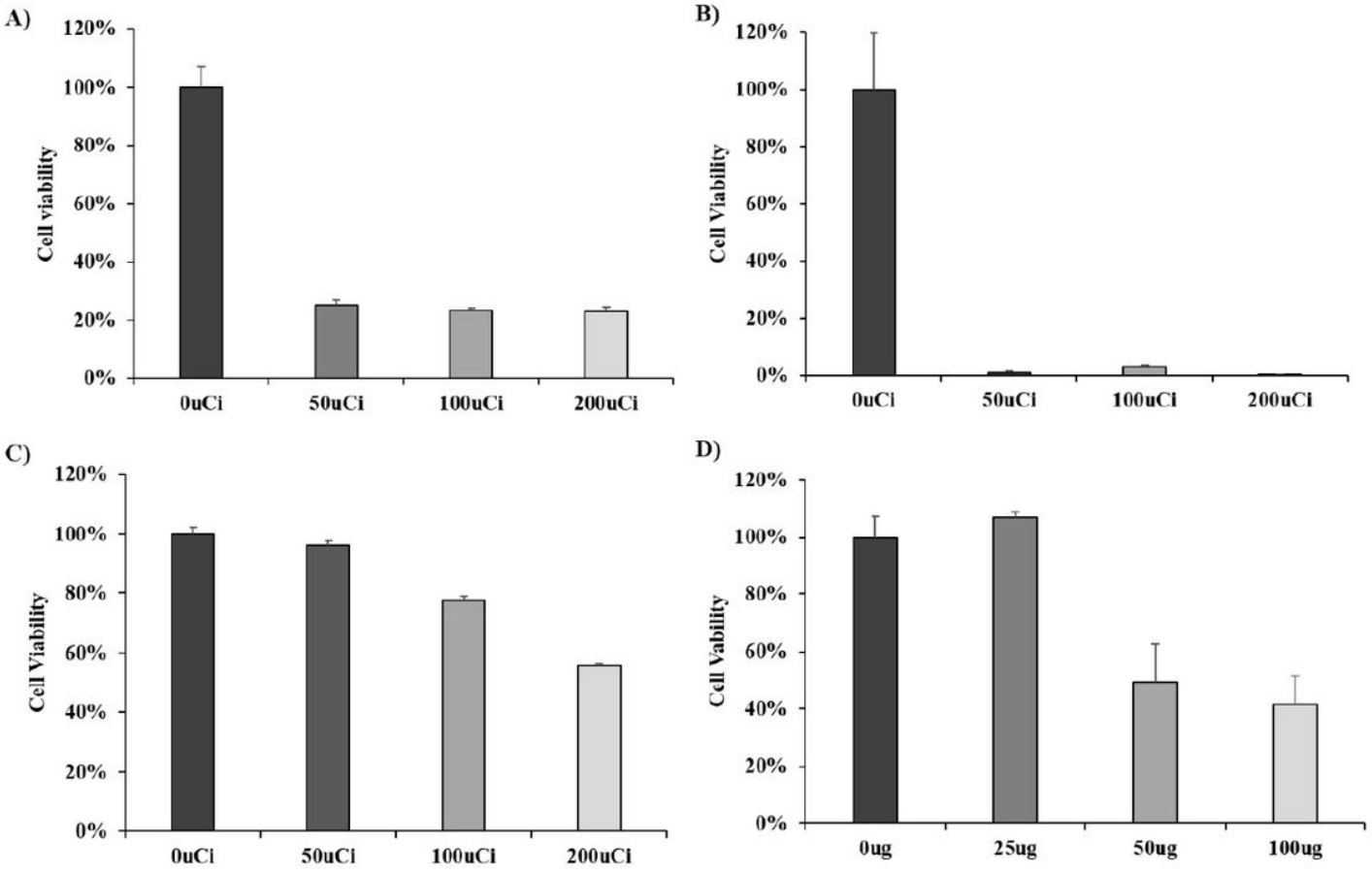


Figure 2

Lanatoside C enhanced 131I trastuzumab RIT in vitro. NCI N87 cells were treated with various activity of 131I trastuzumab RIT (A), 131I trastuzumab RIT combined with lanatoside C (B), 131I alone (C) and trastuzumab alone (D), and cell viability was determined by Ez Cytox assay. Data are presented as percentage of cell viability in which the untreated control sample is set 100%. The average of experimental triplicates ± standard deviation is shown.

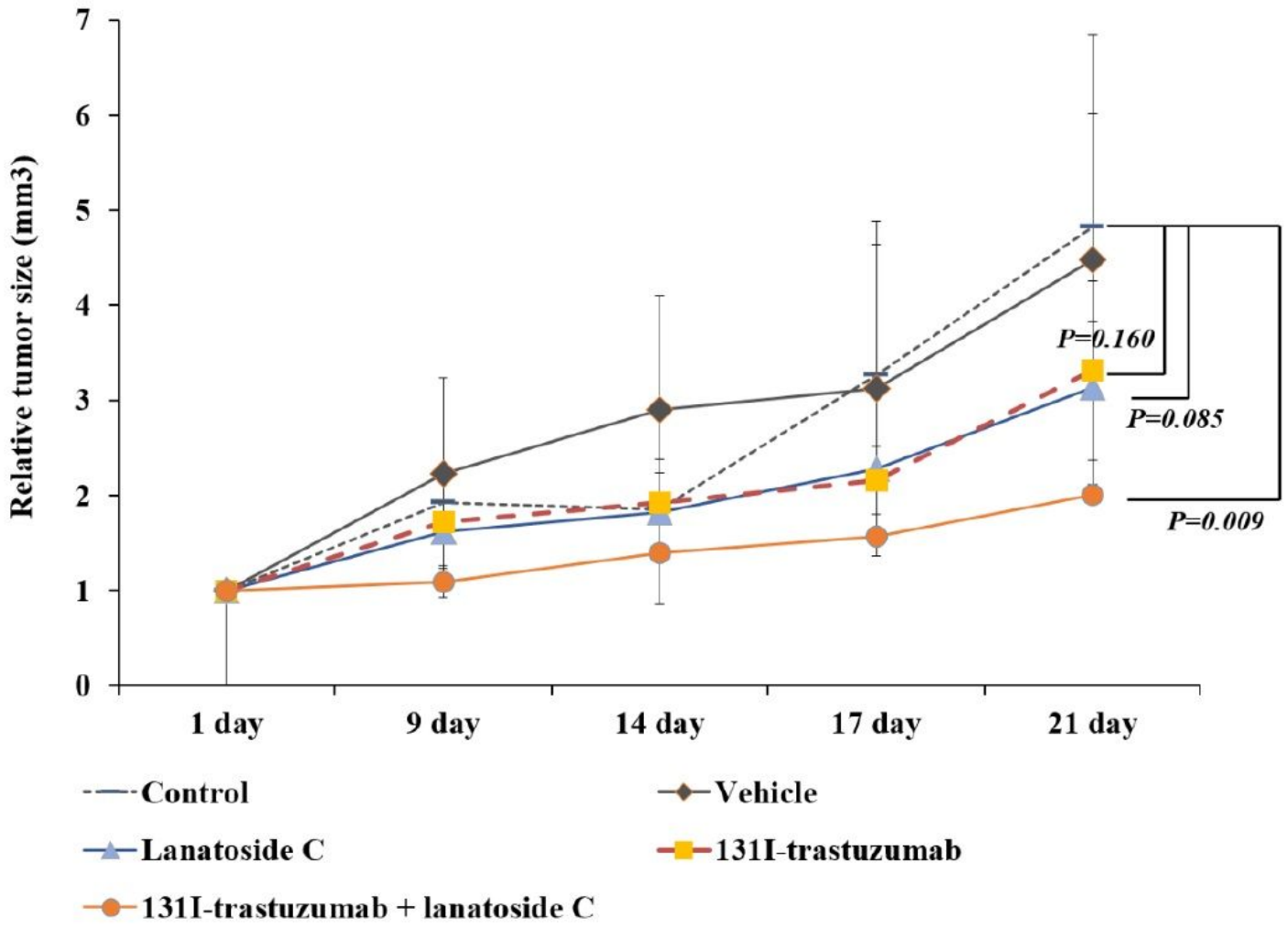


Figure 3

Lanatoside C enhanced 131I trastuzumab RIT in xenograft model. When the NCI N87 tumor volume reached approximately 150 mm3, the BALB/c nude mice were treated with control group (0.01% DMSO in saline, 100 µl, the first 3 days), vehicle group (non treated control), lanatoside C (6 mg/kg body weight, the first 3days intraperitoneal injection), 131I trastuzumab group (400 µCi, once tail vein injection), and combination of 131I trastuzumab and lanatoside C (lanatoside C, 6 mg/kg body weight, the first 3 days intraperitoneal injection, and 131I–trastuzumab, 400 µCi, once tail vein injection).

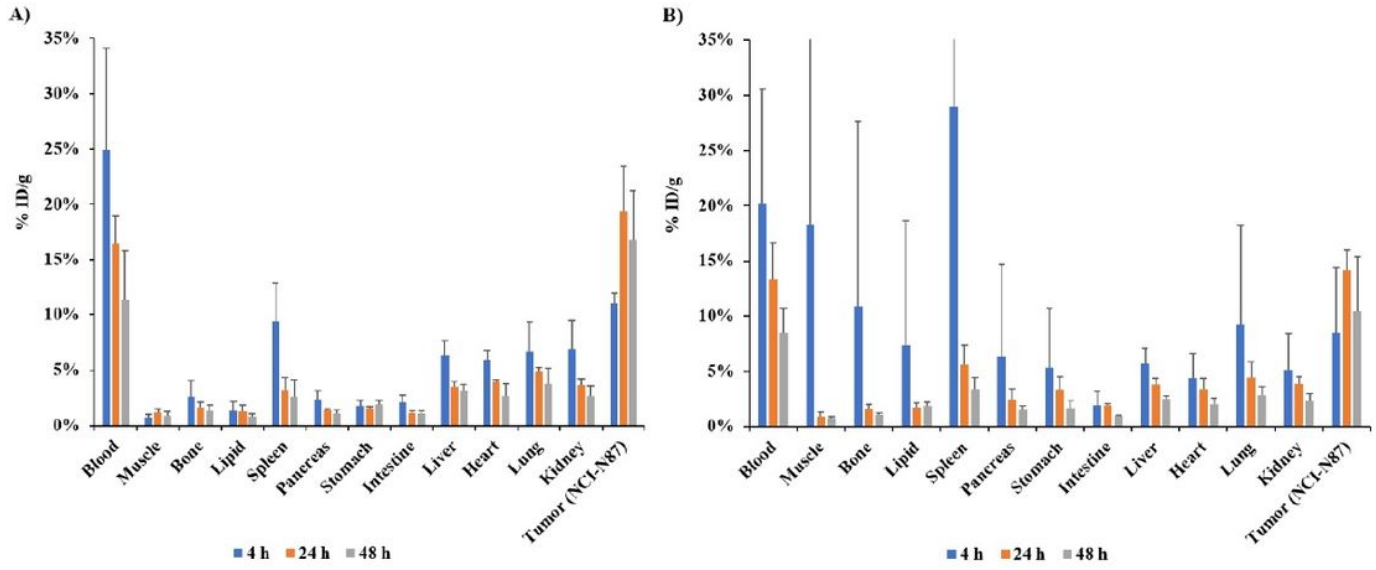


Figure 4

Biodistribution pattern (% ID/g) of 131I trastuzumab (A) or combination of 131I trastuzumab and lanatoside C (B) in tumor (tumor xenograft NCI N87) bearing BALB/c nude mice.