Potential of Three-step Pretargeting Radioimmunotherapy Using Biotinylated Bevacizumab and Succinylated Streptavidin in Triple-negative Breast Cancer Xenograft

Wenchao Gu
Gunma University: Gunma Daigaku

Ryan Yudistiro
Gunma University: Gunma Daigaku

Hirofumi Hanaoka (hanaokah@gunma-u.ac.jp)
Department of Bioimaging Information Analysis, Gunma University Graduate School of Medicine, Maebashi, Japan https://orcid.org/0000-0003-2421-7397

Natsumi Katsumata
Gunma University: Gunma Daigaku

Yoshito Tsushima
Gunma University: Gunma Daigaku

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Abstract

Background Pretargeting radioimmunotherapy (PRIT) is a promising approach that can reduce long-time retention of blood radioactivity and consequently reduce hematotoxicity. Among the PRIT strategies, the combination of biotin-conjugated mAb and radiolabeled streptavidin (StAv) is a simple and convenient method because of ease of preparation. This study performed three-step (3-step) PRIT using the sequential injection of (1) biotinylated bevacizumab (Bt-BV), (2) avidin, and (3) radiolabeled StAv for the treatment of triple-negative breast cancer (TNBC).

Methods Four biodistribution studies were performed using ^{111}In in tumor-bearing mice to optimize each step of our PRIT methods. Further, a therapeutic study was performed with optimized 3-step PRIT using ^{90}Y-labeled StAv.

Results Based on the biodistribution studies, the protein dose of Bt-BV and avidin was optimized to 100 μg and 10 molar equivalent of BV, respectively. Succinylation of StAv significantly decreased the kidney accumulation level (with succinylation (6.96 ± 0.91) vs without succinylation (20.60 ± 1.47) at 1 h after injection, $p < 0.0001$) with little effect on tumor accumulation level. In the therapeutic study, tumor growth was significantly suppressed in treatment groups with optimized 3-step PRIT using ^{90}Y-labeled succinylated StAv compared to that of the no-treatment group ($p < 0.05$).

Conclusion The 3-step PRIT strategy of this study achieved fast blood clearance and low kidney uptake with little effect on tumor accumulation level, and a certain level of therapeutic effect was consequently observed. These results indicated that the pretargeting treatment of the current study may be effective for human TNBC treatment.

Background

Radioimmunotherapy (RIT) has already been used for more than a decade in clinical practice and has shown promising results for lymphoma treatment [1,2]. Monoclonal antibody (mAb) is used as a radionuclide carrier in RIT, which enables high specific accumulation in the tumor. However, the mAb blood clearance is slow because of its high molecular weight (150 kDa) [3,4], causing hematotoxicity, which is dose-limiting toxicity. This hematotoxicity is one of the key factors for the insufficient RIT effect in the treatment of solid tumors. To overcome these limitations, several strategies have been designed to optimize RIT (e.g., smaller size fragment antibodies and pretargeting strategy) [5–7].

The pretargeting concept was proposed more than three decades ago [6,8,9]. In this method, the mAb is injected into the body first. After the mAb is sufficiently accumulated in the tumor, a radiolabeled small molecule targeting the mAb is injected. Thus, pretargeting RIT (PRIT) is a promising approach that can reduce long-time retention of the radioactivity in the blood and consequently reduce the hematotoxicity. One of the major approaches of PRIT is the usage of a high affinity between streptavidin (StAv) and biotin. The combination of biotin-conjugated mAb and radiolabeled StAv is a simple and convenient PRIT method because these molecules are easy to prepare. However, the usefulness of this simple method has

rarely been evaluated because of the relatively large StAv molecular size (53 kDa). In addition, the high renal StAv accumulation evokes renal toxicity.

Bevacizumab, a clinically used anti-vascular endothelial growth factor (VEGF) antibody, is one of the promising RIT carriers [10–12]. However, since bevacizumab has an even longer terminal biological half-life compared to other mAbs (~20 days; range, 11–50 days in patients) [13,14], a blood clearance enhancement strategy is necessary for the application of bevacizumab RIT in clinical settings. In our previous study, avidin injection could accelerate the blood clearance of biotinylated bevacizumab, which improved the therapeutic effect of bevacizumab RIT. Since StAv blood clearance is much faster than that of bevacizumab, PRIT using biotin-conjugated bevacizumab and radiolabeled StAv would be another potential method for bevacizumab RIT.

Biotinylated mAb in the blood would trap radiolabeled StAv immediately after injection, which increases radioactivity in the blood and decreases radioactivity in the tumor. Thus, avidin should be injected as the clearing agent before radiolabeled StAv injection. Succinylation of StAv was reported as an effective method to decrease renal StAv accumulation [15,16]. Therefore, this study tried to elucidate the optimal protocol of the three-step (3-step) PRIT using the sequential injection of (1) biotinylated bevacizumab (Bt-BV), (2) avidin, and (3) radiolabeled succinylated-StAv (succ-StAv). The biodistribution and therapeutic studies in a xenograft model of triple-negative breast cancer (TNBC) were performed.

Materials And Methods

Preparation of tumor xenograft model

All animal experiments were approved by the Animal Experiments Committee of Gunma University. MDA-MB-231, a triple-negative breast cancer cell line, was purchased from American Type Culture Collection (ATTC; Manassas, VA). Cells were grown in RPMI-1640 medium which contains 10% and 1% fetal bovine serum and antibiotics, respectively. The cell suspension was mixed with Matrigel (Corning Life Sciences; Corning, NY) at a 1:1 (*v*/*v*) ratio. Furthermore, the cell suspension (5×10^6 cells/mice) was implanted subcutaneously into the right dorsal flank of a 6-week-old female BALB/c nude mice (Japan SLC, Shizuoka, Japan). The tumor size was measured twice a week using a digital caliper, and the volume was calculated using the $0.5 \times (\text{length} \times \text{width}^2)$ formulation. The biodistribution study was performed after the tumor volume reached approximately 200–300 mm^3.

Preparation of biotinylated and radiolabeled bevacizumab

BV was purchased from Chugai Pharmaceutical Co. (Tokyo, Japan). Bt-BV was prepared with slight modification according to the previously described procedure [17]. BV was reacted with 6-[6-(biotinylamino)hexanoylamin] hexanoic acid *N*-hydroxysuccinimide ester (Dojindo Molecular Technologies; Kumamoto, Japan) in 0.1 M borate buffer (pH 8.5) at the molar ratio of 1:6 overnight at 37°C and purified using a Bio-Spin column (Bio-Rad Laboratories; Hercules, CA) eluted with phosphate-buffered saline (PBS). The average number of biotin molecules per BV was determined as 3.6 according

to the previous report [15]. To obtain ^{111}In-labeled BV, 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid (SCN-Bn-DTPA; Macrocyclics; Dallas, TX) was conjugated to BV and labeled with $^{111}InCl_3$ (Nihon Medi-Physics; Tokyo, Japan) according to the previously described procedure [17].

Preparation of radiolabeled streptavidin analogs

The SCN-Bn-DTPA in dimethylformamide (DMF) was added to StAv (5 mg/mL in 50 mM borate-buffered saline, pH 8.5) at the molar ratio of 5:1 and incubated at 37°C for 24 h. The purification was performed using a Bio-Spin column eluted with 0.1 M borate buffer (pH 8.5). Then, 50 equivalents of succinic anhydride in DMF (8.3 μL) was added to a 200 μL aliquot of a 5 mg/mL solution of DTPA-StAv. After incubating overnight at room temperature, purification was performed using a Bio-Spin column eluted with PBS. For the preparation of ^{111}In- or ^{90}Y-labeled DTPA-StAv and DTPA-succ-StAv, 30 μl (0.37–1.85 MBq) of $^{111}InCl_3$ solution or 20–50 μL (74–185 MBq) of $^{90}YCl_3$ solution (Nucleic; Braunschweig, Germany) was incubated in 0.25 M acetate buffer (pH 5.5, 50 or 100 μL, respectively) for 5 min at room temperature, followed by incubation with DTPA-StAv or DTPA-Succ-StAv (50 or 300 μg in 0.1 M phosphate buffer, respectively) for 1 h at 37°C. To trap unconjugated compounds, 3–5 μL of 100 mM EDTA was added to the reaction mixture. Then, radiolabeled StAv analogs were purified by size-exclusion HPLC using TSKgel SuperSW mAb HR column (7.8 × 300 mm; Tosoh Bioscience, Tokyo, Japan) eluted with 0.1 M phosphate buffer (pH 6.8).

Biodistribution studies

Four types of biodistribution studies were performed in either tumor xenograft mice or normal ddY mice (Japan SLC).

Study 1: Effect of protein dose on biodistribution of ^{111}In-BV in tumor-bearing mice.

To determine the maximum amount of BV binding to the tumor, the biodistribution study of ^{111}In-BVwas performed in tumor-bearing mice. Tumor-bearing mice were injected with 100 μL solution of ^{111}In-BV (20 kBq) with serial amounts of BV (20, 50, 100, 250, and 500 μg/mouse). Mice were sacrificed 24 h after injection, and tissues of interest were excised and weighed. The radioactivity was measured using an automated γ-counter ARC-7001 (Hitachi-Aloka Medical; Tokyo, Japan). The radiotracer uptake was expressed as the percentage of the injected dose per gram of tissue (%ID/g). A reverse sigmoid curve fitting model was used to estimate the nonspecific accumulation of ^{111}In-BV (SigmaPlot software ver. 11.0, Systat Software; San Jose, CA).

Study 2: Comparison between ^{111}In-StAv and ^{111}In-succ-StAv of biodistribution in tumor-bearing mice.

Bt-BV (100 μg/mouse) was injected into tumor-bearing mice, and avidin (44 μg/mouse, a molar equivalent [1 eq]) was injected 24 h later. Three hours after avidin injection, which is 27 h after the Bt-BV injection, ^{111}In-StAv or ^{111}In-succ-StAv (20 kBq/20 μg/mouse) was injected into the tail veins of the

tumor-bearing mice. At 1, 3, 6, 24, and 72 h after radioactivity injection, a radiotracer uptake was evaluated as described above.

Study 3: Effect of avidin dose on biodistribution of ^{111}In-succ-StAv in normal mice.

Normal mice were injected with 100 µL solution of Bt-BV (100 µg/mouse), and the mice were injected with serial amounts of avidin (1, 5, and 10 eq of BV) 24 h later. Three hours after avidin injection, ^{111}In-succ-StAv (20 kBq/20 µg/mouse) was injected. Radiotracer uptake was evaluated as described above at 1, 3, 6, and 24 h after radioactivity injection.

Study 4: Biodistribution of ^{111}In-succ-StAv with final protocol in tumor-bearing mice.

Finally, the pretargeting ^{111}In-succ-StAv biodistribution study was done with an optimized protocol based on studies 1–3 in tumor-bearing mice. Bt-BV (100 µg/mouse) was injected into the mice, and avidin (10 eq) was injected 24 h later. Three hours after avidin injection, ^{111}In-succ-StAv (20 kBq/20 µg/mouse) was injected into the tail veins of the tumor-bearing mice. Radiotracer uptake was evaluated as described above at 1, 3, 6, 24, 72, and 168 h after radioactivity injection.

Therapeutic Study

When the tumors were fully established, animals were randomly divided into four groups (i.e., PRIT with 11.1 MBq (300 µCi, group 1), 9.25 MBq (250 µCi, group 2), and 7.4 MBq (200 µCi, group 3) of ^{90}Y-succ-StAv and no treatment (group 4)). Bt-BV was injected intravenously into tumor-bearing mice as the PRIT strategy of this study. After 24 h, 10 eq of avidin was administered. Lastly, 3 h after avidin injection, ^{90}Y-succ-StAv was administered. The tumor size was measured twice a week. The observation was terminated when the tumor volume exceeded 2,000 mm^3 or the mice were dead.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). GraphPad Prism software version 6.0 (GraphPad Software; La Jolla, CA) was used for statistical analysis. The Student's *t*-test was used for comparing the statistical difference of the two groups, and $p < 0.05$ was considered significant.

Results

Biodistribution study

The tumor accumulation level was decreased in a dose-dependent manner (Fig. 1). Based on the fitting curve, nonspecific accumulation of ^{111}In-BV was estimated at 7.5%ID/g, and BV binding to the target would be saturated by injecting 100 µg BV in the tumor-bearing model of this study.

Using the 3-step PRIT method, both ^{111}In-StAv and ^{111}In-succ-StAv showed relatively rapid blood clearance and tumor accumulation at early time points (Fig. 2). On the other hand, renal accumulation of

^{111}In-succ-StAv was significantly lower than that of ^{111}In-StAv ($p < 0.05$). The accumulation level in some other organs showed a significant difference between ^{111}In-StAv and ^{111}In-succ-StAv, but the difference was not remarkable.

Next, we explored the influence of the amount of avidin on the blood clearance enhancement of ^{111}In-succ-StAv in normal mice. The blood radioactivity was significantly decreased with dose escalation (Fig. 3, $p < 0.05$). On the other hand, other organs showed no significant difference even in 10 eq of avidin dose.

Based on these results, 100 μg was used as the protein dose of Bt-BV and 10 eq of avidin injection in the following studies. A biodistribution study of ^{111}In-succ-StAv in tumor-bearing mice was performed to evaluate the final protocol of the 3-step PRIT. The blood radioactivity was rapidly decreased, and the renal accumulation level was not high. ^{111}In-succ-StAv accumulated in the tumor and remained until 72 h after injection (Fig. 4). The tumor-to-blood ratio became more than 1.0 from 24 h after injection.

Therapeutic Study

Based on the preliminary therapeutic study, the injection of 11.1 MBq as the highest dose was decided on. However, two of three mice treated with 11.1 MBq, four of five mice treated with 9.25 MBq, and one of five mice treated with 7.4 MBq died 2–3 weeks after treatment. Tumor growth was significantly suppressed in all the treatment groups compared to that of the no-treatment group (Fig. 5, $p < 0.05$). The mean tumor volume at day 10 in groups 1, 2, 3, and 4 was 392.3 ± 65.7, 468.4 ± 159.7, 526.8 ± 154.0, and 945.0 ± 474.5 mm^3, respectively.

Discussion

The ideal RIT protocol is to deliver the highest radiation to the tumor with a safe dose. Thus, this study tried to use the 3-step PRIT approaches to increase the therapeutic index. Tumor growth in all three treatment groups was significantly suppressed longer than that in the control group. The result of the current study indicates that the 3-step PRIT has a good therapeutic effect and a good potential for TNBC treatment.

Since the blood clearance of bevacizumab is very slow, the removal of antibodies is necessary before the injection of a radiolabeled molecule. Our previous study showed the usefulness of avidin injection to accelerate the blood clearance of biotinylated bevacizumab [17]. Thus, the 3-step system using biotinylated mAb, avidin, and radiolabeled StAv was selected. In this study, avidin chase can significantly accelerate blood clearance as expected. However, 10 eq of avidin injection showed better results compared with 1 eq avidin injection, unlike our previous data. It may be that most Bt-BV would be cleared from the circulation by injecting 1 eq avidin chase, however, small amounts of Bt-BV remaining in the blood could trap the injected radiolabeled StAv in case of the pretargeting method of the current study. In

addition, a higher dose of Bt-BV (100 μg) increased the radioactivity level in the blood, which would require a higher dose of avidin.

StAv is a 58 kDa protein and has biotin-binding characteristics similar to avidin, but it is not trapped by the liver because it is not glycosylated [18,19]. Thus, radiolabeled StAv is gradually eliminated from the circulation and accumulates in the tumor, then binds to the Bt-BV which is already located on the tumor site. However, high renal accumulation and retention of StAv are of great PRIT concern because renal toxicity is usually a dose-limiting factor in cases of small- to medium-size agents. Radiolabeled peptides and proteins smaller than albumin are usually reabsorbed by the renal cell, and radiometabolite of metal-labeled molecules are retained in the kidney [20,21]. Succinylation of StAv, which was reported to dramatically decrease renal accumulation without impairing affinity to the biotin, was used to decrease the renal uptake [16,22]. The renal uptake of ^{111}In-labeled StAv was significantly decreased by succinylation as expected. Notably, the tumor accumulation level did not impair by succinylation, resulting to a significantly high observation of tumor-to-kidney ratio at 72 h (^{111}In-succ-StAv (1.45 ± 0.10) vs ^{111}In-StAv (0.073 ± 0.20), $p < 0.01$). This result was consistent with the previous report [16,23].

The radioactivity in the blood and the kidney were significantly reduced, while tumor uptake was also reduced by injecting a higher dose of avidin and StAv succinylation. The higher radiation-absorbed dose in the tumor was achieved by increasing the radioactivity dose because the lower tumor uptake would be mainly caused by the faster blood clearance. Notably, the tumor-to-blood and tumor-to-kidney ratio were significantly increased using the PRIT method of this study compared to StAv with 1 eq avidin or succ-StAv with 1 eq avidin (Additional fle 1: Figure S1). Although clinical studies are required to optimize the protocol for human beings, the results of the current study indicated the potential of the 3-step PRIT using bevacizumab.

In the therapeutic study, tumor growth was significantly suppressed by the 3-step PRIT method. The therapeutic effect of the 3-step PRIT method was of a similar level to that of the ^{90}Y-BV but inferior to that of ^{90}Y-Bt-BV with avidin chase [15]. This insufficient therapeutic effect is caused by the unexpectedly low maximum tolerated dose (MTD) of ^{90}Y-succ-StAv. Further studies are required to figure out the cause of death, which is beyond the scope of the current study. The MTD of each method for humans is unknown and an inferior-to-superior relationship between these methods would be changed because the pharmacokinetics of proteins are much different between mouse and human. However, this study at least showed the potential of the 3-step PRIT method. In addition, the 3-step PRIT method of the current study would be beneficial for using α-emitter with a short half-life (e.g., At-211, Pb-212, and Tb-149) because of faster blood clearance of radiolabeled StAv compared with mAb.

Conclusion

The 3-step pretargeting RIT strategy using the avidin–biotin system and succinylated StAv of the current study achieved fast blood clearance and low kidney uptake with little effect on tumor accumulation level.

In addition, a certain level of therapeutic effect was observed. These results indicated that the pretargeting treatment of this study may be effective for human TNBC treatment.

Abbreviations

RIT, radioimmunotherapy; PRIT, pretargeting radioimmunotherapy; StAv, streptavidin; Bt-BV, biotinylated bevacizumab; TNBC, triple-negative breast cancer; mAb, monoclonal antibody; VEGF, vascular endothelial growth factor; succ-StAv, succinylated-StAv; PBS, phosphate-buffered saline; SCN-Bn-DTPA, 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid; DMF, dimethylformamide;

Declarations

Acknowledgements

Not applicable

Authors' contributions

HH participated in the design of the study. Data acquisition was done by GW, RY, HH, and NK. Data analysis was done by GW and RY. GW drafted the manuscript. HH and YT critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Animal studies were performed in accordance with institutional guidelines and were approved by the Local Animal Care Committee of Gunma University (approval number: 17-035).

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

Author details

[1]Department of Diagnostic Radiology and Nuclear Medicine, Gunma University Graduate School of Medicine, Maebashi, Japan.

[2]Department of Bioimaging Information Analysis, Gunma University Graduate School of Medicine, Maebashi, Japan.

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Figures

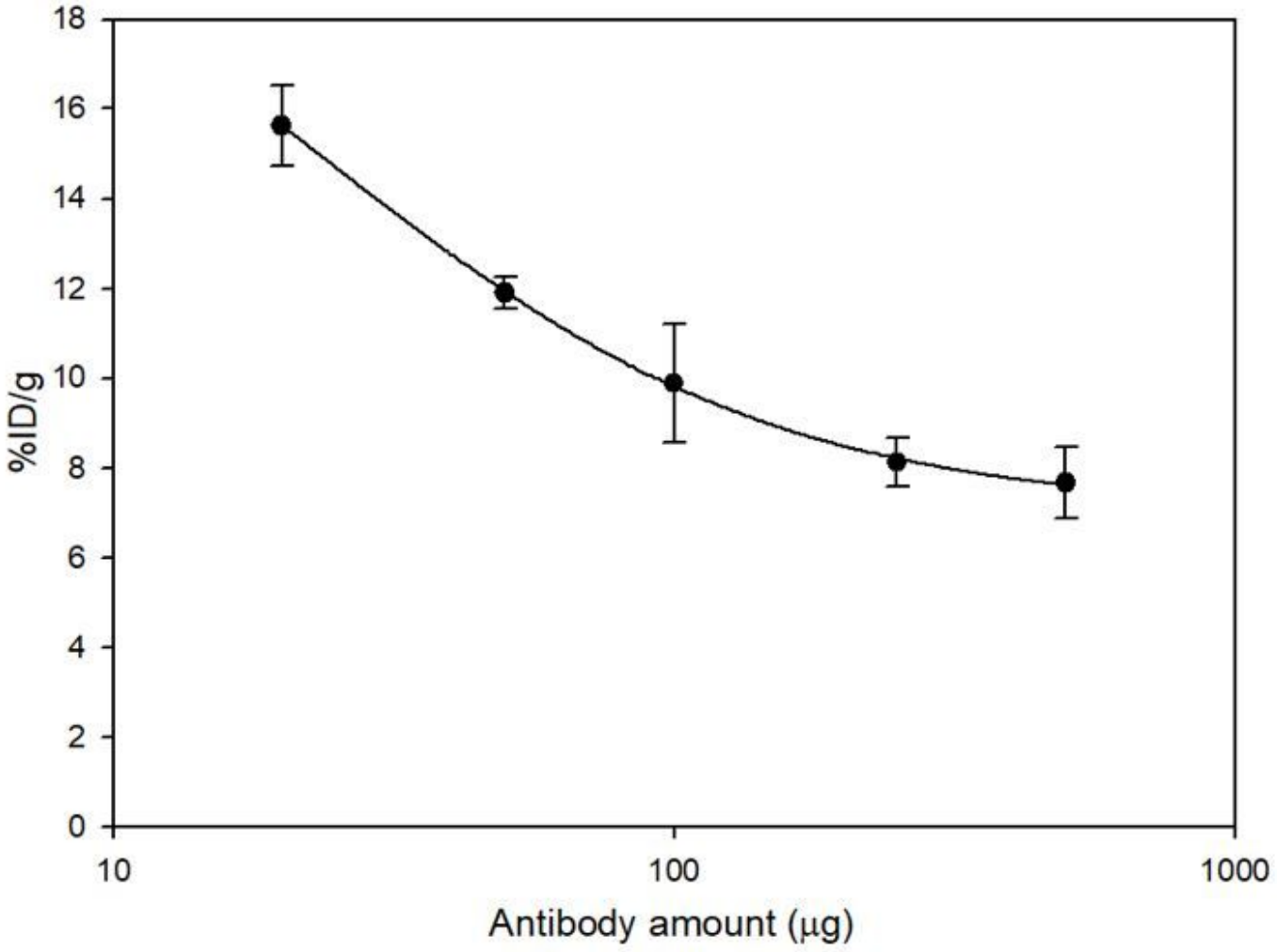

Figure 1

Tumor accumulation level of 111In-BV at 24 h after injection of different doses of antibody in tumor-bearing mice.

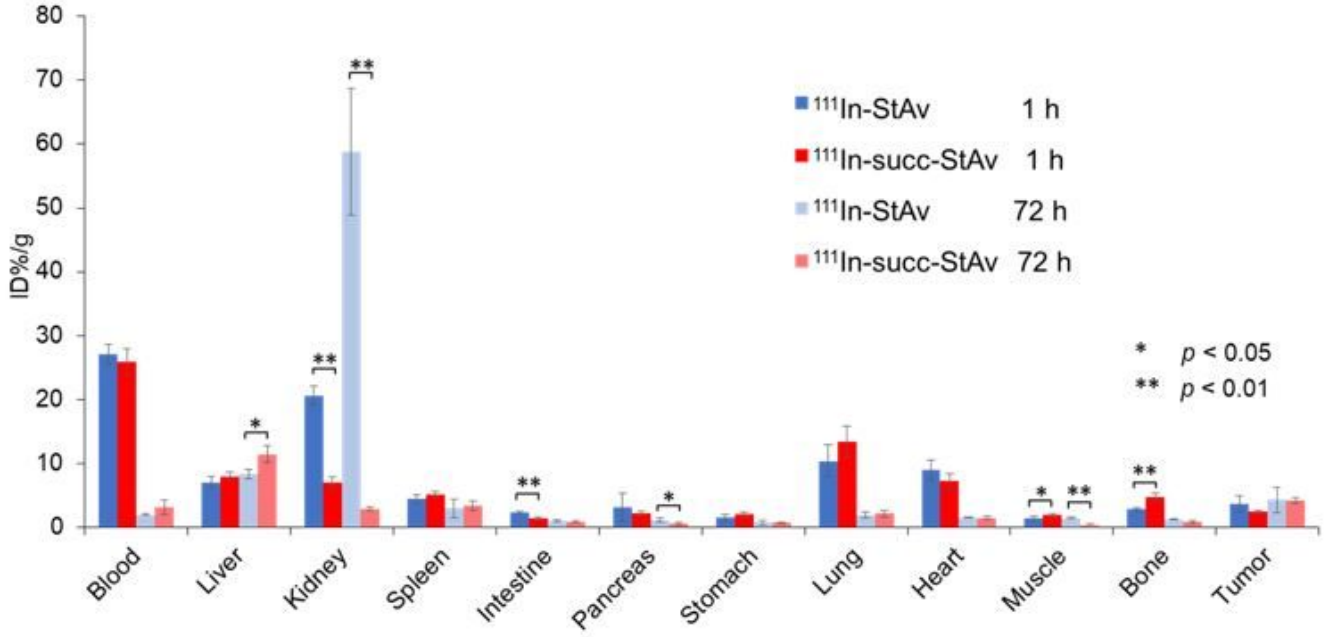

Figure 2

Biodistribution of radioactivity at 1 and 72 h after injection of 111In-StAv or 111In-succ-StAv with 1 eq avidin in tumor-bearing mice. Significant differences between the 111In-succ-StAv group and 111In-StAv group (*p < 0.05, **p < 0.01).

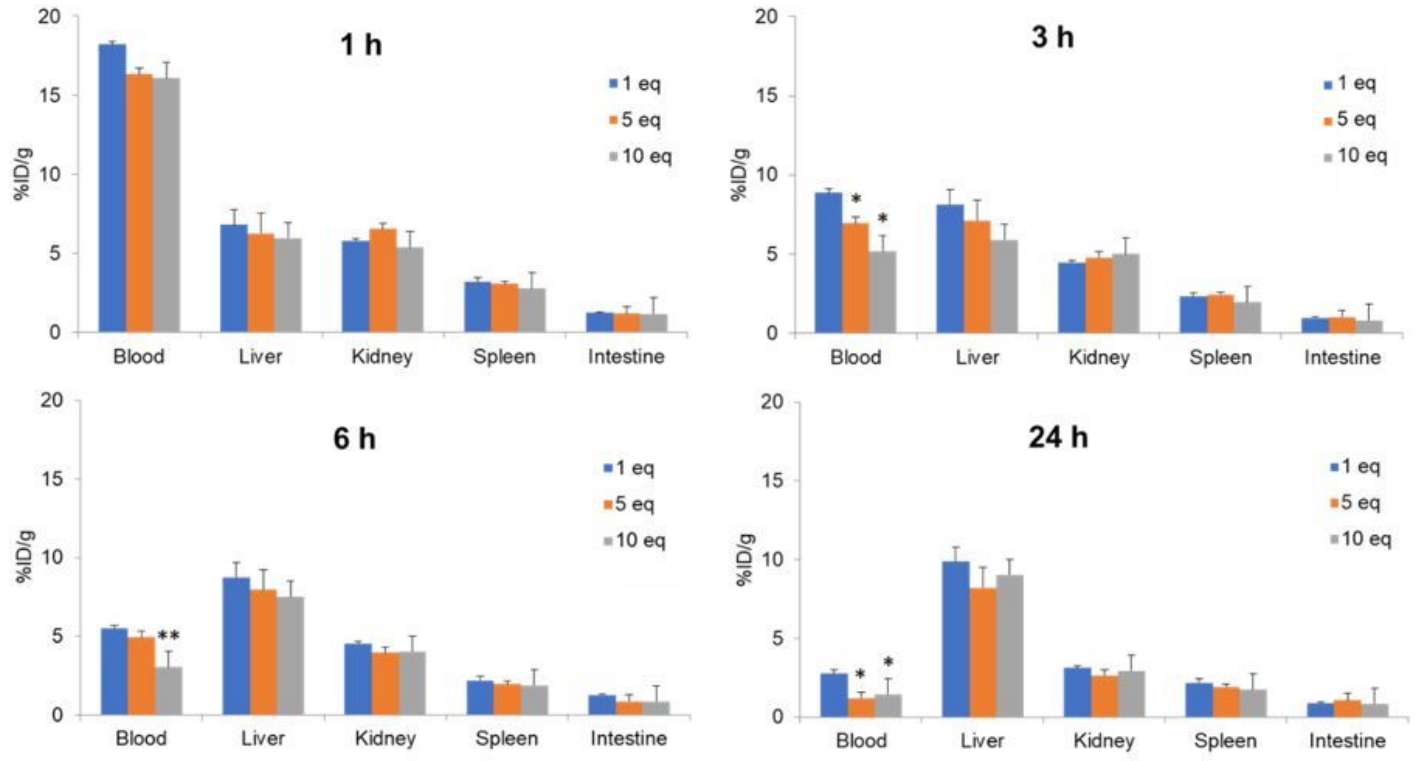

Figure 3

Biodistribution of radioactivity at 1, 3, 6, and 24 h after injection of 111In-succ-StAv with 1, 5, and 10 eq avidin in normal mice. Significant differences from the 1 eq dose group (*p < 0.05, **p < 0.01).

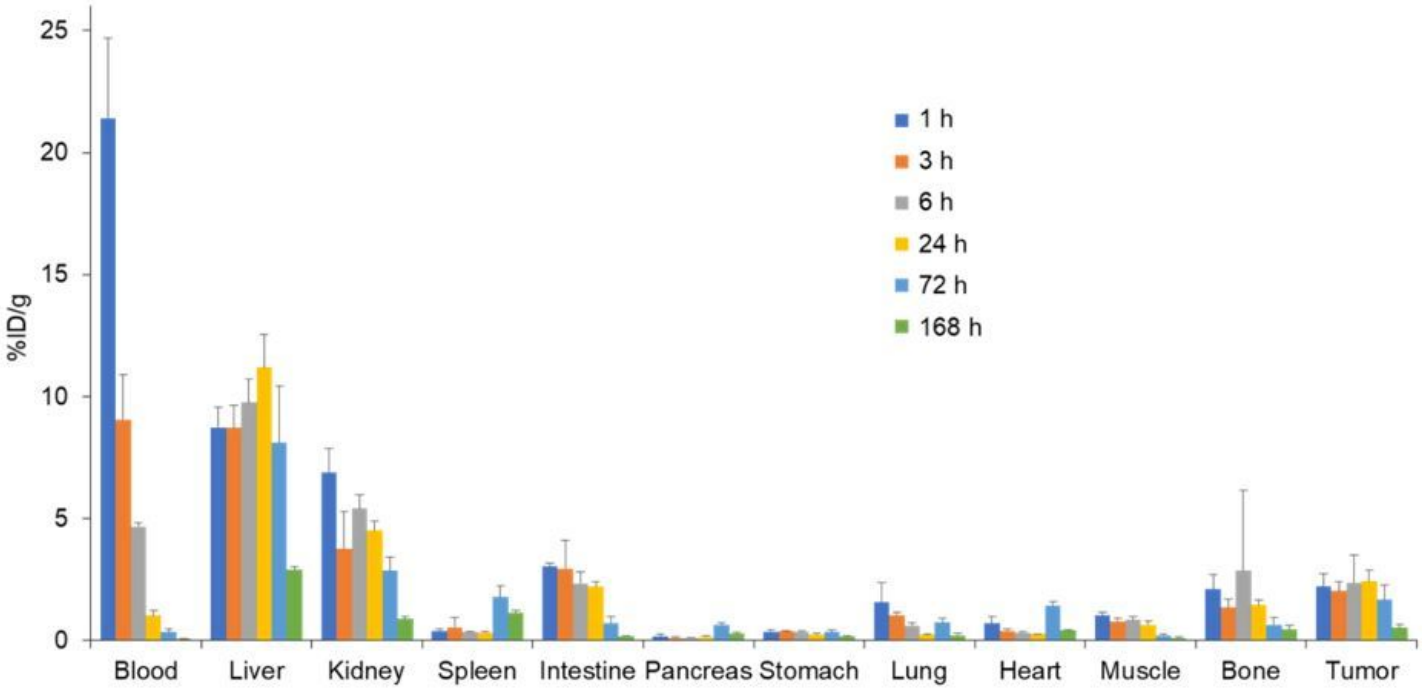

Figure 4

Biodistribution of radioactivity at 1, 3, 6, 24, 72, and 168 h after injection of 111In-succ-StAv with 10 eq avidin in tumor-bearing mice.

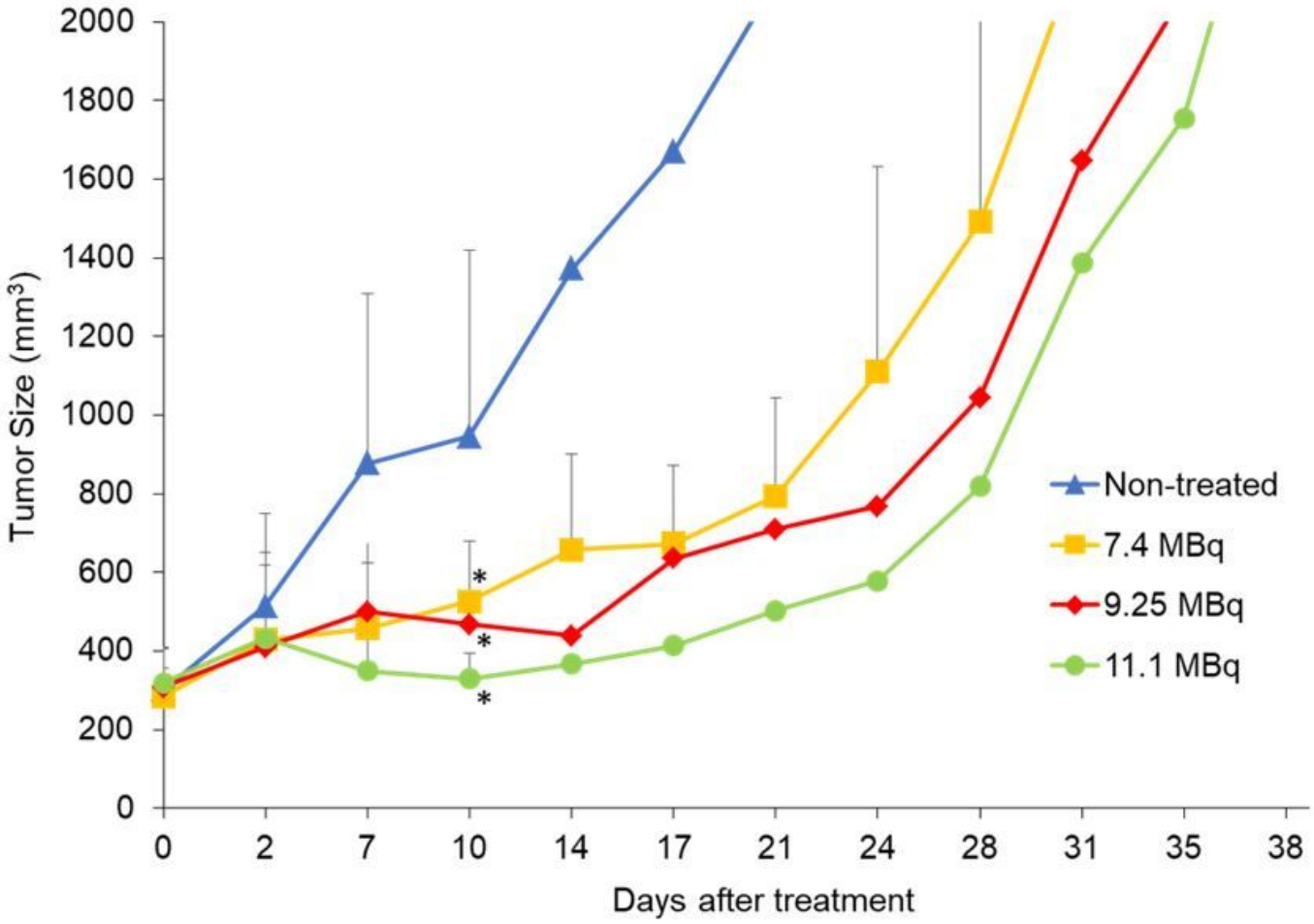

Figure 5

Therapeutic results of 3-step PRIT (Bt-BV + avidin (10 eq) + 90Y-succ-StAv) in tumor-bearing mice. Significantly different from the non-treated group (*p < 0.05).

Supplementary Files

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- FigS1.JPG
- supplmaterial.pdf