\( \alpha_\beta_3 \) Integrin-targeting radionuclide therapy and imaging with monomeric RGD peptide

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The \( \alpha_\beta_3 \) integrin plays a pivotal role in angiogenesis and tumor metastasis. Angiogenic blood vessels overexpress \( \alpha_\beta_3 \) integrin, as in tumor neovascularization, and \( \alpha_\beta_3 \) integrin expression in other microvascular beds and organs is limited. Therefore, \( \alpha_\beta_3 \) integrin is an available receptor for tumor-targeting imaging and therapy. Recently, tetrameric and dimeric RGD peptides have been developed to enhance specificity to \( \alpha_\beta_3 \) integrin. In comparison to the corresponding monomeric peptide, however, these peptides show high levels of accumulation in kidney and liver. The purpose of this study is to evaluate tumor-targeting properties and the therapeutic potential of \( ^{111}\text{In}-\) and \(^{90}\text{Y}\)-labeled monomeric RGD peptides in BALB/c nude mice with SKOV-3 human ovarian carcinoma tumors. DOTA-c(RGDfK) was labeled with \(^{111}\text{In} \) or \(^{90}\text{Y} \) and purified by HPLC. A biodistribution study and scintigraphic images revealed the specific uptake to \( \alpha_\beta_3 \) integrin and the rapid clearance from normal tissues. These peptides were renally excreted. At 10 min after injection of tracers, \( ^{111}\text{In-DOTA-c(RGDfK)} \) and \( ^{90}\text{Y-DOTA-c(RGDfK)} \) showed high uptake in tumors (7.3 \( \pm \) 0.6% ID/g and 4.6 \( \pm \) 0.8% ID/g, respectively) and gradually decreased over time (2.3 \( \pm \) 0.4% ID/g and 1.5 \( \pm \) 0.5% ID/g at 24 hr, respectively). High tumor-to-blood and -muscle ratios were obtained from these peptides. In radionuclide therapeutic study, multiple-dose administration of \( ^{99m}\text{Tc-DOTA-c(RGDfK)} \) (3 \( \times \) 1.1 MBq) suppressed tumor growth in comparison to the control group and a single-dose administration (11.1 MBq). Monomeric RGD peptides, \( ^{111}\text{In-DOTA-c(RGDfK)} \) and \( ^{99m}\text{Tc-DOTA-c(RGDfK)} \), could be promising tracers for \( \alpha_\beta_3 \) integrin-targeting imaging and radiotherapy.

Key words: RGD peptide; radionuclide therapy; \( \alpha_\beta_3 \) integrin; \(^{90}\text{Y} ; ^{111}\text{In} \)

Angiogenesis has a close relationship with tumor proliferation and metastasis. The interactions of cell–cell and cell-matrix are implicated in both angiogenesis and metastasis. Integrins are cell adhesion molecules and have basic roles in angiogenesis and metastasis. There are several isoforms of integrins consisting of \( \alpha \) chains and \( \beta \) chains. The \( \alpha_\beta_3 \) integrin is highly expressed in endothelial cells in angiogenesis and tumor cells (e.g., breast cancer, ovarian cancer, brain tumor, etc.), although the expression of \( \alpha_\beta_3 \) integrin is not restricted to tumor cells and activated endothelial cells.

The \( \alpha_\beta_3 \) integrin recognizes the amino acid sequence of arginine-glycine-aspartic acid (RGD peptide). On the basis of the RGD peptide, many peptidomimetic compounds and peptides have been designed to antagonize the \( \alpha_\beta_3 \) integrin. These compounds and anti-\( \alpha_\beta_3 \) integrin monoclonal antibodies have been reported to inhibit angiogenesis without affecting preexisting vessels. Because of its restricted expression, the \( \alpha_\beta_3 \) integrin is an attractive targeting molecule for tumor imaging and therapy, leading to decreased side effects compared to conventional chemotherapy.

The expression of the \( \alpha_\beta_3 \) integrin has been reported to be associated with metastatic potential in melanoma, breast cancer, and colon cancer. The development of radiopharmaceuticals for targeting the \( \alpha_\beta_3 \) integrin would be clinically beneficial, not only for screening and for treating patients with \( \alpha_\beta_3 \) integrin-positive tumors but also for monitoring therapeutic efficacy.

Many RGD peptides labeled with gamma-emitting and positron-emitting nuclides (\(^{18}\text{F} , ^{64}\text{Cu}, ^{99m}\text{Tc} , ^{125}\text{I} \), etc.) have been reported as angiogenesis-imaging agents. A recent trend in the development of RGD peptides is the multimerization of RGD peptides to improve the high tumor accumulation and retention of RGD peptides. However, this also leads to the enhancement of radioactive accumulation in nontargeted organs such as kidney and liver. Compared with antibody or multimeric RGD peptides, monomeric RGD peptides have a lower molecular mass. Therefore, monomeric RGD peptides labeled with \(^{90}\text{Y} \) are thought to be promising radiopharmaceuticals for tumor therapy causing low radioactivity exposure to normal tissues such as kidney and liver. In this report, we describe the tumor therapeutic and imaging potential of DOTA-conjugated \(^{90}\text{Y} ; ^{111}\text{In} \)-monomeric RGD peptide.

Material and methods

**Radiolabeling of DOTA-c(RGDfK)**

(c(RGDfK)) was synthesized and conjugated with DOTA by Thermo Electron GmbH (Ulm, Germany). \(^{111}\text{InCl}_3 \) and \(^{90}\text{YCl}_3 \) were kindly provided by Chiyoda Technol Corp. (Tokyo, Japan) and Nihon Medi-Physics Co., Ltd. (Nishinomiya, Japan), respectively. Briefly, 40 \( \mu \)L of 3 M acetate buffer (pH 6.0) was added to 18.5 MBq of \(^{111}\text{InCl}_3 \). The mixture was allowed to stand for 5 min at room temperature, and then added to 0.1 mg of DOTA-c(RGDfK). The mixture was heated for 15 min at 100°C. The purification of \(^{111}\text{In-DOTA-c(RGDfK)} \) was performed by RP-HPLC using a Cosmosil 5C18-AR 300 column (4.6 \( \times \) 150 mm, Nacalai Tesque, Kyoto, Japan) eluted with 90% of 0.1% aqueous trifluoroacetic acid and 10% of acetonitrile with 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. The radiochemical purity was determined as described for the purification. \(^{90}\text{Y-DOTA-c(RGDfK)} \) was prepared as described for \(^{111}\text{In-DOTA-c(RGDfK)} \). The radiochemical purity of the radiolabeled DOTA-c(RGDfK) was over 99% (Fig. 1).

**Cell culture**

SKOV-3 human ovarian carcinoma cell line was purchased from American Tissue Culture Collection (ATCC, Manassas, VA) and maintained in DMEM (Sigma, St. Louis, MO) containing 4.5 g/l glucose and 10% FBS. Cells were cultured in a 5% CO\(_2\) humidified atmosphere at 37°C.
The mice were injected via the tail vein with 370 kBq of $^{90}$Y-DOTA-c(RGDfK) for 1-hr postinjection. The highest uptake in organs other than kidney and bone (Fig. 3). The tumor uptake of $^{90}$Y-DOTA-c(RGDfK) showed a distribution pattern similar to $^{111}$In-DOTA-c(RGDfK) (Fig. 1). $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) were eluted in a single peak with a retention time of 9.7 min. Minor peaks were observed with a retention time of 7.0 and 8.5 min. After purification, radiochemical purity of $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) exceeded 99%.

Biodistribution and animal imaging

Biodistribution and animal imaging studies were performed in female BALB/c nude mice with SKOV-3 tumors. On scintigraphic images made at 1- and 4-hr postinjection of $^{111}$In-DOTA-c(RGDfK), kidney and bladder were the organs with high activity, suggesting the renal excretion pattern of $^{111}$In-DOTA-c(RGDfK) (Fig. 2). Kidney was clearly visualized at 24-hr postinjection. Following the clearance of $^{111}$In-DOTA-c(RGDfK) from normal tissues, the SKOV-3 tumors were clearly delineated at 4- and 24-hr postinjection. The images showed high tumor to nontumor ratios (2.33 at 4-hr postinjection and 2.07 at 24-hr postinjection). The tumor and kidney uptake was significantly inhibited by coinjection of 100 µg of DOTA-c(RGDfK).

$^{90}$Y-DOTA-c(RGDfK) showed a distribution pattern similar to that of $^{111}$In-DOTA-c(RGDfK) (Fig. 3). The tumor uptake of $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) was high (2.53% ID/g and 6.28% ID/g at 1-hr postinjection) and remained at 0.94% ID/g and 1.86% ID/g at 48-hr postinjection, respectively. After injection of both peptides, the radioactivity rapidly cleared from the blood and was 0.05% ID/g for $^{90}$Y-DOTA-c(RGDfK) and 0.13% ID/g for $^{111}$In-DOTA-c(RGDfK) at 1-hr postinjection. Kidney also showed the rapid clearance. In some mice, radioactivity in the blood was not detectable at 48-hr postinjection. Various normal tissues such as muscle, liver, and pancreas had relatively lower uptake. These resulted in high tumor-to-blood (T/B) - muscle (T/M) - kidney (T/K) ratios (Fig. 4). The $T/B$ ratios of both tracers rose above 500 and the $T/M$ ratios rose to 41 for $^{90}$Y-DOTA-c(RGDfK) and 50 for $^{111}$In-DOTA-c(RGDfK) at 24-hr postinjection. The $T/K$ ratios of both tracers were more than 1.53 after 1-hr postinjection. The highest $T/K$ ratio for $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) were 2.64 at 24-hr postinjection.

Receptor specificity study

The $\alpha_\beta_3$ integrin-mediated uptake of $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) was investigated by computing the biodistribution of $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) in mice with SKOV-3 tumors in the presence or absence of 100 µg of DOTA-c(RGDfK). Biodistribution was determined as described above at 1-hr postinjection.

Radionuclide therapy

Mice with s.c. SKOV-3 tumors received 11.1 MBq of $^{90}$Y-DOTA-c(RGDfK). One group received $^{90}$Y-DOTA-c(RGDfK) for 1 day, another group for 3 days. As a control group, a third group received saline. Tumor size was measured 3 times weekly. Tumor volume was calculated using the formula: volume = $4/3 \pi (1/2$ length $\times 1/2$ width $\times 1/2$ height).

Radiation dosimetry extrapolation to humans

Estimated human dosimetry was calculated from biodistribution results of $^{111}$In-DOTA-c(RGDfK) in female BALB/c nude mice with SKOV-3 tumors, assuming that the biodistribution of the radiopharmaceuticals in mice is the same as in adult humans. Residence times were calculated by monoexponential extrapolation of the biodistribution data. According to residence times, radiation doses were calculated for male adults using a standard quantitation platform, Organ Level Internal Dose Assessment (OLinda; Vanderbilt University).

Statistical evaluation

Mann-Whitney U test was used for the receptor specificity study. Kruskal-Wallis test followed by Dunn’s post hoc test compared to the control group was used for the radionuclide therapy experiment. The results were considered statistically significant at $p < 0.05$.

Results

Radiolabeling of DOTA-c(RGDfK) with $^{90}$Y and $^{111}$In

$^{90}$Y-DOTA-c(RGDfK) showed a HPLC profile similar to $^{111}$In-DOTA-c(RGDfK) (Fig. 1). $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) were eluted in a single peak with a retention time of 9.7 min. Minor peaks were observed with a retention time of 7.0 and 8.5 min. After purification, radiochemical purity of $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) exceeded 99%.

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Integrin specificity study

Coinjection of 100 µg of DOTA-c(RGDfK) with $^{90}$Y-DOTA-c(RGDfK) or $^{111}$In-DOTA-c(RGDfK) significantly decreased the uptake in various tissues other than kidney and bone (Fig. 5). Tumor uptake of $^{111}$In-DOTA-c(RGDfK) was reduced most...
markedly from 5.76% ID/g to 0.59% ID/g. For $^{111}$In-DOTA-c(RGDfK), kidney and bone showed moderate decrease from 2.97% ID/g to 1.58% ID/g and from 0.31% ID/g to 0.18% ID/g, respectively.

Radionuclide therapy

The growth curves of the 2 groups of mice with $^{90}$Y-DOTA-c(RGDfK) therapy and the control group are shown in Figure 6. Tumor volumes were 32.0 ± 7.0 mm$^3$ for the control group, 45.5 ± 21.4 mm$^3$ for the single-dose group (11.1 MBq × 1), and 39.0 ± 8.3 mm$^3$ for the multiple-dose administration group (11.1 MBq × 3) at day 0. The single-dose administration did not show significant inhibition to tumor growth. In contrast, the multiple-dose administration did inhibit tumor growth. At 23 days after therapy, the tumor volume was 7.2 times greater for the multiple-dose administration group compared to 9.9 times greater for the single-dose group and 13.6 times greater for the control group. Throughout the experiment, no difference in body weight was found among the 3 groups.

Radiation dosimetry

Human absorbed doses to normal organs were estimated from the biodistribution data of $^{111}$In-DOTA-c(RGDfK) in female nude mice, assuming that the biodistribution and pharmacokinetics of $^{111}$In-DOTA-c(RGDfK) in mice and adult human are the same (Table I). The highest absorbed dose was to the kidneys (0.568 mGy/MBq), although all organs had a low level of radiation doses.

Discussion

$^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) showed a high and retentive accumulation in the tumor tissue with a rapid clearance from normal tissues. A trend in the biodistribution of $^{90}$Y-DOTA-c(RGDfK) was similar to that of $^{111}$In-DOTA-c(RGDfK), although the amount of radioactivity in all tissues from $^{90}$Y-DOTA-c(RGDfK) was smaller than that from $^{111}$In-DOTA-c(RGDfK). This could have resulted from measuring the bremsstrahlung from $^{90}$Y because the bremsstrahlung from $^{90}$Y contains low energy spectrum and is easily absorbed by tissues as compared to γ rays. Many studies have been reported using radiolabeled monomeric RGD peptides. In a U-87 MG glioblastoma model, $^{125}$I-c(RGDyK) showed a high accumulation (8.97% ID/g) at 30-min postinjection. However, other studies reported a low accumulation of the RGD peptides in tumors. The tumor uptake of $^{111}$In-DOTA-c(RGDfK) was relatively high (6.28% ID/g at 1-hr postinjection) in our SKOV-3 model, suggesting that the expression of $\alpha_v\beta_3$ integrin in tumor cells, instead of endothelial cells, would mainly contribute to the accumulation of the RGD peptides. Similarly to other monomeric RGD peptides, $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) were rapidly cleared from blood, resulting in remarkably high tumor-to-blood and -muscle ratios at 24- and 48-hr postinjection.

$^{111}$In-DOTA-c(RGDfK) showed a high uptake (8.80% ID/g at 10 min postinjection) in kidney, followed by rapid clearance (2.53% ID/g at 1-hr postinjection). Furthermore, a low accumulation of $^{111}$In-DOTA-c(RGDfK) was observed in liver (1.43% ID/g at 1-hr postinjection). This suggests that $^{111}$In-DOTA-c(RGDfK) as well as other RGD peptides containing lysine is cleared via the renal pathway because of its hydrophilicity.

Multimeric RGD peptides have been developed to increase the affinity to $\alpha_v\beta_3$ integrin, $^{111}$In-DOTA-E-[c(RGDfK)]$_2$ showed a high and retentive uptake in liver and kidney although it also showed high tumor uptake. In contrast, $^{111}$In-DOTA-c(RGDfK) was found to have a lower uptake and faster clearance in liver and kidney. This difference between monomeric and dimeric RGD agrees with the comparison between $^{99m}$Tc-HYNIC-c(RGDfK) and $^{99m}$Tc-HYNIC-E-[c(RGDfK)]$_2$. Dimerization resulted in higher uptake and prolonged retention in kidney, although it led to a higher affinity for $\alpha_v\beta_3$ integrin as well as a longer retention in the tumor in comparison with that of $^{99m}$Tc-HYNIC-c(RGDfK). It has been reported that $T/K$ ratios of $^{99m}$Tc-HYNIC-E-[c(RGDfK)]$_2$ were higher than that of $^{99m}$Tc-HYNIC-E-[c(RGDfK)]$_2$. The enhanced renal clearance of monomeric RGD peptides may improve the delineation of abdominal tumors.

Kidney is the dose-limiting tissue for radionuclide therapy with RGD peptides containing lysine. However, high-dose and multiple-dose administration could be possible for $^{90}$Y-DOTA-c(RGDfK) because of its rapid clearance from kidney and high $T/K$ ratio. Several studies have shown the successful use of alpha particle-emitting nuclides, such as $^{211}$At, $^{211}$Bi and $^{227}$Th for radionuclide therapy due to their higher liner energy transfer and shorter path length as compared to beta particle-emitting nuclides. However, one possible issue is the control of the...
radiation to normal tissues (kidney or bone marrow etc.) due to its high cytotoxicity. DOTA-c(RGDfK) would also be applicable to the radionuclide therapy with a particle-emitting nuclides.

The coinjection of DOTA-c(RGDfK) reduced the tracer uptake in most normal tissues such as liver and spleen. Similar results with regard to other peptides have been reported. The expression of \( \alpha_v\beta_3 \) integrin in microvessels in rat liver and lung as well as in osteoclast and osteoblast has been identified. This fact suggests that there might be an expression of \( \alpha_v\beta_3 \) integrin in microvessels in normal tissues as well as in normal cells. Therefore, these results support the suggestion that \(^{111}\text{In-DOTA-c(RGDfK)}\) and \(^{90}\text{Y-DOTA-c(RGDfK)}\) could be \( \alpha_v\beta_3 \) integrin-targeting tracers.

Janssen reported that 37 MBq of \(^{90}\text{Y-DOTA-E-[c(RGDfK)]_2}\) induced a significant inhibition in tumor growth. However, 57 MBq of a scrambled sequence control peptide, \(^{90}\text{Y-DOTA-E-[c(RGKfD)]_2}\), also elicited a delay in tumor growth in comparison with an untreated group, suggesting that this would not only be caused by \( \alpha_v\beta_3 \) integrin-targeting therapy. Although kidney has relative resistance to radiation, this high radiation dose might impair kidney function. Therefore, the practicality of clinically treating \( \alpha_v\beta_3 \) integrin positive tumors by such a high dose injection remains in question. Injection of 11.1 MBq \(^{90}\text{Y-DOTA-c(RGDfK)}\) did not show any significant delay in tumor growth. A high radiation dose of monomeric RGD peptide could be needed to adequately inhibit tumor growth because of its fast clearance.

**FIGURE 3** – Biodistribution of \(^{90}\text{Y-DOTA-c(RGDfK)}\) (a) and \(^{111}\text{In-DOTA-c(RGDfK)}\) (b) in nude mice bearing SKOV-3 tumors subcutaneously (n = 3–4). Radioactivity in tissues is expressed as % ID/g (mean ± SD).
Multiple-dose administration (11.1 MBq × 3) induced significant inhibition of tumor growth. Although the therapeutic effect by single-dose administration of 33.3 MBq 90Y-DOTA-c(RGDfK) was not investigated in this study, dose fractionation-like effects in addition to an increase in the total radiation dose would enhance the growth inhibition. Some studies have revealed the potential and advantage of dose fractionation in radionuclide therapy.\textsuperscript{28–30} Anderson \textit{et al}. reported that dose fractionation of 15 mCi \textsuperscript{64}Cu-TETA-octreotide into 2 doses (1 or 2 days apart) showed significantly longer inhibition of tumor growth and lower toxicity than a single-dose administration.\textsuperscript{31} Therefore, it is thought that multiple-dose administration rather than single-dose administration of high radiation might achieve the therapeutic effect while regulating the exposure of normal tissues to radiation.

In radionuclide therapy, radiation exposure to normal tissues as well as the absolute accumulated dose in the tumor is an important factor. Kidney is the dose-limiting tissue for radionuclide therapy with 90Y-DOTA-c(RGDfK) or peptides such as octreotide. There are some reports of kidney absorbed dose for 90Y-DOTA-octreotide. Jamar \textit{et al}. and Joerster \textit{et al}. showed that the kidney absorbed dose are 4.4 and 2.73 Gy/GBq, respectively.\textsuperscript{32,33} Lewis \textit{et al}. showed kidney absorbed dose (0.670 mGy/MBq) from \textsuperscript{177}Lu-DOTA-octreotate does not indicate any probability of finding radiation damage in the rats kidneys with an injected activity of 555 MBq, leading to a renal dose of 0.37 Gy.\textsuperscript{34} Compared to those results, 90Y-DOTA-c(RGDfK) has the lower kidney absorbed dosimetry (0.568 mGy/MBq). In our radionuclide therapy, a maximum administrated activity were 33.3 MBq (11.1 MBq × 3), leading to a renal dose of 0.019 Gy. In view of a differ-

**FIGURE 4** – Tumor to nontumor ratios in SKOV-3 tumor-bearing nude mice at 10 min, 1 hr, 4 hr, 24 hr, and 48 hr after injection of 90Y-DOTA-c(RGDfK) and 111In-DOTA-c(RGDfK) (n = 3–4). Data are represented as mean ± SD.

**FIGURE 5** – Biodistribution of 90Y-DOTA-c(RGDfK) (a) and 111In-DOTA-c(RGDfK) (b) in nude mice bearing SKOV-3 tumors at 1 hr with and without coinjection of 100 μg of DOTA-c(RGDfK) as a blocking agent (n = 4). Data are represented as mean ± SD. Significance was determined by Mann-Whitney U test (*p < 0.05 vs. control).
ence in animal models (rats and mice), this renal dose from $^{90}$Y-DOTA-c(RGDfK) would not induce any probability of nephrotoxicity. It is reported that the 5% of probability threshold for radiation nephropathy by $^{90}$Y-DOTA-octreotide is 35 ± 7 Gy, indicating the higher threshold than that by external beam therapy.

For clinical use, therefore, precise absorbed doses in patients should be estimated with $^{90}$Y-DOTA-c(RGDfK) or $^{111}$In-DOTA-c(RGDfK) and administrated activity of $^{90}$Y-DOTA-c(RGDfK) should be decided.

$\alpha_3\beta_1$ integrin is very abundant in bone-residing breast cancer metastase, malignant ovarian carcinoma, metastatic melanoma and invasive prostate cancer. $\alpha_3\beta_1$ integrin is suitable target for the radionuclide therapy when it is highly expressed on tumor cells. However, the relationship between the expression level of $\alpha_3\beta_1$ integrin and the therapeutic efficacy is still not unclear. Further studies in various tumor models are needed to investigate the relationship between tumor uptake of $^{90}$Y-DOTA(c(RGDfK)) or $^{111}$In-DOTA-c(RGDfK) as surrogate markers and the therapeutic efficacy.

Conclusion

Our research indicates the potential of $^{90}$Y-DOTA-c(RGDfK) and $^{111}$In-DOTA-c(RGDfK) as radionuclide therapy and imaging agents. We require further optimization of the therapy by the amount of radiation dose, the number of administrations, and the selection of radionuclide ($\alpha$ emitters as well as auger emitters). These optimizations would lead to an improvement of the therapeutic effect while reducing radiation toxicity to normal tissues.

Acknowledgement

Authors thank Ms. Yoko Kawai for assistance in the animal experiments.

References


TABLE I – HUMAN ABSORBED DOSE ESTIMATES OF $^{90}$Y-DOTA-c(RGDfK) IN SKOV-3 TUMOR-BEARING NUDIE MICE

<table>
<thead>
<tr>
<th>Organ</th>
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<td>Effective Dose</td>
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$^1$Expressed as mSv/MBq.

**Figure 6** – Relative growth curves of the s.c. SKOV-3 tumors in the three groups of mice after injection of 11.1 MBq of $^{90}$Y-DOTA-c(RGDfK), 11.1 MBq of $^{90}$Y-DOTA-c(RGDfK) (3 days), or saline (untreated controls). Data are represented as mean ± SD for 5–6 mice. Significance was determined using the Dunn’s post hoc test followed by the Dunn’s post hoc test (*p < 0.01 vs. control).


