68Ga-NODAGA-RGĐyK for αvβ3 integrin PET imaging
Preclinical investigation and dosimetry

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Keywords
Integrin, αvβ3, RGD-peptide, cyclic RGDyK, 68Ga-NODAGA-RGĐyK, PET imaging

Summary
Aim: To visualize neovascularature and/or tumour integrin αvβ3, we selected the binding moiety Arg-Gly-Asp-D-Tyr-Lys (RGĐyK) coupled to NODAGA for labeling with 68Ga. Methods: NODAGA-RGĐyK (ABX) was labeled with the 68Ga eluate from the 68Ge generator IGG100 using the processor unit PharmTracer. Biodistribution was measured in female Hsd mice sacrificed 10, 30, 60 and 90 min after i.v. injection of 68Ga-NODAGA-RGĐyK for OLINDA dosimetry extrapolated to humans. Tumour targeting was studied in SCID mice bearing A431 and other tumour transplants using microPET and biodistribution measurements. Results: Effective half-life of 68Ga-NODAGA-RGĐyK was 25 min for total body and most organs except liver and spleen that showed stable activity retention. With a bladder voiding interval of 0.5 h the calculated effective dose (ED) was 0.012 and 0.016 mSv/MBq for males and females, respectively. Rapid uptake within 10 min was observed in A431 tumours with dynamic PET followed by a slow release. Biodistribution measurements showed a 68Ga-NODAGA-RGĐyK uptake in A431 tumours of 3.4±0.4 and 2.7±0.3%ID/g at 1 and 2 h, respectively. Similar uptakes were observed in a mouse and human breast and ovarian cancer xenografts.

Co-injection of excess (5 mg/kg) unlabeled NODAGA-RGĐyK with the radiotracer reduced tumour uptake at one hour to 0.23±0.01%ID/g, but similarly decreased uptake in normal organs as well. When unlabeled peptide was injected 15 min after 68Ga-NODAGA-RGĐyK, uptake diminished particularly in tumour and adrenals, suggestive of a different binding mode compared with other normal tissues. Conclusion: NODAGA-RGĐyK was reliably labeled with 68Ga and revealed a predicted ED of 0.014 mSv/MBq. Tumour uptake was rapid and significant and was chased with unlabeled RGĐyK in a similar manner as adrenal uptake.

Schlüsselwörter
Integrin, αvβ3, RGD-Peptid, cyclisches RGĐyK, 68Ga-NODAGA-RGĐyK, PET-Bildgebung

Zusammenfassung
Ziel: Zur Darstellung von Integrin αvβ3 von Tumoren oder Tumorneugefäßen wählten wir das zyklische Bindungspeptid Arg-Gly-Asp-D-Tyr-Lys (RGĐyK) gekoppelt an NODAGA zur Markierung mit 68Ga. Methoden: NODAGA-RGĐyK (ABX) wurde mit dem 68Ge-Eluat des 68Ge-Generators IGG100 markiert mit der Prozessoreinheit PharmTracer. Biowerte wurden in weiblichen Hsd-Mäusen 10, 30, 60 und 90 min nach i.v.-Injektion von 68Ga-NODAGA-RGĐyK gemessen und als OLINDA-Dosimetrie auf den Menschen projiziert. In SCID-Mäusen, die A431 und andere Tumoren trugen, wurde die Tumoraufnahme mit microPET und Bioverteilungen studiert. Ergebnisse: Die effektive Halbwertszeit von 68Ga-NODAGA-RGĐyK war ~25 min im Ganzkörper und den meisten Organen außer Leber und Milz, für die eine stabile Aufnahme gemessen wurde. Die für Frauen und Männer berechneten effektiven Dosis (ED) war 0.012 bzw. 0.016 mSv/MBq mit der Annahme eines Blasenleerungsintervalls von 0.5 h. Mit dem PET wurde eine rasche A431-Tumoraufnahme innerhalb von 10 Minuten beobachtet, gefolgt von einem langsamen Abfluss. Biowerte zeigten eine A431-Tumoraufnahme von 68Ga-NODAGA-RGĐyK von 3.4±0.4 und 2.7±0.3%ID/g nach 1 bzw. 2 Stunden. Ähnliche Tumoranreicherungen in der Maus wurden in einem Maus- und Humankarzinom gemessen sowie in humanem Ovarialkarzinom. Co-Injektion mit einer Überdosis (5 mg/kg) unmarkiertem NODAGA-RGĐyK mit dem Radiopharmazientum reduzierte die Tumoraufnahme auf 0.23±0.01%ID/g nach 1 Stunde, jedoch wurde eine ähnliche reduzierte Aufnahme auch in andern normalen Organen beobachtet. Wenn unmarkiertes Peptid 15 min nach 68Ga-NODAGA-RGĐyK injiziert wurde, vermied sich die Aufnahme besonders in Tumor und Nebennieren. Diese Beobachtung kann im Vergleich zu anderen Normalgeweben als unterschiedliches Bindungsverhalten gedeutet werden. Schlussfolgerung: NODAGA-RGĐyK konnte zuverlässig mit 68Ga markiert werden und zeigte für Menschen eine vorhergesagte ED von 0.014 mSv/MBq. Die Tumoranziehung war schnell und signifikant. Sie wurde aus Tumor und Nebennieren durch nachgespritztes, unmarkiertes NODAGA-RGĐyK in ähnlicher Weise verdrängt.
Integrins are cell surface heterodimers consisting of an α- and β-subunit and 24 different forms have been described (16).

- They mediate cell adhesion, migration and differentiation.
- Integrins are implicated in cancer angiogenesis, invasion and metastasis.
- As transmembrane proteins, they can be expressed in activated and non-activated states and allow cellular outside-in and inside-out signaling (16, 25).
- Viral pathogens may misuse integrins, particularly also αβ₃, as receptors for binding to cells and internalisation (24).

The αβ₃ integrin is of particular interest for oncology since cilegide (EMD 121974) is the first integrin inhibitor in phase III clinical development as anti-cancer agent (21).

In difference to quiescent, established vasculature, endothelial cells of tumour neo-vascularature can express the αβ₃ integrin in high amounts. Molecular imaging with positron emission tomography (PET) has been developed on the αβ₃ recognition motif arginine-glycine-aspartic acid (RGD) presented as pentacysteptide such as RGDV (cilegide), RGDyK, RGDyK or RGDyK (10). Radiolabeled RGD peptides have been studied for tumour imaging by different groups. Direct ¹⁸F-labeled RGD (1, 2, 15) or chelator conjugated mono- and multimeric peptides labeled with ⁶⁴Cu (4, 7), ⁶⁸Ga (11, 12, 14) or ¹¹¹In (6) have been developed.

Radioconjugates targeting αβ₃ integrin have frequently shown, besides high tumour uptake, significant uptake in mouse liver, kidneys and intestines. Co-injection of excess unlabeled peptide together with radiolabeled peptide attenuated uptake of the latter in tumours and in different normal tissues (6, 13, 14).

Currently, it is unclear why RGD pentacysteptide radioconjugates are taken up in mouse liver, kidneys and intestines and other normal tissues which normally express lower to undetectable levels of αβ₃. The results of co-injection experiments are suggestive of a possible specific targeting of non-tumour tissues in mice. A common but not confirmed hypothesis is that RGD peptides such as those used here recognize, albeit with lower affinity, integrins other than αβ₃.

We selected from the literature the RGDyK peptide for coupling with the gallium chelating reagent NODAGA (6, 12) and obtained it in GMP quality for the projected clinical application. We studied here the feasibility of its radiolabeling with ⁶⁷Ga and performed preclinical evaluations including tumour targeting studies in tumour grafted SCID mice.

Material and methods

NODAGA-RGDyK, (cyclo[L-arginylglycyl-L-alpha-aspartyl-D-tyrosyl -N6-{[4,7-bis(carboxymethyl)octahydro-1H-1,4,7-triazonin-1-yl[acetyl]]-L-lysyl}]), i. e. cyclic Arg-Gly-Asp-D-Tyr-Lys-NODAGA or c(R-G-D-y-K)-NODAGA was obtained from ABX, Germany that also prepared it in GMP form. Female outbred (CD-1®) Hsd ICR and female C57BL/6J SCID mice were obtained from Harlan Laboratories (Boxmeer, Netherlands). The human cell line A431 ([α1β1, α1β3, α1β7] (16), epidermoid cancer, ATCC), the mouse mammary tumour 4T1 ([α1β2, α1β3] (8) of Balb/c background (kindly provided by Dr F.R. Miller, Michigan Cancer Foundation, Detroit, MI), the human ovarian cancers IGROV-1 ([17, α1β1, α1β3] (18)) and SKOV-3 [α1β1, α1β3] (18), ATCC) and the human breast adenocarcinomas MDA-MB-231 [α1β7] (16), ATCC) were cultured at 37°C and 5% CO₂ in RPMI 1640 with glutamax I (Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Che- mie Brunschwig AG, Basel, Switzerland), penicillin (50 units/ml) and streptomycin (0.05 mg/ml; Life Technologies Inc., Grand Island, NY, USA). All cells were tested negative for four major potential mycoplasma contaminations (Mycoplasma Detection Kit, F. Hoffmann-La Roche Ltd, Basel, Switzerland) and cultures were renewed every six months after testing.

NODAGA-RGDyK radiolabeling

⁶⁸Ga was eluted with 0.1 mol/l HCl from the ⁶⁷Ge generator IGG100 (Eckert & Ziegler, Germany), using the automatic processor unit Modular-Lab PharmTracer (Eckert & Ziegler). NODAGA-RGDyK (20 μg) was radiolabeled with the high activity ⁶⁷Ga fraction by incubation for 20 min at room temperature. After cartridge purification (part of the automatic processor unit) the ready for use ⁶⁷Ga-NODAGA-RGDyK was eluted in 50% ethanol and for 0.22 μm sterile filter and diluted into NaCl solution. High pressure liquid chromatography analysis was performed on a μ-Bondapak column (Waters C-18) run with trifluoroacetic acid and acetonitrile.

Dosimetry study

All animal experiments were performed according to the principles of laboratory animal care and national ethical guidelines. The animal experiments have been subjected to authorization and control by the official Canton and Swiss veterinary service on surveillance of animal experiments (VD 2013 et VD 2013.3). Animals were kept under specific pathogen-free conditions, in autoclaved filter-topped cages and bedding in a separate room. Mice had access ad libitum to sterilized food and water. Animals were sacrificed after single experiments.

⁶⁸Ga-NODAGA-RGDyK (0.5 MBq) was injected i. v. in female, outbred (CD-1®), Hsd ICR mice for biodistribution studies and OLINDA-based dosimetry extrapolation. Mice were sacrificed 10, 30, 60 and 90 min after injection and blood and organs collected, weighed and radioactivity counted compared with a sample of 5% injected activity. Gastrointestinal tissues were collected with their content. Tissue concentrations of activity were then expressed as % of injected activity per gram (% ID/g). Total body activity was determined by addition of all organs and tumour with the carcass that was also sampled.

⁶⁸Ga-NODAGA-RGDyK biodistribution results were transformed in effective %ID/g and used to calculate single exponential effective half-lives for each organ and the animal. Residence times for each mouse tissue were calculated and maintained for human dosimetry extrapolation without correction for organ masses. Reduced activity was assumed being equal with blood activity with the same half-life while respecting the respective volumes of

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blood and red marrow (18). The urinary bladder model (Miridose) (23) was used for searching optimal bladder voiding intervals. The urinary elimination fraction of $^{68}$Ga-NODAGA-RGDayK was set at 75% with a 40 min biological half-life according the whole-body results. Residence times were introduced into Olinda (22) and radiation doses calculated for female (58 kg) and male (70 kg) human beings.

NODAGA-RGDayK tolerance study

NODAGA-RGDayK was tested at 1000-fold weight excess/kg in five female outbred Hsd ICR mice. No toxicity was expected to occur at this dosage and higher doses were not tested. Mice were weighed from arrival into the lab until tolerance study two months later. Mice with a mean weight of about 35 g were injected 10 µg unlabeled NODAGA-RGDayK (1000-fold excess per kg body weight compared with the maximal projected human dose of 20 µg for a 70 kg patient). Mice were inspected 0.25, 1, 2, 3 and 5 h after injection and then inspected and weighted 2 to 3 times weekly over the next two months. Mice were sacrificed by CO₂ exposure and organs inspected macroscopically by two scientists.

Tumour targeting studies

SCID mice were grafted subcutaneously with human or mouse tumours (1 to 10 x 10⁶ cells/animal, depending on the cell line) and biodistribution was studied with tumours having grown to between 0.1 and 0.3 g, generally two to four weeks after cell transplantation. Mice were injected $^{68}$Ga-NODAGA-RGDayK i.v. (0.5 MBq/animal corresponding to 0.04 to 0.1 µg peptide). Control mice were injected with radio-labeled peptide together with 5 mg/kg non-radioabeled NODAGA-RGDayK. Mice were sacrificed at one or two hours after injection, and biodistribution determined by tissue and blood weighting and radioactivity counting against a standard sample of 5% injected activity. Total body activity represents the sum of all activity measured from injected mice (carcass, organs, blood and tumour).

For microPET imaging tail vein cannulated mice were i.v. injected 4 MBq/animal $^{68}$Ga-NODAGA-RGDayK. PET was performed on a dedicated small-animal PET scanner (LabPET3; Gamma Medic-Ideas, Sherbrooke, Quebec, Canada). Mice were maintained under 1% isoflurane anesthesia in oxygen at 1 l/min for cannulation and during the entire scanning period; temperature and breathing rate were monitored and maintained constant. 50 min list-mode acquisitions were initiated with the i.v. injection of $^{68}$Ga-NODAGA-RGDayK and static imaging recorded 60 to 70 min after injection. An energy window of 250–650 keV and a coincidence timing window of 22.2 ns were used. The list-mode data were sorted into 30 min time frames of 1 min. Images were reconstructed by MLEM iterative method in a cylindrical volume of 46 mm diameters and 3.7 cm length. The voxel was of 0.5 x 0.5 x 1.2 mm giving a typical resolution of 1.2 mm at the center of the field of view. The image data were corrected for nonuniformity of the scanner response, dead time count losses, and physical decay to the time of injection.

Chase experiments

With the intention to chase a supposed low affinity binding of $^{68}$Ga-NODAGA-RGD in normal tissues like liver, kidneys and intestines, we performed different chase experiments in SCID mice bearing A431 tumours. We injected first $^{68}$Ga-NODAGA-RGDayK i.v., followed 15 minutes later by the i.v. injection of moderate amounts (0.1 to 2.0 mg/kg) NODAGA-RGDayK. Chase was performed mostly as a single, non-labeled NODAGA-RGDayK i.v. injection performed for each mouse 15 min after radiolabel injection. In a few experiments chase was performed in two injections, the first at 15 minutes and the second as described in results. Since it was intended not to reduce tumour uptake, the peptide amount used for chase was reduced as compared with competition experiments. Chase experiments were evaluated with biodistribution studies performed one or two hours after radiopptide injection.

Results

$^{68}$Ga-labeling of NODAGA-RGDayK

Labeling of NODAGA-RGDayK with the high activity fraction of $^{68}$Ga eluate was efficient and reliable over the period studied. $^{68}$Ga-NODAGA-RGDayK showed a single peak on HPLC (Fig. 1), contaminants representing <1%. Specific activity in these experiments conducted over one year was between 100 to 250 MBq per 20 µg peptide. This latter amount represents the projected maximal quantity foreseen for injection of patients.

Dosimetry projection for humans

Biodistribution studies were performed after i.v. injection of $^{68}$Ga-NODAGA-RGDayK in normal mice (Fig. 2) and showed effective half-lives of 25 min for whole body within a range of 18 to 35 min for most normal organs except liver and spleen. Radiopptide uptake in liver and spleen remained rather constant over the observation period resulting in effective half-lives of 63 and 53 min, respectively. The human effective dose (ED) and effective dose equivalent (EDE) extrapolated from these results was for men 0.012 and 0.015 mSv/MBq, respectively, and for women 0.016 and 0.020 mSv/MBq.

The highest radiation dose was calculated for the urinary bladder wall with 0.139 and 0.188 mGy/MBq (Fig. 3a) for men and women, respectively, despite choosing an optimized urinary bladder voiding interval of 30 min. In decreasing order organ contributions to the ED (Fig. 3b) of humans would be highest for the urinary bladder wall (58%) followed by liver (10%), stomach (7%), lower large intestinal wall (LLI, 6%), gonads (6%), lungs (4%) and red marrow (3%).

Tolerance towards an excessive dose of NODAGA-RGDayK

The tolerance study showed the absence of toxicity under use of an excessive i. v. overload of animals with peptide. Foreseeing the injection of patients with maximally 20
showed a normal organ appearance in all animals.

**Tumour localization and biodistribution studies**

Tumour uptake in A431 xenografts as measured in biodistribution experiments was $3.4 \pm 0.4$ and $2.7 \pm 0.3\%$ID/g at 1 and 2 hrs, respectively, indicating good retention of peptide over this period of time. Tumour uptake was reduced to $0.2\%$ID/g at 1 hour when using co-injection of excess amount unlabeled NODAGA-RGDyK, however, normal tissue activity uptake, except for kidney, was decreases to a similar degree (Fig. 5a).

Tumour uptake of $^{68}$Ga-NODAGA-RGDyK in different other human cancer xenografts in mice was moderate to high. In human breast cancer MDA MB-231, tumour uptake was similar at 1 and 2 hours post injection with $3.2 \pm 0.6$ and $3.4 \pm 0.7\%$ID/g, respectively (3–4 mice/group). In the mouse mammary tumour 4T1 uptake at 1 and 2 h (3 mice each), was $3.5 \pm 0.4$ and $2.7 \pm 0.3\%$ID/g, respectively (results not shown). In the two human ovarian cancer xenografts IGROV-1 and SKOV-3 uptake at 1 h was $5.8 \pm 1.0$ and $1.6 \pm 0.4\%$ID/g (3 to 5 mice/group), respectively.

MicroPET dynamic imaging of A431 tumour bearing mice showed rapid tumour uptake within 10 min after injection followed by a slow release (result not shown). Imaging 1 h after injection of a mouse bearing on the right side an A431 tumour and on the left a 4T1 tumour showed significant tracer accumulation in A431 while activity in 4T1 remained in the range of abdominal activity (Fig. 5b).

**Chase experiments in vivo**

With the intention to reveal a supposed low affinity binding of $^{68}$Ga-NODAGA-RGDyK in normal tissues like liver, kidneys and intestines, we performed chase experiments. We injected first $^{68}$Ga-NODAGA-RGDyK i.v., followed 15 min later by the i.v. injection of moderate amounts (0.1 to 2.0 mg/kg) unlabeled NODAGA-RGDyK. We observed in a first experiment at 0.1 and
1 mg/kg chase peptide a reduced \(^{68}\)Ga-NODAGA-RGDyK uptake in tumour and adrenals but no uptake inhibition in other normal tissues (Fig. 6a). In a second experiment, the chase with unlabeled peptide at 2 mg/kg given once or twice, showed decreased activity uptake in all tissues compared with controls, however uptake in tumour and adrenals was still reduced to a stronger degree (Fig. 6b).

In a third chase experiment biodistribution was measured two hours after injection of \(^{68}\)Ga-NODAGA-RGDyK, chase being performed with \(2 \times 0.4\) mg/kg unlabeled peptide injected at 15 and 60 minutes. It showed only a modest uptake decrease in tumour and adrenals as compared with unchased controls again without any modification in the other normal mouse tissues (Fig. 6c).

**Discussion**

NODAGA-RGDyK has been synthesized in GMP quality and is currently under review for a clinical study. Towards this aim, we selected from literature (6, 12) the components NODAGA for stable chelation of \(^{68}\)Ga and pentacysl RGDiK for \(\alpha_\beta\), integrin targeting. In RGDiK a single amino acid DTyr is replacing DPh as compared with the original compound present in NODAGA-RGD (c(RGDyK)) of which the synthesis has been described (6, 12). In the meantime the stability of Ga and Cu chelation with NODAGA has also been confirmed by another group (7, 8).

Our in vitro and in vivo results entirely confirmed the published reliable radio-labeling of NODAGA-RGDyK with \(^{68}\)Ga and the tumour localization capacity of this radioconjugate. As was also recently reported, \(^{68}\)Ga-NODAGA-RGD behaved similarly as \(^{18}\)F-galacto-RGD as a reference reagent (20). In agreement with these authors, we consider \(^{68}\)Ga-NODAGA-RGDyK a promising agent for PET imaging of neovascular and tumour \(\alpha_\beta\), integrin expression. The dosimetry projection and absence of toxicity of this peptide at 1000-fold excess amount further confirmed its potential clinical usefulness.

Dosimetry results showed that bladder emptying intervals of 0.5 h optimally reduced bladder wall irradiation. However, bladder wall radiation dose still contributes to close to 60% of the ED as revealed by OLINDA dosimetry. It has been calculated by Monte-Carlo simulations that further bladder wall dose reduction could be obtained by injection of patients without initial bladder voiding (9). Up to 80% bladder wall radiation dose reduction was observed when assuming an initial bladder filling with 0.3 l as compared with the injection of patients with an empty bladder (9).

We selected different tumours available at our institute for uptake studies with \(^{68}\)Ga-NODAGA-RGDyK. While all tumour lines except one (A431), have been described to express integrin \(\alpha_\beta_3\), A431 only expresses \(\alpha_\beta_1\) (19). However, \(\alpha_\beta_3\) is probably also bound by RGDiK (21). Furthermore, when grafted into mice, tumour stroma and notably neovessels can express \(\alpha_\beta_1\) quite abundantly. When

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**Table 1**

<table>
<thead>
<tr>
<th>Target Organ</th>
<th>Organ Doses (mGy/MBq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenals</td>
<td>0.0026 0.0033</td>
</tr>
<tr>
<td>Brain</td>
<td>0.0011 0.0013</td>
</tr>
<tr>
<td>Breasts</td>
<td>0.0012 0.0015</td>
</tr>
<tr>
<td>Gallbladder</td>
<td>0.0037 0.0045</td>
</tr>
<tr>
<td>ULI wall</td>
<td>0.0065 0.0076</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.0174 0.0203</td>
</tr>
<tr>
<td>Stomach wall</td>
<td>0.0075 0.0087</td>
</tr>
<tr>
<td>ULI wall</td>
<td>0.0132 0.0151</td>
</tr>
<tr>
<td>Heart wall</td>
<td>0.0038 0.0049</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.0267 0.0291</td>
</tr>
<tr>
<td>Liver</td>
<td>0.0238 0.0319</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.0040 0.0051</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.0024 0.0035</td>
</tr>
<tr>
<td>Ovaries</td>
<td>0.0047 0.0047</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.0027 0.0034</td>
</tr>
<tr>
<td>Red marrow</td>
<td>0.0032 0.0037</td>
</tr>
<tr>
<td>Osteogenic cells</td>
<td>0.0029 0.0041</td>
</tr>
<tr>
<td>Skin</td>
<td>0.0013 0.0015</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.0133 0.0162</td>
</tr>
<tr>
<td>Testes</td>
<td>0.0022</td>
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<tr>
<td>Thyroid</td>
<td>0.0014 0.0018</td>
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<tr>
<td>Urinary bladder wall</td>
<td>0.1390 0.1880</td>
</tr>
<tr>
<td>Uterus</td>
<td>0.0065</td>
</tr>
<tr>
<td>Total body</td>
<td>0.0033 0.0042</td>
</tr>
<tr>
<td>ED (mSv/MBq)</td>
<td>0.012 0.016</td>
</tr>
</tbody>
</table>
**Table 1**

Dosimetry extrapolation to humans from mouse biodistribution given in Figure 2, extrapolated half-lives, calculated residence times and partition to human organs and tissues.

<table>
<thead>
<tr>
<th>tissue</th>
<th>mouse</th>
<th>human, residence time</th>
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<tbody>
<tr>
<td></td>
<td>g</td>
<td>%ID/g (t = 0)</td>
</tr>
<tr>
<td>liver</td>
<td>1.19</td>
<td>4.84</td>
</tr>
<tr>
<td>kidneys</td>
<td>0.41</td>
<td>6.65</td>
</tr>
<tr>
<td>lung</td>
<td>0.24</td>
<td>4.69</td>
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<tr>
<td>spleen</td>
<td>0.1</td>
<td>3.84</td>
</tr>
<tr>
<td>heart</td>
<td>0.14</td>
<td>2.79</td>
</tr>
<tr>
<td>muscle</td>
<td>9.73*</td>
<td>1.84</td>
</tr>
<tr>
<td>bone</td>
<td>2.78**</td>
<td>2.23</td>
</tr>
<tr>
<td>skin</td>
<td>5.56**</td>
<td>3.42</td>
</tr>
<tr>
<td>stomach</td>
<td>0.41</td>
<td>2.46</td>
</tr>
<tr>
<td>small intestine</td>
<td>1.34</td>
<td>2.57</td>
</tr>
<tr>
<td>large intestine (LI)</td>
<td>0.94</td>
<td>2.42</td>
</tr>
<tr>
<td>upper LI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lower LI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blood</td>
<td>2.78*</td>
<td>3.87</td>
</tr>
<tr>
<td>red marrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>carcass</td>
<td>21.67</td>
<td>2.81</td>
</tr>
<tr>
<td>bladder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total body</td>
<td>27.8</td>
<td>80.79</td>
</tr>
<tr>
<td>rest body</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(total residence time)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*35%, **10%, ***20%, †10% of mouse body weight; ‡Bladder residence time was calculated with Mirdose bladder model: elimination fraction 0.75, biological half-life 0.67h, bladder voiding interval 0.5h.

Red marrow activity concentration is set identical to blood, respecting respective volumes in male and female human beings (19). Rest body residence time is equal to the total body residence time minus all organ residence times introduced into Olinda.

Looking at our results, low tumour uptake was observed in SKOV-3 tumours that expresses α,β, according literature. Stronger tumour uptake of ⁶⁷Ga-NODAGA-RGDyK was, however, observed in the other tumours, namely A431 and the breast and ovarian cancers MDA-MB-231 and IGROV-1. These results therefore suggest that tumour uptake of RGDyK may depend or co-depend from other factors than α,β, integrin expression by the tumour lines themselves.

As also observed with other α,β, targeting reagents, significant background activity was observed in liver, kidneys, the gastrointestinal tract and other normal tissues. Uptake in these normal organs was reduced significantly when using co-injection of excess (5 mg/kg) amount of...
unlabeled peptide with ⁶⁸Ga-NODAGA-RGDyK. Such similar observations made by other groups led to the hypothesis of a low affinity binding to integrins other than αvβ3 in normal mouse tissues. We performed a small series of chase experiments whereby moderate amounts of NODAGA-RGDyK were injected i. v. 15 min post ⁶⁸Ga-NODAGA-RGDyK injection. Overall, the results showed that the low amounts of RGDyK were not able to displace ⁶⁸Ga-NODAGA-RGDyK localization from most normal tissues except adrenals and tumour A431. These results are suggestive of a different binding mode of NODAGA-RGDyK to most normal tissues as compared with tumour and adrenals.

Interestingly, another group observed a particular stable localization of ⁶⁴Cu-NODAGA-c(RGDFK) in tumour and adrenals (7). They mentioned that α,β, was also expressed by normal adrenal tissue (17) similar as by tumour which could explain the particular stability of the RGD peptide uptake in these two tissues.

**Conclusion**

The predicted moderate ED and EDE of 0.014 and 0.018 mSv/MBq, respectively, and the observed tumour uptake and retention qualify ⁶⁸Ga-NODAGA-RGDyK as a promising PET tracer targeting α,β, integrin.

We observed an easy to perform and reliable radiolabeling of NODAGA-RGDyK. NODAGA-RGDyK was also well tolerated in excess amount.

The chase experiments showed that tumour and adrenal ⁶⁸Ga-NODAGA-RGDyK uptake could be selectively reduced, suggestive of a reversible, specific binding in these tissues, while no chase was observed at low NODAGA-RGDyK concentrations in normal tissues other than adrenals, deserving further investigations.

**Fig. 5** Representative biodistribution study (a) and microPET imaging (b) at one hour after i. v. injection of ⁶⁸Ga-NODAGA-RGDyK in SCID mice bearing an A431 human and a 4T1 mouse tumour xenografts

- a) Result of a co-injection of ⁶⁸Ga-NODAGA-RGDyK with unlabeled NODAGA-RGDyK (5 mg/kg) shows uptake inhibition in tumour and in most normal organs.
- b) PET imaging shows the three sections in the orthogonal planes relative to the A431 tumour (crosssection mark); transverse section (upper left), sagittal section (upper right), coronal section (lower left) and the dorsal plain view of the animal (lower right) with the anterior and hind legs at the border of the field of view of microPET. ×, right; l, left; a, anterior; p, posterior; f, front and h, hind of the animal. The marked crosssection and major circled hyperactivity shows on the right anterior side the subcutaneous A431 tumour with strong uptake of ⁶⁸Ga-NODAGA-RGDyK. The 4T1 mouse mammary tumour on the left anterior side appears iso-active with the abdomen indicating moderate uptake of ⁶⁸Ga-NODAGA-RGDyK.
Fig. 6 Chase experiments 1 to 3 (in %ID/g): $^{68}$Ga-NODAGA-RGDyK was injected i. v. and a) chased or not 15 min later with the i. v. injection of 0.1 to 1 mg/kg NODAGA-RGDyK (biodistribution measured 1 h after injection) b) chased once or twice (at 15 and 30 min) with 2 mg/kg NODAGA-RGDyK (biodistribution measured 1 h after injection). c) Animals were chased with $2 \times 0.4$ mg/kg NODAGA-RGDyK i. v. at 15 and 60 min post radiotracer injection and biodistribution measured at two hours.
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