In Vivo PET Imaging of the Cancer Integrin αvβ6 Using 68Ga-Labeled Cyclic RGD Nonapeptides

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Expression of the cellular transmembrane receptor αvβ6 integrin is essentially restricted to malignant epithelial cells in carcinomas of a broad variety of lineages, whereas it is virtually absent in normal adult tissues. Thus, it is a highly attractive target for tumor imaging and therapy. Furthermore, αvβ6 integrin plays an important role for the epithelial–mesenchymal interaction and the development of fibrosis. Methods: On the basis of the 68Ga chelators TRAP (triaza-cyclononane-triphosphinate) and NODAGA, we synthesized mono-, di-, and trimeric conjugates of the αvβ6 integrin–selective peptide cyclo(FRGDLATLRQL) (RGD) via click chemistry. These were labeled with 68Ga and screened regarding their suitability for in vivo imaging of αvβ6 integrin expression by PET and ex vivo biodistribution in severe combined immunodeficiency mice bearing H209 tumor (human lung adenocarcinoma) xenografts. For these, αvβ6 integrin expression in tumor and other tissues was determined by 68Ga immunohistochemistry. Results: Despite the multimers showing higher αvβ6 integrin affinities (23–120 pM) than the monomers (260 pM), the best results—that is, low background uptake and excellent tumor delineation—were obtained with the TRAP-based monomer 68Ga-avebehexin. This compound showed the most favorable pharmacokinetics because of its high polarity (log D = −3.7) and presence of additional negative charges (carboxylates) on the chelator, promoting renal clearance. Although tumor uptake was low (0.65% ± 0.04% injected dose per gram tissue [%ID/g]), it was still higher than in all other organs except the kidneys, ranging from a maximum for the stomach (0.52% ± 0.04 %ID/g) to almost negligible for the pancreas (0.07% ± 0.01 %ID/g). A low but significant target expression in tumor, lung, and stomach was confirmed by immunohistochemistry. Conclusion: Because of highly sensitive PET imaging even of tissues with low αvβ6 integrin expression density, we anticipate clinical applicability of 68Ga-avebehexin for imaging of αvβ6 tumors and fibrosis by PET.

Key Words: positron emission tomography; 68Ga; click chemistry; preclinical imaging

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radiolabeling, the peptide must be equipped with a chelator capable of binding the radiometal ion into a kinetically inert complex. For this purpose, we selected the chelator TRAP (34) (1,4,7-triazacyclononane-1,4,7-tris(methylene(2-carboxyethyl))phosphinic acid (35)), because its extraordinary affinity to 36-38 and selectivity for (39-41) gallium radionuclides enables highly efficient and reliable radiolabeling procedures (42). Because of the presence of 3 independent sites for conjugation, TRAP allows for facile attachment of additional reporter molecules (43) or multimerization of targeting vectors (44), which is particularly conveniently done by means of click chemistry, that is, copper-catalyzed alkylene-azide cycloaddition (CuAAC) (45).

MATERIALS AND METHODS

Syntheses and analytic characterization of the novel compounds are described in the supplemental materials (available at http://jnmm.snjmjournals.org).

Integrin αvβ6 Affinities

Integrin binding assays were performed as described previously (33) by enzyme-linked immunosorbent assays. Briefly, 96-well plates were coated with latency-associated peptide (transforming growth factor β) as extracellular matrix protein. Free binding sites were blocked by incubation with bovine serum albumin. Solutions of the respective compounds were added, followed by a solution of the integrin. Surface-bound integrin was detected by subsequent incubation with a primary antibody (mouse-antihuman) and a second antibody-peroxidase conjugate (antimouse horseradish peroxidase). After addition of the dye antibody (mouse-antihuman) and a second antibody-peroxidase conjugate, surface-incubation with bovine serum albumin. Solutions of the respective compounds were added, followed by a solution of the integrin. Surface-bound integrin was detected by subsequent incubation with a primary antibody (mouse-antihuman) and a second antibody-peroxidase conjugate (antimouse horseradish peroxidase). After addition of the dye tetramethylbenzidine and quenching of the reaction by addition of sulfuric acid, the absorbance signal at A = 405 nm was measured. The determined 50% inhibition concentration value for the inhibitor is referenced to the internal standard RTD Lin (linear helical RTD-peptide RTDLDLSLRT) with an αvβ6-binding affinity of 33 nM.

Radiochemistry

68Ga labeling was done using an automated system (GallElut+; Scintomics) as described previously (36). Briefly, nonprocessed eluate of a 68Ge/68Ga generator with SnO2 matrix (IThembla LABS, SA; 1.25 mL eluent: 1 M HCl, total 68Ga activity 500 MBq) was adjusted to pH 2 by adding 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (450 μL of a 2.7 M solution, prepared from 14.4 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 12 mL water) and used for labeling of 0.5 nmol of the respective chelator conjugate for 3 min at 95°C. Purification was done by passing the reaction mixture over a C8 light solid-phase extraction cartridge (SepPak), which was purged with water (10 mL) and the product eluted with an ethanol–water mixture (1:1 by volumes, 1 mL). The purity of the radiolabeled compounds was determined by radio-thin-layer chromatography (eluents: aqueous acetate solution or citrate solution).

Cell Lines and Animal Models

All animal studies have been performed in accordance with general animal welfare regulations in Germany and the institutional guidelines for the care and use of animals. H2009 human lung adenocarcinoma cells (CRL-5911; American Type Culture Collection) were cultivated as recommended by the distributor. To establish tumor xenografts, 6- to 8-wk-old female CB17 severe combined immunodeficiency mice (Charles River) were inoculated with 10⁶ H2009 cells in Matrigel (CultrexBME, type 3 PathClear; Trevigen, GENTAUER GmbH). Mice were used for biodistribution or PET studies when tumors had grown to a diameter of 6–8 mm (3–4 wk after inoculation).

Biodistribution and PET Imaging

Animals were injected with 12–15 MBq (for PET) or 5–7 MBq (for biodistribution studies) of the radiotracers under isoflurane anesthesia and subsequently allowed to wake up with access to food and water. For blockade, 60 nmol of the respective unlabeled compound was administered 10 min before tracer injection. For biodistribution, animals were sacrificed after 90 min, and organs were harvested and weighed and the activity contained therein counted in a γ-counter (Perkin-Elmer). The injected dose per gram of tissue was calculated from organ weights and counted activities, based on individually administered doses. PET was recorded under isoflurane anesthesia 60 or 75 min after injection for 20 min on a Siemens Inveon small-animal PET system. Images were reconstructed as single frames with Siemens Inveon software, using a 3-dimensional ordered-subset expectation maximum algorithm without scatter and attenuation correction.

Immunohistochemistry

For histology and immunohistochemistry, animals were sacrificed immediately after PET imaging. Tumor tissue and representative organs were fixed in 10% neutral-buffered formalin, routinely embedded in paraffin, and cut in 2-μm sections. Hematoxylin and eosin-stained sections were prepared according to standard protocols to exclude background pathology interfering with experimental results. β6 integrin immunohistochemistry was performed as follows: after enzymatic antigen retrieval (Pro- nase E, 1:20 in tris-buffered saline), unspecific protein and peroxidase binding was blocked with 3% hydrogen peroxide and 3% normal goat serum (Abcam). Immunohistochemistry was performed with a Dako autostainer using an antibody against the β6 subunit (1:50, Calbiochem, 407317). For antibody detection, biotinylated goat-antimouse secondary antibody (Medac Diagnostics, 71-00-29) was used, visualized by a streptavidine-peroxidase system (Medac Diagnostics, 71-00-38) and diaminobenzidine (Immunologic, BS04-500). Counterstaining was done using hematoxylin.

RESULTS

Figure 1 shows that in combination with the previously reported TRAP(azide)3 (45), newly synthesized TRAP derivatives with asymmetrical azide substitution pattern and...
additional polyethyleneglycol (PEG) linkers represent a valuable tool-kit for straightforward click-chemistry synthesis of mono-, di-, and trimeric peptide–chelator conjugates. The complementary terminal alkyne was introduced into the cyclic nonapeptide cyclo(FRGDLAFp(NMe)K) by amide formation with 4-pentyenoic acid on the lysine side chain, resulting in the building block AvB6.

These components were used for straightforward CuAAC synthesis of 4 conjugates (Fig. 1), differing in the type of linker and the number of peptide copies (1–3) per molecule. The Cu, which is inevitably complexed by TRAP in the course of CuAAC, was subsequently removed by transchelation with 1,4,7-triazacyclononane-1,4,7-triacetic acid at pH 2.2 as described previously (45). In addition, functionalization of c(FRGDLAFp(NMe)K) with an aminohexyl linker and NODAGA (46), a bifunctional 1,4,7-triazacyclononane-1,4,7-triacetic acid derivative, afforded the conjugate NODAGA-AvB6 (Fig. 2), which possesses a high degree of structural similarity to the TRAP-based monomer Avebehexin. Thus, a total of 5 c(FRGDLAFp(NMe)K) conjugates was available for evaluation—2 monomers, 1 dimer, and 2 trimers.

αβ6 integrin activity data shown in Table 1 confirm that functionalization of the cyclo(FRGDLAFp(NMe)K) peptide on the lysine side chain indeed does not affect the binding affinity, because 50% inhibition concentration values of both monomers are similar to that of the nondecorated peptide (260 pM) (33). As expected for multimeric systems, activity is increased by a factor of approximately 2 for the dimer 68Ga-TRAP(AvB6)2 and by a factor of approximately 11 for the trimer 68Ga-TRAP(AvB6)3, relative to 68Ga-avebehexin.

Exchange of the free carboxylates of TRAP by peptide substituents also had a marked influence on overall polarity. Although 68Ga-avebehexin is hydrophilic (log D = −3.71), the dimer and trimer show a more lipophilic character (log D of −2.14 and −3.71, respectively; Table 1). The lipophilicity induced by the multiple peptides could only be insufficiently compensated by introduction of PEG10-linkers. 68Ga-TRAP(PEG10-AvB6)3 exhibited a slightly improved log D (−1.94), albeit at the expense of a decreased αβ6 integrin activity (87 vs. 23 pM). A total of 30 PEG units per molecule indeed effected a lower background uptake (Fig. 3).

However, a comparison of PET images for mono-, di-, and trimeric conjugates in the same mouse, bearing a subcutaneous H2009 (human lung adenocarcinoma) αβ6 integrin–expressing tumor (Fig. 4), shows that clearance of the PEG-trimer was still far from being optimal. 68Ga-TRAP(PEG10-AvB6)3 and the dimer 68Ga-TRAP(AvB6)2 show much higher accumulation in the abdominal region (particularly in the liver area) than the monomers 68Ga-NODAGA-AvB6 and 68Ga-avebehexin. A similar pattern was observed for kidney uptake and general background. Unfortunately, the higher affinities of the multimers did not effect a proportional increase of H2009 tumor accumulation, finally resulting in inferior tumor-to-organ contrast and a poor delineation of the tumor lesion (Figs. 4A and 4B). Notwithstanding this, overall polarity appears not to be the only crucial parameter, because in vivo properties of the monomers appear closely related despite their different log D values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>log D</th>
<th>IC50 (pM)</th>
</tr>
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<tbody>
<tr>
<td>68Ga-NODAGA-AvB6</td>
<td>−2.41 ± 0.05</td>
<td>267 ± 31</td>
</tr>
<tr>
<td>68Ga-Avebehexin</td>
<td>−3.71 ± 0.03</td>
<td>260 ± 17</td>
</tr>
<tr>
<td>68Ga-TRAP(AvB6)2</td>
<td>−2.14 ± 0.11</td>
<td>120 ± 23</td>
</tr>
<tr>
<td>68Ga-TRAP(AvB6)3</td>
<td>−1.72 ± 0.04</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>68Ga-TRAP(PEG10-AvB6)3</td>
<td>−1.94 ± 0.13</td>
<td>87 ± 12</td>
</tr>
</tbody>
</table>

Affinities were determined using nonradioactive 69/71GaIII complexes. IC50 = 50% inhibition concentration.

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FIGURE 2. 68Ga-avebehexin and 68Ga-NODAGA-AvB6, 2 68Ga-labeled monomeric chelator conjugates of peptide c(FRGDLAFp(NMe)K) for in vivo mapping of integrin αβ6 expression by PET.

FIGURE 3. PET images (maximum-intensity projections) of severe combined immunodeficiency mice recorded 75 min after administration of 20 MBq (1 nmol, 20 MBq/nmol) of 68Ga-TRAP(AvB6)3 (A) and 68Ga-TRAP(PEG10-AvB6)3 (B), illustrating effect of PEG10 linkers in side chains on overall pharmacokinetics. Bid. = bladder; Kid. = kidneys.
The PET image obtained for the hydrophilic $^{68}$Ga-avebehexin ($\log D = -3.71$, Fig. 4D) is comparable to that of $^{68}$Ga-NODAGA-AvB6 (Fig. 4C), although the $\log D$ value of the latter is much closer to that of $^{68}$Ga-TRAP(AvB6)<sub>2</sub> ($-2.41$ and $-2.14$, respectively).

The virtually complete lack of hepatobiliary excretion and the low background uptake rendered $^{68}$Ga-avebehexin the most attractive compound for further investigation. Figure 5 confirms that the H2009 tumor is clearly delineated despite only a fraction of tumor cells that is positive for $\beta_6$ integrin according to immunohistochemistry (Fig. 6), demonstrating high sensitivity of the tracer. Beyond that, it can be noticed that particularly the tumor cells adjacent to desmoplastic stroma show high $\beta_6$ integrin expression in a membranous and cytoplasmic pattern (Fig. 6B), highlighting the aforementioned link between $\alpha\nu\beta_6$ integrin expression and epithelial–mesenchymal interaction.

PET and immunohistochemistry are well correlated with ex vivo biodistribution data (Fig. 7). Apart from excretion-related elevated activity levels in the kidneys and urinary bladder, the highest uptake is found in the H2009 tumor, whereas the comparatively low absolute value (0.65 percentage injected dose per gram) is explained by the relatively low $\beta_6$ integrin expression. Specificity is proven by a marked decrease of this signal on coinjection of excess unlabeled compound (blockade), which can be seen as well in PET (Fig. 5A). Some other blockable uptake can be explained by a low but not entirely insignificant $\alpha\nu\beta_6$ integrin expression in the epithelial cells of some internal organs. For example, immunohistochemistry confirmed that $\beta_6$ integrin is weakly expressed by bronchial as well as alveolar epithelial cells in the lung (Fig. 6C) and by parietal cells in the glandular part of the mouse stomach (Fig. 6D). On the other hand, the pancreatic tissue, for example, shows no $\beta_6$ integrin expression in any cellular compartment (Fig. 6E), resulting comparably insignificant uptake for both control and blockade. The immunohistochemistry furthermore corresponds essentially to the human expression patterns (7) and suggests that similar imaging results might be obtained in humans.

**DISCUSSION**

To facilitate interpretation of the in vivo behavior of the investigated compounds, it needs to be emphasized that high
increased integrin activity as well as higher uptake in a cyclo(RGDXK) type, to a given reporter (e.g., radionuclide or imaging results were obtained with the monomeric conjugates despite multimers might generally benefit from multimerization. Thus, it for a 36,47 ligands, for example, in numerous studies that tethering multiple copies of receptor aging probes. Concerning receptor affinities, it has been shown via the kidneys and the urinary tract, which is desired for im-
 hydrophilicity promotes a fast excretion from nontarget tissues; however, more detailed investigations will be necessary to fully define the scope and limitations for 68Ga-avebehexin, which will be reported in due course.

**CONCLUSION**

For elaboration of tracers from targeting peptides, the fast, click-chemistry–driven synthesis of substantially different types of conjugates for biologic screening was shown to be an efficient way to identify the structural key parameters for a successful in vivo transfer. In this respect, we like to emphasize that contrary to a large body of previous work, multimerization did not work for the αvβ6 integrin–selective peptide c(FRGDLAfp(NMe)K). Although the multimers showed improved αvβ6 integrin affinities as expected, they did not exhibit improved target (i.e., tumor) accumulation in PET scans but instead possessed inferior pharmacokinetics compared with the respective monomers.

Because of its excellent renal clearance and the resulting low background signal, the monomeric TRAP-conjugate 68Ga-avebehexin enabled highly sensitive PET imaging even of moderate αvβ6 integrin expression levels in subcutaneous H2009 (lung adenocarcinoma) xenografts in mice. It thus allows future in vivo studies on some fundamental questions of tumor biology that recently caused an increasing interest in mapping αvβ6 integrin, such as the exact role of αvβ6 integrin overexpression and integrin-mediated epithelial–mesenchymal transition during tumor invasion, metastasis, and development of resistance to chemotherapies. Furthermore, we anticipate that 68Ga-avebehexin will prove clinically useful for specific PET imaging of cancers with high αvβ6 integrin expression, such as pancreatic, ovarian, lung, and gastric carcinoma as well as invasive head and neck carcinomas.

**DISCLOSURE**

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REFERENCES


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