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Radiolabeled γ-AApeptides: A New Class of Tracers for Positron Emission Tomography†

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Abstract

A γ-AApeptide-based tracer for positron emission tomography imaging of integrin $\alpha_v\beta_3$ is reported. Despite its shorter sequence and linear nature, this tracer had comparable integrin $\alpha_v\beta_3$ binding affinity as the cyclic arginine-glycine-aspartic acid peptide but significantly higher resistance to enzymatic degradation and better stability.

Positron emission tomography (PET) has been widely used in clinical oncology for tumor diagnosis, staging, and treatment monitoring.[1] Development and clinical translation of novel molecularly targeted PET tracers will facilitate future personalized medicine of cancer patients, such as patient stratification and monitoring the therapeutic response for anti-cancer drugs.[2] Non-invasive PET imaging of tumor angiogenesis (i.e. new blood vessel formation) has gained tremendous interest over the last decade,[3] since the development and metastasis of all solid tumors depend on tumor angiogenesis.[4] Among the many proteins that are involved in tumor angiogenesis, integrin $\alpha_v\beta_3$ is one of the most intensively studied and several PET tracers targeting this cell adhesion molecule have entered clinical investigation.[5] Frequently overexpressed on the tumor neovasculature, as well as cancer cells of many tumor types (e.g. lung/prostate/breast cancer and glioblastoma), integrin $\alpha_v\beta_3$ is an attractive target for both cancer diagnosis and therapy.[6]

Since integrin $\alpha_v\beta_3$ binds tightly to extracellular matrix proteins that contain the Arg-Gly-Asp (RGD) tripeptide epitopes, a wide variety of peptides/peptidomimetics based on the RGD motif have been investigated for anti-cancer drug development and/or cancer imaging.[5a, 6] The Achilles' heel of peptides is that they are susceptible to degradation by a variety of enzymes hence are not very stable *in vivo*. Many approaches have been employed to improve the *in vivo* stability of peptide-based imaging/therapeutic agents, such as cyclization and the use of unnatural amino acids.[5a] Recently, a PET tracer based on peptoids (i.e. poly-N-substituted glycines) was reported for PET imaging,[7] which opened the door to a fertile area of future research and development.

We recently designed a new class of peptidomimetics that are termed "γ-AApeptides",[8] which have shown promising potential for various biological applications such as effective

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disruption of protein-protein interactions,[8] recognition of specific nucleic acids,[9] and as novel antimicrobial agents.[10] In addition, these γ-AApeptides are resistant to proteolytic degradation and are amenable for limitless diversification. Since γ-AApeptides project the same number of side chains as that of peptides of the same length, they are expected to be ideal candidates for short peptide mimicry. To further explore their potential applications in biomedical research, and to develop novel molecular imaging agents, herein we report a short and linear γ-AApeptide-based RGD mimetics (Fig. 1), which can be employed for *in vivo* PET imaging of integrin $\alpha_v\beta_3$ expression. Our results demonstrate that this new class of RGD mimetics are comparable to the commonly used c(RGDyK) (where y denotes D-tyrosine) peptide in terms of integrin $\alpha_v\beta_3$ binding affinity and specificity. Furthermore, they are much more protease-resistant, hence are more suitable for PET imaging applications.

γ-AA1 is a γ-AApeptide designed to mimic the tripeptide RGD. An additional phenyl moiety was included in the molecule to provide a balance of hydrophilicity and hydrophobicity, as was found in many RGD-containing peptides for imaging and/or therapeutic applications,[6] which is not expected to interfere with integrin $\alpha_v\beta_3$ binding. To rationalize such design, structural studies were carried out by superimposing the energy-minimized structure of γ-AA1 onto that of c(RGDyK). As shown in Figure 2, the guanidino and carboxyl groups within γ-AA1 superimpose very well with the functional groups of Arg and Asp residues from c(RGDyK), which are responsible for the recognition of integrin $\alpha_v\beta_3$. T hus, computer modeling supports the straightforward and effective design of γ-AApeptides for RGD motif mimicry. Future systematic studies will be carried out to investigate the effect of different functional groups on integrin $\alpha_v\beta_3$ binding of the γ-AApeptide-based RGD mimetics.

To further evaluate the capability of the γ-AApeptides for RGD mimicry, γ-AA1 and the c(RGDyK) peptide, an extensively studied and validated high affinity antagonist for integrin $\alpha_v\beta_3$,[5a] were each conjugated to FITC. After purification by high performance liquid chromatography (HPLC), FITC-γ-AA1 and FITC-c(RGDyK) were compared for integrin $\alpha_v\beta_3$ binding affinity and specificity in U87MG human glioblastoma cells that express high level of integrin $\alpha_v\beta_3$.[11] At a 5 μg/mL concentration which is under a non-saturating condition (i.e. the fluorescence signal was in the 10^2–10^3 range instead of 10^4), FITC-γ-AA1 has similar uptake in the U87MG cells as FITC-c(RGDyK), as evidenced by flow cytometry (Fig. 3a). Blocking the receptor with 2 μM of unconjugated c(RGDyK) significantly reduced the uptake of both FITC-γ-AA1 and FITC-c(RGDyK) to a similar extent. U87MG cell binding assay using ^{64}Cu-DOTA-c(RGDyK) as the radioligand revealed that the IC_{50} values were 831 and 897 nM for γ-AA1 and γ-AA2, respectively (Fig. 3b). These values are slightly lower but comparable to that of c(RGDyK), with an IC_{50} value of 639 nM in the same assay. Together, these findings indicated that γ-AA1/γ-AA2 and the c(RGDyK) peptide have similar binding affinity and specificity to integrin $\alpha_v\beta_3$ *in vitro*.

To enable ^{64}Cu-labeling and PET imaging, DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid) was linked to γ-AA1 via a 6-aminohexanoic acid linker, which was termed γ-AA2 (*i.e.* DOTA-γ-AA1, Fig. 1). ^{64}Cu-labeling of γ-AA2, including final purification with HPLC, took 90 ± 15 min (n = 8). The decay-corrected radiochemical yield was 50 ± 15 %, based on 2 μg of γ-AA2 per 37 MBq of ^{64}Cu, with a radiochemical purity of > 95%. The specific activity of ^{64}Cu-γ-AA2 was measured to be ~9 GBq/mg of γ-AA2. The c(RGDyK) peptide was conjugated with DOTA, purified by HPLC, and labeled with ^{64}Cu in a similar manner.

Before PET imaging was carried out to evaluate the *in vivo* behaviour of ^{64}Cu-labeled γ-AA2, enzymatic stability of the tracer was investigated. Pronase, a mixture of proteinases isolated from the extracellular fluid of Streptomyces griseus, was used to compare the enzymatic stability of the two PET tracers.[8, 12] After incubation with 0.1 mg/mL of pronase at 37 °C in 100 mM ammonium bicarbonate buffer (pH 7.8) for various time periods, stability of the two tracers was compared using radio-HPLC.

^{64}Cu-γ-AA2 exhibited markedly better stability than ^{64}Cu-DOTA-c(RGDyK) (Fig. 4). The stability of c(RGDyK) is expected to be significantly higher than the natural RGD peptide, attributed to the inclusion of a D-Tyr residue and head-to-tail cyclization, both of which are proven strategies for improving the stability of natural peptides. However, ~8% of ^{64}Cu-DOTA-c(RGDyK) was already degraded at 0.5 h post-treatment, with 100% enzyme degradation at 2 h and 8 h post-treatment. In comparison, ^{64}Cu-γ-AA2 only had 4%, 8%, and 15% degradation at 0.5 h, 2 h, and 8 h post-treatment, respectively. The UV traces were similar to the radio-HPLC results (see Supplementary Information). The fact that ^{64}Cu-γ-AA2 is much more enzymatically stable than ^{64}Cu-DOTA-c(RGDyK) makes γ-AApeptides a promising class of targeting ligands for PET imaging applications, which possess exceptional stability.

After demonstrating the excellent stability of ^{64}Cu-γ-AA2 *in vitro*, serial *in vivo* PET imaging was carried out in the U87MG tumor model (which expresses high level of integrin $\alpha_v\beta_3$ on tumor vasculature and tumor cells[11, 13]) after intravenous injection of the tracer. PET scans at various time points post-injection (p.i.), as well as quantitative region-of-interest (ROI) analysis of the PET data, were performed as described previously.[14] Coronal and transaxial PET slices that contain the U87MG tumors are shown in Fig. 5a and the quantitative data are shown in Fig. 5b.

Tumor uptake of ^{64}Cu-γ-AA2 was clearly visible as early as 0.5 h p.i., which remained persistent over time (0.9 ± 0.3, 1.0 ± 0.2, 1.1 ± 0.2, and 0.7 ± 0.2 %ID/g at 0.5, 2, 4, and 24 h p.i. respectively; n = 3; Fig. 5b). Excellent tumor contrast was observed, with tumor/muscle ratio of 3.8 ± 0.9, 4.8 ± 1.8, 5.8 ± 1.4, and 8.3 ± 3.9 at 0.5 h, 2 h, 4 h, and 24 h p.i. respectively (n = 3). Since ^{64}Cu-γ-AA2 undergoes both hepatobiliary and renal clearance, tracer uptake was also observed in the liver/kidneys.

Administering a blocking dose of the c(RGDyK) peptide reduced the U87MG tumor uptake significantly to 0.5 ± 0.1, 0.4 ± 0.1, 0.4 ± 0.1, and 0.4 ± 0.1 %ID/g at 0.5, 2, 4, and 24 h p.i., respectively (n = 3; $P < 0.05$ at 0.5, 2, and 4 h p.i. when compared with mice injected with ^{64}Cu-γ-AA2 only; Fig. 5a–c), which demonstrated integrin $\alpha_v\beta_3$ specificity of the tracer *in vivo*. Liver uptake of ^{64}Cu-γ-AA2 was higher in the blocking group, being 7.2 ± 2.0, 6.1 ± 1.6, 5.3 ± 1.3, and 4.0 ± 0.6 %ID/g at 0.5, 2, 4, and 24 h p.i. respectively (n = 3). Radioactivity in the blood (0.5 ± 0.1, 0.4 ± 0.1, 0.4 ± 0.1, and 0.4 ± 0.1 %ID/g at 0.5, 2, 4, and 24 h p.i., respectively; n = 3) was lower for the blocking group at early time points, indicating faster blood clearance of the tracer (Fig. 5b). After the last PET scans, all mice were euthanized for biodistribution studies to validate the PET data. The %ID/g values of ^{64}Cu-γ-AA2 in the tumor and major organs obtained from biodistribution studies (Fig. 5d) matched well with the results from ROI analysis of the PET scans, confirming that PET can enable accurate quantification of tracer distribution *in vivo*.

In summary, the ^{64}Cu-labeled γ-AApeptide-based RGD mimetic exhibited comparable integrin $\alpha_v\beta_3$ binding affinity as the c(RGDyK) peptide but significantly higher resistance to enzymatic degradation and better *in vivo* stability, despite its shorter sequence and linear nature. Integrin $\alpha_v\beta_3$ specificity, fast blood clearance, and good tumor contrast of ^{64}Cu-γ-

AA2 established γ-AApeptides as a novel class of enzymatically stable targeting ligands for molecular imaging applications.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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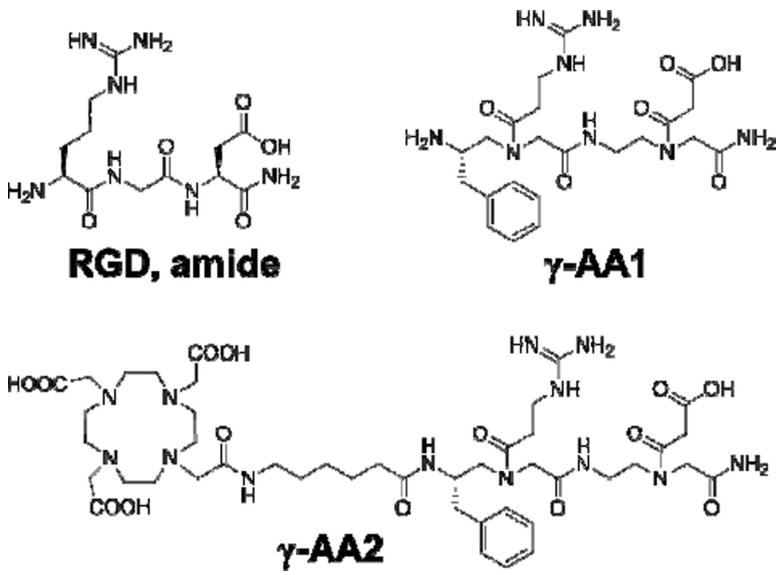

Fig. 1.

The RGD tripeptide and the γ-AApeptides. γ-AA1 is a γ-AApeptide that mimics RGD. γ-AA2 is a DOTA conjugated γ-AA1 for ^{64}Cu-labeling and PET imaging.

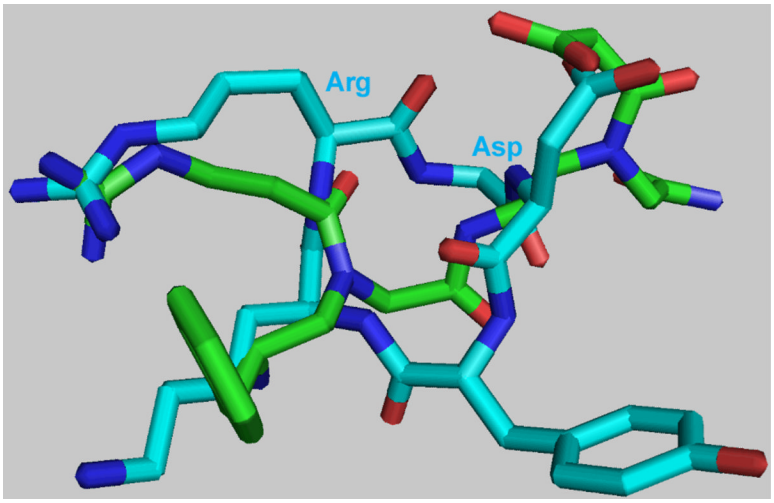

Fig. 2.
The superimposition of energy-minimized γ-AA1 (green) and c(RGDyK) (cyan). The energy minimization and superimposition was carried out using the ChemBioOffice program.

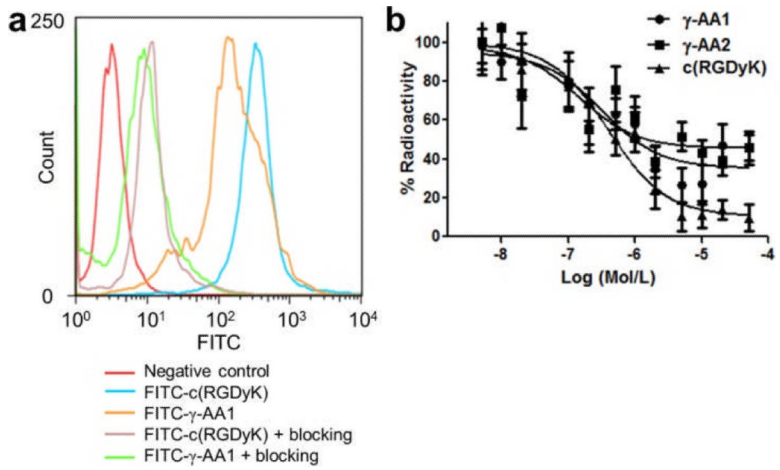

Fig. 3.

(a) Flow cytometry analysis of FITC-conjugated γ-AA1 and c(RGDyK) peptide in U87MG cells at a 5 μg/mL concentration. Blocking experiments with 2 μM of c(RGDyK) peptide were also performed to confirm the specificity for integrin $\alpha_v\beta_3$. (b) U87MG cell binding assay demonstrated that both γ-AA1 and γ-AA2 bind to integrin $\alpha_v\beta_3$, similar to the c(RGDyK) peptide.

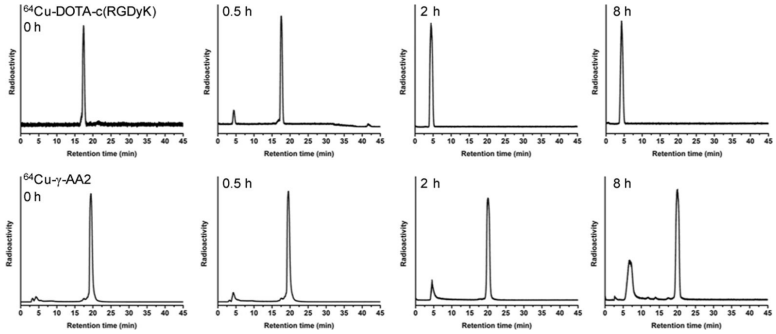

Fig. 4.

Serial radio-HPLC profiles of ^{64}Cu-DOTA-c(RGDyK) and ^{64}Cu-γ-AA2 before and after incubation in pronase at 37 °C.

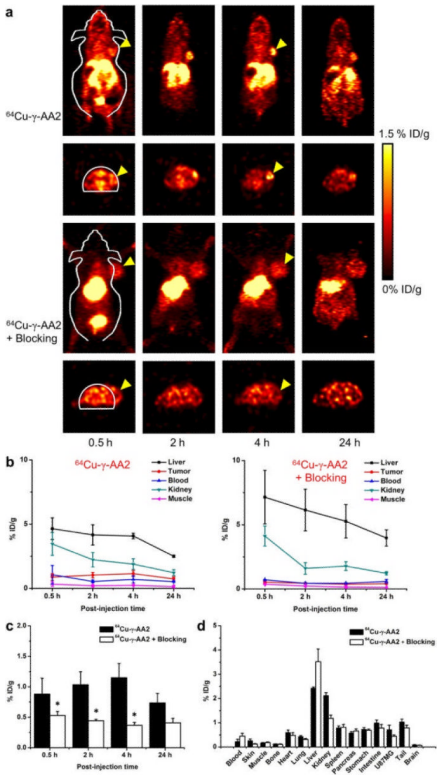

Fig. 5.

Serial PET imaging and biodistribution studies of ^{64}Cu-γ-AA2 in U87MG tumor-bearing mice. (a) Serial coronal and transaxial PET images of U87MG tumor-bearing mice at 0.5, 2, 4, and 24 h after injection of ^{64}Cu-γ-AA2, or co-injection of c(RGDyK) and ^{64}Cu-γ-AA2 (i.e., blocking). Arrowheads indicate tumors. (b) Time-activity curves of the liver, tumor, blood, kidney, and muscle in U87MG tumor-bearing mice after injection of ^{64}Cu-γ-AA2 (left), or co-injection of ^{64}Cu-γ-AA2 and a blocking dose of c(RGDyK) (right). (c) Comparison of U87MG tumor uptake of ^{64}Cu-γ-AA2 between the two groups. (d) Biodistribution data at 24 h post-injection of the tracer. *: $p < 0.05$ ($n = 3$).