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Specific Amyloid Binding of Polybasic Peptides *In Vivo* Is Retained by β -Sheet Conformers but Lost in the Disrupted Coil and All D-Amino Acid Variants

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Abstract

Purpose—The heparin-reactive, helical peptide p5 is an effective amyloid imaging agent in mice with systemic amyloidosis. Analogs of p5 with modified secondary structure characteristics exhibited altered binding to heparin, synthetic amyloid fibrils, and amyloid extracts *in vitro*. Herein, we further study the effects of peptide helicity and chirality on specific amyloid binding using a mouse model of systemic inflammation-associated (AA) amyloidosis.

Procedures—Peptides with disrupted helical structure [p5_(coil) and p5_(Pro3)], with an extended sheet conformation [p5_(sheet)] or an all-D enantiomer [p5_(D)], were chemically synthesized, radioiodinated, and their biodistribution studied in WT mice as well as transgenic animals with severe systemic AA amyloidosis. Peptide binding was assessed qualitatively by using small animal single-photon emission computed tomography/x-ray computed tomography imaging and microautoradiography and quantitatively using tissue counting.

Results—Peptides with reduced helical propensity, p5_(coil) and p5_(Pro3), exhibited significantly reduced binding to AA amyloid-laden organs. In contrast, peptide p5_(D) was retained by non-amyloid-related ligands in the liver and kidneys of both WT and AA mice, but it also bound AA amyloid in the spleen. The p5_(sheet) peptide specifically bound AA amyloid *in vivo* and was not retained by healthy tissues in WT animals.

Conclusions—Modification of amyloid-targeting peptides using D-amino acids should be performed cautiously due to the introduction of unexpected secondary pharmacologic effects.

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Compliance with Ethical Standards

Conflict of Interest

JSW and SJK are inventors on patent applications that describe the use of polybasic peptides for the detection and treatment of amyloidosis. JSW, TR, AS, EBM, and SJK are founders and co-owners of Solex, LLC that sub-licensed intellectual property related to amyloid reactive peptides.

Peptides that adopt a helical structure, to align charged amino acid side chains along one face, exhibit specific reactivity with amyloid; however, polybasic peptides with a propensity for β -sheet conformation are also amyloid-reactive and may yield a novel class of amyloid-targeting agents for imaging and therapy.

Keywords

Peptides; Amyloid binding; AA amyloidosis; SPECT/CT; Biodistribution

Introduction

Amyloidosis is a protein-folding disorder characterized by the aggregation and deposition of proteinaceous fibrils, associated with heparan sulfate proteoglycans (HSPG) and serum-derived proteins, in vital organs and tissues [1]. The unrelenting accumulation of amyloid invariably leads to organ dysfunction, morbidity, or death. Systemic amyloid has a complex and variable presentation and can involve any organ or tissue; however, the kidneys, nerve, liver, spleen, and heart constitute the major deposition sites in patients [1]. In total, more than 30 proteins have been chemically or serologically identified as constituents of fibrils in amyloid deposits. Thus, due to the heterogeneity in disease presentation and extent of organ involvement, there is a clinical need to develop novel amyloid-targeting reagents that can be used for imaging the whole body amyloid burden in patients for the purpose of prognostication, stratification, and monitoring response to therapy.

The major constituents of tissue amyloid, proteinaceous fibrils and HS glycosaminoglycans, are both polyelectrolyte arrays composed of repeating elemental units—the amyloidogenic precursor protein and acidic disaccharides, respectively. Amyloid-associated HS has been shown to be both biochemically and electrochemically distinct from the ubiquitous HS found in healthy (amyloid-free) tissues [2, 3]. The HS found in systemic inflammation-associated (AA) amyloid deposits in mice can be specifically targeted by using a radioiodinated single-chain fragment variable antibody (scFv), NS4F5, which binds hypersulfated HS with N-sulfation, C5-epimerization, and high degrees of 2-O- and 6-O-sulfation [4, 5]. Given that HS is invariably associated with all amyloid deposits and has a distinct pattern of hypersulfation, akin to that of heparin, we developed synthetic heparin-binding peptides as specific amyloid-targeting agents for molecular imaging [3, 6–9]. These peptides have also been shown to bind to synthetic amyloid fibrils, in the absence of HS, via electrostatic interactions with acidic amino acid side chains displayed with a well-defined periodicity on the surface of the fibrils [3, 10].

The peptide, p5, is a non-natural, synthetic, polybasic (+8 net charge), heparin-binding reagent that adopts an α -helical structure in the presence of heparin [9, 11–13], binds synthetic amyloid fibrils and human amyloid extracts *in vitro* [6], and specifically localizes with systemic AA amyloid deposits *in vivo* [7, 8]. Recently, we undertook a study of the structural requirements that govern the amyloid-reactivity of peptide p5 and generated variants using the following: (i) D-amino acids [p5_(D)], (ii) by introducing amino acids to prevent the formation of a helical secondary structure [p5_(coil) and p5_(Pro3)], and (iii) a variant designed to preferentially adopt a β -sheet conformation [p5_(sheet)] [13]. The binding

of these peptides to heparin (as a homolog of amyloid-associated HS), synthetic amyloid fibrils, and human amyloid extracts was studied *in vitro* [13]. The p5 peptide analogs with a decreased propensity for helix formation, p5_(coil) and p5_(Pro3), exhibited decreased reactivity with heparin and amyloid extracts, whereas the D-amino acid peptide and the β -sheet variant, p5_(sheet), were found to bind all amyloid-related substrates as well as the parental p5 peptide [13].

We now describe an investigation of the *in vivo* biodistribution of the four p5 peptide homologs. The ability of each peptide to specifically target amyloid was studied by single-photon emission computed tomography/x-ray computed tomography (SPECT/CT) imaging and tissue biodistribution measurements of radioiodinated peptides administered to healthy wild-type (WT) or AA-amyloid mice. Herein, we show that, in contrast to the p5 peptide, the all-D amino acid variant bound to non-amyloid ligands in the kidney and liver as well as amyloid, whereas disruption of the helical structure prevented amyloid reactivity. Although helicity was necessary for efficient binding of p5, the sheet-structured peptide with different spacing between lysine residues also bound amyloid avidly and specifically *in vivo*. The importance of peptide helicity and chirality is studied in the context of developing novel amyloid-targeting agents that may benefit patients with systemic amyloidosis.

Materials and Methods

Peptides and Proteins

Peptides p5_(D), p5_(sheet), p5_(coil), and p5_(Pro3) (Table 1) were purchased from Keck Laboratories (New Haven, CT) or Anaspec (Fremont, CA) as impure preparations and purified by RP-HPLC (Biologic DuoFlow; BioRad, Hercules, CA) by using a C3 solid phase (Zorbax™300SB; Agilent, Santa Clara, CA). Peptides were eluted using a linear gradient of 1 to 51 % acetonitrile in water with 0.05 % *v/v* trifluoroacetic acid as the mobile phase (flow rate of 4 ml/min). Fractions of 1.8-ml volume were collected and pooled and the integrity of the peptides verified by mass spectrometry [6]. The secondary structure for each peptide was predicted using the iTASSER software (Fig. 1; [14, 15]), and the percent α -helix was predicted based on the amino acid sequence using Agadir (<http://agadir.crg.es/>) with parameters of pH = 7; temperature, 298 K; and an ionic strength of 0.15 [16].

Murine Model of AA Amyloidosis

Systemic AA amyloidosis was induced in H2-L^d-huIL-6 Tg Balb/c transgenic mice (H2/IL-6 mice) that constitutively express the human interleukin-6 (IL-6) transgene [17, 18] by IV injection of 10 μ g of purified, splenic AA amyloid (amyloid-enhancing factor; AEF) suspended in 100 μ l of sterile phosphate-buffered saline, pH 7.4 (PBS). Mice were used for imaging at 4–6 week post-AEF at which time significant amyloid is known to be present in abdominothoracic organs. All animal procedures were performed in accordance with protocols approved by the University of Tennessee Animal Care and Use Committee. The University of Tennessee is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC-I)-accredited institution.

Peptide Radiolabeling

Peptides (10–40 μ g) were radioiodinated with iodine-125 (Perkin Elmer, Waltham, MA) using chloramine T (20 μ g) and the products purified by gel filtration using a Sephadex G-25 size-exclusion matrix (PD10; GE Healthcare) using a mobile phase of 0.1 % w/v gelatin in PBS. The radiochemical purity of all products was determined qualitatively by SDS polyacrylamide gel electrophoresis analyzed by phosphor imaging (Cyclone Storage Phosphor System, PerkinElmer, Shelton, CT).

SPECT/CT Imaging

High-resolution SPECT/CT imaging was performed using an Inveon trimodality SPECT/PET/CT platform (Siemens preclinical, Knoxville, TN) [19]. Image data were acquired and reconstructed using Inveon Acquisition Workplace (IAW) ver. 2.0. The mice ($n = 3$ per peptide, per time point) were administered ~ 100 μ Ci (~ 5 μ g) of iodine-125-labeled peptide intravenously in the lateral tail vein. Five minutes prior to euthanasia, representative mice received 500 μ l of Iohexol CT contrast agent IP (diluted 1:1 w/v in PBS). Groups of animals were euthanized by isoflurane overdose either 2 or 24 h post-injection (pi) of peptide. The animals were placed in the imaging system and SPECT data generated by acquiring 60 16-s projections over a total of 1.5 revolutions. A 1-mm diameter aperture, five-pinhole (mouse whole body) collimator was used at 30 mm from the center of the field of view. The image data were reconstructed using a Maximum A Priori algorithm (MAP-16 iterations, 6 subsets, $\beta = 1$) onto a matrix with x - and y -dimensions of 88 and z -dimension of 312, and 0.5-mm isotropic voxels. *Post hoc* attenuation correction was then applied to the reconstructed data using CT image data. Scatter correction was performed using a SPECT triple energy window (TEW) method [20].

The CT data were acquired for anatomic co-registration and attenuation correction, using an x-ray voltage biased to 80 kVp with a 500 μ A anode current. Two-bed positions were covered with 240 ms exposures and 361 projections collected covering 360° of continuous rotation with low magnification and binning at 4. The data were reconstructed using an implementation of the Feldkamp-filtered cone beam algorithm [21] onto a $256 \times 256 \times 603$ matrix with isotropic 211.4- μ m voxels.

Tissue Radioactivity Biodistribution Measurements

Following SPECT/CT imaging, a necropsy was performed on each mouse. Samples from 11 tissues (muscle, liver, pancreas, spleen, left and right kidneys, stomach, heart, lung, and the lower and upper intestines) were taken for measurement of radioactivity, and a small piece of each tissue was fixed in buffered formalin for microautoradiographic (ARG) studies. For biodistribution measurements, tissue samples were placed in tared vials and the radioactivity quantified using an automated gamma counter (1480 Wizard 3 Gamma Counter, Perkin Elmer). Data were decay-corrected to the time of injection and expressed as % injected dose per gram of tissue (%ID/g).

Microautoradiography and Congo Red Tissue Staining

For microautoradiography, 6- μ m-thick tissue sections were cut from formalin-fixed, paraffin-embedded material. The sections were placed on Plus microscope slides (Fisher

Scientific), dipped in NTB-2 emulsion (Eastman Kodak), stored in the dark, and developed after a 4-day exposure. Each section was counterstained with hematoxylin and eosin. Detection of amyloid was achieved in consecutive tissue sections by staining with an alkaline Congo red solution (0.8 % w/v Congo red, 0.2 % w/v KOH, 80 % ethanol) for 1 h at room temperature followed by counter-stain with Mayer's hematoxylin for 2 min. All tissue sections were examined using a Leica DM500 light microscope fitted with cross-polarizing filters (for Congo red illumination). Digital microscopic images were acquired using a cooled CCD camera (SPOT RT-slider, Diagnostic Instruments, Sterling Heights, MI).

Results

The peptides p5, p5_(sheet), p5_(coil), and p5_(Pro3) were predicted, using the iTASSER algorithm, to have a propensity for helical, sheet, and disordered structures, respectively (Fig. 1). Similarly, the helical content estimated from the amino acid sequence in PBS indicated that p5 (and p5_(D), although D-amino acids cannot be used in either calculation) had ~2.6 % helical structure under these conditions and, in contrast, p5_(sheet) and p5_(coil) had 0.05 and 0.02 %, respectively (Fig. 1).

The biodistribution of radiolabeled peptides was assayed in WT mice and mice with severe systemic AA amyloidosis to evaluate the specific amyloid-targeting capability of each. Qualitative, whole body data were collected by using high-resolution, dual-modality SPECT/CT imaging at 2 and 24 h pi of radiolabeled peptide (Figs. 2 and 3). Additionally, the distribution of each radioiodinated peptide was quantified in 11 organs and tissues by gamma counting of tissue samples obtained at necropsy (Tables 2 and 3). Intraperitoneal injection of CT contrast agent allowed better visualization of the abdominal organ boundaries in the CT images (Figs. 2 and 3).

In WT, amyloid-free mice, two patterns of uptake were observed in the SPECT images. Animals that received [¹²⁵I] p5_(D) had significant retention of the radiolabeled peptide in the kidneys (K) and liver (L), as evidenced by the strong signal in the SPECT images (Fig. 2). The radiolabeled peptide persisted in both anatomic sites for more than 24 h pi. The retention of [¹²⁵I]p5_(D) was ~150 % ID/g in the kidneys and ~20 % ID/g in the liver (Table 2). In contrast, iodine-125-labeled p5_(sheet), p5_(coil), and p5_(Pro3) were not observed in the kidneys or liver in the SPECT data, similar to data obtained with the parent peptide, p5 [7, 22]. Rather, the images were dominated by the stomach (S) and thyroid (T), particularly in the images acquired 2 h pi. At 24 h pi, there was scant radioactivity visible in the WT mice that received these peptides (Fig. 2). This was also reflected in the biodistribution data, where the stomach activity for all three peptides at 2 h pi ranged between 6 and 8 % ID/g but decreased to <0.1 % ID/g at 24 h pi (Table 2). In all other organs and tissues harvested for gamma counting, the activity was <3 % ID/g (the kidney was the second highest activity level) at 2 h pi (Table 2) and decreased 10-fold over the next 24 h.

The H2/IL-6 strain of mice develops significant AA amyloid predominantly in the liver, spleen, pancreas, kidneys, and intestines. The distribution of [¹²⁵I]p5_(D) in the AA mouse was remarkably similar to that in the WT animal (Fig. 3, Table 3), i.e., dominated by >24-h retention of the peptide in the kidneys (100–140 % ID/g) and liver (17 % ID/g), making it

unsuitable for specific amyloid imaging in these anatomic sites. In contrast, SPECT images of [^{125}I]p5_(sheet) in the AA mouse revealed a broad pattern of uptake in the abdominal organs, including the liver (14 % ID/g), spleen (14 % ID/g), pancreas (16 % ID/g), and intestine (6 % ID/g) at 2 h pi (Fig. 3, Table 3). At 24 h pi, [^{125}I]p5_(sheet) was still visible in the liver (6.5 % ID/g) and the spleen (8.4 % ID/g). In contrast, images of the [^{125}I] p5_(coil) in AA mice at 2 h pi revealed radioactivity principally in the stomach (13.5 % ID/g) and to a lesser degree in the kidneys (~7 % ID/g). The liver and spleen had less than 4 and 3 % ID/g, respectively, reflecting the low affinity of p5_(coil) for amyloid *in vitro*. At 24 h pi, all organs had <1.2 % ID/g of peptide [^{125}I]p5_(coil) indicating a lack of retention. Similarly, the [^{125}I]p5_(Pro3) peptide was observed in the kidneys (~4.6 % ID/g) and stomach (13.6 % ID/g) at 2 h pi, but it was not retained in any anatomic site, and by 24 h pi, all organs were <2.5 % ID/g (Fig. 3, Table 3).

The microscopic distribution of radiolabeled p5_(D) and p5_(sheet) was determined to study whether the accumulation of the peptides in the organs was due to specific amyloid binding (Figs. 4 and 5). The appearance of iodine-125-labeled peptide in the ARGs was manifested as black silver grains in the tissue sections. The distribution of p5_(sheet) in the amyloid-laden spleen was shown to correlate with the distribution of perifollicular amyloid, as evidenced in the Congo red-stained tissue section (Fig. 4). Notably, there was no evidence of peptide accumulation in areas of the tissue that were devoid of amyloid. A similar analysis was performed on other amyloid-laden organs harvested at autopsy (Fig. 5). In the kidney, [^{125}I]p5_(D) was associated with the proximal tubules and the Bowman's capsular space or epithelia within the renal cortex (Fig. 5a). In the liver of the WT mouse, [^{125}I]p5_(D) was observed as punctate deposits throughout the sinusoids (Fig. 5a). When hepatic AA amyloid was present, the p5_(D) peptide was observed, in addition to the punctate deposits, as weakly staining the amyloid, notably in the perivascular amyloid (Fig. 5a, AA-ARG). In the spleen, there was no evidence of p5_(D) accumulation in WT mice (Fig. 5a); however, the peptide was associated with perifollicular AA amyloid which exhibited green-gold birefringence in the Congo red-stained tissue section (AA-CR). In contrast to p5_(D), [^{125}I]p5_(sheet) was not observed in any of the amyloid-free WT organs, as evidenced by the lack of black silver grains in the kidney, spleen, liver, heart, stomach, and adrenal (Fig. 5b). However, specific retention of the peptide was seen in the amyloid deposits of the kidney, spleen, liver, heart, stomach wall, and adrenal as shown by the correspondence between black deposits (AA-ARG) and the green-gold birefringent amyloid (AA-CR).

Discussion

Peptide p5 binds synthetic amyloid fibrils as well as human amyloid extracts, and it specifically localizes with AA amyloid in a murine model of AA amyloidosis, the prototypic model of systemic amyloid disease that accurately recapitulates the human disease [7, 8, 10, 22]. The peptide adopts a helical secondary structure in the presence of heparin, and presumably, this conformation is adopted in the presence of hypersulfated HS and amyloid fibrils within the pathologic deposits, likely contributing to the efficacious and selective interaction with these charged arrays. Enhancing the helical propensity of p5 enhanced the affinity for both heparin and amyloid, thus supporting the importance of the helical structure and charge spacing on these interactions [9, 12]. Additionally, disruption of helicity by using

glycine [p5_(coil)] or proline [p5_(Pro3)] amino acid residues abrogated the reactivity with heparin, as evidenced by the lack of structural transition in the presence of heparin when monitored using circular dichroism (CD) spectroscopy [13]. Binding of p5_(coil) and p5_(Pro3) to human amyloid extracts *in vitro* was similarly decreased relative to the parental p5 peptide [13].

Furthermore, when radiolabeled and administered to mice with severe AA amyloidosis, binding of p5_(coil) and p5_(Pro3) to amyloid-laden tissues, notably the liver and spleen, was reduced 3- to 5-fold, as compared to peptide p5 [7]. Neither peptide bound to healthy, amyloid-free tissues in the WT mice indicating that the enhanced structural flexibility of p5_(coil) did not result in increased binding to amyloid or other ligands present in the extracellular matrix and plasma membranes of normal cells. The decreased binding to AA amyloid *in vivo* indicates that a helical secondary structure, which promotes a linear array of basic amino acid side chains, is important for amyloid interaction, as it is for binding to heparin [11, 12]. This is consistent with the reduced affinity of these peptides for amyloid *in vitro* [13].

The incorporation of non-natural D-amino acids into peptides may enhance the circulating half-life *in vivo* as they are resistant as to proteolysis [23]. This strategy has been used to develop longer-lived peptide drugs for the treatment of cancer and hormone abnormalities [23, 24]. We, therefore, had hypothesized that generating the all-D enantiomer of peptide p5 would serve, when radiolabeled, as a more stable and efficacious imaging agent for the detection of amyloid. Indeed, [¹²⁵I]p5_(D) was shown to bind all amyloid-related substrates significantly better than peptide p5 when tested *in vitro* with ~90 % bound material in a pull-down assay, as compared to ~50 % for radioiodinated p5 [13]. Additionally, the reactivity with heparin, evidenced by a change in the molar ellipticity in CD spectra, was indistinguishable from that of p5 [13]. This was a favorable finding given the numerous accounts of decreased bioactivity and immunoreactivity of D-peptides as compared to their L-counterparts [23], as exemplified by the decrease in affinity (10–1000-fold) of the stinging peptide (an analog of bee-venom apamin) for the p53 negative regulator protein, MDM2 [25].

In contrast to the L-amino acid parent p5, when [¹²⁵I]p5_(D) was administered IV into amyloid-free WT mice, there was significant uptake and retention (binding at 2 and 24 h pi, respectively) of peptide in the hepatic sinusoids and renal cortex (Figs. 2, 3, and 4). Uptake was rapid, occurring within 2 h, and the radioactivity persisted for at least 24 h pi. The peptide was neither lost from these sites nor dehalogenated by intracellular deiodinases [26]. The data indicate that peptide p5_(D) binds an extracellular ligand(s) associated with cells in hepatic sinusoids and in the proximal renal tubules. There is considerable heparan sulfate in the liver and kidneys, as evidenced by the binding of HS-reactive basic fibroblast growth factor (bFGF or FGF2) in these organs [27]. However, the distribution of [¹²⁵I]bFGF, which we observed in splenic sinusoids and within renal glomerular tufts, differed from that of [¹²⁵I]p5_(D), indicating that unique ligands for the [¹²⁵I]p5_(D) must exist, at least in the WT kidney and spleen. The binding of [¹²⁵I]bFGF in the liver was reminiscent of that seen for [¹²⁵I]p5_(D), suggesting a common target in this organ.

In mice with AA amyloid, p5_(D) was again observed in the renal tubules within the cortex; however, in the spleen, the perifollicular amyloid was decorated with radiolabeled peptide, and there was weak, but positive, uptake in the hepatic AA amyloid in addition to the non-amyloid bound material in the sinusoids. Uptake of p5_(D) in amyloid is weak, likely due to competition for peptide binding between amyloid and the ligands present in the healthy kidney and liver. Therefore, we have demonstrated that [¹²⁵I]p5_(D) binds tissue amyloid *in vivo*, either through association with the hypersulfated HS or protein fibrils, but it additionally interacts with other ligands that may represent a class of HS not recognized by bFGF or the parental p5 peptide. This gain-of-function property renders p5_(D) unsuitable as an amyloid imaging in patients, assuming that the off-target ligand is present in man also.

To examine the importance of a helical secondary structure, such as adopted by peptide p5, for binding to heparin and amyloid-associated arrays, we compared its reactivity *in vivo* with peptide p5_(sheet), which has a propensity for β -sheet secondary structure. This β -sheet variant was designed using valine and threonine residues, which have low helix propensities [28] and small and polar uncharged side chains, respectively, akin to the alanine and glutamine used in p5. The spacing of lysine residues in p5_(sheet) allowed, theoretically, for the display of charged side chains along one side of the extended sheet (see Table 1 and Fig. 1). During *in vitro* analyses, peptide p5_(sheet) was found to bind heparin with a greater affinity than p5, as evidenced by the formation of a β -sheet-associated minima at 216 nm in the CD spectra using concentrations of heparin that were ten times lower than required for the structural transition of peptide p5 [13]. When radiolabeled, peptide p5_(sheet) also bound numerous amyloid-related substrates *in vitro* including synthetic fibrils and human amyloid extracts and AA-laden mouse liver homogenates, with greater relative affinity relative to p5 (~90 vs ~50 % in a pull-down assay) [13]. When [¹²⁵I]p5_(sheet) was injected IV into AA mice, the peptide localized rapidly to all amyloid-laden organs—principally the liver, spleen, pancreas, and intestines. The hepatosplenic amyloid was decorated with radiolabeled peptide even at 24 h pi, as evidenced in microautoradiographs (Fig. 4), similar to the results with peptide p5 [7]. There was no evidence of uptake in healthy organs or tissues, suggesting that the polybasic sheet conformation retained amyloid-specific interactions *in vivo* that were observed *in vitro*. In contrast to the helical reagent p5, and similar amyloid-reactive peptides [3], peptide p5_(sheet) has only 18 amino acids, rendering it cost-effective to produce for human use relative to peptide p5 and longer reagents [3]. Our initial studies have demonstrated that it exhibits amyloid-specific reactivity *in vivo*, similar to peptide p5, but previous data suggest that it may occupy a novel binding site on the amyloid fibril [13]. Given this, polybasic peptides with a propensity for β -sheet formation might present new opportunities, either alone or as bi-specific reagents joined to α -helical peptides such as p5, that may serve as the basis for novel amyloid-targeting imaging and therapeutic agents.

Conclusions

At present, there are no means for imaging the whole body distribution of amyloid in patients, which would afford a method of confirming diagnosis, defining organ involvement, and monitoring response to therapy. Therefore, we have identified polybasic peptides as reagents that might be used as amyloid imaging agents in patients. To date, all these peptides have a propensity for helical secondary structure. An all-D amino acid peptide exhibited

gain-of-function, off-target reactivity, whereas disruption of the helicity reduced amyloid reactivity *in vivo*. In contrast, a polybasic peptide with a propensity for sheet formation exhibited specific amyloid reactivity *in vivo* that was comparable to the parental p5 peptide. These data suggest that p5_(sheet), or similar peptides, may provide a new class of reagents for amyloid-targeting *in vivo* and serve as agents for amyloid imaging and treatment in patients with systemic amyloidosis.

Acknowledgments

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Abbreviations

AA	Inflammation-associated amyloidosis
SPECT	Single-photon emission computed tomography
CT	x-ray computed tomography
ARG	Microautoradiography
%ID/g	Percent injected dose per gram of tissue
CR	Congo red

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Fig. 1.

Predicted secondary structure of peptides used in this study. Peptide p5 was predicted to be α -helical whereas p5_(sheet) was an extended sheet [13]. Peptide p5_(coil) and p5_(Pro3) were random coils or disrupted helices. Structures were generated using the online prediction program, iTASSER, and rendered using DeepView/Swiss-PDBViewer v4.0.4 (Swiss Institute of Bioinformatics). Helix content of each peptide was predicted using Agadir.

Fig. 2. Biodistribution of radiolabeled peptides in WT mice using SPECT/CT imaging. The uptake of iodine-125-labeled peptides $p5_{(D)}$, $p5_{(sheet)}$, $p5_{(coil)}$, and $p5_{(Pro3)}$ was visualized by using SPECT/CT imaging of WT mice euthanized at 2 and 24 h pi. Coronal and axial views are shown, where the axial slice is at the level of the kidneys. Radioactivity is false colored *yellow-red*. *K* kidney, *L* liver, *S* stomach, *T* thyroid.

Fig. 3. Biodistribution of radiolabeled peptides in mice with AA amyloidosis using SPECT/CT imaging. The uptake of iodine-125-labeled peptides $p5_{(D)}$, $p5_{(sheet)}$, $p5_{(coil)}$, and $p5_{(Pro3)}$ was visualized by using SPECT/CT imaging of AA mice euthanized at 2 and 24 h pi. Coronal and axial views are shown, where the axial slice is at the level of the kidneys. Radioactivity is false colored *yellow-red*. *K* kidney, *L* liver, *S* stomach, *T* thyroid.

Fig. 4. Specific co-localization of $[^{125}\text{I}]\text{p5}_{(\text{sheet})}$ with splenic AA amyloid deposits *in vivo*. **a** The distribution of radiolabeled $\text{p5}_{(\text{sheet})}$ was visualized (*arrow*) in the spleen of a mouse with AA amyloidosis by using microautoradiography. **b** The consecutive tissue section was stained with Congo red to show the presence of birefringent amyloid (*arrow*). Original objective magnifications for autoradiograph and Congo red were $\times 5$ and $\times 10$, respectively.

Fig. 5. Microautoradiographic distribution of [^{125}I]p5_(D) and [^{125}I]p5_(sheet) in WT and AA mice at 24 h pi. Radiolabeled **a** p5_(D) and **b** p5_(sheet) peptides were visualized in 6- μm -thick, formalin-fixed tissue sections from WT and AA mice by using microautoradiography (ARG). Consecutive tissue sections were stained with Congo red (CR) to show the presence of birefringent amyloid. ARG and CR images were taken using objective magnifications of $\times 5$ and $\times 10$, respectively, except for images of the stomach that were acquired with a $\times 10$ objective magnification. The CR image area is noted by the *black box* in the AA-ARG image.

Table 1

Peptide primary structure and physical data

Peptide	Mw	AA	Primary structure
p5	3257.7	31	GGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK Q
p5 _(D)	3257.7	31	[GGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK Q] _D
p5 _(sheet)	2106.6	18	VYKVK TKVKT KVKTK VKT
p5 _(coil)	2491.7	31	GGGYS KGGKG GKGKG KGGKG GKGKG GKGGK G
p5 _(Pro3)	3221.7	31	GGGYS KAQKA PAKQA KQPQK AQKAP AKQAK Q

Table 2Biodistribution (%ID/g) of radioiodinated peptides in WT mice (mean $\pm$ SD)

	WT mice (<i>n</i> = 3) 2 h post-injection					WT mice (<i>n</i> = 3) 24 h post-injection				
	p5(D)	p5(sheet)	p5(coil)	p5(Pro3)	p5(D)	p5(sheet)	p5(coil)	p5(Pro3)	p5(D)	p5(Pro3)
Muscle	0.04 $\pm$ 0.0	0.63 $\pm$ 0.1	0.47 $\pm$ 0.2	0.43 $\pm$ 0.1	0.02 $\pm$ 0.0	0.01 $\pm$ 0.0	0.01 $\pm$ 0.1	0.01 $\pm$ 0.0	0.01 $\pm$ 0.1	0.01 $\pm$ 0.0
Liver	17.42 $\pm$ 2.2	1.27 $\pm$ 0.2	0.94 $\pm$ 0.1	0.99 $\pm$ 0.4	19.27 $\pm$ 1.1	0.07 $\pm$ 0.0	0.06 $\pm$ 0.0	0.04 $\pm$ 0.0	0.04 $\pm$ 0.0	0.04 $\pm$ 0.0
Pancreas	0.09 $\pm$ 0.0	1.04 $\pm$ 0.2	1.12 $\pm$ 0.4	1.04 $\pm$ 0.2	0.04 $\pm$ 0.0	0.01 $\pm$ 0.0	0.02 $\pm$ 0.0	0.01 $\pm$ 0.0	0.01 $\pm$ 0.0	0.01 $\pm$ 0.0
Spleen	2.19 $\pm$ 0.2	0.78 $\pm$ 0.1	0.71 $\pm$ 0.1	0.65 $\pm$ 0.1	2.21 $\pm$ 0.1	0.06 $\pm$ 0.0	0.04 $\pm$ 0.0	0.02 $\pm$ 0.0	0.04 $\pm$ 0.0	0.02 $\pm$ 0.0
L. Kidney	132.13 $\pm$ 41.3	1.38 $\pm$ 0.1	2.45 $\pm$ 0.3	1.85 $\pm$ 0.4	169.7 $\pm$ 15.8	0.10 $\pm$ 0.0	0.16 $\pm$ 0.0	0.11 $\pm$ 0.0	0.16 $\pm$ 0.0	0.11 $\pm$ 0.0
R. Kidney	146.81 $\pm$ 35.9	1.31 $\pm$ 0.1	2.48 $\pm$ 0.0	1.75 $\pm$ 0.3	198.11 $\pm$ 11.3	0.11 $\pm$ 0.0	0.16 $\pm$ 0.0	0.16 $\pm$ 0.0	0.16 $\pm$ 0.0	0.12 $\pm$ 0.0
Stomach	0.75 $\pm$ 0.4	6.34 $\pm$ 4.5	8.90 $\pm$ 2.1	8.50 $\pm$ 3.4	0.14 $\pm$ 0.05	0.10 $\pm$ 0.1	0.07 $\pm$ 0.0	0.07 $\pm$ 0.0	0.07 $\pm$ 0.0	0.07 $\pm$ 0.0
Up. Intestine	0.21 $\pm$ 0.1	0.98 $\pm$ 0.2	1.31 $\pm$ 0.1	1.29 $\pm$ 0.6	0.13 $\pm$ 0.0	0.02 $\pm$ 0.0	0.03 $\pm$ 0.0	0.02 $\pm$ 0.0	0.03 $\pm$ 0.0	0.02 $\pm$ 0.0
Low. Intestine	0.18 $\pm$ 0.1	1.04 $\pm$ 0.3	0.88 $\pm$ 0.1	0.82 $\pm$ 0.4	0.12 $\pm$ 0.0	0.02 $\pm$ 0.0	0.03 $\pm$ 0.0	0.03 $\pm$ 0.0	0.03 $\pm$ 0.0	0.02 $\pm$ 0.0
Heart	0.09 $\pm$ 0.0	0.75 $\pm$ 0.1	0.77 $\pm$ 0.1	0.66 $\pm$ 0.3	0.06 $\pm$ 0.0	0.01 $\pm$ 0.0	0.09 $\pm$ 0.1	0.02 $\pm$ 0.0	0.09 $\pm$ 0.1	0.02 $\pm$ 0.0
Lung	0.39 $\pm$ 0.1	1.53 $\pm$ 0.1	1.35 $\pm$ 0.2	1.40 $\pm$ 0.6	0.35 $\pm$ 0.1	0.15 $\pm$ 0.0	0.07 $\pm$ 0.0	0.05 $\pm$ 0.0	0.07 $\pm$ 0.0	0.05 $\pm$ 0.0

Table 3

Biodistribution (%ID/g) of radioiodinated peptides in AA mice (mean $\pm$ SD)

	AA mice (<i>n</i> = 3) 2 h post-injection					AA mice (<i>n</i> = 3) 24 h post-injection				
	p5 ^(D)	p5 ^(sheet)	p5 ^(coll)	p5 ^(Pro3)	p5 ^(D)	p5 ^(D)	p5 ^(sheet)	p5 ^(coll)	p5 ^(Pro3)	p5 ^(Pro3)
Muscle	0.14 $\pm$ 0.1	1.41 $\pm$ 0.8	0.75 $\pm$ 0.3	0.89 $\pm$ 0.2	0.15 $\pm$ 0.1	0.20 $\pm$ 0.0	0.07 $\pm$ 0.0	0.07 $\pm$ 0.0	0.07 $\pm$ 0.1	0.07 $\pm$ 0.1
Liver	17.86 $\pm$ 1.0	13.85 $\pm$ 2.4	3.78 $\pm$ 2.5	2.42 $\pm$ 0.9	17.21 $\pm$ 2.4	6.51 $\pm$ 1.8	0.31 $\pm$ 0.1	0.26 $\pm$ 0.3	0.26 $\pm$ 0.3	0.26 $\pm$ 0.3
Pancreas	0.28 $\pm$ 0.1	15.58 $\pm$ 5.1	3.21 $\pm$ 0.8	2.03 $\pm$ 0.1	0.37 $\pm$ 0.4	1.79 $\pm$ 0.1	0.14 $\pm$ 0.1	0.27 $\pm$ 0.4	0.27 $\pm$ 0.4	0.27 $\pm$ 0.4
Spleen	6.82 $\pm$ 3.0	14.16 $\pm$ 2.6	2.95 $\pm$ 1.9	1.57 $\pm$ 0.3	7.62 $\pm$ 5.3	8.36 $\pm$ 4.2	0.33 $\pm$ 0.1	0.18 $\pm$ 0.2	0.18 $\pm$ 0.2	0.18 $\pm$ 0.2
L. Kidney	101.18 $\pm$ 14.2	6.04 $\pm$ 1.7	8.41 $\pm$ 6.4	4.65 $\pm$ 1.5	124.3 $\pm$ 27.5	0.92 $\pm$ 0.2	0.36 $\pm$ 0.1	0.33 $\pm$ 0.4	0.33 $\pm$ 0.4	0.33 $\pm$ 0.4
R. Kidney	110.63 $\pm$ 16.2	4.75 $\pm$ 1.6	6.24 $\pm$ 2.5	4.79 $\pm$ 1.1	140.72 $\pm$ 8.8	0.94 $\pm$ 0.1	0.33 $\pm$ 0.0	0.32 $\pm$ 0.3	0.32 $\pm$ 0.3	0.32 $\pm$ 0.3
Stomach	1.48 $\pm$ 0.5	27.54 $\pm$ 2.29	13.48 $\pm$ 4.0	13.65 $\pm$ 0.9	0.47 $\pm$ 0.4	4.66 $\pm$ 0.7	1.13 $\pm$ 0.7	2.28 $\pm$ 3.2	2.28 $\pm$ 3.2	2.28 $\pm$ 3.2
Up. Intestine	0.37 $\pm$ 0.0	5.93 $\pm$ 2.0	2.04 $\pm$ 0.8	1.97 $\pm$ 0.3	0.30 $\pm$ 0.2	0.91 $\pm$ 0.1	0.17 $\pm$ 0.1	0.23 $\pm$ 0.3	0.23 $\pm$ 0.3	0.23 $\pm$ 0.3
Low. Intestine	0.26 $\pm$ 0.0	2.80 $\pm$ 0.7	1.99 $\pm$ 1.2	1.37 $\pm$ 0.2	0.21 $\pm$ 0.1	0.59 $\pm$ 0.2	0.13 $\pm$ 0.1	0.20 $\pm$ 0.3	0.20 $\pm$ 0.3	0.20 $\pm$ 0.3
Heart	0.43 $\pm$ 0.1	2.40 $\pm$ 0.9	1.68 $\pm$ 0.7	1.37 $\pm$ 0.3	0.55 $\pm$ 0.6	0.42 $\pm$ 0.2	0.34 $\pm$ 0.3	0.11 $\pm$ 0.1	0.11 $\pm$ 0.1	0.11 $\pm$ 0.1
Lung	0.56 $\pm$ 0.0	2.33 $\pm$ 0.6	2.74 $\pm$ 1.2	2.34 $\pm$ 0.4	0.32 $\pm$ 0.2	0.54 $\pm$ 0.2	0.20 $\pm$ 0.1	0.27 $\pm$ 0.3	0.27 $\pm$ 0.3	0.27 $\pm$ 0.3