Near-infrared fluorescence molecular imaging of amyloid beta species and monitoring therapy in animal models of Alzheimer’s disease

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="http://dx.doi.org/10.1073/pnas.1505420112">http://dx.doi.org/10.1073/pnas.1505420112</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>National Academy of Sciences (U.S.)</td>
</tr>
<tr>
<td>Version</td>
<td>Final published version</td>
</tr>
<tr>
<td>Accessed</td>
<td>Tue Dec 11 11:44:03 EST 2018</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/101113">http://hdl.handle.net/1721.1/101113</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Article is made available in accordance with the publisher’s policy and may be subject to US copyright law. Please refer to the publisher’s site for terms of use.</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td></td>
</tr>
</tbody>
</table>
Near-infrared fluorescence molecular imaging of amyloid beta species and monitoring therapy in animal models of Alzheimer’s disease

Xueli Zhang1,a, Yanli Tian1, Can Zhang, Xiaoyu Tian1, Alana W. Ross2, Robert D. Moir6, Hongbin Sun1, Rudolph E. Tanzi5, Anna Moore2,2 and Chongzhao Ran2,2

1Molecular Imaging Laboratory, Massachusetts General Hospital/Massachusetts Institute of Technology/Harvard Medical School Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital/Harvard Medical School, Charlestown, MA 02129; 2School of Pharmacy, China Pharmaceutical University, Nanjing 210009, People’s Republic of China; 3Department of Parasitology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, People’s Republic of China; 4Genetics and Aging Research Unit, Department of Neurology, Massachusetts General Hospital, Charlestown, MA 02129; and 5Center for Drug Discovery, Northeastern University, Boston MA 02115

Edited by Gregory A. Petsko, Weill Cornell Medical College, New York, NY, and approved July 1, 2015 (received for review March 18, 2015)

Near-infrared fluorescence (NIRF) molecular imaging has been widely applied to monitoring therapy of cancer and other diseases in preclinical studies; however, this technology has not been applied successfully to monitoring therapy for Alzheimer’s disease (AD). Although several NIRF probes for detecting amyloid beta (Aβ) species of AD have been reported, none of these probes has been used to monitor changes of Aβs during therapy. In this article, we demonstrated that CRANAD-3, a curcumin analog, is capable of detecting both soluble and insoluble Aβ species. In vivo imaging showed that the NIRF signal of CRANAD-3 from 4-mo-old transgenic AD (APP/PS1) mice was 2.29-fold higher than that from age-matched wild-type mice, indicating that CRANAD-3 is capable of detecting early molecular pathology. To verify the feasibility of CRANAD-3 for monitoring therapy, we first used the fast Aβ-lowering drug LY2811376, a well-characterized beta-amyloid cleaving enzyme-1 inhibitor, to treat APP/PS1 mice. Imaging data suggested that CRANAD-3 could monitor the decrease in Aβs after drug treatment. To validate the imaging capacity of CRANAD-3 further, we used it to monitor the therapeutic effect of CRANAD-17, a curcumin analog for inhibition of Aβ cross-linking. The imaging data indicated that the fluorescence signal in the CRANAD-17-treated group was significantly lower than that in the control group, and the result correlated with ELISA analysis of brain extraction and Aβ plaque counting. It was the first time, to our knowledge, that NIRF was used to monitor AD therapy, and we believe that our imaging technology has the potential to have a high impact on AD drug development.

Alzheimer’s amyloid imaging fluorescence curcumin

Alzheimer’s disease (AD) has been considered incurable, because none of the clinically tested drugs have shown significant effectiveness (1–4). Therefore, seeking effective therapeutics and imaging probes capable of assisting drug development is highly desirable. The amyloid hypothesis, in which various Aβ species are believed to be neurotoxic and one of the leading causes of AD, has been considered controversial in recent years because of the failures of amyloid beta (Aβ)-based drug development (1, 3, 5–8). However, no compelling data can prove that this hypothesis is wrong (2, 5), and no other theories indicate a clear path for AD drug development (4). Thus the amyloid hypothesis is still an important framework for AD drug development (1–5, 9–14). Additionally, Kim and colleagues (15) recently reported that a 3D cell-culture model of human neural cells could recapitulate AD pathology. In this study, their finding that the accumulation of Aβs could drive tau pathology provided strong support for the amyloid hypothesis (15).

It is well known that Aβ species, including soluble monomers, dimers, oligomers, and insoluble fibrils/aggregates and plaques, play a central role in the neuropathology of AD (2, 5). Initially, it was thought that insoluble deposits/plaques formed by the Aβ peptides in an AD brain cause neurodegeneration. However, studies have shown that soluble dimeric and oligomeric Aβ species are more neurotoxic than insoluble deposits (16–21). Furthermore, it has been shown that soluble and insoluble species coexist during disease progression. The initial stage of pathology is represented by an excessive accumulation of Aβ monomers resulting from imbalanced Aβ clearance (22, 23). The early predominance of soluble species gradually shifts with the progression of AD to a majority of insoluble species (24, 25). Therefore imaging probes capable of detecting both soluble and insoluble Aβs are needed to monitor the full spectrum of amyloidosis pathology in AD.

Thus far, three Aβ PET tracers have been approved by the Food and Drug Administration (FDA) for clinical applications. However, they are recommended for excluding the likelihood of AD. The fundamental limitation of these three tracers and others under development is that they bind primarily to insoluble Aβs, not the more toxic soluble Aβs (26–32). Clearly, work remains to be done.

Significance

Drug development for Alzheimer’s disease (AD) has been largely unsuccessful to date. Although numerous agents are reportedly effective in vitro, only an inadequate number of them have been tested in vivo, partially because of the lack of reliable and cost-efficient imaging methods to monitor their in vivo therapeutic effectiveness. Several amyloid beta (Aβ)-specific PET tracers have been used for clinical studies. However, their application for monitoring drug treatment in small animals is limited. Near-infrared fluorescence (NIRF) molecular imaging is a cheap, easy-to-use, and widely available technology for small animal studies. In this report we demonstrate, for the first time to our knowledge, that NIRF imaging can be used to monitor the loading changes of Aβs in an AD mouse model.


The authors declare no conflict of interest.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1505420112/-/DCSupplemental.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

1X.Z. and Y.T. contributed equally to this work.

2To whom correspondence may be addressed. Email: amoore@helix.mgh.harvard.edu or cran@nmr.mgh.harvard.edu.

www.pnas.org/cgi/doi/10.1073/pnas.1505420112
in developing imaging probes based on Aβs, and imaging probes capable of diagnosing AD positively are undeniably needed.

Numerous agents reportedly are capable of inhibiting the generation and aggregation of Aβs in vitro; however, only few have been tested in vivo. Partially, this lack of testing arises from the lack of reliable imaging methods that can monitor the agents’ therapeutic effectiveness in vivo. PET tracers, such as 11C-Pittsburgh compound B (11C-PiB) and 18F-AV-45, recently have been adapted to evaluate the efficacy of experimental AD drugs in clinical trials (33). However, they are rarely used to monitor drug treatment in small animals (30–32), likely because of the insensitivity of the tracers for Aβ species [particularly for soluble species (34–36)], complicated experimental procedures and data analysis in small animals, the high cost of PET probe synthesis and scanning, and the use of radioactive material. Therefore, a great demand for imaging agents that could be used in preclinical drug development to monitor therapeutic effectiveness in small animals remains unmet.

Because of its low cost, simple operation, and easy data analysis, near infrared fluorescence (NIRF) imaging is generally more suitable than PET imaging for animal studies. Several NIRF probes for insoluble Aβs have been reported (37–45). It has been almost 10 y since the first report of NIRF imaging of Aβs by Hintersteiner et al. in 2005 (41). However, to the best of our knowledge, successful application of NIRF probes for monitoring therapeutic efficacy has not yet been reported. Our group recently has designed asymmetrical CRANAD-58 to match the hydrophobic (LVFF) and hydrophilic (HHOK) segments of Aβ peptides and demonstrated its applicability for the detection of both insoluble and soluble Aβs in vitro and in vivo (46). In this report, CRANAD-3 was designed to enhance the interaction with Aβs by replacing the phenyl rings of curcumin with pyridyls to introduce potential hydrogen bonds. Additionally, we demonstrated, for the first time to our knowledge, that the curcumin analog CRANAD-3 could be used as an NIRF imaging probe to monitor the Aβ-lowering effectiveness of therapeutic.

Results

Design and Synthesis of CRANAD-3. In our previous studies, we showed that CRANAD-2, a curcumin-based NIRF imaging probe, can differentiate between 19-mo-old wild-type and transgenic mice by in vivo NIRF imaging (47). CRANAD-2 could be considered a “smart” probe because it displayed a significant increase in fluorescence intensity, an emission blue shift, a lifetime change, and quantum yield improvement upon interacting with insoluble Aβ aggregates (47). However, we found that CRANAD-2 was not able to detect soluble Aβ species (46).

Because CRANAD-2 showed a significant fluorescence response to Aβ aggregates/fibrils but not to monomeric Aβ, we speculated that the interaction between monomeric Aβ40 and CRANAD-2 was most likely weak. We reasoned that this weak interaction could be enhanced by replacing the two phenyl rings with pyridyls to introduce hydrogen bonding between the engaged Aβ fragment and the nitrogen atoms of the pyridines (48, 49). To this end, we designed CRANAD-3, which was synthesized by a procedure similar to that used for CRANAD-2 (Fig. 1 A and B); its structure was confirmed by 1H, 13C, and 19F NMR/MS spectra (Fig. S1).

In Vitro Characterization of CRANAD-3. As expected, CRANAD-3 displayed an emission peak around 730 nm (Fig. 1C) in PBS and showed an excellent fluorescence response toward soluble species such as Aβ monomers, dimers, and oligomers (Fig. 2A). It displayed 12.3-, 39.5-, 16.3-, and 16.1-fold increases in fluorescence intensity at 670, 650, 670, and 650 nm for Aβ40 monomers, Aβ42 monomers, Aβ42 dimers, and Aβ42 oligomers, respectively. CRANAD-3 also showed a significant emission wavelength shift upon mixing with these soluble species (Fig. 2B). We found that CRANAD-3 exhibited strong binding with Aβ40/42 monomers, dimers, and oligomers (Kd = 24 ± 5.7 nM, 23 ± 1.6 nM, 16 ± 6.7 nM, and 27 ± 15.8 nM, respectively) (Fig. S2).

Because Aβ oligomers (AβO) have been considered the highly neurotoxic species in AD pathology (16–21), we conducted further comprehensive validation confirming that the fluorescence changes in CRANAD-3 originated from the interaction with AβO. First, we performed immunoprecipitation investigations with AβO-specific antibody A11 (50). We compared the fluorescence changes in two Aβ42O solutions, A and B, before and after immunoprecipitation. Solution A, which contained CRANAD-3 and Aβ42O, was used as control. In solution B, CRANAD-3, Aβ42O, and A11 antibody were mixed. We observed a 92% decrease in fluorescence intensity in solution B after precipitation with magnetic beads but only a 26% decrease in the control solution A (Fig. S3 A and B). These data strongly indicated that Aβ42O is the primary species contributing to the changes in fluorescence intensity. Second, we conducted titration experiments with CRANAD-3 and various Aβs and fragments. (A) Fluorescence spectra of CRANAD-3 with soluble and insoluble Aβs (Left) and with amylin, rat Aβs, and mutated Aβs (Right). (B) Fluorescence spectra of CRANAD-3 with core fragment Aβ16–20 (KLFF) (Left) and noncore fragment Aβ22–35 (Right). (C) 1H NMR spectra of KLFF without (Upper) and with (Lower) CRANAD-3. Asterisks indicate peaks from CRANAD-3.
concentrations of Aβ420 and achieved an excellent linear regression fit (Fig. S3 C and D). Third, we performed dot blotting for various concentrations of Aβ420 with A11 antibody (Fig. S3 E and F) and examined the correlation between the fluorescence signal and the signal from dot blotting. An excellent correlation was achieved between the two groups of signals (Fig. S3G), indicating that the fluorescence intensity can be used to reflect the content of Aβ420. Moreover, we also conducted immunoprecipitation with fibril-specific OC antibody (S1, S2) and observed a significant decrease in signal with Aβ40 aggregates (Fig. S3H). As expected, no significant decrease was observed for Aβ420 (Fig. S3J), indicating the absence of any significant amount of fibrillar Aβs in the tested Aβ420 solution. In addition, thioflavin-T testing, which is sensitive for insoluble fibrillar Aβs but not soluble oligomers, confirmed that the majority of the tested Aβ420 are nonfibrillar Aβs (Fig. S3J). This result was validated further by dot blotting with OC antibody (Fig. S3K).

Studies showed that pyroglutamate Aβ [AβN3(pE)] contributed substantially to AD pathology because of its high neurotoxicity and abundance in AD brains (S3, S4). To investigate whether CRANAD-3 can interact with AβN3(pE), we performed fluorescence spectral testing similar to experiments with other Aβs. We found that the fluorescence intensity of CRANAD-3 increased significantly upon mixing with AβN3(pE) (Fig. S4I), indicating that CRANAD-3 is sensitive for AβN3(pE). In addition, CRANAD-3 displayed a significant increase in intensity and wavelength shift upon interaction with insoluble Aβ40 aggregates (Fig. 2A), suggesting that CRANAD-3 can detect both insoluble and soluble Aβs.

To investigate the specificity of CRANAD-3 for Aβ species, amylin [an aggregation-prone 37-residue peptide secreted together with insulin by pancreatic B cells (S5)] was incubated with CRANAD-3. No significant fluorescence property changes were observed (Fig. 2A), suggesting CRANAD-3’s excellent selectivity toward Aβ species. We also tested the interaction of CRANAD-3 with other amyloidogenic proteins, such as Tau 441 and α-synuclein, and observed no significant increase in fluorescence (Fig. S4B). In addition, we investigated the specificity of CRANAD-3 toward rat Aβ (the differences from human Aβ are R35, Y10F, and H13R) and mutated (H13R) Aβ and found a significant decrease in fluorescence intensity (Fig. 2A), suggesting that these probes are highly specific toward human Aβs.

We also investigated whether CRANAD-3 could interact with the core fragment KLVFF (Aβ16–20) of Aβs. In vitro tests showed that the fluorescence intensities increased with the concentrations of KLVFF fragment (Fig. 2B). This result was consistent with our 1H NMR data, in which apparent changes in the chemical shift of amide protons were observed when CRANAD-3 was incubated with the fragment (Fig. 2C). Therefore, both fluorescence and NMR data suggested that CRANAD-3 interacts with the core fragment. In addition, no significant change in fluorescence property was observed when CRANAD-3 was mixed with a noncore fragment, Aβ22–35 (Fig. 2B). We also investigated this interaction was sequence dependent. In this regard, we tested the changes in the fluorescence intensity of CRANAD-3 with KLVLL, in which two phenylalanines were replaced by hydrophobic leucine. No significant changes in intensity were found (Fig. S4C), indicating that the interaction is sequence dependent and that phenylalanine residues are critical for the binding. Although we believe that CRANAD-3 interactions with Aβ species are sequence specific, conformational dependency also is possible. However, this hypothesis needs a separate investigation, which would be outside of the scope of the present study.

### Two-Photon Imaging of Aβ Deposits with CRANAD-3

As a brain-imaging probe, CRANAD-3 must meet several requirements that include proper lipophilicity (log P) and reasonable penetration of the brain–blood barrier (BBB) (S7). We found that log P for CRANAD-3 was 2.50, which is reasonable for brain imaging. To investigate whether CRANAD-3 is capable of penetrating the BBB, we used two-photon imaging to test its accumulation in the brain. Two-photon imaging is a microscopic technology that has been used widely for visualizing Aβ plaques in live mice (S6, S7). As expected, two-photon images from transgenic AD mice indicated that CRANAD-3 is able to label Aβ plaques and cerebral amyloid angiopathies (CAAs, i.e., Aβ deposits at the exterior of brain blood vessels (S8, S9)) (Fig. 3 A–C), suggesting that this probe can cross the BBB and specifically label Aβ species in vivo. In addition, the probe accumulated gradually in the brain and reached its peak around 10 min after injection, followed by a slow washout (Fig. 3D). Ex vivo histology confirmed that CRANAD-3 clearly labeled Aβ plaques in brain sections, further indicating its ability penetrate the BBB (Fig. 3 E–H and Fig. S5) and its specificity for Aβ species. No apparent plaques were observed in the age-matched wild-type mice by two-photon imaging and ex vivo histology (Fig. 3C and Fig. S5).

#### In Vivo NIRF Imaging of APP/PS1 Transgenic Mice with CRANAD-3

APP/PS1 mice, the most studied transgenic AD mouse model (S24, S56), were used to test the utility of CRANAD-3 for in vivo NIRF imaging. This mouse model possesses double humanized APP/PS1 genes and constantly produces considerable amounts of “human” Aβs. Previous studies indicate that APP/PS1 mice have no significant Aβ deposits or any noticeable behavioral abnormalities before 6 mo of age. It is believed that the majority of Aβ species in mice younger than 6 mo are soluble (S24, S56). To test whether CRANAD-3 was able to detect soluble species in vivo by NIRF imaging, we used 4-mo-old APP/PS1 mice. We found that after i.v. injection the fluorescence signal from the brain of APP/PS1 mice was significantly higher than that from the age-matched wild-type mice at all time points used (Fig. 4). The signals from APP/PS1 mice were 2.29-, 2.04-, 1.98-, 1.60-, and 1.54-fold higher than the signals from wild-type controls at 5, 10, 30, 60, and 120 min after injection (Fig. 4),
suggesting that CRANAD-3 indeed is capable of detecting soluble Aβ species in vivo.

**Proof-of-Concept Application of CRANAD-3 for Therapy Monitoring.**

Our two-photon microscopic imaging and NIRF imaging indicated that CRANAD-3 is able to penetrate the BBB and is specific toward Aβ species. These results encouraged us to investigate CRANAD-3’s capacity for monitoring therapy. To this end, we used two experimental drugs to conduct proof-of-concept experiments.

**Monitoring the rapid Aβ-lowering effect of inhibiting beta-amyloid cleaving enzyme-1.** Developing beta-amyloid cleaving enzyme-1 (BACE-1) inhibitors is one of the current mainstream approaches for AD drug discovery, and several drug candidates have advanced into clinical trials. Although none of the BACE-1 inhibitors is presently approved by the FDA for clinical use, several inhibitors show consistent efficacy in lowering soluble Aβ species in transgenic mice in a short period of treatment. Because our imaging probe can detect soluble Aβs, we conducted NIRF imaging to investigate whether CRANAD-3 can monitor the rapid reduction of soluble Aβs. We first tested the capacity of CRANAD-3 with the well-characterized BACE-1 inhibitor LY2811376, which could lead to a 60% decrease in the soluble Aβs in mouse cortex after a single oral dose (30 mg/kg) (60). As expected, the fluorescence signal of CRANAD-3 from APP/PS1 mice (n = 4) after LY2811376 treatment was 33% lower than the signal from the same mice before treatment (Fig. 5A and B). The difference detected by our near-infrared (NIR) imaging was smaller than that reported from ELISA with brain extracts (33% vs. 60%), very likely because an Aβ species in transgenic mice in a short period of treatment. Because our imaging probe can detect soluble Aβs, we conducted NIRF imaging to investigate whether CRANAD-3 can monitor the rapid reduction of soluble Aβs. We first tested the capacity of CRANAD-3 with the well-characterized BACE-1 inhibitor LY2811376, which could lead to a 60% decrease in the soluble Aβs in mouse cortex after a single oral dose (30 mg/kg) (60). As expected, the fluorescence signal of CRANAD-3 from APP/PS1 mice (n = 4) after LY2811376 treatment was 33% lower than the signal from the same mice before treatment (Fig. 5A and B). The difference detected by our near-infrared (NIR) imaging was smaller than that reported from ELISA with brain extracts (33% vs. 60%), very likely because an Aβ species in transgenic mice in a short period of treatment. Because our imaging probe can detect soluble Aβs, we conducted NIRF imaging to investigate whether CRANAD-3 can monitor the rapid reduction of soluble Aβ species in vivo.

**Discussion**

Fluorescence molecular imaging technologies have advanced very rapidly in the past decade, particularly for peripheral diseases such as cancer and cardiovascular disease. However, its development and applications for CNS diseases has progressed sluggishly. PET imaging has been used widely for CNS diseases; however, its applications in preclinical studies have been limited because of the use of radioisotopes, the short lifetime of the imaging probes, its high cost, and facility requirements. Fluorescence molecular imaging is appealing for small animal studies because of its low cost, easy operation, and stable imaging probes. In the past decade, the development of fluorescent imaging probes for AD has been actively pursued, and several probes showed capacity for imaging Aβs (37–45) and Tau tangles (64) in mice. Nonetheless, none has been used to monitor the effectiveness of drug therapy.

Recent evidence shows that soluble Aβs, such as dimers and oligomers, are more neurotoxic than insoluble deposits and thus lead to a reduction of copper-induced Aβ cross-linking in vitro (46). We performed a preliminary in vivo therapeutic treatment of 4-mo-old APP/PS1 mice with CRANAD-17 for 6 mo. We treated mice (n = 5) with an i.p. injection of CRANAD-17 (2 mg/kg) twice a week, and the control APP/PS1 group (n = 5) was injected with the same volume of saline. After a 6-mo treatment, NIRF imaging with CRANAD-3 indicated that the CRANAD-17-treated group showed significantly lower NIRF signals (25%) than the nontreated group (Fig. 5C and D). This result was consistent with the ELISA analysis showing a 36% reduction in Aβ40 in the brain extracts (Fig. 5E). Our ELISA results were confirmed further by Mesoscale Discovery (MDS) analysis (Fig. S6D) (61–63). We also observed a 25% decrease in Aβ40 in the CRANAD-17–treated group by MDS analysis, although the difference between the two groups did not reach statistical significance (Fig. S6B). In addition, our NIR imaging result was consistent with plaque counting in brain slices stained with thioflavin-S, which showed a 56% reduction with CRANAD-17 treatment (Fig. 5F and G). Interestingly, a comparison of the brain slices from the two groups showed that the number of dimly fluorescent and diffused plaques apparently was lower in the treated group than in the control group (Fig. 5G and Fig. S7), probably because of the anti-cross-linking capability of CRANAD-17.

Our data from both short and chronic treatment studies strongly suggest that CRANAD-3 could be used to monitor the therapeutic effectiveness of drug treatment. Further protocol optimization is ongoing in Run’s group.
potentially could serve as biomarkers for presymptomatic stages of AD (2, 21). In the past decades, drug-development strategies using insoluble Aβ deposits as biomarkers for evaluation of AD have failed; this failure has led to a shift in research toward the targeting of soluble Aβs and to more clinical trials being conducted at the early/presymptomatic stage of AD (14). Imaging probes capable of detecting soluble Aβs are needed both for clinical trials and for preclinical evaluation.

Although both PET and NIFR probes for imaging Aβs are available, they have a fundamental limitation: they primarily detect insoluble Aβ, not the more toxic soluble Aβs. In our previous studies, we designed and tested the NIFR probe CRANAD-58, a curcumin analog, for detecting both soluble and insoluble Aβs (46). However, its sensitivity for in vivo imaging of Aβs is lower than that of CRANAD-3 (the ratios of NIFR signal $F_{\text{APP/PS1}}$/$F_{\text{Akt}}$ are 2.29 vs. 1.71, 2.04 vs. 1.61, and 1.98 vs. 1.82 at 5-, 10- and 30-min, respectively, after the probe injection) (46). Therefore, we decided to use CRANAD-3 to monitor therapeutic effectiveness in the proof-of-concept studies in this report.

The most remarkable advantage of CRANAD-3, compared with other NIFR imaging probes for Aβs, is its ability to detect both soluble and insoluble Aβs, making CRANAD-3 suitable for monitoring the rapid Aβ-lowering effect of BACE-1 inhibitors and other fast-acting drug candidates [i.e., hexareton (63, 65-69) and citadolpram (70)]. This ability is very important, because our imaging method would enable a quick evaluation of the efficacy of some categories of drug candidates, such as BACE-1 inhibitors, gamma-secretase inhibitors/modulators, and others (62, 71).

Our data indicate that CRANAD-3 is suitable for monitoring short-term and chronic treatments. However, more rigorous optimization is needed to make the protocol easily reproducible in other laboratories. Our optimization will focus on formulation, imaging procedure, and data analysis.

Two-photon microscopic imaging has been an important tool for studying amyloidosis of AD (57, 72-74). However, most of the fluorescent probes have short emission, which can limit imaging depth significantly. Although we did not intend to investigate the maximal imaging depth of CRANAD-3 in this report, we believe its NIR emission has the potential to enable deep two-photon imaging of Aβ plaques and CAs.

Available transgenic AD models have different pathological features, with APP23, Tg2576, PSAPP (APPPS1M146L), and APP/PS1 mice being the models most often used (75, 76). Snellman et al. (77) recently reported that different mouse models have different retention capability for 11C-PiB. Among the tested AD models, only APP23 mice showed substantial 11C-PiB binding at the age of 18 mo. This result is consistent with data reported by Maeda et al. (78) and Mori et al. (79). Unlike previous PET imaging studies with PiB (30-32), these studies suggested that 11C-PiB with highly specific radioactivity could be used for mouse imaging, particularly for APP23 mice. Both studies attributed the differences to pyrogglutamate Aβ, which contributes significantly to the Aβ deposits in old APP23 mice and probably plays an important role in the plaque binding with PiB (77-79). Our fluorescence data indicate that CRANAD-3 is responsive to pyrogglutamate Aβs, suggesting that it could be used as an imaging probe with other AD models such as APP23 mice.

In this report, we demonstrated that CRANAD-3 is capable of detecting soluble Aβ both in vitro and in vivo. We believe that CRANAD-3 may be a potential probe for monitoring β-amyloid species at an early/presymptomatic stage of AD. However, the present limitations of fluorescence imaging in human subjects are clearly major obstacles to the clinical use of CRANAD-3. Recently, at least two emerging technologies have shown considerable promise for overcoming the practical difficulties of using CRANAD-3 in humans. First, rapid advances in fluorescence molecular tomography indicate that suitable scanners with penetrating capabilities up to 10 cm may be readily available soon (80, 81). Second, it is likely that the incorporation of isotopes into CRANAD-3 molecules will facilitate the use of the probe for PET imaging, which is a widely used clinical modality. These studies are currently under way in the C.R. laboratory.

Materials and Methods

Reagents used for the synthesis of CRANAD-3 were purchased from Aldrich and were used without further purification. All animal experiments were performed in compliance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee at the Massachusetts General Hospital.

Full descriptions of methods used are available in SI Materials and Methods.

Testing of CRANAD-3 with APP/PS1 Mice, APP23 Mice, and APP/PS1 Aggregates. To test interactions of CRANAD-3 with Aβ species, we used the following three-step procedure. In step 1, 1.0 mL of double-distilled water was added to a quartz cuvette as a blank control, and its fluorescence was recorded with the same parameters as for CRANAD-3. In step 2, fluorescence of a CRANAD-3 solution (1.0 mL, 250 nM) was recorded with excitation at 580 nm and emission from 610–900 nm. In step 3, to the above CRANAD-3 solution, 10 µL of Aβ species [25-µM stock solution in 30% (vol/vol) trifluoroethanol or hexfluoroisopropanol (HFIP) for monomers and dimers and 25-µM stock solution in PBS buffer for APP40 aggregates] was added to make the final Aβ concentration 250 nM. Fluorescence readings from this solution were recorded as described in step 2. A blank control from step 1 was used to correct the final spectra from steps 2 and 3.

In Vivo NIFR Imaging. The IVIS Spectrum animal imaging system (PerkinElmer) was used to perform in vivo NIFR imaging. Images were acquired with a 605-nm excitation filter and a 680-nm emission filter. Living Image 4.2 software (PerkinElmer) was used for data analysis.

Mice (n = 3 or 4 female wild-type control mice) were shaved before background imaging and were i.v. injected with freshly prepared CRANAD-3 [0.5 mg/kg, 15% (vol/vol) DMSO, 15% (vol/vol) cremophor, and 70% (vol/vol) PBS]. Fluorescence signals from the brain were recorded before and 5, 10, 30, 60, 120, and 180 min after i.v. injection of the probe. To evaluate our imaging results, a region of interest (ROI) was drawn around the brain region. Student’s t-test was used to calculate P values.

ACKNOWLEDGMENTS. We thank Pamela Pantazopoulos for proofreading the manuscript. This work was supported by Award K25AG03670 from NIH (to C.R.).