

Development of Novel Amyloid Imaging Agents Based Upon Thioflavin S

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Abstract: To date, small-molecule amyloid-imaging agents for *in vivo* detection and quantitation of amyloid deposits in Alzheimer's disease (AD) have been developed and successfully applied to human subjects. Preliminary studies have indicated that these amyloid-imaging agents were accumulated in the AD brains in a pattern that is relatively consistent with AD pathology, at least in the regions of amyloid-rich grey matter. These studies have also proven the concept that amyloid dyes, normally too hydrophilic to enter the brain, can be chemically modified to enhance brain permeability, binding affinity, as well as improve binding specificity for amyloid deposits. Related studies have suggested that structurally different agents can be developed that bind to different sites on amyloid deposits. In fact, *in vivo* cross-referencing studies based upon different amyloid-imaging agents may permit better characterization of AD pathology. But more importantly, novel amyloid imaging agents are required that will allow direct correlation between the results of animal models and human subjects based upon identical imaging modalities. Thus far, amyloid stains such as Congo red and thioflavin T have been extensively studied. However, another widely used amyloid dye, thioflavin S, has not been previously explored. This is in part due to the fact that thioflavin S exists as a mixture, not a pure chemical entity, albeit that the major component has been characterized. We hypothesized that neutral analogs, based upon the major component, could be developed as novel amyloid imaging agents, that exhibit complementary binding properties and pharmacokinetic profiles compatible with potential human studies.

Keywords: Alzheimer's disease, amyloid, Congo red, thioflavin T, thioflavin S.

INTRODUCTION

Amyloid imaging has become an urgent task in the battle against Alzheimer's disease (AD) [1-3]. It is focused on direct detection and quantitation of amyloid deposits in living patients afflicted by AD [4]. Despite the fact that this chronic and progressively neurodegenerative disorder currently affects over 4.5 million Americans costing the nation nearly 100 billion dollars each year, definitive diagnosis of AD still requires postmortem examination of the brain through histological staining of amyloid plaques and neurofibrillary tangles, which are two hallmarks of AD pathology [5]. Although clinical diagnosis has been significantly improved in accuracy, it still cannot satisfy the needs for early diagnosis [6]. This is because pathological changes in the brain can begin long before the onset of symptoms characteristic of AD occur. Even though AD occurs mainly in people over 60 and represents the most common form of dementia, pathological changes underlying the disease process could begin decades before the onset of symptoms [7]. Presymptomatic screening on a large scale will eventually become an imperative task in terms of disease prevention. Currently, there is still no cure for this devastating mental disease [8].

Tremendous efforts are being made to develop therapies, which are aimed at preventing, halting, or reversing the formation of the amyloid deposits in the brain [9, 10]. Thus, *in vivo* amyloid imaging could function as an important marker to facilitate the efficacious evaluation of therapeutic treatments that are currently under development. In addition, amyloid imaging can also serve as an indispensable tool for the testing of the amyloid cascade hypothesis [11].

To date, amyloid imaging has been explored based upon several imaging modalities currently available, including magnetic resonance imaging (MRI) [12], positron emission tomography (PET) [2, 3], single photon emission computed tomography (SPECT) [13], and multiphoton microscopy [14]. Each modality has its unique features that function complementarily to each other. MRI has high spatial resolution suitable for detection of individual plaques. However, its application in human subjects is limited by the prohibitively long scan times. PET or SPECT, on the other hand, are the *in vivo* imaging modalities that are suitable for the detection of amyloid plaques in near real time. However, the spatial resolution of PET and SPECT is relatively low and individual plaques cannot be detected or observed. Instead, detection and quantification of amyloid deposition can be achieved based upon radioactivity concentration in certain regions. The resolution of multiphoton microscopy is also compatible with the average size of individual plaques. However, its *in vivo* application is limited only to studies in

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animal models due to technical restrictions. Regardless of the pros and cons, application of these imaging modalities is considerably dependent upon the development of suitable molecular probes that can readily penetrate the blood brain barrier and selectively bind to amyloid deposits in the brain.

Over the past decade, significant progress has been made in the development of amyloid-imaging agents for different imaging modalities, particularly for PET imaging [15]. Both relatively large biomolecules (antibodies and peptides) and small-molecular agents have been extensively studied as *in vivo* amyloid-imaging agents [3]. For the purpose of brain uptake, small-molecule probes appear particularly promising as they can passively perfuse across the blood-brain barrier. In addition, their structures can be readily chemically modified for structure-affinity relationship (SAR) studies, which can be used to identify lead compounds that exhibit optimal binding properties and pharmacokinetic profiles.

AMYLOID IMAGING AGENTS BASED UPON CONGO RED

One general approach for the development of small-molecule amyloid-imaging agents is to chemically modify in a systematic fashion the structures of histological amyloid dyes and to develop effective neutral and lipophilic analogs. To date, several research groups have already investigated a wide array of amyloid-binding agents based upon Congo red (Fig. 1). Congo red is a widely used amyloid dye for histological staining of postmortem AD brain tissue sections. Its *in vivo* application is limited by the negative charges caused by the sulphonate groups, which render it too hydrophilic to enter the brain. In order to enhance brain permeability, neutral analogs of Congo red have been developed. Elimination of the negatively charged sulfonate groups and subsequent structural modifications have led to the development of many useful amyloid-imaging agents [16]. Because the brain permeability of this type of amyloid-imaging agents is adequate in rodents, their *in vivo* binding properties have been comprehensively studied in transgenic mouse models of amyloid deposition [17-19]. Some of them were found to readily enter the mouse brain and selectively bind to amyloid deposits *in vivo*. However, clinical application of this type of compounds is still hampered due to the sub-optimal brain

permeability determined in non-human primates [3]. Despite efforts made to improve the brain permeability, the brain uptake is still unable to reach a level acceptable for future human studies. In a move to further increase the flexibility of structural modification, Kung and coworkers [20] developed a series of stilbene analogs, the semi-analogs of Congo red derivative. These stilbene derivatives were found to readily enter the brain and so potentially bind to amyloid deposits. Among these stilbene derivatives, ^{11}C -SB-13 has been evaluated in human AD and control subjects [21]. As shown in (Fig. 2), ^{11}C -SB-13 displayed a good BBB permeability. In addition, ^{11}C -SB-13 showed increased retention in frontal and posterior temporal-inferior parietal association cortices in AD in comparison to the control subjects. These studies indicated that ^{11}C -SB-13 could be used to differentiate AD from healthy control.

AMYLOID IMAGING BASED UPON THIOFLAVIN T

Thioflavin-T (ThT) is another fluorescent dye which has been infrequently used as a histological stain for amyloid [22] (Fig. 1). ThT is a positively charged 2-aryl benzothiazole derivative. Elimination of the positive charge on the benzothiazole ring led to the development of even smaller and more lipophilic derivatives. Compared to neutral Congo red derivatives, uncharged thioflavin T derivatives are found to be more brain permeable [23]. In fact, the brain permeability of uncharged ThT derivatives was found to be well above the level acceptable for human studies. This has led to systematic SAR studies directed toward identifying lead compounds with optimal *in vitro* and *in vivo* properties [24].

Most uncharged ThT derivatives bind to amyloid deposits with affinities greater than that of ThT itself. The exact binding mechanism of ThT and its neutral analogs still remains to be elucidated at the molecular level. Various studies have demonstrated that they bind to other sites of amyloid deposits and do not share the same binding sites as Congo red derivatives [25]. Comprehensive SAR studies have led to the identification of an ^{11}C -labeled amyloid-imaging agent, termed PIB, that has been successfully applied in AD subjects [26] (Fig. 3). Preliminary results indicate that PIB enters the human brain very well. The initial distribution appeared to be proportional to blood flow patterns as expected.

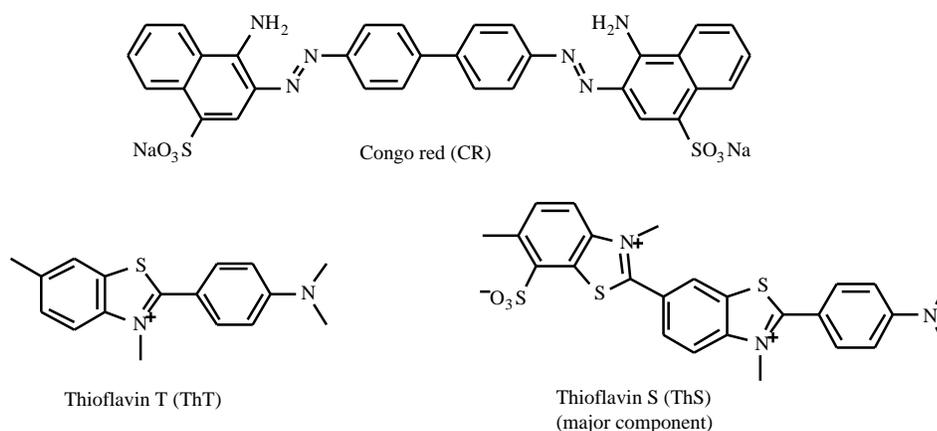


Fig. (1). Structures of Congo red, thioflavin T, and a major component of thioflavin S.

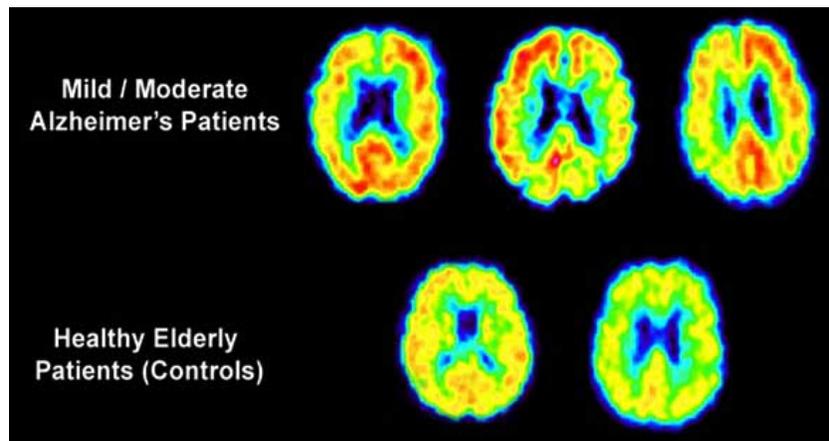


Fig. (2). Parametric images of standardized uptake values obtained by normalizing tissue concentration (nCi/mL) by injected dose per body mass (nCi/g) of positron emission tomography (PET) images summed over 40 to 120 min after injection of 10 mCi of [^{11}C]SB-13. Data are shown for representative Alzheimer's disease patients and comparison subjects (courtesy of Verhoeff NP).

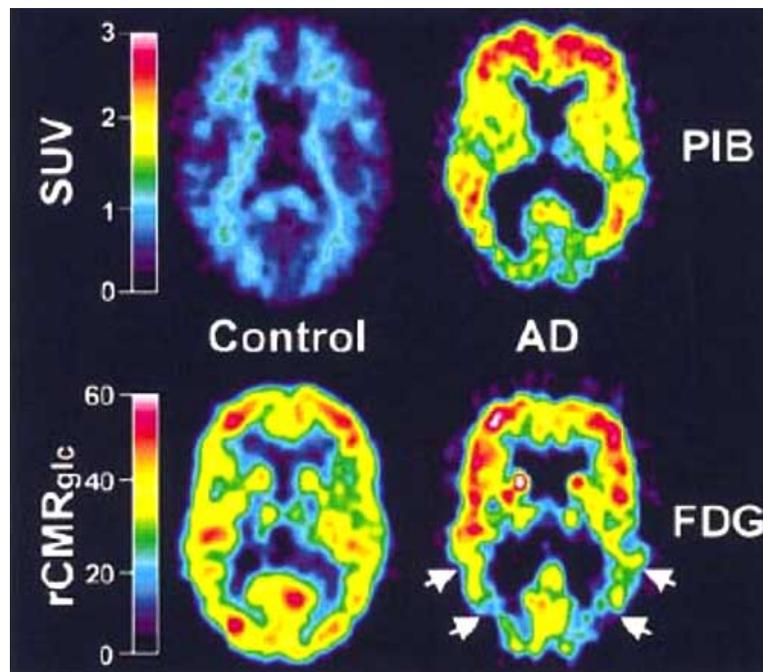


Fig. (3). PIB standardized uptake value (SUV) images demonstrate a marked difference between PIB retention in Alzheimer's disease (AD) patients and healthy control (HC) subjects. PET images of a 67-year-old HC subject (left) and a 79-year-old AD patient (AD6; MMSE= 21; right). (top) SUV PIB images summed over 40 to 60 minutes; (bottom) ^{18}F FDG rCMRglc images (mol/min/100ml). The left column shows lack of PIB retention in the entire gray matter of the HC subject (top left) and normal ^{18}F FDG uptake (bottom left). Nonspecific PIB retention is seen in the white matter (top left). The right column shows high PIB retention in the frontal and temporoparietal cortices of the AD patient (top right) and a typical pattern of ^{18}F FDG hypometabolism present in the temporoparietal cortex (arrows; bottom right) along with preserved metabolic rate in the frontal cortex. PIB and ^{18}F FDG scans were obtained within 3 days of each other. Reproduced with permission from [26].

The time-course of radioactivity uptake was determined in 9 control subjects and 15 AD patients (Fig. 4). As a group, the healthy control subjects showed rapid entry and clearance of PIB in all cortical and sub-cortical gray matter areas, including cerebellar cortex. The uptake and clearance of PIB in the cerebellum were nearly identical in the control and AD subjects. A relatively lower entry and slower clearance were observed in the white matter, but PIB retention was very similar in both groups. In contrast, PIB retention in AD subjects was significantly different from that of the control sub-

jects in areas of the brain known to contain large amounts of amyloid deposits in AD such as parietal and frontal cortices. The AD patients showed a marked retention of PIB compared with control subjects, indicating a quantifiable discrimination between most mild and AD patients and control subjects. In control subjects, there was very little retention of PIB in cortical regions. In AD subjects, the absolute amount of PIB retained in the frontal cortex was over 90% higher than that retained in control frontal cortex or cerebellum of either controls or AD patients.

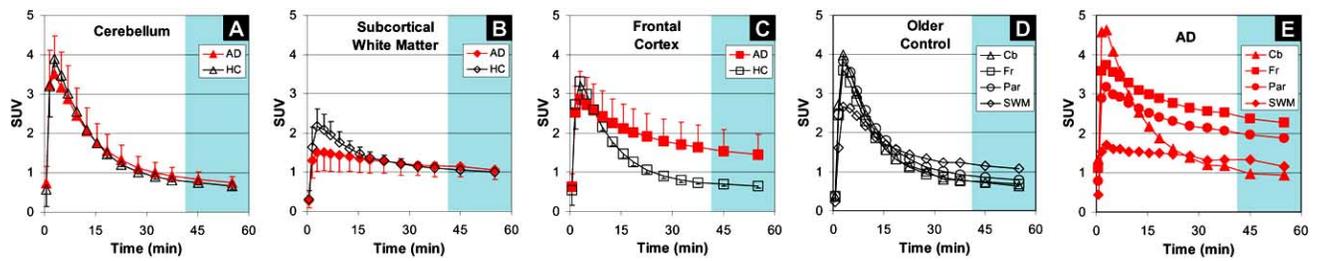


Fig. (4). PIB is differentially retained only in amyloid-laden cortical areas of AD brain. Standardized uptake values (SUVs) demonstrating brain entry and clearance of PIB. A to C represent averaged SUV values for all healthy control subjects (open, black symbols; $n = 9$) and all AD patients (filled, red symbols; $n = 15$) in cerebellum, subcortical white matter, and frontal cortex. D and E show brain entry and clearance in cerebellum (triangles), subcortical white matter (diamonds), frontal cortex (squares), and parietal cortex (circles) for an older control subject (D) and an AD patient (E). Error bars represent one standard deviation (SD) and are too small to be seen in some of the HC subject data in A to C. Asterisks indicate a significant difference between AD and HC values ($p < 0.006$) (reproduced with permission from [26]).

AMYLOID IMAGING AGENTS BASED UPON FDDNP

In addition, a lipophilic ^{18}F -radiolabeled tracer for PET imaging of plaques, NFT's, and diffuse amyloid in the brains of AD patients has been developed [27]. This tracer is a fluorinated derivative of a non-specific cellular membrane dye, 1,1-dicyano-2-[6-(dimethylamino)naphthalen-2-yl] propene (DDNP) (Fig. 5). Compared to C-11 ($T_{1/2}$ 20 min), F-18 has a half-life of 110 min, which allows regional distribution to medical facilities without on-site cyclotron. This agent,

termed FDDNP, is also capable of crossing the BBB and entering brain tissue. The nature and degree of its specific binding to $\text{A}\beta$ -amyloid and NFTs has been characterized in human subjects. The preliminary imaging evidence appears to indicate a brain retention pattern of ^{18}F -FDDNP that could correlate with clinical diagnostic scores both in patients with AD and controls [28]. While the ^{18}F -FDDNP technique has been rapidly applied to studies in humans, no *in vivo* or *ex vivo* studies have been reported validating this technique in transgenic mice with $\text{A}\beta$ deposits.

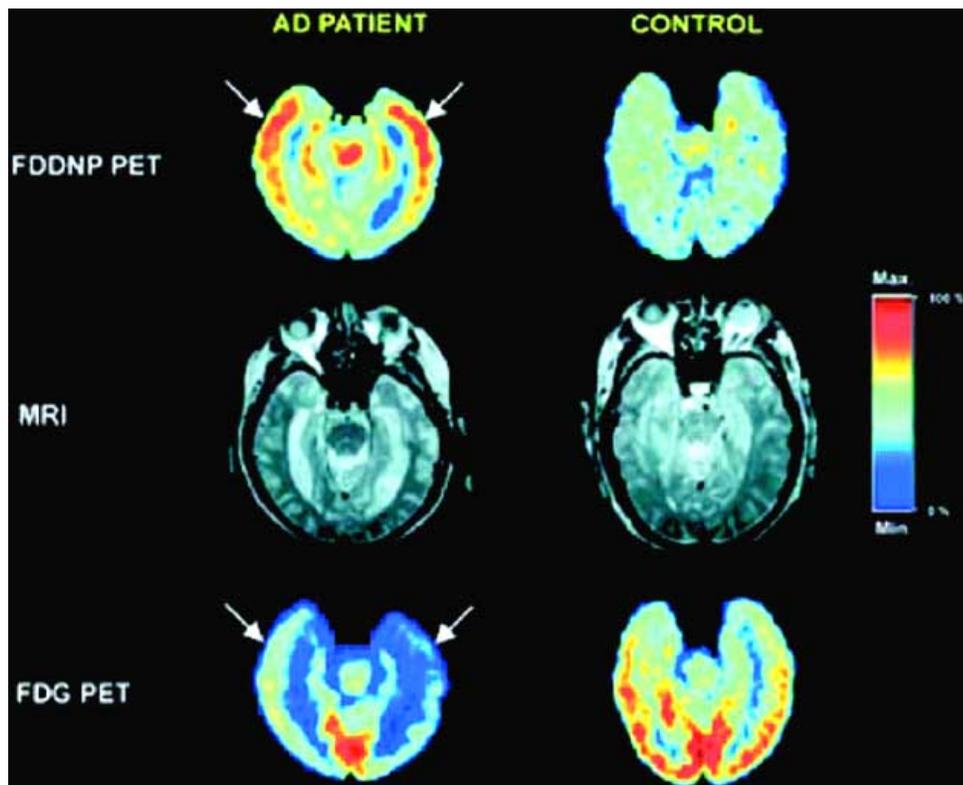


Fig. (5). ^{18}F -FDDNP-PET, MRI, and FDG-PET images of a patient with AD and a normal subject. The ^{18}F -FDDNP and FDG images of each stage are co-registered to their respective MR images. Areas of FDG hypometabolism are matched with the localization of neurofibrillary tangles and APs resulting from ^{18}F -FDDNP binding (arrows). The ^{18}F -FDDNP images represent activity 25-54 min after ^{18}F -FDDNP administration. The FDG images represent activity 20-60 min after FDG injection (reproduced with permission from [28]).

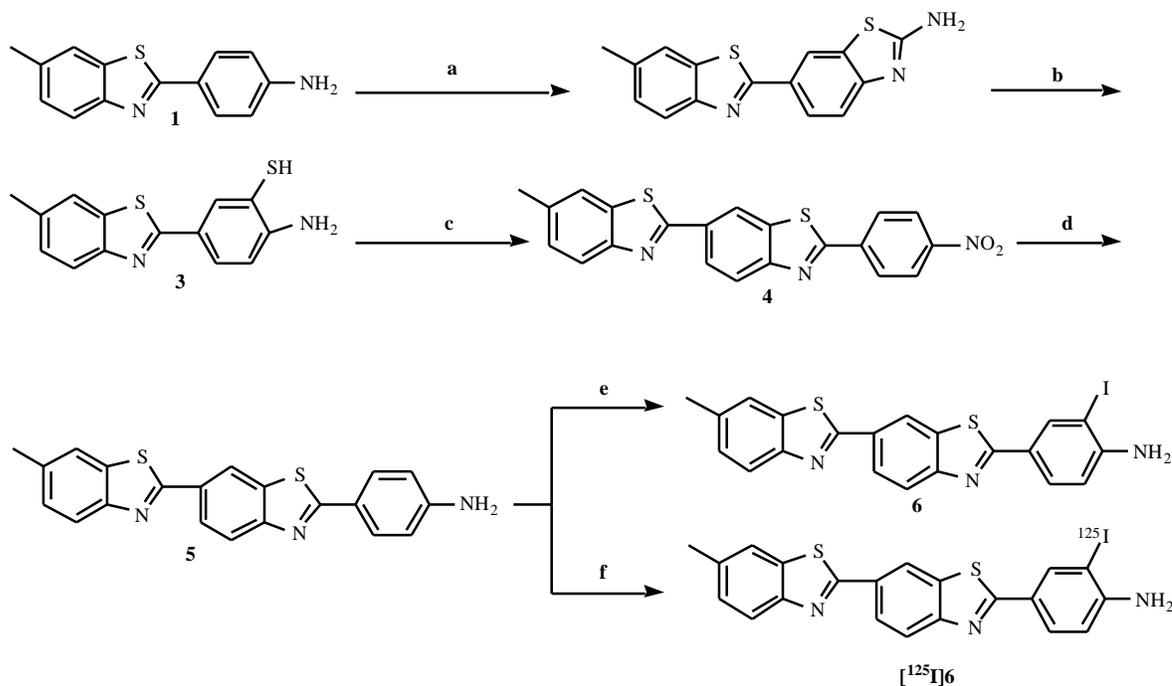
NEEDS FOR NOVEL AMYLOID IMAGING AGENTS

In spite of the progress made so far toward amyloid imaging, many challenges still remain. One of these challenges relates to develop amyloid-imaging agents that permit direct correlation of studies between animal models and human subjects. For the discovery and development of new anti-amyloid therapies, transgenic mice have been created bearing ample amyloid deposits in the brain that mimics the neuropathology of AD [29]. In the meantime, molecular imaging techniques such as microPET and microSPECT have also experienced considerable advancement. This, in principle, makes it possible to detect and quantitate the level of amyloid deposition in the mouse brain for *in vivo* screening of drug candidates. To date, however, no series of amyloid-binding agents have been developed in a CNS model of amyloid deposition using the same tomographic technology that will ultimately be applied to human studies. The amyloid-imaging agents that have been developed for human subjects were found to be inappropriate for use in microPET studies in the mouse models of amyloid deposition. Distinct amyloid-imaging agents have to be developed separately for transgenic mice in order to validate the anti-amyloid effects of therapeutic drug candidates with microPET. *In vivo* microPET studies in transgenic mice would allow accurate prediction of the outcomes in future clinical trials based upon analogous PET studies. Amyloid-imaging agents that directly allow quantitation of the level of amyloid deposits in the brain of living transgenic mice would serve as powerful tools in the discovery and development of anti-amyloid therapies.

SEARCH FOR NOVEL AMYLOID IMAGING AGENTS BASED UPON THIOFLAVIN S

To date, the general approach of chemical structural modification has not yet been applied to thioflavin S, another widely used amyloid dye. This is due to the fact that thioflavin S exists not as a single molecule, but rather as a mixture of at least 6 components. The structure of a major component of thioflavin S has been elucidated (Fig. 1). Despite the fact that the major component in thioflavin S is also a benzothiazole derivative similar to thioflavin T, preliminary studies indicated that thioflavin S does not share the same binding sites with thioflavin T in amyloid deposits. Instead, it competes with Congo red derivatives in binding to amyloid deposits.

Recently, we have set out to develop novel amyloid-imaging agents based upon thioflavin S. We hypothesized that novel amyloid imaging agents can be developed based upon the major component of ThS. For structural design processes, the major component in thioflavin S is used as the prototypical structure and is shown in (Fig. 1). Thus, uncharged, lipophilic analogs of thioflavin S were synthesized and studied for potential amyloid imaging. The synthesis of the ThS-based amyloid imaging is shown in Scheme 1. The brain entry and clearance of the compound were evaluated in normal control mice following I-125 radiolabeling. Following i.v. injection, the brain radioactivity concentration was determined at 2 min, 30 min, and 60 min. As expected, the ¹²⁵I-labelled compound displayed a rapid brain entry at early time points with $1.8\% \pm 0.30\text{ID/g}$ at 2 min. The brain radioactivity concentration decreased sharply to $0.36 \pm 0.05\% \text{ID/g}$



Scheme 1. Synthesis of novel amyloid-imaging agents based upon a major component of thioflavin S. a) KSCN, Br₂, DMF, 61%; b) KOH, H₂O, 64%; c) p-NO₂-PhCOCl, PhCl, quant.; d) SnCl₂, conc. HCl, 49%; e) ICl, CH₃COOH, 53%; f) chloramine-T, CH₃COOH, Na¹²⁵I.

at 30 min and $0.37 \pm 0.07\%$ ID/g at 60 min, with a 2-to-30 min ratio of 5. This indicated good clearance of the compound from the normal mouse brain in the absence of amyloid deposits. Further evaluation of these types of compounds for *in vitro* binding properties and *in vivo* pharmacokinetic profiles is currently in process.

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