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Development of Positron Emission Tomography β-Amyloid Plaque Imaging Agents

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For 100 years, β-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs) have been recognized as the neuropathological hallmarks of Alzheimer's disease (AD), and their presence or absence could only be assessed postmortem using stains and dyes that identified these microscopic structures. Approximately 10 years ago, the first successful Aβ plaque–specific positron emission tomography (PET) imaging study was conducted in a living human subject clinically diagnosed with probable AD using the ^{11}C-labeled radiopharmaceutical Pittsburgh Compound B (PiB). Laboratory studies and preclinical evaluations to design PiB began a decade earlier than the first human PiB PET study and involved chemical modifications of different well-known dyes that bound specifically to the extended β-pleated sheets that comprise the fibrils of amyloid proteins such as Aβ plaques, NFTs, α-synuclein deposits, and prions. These preclinical studies were conducted in our laboratories at the University of Pittsburgh, starting with Congo red derivatives, followed by Chrysamine G derivatives, followed by X-series compounds, and finally with neutral derivatives of thioflavin-T. The in vitro and in vivo evaluations of the different derivatives as candidate PET radioligands for imaging Aβ plaques and neurofibrillary tangles in human brain are described in this review, along with the specific evaluation criteria by which the candidate radioligands were judged. Out of these studies came PiB, a PET radioligand that binds selectively and with high affinity to only fibrillar forms of Aβ. PiB has been used in many different human research protocols throughout the world and has demonstrated the usefulness of assessing the Aβ plaque status of subjects many years before the clinical diagnosis of probable AD. Recently, longer-lived ^{18}F-radiolabeled Aβ-selective radiopharmaceuticals have been developed. It is likely that the full clinical impact of these imaging agents will be realized by identifying presymptomatic subjects who would benefit from early drug treatments with future disease-modifying AD therapeutics.
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Early Efforts With Congo Red Derivatives

For many years, we struggled with manipulating the Congo red (CR) pharmacophore (Fig. 1) into a suitable positron emission tomography (PET) radiotracer to image β-amyloid (Aβ) plaques and hyperphosphorylated tau deposits in the form of neurofibrillary tangles (NFTs) in the brains of Alzheimer's disease (AD) subjects before death.[1-3] CR binds well to both of these aggregated proteins, which have been recognized as the postmortem hallmarks of AD from the time of Alzheimer himself.[4] Our efforts with CR fell short, primarily as a result of the poor brain entry of this class of compounds. Low brain uptake of CR after intravenous (iv) injection in animal studies likely resulted from the low lipophilicity of CR, which derived from its negative (−2) charge at physiological pH. In an effort to increase brain uptake, we examined radiolabeled derivatives of Chrysamine G (CG) (Fig. 1). CG bound with high affinity to both Aβ plaques and NFTs,[5] and CG had a higher lipophilicity than CR as measured by octanol/water partition coefficient (P) or octanol/buffered (pH 7.4) water distribution coefficient (D). Compounds

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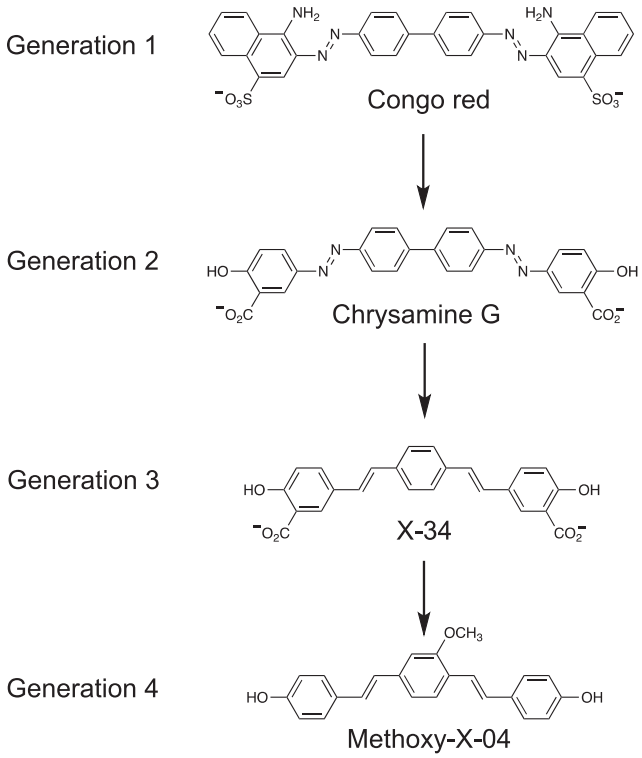


Figure 1 Four generations of Congo Red and derivatives evaluated as potential PET amyloid imaging agents.

with logD values in the range of 1-3 are known to cross the blood–brain barrier (BBB) more readily than compounds outside this range.[6,7] Although CG also displays a negative (−2) charge at pH 7.4, hydrogen bonding imparted by an adjacent -OH group of the salicylic acid moiety likely partially shields the negative charge and provides a compound with a much higher logD value than CR (logD of 1.8 for CG vs −0.2 for CR). Despite their higher logD values, radiolabeled derivatives of CG failed to provide significantly increased rodent brain entry compared with CR.[2] In an effort to decrease the molecular weight and complexity of CG, we synthesized a series of divinyl benzene compounds that we termed X compounds. Among the first X-series compounds was X-34[8] (Fig. 1), similar to CG in some respects but with a single central phenyl group and ring-bridging ethenyl groups rather than the azo groups of CG. Like CG, X-34 binds well to both Aβ plaques and NFTs and has a relatively high logD value (0.4), but does not readily cross the BBB. In an effort to increase logD and BBB penetration, we synthesized several hundred derivatives of X-34 and tested their binding affinities to synthetic Aβ fibrils. We focused on aggregated Aβ over tau binding because we had developed convenient Aβ(1-40) and Aβ(1-42) fibril assays[9] and did not have a readily available tau assay at that time. Out of these studies came the compound methoxy-X-04 (Fig. 1), which possessed a high logD value (2.6), reasonable BBB penetration, and moderately high affinity to Aβ fibrils.

Specific Evaluation Criteria for PET Amyloid Radioligands

During the evaluation of the many X-series derivatives, we considered what properties our ideal in vivo amyloid imaging agent should possess and compiled a list of these desired properties[3] (Table 1). It was clear that the radioligand should bind selectively to amyloid deposits, in the form of Aβ plaques or NFTs or both. It should bind to these deposits with high affinity; typical PET neuroreceptor-binding radioligands in use in the early 1990s such as [^{11}C]raclopride bound to their target protein site with equilibrium dissociation (K_d) or equilibrium inhibition (K_i) constants in the range of 1 nM.[10] Importantly, the amyloid-selective radioligand should readily cross the BBB, and this had proven to be a problem for CR and CG derivatives. A survey of the literature indicated that all the successful PET neuroreceptor radioligands in use in the mid-1990s entered the brain of rats a few minutes after iv tail-vein injection, with an uptake concentration value of ≥0.4% of the injected doses per gram of brain tissue (%ID/g). In mice, this value was ≥4%ID/g. Normalizing these values by the typical body mass (in kg) of the adult rodent provided a targeted minimum brain uptake value of 0.10(%ID/g)×kg or 1.0 standardized uptake value (SUV) unit.[3] In addition to good brain uptake, the PET radioligand should clear rapidly from non-target brain regions. We set the clearance half-time value of the radioligand from normal rodent brain at ≤30 minutes so that a reasonably high target-to-nontarget ratio could be achieved within the relatively short half-lives of typical PET radionuclides such as ^{11}C (20.4 minutes) or ^{18}F (109.8 minutes). Because PET follows the radionuclide distribution (whether incorporated in the parent compound or a radiometabolite), we wanted to ensure that radiolabeled metabolites of the radioligand were not present in the brain, thereby complicating the pharmacokinetic analysis of the radioligand in the brain. Finally, we desired to demonstrate the usefulness of the radioligand in the newly devised transgenic mouse models of AD.[11]

None of the X-series compounds met all of these acceptance criteria. One of the best compounds from this series was methoxy-X-04, and its brain uptake at 2 minutes and binding affinity to Aβ(1-40) fibrils were below the minimally acceptable values we had set (Table 1).[3] Hence, we began to consider other amyloid-binding pharmacophores. However,

Table 1 Ideal Properties of an In Vivo Amyloid Imaging Agent

Selectively binds to only amyloid
High affinity for amyloid (K_d ~ 1 nM)
Crosses the blood–brain barrier well at early times after injection (≥0.4%ID/g in rat brain or ≥4%ID/g in mouse brain; may be expressed as a species-independent value where 0.10 (%ID/g) × kg = 100(% ID/g)×g = 1 SUV unit)
Rapid brain clearance of compound not bound to targeted amyloid (clearance $t_{1/2}$ ≤ 30 minutes in rodents)
No radiolabeled metabolites in brain
Works well in transgenic mice models of AD

before dismissing the CR and X-series of compounds as good in vivo PET amyloid imaging agents, it is important to point out that a variety of useful outcomes derived from this early work with CG and X compounds. The 3 best known are as follows: (1) CG may protect from Aβ-induced neurotoxicity and has been explored as a γ-secretase modulator[12,13]; (2) X-34 is a highly fluorescent compound[8] that is remarkably sensitive for AD pathology in histologic applications[14]; and (3) methoxy-X-04 has proven to be very useful for in vivo imaging in 2-photon microscopy applications in transgenic mouse models of AD,[15-18] as well as in prion disease.[19] It is also worth pointing out that X-34 is the basic structural backbone for (trans,trans)-1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy)styryl-benzene or "BSB," a compound that differs from X-34 only by the addition of 1 bromine atom.[20] BSB, like CR, has been shown to bind to Aβ oligomers (although at solution concentrations many times higher than those attained in PET studies).[21]

Evaluation of Thioflavin-T Analogs

After investing 6 years evaluating several hundred CR and X-series derivatives, our transition away from these compounds began in late 1999. We considered several other amyloid-binding pharmacophores (Fig. 2), and settled on exploring neutral lipophilic thioflavin-T derivatives for several reasons.[22] Amyloid-binding Aβ and tau peptides and antibodies do not have high BBB penetration and were eliminated as candidates. Thioflavin-S is a mixture of several different compounds, and the most abundant compound in the mixture has 2 positive charges and a negative charge at pH 7.4 (Fig. 2). Thioflavin-T is a well-characterized relatively small molecule, and has only 1 positive charge. We therefore chose to synthesize neutral derivatives of thioflavin-T (termed benzothiazole-aniline or BTA derivatives) and assess their BBB penetration and binding affinities to Aβ plaques and NFTs (Fig. 3). Radiolabeling many of these derivatives

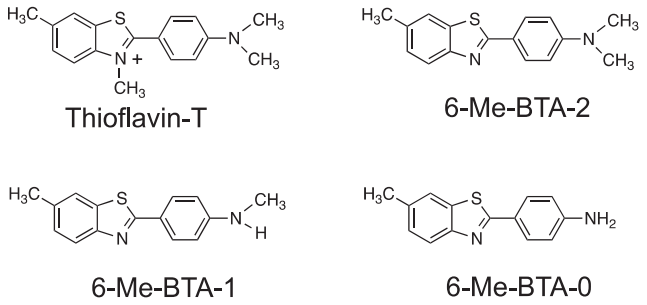


Figure 3 Structures of 3 neutral thioflavin-T analogs.

with ^{11}C for further evaluations proved to be straightforward via methylation of amino or hydroxyl groups with -[^{11}C]CH_3 to form N-methyl amines or methoxy derivatives. These compounds were injected into control mice, and their brain uptake at 2 minutes was evaluated (Fig. 4).[23,24] Of the first 11 ^{11}C -labeled BTA compounds evaluated in mice, 10 exceeded our minimum brain uptake requirement of 1.0 SUV at 2 minutes after iv injection. Although many of the BTA compounds quickly proved to be efficient in penetrating the BBB, an unresolved question was the effect on Aβ and tau binding of the removal of the N-methyl group from the nitrogen of the heterocyclic benzothiazole ring to form the neutral BTA derivatives. Fortunately, the neutral BTA derivatives bound to Aβ(1-40) fibrils with much higher affinity than the parent thioflavin-T compound (Fig. 5),[22,24] and these neutral BTA derivatives bound with very low affinity to aggregated tau. We then synthesized and evaluated several hundred BTA derivatives, and a representative structure–affinity assessment is shown in Figure 6. Several of the BTA derivatives bound to Aβ(1-40) fibrils, with binding affinities approximately equal to the desired value of 1 nM. The next important criterion was to determine the clearance rate of radiotracer from normal rodent brain. This was accomplished relatively easily by comparing the 2-minute brain uptake values in one group of normal mice with the 30-minute brain uptake values in another group of normal mice.[23] The ratio of these 2

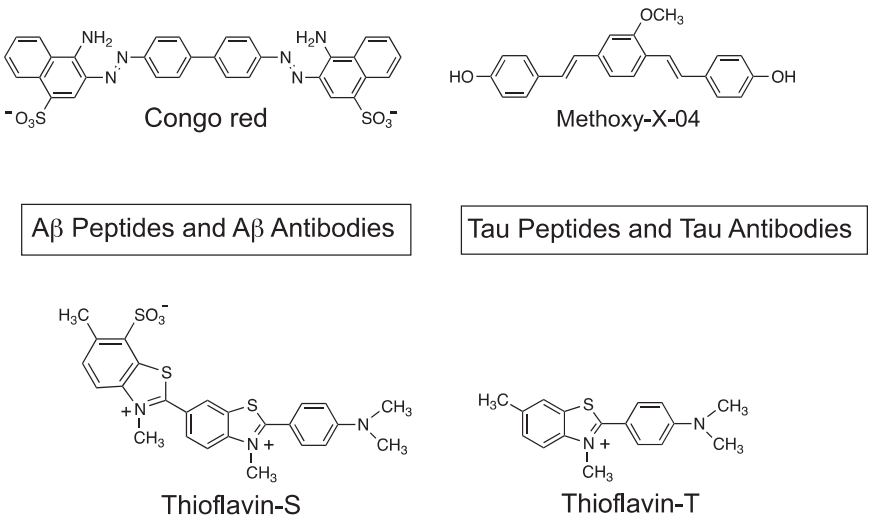


Figure 2 Potential amyloid-binding pharmacophores.

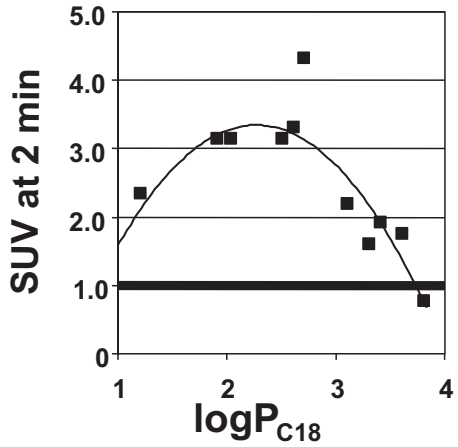
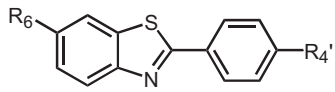


Figure 4 Relationship between normal mouse brain concentration (SUV is the standardized uptake value) for 11 ^{11}C-labeled BTA compounds 2 minutes after iv injection and the lipophilicity of the compounds as assessed by the logarithm of the reverse-phase HPLC–derived partition coefficient (logP_{C18}), which is proportional to the logD value of the derivatives.



Affinity (K_i, nM) for Aβ(1-40) fibrils

		R$_4$'		
		NH_2	$NHCH_3$	$N(CH_3)_2$
R$_6$	CH_3	9.5	10	64
	H	37	10	4.0
	HO	46	4.3	4.4
	CH_3O	7.0	4.9	1.9

Figure 6 Structure–affinity values for representative BTA analogs. The K_i values were determined using [^{3}H]BTA-1 (R_6 = H, R4' = $NHCH_3$) as the radioligand and synthetic Aβ(1-40) fibrils as the binding site protein.

values provides an index of the 30-minute clearance rate: a ratio of 2 indicates approximately a 30-minute clearance half-time and a ratio of 8 indicates approximately a 10-minute clearance half-time. The 2 minute-to-30 minute brain uptake ratios for several BTA compounds are shown in Fig. 7, and it is evident that the 6-OH N-HCH_3 derivative (termed 6-OH-BTA-1) possessed a relatively high 2 minute-to-30 minute ratio of 11. This indicated an in vivo clearance half-life of this compound of approximately 6 minutes from normal mouse brain, and, combined with a K_i value of 4.3 nM for binding to Aβ(1-40) fibrils (Fig. 6), this compound became our lead for further evaluations aimed at assessing how well the compound met the criteria shown in Table 1.[24]

Binding assays indicated that 6-OH-BTA-1 bound selectively with high affinity (K_i and K_d values in the range of 2-4 nM) to aggregated synthetic Aβ(1-40) and Aβ(1-42) fibrils and Aβ plaques in human AD brain tissues and with very poor affinity to NFTs.[25,26] Thus, a nonselective moderate-to-low-affinity pan-amyloid–binding fluorescent dye (thioflavin-T) was chemically modified to produce a neutral lipophilic PET radioligand with high binding affinity and high selectivity only for aggregated Aβ-containing fibrils and plaques. Additional in vivo brain uptake and clearance studies with [^{11}C]6-OH-BTA-1 in baboons demonstrated favorable pharmacokinetics of this compound with respect to the rapid clearance of radiotracer from healthy nonhuman primate brain.[24] We assessed the presence of radiolabeled metabolites in mouse brain after the injection of [^{11}C]6-OH-BTA-1 at times between 2 and 30 minutes after injection and determined that they were negligible.[24] It is interesting to note that other investigators have used rats to evaluate radiometabolites of [^{11}C]6-OH-BTA-1 in brain and have reported a relatively high level of radiolabeled metabolites in postmortem rat brain tissues.[27] We reported that rat appears to be unique in this regard, and that mice, baboons, and humans do not produce significant amounts of radiolabeled metabolites in their brains.[28] Attempts to use transgenic AD mice models that develop Aβ plaques to demonstrate specific binding met with failure initially,[25] and subsequent studies by other groups indicated that this failure was due to the relatively low B_{max} value in transgenic mouse models compared with human AD subjects and the subsequent necessity to use very-high-specific-activity [^{11}C]6-OH-BTA-1 for the transgenic mouse studies.[29,30] Thus, [^{11}C]6-OH-BTA-1 met all the acceptance criteria listed in Table 1, and human studies with this compound were then planned.

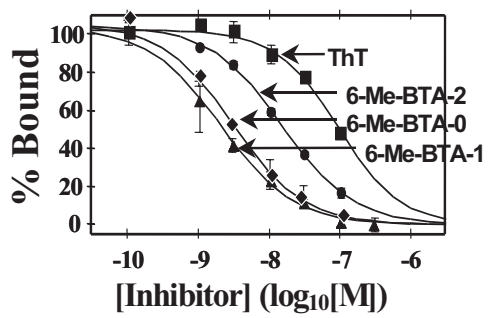
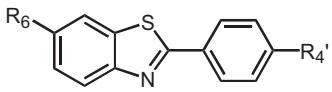


Figure 5 Binding affinity of 3 neutral BTA compounds, the structures of which are shown in Figure 3, relative to thioflavin-T (ThT) for synthetic Aβ(1-40) fibrils.

PET Imaging in Human Subjects With 6-OH-BTA-1

Because the toxicology and pharmacology of 6-OH-BTA-1 was not known, we were compelled by U.S. Food and Drug Administration (FDA) regulations in 2000 to conduct these studies in animals as part of an Investigational New Drug submission package. We sought and received National Institutes of Health/National Institute on Aging funding to conduct these studies at an estimated cost of approximately



Ratio of 2':30' brain uptake values

		R_4'		
		NH_2	$NHCH_3$	$N(CH_3)_2$
R_6	CH_3	-	2.7	0.5
	H	-	7.6	2.5
	HO	-	11	3.0
	CH_3O	3.8	3.2	1.1

Figure 7 Structure–clearance values for representative BTA analogs from normal mice brain. Values are the ratio of the %ID/g concentrations in mouse brain at 2 minutes after injection to the %ID/g concentrations at 30 minutes after injection.

$200,000, and, while waiting for completion of the toxicologic studies, we contacted Bengt Långstrom, director of the Uppsala University PET Centre in Sweden, to discuss initiation of the first human PET study in Sweden using our Aβ radiotracer. We sought out the Uppsala PET Centre group because they had championed the microdosing concept.[31] Because of their very high specific activity and low cold mass, PET radiopharmaceuticals are typically given in "microdoses" of <10 μg, and subjects typically receive only 1 dose per year and seldom >4 doses per year. The microdosing concept holds that the preclinical safety evaluation of high-specific-activity PET radiopharmaceuticals should be tailored to how they are used and not judged by the same requirements originally developed by regulatory agencies interested in assuring the safety of therapeutic drugs typically given in milligram or gram quantities. Långstrom and colleagues led the efforts for these streamlined guidelines, and Swedish regulatory authorities led the way in defining special pathways for microdosing toxicology. The FDA and other agencies have since adopted guidelines similar to those present in Sweden, as evidenced by the current exploratory Investigational New Drug mechanism for new PET and single-photon emission computed tomography imaging agents.[32]

When we first approached the Uppsala group in early 2001 regarding collaborative imaging studies, our lead Aβ imaging compound was not 6-OH-BTA-1, but instead was another compound we designated BTA-1.[23] BTA-1 is a close structural analog of 6-OH-BTA-1 and differs only by the presence of a 6-position H atom rather than the 6-OH group. Långstrom's group designated BTA-1 as "Pittsburgh Compound A" or PiA (the first compound sent to them by our group) in keeping with the tradition at Uppsala of naming compounds by a 3 letter designation.[33] As we conducted additional preclinical evaluations, we realized that 6-OH-BTA-1 was superior to BTA-1 and chose it as our lead compound, based primarily on its very good clearance rate from normal brain in rodents (Fig. 7) as well as in nonhuman primates.[24] The Uppsala group renamed 6-OH-BTA-1 "Pittsburgh Compound B" or PiB.[34]

By January of 2002, the necessary preliminary toxicologic studies and animal physiological studies with PiB were completed to satisfy the microdosing requirements at the Uppsala PET Centre, and the first subject for human PiB studies had been identified and evaluated by Agneta Nordberg. The first human study with PiB was conducted on February 14, 2002, in a relatively young woman whose memory problems had forced her to stop working as a health care professional.[35] From the first images sent to us from Uppsala (Fig. 8), it was immediately clear that the pattern of PiB retention matched the expected regional distribution of Aβ deposits previously reported from many postmortem studies.[36-38] A few weeks later, a negative PiB study in the first cognitively normal subject was conducted and demonstrated the rapid clearance of radiotracer from the same regions of brain that had retained PiB in the first study. A few months later, the preliminary findings from the first 9 AD subjects and 2 elderly and 3 young cognitively normal control subjects were presented at the "Hot Topics" session of the International Conference on Alzheimer's Disease in Stockholm on July 24, 2002, by Engler et al[39] of the Uppsala PET Centre team. The first peer-reviewed article describing this work[34] was published in early 2004, and since that time, >300 manuscripts describing PiB in Aβ studies have been reported.

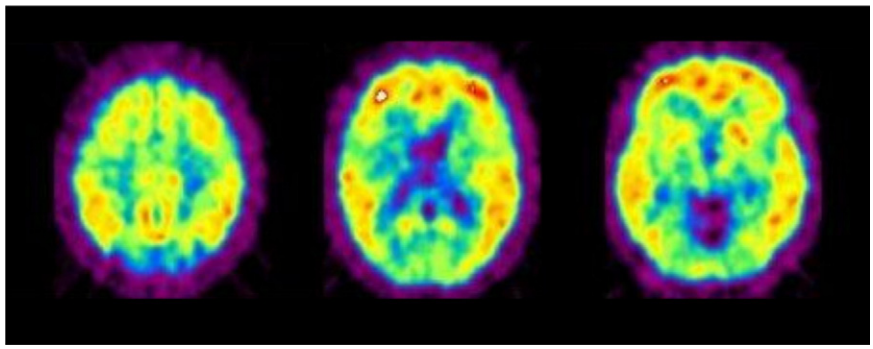


Figure 8 First human PiB PET images obtained on February 14, 2002, by Bengt Långström and colleagues at the Uppsala University PET Centre from the first volunteer (mild AD, MMSE = 25; Reprinted with permission from Klunk and Mathis[34]).

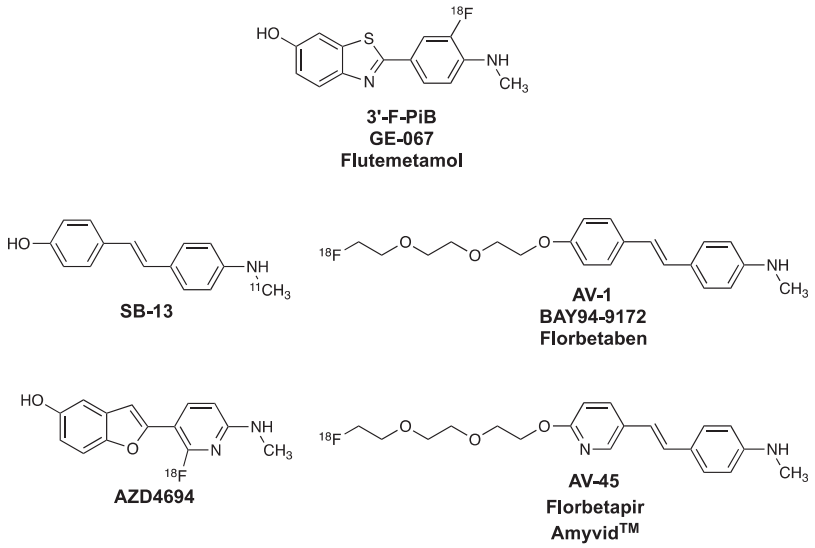


Figure 9 Structures of different PET radioligands selective for fibrillar Aβ.

The woman who provided that first human PiB PET image on Valentine's Day 2002 died in late 2007, and an autopsy confirmed what was suspected in 2002 when the first PiB image was viewed; PiB retention reflected the distribution of Aβ deposits in the brain.[35] This has now been confirmed by several correlative autopsy studies in other patients.[26,40,41] PiB scans are now performed in >60 research centers around the world, and we estimate that more than 10,000 investigational PiB PET scans have been performed. Conditions such as normal aging,[42-45] mild cognitive impairment[46-51] (MCI), AD,[52-55] early-onset familial AD,[56-59] and non-AD dementias[60-66] have been studied with PiB PET. Several current anti-amyloid drug trials have included a PiB component to assess the efficacy of the drug on the Aβ target.[67,68] This will be an important role of amyloid plaque imaging: facilitating the development of effective disease-modifying therapies. The finding that approximately 60% of MCI patients have levels of PiB retention similar to that seen in AD,[69,70] coupled with the finding that approximately 25% of cognitively normal elderly people in their 70s have measurable PiB retention,[71] suggests that one will need to look in asymptomatic people to find the earliest stages of AD pathology.[72] It may be that we have to identify and treat people at this early stage to achieve substantial disease-modifying effects. Our hope is that the benefit of PiB will ultimately be seen best in the development of new effective disease-modifying drugs along with the identification of the people who can best benefit from these drugs even before the first symptoms of AD become clinically apparent.

^{18}F-Labeled Aβ Imaging Agents

Work over the past 6 years at many institutions has focused on developing ^{18}F-labeled PET radiotracers for more widespread availability and routine clinical usefulness than ^{11}C-labeled PiB. At Pittsburgh, we synthesized and evaluated several hundred fluorinated compounds as potential ^{18}F-labeled Aβ imaging agents. These efforts led to the development of an ^{18}F-labeled PiB derivative, 3'-F-PiB (Fig. 9), and this compound is also known as GE-067 and flutemetamol.[73-75] Chemists at the University of Pennsylvania and Avid Radiopharmaceuticals (Philadelphia, PA) explored fluorinated analogs of the stilbene [^{11}C]SB-13[76] (Fig. 9) and derived AV-1 (also known as BAY 94-9172 and florbetaben)[77-79] and AV-45 (also known as florbetapir and Amyvid™ (Eli Lilly and Company, Indianapolis, IN)).[80-83] Chemists at AstraZeneca (Södertälje, Sweden) explored structural analogs of PiB, and developed AZD4694 (Fig. 9), the in vivo properties of which in human subjects have recently been reported.[84] From a chemical homology viewpoint, all of these derivatives are related to the PiB framework as shown in Fig. 10. From a binding perspective, all the compounds shown in Fig. 10 appear to bind selectively with high affinity to aggregated (fibrillar) Aβ and do not bind well to aggregated tau forms such as NFTs. From an in vivo imaging perspective, all the compounds have been shown to readily distinguish subjects with high brain Aβ plaque loads from those with little or no significant Aβ plaque loads. The relative sensitivities and specificities of the compounds for detecting and quantifying different levels of Aβ loads in human subjects remain an area of active investigation.[74,85-87]

Clinical Use of ^{18}F-Labeled Aβ Imaging Agents

Amyvid™ was approved by the FDA for human clinical Aβ imaging in April 2012,[88] and other ^{18}F-labeled agents (flutemetamol, florbetaben, and AZD4694) are currently in phase II or phase III FDA clinical trials in the United States. We believe that the greatest utility of the new ^{18}F-labeled Aβ imaging agents will be similar to that we envisioned for PiB: these agents likely will prove useful in identifying efficacious anti-amyloid therapies that can prevent or delay AD, as well

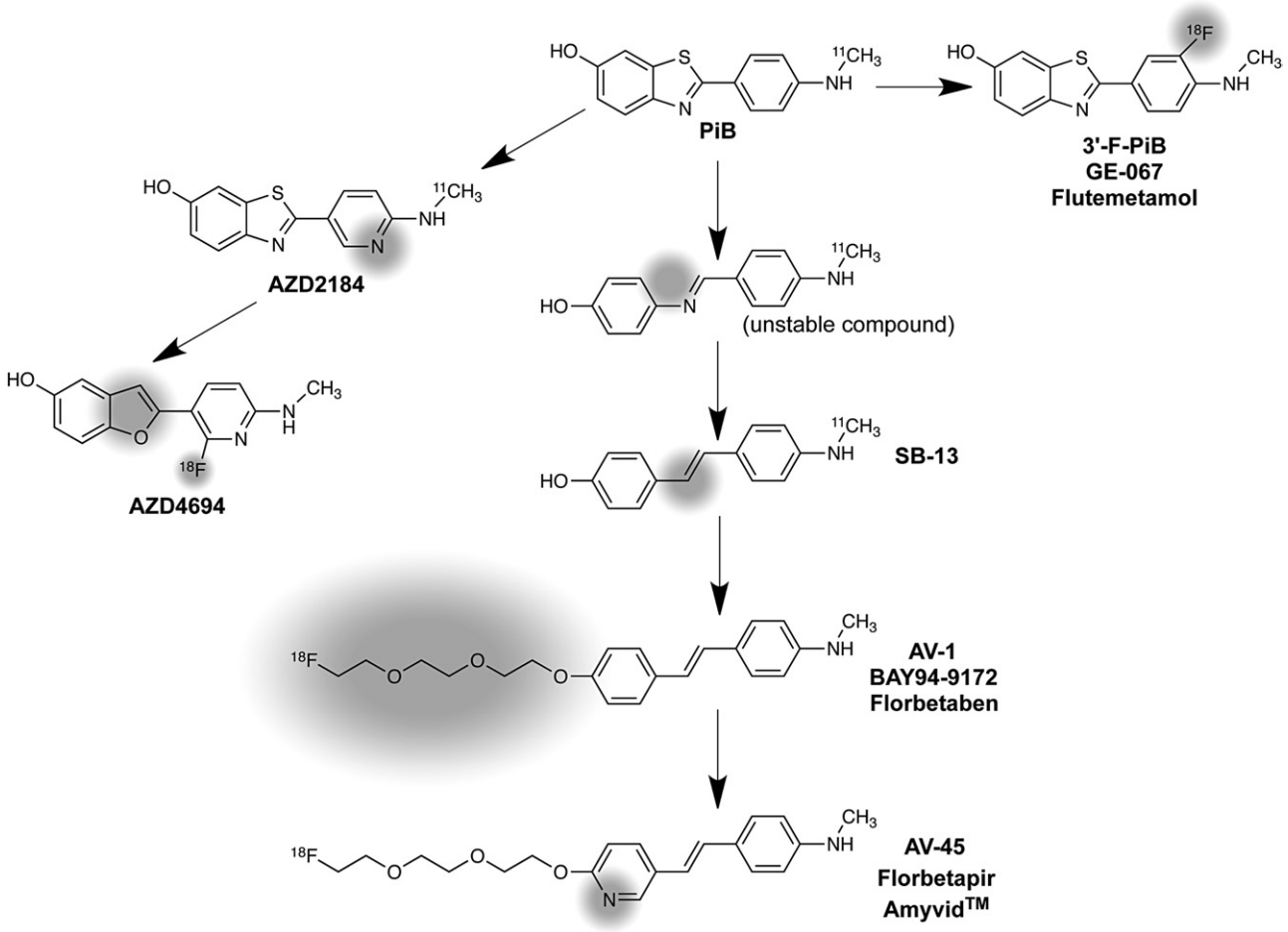


Figure 10 Evolution from PiB of different Aβ-selective PET radioligands, with significant structural changes highlighted in gray shading.

as identifying those subjects who likely will benefit from these therapies long before the clinical diagnosis of probable AD.[89]

The development of PiB as a highly potent and selective PET radioligand for fibrillar Aβ was in good part serendipitous. It resulted from a chemical change in the pan-amyloid fluorescent dye thioflavin-T aimed at increasing brain penetration of neutral BTA derivatives, but that structural change also produced derivatives with both high affinity and selectivity for fibrillar Aβ. This outcome was highly fortuitous for several reasons: (1) interpretation of the specific binding of PiB in human brain is unambiguous, as it binds to only 1 amyloid target; and (2) dysregulation of Aβ is believed to be the first biomarker altered in the course of AD, making a selective imaging agent for Aβ important and useful for early disease detection.[90-92] Other biomarkers that more fully reflect AD progression and disease stage, such as NFTs, regional hypometabolism, and cortical gray matter atrophy, are believed to be altered after (ie, downstream of) Aβ dysregulation. Recently, great progress has been realized in the use of [^{18}F]fluorodeoxyglucose (FDG) and structural magnetic resonance imaging to assess regional brain hypometabolism and cortical atrophy in MCI and AD. In contrast, progress in developing a selective PET radioligand to quantify NFTs in living human brain has lagged, but recent advances are encouraging.[93,94] The combined use of selective Aβ plaque and NFT PET agents, along with FDG and structural magnetic resonance imaging, will likely provide even greater insights into the pathophysiology of AD at all stages of the disease process: from cognitively normal elderly subjects to MCI subjects to AD patients. It is hoped that these imaging insights will assist in the development of effective disease-modifying treatments for AD, and that subjects very early in the course of the disease process will be identified and will benefit most greatly from these future therapeutic treatments.

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GE Healthcare holds a license agreement with the University of Pittsburgh based on the technology described in this manuscript. C.A.M. and W.E.K. are coinventors of this technology and, as such, have a financial interest in this license agreement.

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