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What Amyloid Ligands can tell us about Molecular Polymorphism and Disease

Harry LeVine III^{1,2,3,*} and Lary C. Walker^{4,5}

¹Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40536-0230

²Center on Aging, University of Kentucky, Lexington, KY 40536-0230

³Center for Structural Biology, University of Kentucky, Lexington, KY 40536-0230

⁴Division of Neuropharmacology and Neurologic Diseases, Yerkes National Primate Research Center, Emory University, Atlanta, GA, 30322

⁵Department of Neurology, Emory University, Atlanta, GA, 30322

Abstract

Brain-penetrant PET imaging ligands selective for amyloid pathology in living subjects have sparked a revolution in presymptomatic biomarkers for Alzheimer's disease progression. As additional chemical structures were investigated, the heterogeneity of ligand binding sites became apparent, as did discrepancies in binding of some ligands between human disease and animal models. These differences and their implications have received little attention. This review discusses the impact of different ligand binding sites and misfolded protein conformational polymorphism on the interpretation of imaging data acquired with different ligands. Investigation of the differences in binding in animal models may identify pathological processes informing improvements to these models for more faithful recapitulation of this uniquely human disease. The differential selectivity for binding of particular ligands to different conformational states could potentially be harnessed to better define disease progression and improve the prediction of clinical outcomes.

Keywords

Abeta; Alzheimer; lipids; plaques; proteopathy; strains; tangles; tau

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*CORRESPONDING AUTHOR: Harry LeVine, III, ADDRESS: 800 S. Limestone Street, Lexington, KY 40536-0230, PHONE: 859-218-3329; FAX: 859-323-2866, Harry LeVine, III hlevine@email.uky.edu; Lary C. Walker lcwalke@emory.edu.

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1. Introduction

Neuropathologists distinguish different types of A β deposits in histological sections of brain tissue on the basis of morphological characteristics and staining properties of the lesions. Some have attempted to link diffuse, primitive, classic, and compact A β plaques as well as neurofibrillary (tau) tangles with different stages of Alzheimer's disease (AD) progression. Studies characterizing A β deposit ultrastructure, localization, and molecular composition in humans suggest that the different lesion morphotypes are not necessarily developmentally related to one another (Armstrong, 1998, Armstrong, 2009, Thal, et al., 2006). Multiple factors influence these features (D'Andrea and Nagele, 2010), and the complexity of morphotype formation in the brain obscures their relationship to pathogenicity and disease progression. Variable rates of disease progression and differences in phenotype of idiopathic Alzheimer's disease could result from different relative proportions of the morphotypes. Furthermore, in addition to the classical insoluble fibrillar forms of A β and tau, soluble oligomeric forms of the proteins also are present in the AD brain (Bilousova, et al., 2016, Catalano, et al., 2006, Lue, et al., 1999, McLean, et al., 1999, Wang, et al., 1999). Rapid clinical progression has been connected with a particular A β oligomer size distribution, conformational states, and stabilities (Cohen, et al., 2016, Cohen, et al., 2015).

Attempts to understand the *in vitro* assembly of A β and other amyloidogenic proteins and peptides into soluble oligomers and insoluble fibrils at a structural level have revealed a surprising spectrum of polymorphic forms depending on environmental conditions and starting peptide conformation (Eisenberg and Jucker, 2012, Toyama and Weissman, 2011, Tycko, 2015). These assemblies produced *in vitro* have been structurally characterized at the molecular level by such methods as X-ray crystallography, solid-state NMR, and hydrogen-deuterium exchange (Eisenberg and Jucker, 2012, Kodali, et al., 2010, Tycko, 2015, Zhang, et al., 2009). Because analogous experiments are not feasible on the human AD brain mixture of multimeric A β assemblies, fibril-containing brain extracts from two separate individuals with AD were used to catalyze multiple rounds of seeded growth of fibrils from stable, isotope-labeled monomeric A β . The resultant labeled fibrils were sufficiently homogeneous for solid-state NMR structure to determine that the seeded amyloid fibrils from the two AD cases were structurally different (Lu, et al., 2013). One interpretation of the findings is that only a different single major conformational type of A β amyloid was generated in each case, or that the distinct fibrillar structures resulted from selection through fitness for *in vitro* seeding efficiency. Alternatively, structural variation could be the result of selective amplification of the thermodynamically stable conformation, as demonstrated by the changing conformation of the D23N Iowa mutant A β through repeated cycles of seeding (Qiang, et al., 2011). Selectivity in animal models was maintained during amplification of A β pathology obtained from sporadic human AD, Swedish mutation (K670M/N671L), and Arctic mutation (E193G) brain during propagation in the TgAPP23 mouse model (Watts, et al., 2014).

Support for the presence of different conformational states of A β fibrils *in vivo* comes from staining tissue sections with poly- and oligothiophene probes that bind to a variety of amyloid-like structures including tau tangles. The spectral characteristics of this class of probes depend on the rigidity of their flexible thiophene backbone (Nilsson, et al., 2007).

The structural organization of A β plaques in the brains of APP/PS1 transgenic mice changes with advancing age, and this transformation is reflected by the binding of thiophene probes with different structures (Nystrom, et al., 2013). These observations confirm the polymorphic nature of aggregated A β and other amyloid pathology *in vivo*, and indicate that the assemblies undergo age- and perhaps disease stage-dependent structural changes. In this light, it is important to understand the binding characteristics of clinical imaging ligands to polymorphic forms of A β and tau pathology to know whether these agents are reporting structures relevant to all stages of AD. This review offers a framework for understanding what structurally different small molecule binding probes can tell us beyond the presence of misfolded proteins, as well as caveats about what they do not provide. We suggest additional ways in which these ligands might be employed to aid in identifying and distinguishing underlying biology, improve diagnosis, and develop/monitor therapeutic interventions.

2. Molecular Ligands for Systemic Amyloid

The development of ligands that selectively bind to misfolded protein pathologies has yielded valuable tools for the *in vivo* and *ex vivo* detection and analysis of the lesions. Early studies involved systemic amyloidoses in which misfolded proteins, mainly amyloid A (AA) and antibody light chains (AL), deposit in multiple organs such as the kidney, liver, and heart. The prototypical amyloid visualizing ligand is the diazo cotton dye Congo Red (Elghetany, et al., 1989). When applied as a histological stain, the dye accumulates in amyloid deposits in tissue sections and undergoes a metachromatic shift in its absorbance spectrum. Viewed under illumination through crossed polarizing filters, the ordered, β -sheet-rich structure of the amyloid fibrils is evident in the birefringence of the stained deposits (Howie and Brewer, 2009). In one of the first attempts to use a selective amyloid ligand for diagnosis, Bennhold infused Congo Red intravenously into patients suspected of having a systemic amyloidosis (Bennhold, 1923). Rapid disappearance of the dye from the blood was thought to reflect sequestration of the agent in amyloid deposits; unfortunately, the approach had to be abandoned for safety reasons (Buxbaum and Linke, 2012). In the 1980s, the pentraxin serum amyloid P component was introduced as an imaging ligand for systemic amyloidosis (Pepys, 2001). Serum amyloid P has an intrinsic binding capacity for amyloid fibrils; when labeled with either short half-life radioisotopes ^{131}I or ^{123}I for Single Photon Emission Computed Tomography (SPECT) scintillographic imaging, it is a sensitive and selective tool for detecting amyloid deposits in living patients (Hawkins, et al., 1998, Hawkins and Pepys, 1995). The 25 kDa molecular weight of the labeled serum amyloid P limits its use to deposits outside the central nervous system.

3. Ligands for Imaging Amyloid in the Brain

Imaging probes for use in the brain require a combination of molecular properties (molecular weight, hydrophobicity, resistance to metabolism) to penetrate the blood-brain barrier and reach their targets. Neurodegenerative diseases involving misfolded protein pathology, especially Alzheimer's disease (AD), have been a focus of new ligand development because such agents could provide useful information on the presence of disease in a relatively inaccessible body compartment, thereby enabling the early detection and longitudinal monitoring of the pathogenic process (Vlassenko, et al., 2012). A multitude

of small molecule probes have been prepared and tested for affinity and specificity *in vitro*, with a much smaller number moving into testing *in vivo* for binding potential (reviewed in (Eckroat, et al., 2013)). The great majority have been directed at the A β peptide or at the microtubule-associated protein tau, which form the pathognomonic lesions of AD: senile (A β) plaques and neurofibrillary tangles, respectively. Imaging human subjects with the tau ligand ^{18}F -AV-1451 reveals age-dependent accumulation of tau in regions such as the medial temporal lobe and colocalizes in isocortical regions with PIB binding to A β . The isocortical binding correlates with decline in global cognition. ^{18}F -AV-1451 signal generally recapitulates key features of Braak stage histopathology (Ossenkoppele, et al., 2016, Scholl, et al., 2016, Schwarz, et al., 2016). Targeting other protein aggregates, such as the α -synuclein-containing lesions of Parkinson's disease and Lewy Body dementia, has been challenging due to the difficulty in establishing the requisite selectivity to distinguish α -synuclein from the frequently comorbid A β and tau misfolded protein pathologies (Neal, et al., 2013).

The need for agents to detect amyloid pathology in the brains of living subjects before the onset of the clinical symptoms of AD has driven the search for ligands that can detect and quantify the deposits. The extended conjugated π electron density and aromaticity of the bis-styryl compounds and benzothiazoles confer particularly good histological selectivity of these compounds for amyloid fibrils. In histological preparations, the benzothiazoles Thioflavin S and Thioflavin T selectively bind to amyloid deposits of many different proteins and emit a bright fluorescence (LeVine, 1995). Owing to their selectivity and sensitivity, the molecular motifs of agents such as Congo Red and the Thioflavins appear in various guises in the structures synthesized as ligands for A β deposits in the brain.

Development of high-affinity amyloid-binding ligand structures with appropriate specificity and chemical properties for *in vivo* imaging and/or inhibition of fibril assembly has been undertaken using a variety of screening paradigms and computational approaches. Progress has been limited by the structural heterogeneity and dynamics of full-length A β (1–40) and (1–42) amyloid fibrils. Solid-state NMR structures of the longer peptides have been obtained under certain conditions (Luhres, et al., 2005, Tycko, 2011, Tycko, 2014). Atomic resolution X-ray crystal structures of short synthetic peptides, in particular A β residues KLVFFA, obtained in the absence and presence of small molecule ligands (Landau, et al., 2011), have provided detailed structural information. Molecular modeling and dynamics simulation in docking studies gave insight into interactions with small molecule ligands. These methods identified putative binding sites and accounted for differences in affinity between ligands for the model structures (Biancalana and Koide, 2010, Reinke and Gestwicki, 2011, Wu, et al., 2011, Wu, et al., 2008, Wu, et al., 2007). However, experimental determination of the binding affinity and specificity of resulting ligands in human tissue revealed significant disparities with the binding properties of the proteinaceous aggregates in the AD brain. A β and tau assemblies in the human brain are interact with multiple proteins, lipids, and other molecules whose contribution to fibril structure is difficult to assess. In addition, a significant proportion of the A β of AD brain is truncated, oxidized, crosslinked, isomerized, racemized, and/or pyroglutamylated. The relationship of the pure peptide structures formed *in vitro* to those generated in the living brains of AD patients and animal models of AD remains to be determined.

Transforming a histological dye into a bona fide imaging ligand with high affinity and selectivity is not a trivial undertaking. Creating selective ligands that are sufficiently metabolically stable, blood-brain barrier-penetrant, and that can be rapidly and efficiently radiolabeled with short half-life isotopes for use as brain imaging agents is where many promising compounds fail. The first radioligand applied to imaging A β in patients was ^{18}F -FDDNP (Shoghi-Jadid, et al., 2002). Although it demonstrated the potential of radiolabeled agents for detecting cerebral A β in transgenic mouse models of A β pathology and in humans with AD, the relatively low affinity of FDDNP for A β pathology, a non-negligible affinity for tau pathology shared by bis-styryl ligands, and non-specific accumulation in brain white matter were liabilities (Ossenkoppele, et al., 2012).

The ligand first systematically employed in the clinic to specifically image brain A β deposits was the ^{11}C -labeled benzothiazole Pittsburgh Compound B (PIB) (Klunk, et al., 2004). The basic protocols for obtaining and interpreting brain images of accumulated ligand were developed with this A β -selective agent, and PIB currently remains the gold standard for detecting senile plaques and A β -type cerebral amyloid angiopathy (CAA) *in vivo* (Johnson, et al., 2007, Leuzy, et al., 2014, Mathis, et al., 2012). Other ligands (e.g. Amyvid^{TM} (Florbetapir), Neuraceq^{TM} (Florbetaben), and Vizamy^{TM} (flutemetamol) containing ^{18}F also have been approved by the FDA. ^{18}F has the advantages of a longer, more user-friendly radioactive half-life (2 h vs. 20 min for ^{11}C) as well as lower non-specific uptake than with PIB. These ligands compete for PIB binding in the AD brain (Ni, et al., 2013). Other ligands with different structures also are being evaluated. Herein lies the conundrum – are these different ligands “seeing” the same thing? Probably not, which is the message that this review seeks to convey.

4. Heterogeneity of Ligand Binding Sites

Lockhart and Ye (Lockhart, et al., 2005, Ye, et al., 2005) analyzed the binding of a series of structurally different ligands to synthetic A β (1–40) fibrils and were able to distinguish three sites that could be occupied by different ligands. These sites on a population of fibrils differed with respect to the stoichiometry of the ligand:A β monomer-equivalent, as well as the molecular structure of the ligands that bound to them. The authors defined binding site 1 (BS1) as a Thioflavin T binding site, BS2 as a styrylbenzene (Congo Red-like) binding site, and BS3 as a non-quaternary amine benzothiazole site that binds PIB analogs. *In vitro*-assembled fibrils of synthetic A β (1–40) or A β (1–42) bind ^3H -PIB with ~10-fold lower affinity and with a 100- to 1000-fold lower stoichiometry per A β monomer than does aggregated A β from the AD brain (Klunk, et al., 2005). The structure-activity relationship for displacement of [N-methyl- ^3H]BTA-1 (Klunk, et al., 2003) by a series of PIB analogs and displacement of ^3H -PIB by the same series (Matveev, et al., 2014) is parallel for synthetic fibrils and AD brain, suggesting that the overall structure of the binding site for the ligand is conserved. Various subsequent efforts to characterize ligand binding to synthetic A β fibrils by computational simulations have produced low resolution models of the binding of different types of ligands (Biancalana and Koide, 2010, Cisek and Kuret, 2012, Reinke and Gestwicki, 2011, Wu, et al., 2011). Experimental confirmation of the molecular details of where these ligands bind has not been published.

5. Discrepancies in Ligand Binding Between Human Disease and Animal Models

The picture is intriguingly complicated by the relative lack of high-affinity ^3H -PIB binding in animal models that manifest abundant human-sequence A β pathology when assayed at concentrations of imaging ligand attained in the brain *in vivo* (~1.3 nM) (Fast, et al., 2013, Klunk, et al., 2005, Rosen, et al., 2011). A small amount of high-affinity, *in vivo* binding of PIB to A β deposits in APP-transgenic mouse brains can be detected only when PIB with a specific radioactivity 100-fold greater than that of the ^3H -PIB ligand is used (Maeda, et al., 2007). (Fast, et al., 2013, Klunk, et al., 2005, Rosen, et al., 2011). Scatchard analysis of ^3H -PIB binding to AD brain revealed a stoichiometry of high affinity (1–2 nM) binding of ~500 PIB/1000 molecules of A β . By contrast, APP/PS1 mouse brain high affinity binding accounted for less than 1 PIB/1000 molecules of A β . The small amount of binding was due to a low affinity (242 nM) site (Klunk, et al., 2005). Similar results for the PIB/A β ratio were obtained for several non-human primate species (Rosen, et al., 2011). The presence of ligand binding in most binding studies refers to the amount of nanomolar affinity binding (= high affinity binding) for comparison with *in vivo* imaging studies.

The brains of humans with AD or Down Syndrome with dementia, but not control human brains lacking A β pathology, contain a large amount of high-affinity ^3H -PIB binding (Handen, et al., 2012, Hartley, et al., 2014, Matveev, et al., 2014, Rosen, et al., 2011). Strikingly, similar to APP-transgenic mice (Klunk, et al., 2005), multiple animal models of naturally occurring A β amyloidosis also lack significant high-affinity binding of ^3H -PIB. These include aged nonhuman primates (squirrel monkeys, macaques, and chimpanzees) and aged canines, all of which express human-sequence A β that deposits as senile plaques and/or CAA with advancing age (Fast, et al., 2013, Rosen, et al., 2011). In addition, not all of the A β deposits in human frontal cortex bind ^3H -PIB with high affinity (Matveev, et al., 2014). Hence, to date it appears that high-affinity PIB binding is relatively human-specific. Increasing the confusion is the report of an autopsy-confirmed human case of AD with normal APP, presenilin-1, and presenilin-2 sequences and very high A β levels, yet lacking significant high-affinity ^3H -PIB binding (Rosen, et al., 2010), thus resembling the type of binding in natural and transgenic animal models. Similarly, Ikonomic and colleagues have reported a PIB-refractory case with early AD pathology (Ikonomic et al., 2012), and PIB retention also is greatly diminished in AD patients bearing the Arctic APP mutation (Scholl, et al., 2012). In this regard, it will be instructive to establish whether or not the stoichiometry of high-affinity PIB binding varies generally among human cases of idiopathic AD.

In general, ^3H -PIB binding *in vitro* to tissue homogenates is highly correlated with the diffuse plaque content of the human brain, and somewhat less so with dense-cored plaques (Niedowicz, et al., 2012). Ten micromolar fluorescent PIB analog 6-CN-PIB binds to diffuse and dense-cored plaques in AD brain tissue sections (Ikonomic, et al., 2008). Careful comparative studies with ^{125}I -DRM106, (6-iodo-2-[4-(1H-3-pyrazolyl)phenyl]imidazo[1,2-a]pyridine), a derivative similar to IMPY (2-(4'-dimethylaminophenyl)-6-iodoimidazo[1,2-a]pyridine) that competes for ^{11}C -PIB binding, indicate a selectivity of DRM106 for dense-cored plaques over diffuse plaques (Chen, et al., 2015), a property shared with a 2-ethenyl-

benzoxazole derivative, [^{18}F] FACT (Furumoto, et al., 2013). Chen et al. comment that ^{125}I -DRM106 is distinguished from ^{11}C -PIB by the DRM compound's selectivity for dense-cored plaques while PIB is more promiscuous, labeling both diffuse and dense-cored plaques in autoradiographic analyses of tissue sections. Thus, ligand structure can distinguish histologically distinct plaque types.

The A β that deposits in the vascular wall as CAA can be considered an A β polymorph. Deposition of primarily A β (1–40) in cerebral vessels leads to smooth muscle cell death, disruption of vascular integrity, and an increased risk of hemorrhage (Charidimou, et al., 2012). Deposition in the walls of arterioles, capillaries, and (to a lesser extent) veins is exacerbated by expression of the $\epsilon 4$ isoform of apolipoprotein E (Verghese, et al., 2011). In addition to binding the A β ligands that also label parenchymal plaques, vascular amyloid is distinguished by selectively binding the dye resorufin (Han, et al., 2015, Han, et al., 2011). The molecular basis for this additional specificity remains to be determined. Anti-A β antibodies selective for CAA (M31) or parenchymal A β (M116) deposits also have been generated; after derivatization with polyethylene glycol, these antibodies have been shown to penetrate the blood-brain barrier and are detectable by positron emission tomography (McLean, et al., 2013). The nature of the selectivity for their A β epitopes and the relationship of that epitope to small molecule ligand binding remain to be established.

6. Heterogeneity of Structural States

These observations call for a reassessment of what we know about A β lesions in the brain and how we interpret the way the imaging ligands interact with that pathology. Computational studies indicate that familial mutations within the A β (1–42) sequence generate additional and heterogeneous structural states in the monomeric peptide compared to the wild-type sequence (Chong, et al., 2013). Synthetic wild-type A β assembles into distinct polymorphic fibril structures depending on the assembly conditions, as determined by hydrogen-deuterium exchange kinetics and the pattern of exposure of segments of the A β sequence to solvent (Kodali, et al., 2010). Like crystals, some fibril polymorphs can seed propagation of their particular structure from their soluble monomer (Xiao, et al., 2015) or cross-seed from soluble monomer of a different protein (O'Nuallain, et al., 2004). The property of self-recognition has been used to produce homogeneous fibril preparations of both *in vitro* polymorphs (Petkova, et al., 2005) and polymorphs seeded by amyloid fibrils from the brains of two AD patients (Lu, et al., 2013, Paravastu, et al., 2009) for solid-state NMR structure determination. Brain extracts from these two individuals seeded different synthetic A β fibril structures (Lu, et al., 2013). Seeding may provide an avenue by which conformational strains consistently propagate. The ssNMR structure of the human Iowa mutant D23N A β (1–40) assembled *in vitro* revealed an antiparallel organization of its cross β -sheets, like that found in protofibrils (Liu, et al., 2015), but unlike the parallel arrangement found in other full-length A β peptides (Tycko, et al., 2009). Normally, short A β peptides adopt the antiparallel structure, whereas the full length peptides form parallel β -strand structures (Balbach, et al., 2000, Lansbury, et al., 1995, Nelson, et al., 2005, Petkova, et al., 2004). A later study employing successive generations of seeded fibrils showed that, in subsequent generations of seeding, the descendent fibrils became all parallel β -strand, the more thermodynamically stable structure (Qiang, et al., 2011). A ssNMR study of the

successive stages of assembly of the wild type A β (1–40) peptide confirmed that the all-parallel β -strand arrangement was the most stable configuration (Potapov, et al., 2015).

7. Amyloid Polymorphism Impacts Ligand Binding

Ample evidence now supports the existence of A β fibril structural polymorphs (Agopian and Guo, 2012, Goldsbury, et al., 2005, Petkova, et al., 2004, Spirig, et al., 2014, Tycko, 2014, Tycko, 2015). In fact, the existence of polymorphic fibril strains is considered to be a common feature of amyloid-forming proteins (Eisenberg and Jucker, 2012, Toyama and Weissman, 2011). However, the influence of fibril polymorphism on ligand binding to A β has received little attention. There is both concern and untapped opportunity in these observations of differential binding of imaging ligands to polymorphic A β fibrils. The most immediate concern is the potential for confusion in the interpretation of imaging results obtained with different imaging agents that may bind differently to diverse molecular polymorphs of fibrillar A β . Without establishing the equivalence, or at least understanding the differences in binding, comparing pathology detection, disease progression, or efficacy of therapeutic interventions will lead to misunderstanding and unproductive disagreement.

In addition to the differential binding of distinct imaging agents to a common target, another source of potential confusion is the differential binding of the same imaging agent to β -amyloid deposits in different patients. The discovery of an AD patient with abundant A β yet negligible high-affinity binding of PIB (Rosen, et al., 2010) raises the question of whether there might be more subtle variations in high-affinity binding among the wider population of AD patients. In other words, while PIB has proven to be a useful tool for detecting the presence of A β deposits in most patients, exactly what it is telling us about the quantity and nature of the lesions remains to be clarified. The PIB signal may not be a quantitative indicator of the absolute amount of A β in the brain at all stages of disease progression.

8. Differences in Ligand Selectivity for Tauopathy

The most dramatic difference in ligand binding to misfolded protein pathology is observed with the microtubule-associated protein tau. The various tauopathies, of which AD is only one, clearly have different ligand binding characteristics (Fodero-Tavoletti, et al., 2014, Xia, et al., 2013). Whether this is due to differences in the expression, relative proportion, or assembly of the six isoforms of tau in the different diseases, to differential posttranslational modifications of tau species, or to a combination of factors is currently unclear. The ligand PBB3 has been shown to bind similarly to the lesions in multiple tauopathies (AD, Pick's disease, progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD)). Other ligands (THK523, T807, T808) bind to tau lesions in AD but not in Pick's disease, PSP, or CBD (Fodero-Tavoletti, et al., 2014). Screening for tau ligands using recombinant tau fibrils or transgenic APPSWE-Tau (huP301L) mouse tissue is reported not to be predictive of tau-selective compounds, while screening against tauopathy in AD tissue can identify selective compounds (Xia, et al., 2013).

9. Finding Selective Ligands for other Misfolded Protein Lesions

High-affinity α -synuclein-selective ligands have been difficult to develop, as designing probes using cell culture models and transgenic mouse models that develop α -synuclein aggregates has yielded ligands that do not bind to α -synuclein lesions in the human brain (Neal, et al., 2013). This is suggestive of differences between model systems and human α -synuclein pathology. A moderate affinity ligand, SIL23, binds to recombinant α -synuclein fibrils and to α -synuclein fibrils in postmortem tissues from an α -synuclein-transgenic mouse model and from human Parkinson's disease patients, although it also binds to tau pathology (Bagchi, et al., 2013). Agents also are needed to probe potential molecular polymorphism in other neurodegenerative lesions, such as those involving the heterogeneous ribonucleoprotein TDP-43 and FUS proteopathies.

10. It's Not a Bug, It's a Feature –Advantages of Differential Ligand Binding Selectivity

There is a positive aspect to the uncertainty created by differential ligand binding selectivity. The observation that small molecule ligands can detect differences among misfolded assemblies of the same protein is potentially enabling. The differences for A β are probably conformational, since relative ligand binding differences can be induced in pure, synthetic A β peptides by assembling them under different conditions (Mehta, et al., 2013). For tauopathy and other lesions, the impact of the variety of posttranslational modifications and splice variants on ligand binding needs to be determined. In the disease state, truncation, post-translational modifications, or the presence of auxiliary molecular components on the peptide also could contribute.

Classical prions are assemblies of misfolded prion protein (PrP) that cause fatal neurodegenerative diseases in several mammalian species, including humans (Prusiner, 1998). Functionally variant conformational polymorphisms of prions are referred to as 'strains', in analogy to infectious microbial variants of the same species (Jucker and Walker, 2013, Prusiner, 1982). Prions are infective, and appear to amplify in the brain by a templating mechanism that also has been proposed for the propagation of A β , tau, α -synuclein, and TDP-43 pathologies (Clavaguera, et al., 2015, Frost and Diamond, 2010, Guo and Lee, 2014, Jucker and Walker, 2013, Polymenidou and Cleveland, 2011, Smethurst, et al., 2015, Walker, et al., 2013, Walker and LeVine, 2012). Prions can derive from different conformations of the PrP protein with the same amino acid sequence (Prusiner, 2013). These different prion strains cause dissimilar prion diseases with distinctive pathologies, susceptible brain regions, and clinical symptoms (Aguzzi, et al., 2007, Collinge and Clarke, 2007, Fraser and Dickinson, 1973, Nilsson, et al., 2010). Oligothiophene ligands of defined chain length that bind to a variety of amyloid proteins and detect conformational differences (LeVine, et al., 2014) report the PrP strain differences in their fluorescence spectra (Magnusson, et al., 2014). Multiple prion strains infecting the same mouse brain can be distinguished *in situ* with these probes (Sigurdson, et al., 2007), and a recent study even found that rationally designed long-chain polythiophene ligands can extend the survival of prion-infected mice (Herrmann et al., 2015).

Oligothiophene probes also have shown that the molecular conformation of aggregated A β in a transgenic mouse model evolves during disease progression, and that selective binding *in vitro* as well as conformational reporting are achievable for this structural series of ligands (Nystrom, et al., 2013). This finding suggests that it may be possible to create a scheme to selectively stage the evolution of deposits in living humans, or to monitor the efficacy of a therapeutic regimen longitudinally with appropriately designed ligands whose binding affinity is dependent on the conformational state of a misfolded protein. Analogously, a particularly bioactive conformer of A β could be monitored to probe the biology of the activity, or targeted with a selective ligand.

Bringing these possibilities to fruition will require a more detailed understanding of the interactions of specific ligand structures with different fibrillar and oligomeric forms of misfolded proteins. The A β peptide is featured in this discussion of selective ligands because it has been intensively studied, the assemblies are abundant in the AD brain, and AD is increasing in prevalence in our aging population. Tau ligands are less well developed at this point but promising ligands are moving into the clinic. The situation with multiple isoforms and posttranslational modifications is significantly more complex for tau, but the introduction of new ligands for tau and other pathogenic proteins is a welcome addition to biomedical scientists' toolbox.

11. Probes of Early Assembly Stages of Misfolded Protein Pathologies

Soluble oligomers of A β may be significant toxic multimeric forms of the protein in the disease state (Larson and Lesne, 2012, Lesne, 2014, Lesne, et al., 2013). Oligomeric A β builds up in AD brain and animal models in a different fashion than A β plaques (Cleary, et al., 2005, Fowler, et al., 2014, Kaye, et al., 2003, Liu, et al., 2015). High-affinity small molecule ligands that bind to non-fibrillar oligomeric species of misfolded proteins but not to fibrillar species have yet to be described, although some reagents such as the oligothiophenes can react with both forms (Klingstedt, et al., 2011). The fluorescence spectra of the oligothiophenes bound to oligomers and fibrils are distinguishable, a feature that can be useful for histological studies where immobilized multimers are observed. Low concentrations of soluble oligomers and the lack of localized high concentrations of such small assemblies impede imaging applications. The wealth of non-overlapping, oligomer-specific monoclonal antibodies (Kaye, et al., 2010, Liu, et al., 2015, Murakami, 2014) indicates that multiple conformational states with different properties of these biologically potent assemblies exist and await ligand development.

Outside of imaging applications, probes of the early assembly stages of oligomeric misfolded proteins may provide insight into the mechanisms that lead to the assembly of the biologically active multimers. Such markers would be useful in identifying the features that govern the pathogenicity of the assemblies, and in detecting the trace amounts of these assemblies in accessible biofluids. They may also guide structural analysis of the different complexes and assist in the design of therapeutic interventions.

12. Future Applications of Differential Ligand Binding Selectivity

Polymorphism of A β and other misfolded proteins complicates the interpretation of binding and imaging data because it muddles the connection between classical pathology and functional changes. Until the different molecular forms of proteins can be clearly linked to particular biological events or processes, the power of these biomarkers to diagnose and predict disease is diminished. It is possible that the appearance or disappearance of different polymorphs could signal disease stages or the presence of certain biological activities, thus signifying the therapeutic effectiveness of an intervention. While current clinical applications of ligands are mainly limited to imaging proteopathy in the living brain, at least some of the same biomarkers may be accessible in biofluids (Fiandaca, et al., 2015, Kapogiannis, et al., 2015, Saman, et al., 2012). Evidence that polythiophenes can impede the progress of prion disease (Herrmann et al., 2015) underscores the therapeutic potential of small molecule ligands. Perhaps selective ligands could also be used to manipulate the conformational states of misfolded proteins in a receptor-like fashion to regulate pathophysiological processes.

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ABBREVIATIONS

AA	amyloid A
Aβ	amyloid β -peptide
AD	Alzheimer's disease
AL	Ig light chain amyloid
APP	amyloid precursor protein
APPSwe	APP mutation (Swedish) KM670/671NL; BS1; BS2; BS3 ligand binding sites on A β fibrils
BTA	benzothiazole aniline
CAA	cerebral amyloid angiopathy
CBD	corticobasal degeneration

CN-PIB	6-CN-PIB
FDA	US Food and Drug Administration
FDDNP	[2-(1-{6-[(2-[¹⁸ F]fluoroethyl) (methyl)amino]-2-naphthyl}ethylidene)malononitrile
FUS	fused in sarcoma
PET	Positron Emission Tomography
PBB3	2-((1E,3E)-4-(6-(methylamino) pyridine-3-yl)buta-1,3-dienyl) benz[d]thiazole-6-ol
PIB	Pittsburgh Compound B
PrP	prion protein
PSP	progressive supranuclear palsy
SIL-23	(3-iodoallyl)oxy-phenothiazine
SPECT	single photon emission computed tomography
ssNMR	solid-state NMR
TDP-43	TAR DNA-binding protein 43
[F]-THK523	[2-(4-aminophenyl)-6-(2-fluoroethoxy)quinoline
T807	7-(6-fluoropyridin-3-yl)-5H-pyrido[4,3-b]indole
T808	2-(4-(2-fluoroethyl)piperidin-1-yl)benzo[4,5]imidazo[1,2-a]pyrimidine

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Highlights

- The molecular structure of amyloid fibrils varies
- There are multiple distinct ligand binding sites on amyloid fibrils
- Specific ligand structures bind to different conformational states of amyloid
- Ligand binding to amyloid often differs between human disease and animal models
- Amyloid ligands can help to define strain-like variations in aggregated proteins