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# Tau protein aggregation: key features to improve drug discovery screening

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*Teaser:* From early drug design to clinical trials, what design and therapeutic advances have recently emerged for tau protein aggregation?

## Author biographies

### Johanna Giovannini

Johanna Giovannini obtained her PharmD in 2019 at the University of Caen Normandy and is now a third-year PhD student in molecular modeling and medicinal chemistry at the Centre d'Etudes et de Recherche sur le Médicament de Normandie (CERMN) in Caen in cotutorship with the Faculty of Pharmacy at the University of Bari. Her research focuses on the conception of protein–protein interaction disruptors and the understanding of early protein aggregation processes in Alzheimer's disease.

### Willy Smeralda

Willy Smeralda received his PhD from the University of Caen Normandy in 2019. After a postdoctoral period at CERMN, he is now a postdoctoral fellow at the Laboratoire de Biophysique Moléculaire aux Interfaces (LBMI), a member of the Teaching and Research Center (TERRA) and the BioEcoAgro Joint Research. His research focuses on ligand–membrane interactions, including peptides, proteins, lipopeptides, and molecules, in neurodegenerative diseases and coronavirus.

### Marie Jouanne

Marie Jouanne received her PhD from the University of Caen Normandy in 2015 and is now a postdoctoral fellow of CERMN. Her research interests include the development of protein–protein interaction disruptors and pleiotropic agents with medicinal chemistry, *in vitro* screening, and druggability assessment in oncology and neurodegenerative diseases.

### Anne Sophie Voisin-Chiret

Anne Sophie Voisin-Chiret has been a professor of medicinal chemistry at CERMN since 2015. Her research focuses on medicinal chemistry in the area of protein–protein interactions to design oligo(het)aromatic compounds that mimic proteins. Since 2012, she has been leading research studying protein–protein interfaces to design drug-like modulators of protein–protein interactions, especially in neurodegenerative diseases.

**Tauopathies are neurodegenerative disorders associated with the accumulation of abnormal tubulin associated unit (tau) protein in the brain. Tau pathologies include a broad spectrum of diseases, with Alzheimer's disease (AD) being the most common tauopathy. The pathophysiological mechanisms of AD are still only partially understood. As a consequence, attempts to establish therapeutic approaches have led to numerous clinical trial failures and, to date, no curative treatment is available for AD despite the considerable number of research programs. Therefore, over the past decade, the aggregation of the tau protein in AD has become a therapeutic target of interest. In this review, we gather *in silico*, *in vitro*, and *in vivo* methodologies that are relevant to assess compounds targeting tau aggregation, from early drug design to clinical trials.**

## Introduction

Tauopathies are disorders associated with the accumulation of abnormal tau protein in the brain.<sup>1</sup> Tau pathologies include a broad spectrum of diseases, such as AD, Pick's disease (PD), progressive supranuclear palsy (PSP), and frontotemporal dementia with Parkinsonism (FTDP-17), which are well classified and documented in recent reviews.<sup>2–4</sup> Since its discovery in 1975<sup>5</sup> and its identification in AD neurofibrillary tangles (NFTs),<sup>6</sup> the tau protein has become a major area of interest for therapeutic research related to neurodegenerative diseases.<sup>7</sup>

This major interest for tau protein is mainly explained by AD pathogenesis studies. Tau aggregated species and aggregated amyloid  $\beta$  (A $\beta$ ) peptides constitute the two main protein lesions of AD brains. Pathogenesis studies have shown that, over time, tau-aggregated toxic species have a better correlation with the progression of cognitive

impairments compared with the accumulation of A $\beta$  peptides into amyloid plaques.<sup>8</sup> Moreover, tau can be a pathogen agent without the presence of A $\beta$  toxic species. For instance, in other neurodegenerative diseases, such as PD or FTDP-17, neurodegeneration is caused by tau dysfunction.<sup>9</sup> Finally, tau appears to be essential for neurotoxicity because of its complex relation with A $\beta$  peptide. Indeed, A $\beta$  fibril exposure caused neuronal death of wild-type mouse neurons but not of the tau-knockout mouse neurons.<sup>8–12</sup> Tau can also be related to pathological processes, such as the inflammatory response or synaptic dysfunction. Last but not least, tau is believed to be responsible for disease propagation because of its prion-like properties.<sup>13–17</sup>

In this review, we summarize recent data on the development of compounds targeting tau aggregation. Other approaches, such as immunotherapies, have been reviewed by Cummings *et al.*, who presented the AD drug development pipeline in terms of new agents, targets, biomarkers, and trial design strategies.<sup>18</sup> The mechanisms and pathophysiological functions of tau are complex and remain under debate, but offer a range of therapeutic targets. After brief reminders of tau physiopathology, we present these data and discuss the key drug discovery challenges to developing effective therapeutic agents targeting tau aggregation.

### Generalities of tau protein physiopathology

Tau proteins, also called microtubule-associated protein tau (MAPT) are a group of six protein isoforms involved in the promotion and stabilization of microtubules (MTs). The gene encoding this protein is located on the long arm of chromosome 17 (17q21) and contains 16 exons that spans 100 kbp. In the central nervous system, the alternative splicing of exons 2, 3, and 10 results in six isoforms ranging from 352 to 441 amino acids in length, which are differentially expressed during brain development.<sup>19</sup> These isoforms are differentiated by the presence of three or four tubulin-binding sequences (repeated domains named R1–R4) in the C terminus of tau, and by the presence or absence of one or two inserts in the N terminus (Figure 1a). All these isoforms are not systematically involved in aggregates according to the tauopathy. A classification was proposed by Lebouvier *et al.* showing that these isoforms can be markers of the biochemical heterogeneity of tauopathies.<sup>20</sup> Tau protein is divided in functional domains separated by a proline-rich region (PRR): the MT-binding domain in the C terminus, which contains highly conserved sequences (R1, R2, R3, and R4), and the projection domain located in the N terminus, which extends to interact with other components of the cytoskeleton, such as actin or spectrin, and also surrounding plasma membranes.<sup>20,21</sup>

The MT-binding domain is characterized by the presence of two hexapeptides, PHF6\* (<sup>275</sup>VQIINK<sup>280</sup>) and PHF6 (<sup>306</sup>VQIVYK<sup>311</sup>) in the R2 and R3 domains, respectively (Figure 1a).<sup>21</sup> These two hexapeptides are the main focus of studies on tau aggregation because their removal leads to the disappearance of the aggregative properties of tau<sup>7,22</sup> and several mutations in these regions are known to affect the functional and aggregative properties of this protein.<sup>23,24</sup>

The main function of tau is the regulation of tubulin polymerization, although it has also been studied in terms of transcriptional regulation, DNA/RNA protective function, and signaling functions, especially at the postsynaptic level. These various functions can be associated with tau localization in the brain. Indeed, although most tau proteins are found in axons, a small amount can be found in dendrites, at subsynaptic sites, or in the nucleus.<sup>7,25,26</sup>

Tau is a phosphoprotein the longest form of which contains ~85 potential phosphorylation sites (serine, threonine, and tyrosine). Phosphorylation of tau regulates its binding to MTs and it is itself regulated by kinases and phosphatases, such as glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) and protein phosphatase 2A (PP2A), an essential dynamic for MT stability and neuronal transport.<sup>27</sup> In tauopathies, the balance between the action of kinases and phosphatases is disturbed, which leads to tau hyperphosphorylation, its detachment from MTs, and its aggregation (Figure 1b).<sup>1–3,26</sup> Indeed, many strategies linked to tau properties have been developed and are already well detailed in recent reviews: therapies targeting directly tau aggregation inhibition<sup>16,28–31</sup>; kinase or phosphatase activation inhibition<sup>31–34</sup>; MT stabilization and protein degradation<sup>31,35</sup>; pathological post-translational modifications (hyperphosphorylation, ubiquitination, acetylation, *N*-glycosylation, and truncated protein)<sup>31,36</sup>; and finally tau-directed immunotherapies, the main approach in AD and tauopathies clinical trials.<sup>31,37–40</sup>

### Developing therapeutics towards tau aggregation

NFTs were the first tissue lesions clearly identified in AD,<sup>41</sup> followed by senile plaques (SPs) formed by amyloid cascades. NFTs and SPs remain the two essential criteria for AD postmortem diagnosis. Both NFTs and SPs are associated with protein aggregation disorders.<sup>42</sup>

Regardless of whether the amyloid cascade and tau aggregation occur independently or successively, tau aggregation remains self-propagating and results in stable and highly neurotoxic aggregates (Figure 1b) and is also strongly correlated with cognitive decline. Therefore, pharmacological interventions aiming at the aggregation cascade itself appear as the most appropriate way to deal with NFTs, one of the most relevant AD lesions. The druggability of tau protein aggregation was first explored during the late 1990s with the repurposing of the antimalarial drug methylene blue in AD and FTD,<sup>43</sup> one derivative of which is involved in Phase III clinical trials.<sup>44</sup>

The complexity of tau protein aggregation as a therapeutic target lays in its structural features. Physiologically, tau proteins bind to MTs in monomeric intrinsically disordered protein (IDP) structure.<sup>45</sup> In AD, tau proteins separate from MTs and form different cross- $\beta$ -sheet aggregate structures, leading to filamentous NFTs (Figure 1b). A classic way to explain the transition from monomeric to aggregated tau is the nucleation–elongation mechanism. The nucleation step corresponds to the formation of early small aggregated species and the elongation step corresponds to the addition of monomers leading to polymers, notably paired helical filaments (PHF).<sup>46–48</sup> The

process initiating the PHF formation remains unclear. A classic hypothesis is the prion-like processing of tau protein, in which one tau protein undergoes a conformational change on its repeat domain, followed by another tau protein, until some crucial rate of misfolded tau protein is reached. Finally, once a certain amount of misfolded tau protein is reached, they form stable oligomers.<sup>49</sup> Those oligomers would, by themselves, induce tau protein misfolding in nonaltered neurons. Once misfolded aggregated tau proteins begin to misfold other tau proteins in other neurons, this process would occur exponentially, leading to an 'aggregation cascade'.<sup>50,51</sup> One advantage of this misfolding process is that physiological and pathological tau proteins are conformationally clearly defined. This offers a therapeutic opportunity: potential tau aggregation inhibitors (TAIs) would be selective toward the pathological form of tau protein and would not bind its physiological form. Thus, the main issue with tau aggregation is its time-related behavior. As previously explained, aiming at very early tau protein aggregation or at well-defined tau protein aggregates is aiming at two completely different targets. To date, little structural information is available for the physiological conformation of tau protein and for various reduced-size tau aggregates. For early tau protein aggregates (coming from the first step of dimerization), only *in silico* models with tau protein hexapeptides are available. Besides tau protein, targeting IDP is a challenge mainly because of the presence of various conformational assemblies.<sup>45</sup>

### Early drug conception and preclinical development

Given tau time-related structuration, it is necessary to define precisely what is aimed for in TAI development to use the right assessment methodology. When it comes to aiming at early tau aggregation (first dimerization steps), few investigation methods are available. By contrast, targeting preformed tau aggregates offers a range of various assessment techniques. Here, we review the literature relating to TAI development, from early drug design to preclinical and clinical drug development from 2010 to 2020 (Figure 2).

#### *In silico methodologies*

Computational-aided design for TAI conception, as for any active ingredient conception, widely relies on 3D structural data [X-ray crystallography, solid-state nuclear magnetic resonance (NMR), and cryo-electron microscopy (cryo-EM)] to predict aggregate stability-driving forces and potential hot spots for aggregation disruption.<sup>52</sup>

*In silico* techniques can help predict both early aggregation processes (starting with a monomeric state) and complete aggregated structures (i.e., nucleated and fibril states). This discipline is suited for small molecule and peptide design. Its weakness lies in the size of the considered system, which makes *in silico* methodology almost useless for antibody or vaccine design.

However, the use of *in silico* methodologies is still useful for tau protein aggregation study. Indeed, for tau protein monomers, conformation studies are impossible at high resolution because of their IDP properties. For aggregated forms of tau, several structures of PHF6 and PHF6\*, and extended PHF6 and PHF6\* are available from the Protein Data Bank (PDB: 2ON9 for PHF6; 6HRE or 5O3O for extended PHF6 or PHF6\*).

Recently, cryo-EM has provided advances in determining tau aggregate structures in different tauopathies (reviewed in <sup>53</sup>). In AD, Fitzpatrick *et al.* provided cryo-EM-resolved structures of different filaments sampled directly from the brains of patients with AD.<sup>54</sup> These showed that different isoforms of tau protein are incorporated into the filaments and that they differ compared with filaments extracted from other tauopathies.

Elucidating how the tau protein evolves from a disordered, soluble, and monomeric state to an aggregated state is one of the major challenges of AD research and could help the design of efficient curative drugs. Liu *et al.* reported an innovative way of elucidating the nucleation initiation.<sup>55</sup> From 16 PHF6 peptides randomly disposed in aqueous saline environment, they observed through a 1-ms molecular dynamics simulation how disordered PHF6 peptides tend to arrange together by forming parallel dimers. They provided hot spots to target for the rational design of therapeutics targeting the early aggregation process of tau protein. At the same time, Chen *et al.* used molecular dynamics simulation with a 16-residue tau-derived peptide to highlight how a mutation, P301L, influences the creation of a collapsed structure comprising PHF6.<sup>56</sup> Such insights provided by these types of *in silico* model is of major use for further simulations involving new potential early TAIs.

In terms of preformed aggregated structure modeling, the literature offers a range of investigations with direct applications to TAI design. *In silico* techniques provide two modalities of interest to this end: they can be used directly as a rational design support to perform structure-based drug design and to establish structure–activity relationship (SAR) studies.

In terms of peptide design, Sievers *et al.* and Pandey *et al.* used the PHF6 sequence and structure to directly design synthetic peptides able to covalently interact and prevent PHF6 aggregation, performing structure-based drug design.<sup>57,58</sup> The PHF6 sequence and structure have also been used to study the interaction between previously designed compounds and PHF6.<sup>59,60</sup>

For small-molecule design, various research groups have used PHF6 preformed aggregates to highlight how already known small molecules are able to disrupt tau protein aggregation.<sup>61–66</sup>

As previously mentioned, the use of *in silico* techniques depends on the system size compatible with computer modeling. This explains why, for aggregation process modeling as well as for TAI conception, only the use of reduced models of tau protein, particularly PHF6, has been possible thus far. However, the PDBk<sup>67</sup> also provides models of tau proteins larger than PHF6, predominantly provided by cryo-EM techniques,<sup>53,54,68</sup> which suggests

that it will be possible to carry out 3D simulations of potential new TAIs with more representative models of tau protein in the near future.

#### *In vitro methodologies*

Many investigational techniques are available for the *in vitro* assessment of potential TAIs. These can be divided into two types depending on whether they provide kinetic information or punctual structural information about tau protein aggregation.

Kinetic studies for TAI development are some of the most used proof-of-concept tools. The tau protein model for kinetic aggregation studies based on thioflavin T or S (ThT or ThS) fluorescence is the most common *in vitro* test for TAI development in the literature. It offers a fast assessment of the formation (or not) of tau protein aggregates through ThT/ThS fluorescence intensity, with ThS binding to amyloid fibrils but not monomers compared with ThT. This type of aggregation study can be used for most types of compound, including small molecules,<sup>61,62,64–66,69–81</sup> peptides<sup>57–60,82–85</sup> or plant extracts.<sup>86</sup> Precaution must be taken regarding such assays. If it is a quiet suitable routine assay to set up, false positive results can arise because of potential compound or aggregation inducers, such as heparin interactions with ThT/ThS. Moreover, ThS can be used to identify tau protein insoluble aggregates in a similar way to staining methods, such as Sarkosyl extraction or Gallyas silver staining, which are widely used to study extracts from hippocampal slices, cortical sections, or animal models of AD.<sup>87–89</sup> Besides fluorescence studies, isothermal titration calorimetry methodologies<sup>66</sup> and binding studies<sup>81,90</sup> can also support TAI development for aggregation and interaction kinetic studies (Figure 2).

When developing new TAIs, structural studies highlight the formation of tau protein aggregated species. Various methodologies offer information on structural modification of tau protein models.

High-resolution methods include: (i) microscopy,<sup>57,59,61,62,64,66,69,71,73–75,80–83,85,86,91–94</sup> whereby electron microscopes provide high-resolution images of punctual protein structures. For tau protein, this technique provides structure information for aggregated states (notably fibrils); (ii) NMR spectroscopy<sup>58,60,65,69,81,83,95</sup> and solid-state NMR spectroscopy<sup>96</sup>. Globally, NMR spectroscopy is a reference method in structural biology for the elucidation of structures that do not need any crystal acquisition; (iii) electrophoresis<sup>59,69,71,80,97</sup> is a convenient and well-known technique for size and weight-based protein structure separation; and (iv) X-ray crystallography.<sup>98</sup> This method, combined with NMR spectroscopy, is a key method for structure elucidation. However, X-ray crystallography and NMR spectroscopy offer only a few structures of tau protein at high resolution.

Low-resolution methods include: (i) circular dichroism,<sup>62,64,66,69,71,73–75,78,81,82,85,86,91</sup> a technique that uses the optical activity of protein to determine whether there is any secondary structure (helix and  $\beta$ -sheet), but without providing any location of the secondary structure; (ii) surface plasmon resonance (SPR),<sup>66,74,90,98</sup> which uses a surface-adsorbed substrate and measures the bonding of the substrate to other entities. This can be helpful for assessing the ability of adsorbed-aggregated peptides to bond ligands and can be monitored over time; (iii) dynamic light scattering<sup>57,69,84</sup> uses light intensity to assess protein (and its potential aggregate) size over time; and (iv) attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR),<sup>57,69</sup> which allows secondary structure elucidation after infra-red light absorption.

Similar to kinetic studies, structural studies of small molecules,<sup>61,62,64–66,69,71,73–75,78,80,81,92,95,97</sup> peptides,<sup>57–60,82–85,93,94</sup> antibodies,<sup>90,91,98</sup> or plant extracts<sup>86</sup> can provide evidence of their potential anti-aggregation effects (Figure 2) on either truncated or full-length tau protein.

*In vitro* methodologies are diverse and can provide varied information regarding the *in vivo* behavior of tau protein in the presence of potential TAIs. Compared with *in silico* methodologies, *in vitro* methodologies allow researchers to use full-length tau protein. Consequently, *in vitro* methodologies are a support to visualize potential TAI effects on more representative models of tau protein compared with *in silico* methodologies. However, truncated forms of tau protein are also widely used in *in vitro* characterization of tau protein aggregation.

*In vitro* methodologies represent an essential, if not a necessary, step toward TAI development to orient researchers to more advanced, more complex, and, therefore, more expensive models of tau protein aggregation: *in cellulo* and *in vivo* methodologies.

#### *In cellulo methodologies*

*In cellulo* techniques providing details on TAI effect are varied but are largely overlooked by immunological studies. Thus, we classify *in cellulo* methodologies by whether they constitute an ‘immunological assay’ or a ‘non-immunological assay’.

Immunological assays used in TAI development include: fluorescence-activated cell sorting,<sup>69</sup> immunoprecipitation assays,<sup>99,100</sup> immunoenzymatic assays (e.g., ELISA),<sup>59,84,91,98–101</sup> immunohistochemistry assays,<sup>59,75,80,90,91,97</sup> immune-staining assays,<sup>86,93,94,100,101</sup> immunoblotting assays,<sup>75,80,91,97</sup> or immunoglobulin titration.<sup>91</sup> Globally, these techniques highlight the formation (or not) of tau protein aggregates in the presence of potential TAIs. This kind of technique is suitable for most compounds, including small molecules,<sup>69,71,74,75,78,80,81,97</sup> peptides,<sup>57,59,85</sup> plant extracts,<sup>86</sup> vaccines,<sup>91</sup> and antibodies (Figure 2).<sup>90,98,99,101</sup>

Studies using non-immunological assays for TAI development often refer to non-specific assays highlighting a global health cell condition, such as cytotoxicity assays,<sup>57,71,74,78,97,100</sup> neuronal cultures,<sup>59,85,90,93,94,97–99</sup> or imaging assays.<sup>59,74,84,85,90,91,93,94,97,99,100</sup> Such techniques cannot be a proof-of-concept alone for TAI development and need to be taken into consideration alongside other TAI assessments (Figure 2). A popular cellular model of aggregation developed by Holmes *et al.* helps to predict tauopathy (more precisely, seeding activity) through flow cytometry.<sup>102</sup>

*In cellulo* methodologies require animal (and sometimes human) derived tissue, which is why such studies are often done jointly with *in vivo* methodologies. This methodology, as for *in vitro* methodologies, provides information on tau protein behavior in the presence of potential TAI. However, *in cellulo* methodologies also provide initial insights into the potential ability of the TAI to cross cellular barriers and take into account the complete cellular environment in terms of tau protein and TAI systems. This issue of environment-dependent behavior is vital to understand before a TAI can proceed to full *in vivo* testing.

#### *In vivo methodologies*

We classify *in vivo* methodologies on whether they reflect behavioral assessment in terms of either animal or physiology assessment.

Given that tau protein aggregation is widely neurotoxic and is strongly correlated with issues with memory, behavioral testing on animals for TAI development often relies on cognitive impairment tests, such as spontaneous alternation,<sup>100</sup> novel object recognition,<sup>100</sup> Morris water maze,<sup>78,85,86</sup> or the tail-hanging test.<sup>75</sup> Furthermore, because tau protein aggregation also affects motor coordination, behavioral testing for TAI development also includes motor ability assessment tests, such as limb clasping<sup>91</sup> and the Rotarod test.<sup>75,91</sup> Behavioral and motor coordination testing is suitable for small-molecule,<sup>75,78,80,97</sup> peptide,<sup>59,85</sup> plant extract,<sup>86</sup> vaccine<sup>91</sup> and antibody<sup>90,98,99,101</sup> assessments (Figure 2).

Physiology studies for TAI development include diverse techniques, such as transgenic overexpression<sup>59,75,80,85,90,98,100,101</sup> or wild-type tau protein studies.<sup>86,91,97,101</sup> Globally, those techniques embrace direct and indirect drug effect assessment on animals and provide information about the response of tau protein aggregation as well as non-tau proteins to TAI. Therefore, these studies are suitable for small molecules,<sup>75,80,97</sup> antibodies,<sup>90,98,99,101</sup> peptides,<sup>59</sup> or plant extracts (Figure 2).<sup>86</sup>

*In vivo* techniques are important in terms of proof of concept for the development of any active ingredient. However, when referring to TAI development, this kind of assessment cannot be separated from other assessment methodologies. It is also necessary to take into account the choice of the model, which is usually a transgenic mouse model,<sup>103–106</sup> which has limitations that require practical considerations, as described by Jankowsky *et al.*<sup>107</sup>

#### *Clinical development*

Compared with the preclinical landscape of TAI development, occupied by small molecules and peptides, the clinical development landscape is largely taken up by antibodies (e.g., JNJ-63733657,<sup>108</sup> BIIB076,<sup>109</sup> Lu AF87908,<sup>90</sup> PNT001,<sup>31</sup> gosuranemab,<sup>98</sup> zagotenemab and semorinemab,<sup>110</sup> and ABBV-8E12<sup>101</sup>) and vaccines (e.g., ACI-35<sup>91</sup> and AADvac1<sup>111</sup>). NPT088 is the only representative of fusion proteins currently involved in a clinical trial for tau protein aggregation.<sup>100</sup> To date, only two small molecules have reached clinical trials for direct tau protein aggregation inhibition: LMTM or a Methylene Blue derivative (TauRX Therapeutics) currently in Phase III trials (Figure 2).<sup>97</sup>

### **Design of new active ingredient: drug design strategies**

#### *How to design new molecules targeting tau protein aggregation with drug design tools*

Based on our analysis of data concerning TAIs, we propose both a concise scheme of the drug discovery process for such compounds (Figure 3a), and a decision tree that can serve as a simplified guide for TAI development (Figure 3b).

Many different assessment techniques are available for revealing a tau aggregation-related effect. Some assessments are necessary to move a TAI through the drug discovery process. When starting with a group of potential TAI compounds, high-throughput methodologies are required to highlight activity toward tau protein aggregation. To this end, *in silico* tools provide only reduced-length tau protein models of anti-aggregation activity. In terms of *in vitro* tools, kinetic fluorescence studies using ThS/ThT are a fast and an easy-to-use technique to provide a first proof of action, as long as the potential TAI does not interact in any way with ThS/ThT. If a potential TAI interacts with ThS/ThT, then a structural study (microscopy, spectroscopy, etc.) must show evidence of tau aggregation activity before the potential TAI can be moved on to preclinical studies. Once evidence is provided of tau aggregation activity, the TAI can undergo more complex tests (*in cellulo* and *in vivo* assessments) to provide more detailed information on its mechanism of action and pharmacokinetic and pharmacodynamic parameters. Then, if all the parameters are encouraging (activity and nontoxicity), then the potential TAI can be moved to clinical trials (Figure 3).

#### *Small-molecule development*

We gathered structural information on small-molecule development targeting tau protein aggregation (Figure 4). Three main scaffolds emerge as scaffold-leading small-molecule designs: curcumin-based; naphthoquinone-based; and methylene blue-based derivatives.

Curcumin (Figure 4) is a compound bearing a double ketone alkenic system containing two phenolic moieties. With its two ketones on an alkene chain, curcumin exists in a keto-enol equilibrium. Pharmacomodulation of curcumin over the past decade led to a variety of analogs. All curcumin derivatives in Figure 4 went through chemical simplification with the conservation of one phenol fragment carrying a carbon chain, itself carrying a hydroxyl (usnic acid **1**<sup>78</sup> and resveratrol **5**<sup>80</sup>), a carbonyl (nitrocatechol derivative **2**<sup>62</sup>, xanthohumol **3**,<sup>81</sup> oleocanthal **4**<sup>95</sup>, Okuda's curcumin derivative **6**<sup>76</sup>, rosmarinic acid **8**<sup>61</sup>, morin **9**<sup>80</sup>, acridone analog **10**,<sup>74</sup> and shogaol-huprine

hybrid **11**<sup>77</sup>), or a carbonyl bioisostere imine (PE859 **7**<sup>75</sup>). This common trait is shown in pink in Figure 4. Curcumin derivatives then went through various medicinal chemistry modulations. The whole structure of compound **1** is condensed on a dibenzofuran basis, which holds the chemical scaffold into one unique conformation. Compounds **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, and **11** have undergone expansion of the chemical structure. Compound **2** is expanded with nitrogen entities, making it more water soluble than curcumin because of the ionizable nitrogen. Compound **4** is the product of esterification of the phenolic ethanol fragment and elenolic dialdehyde acid. Compound **8** corresponds to the dihydroxyphenylacetic acid curcumin fragment esterified by salvianic acid. Both compounds **4** and **8** are  $\pi$ -conjugated systems but in a smaller size compared with curcumin, and their ester moiety provides them with a retarded-metabolism action. Compounds **3**, **5**, **7**, and **9** are expanded by carbon chain cyclization into either a phenol (**3**, **5**), indole (**6**), pyrazole (**7**), or benzopyranone (**9**). This modulation provides the chemical structure with a more rigid conformation and makes it more resilient to metabolism.

Polyphenolic compounds have attracted much interest through their diverse properties (particularly their antioxidant, anticancer and anti-inflammatory actions).<sup>112</sup> Given that polyphenolic compounds are able to trigger oxidative stress,<sup>113</sup> they are now considered as potential modulators of degenerative disorders.<sup>114</sup> They also appear to have a strong role as anti-amyloidogenics. Polyphenolic compounds can take part in  $\pi$ - $\pi$  stacking and H-bonding interactions with residues constituting  $\beta$ -sheet aggregated structures. Therefore, curcumin and its derivatives naturally constitute one of the predominant scaffolds among small molecules targeting tau protein aggregation.<sup>115</sup> However, curcumin scaffolds are pan-assay interference compounds (PAINS) scaffolds,<sup>116,117</sup> indicating that curcumin can bind covalently to proteins, chelate metals, show oxidizing-reducing activity, inhibit aggregation process, disrupt membranes, and interfere in fluorescence assays simultaneously.<sup>118</sup> Thus, the activity of curcumin derivatives toward tau aggregation could also be linked to their false-positive testing behavior rather than to an authentic aggregation inhibition capacity. Several different biological evaluation processes must be performed to attribute a given activity to a real desired effect (from *in vitro* to *in vivo* testing).

The second class arising from small molecules with activity against tau aggregation are 1,4-naphthoquinone-derived compounds. Naphthoquinone is an aromatic bicyclic compound derived from naphthalene. All naphthoquinone derivatives detailed in Figure 4 show structural growth but with a conserved naphthoquinone basis (morin **9**,<sup>80</sup> acridone analog **10**,<sup>74</sup> naphthoquinone hybrid **12**,<sup>64</sup> purpurin **13**,<sup>66</sup> and rhein-huprine hybrid **14**<sup>79</sup>). This common trait is represented in orange in Figure 4. Compound **12** is a naphthoquinone entity hybridized with tryptophan through a simple carbon–nitrogen bond. Given that tryptophan is a precursor of several neurotransmitters and is able to cross the blood–brain barrier (BBB), this hybridization might result in this molecule having various capabilities in terms of physicochemical and biological properties, such as enhanced bioavailability and druggability. Compound **13** is a tricyclic molecule bearing a naphthoquinone entity with a phenol condensed on it, providing both naphthoquinone and polyphenolic capabilities on a single scaffold. Compound **14** is hybridized to an acridine Methylene Blue-based moiety through a peptide linker, which is easily removed through metabolism and which provides two entities with anti-aggregation activity. This property could be related to the multitarget-directed ligand strategy.<sup>119</sup>

Similar to polyphenolic compounds, this class has several properties that are well documented in human health. The first is blood coagulation, because quinone compounds are in the center of fat-soluble vitamin K structures and have a role in antiparasitic therapy<sup>120</sup> and osteoporosis.<sup>121</sup> Several derivatives (benzoquinones, naphthoquinones, and anthraquinones) have proven action against amyloid fibril formation.<sup>122,123</sup> However, similar to the curcumin scaffold, both the naphthoquinone scaffold and quinone motif are listed as PAINS scaffolds.<sup>117</sup> Even though naphthoquinone derivatives appear able to interact with tau aggregates with  $\pi$ - $\pi$  stacking and H-bond interactions,<sup>124</sup> their PAINS scaffolds could lead to aberrant identification of leads and, thus, crossed biological tests are required before any hit-to-lead considerations.

The third class of small molecules are perhaps the most classic with activity toward tau protein aggregation: Methylene Blue derivatives. Methylene Blue is one of only two small-molecule scaffolds to reach clinical trials in terms of direct tau aggregation inhibition. Methylene Blue is a dimethylanilinothiazine. Its derivatives in Figure 4 were subjected to either chain expansion or hybridization. Compounds **11**, **16** (naphthyridine-chlorotacrine hybrid<sup>125</sup>) and **14** (rhein-huprine hybrid<sup>79</sup>) are Methylene Blue-expanded structures of acridine with a chlorotacrine moiety (compound **16**) or a naphthoquinone-based fragment rhein (compound **14**). Compound **15** (palmatine chloride<sup>73</sup>) is a condensed tetracyclic structure bearing a shifted nitrogen compared with an authentic acridone scaffold.

Similar to the two small-molecule classes previously detailed, this class has other effects on human health in addition to its antiaggregation capabilities, mainly as a neuropsychiatric disorder therapy<sup>126</sup> and malaria therapy.<sup>127</sup> However, the Methylene Blue scaffold is not a PAINS compound, which might explain why it has been entered into clinical trials.

Acridone derivatives (compound **10**) are hybrids of the three chemical scaffolds previously listed: it bears a phenol conjugated to an alkene chain, such as curcumin; it carries a simplified naphthoquinone skeleton; and it comprises a tricyclic acridine scaffold, such as Methylene Blue.

Other small molecules with activity against tau aggregation represent multiple and diverse scaffolds: (i) compound **17** (phenylthiazolyl-hydrazide inhibitor<sup>63</sup>), compound **20** (NO-donor thiocarbocyanine<sup>92</sup>), and compound **21** (thiophene dye<sup>72</sup>) are thiophene-based (**21**) or thiazoline-based scaffolds (**17** and **20**); (ii) compound **18** (depside tenuiorin<sup>65</sup>) is a polyester structure; (iii) compound **19** (linolenic acid<sup>71</sup>) is an omega-3 fatty acid; (iv) Compound **22**

(dantrolene<sup>70</sup>) is an already-marketed structure repurposed for its involvement in multiple mechanisms against protein aggregation; and (v) compound **23** (PcTS<sup>69</sup>) is a porphyrin, a class well known for inhibiting protein aggregation.<sup>128</sup>

Globally, these compounds all bear either an aromatic cycle, or a pi-conjugated system. This common feature could be key for tau aggregation inhibition.

#### *PROTACs as a new tau aggregation inhibition therapies*

Proteolysis targeting chimeras (PROTACs) are bi-functional small molecules able to target one protein and one ubiquitin ligase enzyme simultaneously. By targeting these two partners at the same time, a PROTAC allows their interaction, resulting in the targeting protein to be both ubiquitinated and degraded. The PROTAC proof-of-concept was first demonstrated in 2001 by Crews, Deshaies and coworkers, with potential applications in cancer therapeutics via angiogenesis inhibition.<sup>129</sup> For neurodegenerative diseases, and more precisely for tau protein, PROTAC research is more recent. In 2018, Lu *et al.* designed a peptide PROTAC able to target tau protein and keap1, an ubiquitin-proteasome system target.<sup>130</sup> PROTACs targeting tau protein is a promising new approach for neurodegenerative therapy: low quantities of active ingredient are necessary, which is an advantage for any therapeutic molecule. However, it might encounter some pitfalls: the larger the molecule is, the more poor-solubility and permeability issues it might experience. In addition, as mentioned earlier, the tau protein shows different behaviors depending on whether it is disordered or aggregated. Thus, PROTAC molecules targeting the tau protein should be well balanced so that only its pathological form is targeted.

#### **Key points for tau physiopathological studies**

Most clinical trials that have followed the development of anti-aggregating compounds have not yielded any convincing therapeutic benefit, especially in the case of A $\beta$  aggregation.<sup>131</sup> Few reports have focused on the causes of these failures, with most proposing that the initial assumption, the amyloid cascade, was probably wrong. However, it is important to take advantage of this acquired experience to increase the chances of developing effective drug candidates. In particular, it is possible to make improvements from not only a technical aspect, but also in terms of understanding concepts linked to the issues surrounding such compound development. For example, the choice of appropriate methods according to the development stage of the compound and the results obtained lay the foundations of a rational approach to the design of the drug.

Cryo-EM results have shown that a molecular signature exists in the different tau protein filaments, and that they differ according to the disease in which they are involved.<sup>132</sup> In addition, tau protein monomers do not appear to have the same capacity to enroll in aggregation in terms of the environment in which they occur.<sup>133</sup> These two concepts highlight the need to increase our understanding to develop successful approaches to treat tau protein aggregation. However, *in vitro* studies with amyloids require specific technical precautions, which often appear lacking.<sup>134</sup> Unwanted interference of amyloid with the experimental environment, complex isolation of the toxic species, or incomplete knowledge of amyloid physiology widely delay research advances in TAI development (Table 1). Moreover, although there are some physicochemical differences between A $\beta$  and tau (e.g., the *in vitro* ability of A $\beta$  to self-aggregate versus the need to add a co-factor, such as heparin or RNA for tau), the different points specified for A $\beta$  can be adapted to tau with regard to its specificity.<sup>135</sup> Thus, we propose leads and key points that, in our opinion, are part of the answer to the technical issues detailed in Table 1. These include complete homogenization during sample preparation rather than laboratory-related methodologies, the use of mutants instead of wild-type peptides, or multimodal analyses for interaction studies rather than monomodal ones (Table 1).

However, even by taking maximum precautions from a technical point of view, it is also necessary to bear in mind that data interpretation can be biased by many parameters (heterogeneity of samples, different populations in solution, artefacts, etc.) (Table 1). In addition, the impact of physiological parameters, such as the redox effect, should systematically be taken into account when analyzing the results, in particular because of the potential role of cysteines in tau protein aggregation.<sup>136–138</sup>

In terms of conceptual issues, the most important outcome is the fact that clinical trials are probably done when the disease is too advanced. This issue highlights the importance of improving the early detection of AD. This involves the precise characterization of known biomarkers, the establishment of standards to be able to collect and corroborate more data, and the addition of new biomarkers to the AD biomarker toolbox, as described by Blennow *et al.*<sup>139,140</sup>

Moreover, it appears necessary to include the plurality of AD, whether for diagnostics development or for therapeutic approaches. As an example, early-onset AD (EOAD) has been described as having more effects on executive, visuospatial, and motor functions and displaying less memory loss compared with late-onset AD (LOAD).<sup>141</sup> Given that AD is a multifactorial disease (aging, genetic alterations, biochemical and environmental factors), it appears important to not only develop a precise and early diagnostic test, but also to be able to classify different subtypes of AD according to the factors involved and to apply adapted therapeutic approaches, as recently described by Golde *et al.*<sup>142</sup> (Figure 5).

The last important concept to keep in mind is the amyloid aggregation itself. Hypotheses are constantly evolving regarding the species responsible for toxicity (fibers versus oligomeric species).<sup>131</sup> Amyloid aggregation is also a complex process involving different pathways, such as the dependence ('on' pathway) or not ('off' pathway) of the formation of intermediates within the fibrillation process.<sup>143</sup> Thus, pharmacologists should not look only for



inhibitors, but also modulators, of aggregation, with the objective of promoting pathways that do not lead to toxic species formation.

### Concluding remarks

TAI development is a key research approach for developing effective AD and tauopathy treatments. If small compounds and peptides widely account for the early TAI development spectrum, it is clear that they fail to move into preclinical and clinical studies. So far, drug discovery for tauopathies treatment remains challenging at every development step. Early development step methodologies suffer: (i) the lack of full-length suitable 3D models for *in silico* efficient predictions; (ii) the probable interference of tested compounds with the tau protein environment rather than with the tau protein itself in *in vitro* and *in cellulo* assessments; and (iii) the multifactorial aspects of AD and tauopathies that make the effects of tested compounds difficult to attribute to a single tau aggregation stopping effect in *in vivo* assessments.

Finally, because of tau protein localization, compounds under development have to be designed that are able to cross the BBB. This is a major challenge and might be a main reason for the failure of promising drug molecules. Other early-development issues might also be plausible explanations for clinical trial failure. We have also listed several solving-problem approaches that we hope could explain the lack of *in vivo* efficacy and to upgrade compounds that might have failed before but could be modified structurally and/or in terms of their administration to become potential TAIs. In addition, we have presented conceptual issues to highlight key points of drug candidate development for therapeutic purposes in the context of AD and other tauopathies.

### Authors' contributions

All authors contributed to writing the manuscript and have given their approval to the final version.

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**Figure 1.** Title. (a) Tau protein structure. (b) Tau protein aggregation general mechanism.

**Figure 2.** Overview of tau aggregation inhibitors (TAIs) detailed in the literature between 2010 and 2020. (a–c) Methodologies used to characterize a potential TAI. (d) TAIs described in the literature<sup>57–66,69–86,92–95,99,125</sup>, classified by their type and by how they were characterized if known [refer to the symbol color related to colors used in (a–c)]. When there is no information on the characterization method, the symbol was kept in black.

**Figure 3.** Title. (a) Drug discovery process steps. (b) Decision tree for TAI development. Abbreviations: ThS, thioflavin S; ThT, thioflavin T.

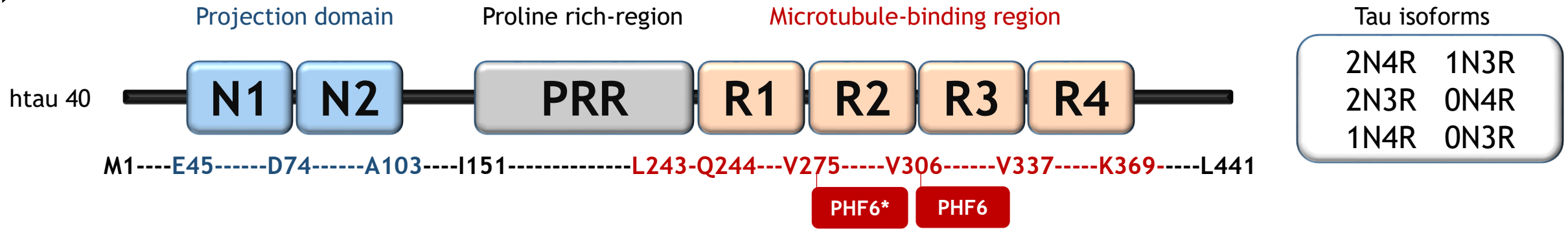
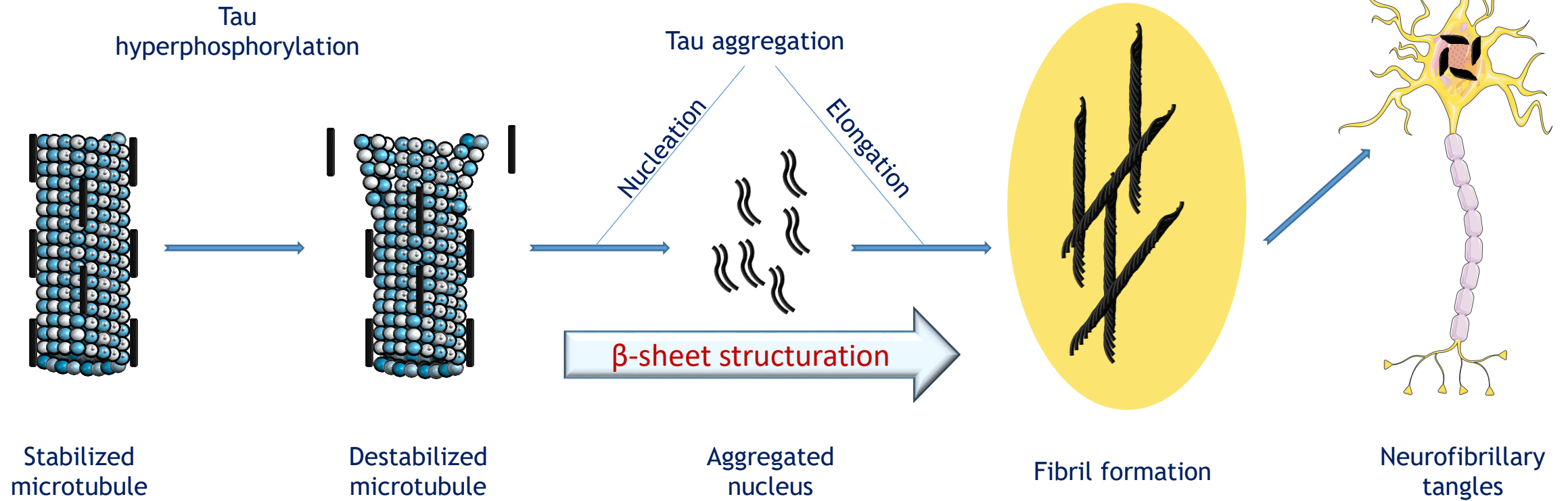
**Figure 4.** Summary of small molecules with a direct inhibitory action of tau protein aggregation published between 2010 and 2020.<sup>57,59-62,67-79,87,90,112</sup>

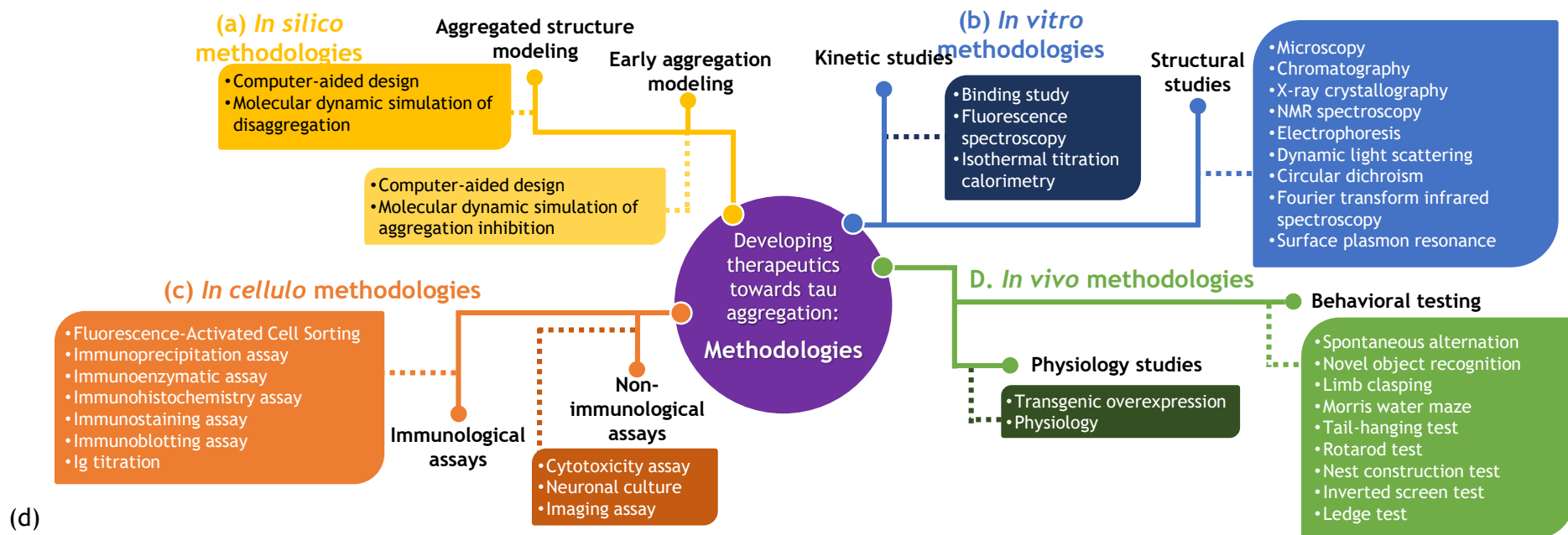
**Figure 5.** 'A framework for selecting the right time for the right drug'. Adapted from <sup>142</sup>.

**Table 1. Technical and conceptual issues associated with an amyloid therapeutic approach<sup>a</sup>**

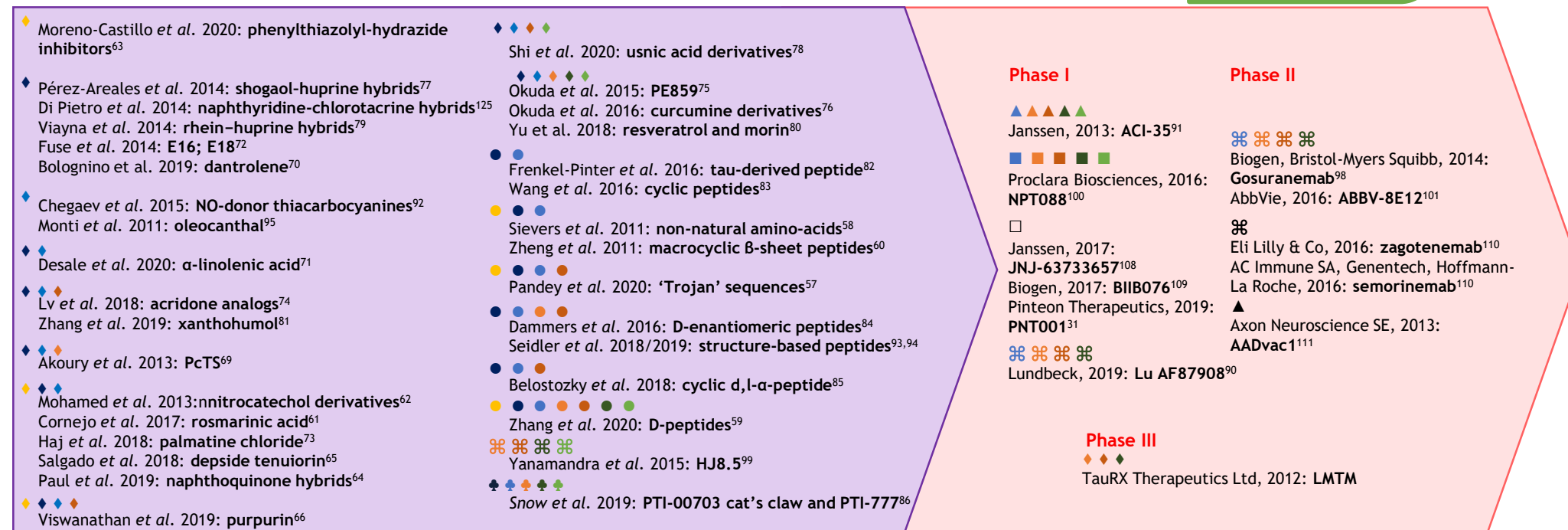
Issue	Problem-solving approach
Peptide concentrations used in models are too high ( $\mu\text{M}$ versus $\text{nM}$ or less in human brain)	Use of low concentration with adapted methods (microplates, ATR-FTIR, AFM)
Low reproducibility of experiments (nonspecific interactions, well-defined sample preparation is needed)	Sample homogenization, impact of physicochemical parameters (buffer, redox effect, temperature, agitation, presence of metal ions, neuronal membrane, dye effect), use of low-binding material to develop experiments with amyloid
Tested drugs can interfere with fluorescence of probes commonly used to monitor $\text{A}\beta$ peptide aggregation, such as ThT/ThS	Multimodal analysis with methods such as DLS
Toxic oligomeric species are poorly defined and complicated to isolate	Purification of toxic species, use of mutants
Microscopy images might not be sufficiently representative of peptide populations	More complete analysis; keep in mind that the observation might not be representative of all populations
Heterogeneity of nonfibrillar aggregates	
Results depend on $\text{A}\beta$ isoform studied ( $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ )	
Small molecules can only weakly bind to random coil $\text{A}\beta$ monomers	Better identification of species analyzed in interaction with the molecules (DLS, ITC, microscopy, fluorescence, AFM...)
Besides $\text{A}\beta$ , many other proteins are involved in AD (tau protein)	More physiopathological studies
Animal models are not sufficiently representative of AD	
Human genome-wide studies have not yet revealed all genes involved in AD	
No available high-resolution structure of $\text{A}\beta$ or tau protein interacting with key cell players	
New APP metabolites, such as $\text{A}\eta\text{-}\alpha$ and $\text{A}\eta\text{-}\beta$ , are not yet fully characterized	
Lack of target/substrate specificity and of efficiency of developed compounds	Adapted <i>in vitro</i> studies; multiparametric analysis; more demanding specifications
Clinical trials too late in the disease process	Earlier clinical trials; development of efficient diagnosis

<sup>a</sup>Abbreviations: AFM, atomic force microscopy; DLS, dynamic light scattering; ITC, isothermal titration calorimetry.

**(a)****(b)**

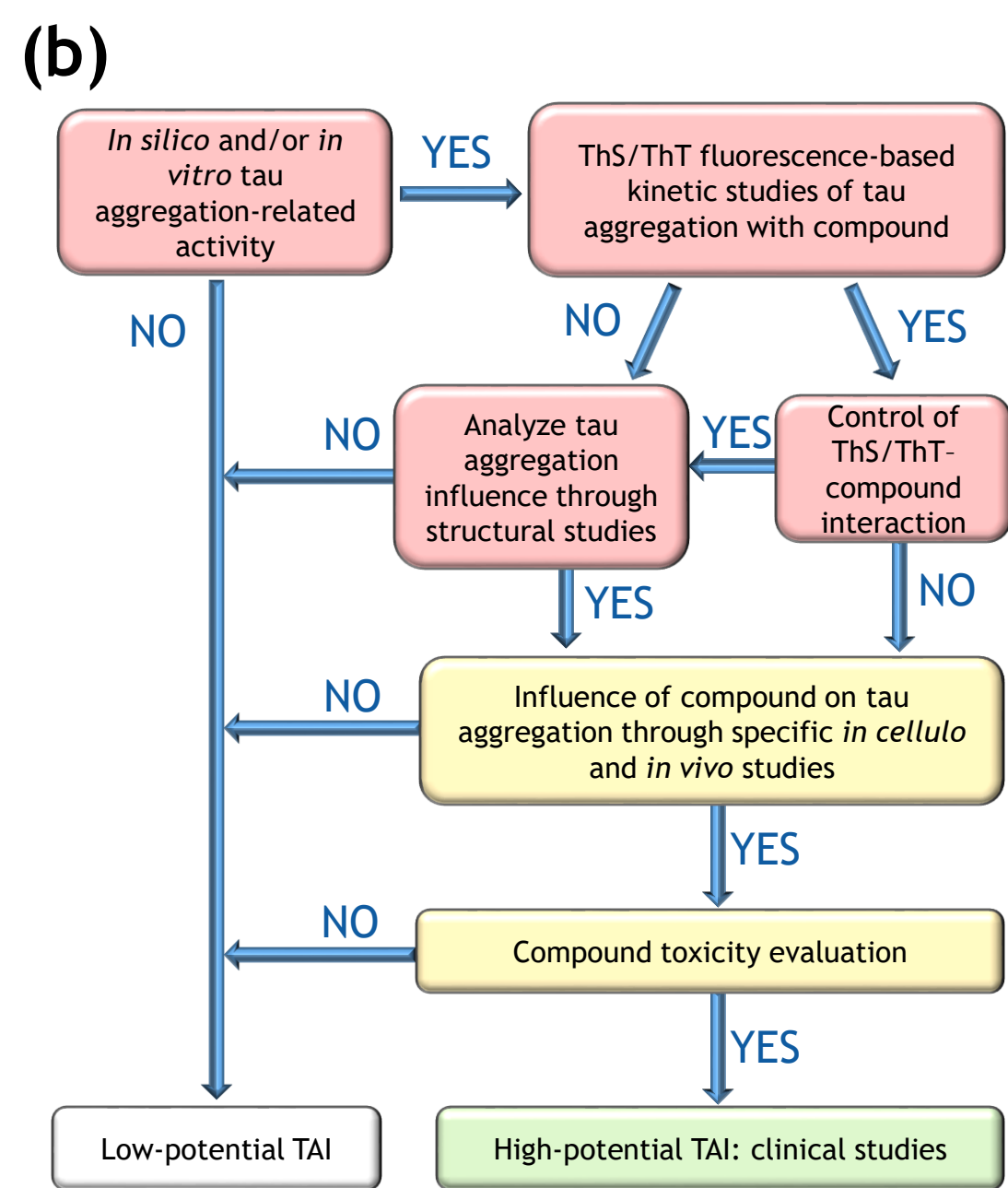
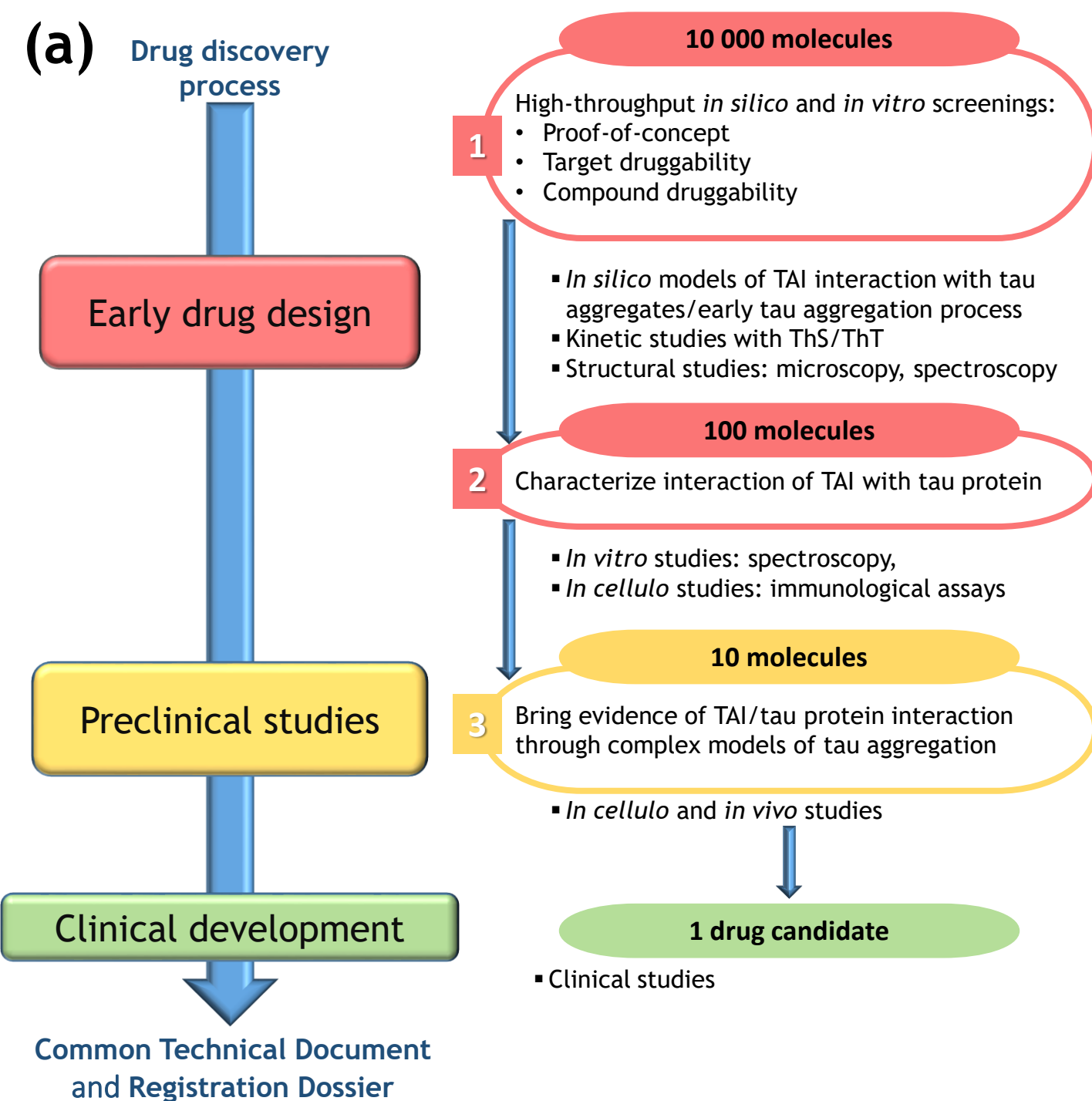


(d)



**Early drug conception and preclinical development**      **Clinical development**

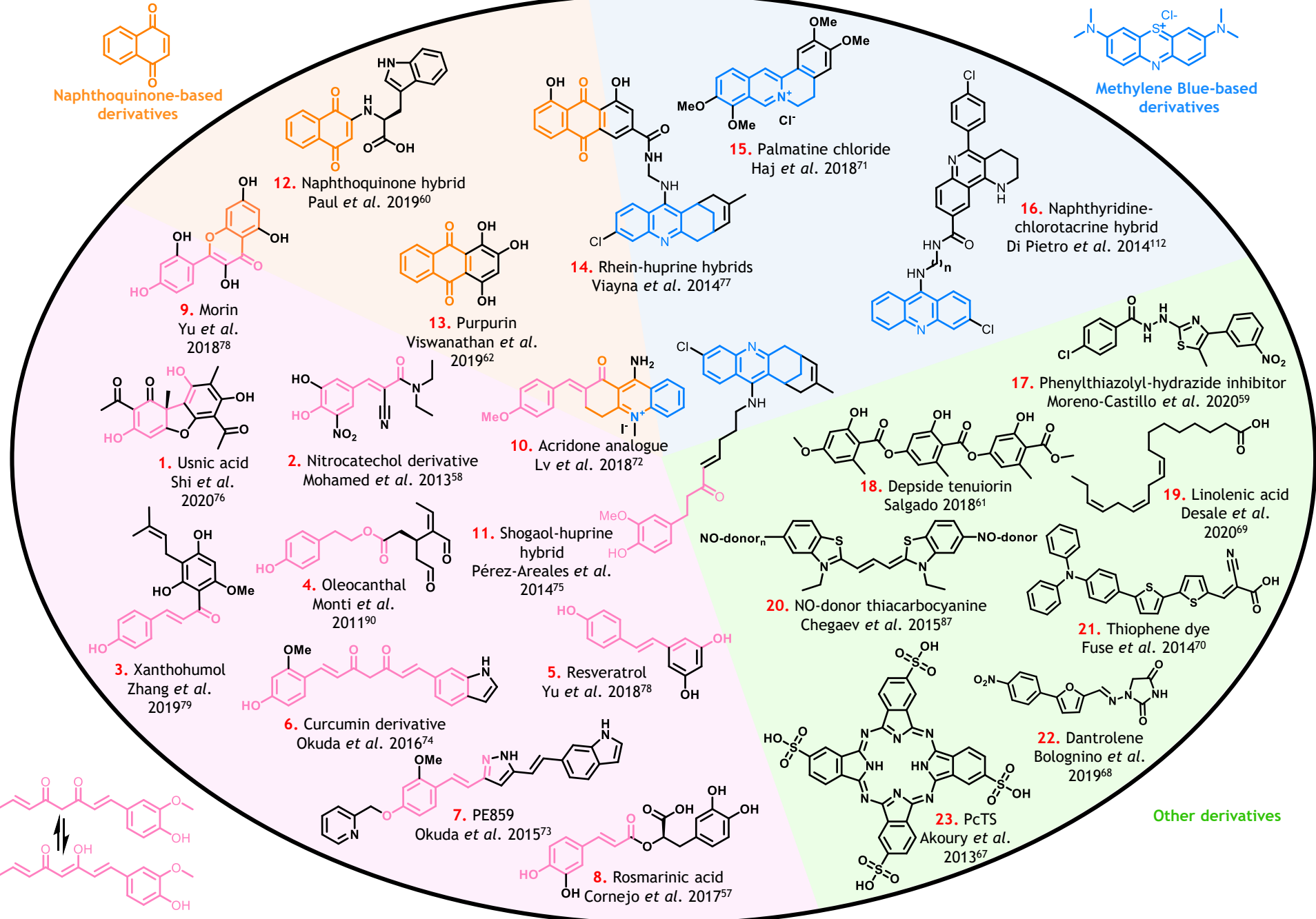
⌘ Antibody      ■ Fusion protein      ▲ Vaccine      ◆ Small molecule      ● Peptide      ♣ Plant extract





**Naphthoquinone-based derivatives**

**Methylene Blue-based derivatives**



**Curcumin-based derivatives**

**Other derivatives**

5.

