Drug targets for amyloidosis

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Abstract

The amyloid hypothesis indicates that protein misfolding is at the root of many neurodegenerative disorders. Small molecules targeting the formation, clearance, aggregation to toxic oligomers or SOD (superoxide dismutase)-like activities of Aβ (amyloid β-peptide) 1–42 have provided encouraging candidates for AD (Alzheimer’s disease) medicines in animal models, although none have yet proved to be effective in human trials. We have been investigating approaches to treat systemic amyloidoses, conditions that show common features with some CNS (central nervous system) disorders. For TTR (transthyretin) amyloidosis, we are seeking small molecule compounds that stabilize the amyloidogenic protein and either prevent its structural transition to the crossed β fibres deposited in diseased tissues, or promote its clearance from circulation. Effective stabilizer compounds that simultaneously bind to both thyroxine-binding sites have been developed. A more generic approach involves targeting the plasma glycoprotein SAP (serum amyloid P component). This protein recognizes the misfolded polypeptide structures of amyloid deposits wherever they occur, and acts as a powerful anti-opsonin. We have developed a bivalent drug called CPHPC ((R)-1-6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]-pyrrolidine-2-carboxylic acid) that cross-links pairs of pentameric SAP molecules and causes their rapid elimination from the circulation. This strategy raises the prospect of encouraging natural mechanisms to clear amyloid and recent work suggests that this approach extends to the CNS.

The ideal drug development project involves the elucidation of pathological biochemical or cellular processes in order to identify and target influential molecules; although this ideal can rarely be followed. Rather the pressing need for effective medicines directs efforts towards a myriad of emerging hypotheses based on the latest experimental data from a variety of sources.

The association of amyloid formation with neurodegenerative conditions such as AD (Alzheimer’s disease), where synaptic failure is a central issue, has captured the attention of drug developers in recent years [1]. This especially followed the reproduction of tissue pathology in the brains of transgenic mice carrying the gene for human amyloid precursor protein, including a mutation linked with early onset AD [2]. Guilt by association for Aβ (amyloid β-peptide) 1–42, initially in fibre form but more recently in a cytotoxic soluble oligomeric form, has spurred investigations of active and passive immunization, aggregation inhibitors and, at the extremes, Methylene Blue, curcumin, green tea and coffee [3]. In the systemic amyloidoses, it is clearer that the burden of deposited fibre mass is a substantial contributor to organ failure [4]; however, the possibility that organs are much sturdier than neurons to toxic effects of misfolded protein has yet to be ruled out. Pre-fibrillar TTR (transthyretin) aggregates are toxic to neural cells [5], however, large quantities are present in a number of organs without recognized cyttotoxicity [6]. Apart from this unresolved toxicity issue, there are a number of similarities between neurological and systemic diseases involving amyloidosis. Mutations giving predisposition to disease are common in all amyloidoses as is the failure of the immune system to remove amyloid deposits. Protein misfolding, fibre formation and the decoration by SAP (serum amyloid P component) are also common [7].

We have been searching for new drug compounds that interfere with the deposition of systemic amyloid involving (i) stabilizing or clearance of a specific precursor protein in the TTR amyloidoses, and (ii) developing CPHPC ((R)-1-6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]-pyrrolidine-2-carboxylic acid), a drug that strips SAP from amyloid deposits and removes it from the circulation, leading to the subsequent natural clearance of amyloid in mice [8].

TTR amyloidosis

TTR is the normal plasma protein that transports thyroid hormone and retinol-binding protein. It is inherently amyloidogenic and forms microscopic amyloid deposits in the elderly [9]. Massive deposits in the heart can also occur causing fatal senile cardiac TTR amyloidosis. The amyloidogenicity of TTR is considerably enhanced by most of the reported >80 different point mutations that encode single residue substitutions in the TTR sequence [10]. These mutations cause autosomal dominant adult onset hereditary amyloidosis, a fatal condition affecting about 10 000 patients worldwide. The usual clinical presentation is familial amyloid
polyneuropathy with amyloid deposition in peripheral nerves, but there is often also serious damage to the heart, kidneys and eyes. The condition is usually diagnosed after the gene has been transmitted to offspring, ensuring persistence of the disease. TTR amyloidosis predominantly affecting the heart is particularly associated with the V122I variant, which is very rare in Caucasians but is carried by 4% of African-Americans including 13,000 individuals homozygous for the mutation [11]. It is the second most common pathogenic mutation in that population after sickle cell haemoglobin. Cardiac TTR amyloidosis causes progressive heart failure and is usually misdiagnosed as coronary heart disease.

TTR is a homotetramer of $M_r 14,500$ subunits of 129 amino acids. It is synthesized in the liver and the choroid plexus, and circulates at a concentration of 1.6–3 mg/ml in plasma. The native three-dimensional structure of the TTR subunit is made up mostly of $\beta$ structure organized as a highly twisted two-layered sandwich [12]. Two pairs of subunits interact via an intermolecular extension of these sheets between two-fold axis related molecules bound head-to-head. Two such dimers come together to form the tetramer with the hormone-binding site between them (Figure 1). Fibre X-ray diffraction work of Blake and co-workers suggested that the TTR fibre was composed of an extended $\beta$-helix with polypeptide strands running nearly normal to the fibre axis [13]. However, others have suggested a more explicit involvement of a native-like TTR dimer as a repeat motif for fibre assembly [14]. Incorporation of nitroxide spin labels into edge strand side chains has provided evidence that at least part of the inter-subunit $\beta$-sheet of native TTR is maintained in TTR amyloid but that a strand on the other side of the sheet must be displaced to enable a new interface for the docking of the next subunit [15,16]. The crystal structure of a highly amyloidogenic TTR triple mutant has shown a packing arrangement of TTR tetramers in a trigonal unit cell, where crystal symmetry generated a double-helical $\beta$-helix reminiscent of that derived from the fibre diffraction experiments [17]. A conformational adjustment where the register of an edge strand shifts by three residues (a $\beta$-slip) to create a novel inter-subunit contact is believed to be of central importance to the particular packing observed. Amyloid formation is believed to involve destabilization of the TTR homotetramer, leading to the formation of an amyloidogenic monomeric species that subsequently aggregates to form the pathologically significant fibres.

One approach to the potential treatment of TTR amyloidosis initiated by Kelly and co-workers has been to identify small molecule ligands that are specifically bound in the thyroid hormone-binding pocket. These stabilize the native TTR tetrameric structure and fold in order to prevent protomer formation and subsequent aggregation [18]. Although this method of stabilization prevents TTR binding to its natural ligand thyroxine, this approach is not harmful due to the presence of other thyroxine carriers in the serum. A number of research groups have followed broadly similar paths of synthesizing focused libraries of compounds targeting the thyroxine-binding site, using crystallography to image the bound conformation and guide further attempts to fill the space in the hormone-binding site [19]. The relative affinity of these compounds can be estimated in competition assays of radioactive thyroxine displacement. Extensions of such assays in serum enable the selection of candidates that are not bound efficiently by plasma proteins, a phenomenon that may not be ideal for an effective medicine. True affinity for TTR is measured by isothermal titration calorimetry (when drug solubility permits) while the ability of compounds to stabilize TTR tetramers can be estimated by differential scanning calorimetry estimates of protein denaturation temperature. Assays showing a compound’s ability to reduce the aggregation and fibre forming tendency of TTR at reduced pH takes us a step closer to estimating the likely efficacy of a compound in reducing amyloid formation (S.E. Kolstoe, P.P. Mangione, V. Bellotti, G.W. Taylor, G.A. Tennent, S. Deroo, A.J. Morrison, A.J.A. Cobb, A. Coyne, M.G. McCammon, R. Gill, M.D. Smith, S.V. Ley, C.V. Robinson, S.P. Wood and M.B. Pepys, unpublished work).

Targeting the binding site of a natural ligand in drug development has long been a prominent approach but brings with it the need to design away from natural activities. In the case of TTR it is quite important that stabilizers are not able to displace thyroxine from the major carrier protein, TBG (thyroxine-binding globulin), as drug-induced release of this powerful hormone would probably have severe consequences. Likewise it is important that potential drugs do not activate receptors for thyroxine. Ideally drugs should be delivered in tablet form and the active constituents efficiently absorbed into the circulation where they should not be degraded too quickly or in such a way as to generate toxic metabolites. The accumulated experience of medicinal chemistry can point to paths through this range of hurdles but adds additional constraints to the design process. For instance, the Lipinski rule tells us that we should also keep

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**Figure 1** | TTR tetramer with Fx-1006a bound in the hormone-binding site
The structure of Fx-1006a is shown on the right. Figure prepared using PyMOL (DeLano Scientific, http://www.pymol.org).
Figure 2 | Schematic diagram of the two TTR hormone-binding sites on either side of the protein

Ligand ‘headgroups’ that bind in each hormone-binding site are joined together by a ‘linker’, resulting in a small molecule that binds simultaneously to both hormone sites. Such compounds bind irreversibly, prevent protomer dissociation, and inhibit amyloid deposition by depleting the pool of protein precursors. Reprinted with permission from [24]. © 2003 American Chemical Society.

the molecular mass low, not have too many hydrogen bond donors and acceptors, and appropriate polarity for efficient absorption [21].

Recent studies have shown that halogenated biarylamine compounds have high affinity for the binding pocket in TTR and can stabilize the tetrameric form of the protein and prevent dissociation and subsequent aggregation [22]. This approach has been used by Kelly and co-workers to develop a novel drug compound, Fx-1006a (Figure 1), which is currently in Phase III clinical trials [23]. Numerous groups are working on parallel drug design projects. Our specific approach is to examine the possibility of small molecule ligand cross-linking in order to stabilize or promote the clearance of TTR. We have revisited the concept of simultaneous occupation of both thyroxine sites (Figure 2) [24] within the TTR tetramer with some success (S.E. Kolstoe, P.P. Mangione, V. Bellotti, G.W. Taylor, G.A. Tennent, S. Deroo, A.J. Morrison, A.J.A. Cobb, A. Coyne, M.G. McCammon, R. Gill, M.D. Smith, S.V. Ley, C.V. Robinson, S.P. Wood and M.B. Pepys, unpublished work), producing compounds that bind less rapidly than thyroxine but dissociate very slowly indeed. These compounds inhibit aggregation of TTR very effectively and in spite of their size and chemical character behave in a very drug-like manner during initial pharmacokinetic characterization.

SAP and CPHPC

SAP is a pentameric plasma glycoprotein of $M_r$ 25,000 subunits first identified as the pentagonal constituent of in vivo pathological amyloid deposits [25]. SAP can make up 14% of the dry mass of amyloid and is thought to decorate and stabilize fibres, inhibiting their removal by normal scavenging mechanisms [26]. This association is utilized in the clinical diagnostic technique of SAP scintigraphy where radiolabelled protein is injected into patients to locate areas of amyloid deposition [27]. SAP is a member of the pentraxin family, characterized by calcium-dependent ligand binding and a distinctive flattened $\beta$-jellyroll structure similar to that of the legume lectins [28]. Human SAP has 51% sequence homology with CRP (C-reactive protein), a classical acute phase response plasma protein, and is a more distant relative of the ‘long’ pentraxins such as PTX3 and several neuronal pentraxins [7]. No deficiency of SAP or polymorphism has been reported in humans. It is synthesized primarily by hepatocytes, although there is evidence that both CRP and SAP mRNA are present in pyramidal neurons of the brain [29]. SAP is highly resistant to proteolytic cleavage in the presence of calcium, while its protomers are tightly associated, requiring boiling in SDS or other strong denaturants to separate [7]. Each protomer is composed of two anti-parallel $\beta$-sheets with short connecting loops and an $\alpha$-helix forming the ‘A’ face of the protein, while a calcium-binding site is located on the opposite side of the molecule, referred to as the ‘B’ face. The calcium-binding site consists largely of polar residues forming a long irregular loop thought to be responsible for amyloid recognition and binding. SAP is present in human serum at a concentration of 40 mg/l, remaining fairly constant and never exceeding 100 mg/l during an acute phase response, despite a corresponding increase in CRP levels of over 1000-fold. It forms stable pentamers in physiological conditions [30] but in the absence of calcium (or in the presence of the chelator EDTA) forms decamers as two pentamers interact face to face. Larger ‘stacks’ of SAP have also been observed on the surface of amyloid fibres [31] while even longer strings of decamers have been observed using electron microscopy in the absence of calcium [32]. The calcium-dependent binding of SAP on to amyloid fibres stabilizes the deposits against proteolysis and attack by phagocytic cells, and contributes to the invisibility of amyloid to the immune system [26]. In order to intervene
in this process we have developed CPHPC (Figure 3), as a competitive inhibitor of SAP binding to amyloid fibrils [8]. The molecule was discovered from a high throughput screen of competitors for SAP binding to immobilized Aβ 1–42 and consists of two D-proline residues joined by a six-carbon aliphatic linker. The palindromic nature of this molecule results in each CPHPC binding to two different SAP pentamers, resulting in a 1000-fold increase in binding affinity compared with a single D-proline ligand. X-ray crystallographic studies of the resulting complex have confirmed a complex consisting of two SAP pentamers cross-linked by five CPHPC molecules. Trials of the compound found that this binding also occurs \textit{in vivo} and causes rapid clearance of SAP by the liver and a remarkable depletion of circulating SAP levels (which are normally tightly regulated), resulting in a redistribution of SAP from the amyloid deposits into the circulation. Because of this novel mechanism, CPHPC does not require direct access to the affected tissues in order to exert an effect and thus may be applicable to all forms of amyloidosis. Recent results have shown the rapid depletion of SAP in the CSF (cerebrospinal fluid), suggesting the use of this compound as a novel treatment for all types of amyloid-related neurological pathologies [33]. SAP binds well to o-phosphothreonine, and multipoint attachment through this ligand may explain why SAP decorates hyperphosphorylated tau in neurofibrillary tangles.

This work provides examples of both rational design and screening methods to identify lead compounds. In both cases, target binding is developed through detailed understanding of the structure and properties of the target and the small molecule, although considerable gaps still remain in our knowledge about the underlying biology and how these compounds might function \textit{in vivo}. With TTR the rate of biosynthesis is clearly regulated and molecules that stabilize and prolong the lifetime of the protein will probably ramp down this process perhaps producing unforeseen effects. Conversely drugs that accelerate TTR clearance may switch on biosynthesis and perhaps the overproduction of protein, although hopefully clearance and degradation will occur at a quicker rate than dissociation and pathological aggregation. Interestingly, TTR is present in the CSF where it is reported to interact with Aβ and retard its progression to the fibre state [34]. TTR does not appear to form clinically relevant amyloid in the CSF where it is a major carrier of thyroxine, however, the protein’s interaction with Aβ suggests it may become a potential drug target.

Regarding SAP, we do not know if it’s binding to amyloid is an accident that potentiates amyloid formation or if the protein has evolved to recognize misfolded proteins and escort them towards insoluble and perhaps inactive deposits. While it is clear that CPHPC does lead to amyloid clearance in mice, it does not appear to be of sufficiently high affinity to completely strip SAP from fibres in humans in the face of ongoing biosynthesis of SAP. Interestingly a number of homologues of SAP and its close relative CRP are found throughout the nervous system and participate in modulating plasticity through effects on the complement system. We have shown that CPHPC enters the CSF and lowers SAP levels in this compartment [33]. Small-scale trials have given confidence that there are no detrimental affects of CPHPC in AD patients or those suffering systemic amyloidosis, and in some cases clear benefit has been derived.

References
20. Reference deleted

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