Drug Development Based on the Metals Hypothesis of Alzheimer’s Disease

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Abstract. The recent report of positive results from a Phase IIa clinical trial of PBT2, a novel drug that targets amyloid-β-metal interactions, underscores the value of abnormal transition metal metabolism as a potential therapeutic target in Alzheimer’s disease. The Metals Hypothesis of Alzheimer’s disease is based upon observations of the precipitation of amyloid-β by zinc and its radicalization by copper. Both metals are markedly enriched in plaques. The Hypothesis involves the perturbance of these endogenous brain metals, and it does not consider toxicological exposure part of pathogenesis. Recent descriptions of the release of ionic zinc and copper in the cortical glutamatergic synapse, modulating the response of the NMDA receptor, may explain the vulnerability of amyloid-β to abnormal interaction with these metal ions in the synaptic region leading to aggregation and fostering toxicity. Increasingly sophisticated medicinal chemistry approaches are being tested which correct the abnormalities without causing systemic disturbance of these essential minerals. PBT2, clioquinol and related compounds are ionophores rather than chelators. PBT2 is a once per day, orally bioavailable, second generation 8-OH quinoline derivative of clioquinol. It has performed very satisfactorily in toxicology and Phase I clinical trials and is advancing as a disease-modifying candidate drug for Alzheimer’s disease.

Keywords: Alzheimer’s disease, amyloid-β, clinical trials, copper, iron, metals, oligomer, oxidation, zinc

INTRODUCTION

While an involvement of amyloid-β in the pathogenesis of Alzheimer’s disease (AD) is almost indisputable, considerable evidence indicates that Aβ is not the sole culprit in the pathogenesis of AD. This problem is central to the ability to target disease-modifying drugs against the disorder as there is no certainty as to how Aβ accumulates or how it induces dementia. Aβ is neurotoxic at non-physiological (micromolar) concentrations in vitro, but it is also produced in health [127] and, at physiological (nanomolar) concentrations, is neurotrophic in cell culture [155,156,158].

Synthetic Aβ1–42 appears to be more self-aggregating than Aβ1–40 in solution [63,72]. This led to the canonical “Amyloid Cascade Hypothesis”, where the mere generation of Aβ is regarded as the cause of the disease. But the self-aggregating properties of Aβ are insufficient to explain the association of the peptide with AD. Indeed, there is considerable evidence that the soluble, but not the fibrillar, forms of Aβ correlate with both mortality, and dementia-associated neuropathological features like tangles and neuritic changes [84, 92,153]. However, not all forms of soluble Aβ are toxic, since healthy people without AD normally have soluble Aβ in their brains, and Aβ is a soluble component of all biological fluids. Therefore, there may be abnormally modified forms of soluble Aβ that are toxic in AD.

Based upon the implication of Aβ as the culprit protein in AD, the major approaches for developing therapeutics for AD have attempted either to prevent Aβ...
production (secretase inhibitors) or to clear Aβ (immunotherapy). However, there is strong evidence that other neurochemical reactions apart from Aβ production must contribute to amyloid formation in AD. Were elevated cortical Aβ concentrations solely responsible for the initiation of amyloid, it would be difficult to explain why the amyloid deposits are focal (related to synapses, and the cerebrovascular lamina media) and not uniform in their distribution since Aβ is ubiquitously expressed. To attribute amyloid initiation to the presence of Aβ₁₋₄₂ alone is problematic since the peptide is a normal component of healthy cerebrospinal fluid (CSF) [152]. Finally, amyloid deposition is an age-dependent phenomenon, and if Aβ production does not increase with age, other age-related stochastic neurochemical changes would play an essential role in the reaction that causes Aβ to accumulate. The age-dependent changes are closely associated with oxidative damage to neuronal cells, which precedes Aβ deposition [108–110].

We first observed in 1994 that Aβ becomes amyloidogenic upon reaction with stoichiometric amounts of Zn²⁺ and Cu²⁺ [22,23]. In subsequent years, it has become clear that Aβ is a metalloprotein [45,111], whose combination with the brain’s intrinsic supply of Cu²⁺ and Zn²⁺ (and possibly Fe³⁺) mediates the toxicity of the peptide (through radical and hydrogen peroxide production) and aggregation. On the basis of these findings, we embarked on a program to develop a new class of drug therapy with the major collaborators being Rudy Tanzi, Rob Moir, Colin Masters, Robert Cherny, Kevin Barnham, Robert Cappai, and myself, in partnership with Prana Biotechnology Ltd. Our efforts have lead to the completion of two Phase II clinical trials in AD, and we currently have a favorable candidate for large-scale clinical trials.

**METALLOCHEMISTRY MEDIATES THE AGGREGATION AND TOXICITY OF AMYLOID-β**

Aβ is rapidly precipitated by Zn²⁺ [22,23,58]. Cu²⁺ and Fe³⁺ also induce marked Aβ aggregation but only under mildly acidic conditions (e.g., pH 6.8–7.0) [22,23,58], such as those believed to occur in AD brain. Cu²⁺ induces greater precipitation of Aβ than Fe³⁺, and even the trace (nanomolar) concentrations of Zn²⁺, Cu²⁺ or Fe³⁺ in common laboratory buffers is sufficient to induce nucleation of Aβ, which can then lead to fibrillization of the peptide solution [9,11,68]. Significantly, rat/mouse Aβ has amino acid substitutions that decrease metal interactions [23], perhaps explaining why these animals are exceptional among mammals for not forming cerebral Aβ with age [151]. Aβ possesses selective high and low affinity metal binding sites, which are histidine-mediated [11,40,145].

The original reported Kᵅ of high affinity Zn²⁺ binding was ≈ 100 nM, and for low affinity binding ≈ 5 μM [22,23]. Over the years there has been some contention about the exact Kᵅ values for both Zn²⁺ and Cu²⁺, but it is now understood that both the buffer conditions (e.g., the presence of NaCl [69]), the aggregation state of the peptide [22,55,73], and the means used for assaying the bound and free metal ions [148] are critical for the observed values. However, a consensus has emerged that the μmolar concentrations of both Zn²⁺ and Cu²⁺ that are released from cortical synapses are sufficient to induce Aβ aggregation [58,73,137,148]. Low affinity Zn²⁺ binding mediates the precipitation of the peptide, as well as its resistance to tryptic (α secretase-like) cleavage [22]. Aβ also possesses high and low affinity Cu²⁺ binding sites [9,11]. Although the affinity of the low affinity Cu²⁺ binding site is similar between Aβ₁₋₄₀ and Aβ₁₋₄₂ (5.0 × 10⁻⁹ M), the apparent affinity of the high affinity site on Aβ₁₋₄₂ has been reported as 7.0 × 10⁻¹⁸ M, which may be the product of a perturbed equilibrium caused by precipitated Aβ withdrawing Cu²⁺ from solution. This is much greater that the highest observed affinity of Aβ₁₋₄₀ for Cu²⁺ (5.0 × 10⁻¹¹ M) [11]. The higher affinity of Aβ₁₋₄₂ than Aβ₁₋₄₀ for Cu²⁺ correlates with enhanced precipitation of Aβ₁₋₄₂ by Cu²⁺ [9,11], increased SDS-resistant dimerization of Aβ₁₋₄₂ by Cu²⁺ [9,9], and with increased redox activity and toxicity of the Cu²⁺:Aβ₁₋₄₂ complex (see below).

Aβ binds equimolar amounts of Cu²⁺ and Zn²⁺ at pH 7.4, but under conditions representing acidosis (pH 6.6) Cu²⁺ completely displaces Zn²⁺ from Aβ [11]. Aβ binds up to 2.5 equivalents of either Cu²⁺ or Zn²⁺, the fractional stoichiometry indicating that metal binding is possibly coordinated by oligomers [11]. This would have implications for utilizing hexafluroisopropanol, a popular means of monomerizing Aβ in vitro. The positive cooperativity in Cu²⁺ binding observed for Aβ may be greater for Aβ₁₋₄₂ than for Aβ₁₋₄₀ because of the enhanced ability of the longer peptide to form a Cu²⁺ coordinating oligomer [36]. Intriguingly, the apolipoprotein E isoforms prevent metal mediated aggregation of Aβ in a manner that correlates with their risk for AD [98]. Importantly, the precipitation of Aβ by Zn²⁺ and Cu²⁺ is reversible with chelation [9,27,66,69], in contrast with fibrillization, which is irreversible.
Cu, Fe and Zn play more of a role than merely assembling Aβ. We also found that when binding Cu²⁺ or Fe³⁺, Aβ reduces the metal ions and produces H₂O₂ by double electron transfer to O₂ (there is no evidence of O₂ formation as an intermediate) [111], a reaction that has since been repeatedly confirmed [44,105,146]. This electrochemistry is critical for Aβ-induced oxidative stress and toxicity in cell culture and partly mediated by methionine 35 [5,30] and tyrosine 10 [14,59]. H₂O₂ is also formed catalytically by the cycling of Cu or Fe bound to Aβ using biological reducing agents as electron donors without net oxidation of the Aβ peptide. The most likely electron donors pathologically are cholesterol and long-chain fatty acids [59,103,105,111,117,129], consistent with the toxicity of Aβ being mediated by adherence to the cell membrane [30] and the consequent production of toxic lipid oxidation products oxyysterols and 4-hydroxynonenal (HNE), which are elevated in affected brain tissue in AD and in amyloid-β protein precursor transgenic mice [59,105,111,117,129]. Aβ promotes the Cu-mediated generation of HNE from polyunsaturated lipids, and in turn, HNE can covalently modify the histidine side chains of Aβ [102]. HNE-modified Aβ has an increased affinity for lipid membrane and an increased tendency to aggregate into amyloid fibrils [102]. Thus, the prooxidant activity of Aβ leads to its own covalent modification and to accelerated amyloidogenesis. Catecholamines are also oxidized by Aβ:Cu complexes [38,39,111].

These reactions are important because there is overwhelming evidence in the literature for oxidation injury in AD that is mediated by H₂O₂ and peroxidation products. H₂O₂ is freely permeable across all tissue boundaries and will react with reduced metal ions (Fe²⁺, Cu⁺) to generate OH by Fenton chemistry, which in turn, generates lipid peroxidation adducts, protein carbonyl modifications, and nucleic acid adducts such as 8-OH:guanosine, in all cellular compartments, which typify AD neuropathology [89,130,131]. In AD, the H₂O₂ scavenging defenses, e.g., catalase and glutathione peroxidase, may be overwhelmed by the catalytic generation of H₂O₂ from the Aβ metalloprotein mass. The redox activity (metal reduction, OH and H₂O₂ formation) of Aβ variants is greatest for Aβ42human > Aβ40human > Aβ40mouse ≈ 0 [70], which is a strikingly relevant relationship since Aβ1-42 is most involved in AD and is overproduced as a consequence of some mutations that cause familial forms of AD. This redox relationship also corresponds to the neurotoxicity of the respective peptide in neuronal culture, which is largely mediated by the Cu²⁺:Aβ interaction [70,111]. Notably, the interaction of Aβ with the cell membrane is promoted by binding Cu²⁺ and Zn²⁺ [36,37]. Conversely, Cu and Fe chelators like TETA and clioquinol (CQ) block these electrochemical reactions and attenuate Aβ toxicity in cell culture [1,117].

Aβ coordination of Cu leads to the generation of reactive oxygen species (ROS) involving the reduction of the coordinated Cu²⁺ to Cu⁺ and when this reduction is not accompanied by the oxidation of another moiety, such as cholesterol [117], Aβ side-chains can become oxidized. This can lead to a variety of oxidized Aβ species, and to covalent oligomerization. Mass spectrometry has shown that Cu²⁺ ions are able to oxidize Aβ and NMR data shows that the most likely candidate being the sulphur atom of methionine 35 [5]. In addition to methionine sulfoxide, a number of other adducts resulting from Cu-mediated redox actions can be generated. Products include aldehyde adducts to the lysine residues [26] and tyrosine modified with adducts such as DOPA, dopamine, dopamine quinone, dihydroxyindol and isodityrosine [4,5]. 2-oxo-histidine adducts of Aβ have also been extracted from AD plaques [95] as have N3-pyroglutamate modified forms of Aβ that are the main ligands for the amyloid PET ligand PIB [87]. Tyrosine is particularly susceptible to free radical attack due to the conjugated aromatic ring. Elevated dityrosine and 3-nitrotirosine within the neuronal lesions in AD brain have been reported. In vitro Aβ42 in the presence of Cu²⁺ and H₂O₂ forms dityrosine cross-linked oligomers, a modification that is resistant to proteolysis [10]. The formation of dityrosine linkage in Aβ facilitates further peptide aggregation, leading to the formation of higher order oligomers [14]. In addition, Aβ radicals formed after reduction of Cu can form covalent adducts onto other proteins. Peroxidases like cyclooxygenase 2 (COX2) are particularly vulnerable because of the formation of dityrosine bridges, and indeed we found that levels of COX2-Aβ covalent complexes are elevated in AD brain [104].

While diverse hypotheses have been proposed for the mechanism by which Aβ induces its neurotoxic effects, the one point of consensus shared by most hypotheses is the requirement for Aβ to aggregate. Nearly every conceivable aggregate from dimer to fully formed fibrillar structures have been reported as toxic. In general the various aggregates have been poorly characterized, although there is a growing interest in soluble oligomers, which appear to be particularly toxic [81]. It is possible that covalent crosslinking of Aβ (e.g., dityrosine for-
mation generated by Cu oxidation) contributes to the formation of these toxic soluble species [14].

Interactions with metal ions may explain the increased involvement of soluble oligomeric species in pathology. Zn\(^{2+}\) and Cu\(^{2+}\) more readily precipitate A\(\beta\) oligomers, but monomeric A\(\beta\) is relative resistant to precipitation [22,55]. One recent report describes how the N-terminal region of A\(\beta\) can access a range of metal-coordination structures, and that there is a correlation between A\(\beta\)-Cu\(^{2+}\) coordination, peptide self-assembly, and neuronal viability [46].

**ZINC AND COPPER INDUCE AMYLOID-\(\beta\) AGGREGATION IN VIVO**

AD is characterized by the deposition of amyloid plaques, the major constituent being A\(\beta\) that is cleaved from the membrane-bound amyloid-\(\beta\) protein precursor (A\(\beta\)PP). Several reports, involving various techniques, have now reported that Zn, Cu and Fe are markedly enriched within A\(\beta\) plaques and congophilic angiopathy in AD and in A\(\beta\)PP transgenic mice [45,54,79,83,96,138,139,144]. Cu (390 \(\mu\)M), Zn (1055 \(\mu\)M) and Fe (940 \(\mu\)M) have been reported to be several-fold elevated compared to the normal age-matched neuropil [Cu (70 \(\mu\)M), Zn (350 \(\mu\)M) and Fe (340 \(\mu\)M)] [83]. A\(\beta\) directly coordinates Cu and Zn, but not Fe or other metal ions, within plaques [45,111]. In the plaque vicinity, Fe is found primarily complexed with ferritin in the plaque-associated neuritic processes [57] and is also found together with Cu and Zn within neurons and NFTs [21,82,100].

Consistent with the metal ions in plaques playing a primary role in A\(\beta\) aggregation, experiments in ZnT3 knockout mice have established that presynaptic Zn release causes amyloid formation in A\(\beta\)PP transgenic mice. ZnT3 is responsible for concentrating Zn ions into glutamatergic synaptic vesicles (Fig. 1A). ZnT3 knockout mice have about 15% less Zn in their cortex, but are otherwise phenotypically subtle [31]. These mice were crossed with Tg2576 A\(\beta\)PP transgenic mice, and the progeny characterized. These experiments showed that ZnT3 genetic ablation markedly inhibits amyloid pathology and congophilic angiopathy [54,77], increasing the concentration of soluble A\(\beta\) [77]. This suggests that soluble A\(\beta\) and soluble Zn exist in a dissociable equilibrium with insoluble plaque A\(\beta\) (containing incarcerated Zn) [24]. The increased amyloid deposition in women and female A\(\beta\)PP transgenic mice also may be explained by an estrogen-dependent increase in ZnT3 expression [78].

The possibility that A\(\beta\) may be dissociated from plaque by ionic chemistry encouraged therapeutic approaches that were initially chelators. Reproducing effects seen with synthetic A\(\beta\), Zn/Cu-selective chelators were found to markedly enhance the resolubilization of A\(\beta\) deposits from postmortem AD brain samples [28]. The observed increase in extractable A\(\beta\) from postmortem human brain specimens correlated with significant depletion in Zn (30%) and to a lesser extent, Cu [28]. The ability of a chelator to extract A\(\beta\) depended upon the presence of Mg\(^{2+}\) and Ca\(^{2+}\), hence the chelating compound needed to be far more selective for Zn\(^{2+}\) and Cu\(^{2+}\), than Ca\(^{2+}\) and Mg\(^{2+}\) [28]. These results fostered the first generation of attempts to inhibit amyloid pathology in A\(\beta\)PP transgenic mice, and are discussed further below.

Apolipoprotein E (ApoE) allelic status is the major risk factor for AD [34], and the ApoE isoforms bind Cu and Zn [97] in a manner that modulates A\(\beta\) precipitation, with the ApoE2 isoform being most protective and the ApoE4 isoform least protective [98], in concordance with their respective risks for AD. This is believed to be due to the coordination of the metals by cysteine, which is a transition metal ligand. E4 differs from E3 by a cys-to-arg change at position 112, E2 is an arg158-to-cys substitution.

**THE NEUROCHEMISTRY OF TRANSITION METAL IONS IN THE CORTEX AND GLUTAMATERGIC SYNAPSE**

One of the most common misunderstandings that is ventilated is that the neurological syndromes where metals is implicated are hypothetically caused by toxicological exposure to Cu, Fe, Zn and Mn. In other words, ingestion or exposure to the metals causes an abnormal protein interaction, which then causes the disease. This misconception is probably a legacy of the defunct [49] suspicion that aluminum exposure can cause AD pathology. In terms of total concentrations, the brain has more than enough transition metal ions to damage or dysregulate numerous proteins and metabolic systems. For example, the concentration of Zn\(^{2+}\) that is released during neurotransmission is \(\approx 300 \mu\)M, which is more than sufficient to be rapidly neurotoxic in neuronal cell culture [51]. Therefore, the brain must have efficient homeostatic mechanisms and buffers in place to prevent the abnormal dyscompartmentalization
A) The healthy synapse. Zn$^{2+}$ is concentrated in the presynaptic bouton by the activity of ZnT3, where it is co-compartmentalized with glutamate, and achieves concentrations up to 300 µM in the cleft upon release. Cu$^{2+}$ is released post-synaptically following NMDA-induced activation, which causes the translocation of the Menkes Cu7aATPase and its associated Cu-laden vesicles to the synaptic cleft. Cu$^{2+}$ concentrations reach 15 µM in the synaptic cleft. Both Cu and Zn can quench the response of the NMDA receptor, which may feedback to prevent further Cu from being released into the cleft. Aβ is constitutively released into the synaptic cleft and normally would be cleared by movement into the periphery or degradation by extracellular proteases. Despite high peak concentrations, the average concentrations of synaptic Cu and Zn is kept low over time by putative energy-dependent reuptake mechanisms as well as buffering by metallothionein III (MT3) released by astrocytes. B) Alzheimer’s disease. Decreased mitochondrial energy leads to reduced metal reuptake, which causes the average concentration of metals to rise over time. This allows Cu and Zn to react with extracellular Aβ to form oxidized, cross-linked soluble aggregates and precipitated amyloid. These aggregates generate H$_2$O$_2$ and radicals, and foster further Aβ cross-linking. MT3 is decreased in AD, so promoting abnormal metal-Aβ interaction. Sequestering of Zn and Cu by amyloid allows unopposed glutamate activation of the NMDA receptor, which could increase the release of post-synaptic Cu.
free Fe, the labile Fe pool, has been well established to modulate the expression of various proteins, including AβPP [122]. Intracellular Cu is considered largely bound, but is clearly exchangeable and transferred from protein to protein (e.g. by the Cu chaperone of superoxide dismutase 1, CCS1) [133].

In the last few years, there have been several important basic discoveries about Cu and Zn release and flux at the glutamatergic synapse in the cortex and hippocampus (Fig. 1A). This synapse is important not just because it is the site of long-term potentiation, a physical substrate of memory formation, but also because it is here that amyloid first deposits in AD and is probably the most important site for dysfunction in this disorder [147]. There has been interest in the presence of Zn and Cu released by hippocampal tissue for about the last 20 years (reviewed in [53]). Considerable evidence has supported the release of Zn either as a free or an exchangeable ionic species into the extracellular space [52]. This pool of vesicular Zn is formed by the activity of ZnT3, which is found in the membrane of glutamatergic vesicles, and not found elsewhere; a rather good explanation for why amyloid forms there and not in peripheral tissue. This Zn, released possibly with glutamate during neurotransmission [42], suppresses the response of the NMDA receptor and may prevent seizure activity.

Post-synaptic NMDA neurites were recently reported to release free ionic Cu upon NMDA activation [125]. Activation of synaptic NMDA receptors in hippocampal neurons results in trafficking of Menkes ATPase and an associated efflux of Cu [125]. Catalytic amounts of Cu can function as electron acceptors promoting the reaction of nitric oxide with thiols, and possibly the release of Cu could function as a molecular switch to control extracellular S-nitrosylation of the NMDA receptor, a post-translational mechanism shown to be critical for modulating receptor function [125]. Cu has also been reported to be specifically protective against NMDA-mediated excitotoxic cell death in primary hippocampal neurons, and that this protective effect of Cu depends on endogenous nitric oxide production in hippocampal neurons [126]. Menkes ATPase expression is developmentally regulated, peaking during synaptogenesis, and playing a role in the endothelial cells of the BBB [48,107].

The average concentration of metals ions in the synapse is not just a product of release from presynaptic boutons but also a product of reuptake mechanisms both pre- and post-synaptically (Fig. 1A). While Zinc Importing Proteins (ZIPs) and the main Cu uptake protein, Ctrl, are known to be present in neurons, the specific mechanisms involved in the reuptake of synaptic metals are not known. However, they must be powerful and rapid pumps to avoid the toxicity that is caused by sustained high micromolar concentration of the metals. Indeed, avid reuptake mechanisms have been described for synaptic Cu and Zn [56,65]. For Zn, this process is energy-dependent; for Cu, it is not known but likely to be energy-dependent. The significance of this is that mitochondrial energy failure is a feature of aging and AD, and may lead to pooling of synaptic Zn and Cu leading to abnormal Aβ metallation (Fig. 1B).

Taken together, the developing literature has described the glutamatergic synapse as containing an extraordinary confluence of chemically exchangeable Zn and Cu (Fig. 1), which is unique in the body to our knowledge. This may be an explanation for why Aβ, with its vulnerability to precipitation and cross-linking induced by these metal ions, initially precipitates in this site in AD (Fig. 1B). One final component in this vicinity that will modulate the availability of Zn and Cu ions to bind to the NMDA receptor or to Aβ is the release of metallothionein-3 (MT3 or GIF, Growth Inhibitory Factor) [93] by the neighboring astrocytes [149] (Fig. 1A), which is decreased in AD [150] (Fig. 1B).

ABNORMAL METAL HOMEOSTASIS IN THE AGING BRAIN AND IN ALZHEIMER’S DISEASE

The metal ion content of the brain is stringently regulated and there is no passive flux of metals from the circulation to the brain: movement of metals across the BBB is highly regulated. While Fe, Cu and Zn are being increasingly implicated in interactions with the major protein components of neurodegenerative disease, this is not merely due to increased (e.g., toxicological) exposure to metals, but rather due to a breakdown in the homeostatic mechanisms that compartmentalize and regulate metals.

The dominant risk factor associated with the neurodegenerative diseases is increasing age. Several studies in animals and humans have reported a rise in the levels of brain Cu from youth to adulthood (reviewed in [2]). However, from middle age onwards, Cu levels drop markedly, and are accompanied by a loss of Cu-dependent enzyme activities, e.g. cytochrome c oxidase, superoxide dismutase 1, ceruloplasmin [119]. Age-related increases in brain Fe have been documented in all species examined, including humans [15,60],
non-human primates [61], rodents [91,123,132,143], and even drosophila [90]. This increase in Fe content is due to the accumulation of surplus Fe stored partly in ferritin, which correspondingly increases with age [32,33,160]. Indeed failure of ubiquitous ferroxidases ceruloplasmin, ferritin [29] and frataxin [88] cause neurodegenerative diseases, underscoring the vulnerability of the brain to abnormal Fe regulation [161]. Elevated Fe may be a relevant pharmacological target in AD, but needs to be differentiated from the changes in tissue Cu and Zn distribution that involve amyloid.

An interesting feature of the mechanism of increased AD pathology in sod+/- x A/βPP transgenic mice is that brain Cu, Zn and Fe levels are decreased by the mitochondrial lesion [94]. This recapitulates a feature of the pathology of AD, where Cu levels decrease with advanced pathology [119]. Both dietary and genetics manipulations that increase brain Cu levels ameliorated amyloid pathology in two strains of A/βPP transgenic mice [16,113]. However, there are also reports that exposure to Cu in combination with a high fat diet increases the risk for AD [101], a possibility that has found support in studies of rabbits exposed to Cu and cholesterol [134,135]. In contrast, Zn levels increase in advanced AD, correlating with brain A/β burden in humans but not A/βPP transgenic mice [119]. Zn nutritional deficiency is common in advanced age, and a recent report indicated that Zn deficiency in A/βPP transgenic mice increased the volume of amyloid plaques [139]. These data indicate the complexity of the disordered metal metabolism in AD. The consensus that has emerged is that Zn and Cu are enriched in amyloid where they coordinate Aβ, Fe is enriched in the tissue and neuritic pathology, and there is evidence of functional Cu deficiency. Therefore, pharmacotherapy that targets abnormal A/β metabolism is best geared to be not merely a chelation approach. Ideally the drug should release the metals trapped by A/β, and return them to normal metabolism, hence our interest in ionophores.

PHYSIOLOGICAL INTERACTIONS OF A/βPP AND ITS PROCESSING WITH ZINC AND COPPER

While the function of A/βPP is unknown, recent evidence suggests it has a role to play in maintaining Cu homeostasis [8,17,18,91]. A/βPP coordinates Cu+ at its amino-terminus, and A/βPP expression promotes the export of neuronal Cu [17]. A functional role for A/βPP in Cu homeostasis is supported by reports that cellular Cu drives the expression of A/βPP mRNA [8,18].

BACE1 (β-secretase) possesses a Cu+ binding site in its C-terminal cytoplasmic domain through which it interacts with domain I of the Cu chaperone of SOD1 (CCS1) [7]. The functional implications of this interaction are unknown but imply that Cu levels can impact upon A/β generation. Similarly, γ-secretase activity has been recently reported to be inhibited by low concentrations of Zn2+ but the physiological implications are unclear [64].

In AD, there is abnormal brain Cu distribution, with accumulation of Cu in amyloid plaques and a deficiency of Cu in neighboring cells. In vitro, excess Cu inhibited A/β production from A/βPP-transfected CHO cells [20], but the effects of deficiency were not previously explored. A recent report has studied the effects of modulating intracellular Cu levels upon the processing of A/βPP and the production of A/β [25]. Human fibroblasts genetically disposed to Cu accumulation secreted higher levels of sA/βPP into their medium, while fibroblasts genetically manipulated to be profoundly Cu deficient secreted predominantly sA/βPP and produced more amyloidogenic C-termini (C99). The level of A/β secreted from Cu deficient fibroblasts was, however, regulated and limited by α-secretase cleavage. In this system, A/βPP was found to be processed simultaneously by both α- and β-secretase, as Cu deficient fibroblasts secreted sA/βPP exclusively but produced primarily α-cleaved C83 [25]. Cu deficiency also markedly reduced the steady-state level of A/βPP mRNA while A/βPP protein level remained constant, indicating that Cu deficiency may accelerate A/βPP translation [25]. Cu deficiency in human neuroblastoma cells significantly increased the level of A/β secretion, but did not affect the cleavage of A/βPP. Therefore, Cu deficiency markedly alters A/βPP metabolism and can elevate A/β secretion by either influencing A/βPP cleavage or by inhibiting its degradation, with the mechanism dependent on cell type [25].

Several activities that degrade A/β in the extracellular milieu are Zn metalloproteinases, such as nepri bysin, insulin degrading enzyme, and matrix metalloproteinases (reviewed in [2]). This may explain why there is an inverse correlation between CSF Zn and Cu levels and CSF Aβ42 levels in normal men [141]. This possibility was supported by the observation that adding low micromolar concentrations of Zn or Cu to ex vivo CSF samples accelerated the degradation of A/β [141].

Taken together, our team believes that the biochemistry of A/β metal complexes is pathophysio logically
relevant, and that the Metal Hypothesis of AD has several advantages in explaining AD pathology that remain unexplained by the Amyloid Cascade Hypothesis. We have focused on blocking these metal-mediated reactions as a pharmacological maneuver.

METALS AND TAU PATHOLOGY

The microtubule-associated protein tau is the principal component of the paired helical filaments that comprise neurofibrillary tangles (NFTs), the other pathological hallmark of AD, and also a number of neurodegenerative disorders, such as frontotemporal dementia. While the evidence linking tau to metal neurochemistry is not yet as developed as the literature about Aβ or Aβ interactions with metals, there are a growing number of reports that indicate that tau may well participate in the metal-related abnormalities observed in AD, although it is still unclear how the Aβ and tau lesions may be linked by metal interactions. Tau and two peptides corresponding to the second and third repeat region of the tau microtubule binding domain both bind Cu (Cu²⁺) in a pH- and stoichiometric-dependent fashion and results in a conformational change in tau that may be important in the formation of paired helical filaments [85,86,162]. Prior to these reports, NFTs were reported as capable of binding adventitious Cu and Fe in a redox-competent manner, acting as a source for ROS within the neuron [124]. Recent data indicate that tau interaction with Cu²⁺ can mediate the generation of H₂O₂ in a manner analogous to Aβ [142].

In addition to this direct interaction, it is also known that many of the kinases that phosphorylate tau (ultimately resulting in hyperphosphorylation and dissociation from the microtubules), such as the extracellular signal-regulated kinase (ERK1/2), are induced by metal ions such as Zn [6,62]. Indeed, tau hyperphosphorylation has been induced in both SH-SY5Y and N2a cells by Zn [19]. This is important since Zn levels rise dramatically in AD-affected neocortex [41,83,112,119], and tangle-bearing neurons fill with Zn [144]. Conversely, H₂O₂ generated by Cu/Fe-Aβ complexes [67,111] may inhibit phosphatases [43]. In contrast, the treatment of hippocampal neuron cultures with Fe citrate results in a decrease in tau phosphorylation at AD-related epitopes, possibly from a decrease in the activity of the Cdk5/p25 complex, where p25 may be a regulator of the activity of protein kinase Cdk5 [47]. Furthermore, Fe(III), but not Fe(II), induces an aggregation of hyperphosphorylated tau that can be reversed by the reduction of Fe(III) to Fe(II) [157]. Indeed, the treatment of tau aggregates from the AD brain with reducing agents results in the resolubilization of tau and a release of Fe(II), further demonstrating the potential role of Fe in NFT formation [157].

While tau is the principal component of NFT, there are other cytoskeletal components found within NFTs that are also known to interact with metals. Neurofilaments (NF), for example, show phosphorylation-dependent alterations very early in the AD cascade and are found within the NFT and in association with the dystrophic neurites surrounding Aβ plaques [75]. Purification of this multi-subunit protein demonstrated that it stoichiometrically binds at least one mole of Cu and four moles of Zn [114]. The assembly of NFs may be mediated, in part, by metals ions such as Cu, which foster the assembly of the light NF subunit (NF-L). NFs are also phosphorylated by a number of the tau-kinases which, as discussed above, can be modulated by metal ions and indeed, Zn can induce the phosphorylation of NFs at various sites [19].

THERAPEUTIC INVENTIONS BASED ON THE METALS HYPOTHESIS

A common mistake in envisioning of therapeutic approaches based on these findings is forming the belief that chelation, meaning the removal of metal ions from tissue, is the obvious intervention. While there are several medical chelators, their approved use is confined to genuine overexposure situations (e.g., Wilson’s disease, or lead toxicity), or to rheumatoid arthritis. There is no evidence that AD will respond to traditional medical chelation. The risk, however, is in the removal of essential metal ions with subsequent side effects, e.g., Fe-deficiency anemia. These problems can be to some extent overcome by engineering small molecules to target specific compartments or organelles. However, even so, complex situations such as the pooling of metals in plaques and their relative deficiency within neighboring cells, call for the development of small molecules with sophisticated properties, e.g., ionophores.

A logical property of such neurotherapeutic small molecules is to target the initiating event in the generation of free radicals, that is to employ metal complexing agents to prevent the metal ions from participating in redox chemistry. Another important property of a potential neurodegenerative therapeutic will be its ability to cross the BBB. This excludes a large number of common metal chelators as possibilities
due to their hydrophilic nature. Nevertheless, there have been two published placebo-controlled clinical trials in AD of chelators, both of which reported some clinical or biochemical benefits (reviewed in Table 1). In 1991, Crapper-McLachlan and colleagues reported benefit over a two-year period in AD patients treated with intramuscular desferrioxamine twice daily [35]. Desferrioxamine treatment led to significant reduction in the rate of decline of daily living skills, which the authors originally attributed to chelation of aluminum. However, desferrioxamine also chelates Zn, Fe and Cu. A small double-blind trial of 34 AD subjects with d-penicillamine or placebo reported a decrease in serum oxidative markers over a six-month period but no change in cognitive decline [136]. There was a large dropout rate in the study, so the results are inconclusive.

For AD, a range of metal-complexing agents have been tested in a variety of preclinical systems. The bicyclam analogue JKL169 (1,1′-xylyl bis-1,4,8,11 tetraaza cyclotetradecane) was effective in reducing Cu levels in brain cortex and is able to maintain normal Cu levels in the blood, CSF and corpus callosum of rats [99]. The lipophilic chelator DP109 reduced the levels of aggregated insoluble Aβ and conversely increased soluble Aβ forms in a mouse model [80].

Oral treatment with the chelator clioquinol (CQ, 5-chloro-7-iodo-8-hydroxyquinoline) in Tg2576 mice resulted in a reduction of cortical deposition of amyloid (49%) with an improvement or stability in the general health and weight parameters compared to untreated mice [27]. This quinoline compound is able to cross the BBB and increased brain Cu and Zn levels in treated mice [27]. CQ has only a modest (nanomolar) affinity for Cu2+ and Zn2+ [112], which is sufficient to facilitate dissociation of these metal ions from the low affinity metal binding sites of Aβ. Upon peripheral dosing, CQ was demonstrated in Tg2576 mice to cross the BBB and complex to amyloid plaques, and to complex with Zn2+-metallated Aβ from postmortem AD-affected brain specimens [112]. In a pilot Phase II human trial, the oral administration of CQ in moderately severe AD patients for 36 weeks slowed the rate of cognitive decline (significant at week 4 and week 16, with a trend to improvement at other intervals) and caused a reduction in plasma Aβ42 levels as compared to the placebo controls [121].

The second generation 8-OH quinoline derivative of CQ, PBT2, which has greater BBB penetration recently completed its first double-blind, placebo-controlled Phase II clinical trial in 78 subjects over 12 weeks for the treatment of early AD. The results revealed that the drug was safe and well tolerated at 50 mg and 250 mg daily doses for 12 weeks, that CSF Aβ levels were significantly lowered by the 250 mg dose at 12 weeks, and that there was a significant improvement above baseline in performance on executive tests of the Neuropsychological Test Battery (NTB) at 12 weeks [76]. These results appear to be the basis for proceeding with further Phase IIb or Phase III testing of what may be a disease-modifying drug based on The Metals Hypothesis. The rationale for studying more mildly-affected AD cases was because the tests on AβPP transgenic mice gave strikingly impressive improvement within days of commencing treatment [3]. We therefore reasoned that it would be reasonable to study patients who qualify for a diagnosis of AD but with minimal neuronal loss, since the transgenic mice do not exhibit much neuronal loss despite the heavy amyloid burden and made surprising recoveries. In the Phase II CQ trial, the most statistically robust signal on ADAS-cog was achieved in the more severely affected group, but the study was small in size, and we did not believe that the benefits of the drug would be confined to more severely affected patients on the basis of the results of such a small sample. In the first PBT2 Phase II study, the primary readout was safety and tolerability, and efficacy readouts were secondary. In a 12-week readout, there is not a great chance of changes with a disease-modifying drug being detected by ADAS-cog, although the instrument can detect improvement in cognition in adequately powered clinical trials of stimulants, e.g., acetylcholinesterase inhibitors, but the NTB may be better suited to detect more subtle cognitive changes and in different realms of brain function [140]. Both the ADAS-cog and the NTB were utilized in the PBT2 Phase Ila trial, but ADAS-cog results were not significant at 12 weeks [76]. In future studies, it would very interesting to investigate the impact of such disease-modifying drugs upon PIB imaging signals of brain amyloid burden [115], which would be a more direct marker of pathology than CSF Aβ.

The mechanism of action of CQ and PBT2 is still to be confirmed. In mice, CQ is understood to enter the brain and to combine with metallated Aβ in plaques and possibly diffuse Aβ collections [112]. CQ treatment of transgenic mice modestly increases brain Zn and Cu levels [27], and in the Phase II clinical trial in AD patients, the plasma Zn levels significantly increased (normalized from a baseline of deficiency) [121], therefore CQ does not act as a simple chelator. In cell culture, CQ-Cu complexes enter cells where they markedly inhibit the secretion of Aβ by a mechanism where...
### Table 1
Summary of current and completed AD clinical trials of chelators and metal-complexing agents

<table>
<thead>
<tr>
<th>Drug candidate</th>
<th>Mode of action</th>
<th>Population</th>
<th>Study design</th>
<th>Outcome measures</th>
<th>Follow-up and outcomes</th>
<th>Refs</th>
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<tr>
<td>Desferrioxamine mesylate: 125 mg DFO given intra-muscularly b.i.d., 5 days per week</td>
<td>Multiple metal chelator: Fe, Al, Zn, Cu (thought originally to target only Al)</td>
<td>Probable AD by NINCDS/ADRDA criteria, Hachinski score &lt; 4. Mean age ≈ 63.</td>
<td>Single-blind. 48 patients were assigned to three different groups: DFO (n = 25), oral placebo (500 mg oral lecithin twice daily, n = 9) and no treatment (n = 14, merged n = 23). Video appraisal of activities of daily living.</td>
<td>Activities of daily living monitored and recorded over 24 months. No differences were observed in the rate of deterioration of patients receiving either placebo or no treatment. DFO treatment led to a significant reduction (50%) in the rate of decline in daily living activities.</td>
<td>2 years. Well-tolerated. DFO group: no subjects died, 5 were lost to follow up at month 24. No-treatment group: 5 died, 4 were lost to follow up at month 24.</td>
<td>[35]</td>
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<td>Clioquinol (CQ) 20 mg or 80 mg orally daily</td>
<td>Cu/Zn ionophore, hydrophobic with modest chelating properties</td>
<td>Probable AD by NINCDS/ADRDA criteria, MMSE 10-24. Mean age ≈ 75.</td>
<td>Single-site, Phase I clinical trial of CQ with B12 supplementation. Open-label study of 20 patients with a diagnosis of probable AD. Patients randomized to receive 20 or 80 mg CQ a day with cyanocobalamin 1 mg and folic acid 5 mg.</td>
<td>Baseline, day 7 and 21: MMSE, ADAS-Cog and GBS, CSF: Aβ 42, GAP-43 and tau, serum Cu and Zn.</td>
<td>21 days. No adverse systemic or neurological events reported. Combined treatment groups improved slightly (2.7 points) on ADAS-cog (P = 0.07) and on the ADAS-cog naming, instructions, and comprehension sub-tests (P&lt;0.05). The high-dose treatment group showed improvement on ADAS-cog comprehension (P &lt; 0.05), whereas the low-dose treatment group did not. No changes on MMSE and GBS. CSF: no changes in Aβ, significant increase in tau and GAP43 between baseline and day 7, which was followed by a decrease between day 7 and day 21. No changes in serum Cu and Zn.</td>
<td>[118]</td>
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<td>D-penicillamine: 600 mg orally per day.</td>
<td>Chelator: selective for Cu, but also other metals</td>
<td>Probable AD by NINCDS/ADRDA criteria, MMSE 10-26. Mean age ≈ 74.</td>
<td>34 AD patients (17 active, 17 placebo). 6-month, double-blind, randomized, placebo-controlled, single-site. Supplemented in both arms with vitamin B6 75 mg/day to prevent side effects.</td>
<td>Primary: Mental Deterioration Battery scale; secondary outcome measures MMSE, NeuroPsychiatric Inventory, Geriatric Depression Scale; Gottfries Brane Skem (GBS) scale. Serum peroxides and lipoperoxides, total radical trapping antioxidant (TRAP) capacity, Cu, Fe, transferrin, 24 h Cu excretion.</td>
<td>6 months. Trial was halted due to one death (cardiac arrest) in the treatment group. Other serious adverse events in the treatment group: leucopenia (two cases), gout (one case). 9 patients from each group completed the trial. No significant difference in the rate of AD progression was found between D-penicillamine and placebo groups. Significant decrease in serum peroxides and TRAP, increased urinary Cu excretion. No change in serum Cu.</td>
<td>[136]</td>
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Table 1, continued

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<td>Clioquinol 125 mg b.i.d. 12 weeks, then 250 mg b.i.d. 12 weeks, then 375 mg b.i.d. 12 weeks.</td>
<td>As above</td>
<td>Probable AD by NINCDS/ADRDA criteria, MMSE 10-24. Donepezil at max. beneficial dose for ≥ 6 months. Mean age ≈ 75.</td>
<td>36 AD patients (18 active, 18 placebo), 9-month, double-blind, randomized, placebo-controlled, single-site, dose escalation. Supplemented in both arms with vitamin B12, and maintained on donepezil. Groups analyzed as more or less severe at baseline on the basis of median baseline ADAS-cog score.</td>
<td>Change from baseline in ADAS-cog, plasma Aβ42, Zn and Cu levels at 4, 12, 24, 36 weeks.</td>
<td>N = 15 CQ, N = 16 placebo completed. Well tolerated, but one subject on CQ reported temporary loss of color vision. More severe group significantly less ADAS-cog deterioration at 4 and 16 weeks; trends to improvement at other time points. Less severe group did not significantly deteriorate from baseline. At 500 mg/day the less severe cohort showed a significant and sustained decrease in Aβ42. Plasma Zn but not Cu significantly increased in the CQ group.</td>
<td>[121]</td>
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<td>Clioquinol 250 mg orally b.i.d. with vitamin B12 IM monthly.</td>
<td>As above</td>
<td>Probable AD by NINCDS/ADRDA criteria. 47 year old male with familial AD London mutation MMSE 23, 48 year old female, MMSE 21.</td>
<td>Case reports on two patients who were treatment failures on acetylcholine esterase inhibitors and memantine.</td>
<td>Fluorodeoxyglucose PET scans showed hypometabolism at baseline, repeated after prolonged treatment. Repeat lumbar punctures with CSF analysis of tau, Aβ40 and Aβ42 at 4 months and end-point. The other patient did not comply with repeat lumbar puncture.</td>
<td>FAD patient observed 9 months, sporadic patient observed 14 months. Treatment was well tolerated. In both cases, MMSE declined after 3 months and then stayed stable. Both showed improved (+11–14%) glucose utilization at final observation. ≈ 20-50% variation in tau, Aβ40 and Aβ42 levels, with no consistent direction in the FAD patient.</td>
<td>[71]</td>
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<td>PBT2 orally 50 mg and 250 mg daily. Similar to clioquinol</td>
<td>Probable AD by NINCDS/ADRDA criteria. Recently diagnosed (MMSE 20-26), treatment failures on acetylcholine esterase inhibitors.</td>
<td>Phase 2a, with 3 arms. 78 AD patients. Double-blind, randomized, placebo-controlled, multiple-site (Sweden, Australia).</td>
<td>Safety and tolerability, CSF Aβ42 and tau, plasma Cu and Zn, cognitive performance change from baseline on Neuropsychological Test Battery (NTB) and ADAS-cog.</td>
<td>12 weeks. Satisfactory safety and tolerability. Significant decrease in CSF Aβ42. No significant changes in tau, plasma Cu or Zn, ADAS-cog. Significant improvement over baseline in frontal lobe tests in NTB.</td>
<td>[76,116]</td>
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the peptide is degraded through upregulation of matrix metalloprotease (MMP)-2 and MMP-3. MMP activity was increased through activation of phosphoinositol 3-kinase and JNK. CQ-Cu also promoted phosphorylation of glycogen synthase kinase-3 (GSK-3) and this potentiated activation of JNK and degradation of Aβ1−40 [154]. We contemplate a mechanism of action for AD where CQ or PBT2 enters the brain and is attracted to the extracellular pool of metals that are in a dissociable equilibrium in amyloid (Fig. 2). The molecule then binds Zn or Cu in the amyloid, possibly forming a ternary complex with Aβ, and the drug-metal complex then enters the cell. This activates MMPs and facilitates the clearance of Aβ in the synapse. At the same time the oxidative oligomerization of Aβ and the toxic redox activity of Aβ oligomers is blocked by the drug.

The goal with this class of “metal-protein attenuating compound” is to remove the essential metal Cu and Zn from where they do harm (i.e., coordinated to Aβ) and relocate the metal to a place where they will do good (i.e., restore dysregulated metal homeostasis). CQ has also been trialed in other neurodegenerative disease models with efficacy shown in both PD [74] and HD [106] animal models, where both diseases have been associated with Fe or Cu overload [50] leading to oxidative stress.

Another interesting approach to metal-based therapeutics for AD targets the increase in brain Fe in the disease. This is a more traditional Fe chelation therapy, with molecules that pass the BBB and are designed to be multifunctional e.g., by attaching a propargylamine moiety that inhibits acetylcholinesterase, or by possessing antioxidant or monoamine oxidase inhibitor activity [12,13,159]. By decreasing the labile Fe pool, these drugs attempt to decrease AβPP translation and hence Aβ generation [120]. A further salutary effect of Fe depletion could arise from inhibition of hypoxia-inducible factor (HIF) prolyl 4-hydroxylases, which has been shown to be neuroprotective [128]. Like all metal complexing agents, it is very difficult to achieve complete metal ion specificity with any structure, and it is likely that these molecules that are thought to target Fe will also interact with Cu, Zn and other metal ions. This may have advantages in the dissolution of Aβ aggregates, but, as noted above, excess depletion of Cu and Zn may paradoxically exaggerate AD pathology. Only empirical testing can determine whether this will be of value in the clinical situation.

Looking into the future, it seems likely that pharmacotherapeutic approaches to AD will involve combina-
tion therapies, and there appears no biochemical barrier to the potential combination of metal-complexing agents with other potentially disease-modifying interventions such as cholinergic modulators, secretase inhibitors or immunotherapy.

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References

A.I. Bush / Drug Development Based on the Metals Hypothesis of Alzheimer’s Disease

236


