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EDITORIAL

Tau aggregation inhibitors: the future of Alzheimer's pharmacotherapy?

1. Introduction

Neuropathogical hallmarks of Alzheimer's disease (AD) are intracellular neurofibrillary tangles (NFTs) composed of paired helical filaments (PHFs) and straight filaments partly constituted of hyperphosphorylated tau protein, neuropil threads, dystrophic neuritis, and extracellular deposits of β-amyloid (Aβ) as the major component of senile plaques in the brain. These neuropathological hallmarks of AD strongly influenced recent therapeutic approaches, with many therapeutic approaches under development for AD treatment directed against the production and accumulation of Aβ.[1] However, several drugs targeting Aβ with different mechanisms of action have failed to demonstrate efficacy in randomized clinical trials or their development has been halted.[1,2] In recent years, tau-based treatments for AD have become a point of increasing focus and current and previous investigational therapies can be grouped into four categories including tau-centric active and passive immunotherapeutics, microtubule-stabilizing agents, tau-protein kinase inhibitors, and tau-aggregation inhibitors (TAIs). Among different tau-directed approaches in AD, small molecular weight compounds developed to inhibit formation of tau oligomers and fibrils by blocking tau–tau aggregation have already been tested in humans.[2–4] In cell-based and/or *in vitro* screening assays, several classes of agents that may act to prevent tau aggregation have been identified, including but not limited to polyphenols, porphyrins, phenothiazines, benzothiazoles/cyanines, *N*-phenylamines, thioxothiazolidinones (rhodanines), phenylthiazole-hydrazides, anthraquinones, and aminothienopyridazines.[4–6] However, the efficacy for inhibiting tau aggregation *in vivo* for many TAIs has not yet been tested. On the other hand, several TAIs have toxic profiles that would preclude their use *in vivo*. Currently, TAIs fall into two mechanistic classes depending on their way to interact with tau protein, that is covalent and non-covalent molecules.[4] Covalent TAIs can attack any or all species in an aggregation pathway, but appear to be especially efficacious modifiers of tau monomers.[4] Natural polyphenols are covalent TAIs, such a as oleocanthal, a natural product aldehyde reacting with epsilon amino groups of lysine residues, oleuropein aglycone, abundant in the extra virgin olive oil, or the green tea-derived (–)-epigallocatechin gallate.[4] Other redox-active compounds, including the non-neuroleptic phenothiazine methylene blue (MB) [methylthioninium chloride (MTC), Rember™, TRx-0014, TauRx Therapeutics, Singapore, Republic of Singapore] can also modulate cysteine oxidation when incubated in the absence of exogenous reducing agents.[7] In general, covalent mechanisms of tau-aggregation inhibition in AD are predicted to have low utility *in vivo*.[8] However, dimethylfumarate, an electrophile capable of reacting covalently with cysteine sulfhydryls, was approved for oral treatment of multiple sclerosis,[9] suggesting that electrophilic compounds acting through covalent inhibitory mechanisms can be useful therapeutic agents.

2. TAIs for the treatment of AD: preclinical studies of methylthioninium and derivatives

The second broad class of TAIs interacts with tau species non-covalently, through multiple mechanisms and with different structures.[4] Among different mechanisms, small molecules can interact directly but transiently with natively unfolded tau-protein monomer.[4] Structure–activity relationships were established within specific chemical series.[10] Like common dyes, most TAIs absorb electromagnetic radiation in the visible spectrum, a property linked to the property of delocalizing π-electron distribution. Ligand polarizability correlates with tau-aggregation inhibitory potency within specific chemical series of cyanine, phenothiazine, arylmethine, and rhodanine derivatives.[8] MB or MTC is an old dye, repurposed as medical treatment of tau pathologies.[11] Chemically, MTC is a tricyclic phenothiazine derivative and exists in equilibrium between a reduced (leuco-methylthionium [LMT]) and oxidized form (MT^+).[12] In an ambient oxygen atmosphere, it is present as a cation (MT^+) and formulated as a chloride salt (commonly known as MB). MTC may be reduced by nicotinamide adenine dinucleotide phosphate or thioredoxin to give LMT (leuco-MB), an uncharged colorless compound. MTC is excreted in the urine as a mixture of MTC, LMT, and demethylated metabolites, for example azure B and azure A.[13] MTC has been used to treat malaria, methemoglobinemia, and depression.[11] Intravenous administration results in higher MT concentrations in the brain.[14] Therefore, MT can permeate the blood-brain barrier in rats irrespective of the administration route [14] and selectively penetrate certain neuronal cell types after systemic administration, particularly hippocampal cells.[15] At present, MTC and its derivatives represent the most advanced TAIs in clinical development for the treatment of AD. MTC has been shown to interfere with the tau–tau binding necessary for aggregation.[6] In a cell-based model of inducible tau aggregation, the inhibitory constant of MT was found to be 123 nM.[16] Other studies reported quite different *in vitro* inhibitory potency (IC_{50}) varying from 1.9 μM [5] to 3.5 μM.[17] The estimated concentration of MT and its active metabolites in the human brain at the 138 mg/day dose was 0.18 μM.[12] This value appears to be in the range of the *in vitro* IC_{50} values for dissolution of PHFs (0.16 μM) and the

calculated intracellular Ki for TAI activity (0.12 μM),[16] but not in the range of IC_{50}s of other *in vitro* [5] and cell-based [17] studies. In tau transgenic mouse models, MT levels in the brain followed a sigmoidal concentration–response relationship over a 10-fold range (0.13–1.38 μM) after oral administration of 5–75 mg/kg for 3–8 weeks.[18] Alternative mechanisms of action have been proposed for MT [2] including inhibition of microtubule assembly [19] that requires an IC_{50} of 50 μM.[2,19] However, the dose required to achieve inhibition of microtubule assembly with MTC would be about 50 g of MTC/day,[2] exceeding the median lethal dose (LD_{50}) for MTC in several species. Similarly, it has been proposed that MTC may reduce endogenous production of tau protein,[20] but the EC_{50} for this effect is 10 μM, requiring a human clinical dose of 9 g of MTC/day, a dose that could not safely be administered in humans. It has been also proposed that MTC could affecting tau phosphorylation via inhibition of Hsp70 ATP-ase,[21] but again the EC_{50} for this effect is 83 μM, with a theoretical dose in humans of 75 g MTC/day.

Recent *in vivo* and *in vitro* studies have suggested that MTC may reduce tau-protein aggregates in AD through proteasomal [22] and macroautophagic [23] degradation of the protein. Other potential effects of MTC are oxidation of cysteine sulfhydryl groups in the tau-repeat domain preventing formation of disulphide bridges to keep tau monomeric, acetylcholinesterase inhibition, nitric oxide synthase inhibition, noradrenaline uptake inhibition, glutamatergic inhibition, monoamine oxidase B inhibition, guanylate cyclase inhibition, and inhibition of the aggregation of Aβ peptides, stimulation of Aβ clearance, improvement of brain metabolism, improvement of astrocyte cellular respiration, improvement of brain mitochondrial amyloid-binding alcohol dehydrogenase functions, improvement of mitochondrial antioxidant properties, improvement of the Nrf2/antioxidant response element, antagonism of α7-nicotinic acetylcholine receptors, inhibition of β-secretase activity, enhancement of mitochondrial oxidation, and inhibition of monoamine oxidase A.[2,5,6,24–28] However, the clinical relevance of these potential effects is doubtful. Studies on the activity of MTC on tau aggregation *in vivo* are controversial.[19,20,26–28] In zebrafish, MTC did not alter abnormal tau phosphorylation and failed to inhibit tau-dependent neuronal cell toxicity.[29] However, in this study, there was no information provided regarding the actual concentration of MT in the brain of the zebrafish, what form this was in (i.e. parent MT or inactive conjugates), and whether the concentration at the site of action was sufficient to reach the Ki threshold for TAI activity. In P301L tau-transgenic mice, MTC reduced detergent-insoluble brain-phosphorylated tau levels in one study,[30] while in another study, the drug affected only soluble tau levels without affecting insoluble forms.[26] In rTg4510 tau-transgenic mice, MTC prevented behavioral deficits and reduced soluble brain tau levels when given before formation of NFTs in the brain,[25] but one study suggested that it could not be able to reverse established NFT pathology.[31] Other studies reported inhibitory activities of MTC on aggregation processes of prion protein,[32] α-synuclein, transactive response DNA-binding protein 43,[33,34] and huntingtin [35] with potential applications in other neurodegenerative diseases.

3. Clinical efficacy and safety of methylthioninium and derivatives

A double-blind, placebo-controlled study evaluated the safety and efficacy of MT at 69 mg, 138 mg, and 228 mg/day for 24 weeks in 321 mild-to-moderate AD patients who were not taking acetylcholinesterase inhibitors or memantine (ClinicalTrials.gov Identifier: NCT00515333).[36] The primary efficacy outcome of the study was the change in the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) at 24 weeks relative to baseline. The effects of treatments on regional cerebral blood flow (rCBF) were evaluated in a subgroup of 135 patients.[36] Wischik and colleagues did not present results for the overall patient group but made a statistical analysis by disease severity at baseline ('mild' and 'moderate' subgroups, according to baseline Clinical Dementia Rating scale value of 1 or 2, respectively).[36] They introduced in the statistical model a covariate for the interaction between 'treatment' and 'disease severity at baseline' but, unfortunately, they did not disclose if this interaction was statistically significant. At 24 weeks, Wischik and colleagues found in the subgroup of moderately affected patients (n = 69) a significant difference in favor of the intermediate dose of 138 mg/day (n = 17) compared to placebo (n = 20) on the ADAS-cog scale, the primary outcome measure of efficacy of the study (5.42 points, p = 0.047). The mildly affected patients did not show significant changes from baseline in the ADAS-Cog in any treatment groups, including placebo (−0.14 points). Nevertheless, in the mildly affected patients undergoing the rCBF analysis, a significant decline compared to baseline in the placebo group (−2.16%, p< 0.001) was found, a difference that resulted significant when compared to that observed in the 138 mg/day group (−0.19%, p< 0.001).[36]

A number of 227 patients entered a 26-week open-label extension, and 111 of them completed this phase (ClinicalTrials.gov Identifier: NCT00684944). At 50 weeks, the mean change of ADAS-cog score of the 138 mg/day dose group was better than the mean change of patients initially receiving placebo for 24 weeks and then 152 mg/day for 26 weeks (2.8 and 5.2 points in mild and moderate patients, respectively). The most commonly reported adverse events (incidence ≥5%) in MTC-treated subjects included gastrointestinal disorders (primarily diarrhea), renal and urinary disorders (primarily dysuria and frequency), and falls.[36] Treatment with MTC produced dose-dependent decreases in red cell count and hemoglobin and increases in methemoglobin with peak decreasing effects on red blood cells of about 400,000/μL at 12 weeks compared to baseline in the 228 mg/day group. There were 4 cases (of 307 exposed to MTC) with methemoglobin greater than 3.5% (a threshold set for withdrawal of treatment) which resolved on cessation of treatment.[36] The authors of the study reported that the delivery of the highest dose was impaired due to dose-dependent dissolution and absorption factors of the 100 mg MTC gelatin capsule

formulation.[12] MTC (Rember™) was later discontinued for the treatment of AD.

4. Pharmacokinetic, preclinical, and clinical studies with leuco-methylthioninium and derivatives

A stabilized, reduced form of MTC, TRx 0237 (LMTX™), is being developed by TauRx Therapeutics (Singapore, Republic of Singapore).[16] An *in vitro* study showed the ability of TRx 0237 in disrupting PHFs isolated from AD brain tissues at the concentration at 0.16 μM.[16] This value is identical to what found for MT (0.16 μM).[16] The *in vivo* effects of MTC and TRx0237 (5–75 mg/kg orally for 3–8 weeks) were compared in two novel mouse models overexpressing different human tau-protein constructs (L1 and L66).[37] Both MTC and TRx0237 dose-dependently rescued the learning impairment and restored behavioral flexibility in a spatial problem-solving water-maze task in L1 (minimum effective dose: 35 mg MT/kg for MTC, 9 mg MT/kg for TRx0237) and corrected motor learning in L66 (effective doses: 4 mg MT/kg).[18] Both compounds reduced the number of tau-reactive neurons, particularly in the hippocampus and entorhinal cortex in L1 and in a more widespread manner in L66.

A safety and tolerability study of TRx0237 (250 mg/day for 4 weeks) in nine patients with mild-to-moderate AD began in September 2012 but it was terminated in April 2013, reportedly for administrative reasons (ClinicalTrials.gov Identifier: NCT01626391) (Table 1). Three Phase III placebo-controlled studies with TRx0237 are ongoing (Table 1). The first study is evaluating the 200-mg/day dose in 700 patients with a diagnosis of either all-cause dementia and probable AD and adopted the cognitive ADAS-Cog 11 scale and the clinical Alzheimer's Disease Cooperative Study – Clinical Global Impression of Change (ADCS-CGIC) scale as primary efficacy variables (ClinicalTrials.gov Identifier: NCT01689233). The second study is evaluating the doses of 150 and 250 mg/day in 833 patients with mild-to-moderate AD and is using the ADAS-Cog 11 and ADCS-CGIC as primary endpoints (ClinicalTrials.gov Identifier: NCT01689246). The third Phase III trial is evaluating the 200 mg/day dose in 220 patients affected by the behavioral variant of frontotemporal dementia (bvFTD) (ClinicalTrials.gov Identifier: NCT01626378). This trial adopted a modified version of the ADCS-CGIC scale as measure of clinical efficacy and the revised Addenbrooke's Cognitive Examination as cognitive measure. Finally, an open-label extension study in subjects who have completed participation in a Phase II or Phase III trials with TRx0237 is evaluating the long-term safety of the compound (ClinicalTrials.gov Identifier: NCT02245568) (Table 1). With the hope of maintaining blinding, the Phase III studies are using 'active placebo' tablets that include 4 mg of TRx0237 as a urinary and fecal colorant. Overall, these Phase III trials are recruiting 1753 patients at 250 centers in 22 countries and results are expected in the first half of 2016 for one of these trials (ClinicalTrials.gov Identifier: NCT01689246) and in the second half of 2016 for the other two studies (ClinicalTrials.gov Identifiers: NCT01689233 and NCT01626378).

5. Expert opinion

In the last 10 years, several clinical trials with anti-Aβ agents failed, challenging the hypothesis that Aβ accumulation is the initiating event in the pathological AD cascade, and underscoring the need for novel therapeutic approaches and targets. Among TAIs, MT belongs to a class of diaminophenothiazines that have TAI activity *in vitro*.[6,16] MTC, in which MT is dosed as the oxidized form MT^{+}, was investigated in an exploratory Phase II dose-ranging double-blind clinical trial in 321 patients with mild-to-moderate AD.[36] The minimum effective dose was identified as 138 mg MT/day at both clinical and molecular imaging endpoints at 24 weeks. Treatment at this dose was found to prevent the decline in regional cerebral blood flow, particularly in medial temporal lobe structures and temporoparietal regions. Given that the delivery of the highest dose of MT was impaired due to dose-dependent dissolution and absorption limitations, four Phase I studies [12] and two preclinical *in vitro* [16] and *in vivo* studies [18] were required to get to the bottom of the bioavailability limitations of the form of MT tested in the Phase II trial,[36] setting out the basis for proceeding into Phase III trials with TRx0237 for AD treatment. TRx0237 is claimed to have a better pharmacokinetic and tolerability profile than MTC, but not convincing evidences have been provided to support this. The better oral absorption of TRx0237 compared to MTC in the presence of food showed in healthy volunteers did not translate in higher CNS levels since drug brain levels in minipigs are almost identical after 33 mg/kg (about 5 μM) of MT or TRx0237.[12] On the other hand, no data on TRx0237 CSF concentrations in humans are available.[12] No robust data on safety and tolerability of TRx0237 in humans are available to make direct comparison with MTC. Comparative *in vitro* data showed a therapeutic index (ratio of LD_{50}/EC_{50}) of 92 for LMT-dihydrobromide and 179 for LMT-dihydromesylate compared to value of 110 for MTC.[16] We believe that these *in vitro* differences were not so dramatic to necessarily translating in pharmacological or clinical differences. In terms of efficacy, pharmacological studies in transgenic mouse tauopathy models did not show dramatic differences between the two compounds.[18] Indeed, a dose of 45 mg/kg of MTC or TRx0237 produced identical behavioral effects.[18]

The lack of Phase II data on TRx0237 appears to be a risk for the proper design of the Phase III trials. We noted that in one of the two Phase III studies in AD patients, mildly affected patients have been recruited on the basis of the apparent encouraging signals of cognitive efficacy of MTC in AD, although these results were observed in moderately affected patients.[36] A dose of 100 mg administered twice daily of TRx027 was selected in this Phase III study (ClinicalTrials.gov Identifier: NCT01689233). The second Phase III study in AD patients has recruited both mild and moderate patients and is evaluating two doses (75 mg and 125 administered twice daily) (ClinicalTrials.gov Identifier: NCT01689246), a choice not consistent with the first trial. Nevertheless, the overall results of these Phase III studies will tell us something on the clinical potential of TAIs in treating AD and bvFTD. More importantly, we still need to fully understand the role of tau protein in AD pathogenesis. We have to clarify the mechanisms of tau

degradation, the role of soluble, non-aggregated forms of tau, the link between tau and Aβ toxicity, and the mechanisms by which tau may damage mitochondrial activity.

Declaration of Interest

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This article described two transgenic mouse models developed to better understanding tau pathology.

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