Administration and dosage of diaminophenothiazines.

Technical field

The present invention relates generally to methods and materials for use in the treatment or prophylaxis of diseases of protein aggregation, for example cognitive disorders, using diaminophenothiazines.

Background art

Aberrant protein aggregation is believed to be a proximal cause of numerous disease states, which may be manifested as neurodegeneration, clinical dementia, and other pathological symptoms.

In general, the aberrant protein aggregation is that which arises from an induced conformational polymerisation interaction, i.e., one in which a conformational change of the protein, or in a fragment thereof, gives rise to templated binding and aggregation of further (precursor) protein molecules in a self-propagating manner.

Once nucleation is initiated, an aggregation cascade may ensue which involves the induced conformational polymerisation of further protein molecules, leading to the formation of toxic product fragments in aggregates which are substantially resistant to further proteolysis.

For example certain conditions of dementia may be characterised by a progressive accumulation of intracellular and/or extracellular deposits of proteinaceous structures such as β-amyloid plaques and neurofibrillary tangles (NFTs) in the brains of affected patients. The appearance of these lesions largely correlates with pathological neurofibrillary degeneration and brain atrophy, as well as with cognitive impairment (see, e.g., Mukaetova-Ladinska, E.B. et al., 2000, Am. J. Pathol., Vol. 157, No. 2, pp. 623-636).

Current approved treatments for Alzheimer's disease include acetylcholinesterase inhibitors (AChEIs) and the N-methyl-D-aspartate receptor antagonist memantine. These are symptomatic and do not address the underlying disease pathology. Therapies targeting the amyloid pathology have so far proved unsuccessful in late stage clinical trials (Geerts et al., 2013; Mullane and Williams, 2013). According to a recent Lancet Neurology Commission, "an effective treatment for AD is perhaps the greatest unmet medical need facing modern medicine", (Winblad et al., 2016) not least because the global economic cost of dementia is estimated to be $818 billion, or 0.65% of global gross domestic product (Alzheimer's Disease International, 2015).

NFTs (the pathology discovered by Alois Alzheimer, (Alzheimer, 1907)) are made up of paired helical filaments (PHFs), composed predominantly of a 12-kDa repeat-domain fragment of the microtubule-associated protein tau (Wischik et al., 1985; Wischik et al., 1988a,b). Numerous studies have confirmed a quantitative link for the spread of neurofibrillary tangle pathology and the quantity of aggregated tau with both the extent of

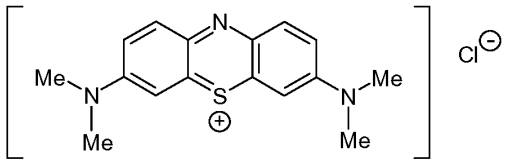
clinical dementia and functional molecular imaging deficits in Alzheimer's disease (Arriagada et al., 1992; Brier et al., 2016; Giannakopoulos et al., 2003; Josephs et al., 2003; Maruyama et al., 2013). Since pathological aggregation of tau protein begins at least 20 years prior to any of the clinical manifestations, (Braak and del Tredici, 2013) targeting this pathology offers a rational approach to both treatment and prevention of AD and related tau aggregation disorders (Huang and Mucke, 2012; Wischik et al., 2014; Wischik et al., 2010).

The tau fragment originally identified as an intrinsic structural constituent of the PHF core has prion-like properties *in vitro* in that it captures normal tau protein with very high affinity (Lai et al., 2016) and converts it to a proteolytically stable replicate of itself (Wischik et al., 1996; Harrington et al., 2015) in a process which is self-propagating and autocatalytic. Phosphorylation is inhibitory to aggregation (Lai et al., 2016) and is unlikely to drive of the cascade (Mukaetova-Ladinska et al., 2000; Schneider et al., 1999; Wischik et al., 1995). Direct inhibition of tau aggregation represents a plausible point for therapeutic intervention.

Methylthioninium (MT) acts as a tau aggregation inhibitor (TAI) *in vitro*, (Wischik et al., 1996; Harrington et al., 2015) dissolves PHFs from Alzheimer's disease brain tissue, (Wischik et al., 1996) and reduces tau pathology and associated behavioural deficits in transgenic mouse tau models at brain concentrations consistent with human oral dosing. (Melis et al., 2015; Baddeley et al., 2015) MT has also been shown to inhibit other disease-associated protein aggregation (see e.g. WO2007/110629).

MT is a redox molecule and, depending on environmental conditions (e.g., pH, oxygen, reducing agents), exists in equilibrium between a reduced [leucomethylthioninium (LMT)] and oxidized form (MT^+).

WO96/30766 describes such MT containing compounds for use in the treatment and prophylaxis of various diseases, including AD and Lewy Body Disease. One example compound was methylthioninium chloride ("MTC") commonly known as methylene blue, which is the chloride salt of the oxidized form of methylthioninium (MT) i.e. MT^+.

MTC	 methyl-thioninium chloride

WO96/30766 describes, in the case of oral administration, a daily dosage of about 50 mg to about 700 mg, preferably about 150 mg to about 300 mg, divided in preferably 1-3 unit doses.

WO2007/110630 discloses certain specific diaminophenothiazine compounds related to MTC, including (so-called) ETC, DEMTC, DMETC, DEETC, MTZ, ETZ, MTI, MTILHI, ETI, ETLHI, MTN, and ETN, which are useful as drugs, for example in the treatment of Alzheimer's disease.

WO2007/110630 describes dosage units comprising 20 to 300 mg of 3,7-diaminophenothiazine (DAPTZ) compounds described therein e.g. 30 to 200 mg, for example 30 mg, 60 mg, 100 mg, 150 mg, 200 mg. A suitable dose of the DAPTZ compound is suggested in the range of about 100 ng to about 25 mg (more typically about 1 µg to about 10 mg) per kilogram body weight of the subject per day e.g. 100 mg, 3 times daily, 150 mg, 2 times daily, 200 mg, 2 times daily. A dosage of 50mg 3 or 4 times daily is also discussed.

A preliminary pharmacokinetic model for methylene blue, based on studies of urinary excretion data sets in humans, dogs and rats, was proposed by DiSanto and Wagner, J Pharm Sci 1972, 61:1086-1090 and 1972, 61:1090-1094 and Moody et al., Biol Psych 1989, 26: 847-858.

Peter *et al.* (2000) Eur J Clin Pharmacol 56: 247-250 provided a model which integrated blood level data, which contradicted the earlier data from DiSanto and Wagner as regards terminal elimination half-life.

May et al. (Am J Physiol Cell Physiol, 2004, Vol. 286, pp. C1390-C1398) showed that human erythrocytes sequentially reduce and take up MTC i.e. that MTC itself is not taken up by the cells but rather that it is the reduced from of MT that crosses the cell membrane. They also showed that the rate of uptake is enzyme dependent; and that both oxidised and reduced MT are concentrated in cells (reduced MT re-equilibrates once inside the cell to form oxidised MT).

Based on these and other disclosures, it is believed that orally administered MTC and similar drugs are taken up in the gut and enter the bloodstream, with unabsorbed drug percolates down the alimentary canal, to the distal gut. One important undesired side-effect is the effect of the unabsorbed drug in the distal gut, for example, sensitisation of the distal gut and/or antimicrobial effects of the unabsorbed drug on flora in the distal gut, both leading to diarrhoea.

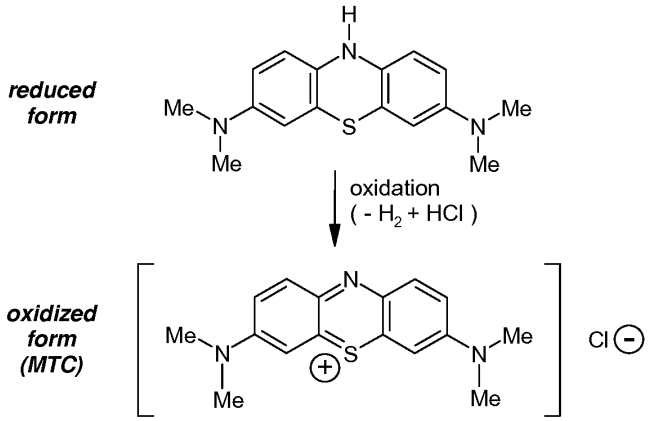
MTC was tested clinically in a phase 2 study (Wischik et al., 2015). Although the minimum safe and effective dose was identified as 138 mg/day, a higher dose of 218 mg/day had limited efficacy due to absorption limitations, most likely due to the need for the MT^+ to be reduced to the leuco-MT (LMT) form to permit efficient absorption by passive diffusion.

WO2009/044127 disclosed the results of a phase 2 clinical trial, which indicated that MTC had two systemic pharmacological actions: cognitive effects and haematological effects, but that these actions were separable. Specifically the cognitive effects did not show a monotonic dose-response relationship, whereas the haematological effects did. It was

proposed that two distinct species were responsible for the two types of pharmacological activity: MTC absorbed as the uncharged Leuco-MT form being responsible for the beneficial cognitive activity, and MTC absorbed as an oxidised dimeric species being responsible for the oxidation of haemoglobin. WO2009/044127 described how dosage forms could be used to maximise the bioavailability of the therapeutically active (cognitively effective) species whether dosing with oxidised or leuco-DAPTZ compounds.

Since it is the reduced form of MT that is taken up by cells, it has been proposed to administer a reduced form to patients. This may also reduce reliance on the rate limiting step of enzymatic reduction.

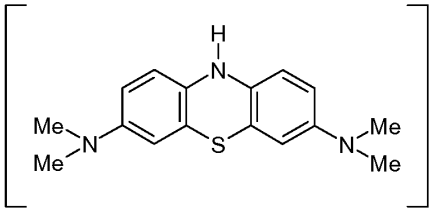
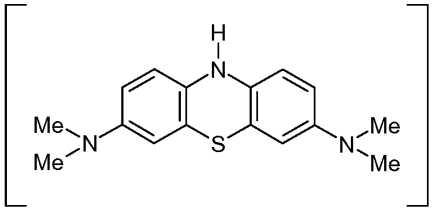
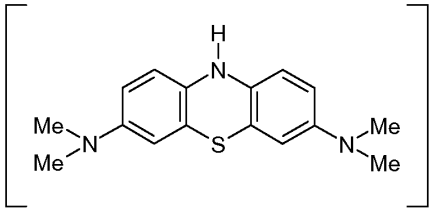
MTC, a phenothiazin-5-ium salt, may be considered to be an "oxidized form" in relation to the corresponding 10H-phenothiazine compound, N,N,N',N'-tetramethyl-10H-phenothiazine-3,7-diamine, which may be considered to be a "reduced form":



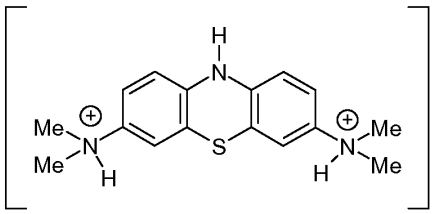
The "reduced form" (or "leuco form") is known to be unstable and can be readily and rapidly oxidized to give the corresponding "oxidized" form.

WO 02/055720 discloses the use of reduced forms of certain diaminophenothiazines for the treatment of protein aggregating diseases, primarily tauopathies. Based on in vitro activity for the reduced forms of diaminophenothiazines therein, a suggested daily dosage was 3.2-3.5 mg/kg, and dosages of 20 mg t.d.s., 50 mg t.d.s. or 100 mg t.d.s., combined with 2x mg ratio of ascorbic acid in such a manner as to achieve more than 90% reduction prior to ingestion were also described.

WO2007/110627 disclosed certain 3,7-diamino-10H-phenothiazinium salts, effective as drugs or pro-drugs for the treatment of diseases including Alzheimer's disease. These compounds are also in the "reduced" or "leuco" form when considered in respect of MTC. These leucomethylthioninium compounds were referred to as "LMTX" salts, and included the following salts:

	HCl HCl	N,N,N',N'-tetramethyl-10H-phenothiazine-3,7-diaminium di(hydrochloride), (LMT.2HCl)
	HBr HBr	N,N,N',N'-tetramethyl-10H-phenothiazine-3,7-diaminium di(hydrobromide), (LMT.2HBr)
	HI HI	N,N,N',N'-tetramethyl-10H-phenothiazine-3,7-diaminium di(hydroiodide), (LMT.2HI)

WO2012/107706 described other LMTX salts having superior properties to the LMTX salts listed above, including leuco-methylthioninium bis(hydromethanesulfonate) (LMTM):

	$MeSO_3^-$ $MeSO_3^-$	*N,N,N',N'*-tetramethyl-10*H*-phenothiazine-3,7-diaminium bis(hydromethanesulfonate). LMT.2MsOH / LMTM

Specifically LMTM retains TAI activity *in vitro* and *in vivo* (Wischik et al, 1996; Harrington et al., 2015; Melis et al., 2015) has superior pharmaceutic properties in terms of solubility and pKa, and is not subject to the absorption limitations of the MT^+ form.[24] (Baddeley et al., 2015)

WO2007/110627 and WO2012/107706 describes dosage units comprising 20 to 300 mg of the DAPTZ compounds described therein e.g. 30 to 200 mg, for example 30 mg, 60 mg, 100 mg, 150 mg, 200 mg. A suitable dose of the DAPTZ compound is suggested in the range of about 100 ng to about 25 mg (more typically about 1 µg to about 10 mg) per kilogram body weight of the subject per day e.g. 100 mg, 3 times daily, 150 mg, 2 times daily, 200 mg, 2 times daily.

Disclosure of the invention

The present inventors have conducted a 15-month double-blind randomised controlled phase 3 trial in mild to moderate AD patient to test the safety and efficacy of LMTM.

Doses of LMTM at 75 mg and 125 mg given twice daily (b.i.d.) were compared with a control dose of 4 mg b.i.d. The control dosage was determined from prior repeat-dose phase 1 studies as the minimum needed to maintain blinding with respect to discolouration of excreta.

Unexpectedly, the 4 mg b.i.d. dose showed therapeutic benefits. The efficacy profiles were similar in mild and moderate subjects for most of the measured outcomes.

The reason for the efficacy of this low dose of MT compound, is unclear. It has previously been shown that the absorption and distribution of MT to the brain is complex, and likely to be mediated via red cells rather than plasma (Baddeley et al., 2015) providing a route which protects MT from first-pass metabolism. In the same study MT uptake into red cells was approximately 20-fold higher *in vivo* when as administered intravenously as LMTM compared with MTC, most likely due to direct red cell uptake of LMT by passive diffusion without need for prior reduction of MT^+ as is the case for MTC (Baddeley et al., 2015; May et al., 2004). Without wishing to be bound by theory, the results of the present study suggest that MT uptake and distribution are capacity-limited by the amount that red cells can take up whilst within the portal circulation.

Unlike an earlier phase 2 study, which was conducted in subjects not taking AD-labelled co-medications, patients in the phase 3 trial were permitted to enter whether or not they were not taking these medications, as it was considered infeasible for them to be restricted given their extensive use.

Unexpectedly, treatment benefit in AD (according to the trial criteria) was restricted to patients taking LMTM as monotherapy. By contrast, the decline seen at corresponding doses in patients taking LMTM in combination with AD-labelled treatments (acetylcholinesterase inhibitors [AChEIs] and\or memantine) who were the majority, was indistinguishable on all parameters from that seen in the control arm.

The reason for the loss of benefit on clinical and volumetric outcomes in AD when LMTM is combined with symptomatic AD treatments is unclear, although a possible contributory factor may be induction of the multidrug resistance protein 1 (MDR1), a transporter which is upregulated by AChEIs and memantine. This transporter may directly or indirectly lead to reduction of the levels of MT from the site of action.

Irrespective of the mechanism, the disclosure herein indicates that much lower doses of MT than previously envisaged can produce substantial clinical benefits whilst being well tolerated and having fewer side effects than the higher doses.

In respect of AD treatments, such treatments would preferably be a monotherapy, or at least introduced either prior to or following cessation of the currently available AD treatments AChEIs and memantine,

Although the term “low dose” or “low dosage” has been used in relation to MT-containing compounds in prior art publications, there is no teaching or suggestion in those publications of the utility of the present invention.

For example:

Telch, Michael J., et al. "Effects of post-session administration of methylene blue on fear extinction and contextual memory in adults with claustrophobia." American Journal of Psychiatry 171.10 (2014): 1091-1098: this publication refers to the use of “low-dose methylene blue” on retention of fear extinction and contextual memory following fear extinction training. The paper reports that “Methylene blue is a diamino phenothiazine drug that at low doses (0.5–4 mg/kg) has neurometabolic-enhancing properties. The dosages used in the publication were 260mg/day for adult participants, corresponding to a 4mg/kg dose.

Gonzalez-Lima F and Auchter A (2015) “Protection against neurodegeneration with low-dose methylene blue and near-infrared light”. Front. Cell. Neurosci. 9:179. doi: 10.3389/fncel.2015.00179: this publication discusses the cellular mechanisms mediating the neuroprotective effects of low doses of methylene blue and near-infrared light. It refers to earlier work citing 0.5–4mg/kg of methylene blue as safe and effective.

Alda, Martin, et al. "Methylene blue treatment for residual symptoms of bipolar disorder: randomised crossover study." *The British Journal of Psychiatry* (2016): doi: 10.1192/bjp.bp.115.173930: this publication described the use of a 15 mg “low dose” of methylene blue as a placebo in a 6 month trial. The “active dose” was 195 mg. In each case the dose was split three times daily.

Rodriguez, Pavel, et al. "Multimodal Randomized Functional MR Imaging of the Effects of Methylene Blue in the Human Brain." *Radiology* (2016): 152893: this publication also refers to the ‘known’ pharmacokinetic and side effects of “low-dose” (0.5–4.0 mg/kg) methylene blue, which are contrasted with the effects of dosages greater than 10 mg/kg. The dosages used in the publication were 280mg/day for adult participants, approximating to a 4mg/kg dose.

Naylor et al. (1986) “A two-year double-blind crossover trial of the prophylactic effect of methylene blue in manic-depressive psychosis”. Biol. Psychiatry 21:915-920 and Naylor et al. (1987) A controlled trial of methylene blue in severe depressive psychosis. Biol. Psychiatry 22:657-659: these studies used 15mg/day methylene, nominally as a placebo vs. a treatment of 300 mg/day methylene blue. However in the latter paper the authors proposed that the placebo dosage may act as an antidepressant.

Thus in one aspect there is disclosed a method of treatment of a neurodegenerative disorders of protein aggregation in a subject,

which method comprises orally administering to said patient an methylthioninium (MT) containing compound,

wherein said administration provides a total of between 0.5 and 20mg of MT to the subject per day, optionally split into 2 or more doses.

The total dose may be from around any of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 mg to around any of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20mg.

An example dosage is 1 to 20mg.

A further example dosage is 2 to 15 mg.

A further example dosage is 3 to 10 mg.

A further preferred dosage is 3.5 to 7 mg.

A further preferred dosage is 4 to 6 mg.

As explained below, when administering MT in the reduced (LMT) form, it may be desired to use a smaller total amount within the recited range, compared to the oxidised (MT^+) form.

As explained below, when administering the MT dose split in a larger number of doses/day it may be desired to use a smaller total amount within the recited range, compared to a single daily dosing, or a smaller number of doses per day.

In other embodiments the neurodegenerative disorder may be AD.

As explained herein, in some embodiments the treatment will be a monotherapy, or at least will exclude co-medication with AChEIs and memantine,

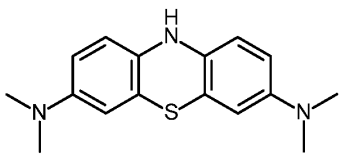
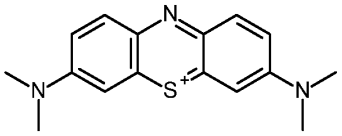
In other embodiments the neurodegenerative disorder may be a neurodegenerative disorder other than AD.

Also provided herein are methods of prophylactic treatment of neurodegenerative disorders of protein aggregation.

Also provided herein are novel dosage forms containing low unit doses of MT compounds, for example high purity MT compounds, for example MT^+ compounds or LMTX compounds.

These aspects and embodiments will now be described in more detail:

Methylthioninium moiety

Structure		
IUPAC	N3,N3,N7,N7-tetramethyl-10H-phenothiazine-3,7-diamine	N3,N3,N7,N7-tetramethylphenothiazin-5-ium-3,7-diamine
Composition	Formula Weight : 285.41(1) Exact Mass : 285.1299683(1) Formula : $C_{16}H_{19}N_3S$ Composition : C 67.33% H 6.71% N 14.72% S 11.23%	Formula Weight : 284.40(1) Exact Mass : 284.1215947(1) Formula : $C_{16}H_{18}N_3S$ Composition : C 67.57% H 6.38% N 14.78% S 11.27%
Synonym	leucomethylthioninium (LMT)	oxidized methylthioninium (MT^+)

The MT^+-containing compounds used in the present invention can contain MT in either reduced or oxidised form. The "MT" is the active ingredient, which is to say that it is present to provide the recited therapeutic effect. Specifically, the compounds may comprise either of the MT moieties described above. The MT moieties *per se* described above are not stable. They will therefore be administered as MT compounds – for example LMT or MT^+ salts.

MT^+ salts will generally include include one or more anionic counter ions (X^-) to achieve electrical neutrality. The compounds may be hydrates, solvates, or mixed salts of the MT^+ salt.

LMT containing compounds will generally be stabilised, for example by the presence of one or more protic acids e.g. two protic acids.

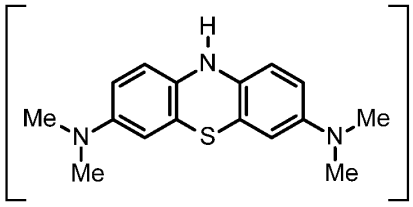
The MT content of such salts can be readily calculated by those skilled in the art based on the molecular weight of the compound, and the molecular weight of the MT moiety. Examples of such calculations are given herein.

LMT compounds

Preferably the MT compound is an LMT compound.

Preferably the MT compound is an "LMTX" compound of the type described in WO2007/110627 or WO2012/107706.

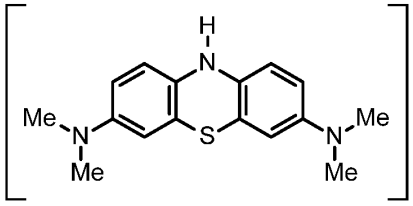
Thus the compound may be selected from compounds of the following formula, or hydrates or solvates thereof:

	$p(H_nA)$ $q(H_nB)$	Options: p = 1, 2 q = 0, 1 n = 1, 2 (p + q) × n = 2

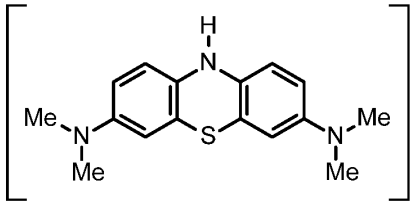
Each of H_nA and H_nB (where present) are protic acids which may be the same or different.

By "protic acid" is meant a proton (H^+) donor in aqueous solution. Within the protic acid A^- or B^- is therefore a conjugate base. Protic acids therefore have a pH of less than 7 in water (that is the concentration of hydronium ions is greater than 10^{-7} moles per litre).

In one embodiment the salt is a mixed salt that has the following formula, where HA and HB are different mono-protic acids:

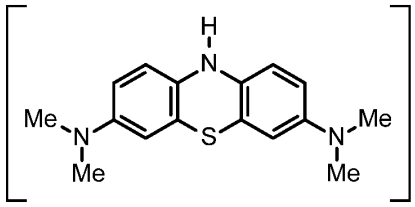
	HA HB	when: p = 1 q = 1 n = 1 (1 + 1) × 1 = 2

However preferably the salt is not a mixed salt, and has the following formula:

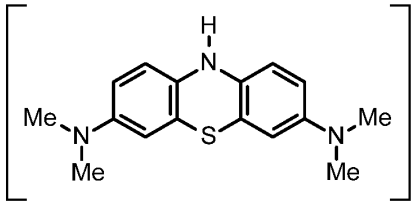
	$p(H_nX)$	when: p = 1, 2 n = 1, 2 p × n = 2

wherein each of H_nX is a protic acid, such as a di-protic acid or mono-protic acid.

In one embodiment the salt has the following formula, where H_2A is a di-protic acid:

	H_2A	when: p = 1 q = 0 n = 2 (1 + 0) × 2 = 2

Preferably the salt has the following formula which is a bis monoprotic acid:

	2(HA)	when: p = 2 q = 0 n = 1 (2 + 0) × 1 = 2

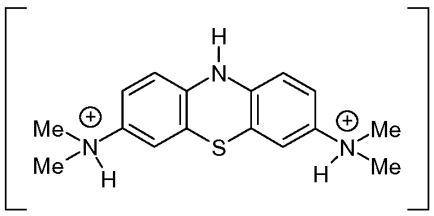
Examples of protic acids which may be present in the LMTX compounds used herein include:

Inorganic acids: hydrohalide acids (e.g., HCl, HBr), nitric acid (HNO_3), sulphuric acid (H_2SO_4)

Organic acids: carbonic acid (H_2CO_3), acetic acid (CH_3COOH), methanesulfonic acid, 1,2-Ethanedisulfonic acid, ethansulfonic acid, Naphthalenedisulfonic acid, p-toluenesulfonic acid,

Preferred acids are monoprotic acid, and the salt is a bis(monoprotic acid) salt.

A preferred MT compound is LMTM:

1		LMT.2MsOH (LMTM)	477.6 (1.67)

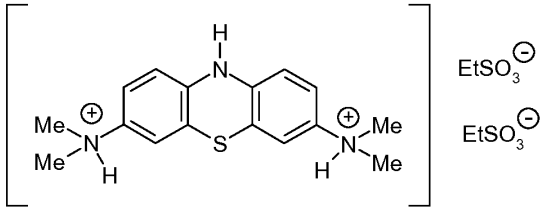
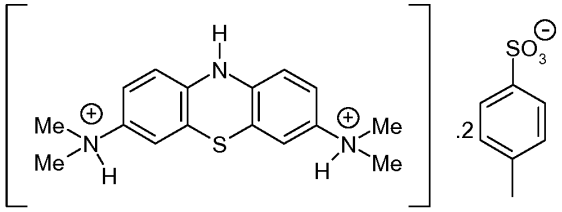
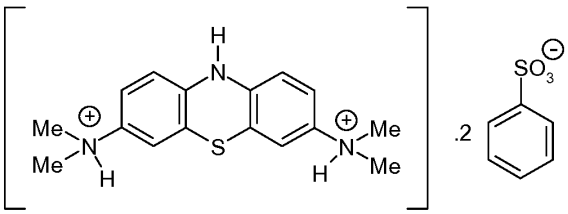
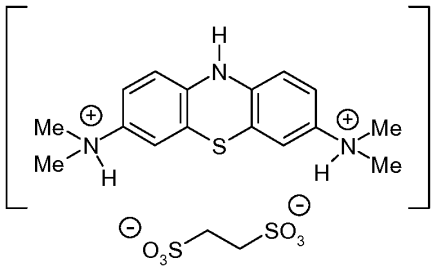
The anhydrous salt has a molecular weight of around 477.6. Based on a molecular weight of 285.1 for the LMT core, the weight factor for using this MT compound in the invention is 1.67. By “weight factor” is meant the relative weight of the pure MT containing compound vs. the weight of MT which it contains.

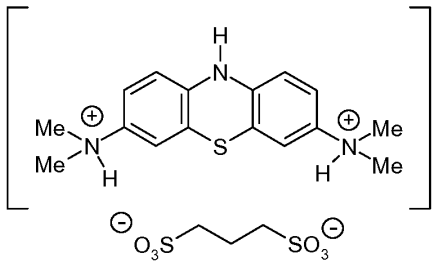
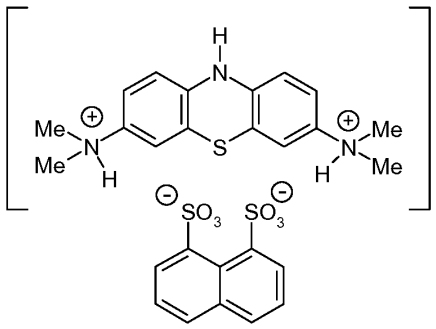
Other weight factors can be calculated for example MT compounds herein, and the corresponding dosage ranges can be calculated therefrom.

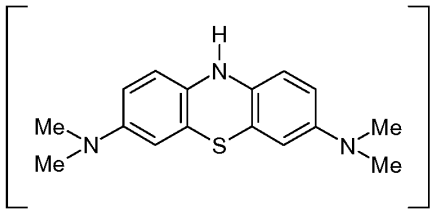
Therefore the invention embraces a total daily dose of around 0.8 to 33 mg/day of LMTM.

More preferably around 6 to 12 mg/day of LMTM total dose is utilised, which corresponds to about 3.5 to 7 mg MT.

Other example LMTX compounds are as follows. Their molecular weight (anhydrous) and weight factor is also shown:

2		LMT.2EsOH	505.7 (1.77)
3		LMT.2TsOH	629.9 (2.20)
4		LMT.2BSA	601.8 (2.11)
5		LMT.EDSA	475.6 (1.66)

6		LMT.PDSA	489.6 (1.72)
7		LMT.NDSA	573.7 (2.01)

8	 HCl HCl	LMT.2HCl	358.33 (1.25)

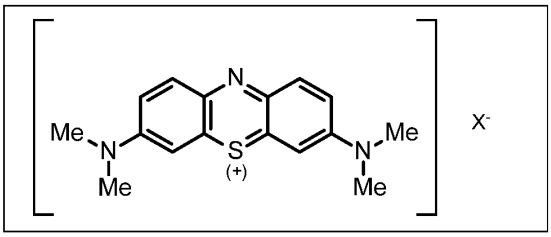
The dosages described herein with respect to MT thus apply *mutatis mutandis* for these MT containing compounds, as adjusted for their molecular weight.

Oxidised MT compounds

In another embodiment the MT compound is an MT^+ compound.

Preferably the MT compound is an MT^+ compound of the type described in WO96/30766 or WO2007/110630.

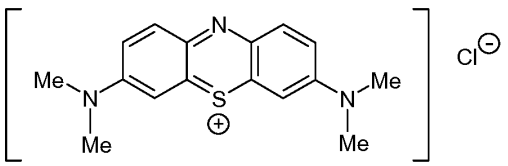
Thus the compound may be selected from compounds of the following formula, or hydrates, solvates, or mixed salts thereof:



Where X^- is an anionic counter ion.

In some embodiments of the present invention the MT^+ compound is MTC, for example a "high purity" MTC as described below.

In some embodiments of the present invention the MT^+ compound is not MTC.

9	 methyl-thioninium chloride	MTC

As explained in WO2011/036561 and WO2011/036558, MTC occurs in a number of polymorphic forms having different levels of hydration.

In some embodiments of the present invention, the MT^+ compound is a high purity MTC. In this context 'high purity' is defined by one or more of the criteria set out below.

In some embodiments, the MTC has a purity of greater than 97%.
In some embodiments, the MTC has a purity of greater than 98%.
In some embodiments, the MTC has a purity of greater than 99%.

In some embodiments, the MTC has less than 2% Azure B as impurity.
In some embodiments, the MTC has less than 1% Azure B as impurity.
In some embodiments, the MTC has less than 0.5% Azure B as impurity.
In some embodiments, the MTC has less than 0.1% Azure B as impurity.

In some embodiments, the MTC has less than 0.15% Azure A as impurity.
In some embodiments, the MTC has less than 0.10% Azure A as impurity.
In some embodiments, the MTC has less than 0.05% Azure A as impurity.

In some embodiments, the MTC has less than 0.15% Azure C as impurity.
In some embodiments, the MTC has less than 0.10% Azure C as impurity.
In some embodiments, the MTC has less than 0.05% Azure C as impurity.

In some embodiments, the MTC has less than 0.13% MVB (Methylene Violet Bernstein) as impurity.
In some embodiments, the MTC has less than 0.05% MVB as impurity.
In some embodiments, the MTC has less than 0.02% MVB as impurity.

All percentage purities recited herein are by weight unless otherwise specified.

In some embodiments, the MTC has an elementals purity that is better than that specified by the European Pharmacopeia (EP).

As used herein, the term 'elementals purity' pertains to the amounts of the twelve (12) metals specified by the European Pharmacopeia: Al, Cd, Cr, Cu, Sn, Fe, Mn, Hg, Mo, Ni, Pb, and Zn. The current edition of the European Pharmacopeia (8th Edition, supplementum 8.8) specifies the following limits for these metals:

European Pharmacopeia 8.8 (EP8.8)	
Element	**Maximum content (µg/g)**
Aluminium (Al)	100
Cadmium (Cd)	1
Chromium (Cr)	100
Copper (Cu)	300
Tin (Sn)	10
Iron (Fe)	200
Manganese (Mn)	10
Mercury (Hg)	1
Molybdenum (Mo)	10
Nickel (Ni)	10
Lead (Pb)	10
Zinc (Zn)	100

In one embodiment, the MTC has an elementals purity (e.g. for each of Al, Cd, Cr, Cu, Sn, Fe, Mn, Hg, Mo, Ni, Pb, and Zn) which is equal to or better than (i.e. lower than) the EP8.8 values set out in the table above.

In one embodiment, the MTC has an elementals purity which is equal to or better than 0.9 times the EP8.8 values set out in the table above.

In one embodiment, the MTC has an elementals purity which is equal to or better than 0.8 times the EP8.8 values set out in the table above.

In one embodiment, the MTC has an elementals purity which is equal to or better than 0.7 times the EP8.8 values set out in the table above.

In one embodiment, the MTC has an elementals purity which is equal to or better than 0.5 times the EP8.8 values set out in the table above.

(For example, 0.5 times the EP8.8 values as set out above are 50 µg/g Al, 0.5 µg/g Cd, 50 µg/g Cr, etc.)

In one embodiment the MTC has a chromium level that is equal to or better than (i.e. lower than) 100 µg/g.

In one embodiment the MTC has a chromium level that is equal to or better than (i.e. lower than) 10 µg/g.

In one embodiment the MTC has a copper level that is equal to or better than (i.e. lower than) 300 µg/g.

In one embodiment the MTC has a copper level that is equal to or better than (i.e. lower than) 100 µg/g.

In one embodiment the MTC has a copper level that is equal to or better than (i.e. lower than) 10 µg/g.

In one embodiment the MTC has an iron level that is equal to or better than (i.e. lower than) 200 µg/g.

In one embodiment the MTC has an iron level that is equal to or better than (i.e. lower than) 100 µg/g.

All plausible and compatible combinations of the above purity grades are disclosed herein as if each individual combination was specifically and explicitly recited.

In particular embodiments, the MTC is a high purity MTC wherein 'high purity' is characterised by a purity of greater than 98% and one or more of the following:
(i) less than 2% Azure B as impurity;
(ii) less than 0.13% MVB (Methylene Violet Bernstein) as impurity; or
(iii) an elementals purity better than the European Pharmacopeia limits of less than 100 µg/g Aluminium (Al); less than 1 µg/g Cadmium (Cd); less than 100 µg/g Chromium (Cr); less than 300 µg/g Copper (Cu); less than 10 µg/g Tin (Sn); less than 200 µg/g Iron (Fe); less than 10 µg/g Manganese (Mn); less than 1 µg/g Mercury (Hg); less than 10 µg/g Molybdenum (Mo); less than 10 µg/g Nickel (Ni); less than 10 µg/g Lead (Pb); and less than 100 µg/g Zinc (Zn).

In particular embodiments, the MTC is a high purity MTC wherein high-purity is characterised by a purity of greater than 98% and one or more of the following:
(i) less than 1% Azure B as impurity;
(ii) less than 0.15% Azure A as impurity;
(iii) less than 0.15% Azure C as impurity;
(iv) less than 0.13% Methylene Violet Bernthsen (MVB) as impurity;
(v) an elementals purity better than the European Pharmacopeia limits of less than 100 µg/g Aluminium (Al); less than 1 µg/g Cadmium (Cd); less than 100 µg/g Chromium (Cr);

less than 300 µg/g Copper (Cu); less than 10 µg/g Tin (Sn); less than 200 µg/g Iron (Fe); less than 10 µg/g Manganese (Mn); less than 1 µg/g Mercury (Hg); less than 10 µg/g Molybdenum (Mo); less than 10 µg/g Nickel (Ni); less than 10 µg/g Lead (Pb); and less than 100 µg/g Zinc (Zn).

In particular embodiments, the MTC is a high purity MTC wherein high-purity is characterised by a purity of greater than 98% and one or more of the following:
(i) less than 1% Azure B as impurity;
(ii) less than 0.15% Azure A as impurity;
(iii) less than 0.15% Azure C as impurity;
(iv) less than 0.05% Methylene Violet Bernthsen (MVB) as impurity; or
(v) an elementals purity better than the European Pharmacopeia limits of less than 100 µg/g Aluminium (Al); less than 1 µg/g Cadmium (Cd); less than 100 µg/g Chromium (Cr); less than 300 µg/g Copper (Cu); less than 10 µg/g Tin (Sn); less than 200 µg/g Iron (Fe); less than 10 µg/g Manganese (Mn); less than 1 µg/g Mercury (Hg); less than 10 µg/g Molybdenum (Mo); less than 10 µg/g Nickel (Ni); less than 10 µg/g Lead (Pb); and less than 100 µg/g Zinc (Zn).

In particular embodiments, the MTC is a high purity MTC wherein high-purity is characterised by at least 98% purity and less than 1% Azure B as impurity.

In particular embodiments, the MTC is a high purity MTC wherein high-purity is characterised by:
(i) at least 98% purity
(i) less than 1% Azure B as impurity; and
(ii) an elementals purity better than the European Pharmacopeia limits of less than 100 µg/g Aluminium (Al); less than 1 µg/g Cadmium (Cd); less than 100 µg/g Chromium (Cr); less than 300 µg/g Copper (Cu); less than 10 µg/g Tin (Sn); less than 200 µg/g Iron (Fe); less than 10 µg/g Manganese (Mn); less than 1 µg/g Mercury (Hg); less than 10 µg/g Molybdenum (Mo); less than 10 µg/g Nickel (Ni); less than 10 µg/g Lead (Pb); and less than 100 µg/g Zinc (Zn).

In particular embodiments, the MTC is a high purity MTC wherein high-purity is characterised by at least 98% purity and an elementals purity better than the European Pharmacopeia limits of less than 100 µg/g Aluminium (Al); less than 1 µg/g Cadmium (Cd); less than 100 µg/g Chromium (Cr); less than 300 µg/g Copper (Cu); less than 10 µg/g Tin (Sn); less than 200 µg/g Iron (Fe); less than 10 µg/g Manganese (Mn); less than 1 µg/g Mercury (Hg); less than 10 µg/g Molybdenum (Mo); less than 10 µg/g Nickel (Ni); less than 10 µg/g Lead (Pb); and less than 100 µg/g Zinc (Zn).

Methods for the production of ‘high purity’ diaminophenothiazinium compounds, including MTC, are described, for example, in WO2006/032879 and WO2008/007074 (WisTa Laboratories Ltd) and in WO2008/006979 (Provence Technologies).

A preferred MTC polymorph for use in the methods and compositions described herein is ‘form A’ described in WO2011/036561 which is a pentahydrate, at a “high purity”

described above. That has a molecular weight of around 409.9. Based on a molecular weight of 284.1 for the MT^+ core, the weight factor for using this MT compound in the invention is 1.44.
Other weight factors can be calculated for example MT compounds herein, and the corresponding dosage ranges can be calculated therefrom.

Therefore the invention embraces a total daily dose of around 0.7 to 29 mg/day of $MTC.5H_2O$.

More preferably around 5 to 10 mg/day of $MTC.5H_2O$ total dose is utilised, which corresponds to about 3.5 to 7 mg MT.

Other example MT compounds are described in WO2007/110630. Their molecular weight (anhydrous) and weight factor is also shown:

	Compound	Molecular weight	MT^+ anhydrous weight factor
10	MTC. $0.5ZnCl_2$	388.0	1.36
11	MTI	411.3	1.45
12	MTI.HI	539.2	2.73
13	$MT.NO_3$	346.4	1.22

The dosages described herein with respect to MT thus apply *mutatis mutandis* for these MT containing compounds, as adjusted for their molecular weight, and for choice of hydrate if used. For example $MTC.0.5ZnCl_2$ (also referred to as 'METHYLENE BLUE ZINC CHLORIDE DOUBLE SALT; CI 52015) may be obtained commercially as a monohydrate by several suppliers, which would have a molecular weight higher by 18, and correspondingly altered weight factor. MTI is reportedly available as a hemihydrate,

Adsorption factors

As explained herein, the present inventors have determined that, unexpectedly, low doses of MT salts showed therapeutic benefits in a neurodegenerative disorder of protein aggregation. This was demonstrated using an example LMTX salt. This finding has implications for the dosing of both LMT and MT^+ salts.

The present inventors have determined that dosing with LMTX salts permits more efficient adsorption, compared with MT^+ salts. Typically MT adsorption may be around 1.5x greater when delivered as an LMTX salt as opposed to an MT^+ salts. This 1.5 factor may be termed herein an "adsorption factor".

Therefore in certain embodiments of the invention, the dosed amount of MT^+ salt may be higher than when using LMTX salt to achieve a similar plasma concentration.

Thus one preferred dosage of MT^+ salt may be about 5.25 to 10.5 mg MT, which is expected to provide a similar adsorbed dosage as 3.5 to 7 mg MT when delivered as LMTX.

Any of the MT compounds described herein, may be formulated with a reducing agent. In particular, MT^+ salts such as MTC may be formulated with a reducing agent such as ascorbate, and then lyophilized (as described in WO02/055720). This is expected to improve adsorption of the MT delivered by the compound.

In the various aspects of the invention described herein (as they relate to an MT-containing compound) this may optionally be any of those compounds described above:

In one embodiment, it is compound 1.
In one embodiment, it is compound 2.
In one embodiment, it is compound 3.
In one embodiment, it is compound 4.
In one embodiment, it is compound 5.
In one embodiment, it is compound 6.
In one embodiment, it is compound 7.
In one embodiment, it is compound 8.
In one embodiment, it is compound 9.
In one embodiment, it is compound 10.
In one embodiment, it is compound 11.
In one embodiment, it is compound 12.
In one embodiment, it is compound 13.

Or the compounds may be a hydrate, solvate, or mixed salt of any of these.

Accumulation factors

As will be appreciated by those skilled in the art, for a given daily dosage, more frequent dosing will lead to greater accumulation of a drug.

The present inventors have derived estimated accumulation factors for MT as follows:

Dosing	Observed plasma accumulation for MT	Relative accumulation
Once daily	$1.29^{extrapolated}$	1
Twice daily	1.47	1.13
Three-times daily	1.65	1.28

For example, considering a total daily dose of 3.5 to 7 mg MT:

When given as a single daily dose, this may equate to an accumulation of MT in plasma of 4.5 to 8

When split b.i.d., this may equate to an accumulation of MT in plasma of 5.1 to 10.3

When split t.i.d., this may equate to an accumulation of MT in plasma of 5.8 to 11.6

Therefore in certain embodiments of the invention, the total daily dosed amount of MT compound may be lower, when dosing more frequently (e.g. twice a day [bid] or three times a day [tid]).

In one embodiment , LMTM is administered around 9 mg/once per day; 4 mg b.i.d.; 2.3 mg t.i.d (based on weight of LMTM)

In one embodiment, MTC:5H20 is administered around 10.6 mg/once per day; 6 mg b.i.d.; 2.8 mg t.i.d (based on weight of MTC:5H20).

Treatment and prophylaxis

The term “treatment,” as used herein in the context of treating a condition, pertains generally to treatment and therapy, whether of a human or an animal (e.g., in veterinary applications), in which some desired therapeutic effect is achieved, for example, the inhibition of the progress of the condition, and includes a reduction in the rate of progress, a halt in the rate of progress, regression of the condition, amelioration of the condition, and cure of the condition.

The term “therapeutically-effective amount,” as used herein, pertains to that amount of a compound of the invention, or a material, composition or dosage from comprising said compound, which is effective for producing some desired therapeutic effect, commensurate with a reasonable benefit/risk ratio, when administered in accordance with a desired treatment regimen. The present inventors have demonstrated that a therapeutically-effective amount of an MT compound in respect of the diseases of the invention can be much lower than was hitherto understood in the art.

The invention also embraces treatment as a prophylactic measure is also included.

Thus the invention also provides

a method of prophylaxis of a neurodegenerative disorders of protein aggregation in a subject,

which method comprises orally administering to said patient an MT containing compound,

wherein said administration provides a total of between 0.5 and 20mg of MT to the subject per day, optionally split into 2 or more doses.

The term “prophylactically effective amount,” as used herein, pertains to that amount of a compound of the invention, or a material, composition or dosage from comprising said compound, which is effective for producing some desired prophylactic effect, commensurate with a reasonable benefit/risk ratio, when administered in accordance with a desired treatment regimen.

"Prophylaxis" in the context of the present specification should not be understood to circumscribe complete success i.e. complete protection or complete prevention. Rather prophylaxis in the present context refers to a measure which is administered in advance of detection of a symptomatic condition with the aim of preserving health by helping to delay, mitigate or avoid that particular condition.

Combination treatments and monotherapy

The term "treatment" includes "combination" treatments and therapies, in which two or more treatments or therapies for the same neurodegenerative disorder of protein aggregation, are combined, for example, sequentially or simultaneously. These may be symptomatic or disease modifying treatments.

The particular combination would be at the discretion of the physician.

In combination treatments, the agents (i.e., an MT compound as described herein, plus one or more other agents) may be administered simultaneously or sequentially, and may be administered in individually varying dose schedules and via different routes. For example, when administered sequentially, the agents can be administered at closely spaced intervals (e.g., over a period of 5-10 minutes) or at longer intervals (e.g., 1, 2, 3, 4 or more hours apart, or even longer periods apart where required), the precise dosage regimen being commensurate with the properties of the therapeutic agent(s).

An example of a combination treatment of the invention would be an agent which is MT-containing compound at the specified dosage in combination with an agent which is an inhibitor of amyloid precursor protein to beta-amyloid (e.g., an inhibitor of amyloid precursor protein processing that leads to enhanced generation of beta-amyloid).

In other embodiments the treatment is a "monotherapy", which is to say that the MT-containing compound is not used in combination (within the meaning discussed above) with another active agent for treating the same neurodegenerative disorder of protein aggregation in the subject.

As explained below, in the present invention, when treating AD at least, it is preferred that the treatment does not include administration of either or both of: an acetylcholinesterase inhibitor or an N-methyl-D-aspartate receptor antagonist. The MT-compound based treatment of AD may optionally be a monotherapy.

Duration of treatment

For treatment of the neurodegenerative disorder of protein aggregation described herein, a treatment regimen based on the low dose MT compounds will preferably extend over a sustained period of time. The particular duration would be at the discretion of the physician.

For example, the duration of treatment may be:

At least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months, or longer.

At least 2, 3, 4, 5 years, or longer.

Between 6 and 12 months.

Between 1 and 5 years.

Where the disorder is AD, duration may be such as to achieve any one or more of:

A 3, 4 or 5-point improvement on the 11-item Alzheimer's Disease Assessment Scale – cognitive subscale (ADAS-cog) over a 52-week period;
4, 5 or 6 point improvement on the 23-item Alzheimer's Disease Cooperative Study Activities of Daily Living (ADCS-ADL) over a 52-week period;
A reduction in the increase of Lateral Ventricular Volume (LVV), as measured by the Ventricular Boundary Shift Integral (VBSI) of 1 or 2 cm^3 over a 52-week period.

For prophylaxis, the treatment may be ongoing.

In all cases the treatment duration will generally be subject to advice and review of the physician.

Oral dosage forms

The MT compound of the invention, or pharmaceutical composition comprising it, is administered to a subject/patient orally.

Pharmaceutical dosage forms

Another aspect of the invention therefore provides a composition comprising a compound as described herein, and a pharmaceutically acceptable carrier or diluent.

In some embodiments, the composition is a pharmaceutical composition (e.g., formulation, preparation, medicament) comprising a compound as described herein, and a pharmaceutically acceptable carrier, diluent, or excipient.

The term "pharmaceutically acceptable," as used herein, pertains to compounds, ingredients, materials, compositions, dosage forms, etc., which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of the subject in question (e.g., human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each

carrier, diluent, excipient, etc. must also be “acceptable” in the sense of being compatible with the other ingredients of the formulation.

In some embodiments, the composition is a pharmaceutical composition comprising at least one compound, as described herein, together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, including, but not limited to, pharmaceutically acceptable carriers, diluents, excipients, adjuvants, fillers, buffers, preservatives, anti-oxidants, lubricants, stabilisers, solubilisers, surfactants (e.g., wetting agents), masking agents, colouring agents, flavouring agents, and sweetening agents.

In some embodiments, the composition further comprises other active agents, for example, other therapeutic or prophylactic agents.

Suitable carriers, diluents, excipients, etc. can be found in standard pharmaceutical texts. See, for example, Handbook of Pharmaceutical Additives, 2nd Edition (eds. M. Ash and I. Ash), 2001 (Synapse Information Resources, Inc., Endicott, New York, USA), Remington's Pharmaceutical Sciences, 20th edition, pub. Lippincott, Williams & Wilkins, 2000; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

One aspect of the present invention pertains to a dosage unit (e.g., a pharmaceutical tablet or capsule) comprising an MT compound as described herein (e.g., obtained by, or obtainable by, a method as described herein; having a purity as described herein; etc.), and a pharmaceutically acceptable carrier, diluent, or excipient.

The “MT compound”, although present in relatively low amount, is the active agent of the dosage unit, which is to say is intended to have the therapeutic or prophylactic effect in respect of a neurodegenerative disorder of protein aggregation. Rather, the other ingredients in the dosage unit will be therapeutically inactive e.g. carriers, diluents, or excipients. Thus, preferably, there will be no other active ingredient in the dosage unit, no other agent intended to have a therapeutic or prophylactic effect in respect of a disorder for which the dosage unit is intended to be used.
In some embodiments, the dosage unit is a tablet.
In some embodiments, the dosage unit is a capsule.

In some embodiments, said capsules are gelatine capsules.
In some embodiments, said capsules are HPMC (hydroxypropylmethylcellulose) capsules.

In some embodiments, the amount of MT in the unit is 0.5 to 10 mg.

An example dosage unit may contain 1 to 10mg of MT.

A further example dosage unit may contain 2 to 9 mg of MT.

A further example dosage unit may contain 3 to 8 mg of MT.

A further preferred dosage unit may contain 3.5 to 7 mg of MT.

A further preferred dosage unit may contain 4 to 6 mg of MT.

In some embodiments, the amount is about 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10 mg of MT.

Using the weight factors described or explained herein, one skilled in the art can select appropriate amounts of an MT containing compound to use in oral formulations.

As explained above, the MT weight factor for LMTM is 1.67. Since it is convenient to use unitary or simple fractional amounts of active ingredients, non-limiting example LMTM dosage units may include 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 15, 16, 17, 18 mg etc.

As explained above, the MT weight factor for MTC.5$H_2$0 is 1.44. Since it is convenient to use unitary or simple fractional amounts of active ingredients, non-limiting example MTC.5$H_2$0 dosage units may include 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 15, 18, 20 mg etc.

Nutraceutical compositions

The "nutraceutical compositions" of the invention comprise a low dose of MT compound, as described herein, in combination with one or more nutrients in an edible form (for example an oral dosage form).

The novel nutraceutical compositions of the invention can find use as supplements to food and beverages, and as pharmaceutical compositions.

"Nutrients" as used herein refers to the components of nutraceutical compositions that serve a biochemical and/or physiological role in the human or animal body. "Nutrients" includes such substances as vitamins, minerals, trace elements, micronutrients, antioxidants and the like, as well as other bioactive materials, such as enzymes, or compounds biosynthetically produced by human or animal enzymes; as well as herbs and herbal extracts; fatty acids, amino acids and derivatives thereof.

"Edible form" denotes a composition that can be ingested directly or converted to an ingestible form, such as, by dissolving in water.

Alternatively, the nutraceutical composition can be in the form of a food or drink, such as a defined portion of a foodstuff (which term includes both food or drink) supplemented with the defined dosage of MT compound. These foodstuffs will typically comprise one or more of a fat, a protein, or a carbohydrate.

The term “nutraceutical' as used herein denotes a usefulness in both the nutritional and pharmaceutical field of application, and the disclosure herein relating to pharmaceutical dosage forms applies *mutatis mutandis* to the nutraceutical compositions.

Oral dosage forms particularly suitable for nutraceutical compositions are well known in the art and described in more detail elsewhere herein. They include powders, capsules, pills, tablets, caplets, gelcaps, and defined portions of edible food items. Liquid forms include solutions or suspensions. General examples of dosage forms and nutraceutical forms are given, for example in WO2010/078659.

Some examples of nutrients useful in the compositions of the present invention are as follows. Any combination of these nutrients is envisaged by the present invention:

Vitamins

It is reported that B-vitamin supplementation (folic acid [folate, vitamin B_9], vitamin B_{12}, vitamin B_6) can slow the atrophy of specific brain regions that are a key component of the AD process and that are associated with cognitive decline. This is particularly the case for elderly subjects with high homocysteine levels (Douaud, Gwenaëlle, et al. "Preventing Alzheimer’s disease-related gray matter atrophy by B-vitamin treatment." Proceedings of the National Academy of Sciences 110.23 (2013): 9523-9528; see also Quadri, Pierluigi, et al. "Homocysteine, folate, and vitamin B_{12} in mild cognitive impairment, Alzheimer disease, and vascular dementia." *The American journal of clinical nutrition* 80.1 (2004): 114-122; Rosenberg IH, Miller JW. Nutritional factors in physical and cognitive functions of elderly people. The American journal of clinical nutrition. 1992 Jun 1;55(6):1237S-1243S.).

It has been suggested that, along with other antioxidants (see below), vitamin C may have utility in protecting neural tissue, as well as potentially decreasing β-amyloid generation and acetylcholinesterase activity and prevents endothelial dysfunction by regulating nitric oxide (see e.g. Heo JH, Hyon-Lee, Lee KM. The possible role of antioxidant vitamin C in Alzheimer’s disease treatment and prevention. American Journal of Alzheimer's Disease & Other Dementias. 2013 Mar;28(2):120-5).

It has also been suggested that Vitamin E supplementation may have a role to play in AD treatment (see e.g. Mangialasche, Francesca, et al. "Serum levels of vitamin E forms and risk of cognitive impairment in a Finnish cohort of older adults." *Experimental gerontology* 48.12 (2013): 1428-1435).

Micronutrients, antioxidants

Micronutrients or antioxidants, such as polyphenols, have been reported to have benefits in relation to protection or treatment of age-related diseases including neurodegenerative ones, particularly cognitive impairment and AD.

Micronutrients and\or antioxidants which may be utilised in the nutraceutical compositions described herein include the flavonoids shown in the Table below (reproduced from Mecocci, Patrizia, et al. "Nutraceuticals in cognitive impairment and Alzheimer's disease." *Frontiers in pharmacology* 5:147 (2014)):

Flavonoid chemical subgroups and relative food sources:

Groups	Molecules	Food source
FLAVANOLS	Catechin, epicatechin, epigallocathechin, epigallocatechin gallate (EGCG)	Cocoa and chocolate, green tea, grapes
FLAVONOLS	Kaempferol, quercetin	Onions, apples, green tea, capers, leeks, broccoli
FLAVONES	Luteolin, apigenin	Celery, parsley, rosemary
ISOFLAVONES	Daidzein, genistein	Soy
FLAVANONES	Hesperetin, naringenin	Citrus fruit, tomatoes
ANTHOCYANIDINS	Pelargonidin, cyanidine, malvidin	Berry fruits, red wine

Other micronutrients having potential utility in relation to protection or treatment of age-related diseases, and described by Mecocci *et al* include:

- Non-flavonoid polyphenols: resveratrol and curcumin,
- Carotenoids: lycopene, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and the most prominent carotenoid, β-carotene,
- Crocin (the main chemical compound identified in saffron),
- Diterpenes: for example carnosic and rosmarinic acids are two of the most important antioxidant compounds in rosemary.

Herbs and plant extracts

In addition to the plants described or cross-referenced above in relation to micronutrients and antioxidants, other plant extracts and herbs are reported to have benefit in CNS disorders – see Kumar, Vikas. "Potential medicinal plants for CNS disorders: an overview." *Phytotherapy Research* 20.12 (2006): 1023-1035. These include *Ginkgo biloba, Hypericum perforatum* (St John's wort), *Piper methysticum* Forst. (Family Piperaceae) also called kava kava, *Valeriana officinalis* L. (Valerian), *Bacopa monniera*

(which in India is locally known as Brahmi or Jalanimba), *Convolvulus pluricaulis* (also known as Shankhpushpi or shankapushpi)

Oils and fats

It is reported that ω-3 poyunsaturated fatty acid (PUFA), for example, may be a promising tool for preventing age-related brain deterioration. Sources of PUFA such as (docosahexaenoic acid (DHA, 22:6) and eicosapentenoic acid (EPA, 20:5) include fish oils (Denis, I., et al. "Omega-3 fatty acids and brain resistance to ageing and stress: body of evidence and possible mechanisms." *Ageing research reviews* 12.2 (2013): 579-594.)

Immediate release dosage units

The formulations and compositions (especially pharmaceutical compositions) may be prepared to provide for rapid or slow release; immediate, delayed, timed, or sustained release; or a combination thereof.

An immediate release product allows the ingredient or active moiety to dissolve in the gastrointestinal tract, without causing any delay or prolongation of the dissolution or absorption of the drug. Requirements for dissolution testing of immediate release products are set out in the Guidance for Industry (CDER 1997) "Dissolution testing for immediate release solid oral dosage forms", (CDER 1997) "Immediate release solid oral dosage forms - Scale up and Post approval Changes", ICH Guidance Q6A, Specifications: Test Procedures and Acceptance Criteria For New Drug Substances And New Drug Products. The most commonly employed dissolution test methods as described in the USP and European Pharmacopeia (6th edition) are the basket method (USP 1) and the paddle method (USP 2). The described methods are simple, robust, well standardized, and used worldwide. They are flexible enough to allow dissolution testing for a variety of drug products. The following parameters influencing the dissolution behaviour may for example be relevant for selecting the appropriate in vitro dissolution test conditions for an immediate release solid oral product: apparatus, stirring speed, dissolution medium and temperature. Because of the biopharmaceutical properties of MTC and its expected desirable absorption characteristics in the upper gastrointestinal tract, it was preferable to produce rapidly dissolving tablets of MTC.

Compositions according to the invention can be dissolution tested in a USP-2 apparatus in 900ml of 0.1N HCl, with paddles rotating at 50-75 rpm. Compositions according to the invention exhibit at least the acceptance criteria cited for Stage 1 (S1) testing in the USP 32 (The United States Pharmacopeia, edited by the United States Pharmacopeial Convention, Inc., 12601 Twinbrook Parkway, Rockville, MD 20852; Published by Rand McNally, Inc., 32nd Edition, 2008):

Acceptance Criteria: Each tablet achieved 85% dissolution of MTC within 30 minutes after insertion of the coated tablet into the 0.1N HCl.

Thus in some embodiments, the MTC based formulations of the invention, when evaluated using this method, provide at least:

75% dissolution of MTC within 45 minutes after insertion of the coated tablet into the 0.1N HCl; or
85% dissolution of MTC within 30 minutes after insertion of the coated tablet into the 0.1N HCl;
85% dissolution of MTC within 15 minutes after insertion of the coated tablet into the 0.1N HCl.

Another aspect of the present invention pertains to methods of making a low dosage MT compound pharmaceutical composition comprising admixing at least one MT compound, as defined herein, together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, e.g., carriers, diluents, excipients, etc. If formulated as discrete units (e.g., tablets, etc.), each unit contains a predetermined amount (dosage) of the compound.

The formulations may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the compound with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the compound with carriers (e.g., liquid carriers, finely divided solid carrier, etc.), and then shaping the product, if necessary.

In some embodiments, the pharmaceutically acceptable carrier, diluent, or excipient is or comprises one or both of a glyceride (e.g., Gelucire 44/14 ®; lauroyl macrogol-32 glycerides PhEur, USP) and colloidal silicon dioxide (e.g., 2% Aerosil 200 ®; Colliodal Silicon Dioxide PhEur, USP).

Preferably the pharmaceutical compositions comprising a compound of the invention, in solid dosage form. The composition preferably further comprises at least one diluent suitable for dry compression. The pharmaceutical composition is characterised in that the compound exists in a substantially stable form.

The pharmaceutical composition will generally also include a lubricant. Examples of lubricants include magnesium stearate, calcium stearate, sodium stearyl fumarate, stearic acid, glycerylbehaptate, polyethylene glycol, ethylene oxide polymers (for example, those available under the registered trademark Carbowax from Union Carbide, Inc., Danbury, CT), sodium lauryl sulphate, magnesium lauryl stearate, mixtures of magnesium stearate with sodium lauryl sulphate, and hydrogenated vegetable oil. Preferred lubricants include calcium stearate, magnesium stearate and sodium stearyl fumarate. Most preferred as the lubricant is magnesium stearate. Lubricants generally comprise from about 0.5 to about 5.0% of the total (uncoated) tablet weight. The amount of lubricant employed is generally from about 1.0 to about 2.0%, preferably 0.5 to 2.0% w/w.

In addition to the diluent(s) and lubricant(s), other conventional excipients may also be present in the pharmaceutical compositions of the invention. Such additional excipients

include disintegrants, binders, flavouring agents, colours and glidants. Some excipients can serve multiple functions, for example as both binder and tablet disintegrant.

A tablet disintegrant may be present in an amount necessary to achieve rapid dissolution. Disintegrants are excipients which oppose the physical forces of particle bonding in a tablet or capsule when the dosage form is placed in an aqueous environment. Examples of disintegrants include crosslinked polyvinylpyrrolidone (crospovidone), sodium starch glycolate, crosslinked sodium carboxymethyl cellulose (sodium croscarmellose), and pregelatinized starch. Generally the amount of disintegrant can be from 0 to about 25% w/w, more commonly from about 1% to about 15% w/w, and usually less than 10% or less than 5% w/w, of the composition.

Binders are excipients which contribute to particle adhesion in a solid formulation. Examples of binders include cellulose derivatives (carboxymethylcellulose, hydroxypropyl methylcellulose, hydroxypropyl cellulose, hydroxyethylcellulose, ethylcellulose, microcrystalline cellulose) and sugars such as lactose, sucrose, dextrose, glucose, maltodextrin, and mannitol, xylitol, polymethacrylates, polyvinylpyrrolidone, sorbitol, pregelatinized starch, alginic acids, and salts thereof such as sodium alginate, magnesium aluminum silicate, polyethylene glycol, carrageenan and the like. Generally, the amount of binder can vary widely, eg from 0% to 95% w/w of the composition. As noted above, excipients may serve multiple functions. For instance, the tabletting diluent may also serve as a binder.

Glidants are substances added to a powder to improve its flowability. Examples of glidants include magnesium stearate, colloidal silicon dioxide (such as the grades sold as Aerosil), starch and talc. Glidants may be present in the pharmaceutical composition at a level of from 0 to about 5% w/w. Again, however, it should be noted that excipients may serve multiple functions. The lubricant, for example magnesium stearate, may also function as a glidant.

Examples of colours that may be incorporated into the pharmaceutical compositions of the invention include titanium dioxide and/or dyes suitable for food such as those known as FD&C dyes and natural colouring agents. A colouring agent is unlikely to be used in the powder mixture that is compressed in accordance with the aspects of the invention discussed above, but may form part of a coating applied to the composition, as described below, in which case the colouring agent may be present in the film coat in an amount up to about 2.0% w/w.

The tablet is desirably coated with a conventional film coating which imparts toughness, ease of swallowing, and an elegant appearance to the final product. Many polymeric film-coating materials are known in the art. A preferred film-coating material is hydroxypropylmethylcellulose (HPMC) or polyvinyl alcohol-part hydrolysed (PVA). HPMC and PVA may be obtained commercially, for example from Colorcon, in coating formulations containing excipients which serve as coating aids, under the registered trademark Opadry. Opadry formulations may also contain talc, polydextrose, triacetin, polyethyleneglycol, polysorbate 80, titanium dioxide, and one or more dyes or lakes.

Other suitable film-forming polymers may also be used, including hydroxypropylcellulose, vinyl copolymers such as polyvinyl pyrollidone and polyvinyl acetate, and acrylate-methacrylate copolymers. Use of a film coating is beneficial for ease of handling and because a blue coloured uncoated core may stain the inside of the mouth during swallowing. Coating also improves light stability of the dosage form.

Coating of the tablets may conveniently be carried out using a conventional coating pan. In preferred embodiments of the process, the coating pan is pre-heated using heated inlet air until the exhaust temperature reaches 35°-55°C, more preferably 40-50°C. This may typically require application of heated inlet air at an inlet temperature of 45-75°C, preferably 50-65°C, for 10-15 minutes. The tablet cores containing the active ingredient (e.g. LMTM) are then added to the coating pan and the aqueous film coat applied. The spray rate is controlled such that the bed temperature is maintained at 38-48°C, more preferably 42-44°C, until the desired weight gain (coating weight) has been achieved.

Subjects, patients and patient groups

The subject/patient may be an animal, a mammal, a placental mammal, a rodent (e.g., a guinea pig, a hamster, a rat, a mouse), murine (e.g., a mouse), a lagomorph (e.g., a rabbit), avian (e.g., a bird), canine (e.g., a dog), feline (e.g., a cat), equine (e.g., a horse), porcine (e.g., a pig), ovine (e.g., a sheep), bovine (e.g., a cow), a primate, simian (e.g., a monkey or ape), a monkey (e.g., marmoset, baboon), a monotreme (e.g. platypus), an ape (e.g., gorilla, chimpanzee, orangutan, gibbon), or a human.

In preferred embodiments, the subject/patient is a human who has been diagnosed as having one of the cognitive or CNS disorders described herein, or (for prophylactic treatment) assessed as being susceptible to one of the neurodegenerative disorders of protein aggregation (e.g. cognitive or CNS disorder) described herein – for example based on familial or genetic or other data.

The patient may be an adult human, and the dosages described herein are premised on that basis (typical weight 50 to 70kg). If desired, corresponding dosages may be utilised for subjects outside of this range by using a subject weight factor whereby the subject weight is divided by 60 kg to provide the multiplicative factor for that individual subject.

The low dosage treatments of the present invention increase the feasibility of purely prophylactic treatments, since the reduced concentration of active ingredients inevitably reduces risk of any adverse side effects (and increases the safety profile) and hence increases the risk/benefit ratio for such prophylactic treatments.

Thus, for example, for diagnosis of AD, and assessment of severity, the initial selection of a patient may involve any one or more of: rigorous evaluation by experienced clinician; exclusion of non-AD diagnosis as far as possible by supplementary laboratory and other investigations; objective evaluation of level of cognitive function using neuropathologically validated battery.

Diagnosis of AD and other disorders described herein can be performed by physicians by methods well known to those skilled in the art.

As explained herein, in the present invention it is preferred that the subject or patient group, if they are being treated in respect of AD, is one who is not receiving treatment with any of: an acetylcholinesterase inhibitor or an N-methyl-D-aspartate receptor antagonist. Examples of acetylcholinesterase inhibitors include Donepezil (Aricept™), Rivastigmine (Exelon™) or Galantamine (Reminyl™). An examples of an NMDA receptor antagonist is Memantine (Ebixa™, Namenda™).

Based on the findings described herein, these selection criteria are likely to be applicable when treating AD with MT^+ compounds even at higher doses than those described herein e.g. between 0.5 mg and 300 mg total daily dose per day.

Thus in one aspect the present invention provides a method of treatment (or prophylaxis) of AD in a subject,

which method comprises orally administering to said subject a methylthioninium (MT) containing compound,

with the proviso that said treatment does not include administration of either or both of an acetylcholinesterase inhibitor or an N-methyl-D-aspartate receptor antagonist.

Unless context demands otherwise, the disclosure made herein regarding the “low dose” treatments of neurodegenerative diseases more generally applies *mutatis mutandis* to these aspects of the invention.

For example the AD subject or patient group may be entirely naïve to these other treatments, and have not historically received one or both of them.

For example the AD subject or patient group may have historically received one or both of them, but ceased that medication at least 1, 2, 3, 4, 5, 6, 7 days, or 2, 3, 4, 5, 6, 7, 8, 12, or 16 weeks, or more preferably at least 1, 2, 3, 4, 5 or 6 months etc. prior to treatment with an MT compound according to the present invention.

Any aspect of the present invention may include the active step of selecting the AD subject or patient group according to these criteria, or selecting an AD subject or patient group who is or are receiving treatment with either or both an acetylcholinesterase inhibitor or an N-methyl-D-aspartate receptor antagonist, and discontinuing that treatment (instructing the subject or patient group to discontinue that treatment) prior to treatment with an MT compound according to the present invention.

Labels, instructions and kits of parts

The unit dosage compositions described herein (e.g. a low dose MT containing compound plus optionally other ingredients, or MT composition more generally for treatment in AD) may be provided in a labelled packet along with instructions for their use.

In one embodiment, the pack is a bottle, such as are well known in the pharmaceutical art. A typical bottle may be made from pharmacopoeial grade HDPE (High-Density Polyethylene) with a childproof, HDPE pushlock closure and contain silica gel desiccant, which is present in sachets or canisters. The bottle itself may comprise a label, and be packaged in a cardboard container with instructions for us and optionally a further copy of the label.

In one embodiment, the pack or packet is a blister pack (preferably one having aluminium cavity and aluminium foil) which is thus substantially moisture-impervious. In this case the pack may be packaged in a cardboard container with instructions for us and label on the container.

Said label or instructions may provide information regarding the neurodegenerative disorders of protein aggregation (e.g. cognitive or CNS disorder) for which the medication is intended.

Where the medication is indicated for AD, said label or instructions may provide information instructing the user that the compositions should not be used in conjunction with any of: an acetylcholinesterase inhibitor or an N-methyl-D-aspartate receptor antagonist.

Said label or instructions may provide information regarding the maximum permitted daily dosage of the compositions as described herein – for example based on once daily, b.i.d., or t.i.d.

Said label or instructions may provide information regarding the suggested duration of treatment, as described herein.

Reversing and/or Inhibiting the Aggregation of a Protein

One aspect of the invention is the use of an MT compound or composition as described herein, to regulate (e.g., to reverse and/or inhibit) the aggregation of a protein, for example, aggregation of a protein associated with a neurodegenerative disease and/or clinical dementia. The aggregation will be associated with a disease state as discussed below.

Similarly, one aspect of the invention pertains to a method of regulating (e.g., reversing and/or inhibiting) the aggregation of a protein in the brain of a mammal, which aggregation is associated with a disease state as described herein, the treatment comprising the step of administering to said mammal in need of said treatment, a prophylactically or therapeutically effective amount of an MT compound or composition as described herein, that is an inhibitor of said aggregation.

Disease conditions treatable via the present invention are discussed in more detail below.

Methods of Treatment

Another aspect of the present invention, as explained above, pertains to a method of treatment comprising administering to a patient in need of treatment a prophylactically or therapeutically effective amount of a compound as described herein, preferably in the form of a pharmaceutical composition.

Use in Methods of Therapy

Another aspect of the present invention pertains to a compound or composition as described herein, for use in a method of treatment (e.g., of a disease condition) of the human or animal body by therapy.

Use in the Manufacture of Medicaments

Another aspect of the present invention pertains to use of an MT compound or composition as described herein, in the manufacture of a medicament for use in treatment (e.g., of a disease condition).

In some embodiments, the medicament is a composition e.g. a low-dose unit dose composition as described herein.

Diseases of Protein Aggregation

The compounds and compositions of the present invention are useful in the treatment or prophylaxis of diseases of protein aggregation.

Thus, in some embodiments, the disease condition is a disease of protein aggregation, and, for example, the treatment is with an amount of a compound or composition as described herein, sufficient to inhibit the aggregation of the protein associated with said disease condition.

The following Table lists various disease-associated aggregating proteins and the corresponding neurodegenerative disease of protein aggregation. The use of the compounds and compositions of the invention in respect of these proteins or diseases is encompassed by the present invention.

Diseases of protein aggregation				
Protein	**Disease**	**Aggregating domain and/or mutations**	**Fibril subunit size (kDa)**	**Reference**
Neurodegenerative disorders				
Prion protein	Prion diseases	*Inherited and sporadic forms*	27	Prusiner (1998)

Diseases of protein aggregation				
Protein	**Disease**	**Aggregating domain and/or mutations**	**Fibril subunit size (kDa)**	**Reference**
	(CJD, nvCJD, Fatal familial insomnia, Gerstmann-Straussler-Scheinker syndrome, Kuru)	PrP-27-30; many mutations.	27	Prusiner (1998)
		Fibrillogenic domains: 113-120, 178-191, 202-218.		Gasset et al. (1992)
Tau protein	Alzheimer's disease, Down's syndrome, FTDP-17, CBD, post-encephalitic parkinsonism, Pick's disease, parkinsonism with dementia complex of Guam	*Inherited and sporadic forms*	10-12	Wischik et al. (1988)
		Truncated tau (tubulin-binding domain) 297-391.	10-12	Wischik et al. (1988)
		Mutations in tau in FTDP-17.		Hutton et al. (1998)
		Many mutations in presenilin proteins.		Czech et al. (2000)
Amyloid β-protein	Alzheimer's disease, Down's syndrome	*Inherited and sporadic forms*	4	Glenner & Wong, (1984)
		Amyloid β-protein; 1-42(3).	4	Glenner & Wong, (1984)
		Mutations in APP in rare families.		Goate et al. (1991)
Huntingtin	Huntington's disease	N-termini of protein with expanded glutamine repeats.	40	DiFiglia et al. (1997)
Ataxin)	Spinocerebellar ataxias (SCA1, 2, 3, 7)	Proteins with expanded glutamine repeats.		Paulson et al. (1999)

Diseases of protein aggregation				
Protein	**Disease**	**Aggregating domain and/or mutations**	**Fibril subunit size (kDa)**	**Reference**
Atrophin	Dentatorubropallidoluysian atrophy (DRPLA)	Proteins with expanded glutamine repeats.		Paulson et al. (1999)
Androgen receptor	Spinal and bulbar muscular atrophy	Proteins with expanded glutamine repeats.		Paulson et al. (1999)
Neuroserpin	Familial encephalopathy with neuronal inclusion bodies (FENIB)	Neuroserpin; S49P, S52R.	57	Davis et al. (1999)
α-Synuclein	Parkinson's disease, dementia with Lewy bodies, multiple system atrophy	*Inherited and sporadic forms*	19	Spillantini et al. (1998) also PCT/GB2007/001105
		A53T, A30P in rare autosomal-dominant PD families.		Polymeropoulos et al. (1997)
TDP-43	FTLD-TDP	Several TDP-43 mutations	10-43	Mackenzie et al. (2010)
	Amyotrophic lateral sclerosis	Several TDP-43 mutations	10-43	Mackenzie et al. (2010)
Cystatin C	Hereditary cerebral angiopathy (Icelandic)	Cystatin C less 10 residues; L68Q.	12-13	Abrahamson et al. (1992)
Superoxide dismutase 1	Amyotrophic lateral sclerosis	SOD1 mutations.	16	Shibata et al. (1996)

As described in WO 02/055720, WO2007/110630, and WO2007/110627, diaminophenothiazines have utility in the inhibition of such protein aggregating diseases.

Thus it will be appreciated that, except where context requires otherwise, description of embodiments with respect to tau protein or tau-like proteins (e.g., MAP2; see below), should be taken as applying equally to the other proteins discussed herein (e.g., β-amyloid, synuclein, prion, etc.) or other proteins which may initiate or undergo a similar pathological aggregation by virtue of conformational change in a domain critical for propagation of the aggregation, or which imparts proteolytic stability to the aggregate thus formed (see, e.g., the article by Wischik et al. in “Neurobiology of Alzheimer’s Disease”, 2nd Edition, 2000, Eds. Dawbarn, D. and Allen, S.J., The Molecular and Cellular

Neurobiology Series, Bios Scientific Publishers, Oxford). All such proteins may be referred to herein as "aggregating disease proteins."

Likewise, where mention is made herein of "tau-tau aggregation", or the like, this may also be taken to be applicable to other "aggregating-protein aggregation", such as β-amyloid aggregation, prion aggregation, synuclein aggregation, etc. The same applies for "tau proteolytic degradation" etc.

Preferred Aggregating Disease Target Proteins

Preferred embodiments of the invention are based on tau protein. The term "tau protein," as used herein, refers generally to any protein of the tau protein family. Tau proteins are characterised as being one among a larger number of protein families which co-purify with microtubules during repeated cycles of assembly and disassembly (see, e.g., Shelanski et al., 1973, Proc. Natl. Acad. Sci. USA, Vol. 70, pp. 765-768), and are known as microtubule-associated-proteins (MAPs). Members of the tau family share the common features of having a characteristic N-terminal segment, sequences of approximately 50 amino acids inserted in the N-terminal segment, which are developmentally regulated in the brain, a characteristic tandem repeat region consisting of 3 or 4 tandem repeats of 31-32 amino acids, and a C-terminal tail.

MAP2 is the predominant microtubule-associated protein in the somatodendritic compartment (see, e.g., Matus, A., in "*Microtubules*" [Hyams and Lloyd, Eds.] pp. 155-166, John Wiley and Sons, New York, USA). MAP2 isoforms are almost identical to tau protein in the tandem repeat region, but differ substantially both in the sequence and extent of the N-terminal domain (see, e.g., Kindler and Garner, 1994, Mol. Brain Res., Vol. 26, pp. 218-224). Nevertheless, aggregation in the tandem-repeat region is not selective for the tau repeat domain. Thus it will be appreciated that any discussion herein in relation to tau protein or tau-tau aggregation should be taken as relating also to tau-MAP2 aggregation, MAP2-MAP2 aggregation, and so on.

In some embodiments, the protein is tau protein.

In some embodiments, the protein is a synuclein, e.g., α- or β-synuclein.

In some embodiments, the protein is TDP-43.

TAR DNA-Binding Protein 43 (TDP-43) is a 414 amino acid protein encoded by *TARDBP* on chromosome 1p36.2. The protein is highly conserved, widely expressed, and predominantly localised to the nucleus but can shuttle between the nucleus and cytoplasm (Mackenzie *et al* 2010). It is involved in transcription and splicing regulation and may have roles in other processes, such as: microRNA processing, apoptosis, cell division, stabilisation of messenger RNA, regulation of neuronal plasticity and maintenance of dendritic integrity. Furthermore, since 2006 a substantial body of evidence has accumulated in support of the TDP-43 toxic gain of function hypothesis in amyotrophic lateral sclerosis (ALS). TDP-43 is an inherently aggregation-prone protein

and aggregates formed *in vitro* are ultrastructurally similar to the TDP-43 deposits seen in degenerating neurones in ALS patients (Johnson et al 2009). Johnson et al (2008) showed that when TDP-43 is overexpressed in a yeast model only the aggregated form is toxic. Several *in vitro* studies have also shown that C-terminal fragments of TDP-43 are more likely than full-length TDP-43 to form insoluble cytoplasmic aggregates that become ubiquitinated, and toxic to cells (Arai et al 2010; Igaz et al 2009; Nonaka et al 2009; Zhang et al 2009). Though Nonaka et al (2009) suggested that these cytoplasmic aggregates bind the endogenous full-length protein depleting it from the nucleus, Zhang et al (2009) found retention of normal nuclear expression, suggesting a purely toxic effect for the aggregates. Yang et al (2010) have described the capture of full-length TDP-43 within aggregates of C- and N-terminal fragments of TDP-43 in NSC34 motor neurons in culture. Neurite outgrowth, impaired as a result of the presence of such truncated fragments, could be rescued by overexpression of the full-length protein. Although the role of neurite outgrowth *in vivo* has not been established, this model would support the suggestion made by Nonaka and colleagues for a role of TDP-43 aggregation in ALS pathogenesis.

Mutant TDP-43 expression in cell cultures has repeatedly been reported to result in increased generation of C-terminal fragments, with even greater cytoplasmic aggregation and toxic effects than the wild-type protein (Kabashi et al 2008; Sreedharan et al 2008; Johnson et al 2009; Nonaka et al 2009; Arai et al 2010; Barmarda et al 2010; Kabashi et al 2010).

Where the protein is tau protein, in some embodiments of the present invention, there is provided a method of inhibiting production of protein aggregates (e.g. in the form of paired helical filaments (PHFs), optionally in neurofibrillary tangles (NFTs) in the brain of a mammal, the treatment being as described above.

Preferred Indications - Diseases of Protein Aggregation

In one embodiment the present invention is used for the treatment of Alzheimer's disease (AD) – for example mild, moderate or severe AD.

Notably it is not only Alzheimer's disease (AD) in which tau protein (and aberrant function or processing thereof) may play a role. The pathogenesis of neurodegenerative disorders such as Pick's disease and progressive supranuclear palsy (PSP) appears to correlate with an accumulation of pathological truncated tau aggregates in the dentate gyrus and stellate pyramidal cells of the neocortex, respectively. Other dementias include fronto-temporal dementia (FTD); FTD with parkinsonism linked to chromosome 17 (FTDP-17); disinhibition-dementia-parkinsonism-amyotrophy complex (DDPAC); pallido-ponto-nigral degeneration (PPND); Guam-ALS syndrome; pallido-nigro-luysian degeneration (PNLD); cortico-basal degeneration (CBD) and others (see, e.g., the article by Wischik et al. in "Neurobiology of Alzheimer's Disease", 2nd Edition, 2000, Eds. Dawbarn, D. and Allen, S.J., The Molecular and Cellular Neurobiology Series, Bios Scientific Publishers, Oxford; especially Table 5.1). All of these diseases, which are characterized primarily or partially by abnormal tau aggregation, are referred to herein as "tauopathies".

Thus, in some embodiments, the disease condition is a tauopathy.
In some embodiments, the disease condition is a neurodegenerative tauopathy.

In some embodiments, the disease condition is selected from Alzheimer's disease (AD), Pick's disease, progressive supranuclear palsy (PSP), fronto temporal dementia (FTD), FTD with parkinsonism linked to chromosome 17 (FTDP 17), frontotemporal lobar degeneration (FTLD) syndromes; disinhibition-dementia-parkinsonism-amyotrophy complex (DDPAC), pallido-ponto-nigral degeneration (PPND), Guam-ALS syndrome, pallido nigro luysian degeneration (PNLD), cortico-basal degeneration (CBD), dementia with argyrophilic grains (AgD), dementia pugilistica (DP) or chronic traumatic encephalopathy (CTE), Down's syndrome (DS), dementia with Lewy bodies (DLB), subacute sclerosing panencephalitis (SSPE), MCI, Niemann-Pick disease, type C (NPC), Sanfilippo syndrome type B (mucopolysaccharidosis III B), or myotonic dystrophies (DM), DM1 or DM2, or chronic traumatic encephalopathy (CTE).

In some embodiments, the disease condition is a lysosomal storage disorder with tau pathology. NPC is caused by mutations in the gene *NPC1*, which affects cholesterol metabolism (Love et al 1995) and Sanfilippo syndrome type B is caused by a mutation in the gene *NAGLU*, in which there is lysosomal accumulation of heparin sulphate (Ohmi et al. 2009). In these lysosomal storage disorders, tau pathology is observed and its treatment may decrease the progression of the disease. Other lysosomal storage disorders may also be characterised by accumulation of tau.

Use of phenothiazine diaminium salts in the treatment of Parkinson's disease and MCI is described in more detail in PCT/GB2007/001105 and PCT/GB2008/002066.

In some embodiments, the disease condition is Parkinson's disease, MCI, or Alzheimer's disease.

In some embodiments, the disease condition is Huntington's disease or other polyglutamine disorder such as spinal bulbar muscular atrophy (or Kennedy disease), and dentatorubropallidoluysian atrophy and various spinocerebellar ataxias.
In some embodiments, the disease condition is an FTLD syndrome (which may for example be a tauopathy or TDP-43 proteinopathy, see below).

In some embodiments, the disease condition is PSP or ALS.

TDP-43 proteinopathies include amyotrophic lateral sclerosis (ALS; ALS-TDP) and frontotemporal lobar degeneration (FTLD-TDP).

The role of TDP-43 in neurodegeneration in ALS and other neurodegenerative disorders has been reviewed in several recent publications (Chen-Plotkin *et al* 2010; Gendron *et al* 2010; Geser *et al* 2010; Mackenzie *et al* 2010).

ALS is a neurodegenerative disease, characterised by progressive paralysis and muscle wasting, consequent on the degeneration of both upper and lower motor neurones in the primary motor cortex, brainstem and spinal cord. It is sometimes referred to as motor neuron disease (MND) but there are diseases other than ALS which affect either either upper or lower motor neurons. A definite diagnosis requires both upper and lower motor neurone signs in the bulbar, arm and leg musculature with clear evidence of clinical progression that cannot be explained by any other disease process (Wijesekera and Leigh 2009).

Although the majority of cases are ALS-TDP, there are other cases where the pathological protein differs from TDP-43. Misfolded SOD1 is the pathological protein in ubiquitin-positive inclusions in ALS with *SOD1* mutations (Seetharaman *et al* 2009) and in a very small subset (approximately 3-4%) of familial ALS, due to mutations in FUS (fused in sarcoma protein), the ubiquitinated pathological protein is FUS (Vance *et al* 2009; Blair *et al* 2010). FUS, like TDP-43, appears to be important in nuclear-cytoplasmic shuttling although the ways in which impaired nuclear import of FUS remains unclear. A new molecular classification of ALS, adapted from Mackenzie *et al* (2010), reflects the distinct underlying pathological mechanisms in the different subtypes (see Table below).

New Molecular Classification of ALS (modified from Mackenzie *et al* 2010). In the majority of cases, TDP-43 is the pathological ubiquitinated protein found in ALS.

Ubiquitin-positive inclusions in ALS			
Ubiquitinated disease protein	**TDP-43**	**FUS**	**SOD1**
Clinico-pathologic subtype	ALS-TDP	ALS-FUS	ALS-SOD1
Associated genotype	*TARDBP*	*FUS*	*SOD1*
Frequency of ALS cases	Common	Rare	Rare

Amyotrophic lateral sclerosis has been recognised as a nosological entity for almost a century and a half and it is recognised in ICD-10 is classified as a subtype of MND in ICD 10 (G12.2). Reliable clinical diagnostic are available for ALS, which differ little from Charcot's original description, and neuropathological criteria, reflecting the underlying molecular pathology, have also been agreed.

While ALS is classified pathologically into three subgroups, ALS-TDP, ALS-SOD1 and ALS-FUS, both latter conditions are rare. The largest study to date showed all sporadic ALS cases to have TDP-43 pathology (Mackenzie et al 2007). Only around 5% of ALS is familial (Byrne et al 2010) and mutations in *SOD1,* the commonest mutations found in FALS, account for between 12-23% of cases (Andersen et al 2006). *SOD1* may also be

implicated in 2-7% of SALS. Mutations in *FUS* appear to be far less common, accounting for only around 3-4% of FALS (Blair et al 2010). So it can be reliably predicted that a clinical case of SALS will have TDP-43 based pathology. Similarly this can be reliably predicted in FALS due to mutations in *TDP-43*, which account for around 4% of cases (Mackenzie et al 2010). ALS with mutations in: *VCP,* accounting for 1-2% of FALS (Johnson et al 2010), *ANG* (Seilhean et al 2009), and *CHMP2B* (Cox et al 2010) have also been reported to be associated with TDP-43 positive pathology. Although *SOD1, FUS* and *ATXN2* mutations have not been found to be associated with TDP-43 positive aggregates, it has however been reported that TDP-43 is implicated in the pathological processes putatively arising from these mutations (Higashi et al 2010; Ling et al 2010; Elden et al 2010).

It is therefore established that TDP-43 has an important, and potentially central role, in the pathogenesis of the vast majority of SALS cases and may be implicated in the pathogenesis of a significant proportion of FALS. ALS is now widely considered to be a TDP-43 proteinopathy (Neumann et al 2009) and numerous *in vitro*, and *in vivo* studies provide support to the hypothesis that toxic gain of function, due to TDP-43 aggregation is responsible for at least some of the neurotoxicity in the disease.

FTLD syndromes are insidious onset, inexorably progressive, neurodegenerative conditions, with peak onset in late middle age. There is often a positive family history of similar disorders in a first degree relative.

Behavioural variant FTD is characterised by early prominent change in social and interpersonal function, often accompanied by repetitive behaviours and changes in eating pattern. In semantic dementia there are prominent word finding problems, despite otherwise fluent speech, with degraded object knowledge and impaired single word comprehension on cognitive assessment. Progressive non-fluent aphasia presents with a combination of motor speech problems and grammatical deficits. The core clinical diagnostic features for these three FTLD syndromes are shown in the Table below and the full criteria in Neary *et al* (1998).

Clinical Profile and Core Diagnostic Features of FTLD Syndromes

FTLD Syndrome -Clinical Profile	Core Diagnostic Features
Frontotemporal Dementia Character change and disordered social conduct are the dominant features initially and throughout the disease course. Instrumental functions of perception, spatial skills, praxis and memory are intact or relatively well preserved.	1. Insidious onset and gradual progression 2. Early decline in social interpersonal conduct 3. Early impairment in regulation of personal conduct 4. Early emotional blunting 5. Early loss of insight
Semantic Dementia Semantic disorder (impaired understanding of word meaning and/or object identity) is the dominant feature initially and throughout the disease course. Other aspects of cognition, including autobiographic memory, are intact or relatively well preserved.	A) Insidious onset and gradual progression B) Language disorder characterised by 1. Progressive, fluent empty speech 2. Loss of word meaning manifest by impaired naming and comprehension 3. Semantic paraphasias and / or 4. Perceptual disorder characterised by 1. Prosopagnosia: impaired recognition of identity of familiar faces and /or 2. Associative agnosia: impaired recognition of object identity C) Preserved perceptual matching and drawing reproduction D) Preserved single word repetition E) Preserved ability to read aloud and write to dictation orthographically regular words
Progressive Non-fluent Aphasia Disorder of expressive language is the dominant feature initially and throughout the disease course. Other aspects of cognition are intact or relatively well preserved.	A) Insidious onset and gradual progression B) Non-fluent spontaneous speech with at least one of the following: agrammatism, phonemic paraphasias or anomia

The discovery that TDP-43-positive inclusions characterize ALS and FTLD-TDP (Neumann *et al* 2006) was quickly followed by the identification of missense mutations in the *TARDBP* gene in both familial and sporadic cases of ALS (Gitcho *et al* 2008; Sreedharan et al., 2008). So far, 38 different *TARDBP* mutations have been reported in

79 genealogically unrelated families worldwide (Mackenzie et al 2010). *TARDBP* mutations account for approximately 4% of all familial and around 1.5% of sporadic ALS cases.

As of December 2010, mutations in thirteen genes which are associated with familial and sporadic ALS have been identified. Linkage of ALS to five other chromosome loci has been demonstrated but thus far specific mutations have not been identified.

TDP-43 proteinopathies

MT has a mode of action which targets and can reduce TDP-43 protein aggregation in cells, which is a pathological feature of the vast majority of both familial and sporadic ALS and is also characteristic of FTLD-P.

In addition laboratory data shows that methylthioninium inhibits the formation of TDP-43 aggregates in SH-SY5Y cells. Following treatment with 0.05 μM MT, the number of TDP-43 aggregates was reduced by 50%. These findings were confirmed by immunoblot analysis (Yamashita et al 2009).

The compounds and compositions of the invention may therefore be useful for the treatment of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD).

Huntington's disease and polyglutamine disorders

MT can reduce polyglutamine protein aggregation in cells, which is a pathological feature of Huntington's disease. Huntington's disease is caused by expansion of a translated CAG repeat located in the N-terminus of huntingtin. Wild-type chromosomes contain 6–34 repeats whereas, in Huntington's disease, chromosomes contain 36–121 repeats. The age of onset of disease correlates inversely with the length of the CAG tracts that code for polyglutamine repeats within the protein.

Laboratory data shows that methylthioninium inhibits the formation of aggregates of a huntingtin derivative containing a polyglutamine stretch of 102 residues in zebrafish (van Bebber et al. 2010). MT, when tested at 0, 10 and 100 μM, prevented the formation of such aggregates in zebrafish in a dose dependent manner.

The compounds and compositions of the invention may therefore be useful for the treatment of Huntington's disease and other polyglutamine disorders such as spinal bulbar muscular atrophy (or Kennedy disease), and dentatorubropallidoluysian atrophy and various spinocerebellar ataxias (Orr & Zoghbi, 2007).

Mitochondrial Diseases and Lafora Disease

The organ most frequently affected in mitochondrial disorders, particularly respiratory chain diseases (RCDs), in addition to the skeletal muscle, is the central nervous system (CNS). CNS manifestations of RCDs comprise stroke-like episodes, epilepsy, migraine, ataxia, spasticity, movement disorders, psychiatric disorders, cognitive decline, or even dementia (mitochondrial dementia). So far mitochondrial dementia has been reported in MELAS, MERRF, LHON, CPEO, KSS, MNGIE, NARP, Leigh syndrome, and Alpers–Huttenlocher disease (Finsterer, 2009). There are four complexes in the mitochondrial respiration chain, involving a series of electron transfers. Abnormal function of any of these complexes can result in mitochondrial diseases secondary to an abnormal electron transport chain and subsequent abnormal mitochondrial respiration. Complex III of the mitochondrial respiration chain acts to transfer electrons to cytochrome c.

Compounds and compositions of the invention may also be used to treat mitochondrial diseases which are associated with a deficient and/or impaired complex III function of the respiration chain. The compounds have the ability to act as effective electron carrier and/or transfer, as the thioninium moiety has a low redox potential converting between the oxidised and reduced form. In the event of an impaired and/or deficient function of Complex III leading to mitochondrial diseases, compounds of the invention are also able to perform the electron transportation and transfer role of complex III because of the ability of the thioninium moiety to shuttle between the oxidised and reduced form, thus acting as an electron carrier in place of sub-optimally functioning complex III, transferring electrons to cytochrome c.

Compounds and compositions of the invention also have the ability to generate an active thioninium moiety that has the ability to divert misfolded protein/amino acid monomers/oligomers away from the Hsp70 ADP-associated protein accumulation and/or refolding pathways, and instead rechannel these abnormal folded protein monomers/oligomers to the pathway that leads directly to the Hsp70 ATP-dependent ubiquitin-proteasome system (UPS), a pathway which removes these misfolded proteins/amino acid monomers/oligomers via the direct route (Jinwal et al. 2009).

Lafora disease (LD) is an autosomal recessive teenage-onset fatal epilepsy associated with a gradual accumulation of poorly branched and insoluble glycogen, termed polyglucosan, in many tissues. In the brain, polyglucosan bodies, or Lafora bodies, form in neurons. Inhibition of Hsp70 ATPase by MT (Jinwal et al. 2009) may upregulate the removal of misfolded proteins. Lafora disease is primarily due to a lysosomal ubiquitin-proteasomal system (UPS) defect because of a mutation in either the Laforin or Malin genes, both located on Chromosome 6, which result in inclusions that may accelerate the aggregation of misfolded tau protein. Secondary mitochondrial damage from the impaired UPS may further result in a suppressed mitochondrial activity and impaired electron transport chain leading to further lipofuscin and initiating the seizures that are characteristic of Lafora disease.

The MT moiety may disaggregate existing tau aggregates, reduce more tau accumulating and enhance lysosomal efficiency by inhibiting Hsp70 ATPase. MT may lead to a reduction in tau tangles by enhancing the ubiquitin proteasomal system removal of tau monomers/oligomers, through its inhibitory action on Hsp70 ATPase.

Thus compounds and compositions of the present invention may have utility in the treatment of Lafora disease.

Mixtures of oxidised and reduced MT compounds

MT compounds for use in the present invention may include mixtures of the oxidised and reduced form.

In particular, the LMT-containing compounds may include oxidised (MT^+) compounds as 'impurities' during synthesis, and may also oxidize (e.g., autoxidize) after synthesis to give the corresponding oxidized forms. Thus, it is likely, if not inevitable, that compositions comprising the compounds of the present invention will contain, as an impurity, at least some of the corresponding oxidized compound. For example an "LMT" salt may include 10 to 15% of MT^+ salt.

When using mixed MT compounds the MT dose can be readily calculated using the molecular weight factors of the compounds present.

Salts and solvates

Although the MT containing compounds described herein are themselves salts, they may also be provided in the form of a mixed salt (i.e., the compound of the invention in combination with another salt). Such mixed salts are intended to be encompassed by the term "and pharmaceutically acceptable salts thereof". Unless otherwise specified, a reference to a particular compound also includes salts thereof.

The compounds of the invention may also be provided in the form of a solvate or hydrate. The term "solvate" is used herein in the conventional sense to refer to a complex of solute (e.g., compound, salt of compound) and solvent. If the solvent is water, the solvate may be conveniently referred to as a hydrate, for example, a mono-hydrate, a di-hydrate, a tri-hydrate, a penta-hydrate etc. Unless otherwise specified, any reference to a compound also includes solvate and any hydrate forms thereof.

Naturally, solvates or hydrates of salts of the compounds are also encompassed by the present invention.

A number of patents and publications are cited herein in order to more fully describe and disclose the invention and the state of the art to which the invention pertains. Each of these references is incorporated herein by reference in its entirety into the present disclosure, to the same extent as if each individual reference was specifically and individually indicated to be incorporated by reference.

Throughout this specification, including the claims which follow, unless the context requires otherwise, the word “comprise,” and variations such as “comprises” and “comprising,” will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

It must be noted that, as used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a pharmaceutical carrier” includes mixtures of two or more such carriers, and the like.

Ranges are often expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by the use of the antecedent “about,” it will be understood that the particular value forms another embodiment.

Any sub-titles herein are included for convenience only, and are not to be construed as limiting the disclosure in any way.

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

The disclosure of all references cited herein, inasmuch as it may be used by those skilled in the art to carry out the invention, is hereby specifically incorporated herein by cross-reference.

Figures

Figure 1. (A) Screening, safety and efficacy populations. (B) Patient categorisation by randomised treatment, AD-approved co-medication status and withdrawal rates at 65 weeks.

Figure 2. Least squares estimates of change from baseline in (A) ADAS-cog and (B) ADCS-ADL using prespecified repeat of primary analysis with stratification factor AChEI / Mem as an interaction term in the model.

Figure 3. Least squares estimates of change from baseline for (A) ADAS-cog and (B) ADCS-ADL in patients restricted to those randomised to the control arm and receiving 4 mg b.i.d. of LMTM, either alone or in combination with AD-labelled medications.

Figure 4. Least squares estimates of LVV change over time using prespecified repeat of primary analysis with stratification co-variate AChEI / Mem as an interaction term in the model.

Figure 5. Least squares estimates of TPV change from baseline using prespecified repeat of primary analysis with stratification factor AChEI / Mem as an interaction term in the model.

Figure 6. Least squares estimates of HV change from baseline using prespecified repeat of primary analysis with stratification factor AChEI / Mem as an interaction term in the model.

Figure 7. Least squares estimates of responses for change from baseline in (A) LVV, (B) TPV and (C) HV in subjects restricted to those randomised to the control arm and receiving 4 mg b.i.d. of LMTM either alone or in combination with AD-labelled medications.

Examples

Example 1 – provision of MT-containing compounds

Methods for the chemical synthesis of the MT-containing compounds described herein are known in the art. For example:

Synthesis of compounds 1 to 7 can be performed according to the methods described in WO2012/107706, or methods analogous to those.

Synthesis of compound 8 can be performed according to the methods described in WO2007/110627, or a method analogous to those.

Synthesis of compound 9 (MTC) is well known in the art. Examples syntheses of highly pure MTC are provided in WO2006/032879 and WO2008/007074.

Synthesis of compounds 10 to 13 can be performed according to the methods described in WO2007/110630, or methods analogous to those.

Example 2 – formulation of MT-containing compounds

Methods for the chemical synthesis of the MT-containing compounds described herein are known in the art. Example methods using dry compression, for example, are provided in WO2012/072977.

Example 3 – phase 3 clinical trial in mild to moderate AD

Methods

Outcomes and measures

The co-primary efficacy outcomes were change from baseline in 11-item Alzheimer's Disease Assessment Scale – cognitive subscale (ADAS-cog) and the 23-item Alzheimer's

Disease Cooperative Study Activities of Daily Living (ADCS-ADL). Magnetic resonance imaging (MRI) volumetry was chosen as the principal secondary outcome to support a potential effect on the rate of brain atrophy (Fox et al., 1997; Ridha et al., 2008). Change in Lateral Ventricular Volume (LVV), as measured by the Ventricular Boundary Shift Integral (VBSI), (Salloway et al., 2014) was chosen as the principal measure as it provides high-contrast boundaries for measurement and is less affected by motion artefacts than whole brain or smaller structures. Key supportive topographical measures (temporo-parietal (TPV) and hippocampal volume (HV)) are also reported.

Patients

Patients were recruited at 115 sites across 16 countries between January 2013 and August 2014. Enrolment of approximately 833 patients was targeted, with 891 patients actually recruited, in order to obtain data on approximately 500 patients completing the study, assuming a 30–40% drop-out rate. Patients aged <90 meeting a diagnosis of all cause dementia and probable AD according to National Institute of Aging (NIA) and Alzheimer's Association (AA) criteria were included if they had a Clinical Dementia Rating (CDR) total score of 1 or 2 and Mini-Mental State Examination (MMSE) score of 14–26 inclusive. Adult caregiver(s) were required to participate. Concomitant use of AChEIs and/or memantine was permitted, provided the patient had been taking the medication(s) for ≥3 months, with no changes to the dosage for ≥6 weeks prior to screening. Concomitant use of serotonergic antidepressant medication was permitted, but patients were monitored closely using a targeted rating scale derived from 4 published diagnostic criteria (Alusik et al., 2014) due to a theoretical potential for serotonin syndrome (Ramsay et al., 2007). Patients were excluded from the study if they had a significant central nervous system disorder other than AD or significant focal or vascular intracranial pathology on brain magnetic resonance imaging (MRI) performed within 6 weeks prior to baseline. Because MT^+ in high doses can induce methaemoglobinaemia, subjects with glucose-6-phosphate dehydrogenase deficiency or who were otherwise at haematological risk were excluded. Other inclusion/exclusion criteria are provided in the Supplementary Materials.

Randomisation and masking

Patients were randomised at baseline to LMTM 75 mg b.i.d. or 125 mg b.i.d. (expressed as MT base equivalent) or control in a 3:3:4 ratio. The randomisation was stratified according to geographical region (3 levels: North America, Europe, rest of world), use of AD-labelled co-medications (2 levels, using or not using) and severity (2 levels, moderate MMSE 14 – 19 inclusive, mild MMSE 20 – 26 inclusive). Patients in the control arm received a dose of 4 mg b.i.d. to maintain blinding.

Ethical conduct of the study

All patients provided written informed consent prior to enrolling in the study; legally acceptable representatives provided consent on behalf of patients with reduced decision-making capacity. Adult caregivers also provided consent for their own involvement. The study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonisation Guidelines for Good Clinical Practice, and approval of the study protocol and all related documents was obtained from the appropriate Independent Ethics Committees and Institutional Review Boards for all study sites. An independent Data and Safety Monitoring Board was established for oversight of accruing safety information. The trial is registered at www.clinicaltrials.gov (NCT01689246) and the European Union Clinical Trials Registry (2012-002866-11).

Clinical and imaging assessments

ADAS-cog and ADCS-ADL assessments were performed at baseline and every 13 weeks thereafter, with change at 65 weeks (the final on-treatment visit) the primary efficacy measures. These were repeated at the final off-treatment follow-up visit at Week 69. Secondary efficacy measures included Clinical Global Impression of Change (ADCS-CGIC, administered by an independent rater); MMSE, administered on screening and at Weeks 26, 52, 65 and 69. Brain MRI scans were performed at baseline/screening and every 13 weeks using a standardized protocol including volumetric 3D T1-weighted data consistent with ADNI recommendations, FLAIR, T2* Gradient Echo and T2-weighted sequences. Diffusion-Weighted Imaging was also available at screening in order to exclude patients with co-existing pathology that would lead to a diagnosis other than probable AD. Each site was first qualified ensuring standardised acquisition protocol, patient handling and data management. MRI data were centrally collected by Imaging Corelab (Bioclinica). Data were centrally reviewed by RadMD for eligibility and safety (Amyloid Related Imaging Abnormalities, or ARIA monitoring) on an on-going basis. Volumetric 3DT1 data were also reviewed centrally in order to measure (using a boundary shift integral (BSI) technique; Salloway et al., 2014) change in LVV as principal secondary outcome measure, and as exploratory endpoints temporo-parietal volume (TPV), whole brain volume (WBV) and hippocampal volume (HV, estimated as the mean of left and right) (Wischik et al., 2016). Additional exploratory endpoints included change in glucose uptake in the temporal lobe, assessed using ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) performed during screening and at Weeks 39 and 65 in a subset of patients in sites with this imaging capability. Changes in cerebrospinal fluid biomarkers of Alzheimer's disease, including total tau, phospho-tau and amyloid-β_{1-42}, were explored using cerebrospinal fluid samples that were collected at baseline and Week 65 (or early termination visit). Lumbar punctures were performed only in subjects who were themselves able to provide consent specifically for this procedure.

Patients were monitored throughout for adverse events (AEs) and clinical laboratory testing, physical and neurological examinations and 12-lead electrocardiograms were performed at all clinic visits (screening, baseline and Weeks 2, 6, 13, 26, 39, 52, 65 and 69). Patients were also assessed at all visits for suicidal ideation and intent, and were systematically assessed for signs of serotonin toxicity.

Statistical methods

The primary efficacy analyses of change from baseline in ADAS-cog and ADCS-ADL scores were conducted in the modified intent-to-treat (mITT) population (all randomised patients who took at least one dose of study treatment and had at least one post-baseline, on-treatment efficacy assessment). The primary analysis was specified as a mixed model repeated-measures (MMRM) analysis with an unstructured covariance matrix and no imputation for missing data. The model included categorical visit (5 levels corresponding to assessments at weeks 13, 26, 39, 52 and 65), treatment (3 levels corresponding to control, 75 mg b.i.d. and 125 mg b.i.d.), treatment-by-visit interaction, and the stratification variables as additive terms, and baseline ADAS-cog or ADCS-ADL as a covariate. A similar exploratory analysis was specified in the Statistical Analysis Plan (SAP) with the AChEI / Mem covariate as an interaction term with treatment and as an interaction term with visit. Westfall's method for multiple comparison correction was used in each step to ensure strong control of the familywise error with alpha 0.05 (Westfall et al., 1997) The same analyses were implemented for change in LVV, WBV, TPV, HV and TPV.

Results

Patients

The patient disposition and trial design is shown in Figure 1. Of 891 patients randomised, 885 received at least one dose of study drug and comprised the safety population, and 855 patients were in the mITT population Figure 1(A). Patient disposition by randomised treatment and treatment with or without AChEI / Mem is shown in Figure 1(B). The baseline demographics and clinical characteristics of the safety population are shown in Table 1. There were 618 patients completing the study to 65 weeks (with 579 remaining on treatment), an overall study withdrawal rate of 31%. MRI scans from all scheduled visits were available from 880 patients pre-treatment and 554 at 65 weeks. FDG-PET data were available from 101 patients at 65 weeks, of whom only 6 were not taking AChEI / Mem treatments.

Table 1. Patient baseline demographics and clinical characteristics (safety population)

Characteristic	Control LMTM 4 mg b.i.d.	LMTM 75 mg b.i.d.	LMTM 125 mg b.i.d.	Total
	n=354	n=267	n=264	n=885
Age (years)				
Mean (SD)	70·7 (8·5)	71·0 (9·3)	70·1 (9·3)	70·6 (9·0)
Median (min; max)	72·0 (40; 89)	72·0 (39; 88)	71·0 (32; 89)	72·0 (32; 89)
Sex				
Male, n (%)	134 (38)	93 (35)	113 (43)	340 (38)
Female, n (%)	220 (62)	174 (65)	151 (57)	545 (62)
Race				
American Indian or Alaska Native, n (%)	2 (0·6)	3 (1·1)	2 (0·8)	7 (0·8)
Asian, n (%)	41 (11·6)	32 (12·0)	30 (11·4)	103 (11·6)
Black or African American, n (%)	3 (0·8)	3 (1·1)	4 (1·5)	10 (1·1)
White, n (%)	307 (86·7)	226 (84·6)	225 (85·2)	758 (85·6)
Other, n (%)	1 (0·3)	0	2 (0·8)	3 (0·3)
Multiple Race, n (%)	0	3 (1·1)	1 (0·4)	4 (0·5)
Years since diagnosis				
Mean (SD)	2·8 (2·4)	2·9 (2·3)	2·8 (2·2)	2·8 (2·3)
Dementia severity				
CDR 0·5, n (%)	4 (1·1)	1 (0·4)	2 (0·8)	7 (0·8)
CDR 1, n (%)	261 (73·7)	209 (78·3)	192 (72·7)	662 (74·8)
CDR 2, n (%)	89 (25·1)	57 (21·3)	70 (26·5)	216 (24·4)
MMSE				
Mean (SD)	18·6 (3·45)	18·8 (3·44)	18·5 (3·40)	18·6 (3·43)
Median (min; max)	18·0 (14; 26)	19·0 (14; 26)	18·0 (14; 26)	18·0 (14; 26)
MMSE severity				
MMSE ≥20, n (%)	134 (38)	105 (39)	98 (37)	337 (38)
MMSE <20, n (%)	220 (62)	162 (61)	166 (63)	548 (62)
ADAS-Cog:				
Mean (SD)	27·2 (10·1)	26·5 (9·4)	26·7 (9·7)	26·9 (9·8)
Median (min; max)	26·3 (7; 57)	26·3 (8; 54)	26·3 (8; 56)	26·3 (7; 57)
ADCS-ADL:				
Mean (SD)	55·9 (12·7)	58·0 (11·1)	57·5 (12·7)	57·0 (12·3)
Median (min; max)	58·0 (17; 78)	58·5 (16; 78)	60·0 (13; 78)	59·0 (13; 78)
Whole brain volume (cm^3)				
Mean (SD)	927 (108)	922 (115)	939(101)	929 (108)
Median (min; max)	917 (681; 1,233)	922 (602; 1,207)	934 (682; 1,264)	925 (602; 1,264)
Lateral ventricular volume (cm^3)				
Mean (SD)	52 (23)	52 (26)	51 (23)	52 (24)
Median (min; max)	49 (15; 154)	44 (12; 160)	47 (15; 138)	47 (12; 160)
Hippocampal volume (mm^3)				
Mean (SD)	2·3 (0·6)	2·7 (0·6)	2·9 (0·6)	2·8 (0·6)
Median (min; max)	2·7 (1·4; 4·5)	2·7 (1·4; 4·4)	2·8 (1·5; 5·0)	2·7 (1·4; 5·0)
AD-approved co-medications				
AChEI only, n (%)	183 (52)	151 (57)	150 (57)	484 (55)
Memantine only, n (%)	32 (9)	16 (6)	15 (6)	63 (7)
AChEI and memantine, n (%)	93 (26)	60 (23)	61 (23)	214 (24)
CSF biomarkers (ng/L)				
Total tau, mean (SD) [n]	143·9 (68·4)	156·4 (72·5)	113·2 (54·7) [5]	144·8 (68·2)

	[19]	[15]		[39]
Phospho-tau, mean (SD) [n]	59·2 (25·3) [20]	61·2 (20·3) [15]	58·1 (12·8) [5]	59·8 (21·9) [40]
Aβ1-42, mean (SD) [n]	264·7 (96·6) [20]	276·0 (85·9) [15]	235·8 (62·1) [5]	265·3 (88·0) [40]
***APOE* genotype**				
ε4 allele present, n (%)	144 (47.5)	91 (41.9)	114 (52.5)	349 (47.4)
ε4 allele absent, n (%)	159 (52.5)	126 (58.1)	103 (47.5)	388 (52.6)

Efficacy analyses of primary clinical outcomes

Table 2 reports change in ADAS-cog and ADCS-ADL scores, decline in the control arm and difference with respect to control by treatment arm. The ADAS-cog decline at 12 months (3.85 ± 0.47, mean ± se) was indistinguishable from a meta-analysis of recent studies (4.58, 95% CI: 3.69 – 5.47) at 12 months (Salloway et al., 2014; Doody et al., 2014). Likewise, the ADCS-ADL decline at 12 months (-6.51 ± 0.63) was similar to that of the only available recent study (Doody et al., 2014) in mild/moderate AD (-5.79, 95% CI: -7.10 – -4.48).

As can be seen from Table 2, neither of the primary analyses yielded significant effects for either 75 mg b.i.d. or for 125 mg b.i.d. at 65 weeks. However, the AChEI / Mem factor was significant for both ADAS-cog and ADCS-ADL, with p-values 9.4e-05 and 0.0174 respectively after correction for multiple comparisons. This implies a mean additive treatment benefit in patients not taking AD treatments of -2.30 (95% CI: -3.35 – -1.25) ADAS-cog units and 2.00 (95% CI: 0.65 – 3.35) ADCS-ADL units. Likewise, mild subjects had lower rates of progression for both ADAS-cog and ADCS-ADL overall (p values: 8.6e-04 and 9.6e-07, respectively). There was no overall effect of region on either outcome.

Table 2. Primary efficacy analyses for ADAS-cog (A) and ADCS-ADL (B) at 65 weeks. In this and in all following tables, "pval-adj" signifies the p values corrected for multiple comparisons using the Westfall procedure. All stratification covariates are additive terms.

	Estimate	**SE**	**2.50%**	**97.50%**	**p value**	**pval-adj**
ADAS-cog						
Control	6.32	0.52	5.31	7.34		
75 mg b.i.d.	-0.02	0.80	-1.60	1.56	0.983	0.983
125 mg b.i.d.	-0.43	0.83	-2.06	1.20	0.602	0.932
Not using AChEI /Mem	-2.30	0.53	-3.35	-1.25	1.78e-05	9.4e-05
Mild	-1.03	0.28	-1.57	-0.59	1.85e-04	8.6e-04
North America	-0.08	0.26	-0.58	0.43	0.769	0.947
Rest of world	-0.47	0.44	-1.32	0.39	0.285	0.724
ADCS-ADL						
Control	-8.22	0.72	-9.63	-6.82		
75 mg b.i.d.	-0.93	1.12	-3.12	1.26	0.407	0.866

125 mg b.i.d.	-0.34	1.16	-2.61	1.93	0.770	0.948
Not using AChEI /Mem	2.00	0.69	0.65	3.35	0.00365	0.0174
Mild	1.62	0.31	1.02	2.23	2.0e-07	9.6e-07
North America	0.08	0.34	-0.58	0.74	0.814	0.948
Rest of world	-0.27	0.56	-1.37	0.83	0.632	0.948

In order to understand better the role of the AChEI / Mem factor, a pre-specified analysis was undertaken which included this as an interaction term with visit and as an interaction term with LMTM treatment in the model rather than only as an overall additive term as in the primary model. The results by dose-arm and AChEI / Mem status are shown in Table 3 and Figure 2 for both ADAS-cog and ADCS-ADL.

Table 3. Prespecified repeat of primary analysis of ADAS-cog and ADCS-ADL with stratification covariate AChEI / Mem as an interaction term in the model.

	Estimate	SE	2.50%	97.50%	p value	pval-adj
ADAS-cog						
Control	5.98	0.51	5.00	6.98	0.000	
75 mg b.i.d.	-6.25	1.36	-8.92	-3.59	4.15e-06	1.5e-05
125 mg b.i.d.	-5.79	1.37	-8.47	-3.11	2.35e-05	6.8e-05
75 mg b.i.d. + AChEI / Mem	1.02	0.81	-0.58	2.61	0.212	0.362
125 mg bi.d. + AChEI / Mem	0.50	0.84	-1.15	2.14	0.555	0.555
ADCS-ADL						
Control	-7.92	0.70	-9.29	-6.55	0.000	
75 mg b.i.d.	6.48	1.84	2.87	10.09	4.3e-04	0.00138
125 mg b.i.d.	6.93	1.86	3.29	10.57	1.9e-04	0.00711
75 mg b.i.d. + AChEI / Mem	-2.16	1.13	-4.37	0.05	0.0553	0.103
125 mg bi.d. + AChEI / Mem	-1.62	1.17	-3.91	0.68	0.167	0.167

Given the effect of AD treatments on the efficacy of LMTM given at higher doses, the same effect was explored further in *post-hoc* analyses restricted to subjects randomised to the control arm receiving 4 mg b.i.d. of LMTM. The results are shown in Table 4 and Figure 3 for ADAS-cog and ADCS-ADL.

Table 4. *Post-hoc* analysis of ADAS-cog and ADCS-ADL restricted to subjects randomised to the control arm and receiving 4 mg b.i.d. with or without AD-labelled medications.

	Estimate	SE	2.50%	97.50%	p value

ADAS-cog					
4 mg b.i.d. + AChEI / Mem	6.79	0.53	5.75	7.83	
4 mg b.i.d.	-5.90	1.14	-8.13	-3.66	2.3e-07
ADCS-ADL					
4 mg b.i.d. + AChEI / Mem	-8.90	0.73	-10.34	-7.47	
4 mg b.i.d.	7.19	1.55	4.15	10.23	3.5e-06

MRI volumetric analysis of lateral ventricular volume (LVV), temporo-parietal volume (TPV) and hippocampal volume (HV)

Using the same initial analysis model as for the primary clinical outcomes, no effect of treatment was seen (data not shown). In the prespecified analysis model with the AChEI / Mem factor as an interaction term in the model, results similar to those shown for the clinical outcomes were found for LVV, with p values 4.0e-05 and 3.8e-04, respectively for the 75 mg b.i.d. or 125 mg b.i.d. doses. The results were similar in the TPV analysis, with p values 2.4e-04 and 0.00160 for 75 mg b.i.d. and 125 mg b.i.d. doses respectively. In the HV analysis, only the 125 mg b.i.d. dose taken alone approached nominal significance (p=0.0558) in the mixed mild/moderate population. The data are shown in Table 5 and Figures 4, 5 and 6.

Table 5. Prespecified repeat of primary analysis of LVV, TPV and HV (mm^3) with stratification co-variate AChEI / Mem as an interaction term in the model.

	Estimate	**SE**	**2.50%**	**97.50%**	**p value**	**pval-adj**
LVV						
Control	7,187	279	6,641	7,733		
75 mg b.i.d.	-2,707	659	-3,999	-1,416	4.0e-05	1.9e-04
125 mg b.i.d.	-2,347	660	-3,641	-1,052	3.8e-04	0.00124
75 mg b.i.d. + AChEI / Mem	-273	443	-1,141	596	0.538	0.733
125 mg b.i.d. + AChEI / Mem	-306	453	-1,194	583	0.500	0.733
TPV						
Control	-1529	46	-1620	-1438		
75 mg b.i.d.	472	117	244	701	5.2e-05	2.4e-04
125 mg b.i.d.	405	117	176	634	5.2e-04	0.00160
75 mg b.i.d. + AChEI / Mem	-39	74	-183	106	0.597	0.825
125 mg b.i.d. + AChEI / Mem	-12	75	-160	135	0.869	0.869
HV						
Control	-115.3	4.0	-123.1	-108.5		
75 mg b.i.d.	9.5	10.4	-10.8	29.8	0.360	0.571
125 mg b.i.d.	20.0	10.4	-0.5	40.5	0.0558	0.187

75 mg b.i.d. + AChEI / Mem	-2.0	6.4	-14.5	10.5	0.754	0.754
125 mg b.i.d. + AChEI / Mem	-7.4	6.5	-20.2	5.3	0.253	0.555

The effect of AD-labelled co-medications on the LVV, TPV and HV efficacy of low dose LMTM was explored further in the *post-hoc* analysis restricted to subjects randomised to the control arm receiving LMTM 4 mg b.i.d. either alone or in combination with AChEI / Mem. The results are shown in Table 6 and Figure 7.

Table 6. *Post-hoc* analysis of LVV, TPV and HV (mm^3) restricted to subjects randomised to the control arm and receiving 4 mg b.i.d. of LMTM either alone or in combination with AD-labelled medications.

	Estimate	SE	2.50%	97.50%	p value
LVV					
4 mg b.i.d. + AChEI / Mem	7,426	289	6860	7,993	
4 mg b.i.d.	-1,894	549	-2,972	-818	0.000564
TPV					
4 mg b.i.d. + AChEI / Mem	-1,596	48	-1,691	-1,501	
4 mg b.i.d.	534	98	342	725	4.9e-08
HV					
4 mg b.i.d. + AChEI / Mem	-117.8	4.1	-125.9	-109.7	
4 mg b.i.d.	19.5	8.7	2.3	36.6	0.0259

Mild and moderate AD

The same analyses were repeated in mild and moderate subjects separately as disease severity was a highly significant covariate in the primary model (Table 7). The results for treatment with LMTM as monotherapy are shown only for the 75 mg b.i.d. and 125 mg b.i.d. doses compared with the control arm as randomised. The analysis of the 4 mg b.i.d. dose was restricted to subjects randomised to the control arm and receiving 4 mg b.i.d. with or without AChEI / Mem.

Table 7. Analyses of ADAS-cog, ADCS-ADL, LVV, TPV and HV in mild AD and moderate AD with stratification covariate AChEI / Mem as an interaction term in the model. Treatment with LMTM alone is compared with the control arm as randomised for the 75 mg b.i.d. and 125 mg b.i.d. doses. The analysis of the 4 mg b.i.d. dose was restricted to subjects randomised to the control arm and taking or not taking AD-labelled co-medications.

	Estimate	SE	2.50%	97.50%	p value
ADAS-cog					
Mild AD					
Control	2.27	0.77	0.77	3.77	
75 mg b.i.d.	-8.89	1.76	-12.37	-5.41	5.5e-07

125 mg b.i.d.	-5.03	1.81	-8.58	-1.47	0.00557
4 mg b.i.d. + AChEI / Mem	3.24	0.80	1.67	4.81	
4 mg b.i.d.	-6.41	1.55	-9.44	-3.37	3.5e-05
Moderate AD					
Control	7.89	0.68	6.55	9.23	
75 mg b.i.d.	-2.01	1.94	-5.81	1.80	0.301
125 mg b.i.d.	-5.75	1.92	-9.51	-1.98	0.00277
4 mg b.i.d. + AChEI / Mem	8.49	0.71	7.09	9.88	
4 mg b.i.d.	-4.74	1.58	-7.83	-1.65	0.00267
ADCS-ADL					
Mild AD					
Control	-3.20	0.96	-5.08	-1.33	
75 mg b.i.d.	4.65	2.35	0.04	9.25	0.0480
125 mg b.i.d.	7.77	2.39	3.10	12.44	0.00112
4 mg b.i.d. + AChEI / Mem	-3.86	1.01	5.83	-1.88	
4 mg b.i.d.	5.20	2.03	1.23	9.17	0.0103
Moderate AD					
Control	-10.93	0.95	-12.80	-9.07	
75 mg b.i.d.	6.89	2.67	1.66	12.13	0.00989
125 mg b.i.d.	5.53	2.66	0.31	10.75	0.0377
4 mg b.i.d. + AChEI / Mem	-11.91	0.99	-13.85	-9.97	
4 mg b.i.d.	7.70	2.20	3.39	12.01	4.6e-04
LVV					
Mild AD					
Control	5,789	374	5,056	6,522	
75 mg b.i.d.	-2,549	835	-4,185	-913	0.00226
125 mg b.i.d.	-2,695	841	-4,343	-1,047	0.00135
4 mg b.i.d. + AChEI / Mem	6,167	389	5,355	6,879	
4 mg b.i.d.	-2,655	695	-4,021	-1,297	1.3e-04
Moderate AD					
Control	8,023	372	7,293	8,753	
75 mg b.i.d.	-2,251	925	-4,065	-438	0.0150
125 mg b.i.d.	-1,703	916	-3,499	92	0.0630
4 mg b.i.d. + AChEI / Mem	8,143	386	7,386	8,901	

4 mg b.i.d.	-940	777	-2,463	583	0.226
TPV					
Mild AD					
Control	-1,309	73	-1,452	-1,166	
75 mg b.i.d.	392	170	60	726	0.0208
125 mg b.i.d.	439	170	106	772	0.00978
4 mg b.i.d. + AChEI / Mem	-1,398	76	-1,547	-1,249	
4 mg b.i.d.	722	144	440	1,005	5.5e-07
Moderate AD					
Control	-1,649	59	-1,764	-1,534	
75 mg b.i.d.	469	155	164	773	0.00257
125 mg b.i.d.	293	154	-9	895	0.0570
4 mg b.i.d. + AChEI / Mem	-1,697	61	-1,817	-1,576	
4 mg b.i.d.	370	129	116	623	0.00425
HV					
Mild AD					
Control	-115.8	6.6	-126.7	-100.8	
75 mg b.i.d.	16.9	16.0	-14.7	48.1	0.267
125 mg b.i.d.	40.5	16.0	9.2	71.9	0.0113
4 mg b.i.d. + AChEI / Mem	-119.5	6.9	-113.1	-106.0	
4 mg b.i.d.	46.7	13.9	19.6	73.9	7.5e-04
Moderate AD					
Control	-116.9	4.9	-126.5	-107.3	
75 mg b.i.d.	3.6	13.6	-23.1	30.4	0.789
125 mg b.i.d.	2.2	13.7	-24.8	29.1	0.875
4 mg b.i.d. + AChEI / Mem	-116.6	5.1	-126.6	106.6	
4 mg b.i.d.	-2.4	11.2	-24.4	19.6	0.829

Comparison at baseline of patients taking or not-taking AD-labelled medications

Given the differences in outcomes according to whether LMTM was taken as monotherapy or not, differences at baseline between these groups were analysed according to clinical severity (Table 8). No difference was found in age or sex distribution. There was a significant regional difference in mild (but not moderate) patients, in that patients not prescribed AD-labelled co-medications were found to be at sites located predominantly in Russia, Eastern Europe (Poland and Croatia) and Malaysia. There was no difference in baseline ADAS-cog or MMSE. Mild patients not taking these medications were marginally worse on the ADCS-ADL scale, but there was no difference for moderate patients. In terms of the MRI parameters at baseline, mild (but not moderate) patients not

taking these medications had a slightly larger HV and LVV, but there was no difference in WBV, TPV or in extent of vascular pathology burden as indicated by Fazekas score at baseline (Murray et al., 2005). Likewise no differences were found for baseline bilirubin or creatinine clearance would might suggest differences in metabolism or excretion of LMTM.

Table 8. Differences at baseline between patients taking or not taking AD-labelled co-medications.

	Mild (not taking)	Mild (taking)	p-value	Moderate (not taking)	Moderate (taking)	p-value
Sex			0.142			0.759
Age	70.6 (5.8)	71.7 (8.3)	0.251	72.2 (9.8)	70.1 (8.6)	0.555
Region			**0.00763**			0.212
ADAS-cog	20.1 (5.6)	20.2 (7.1)	0.366	33.2 (9.8)	31.2 (9.2)	0.846
ADCS-ADL	60.7 (7.3)	61.6 (9.7)	**0.00106**	47.7 (16.6)	53.2 (12.8)	0.147
MMSE	22.3 (1.8)	22.2 (2.2)	0.705	16.5 (1.7)	16.4 (1.9)	0.585
WBV	946,295 (97,194)	950,840 (116,924)	0.664	925,032 (111,971)	912,214 (100,101)	0.357
TPV	41,841 (6,065)	41,911 (5,733)	0.488	38,208 (5,366)	38,069 (5,101)	0.229
HV	3,153 (647)	2,775 (534)	**7.2e-05**	2,654 (610)	2738 (508)	0.0893
LVV	37,975 (17,346)	51,732 (24,072)	**0.00196**	56,541 (25,660)	53,719 (23,021)	0.600
Fazekas score			0.149			0.463
Creatinine clearance	66.0	67.2	0.701	68.6	65.9	0.332
Bilirubin	0.51	0.56	0.235	0.53	0.52	0.772

Safety outcomes

The gastrointestinal and urinary tracts were the body systems most commonly affected by adverse events. These were also the most common reasons for discontinuing high dose LMTM (9% and 3% of patients, respectively); in comparison, only 1-2% of control patients discontinued for these events. Of note, the incidence of gastrointestinal adverse events was about two-fold higher in patients also receiving acetylcholinesterase inhibitors (data not shown). The treatment emergent adverse events occurring in ≥5% on high dose LMTM and greater than in the control arm are shown in Table 9.

Table 9. Most common treatment emergent adverse events occurring in ≥5% on 75 mg b.i.d. or 125 mg b.i.d. LMTM and greater than in control arm.

MedDRA System Organ Class / Preferred term	Control 4 mg b.i.d. (n=354)	High dose LMTM 75 mg b.i.d. (n=267)	125 mg b.i.d. (n=264)
At least one TEAE	296 (83.6%)	224 (83.9%)	229 (86.7%)
Blood and lymphatic system disorders	17 (4.8%)	29 (10.9%)	25 (9.5%)
Anemia	10 (2.8%)	22 (8.2%)	15 (5.7%)
Gastrointestinal disorders	87 (24.6%)	105 (39.3%)	111 (42.0%)
Diarrhea	33 (9.3%)	63 (23.6%)	67 (25.4%)
Nausea	14 (4.0%)	22 (8.2%)	19 (7.2%)
Vomiting	2 (0.6%)	25 (9.4%)	18 (6.8%)
Infections and infestations	88 (24.9%)	83 (31.1%)	76 (28.8%)
Urinary tract infection	29 (8.2%)	29 (10.9%)	26 (9.8%)
Investigations	80 (22.6%)	87 (32.6%)	80 (30.3%)
Blood folate decreased	21 (5.9%)	18 (6.7%)	19 (7.2%)
Renal and urinary disorders	29 (8.2%)	61 (22.8%)	65 (24.6%)
Dysuria	3 (0.8%)	7 (2.6%)	27 (10.2%)
Pollakiuria	6 (1.7%)	15 (5.6%)	18 (6.8%)
Urinary incontinence	9 (2.5%)	18 (6.7%)	12 (4.5%)
Respiratory, thoracic and mediastinal disorders	28 (7.9%)	32 (12.0%)	22 (8.3%)
Cough	12 (3.4%)	14 (5.2%)	11 (4.2%)

Adverse events of special interest (AESIs) were identified based on the known pharmacology of the MT moiety (specifically, ARIA). When the various adverse event terms are grouped, anaemia-related terms were reported in 22% of patients receiving high dose LMTM (as compared to 16% receiving control). The maximum mean changes in haemoglobin from baseline were at 6 weeks and were respectively (in g/L) -0.01 (95% CI: -0.23 – 0.21, p-value = 0.914), -0.47 (95% CI: -0.73 – -0.22, p-value = 6.4e-04) and -0.93 (95% CI: -1.18 – -0.68, p-value = 1.2e-12) g/L at doses of 4, 75 and 125 mg b.i.d. respectively. There was no case of haemolytic anemia. Twenty two percent of patients entered the study taking an SSRI (selective serotonin reuptake inhibitor). Only 2 patients had transient symptoms consistent with serotonergic excess but the temporal course and presentation were not consistent with serotonin toxicity; both patients were treated with LMTM 75 mg b.i.d. and neither received a concomitant serotonergic drug. In total, 8 patients developed ARIA during the study (<1%), with no dose relationship.

Based on the Columbia Suicide Severity Rating Scale, 26 patients had transient responses indicating a wish to be dead. There was one suicide attempt. With respect to other significant events, 9 patients who participated in the study died, the most common reasons being progression of AD or cancer; none was related to treatment. A total of 97 patients had one or more other non-fatal serious adverse events (SAEs), consistent with the nature of the patient population and evenly distributed between the three treatment groups. These were possibly related to treatment in only 14% of the cases, the most common being convulsion (all 4 occurring in the control arm).

Category	Control 4 mg b.i.d. (n=354)	High dose LMTM 75 mg b.i.d. (n=267)	High dose LMTM 125 mg b.i.d. (n=264)
Deaths, n (%)	3 (0.8)	3 (1.1)	3 (1.1)
Adverse Events of Special Interest (AESIs)			
Methemoglobinemia, hemolytic anemia, and/or Heinz bodies, n (%)	1 (0.3)	0 (0)	1 (0.4)
"Serotonin syndrome", n (%)	0 (0)	2 (0.7)	0 (0)
ARIA, n (%)	3 (0.8)	4 (1.5)	1 (0.4)
Other SUSARs, n (%)	2 (0.6)	2 (0.7)	4 (1.5)

Table 10. Significant treatment emergent adverse events

Discussion

The purpose of the present study was to confirm the efficacy reported in the earlier phase 2 study using a total daily dose of 150 mg/day and to determine whether 250 mg/day could achieve superior benefit using a newly developed stabilised reduced form of MT as LMTM. The study used a low dose of LMTM (4 mg b.i.d.) in the control arm rather than a true placebo to ensure blinding with respect to discolouration of excreta. The rates of decline on the ADAS-cog and ADCS-ADL scales seen in the control arm were linear and indistinguishable from those reported in recent studies. The same was found to be true for the rate of progression of brain atrophy in the mild AD group measured by change of LVV in comparison with data available from the ADNI program (Frisoni et al., 2010; Nestor et al., 2008) These comparisons support the face validity of the present study.

The AChEI / Mem factor was defined as a stratification variable, along with baseline severity and geographical region, thereby ensuring that the randomised treatment arms were equally represented in all three strata. It was assumed that it would be sufficient to account for the AChEI / Mem effect by including it as an additive term in the model, along with the other stratification factors. The primary efficacy analysis as prespecified did not, however, demonstrate statistical significance on either of the primary efficacy outcomes at either 75 mg b.i.d. or 125 mg b.i.d. using this model. The same analysis showed that the AChEI / Mem factor was a statistically significant determinant of efficacy, such that those not taking AD-labelled drugs experienced a mean overall benefit relative to controls of -2.30 ADAS-cog units and 2.00 ADCS-ADL units, effects that remained statistically significant in the whole-population analysis after full correction for multiple comparisons.

Since the baseline values were by definition zero, such an overall benefit could only occur if the intended active treatments produced a difference in the rate of progression relative to controls in patients taking LMTM as monotherapy.

To confirm this, the effect of LMTM treatment in the whole population was re-examined using a prespecified analysis model in which the AChEI / Mem term was included as an interaction term with visit and an interaction term with LMTM treatment, rather than only as an additive term. This analysis confirmed that treatment benefit was restricted to patients taking LMTM as monotherapy. LMTM at doses of 75 mg b.i.d. or 125 mg b.i.d. produced treatment effects of -6.25 and -5.79 ADAS-cog units respectively at 65 weeks, or 103% ± 23% and 83% ± 23% (mean ± SE) of the decline over 65 weeks seen in the control arm. The corresponding effect sizes on the ADCS-ADL scale were 6.48 and 6.92, or 82% ± 23% and 88% ± 23% of the decline seen over 65 weeks in the control arm. An identical profile was found for MRI measures of progression of neocortical atrophy, with reductions of 38% ± 9% and 33% ± 9% in LVV and increases of 31% ± 8% and 26% ± 8% in TPV for the 75 mg b.i.d. and 125 mg b.i.d. doses. All of these effects were statistically robust after appropriate correction for multiple comparisons. By contrast, the decline seen at the same doses in patients taking LMTM in combination with AD-labelled treatments, who were the majority, was indistinguishable on all parameters from that seen in the control arm.

Given that the higher dose did not result in greater efficacy, we examined whether differential efficacy for LMTM as monotherapy or not might also be present at the 4 mg b.i.d. dose originally intended as a urinary and faecal discolourant. A *post hoc* analysis showed that 4 mg b.i.d. as monotherapy showed effects of -5.90 ADAS-cog units, 7.19 ADCS-ADL units, as well as benefits in LVV and TPV similar to those seen at the higher doses relative to patients taking the same dose in combination with standard AD treatments, and in whom the decline was again indistinguishable from the decline seen either in the control arms of recently reported studies or ADNI data.

The efficacy profiles were also similar in mild and moderate subjects. The only difference was that, in hippocampus, benefit was seen in mild patients (increased by 35.5 mm^3) but not in moderate patients. This is consistent with the known staging of tau aggregation pathology, whereby damage in medial temporal lobe structures occurs earlier and is more severe than in neocortex. The general concordance of benefit on the volumetric measures of rate of progression of brain atrophy by LVV, TPV and HV, particularly in mild AD, argues against the possibility that the LVV measure is simply reporting treatment-related fluid shifts. Therefore TAI therapy has potential to benefit patients at both the mild and moderate stages of the disease, and not just at more advanced stages of AD as has been supposed. Indeed, the mean treatment benefit for LMTM monotherapy relative to control was 240% ± 41% and 156% ± 39% on the ADAS-cog and ADCS-ADL in mild patients.

The benefit seen with LMTM monotherapy at doses of 4, 75 and 125 mg b.i.d. is comparable to that seen on the ADAS-cog scale at 12 months in the phase 2 study where MTC was also given as monotherapy at 47 mg MT t.i.d. (Wischik et al., 2015). We have

recently reported that the absorption and distribution of MT to the brain is complex, and likely to be mediated via red cells rather than plasma, (Baddeley et al., 2015) providing a route which protects MT from first-pass metabolism. In the same study MT uptake into red cells was approximately 20-fold higher *in vivo* when as administered intravenously as LMTM compared with MTC, most likely due to direct red cell uptake of LMT by passive diffusion without need for prior reduction of MT^+ as is the case for MTC (Baddeley et al., 2015; May et al., 2004). The results of the present study suggest that MT uptake and distribution are capacity-limited by the amount that red cells can take up whilst within the portal circulation.

The reason for the loss of benefit on clinical and volumetric outcomes when LMTM is combined with symptomatic AD treatments remains to be explained, and studies are ongoing which aim to understand this better. To date, an interference at the site of action at the high affinity tau-tau binding site has been ruled out *in vitro* in both cell-free and cellular assays (Harrington et al., 2015). Likewise a direct effect on dissolution of LMTM tablets or complexing of LMTM with the AD medications or their excipients has been ruled out (unpublished data). The interference does not occur in certain other neurodegenerative disorders of protein aggregation (unpublished data), implying that the interference effect shown in AD is not applicable to all MT treatments of neurodegenerative disorders and may indeed be disease-specific. One possible contributory factor may be induction of the multidrug resistance protein 1 (MDR1), a transporter which is upregulated by AChEIs and memantine (Mohamed et al., 2015, 2016). We have shown that MT is a pH-dependent substrate for this pathway (unpublished data). The net effect could be enhanced efflux of MT from the brain, enhanced liver uptake leading to conjugation and inactivation of MT and faecal excretion, and also enhanced excretion of MT via the kidney. This may lower the concentration of MT at the site of action below a critical level required for efficacy in AD. Further studies to confirm this or other hypotheses are in progress.

The overall safety of LMTM as monotherapy is consistent with prior experience with MTC (Wischik et al., 2015). Adverse events affecting the gastrointestinal and urinary systems were the most common, and were also the most common reason for discontinuing high dose LMTM. Reporting of reductions in red cell indices was greater in patients receiving higher doses of LMTM, consistent with effects previously described for MTC, (Baddeley et al., 2015) although there was no significant reduction in haemoglobin at the 4 mg b.i.d. dose. Although 22% of patients were taking SSRIs, only 2 patients had transient symptoms meeting any of the criteria for serotonin toxicity, but neither of these was taking an SSRI (or any other serotonergic drug). Out of the 9 deaths that occurred during the study, none was related to treatment. Eight developed ARIA during the study (<1%) but there was no dose relationship. This frequency is consistent with the placebo rates reported in recent trials (Doody et al., 2014).

In conclusion, the results herein demonstrate the potential benefits of adding a tau-based approach to those currently available or planned for the treatment of diseases such as mild and moderate AD. A dose of LMTM as low as 4 mg b.i.d. as monotherapy may be the optimal dose in mild AD, able to produce substantial clinical benefits whilst being well

tolerated and having fewer side effects than the higher doses. Such treatment would need to be introduced either prior to or following cessation of the currently available AD treatments, as the combination appears to eliminate benefit.

Example 4 – further phase 3 clinical trial in mild AD

Objectives

To examine the potential efficacy of LMTM as monotherapy in non-randomised observational cohort analyses as modified primary outcomes in an 18-months Phase 3 trial in mild AD.

Methods

Mild AD patients (n=800) were randomly assigned to 100 mg twice a day or 4 mg twice a day.

The Statistical Analysis Plan was revised in light of Example 3 (which completed earlier) prior to database lock and unblinding, to compare the 100 mg twice a day as monotherapy subgroup (n=79) versus 4 mg twice a day as randomised (n=396), and 4 mg twice a day as monotherapy (n=76) versus 4 mg twice a day as add-on therapy (n=297), with strong control of family-wise type I error.

Results

The revised analyses were statistically significant at the required threshold of $p < 0.025$ in both comparisons on the co-primary clinical efficacy endpoints (ADAS-cog and ADCS-ADL), MRI atrophy and glucose uptake. Whole brain atrophy progressed initially as expected for mild AD in both add-on and monotherapy groups, but diverged significantly after 9 months of treatment, with the final atrophy rate in monotherapy patients typical of normal elderly controls. Differences at baseline between monotherapy and add-on patients did not account for significant differences in favour of monotherapy. Treatment response to LMTM as add-on was inversely correlated with relative basal forebrain atrophy.

Conclusions

The as-randomised analyses of two Phase 3 trials using LMTM are described herein: the first in mild to moderate AD (Example 3) and the second in mild AD (this Example). Both studies were originally designed to compare higher doses of LMTM in the range 150 – 250 mg/day with a low dose of 4 mg twice a day intended as a urinary discolourant to maintain blinding. It was assumed that this low dose would be ineffective, since a dose of 69 mg MT/day as MTC was found to have minimal efficacy in the Phase 2 study.

Neither Phase 3 trial study showed any difference on primary or secondary outcomes between the high doses and 4 mg twice a day. In the first study (Example 3) treatment status with cholinesterase inhibitors and/or memantine was found to be a significant

covariate in the primary analysis model. Exploratory analyses showed that this was due to significantly lower rates of progression on clinical and brain atrophy endpoints in patients receiving any of the LMTM doses as monotherapy, including 4 mg twice a day, which did not appear to be explicable by cohort differences in severity at baseline.

The results of the Example 3 study raised the possibility LMTM might be most effective as a monotherapy and that the minimum effective dose might be substantially lower for LMTM than that previously expected for MTC (see e.g. WO2009/044127).

We therefore modified the primary analyses and treatment comparisons in the study described in this Example (prior to database lock and unblinding) to investigate whether the monotherapy differences could be confirmed as observational cohort comparisons defined as primary outcomes with strong control of family-wise type I error in the second independent study. The monotherapy cohort comparisons which were of particular interest in light of the earlier study were: (A) 100 mg twice a day a monotherapy compared with the controls as originally randomised, and (B) 4 mg twice a day as monotherapy compared with the same dose as add-on to standard AD treatments.

Both primary Comparisons A and B met the required statistical threshold of $p < 0.025$ for both co-primary clinical outcomes (ADAS-cog and ADCS-ADL), as well as for volumetric MRI and glucose uptake biomarker outcomes. Specifically, patients receiving LMTM as monotherapy at either of the two doses tested had consistently better outcomes than patients receiving the same doses as add-on to cholinesterase inhibitors and/or memantine.

This confirmation of the same pattern of results in this second, independent, study argues against either the present findings or those reported as *post hoc* findings from the earlier mild/moderate AD study of Example 3 being the result of chance in small subgroups, although the monotherapy subgroups remain small in the present study (155 or 20% in total in the mITT analyses).

It is also unlikely that the earlier findings of Example 3 are explicable by inclusion of non-western geographies, since the present study was conducted in north America, western Europe and Australia. A clinical placebo effect in patients coming into a trial setting after previously not receiving active treatment cannot explain the same pattern of results seen in both the MRI brain atrophy and ^{18}F-FDG-PET functional data as seen in the clinical data. A difference in withdrawal rates between patients taking or not taking standard AD treatments is also unlikely, since the overall retention rates over 18 months were similar in monotherapy (65%) and add-on (69%) treatment groups.

The pattern of atrophy at baseline in patients receiving LMTM as monotherapy was typical of mild AD and significantly different from a cohort of well characterised normal elderly controls. The annualised rate of whole brain atrophy in these patients over the first 6 months was also similar to that reported for mild AD and significantly different from normal elderly controls. Likewise glucose uptake in inferior temporal gyrus was comparable in monotherapy patients to that reported for mild AD and significantly

different from MCI or normal elderly controls. In addition to meeting clinical diagnostic criteria for mild AD, the baseline imaging data therefore confirm that the patients not prescribed cholinesterase inhibitors or memantine can be taken as typical of mild AD.

Patients not receiving standard AD treatments were somewhat less impaired at study entry on the ADAS-cog, ADCS-ADL, MMSE scales, as well as in ventricular, temporoparietal and hippocampal atrophy, and temporal lobe glucose uptake. It is therefore possible that this difference in severity at baseline might have accounted for significant differences in progression. However, baseline severity was included as an additive term in the primary analysis models and was therefore corrected for. We further tested whether baseline severity or other patient characteristics could explain differences in rate of progression by undertaking sensitivity analyses with additional rate-correction terms in the analysis model. If differences in baseline characteristics explain the differences seen over 18 months, then the significant differences in favour of LMTM as monotherapy would be expected to disappear when corrected for baseline effects, as they did in a similar analysis examining differences in rate of progression according to AD treatment status in patients with MMSE 20 – 26 in the currently available ADNI data set (unpublished observation). Rate-correction for differences in clinical severity at baseline, APO ε4 frequency, vascular pathology load, hippocampal atrophy, temporoparietal atrophy and glucose uptake in the temporal lobe did not eliminate the significant differences in favour of LMTM monotherapy for ADAS-cog, ADCS-ADL or lateral ventricular volume. We further examined whether the differences in favour of LMTM as monotherapy depend on inclusion of patients receiving a cholinesterase inhibitor and memantine in combination, which could be taken to reflect a potential prescriber perception of risk of more rapid decline. Removing them had minimal effect on the estimates or significance of the treatment differences. It therefore appears unlikely that the relatively minor differences in severity or the other characteristics at baseline explain the significant outcome differences in favour of LMTM monotherapy over 18 months.

An analysis that is free of between-cohort confounding effects is the within-cohort comparison of annualised rate of whole brain atrophy at study entry and after 9 months of treatment with LMTM. We found that in patients receiving LMTM as monotherapy there was a significant delayed reduction in the annualised rate of whole brain atrophy. As noted above, monotherapy patients entered the study with an initial whole brain atrophy rate typical of mild AD and significantly different from that reported for normal elderly controls. After receiving LMTM as monotherapy for 9 months, the rate was reduced to that reported for normal elderly controls and significantly different from mild AD. These changes were not seen in the patients receiving LMTM as add-on therapy, who continued to decline as expected for mild AD. Similarly, the decline in temporal lobe glucose uptake in patients receiving LMTM as monotherapy was significantly less than reported for mild AD.

Decline on the ADAS-cog scale in patients receiving LMTM in combination with a cholinesterase inhibitor was found to vary inversely with atrophy in the nucleus basalis and nucleus accumbens relative to whole brain volume. A similar effect was also seen for cortical glucose uptake. The corresponding effect of basal forebrain atrophy was weak or

absent for the LMTM/memantine combination and was also absent for LMTM monotherapy. Both of these basal forebrain nuclei are known to be affected by tau aggregation pathology. The role of nucleus basalis in determining treatment response may help to provide some insight into a possible mechanism underlying the negative interaction with cholinesterase inhibitors. Ascending cholinergic projections originating predominantly from nucleus basalis provide both direct activation and indirect inhibitory modulation of cortical pyramidal cells (Huang M, Felix AR, Flood DG, Bhuvaneswaran C, Hilt D, Koenig G, Meltzer HY (2014) The novel α7 nicotinic acetylcholine receptor agonist EVP-6124 enhances dopamine, acetylcholine, and glutamate efflux in rat cortex and nucleus accumbens. *Psychopharmacology* **231**, 4541-4551.; Picciotto Marina R, Higley Michael J, Mineur Yann S (2012) Acetylcholine as a Neuromodulator: Cholinergic Signaling Shapes Nervous System Function and Behavior. *Neuron* **76**, 116-129). Memantine also increases release of acetyl choline in nucleus accumbens (Shearman E, Rossi S, Szasz B, Juranyi Z, Fallon S, Pomara N, Sershen H, Lajtha A (2006) Changes in cerebral neurotransmitters and metabolites induced by acute donepezil and memantine administrations: A microdialysis study. *Brain Res Bull* **69**, 204-213) which modulates cortical activity indirectly. Long-term inhibition of cholinesterase activity combined with loss of inhibitory modulation may therefore result in chronic hyperactivation of pyramidal cells in cortex (and in CA 1-3 of hippocampus) which are the principal sites of neurofibrillary degeneration in AD (Lewis DA, Campbell MJ, Terry RD, Morrison JH (1987) Laminar and regional distributions of neurofibrillary tantles and neuritic plaques in Alzheimer's disease: a quantitative study of visual and auditory cortices. *J Neurosci* **7**, 1799-1808). It is therefore possible that the relative severity of basal forebrain pathology together with chronic choline esterase inhibition may determine the degree of hyperactivation of cortical pyramidal cells and that this impairs the action of MT even at high dose. We show that whereas the treatment response to LMTM as add-on to approved treatments for AD varies according to the severity of relative basal forebrain atrophy, the effect of LMTM as monotherapy does not. The difference in treatment response between LMTM monotherapy and add-on therapy cannot therefore be attributed simply to cohort differences between patients prescribed or not prescribed such treatments. It also cannot be attributed to relative lack of pathology, since patients with the greatest basal forebrain atrophy responded significantly better to monotherapy than to combination treatment.

The potential for LMTM to be active at the low dose of 4 mg twice a day and the lack of dose-response are at first sight surprising given the results of an earlier Phase 2 placebo-controlled study using the oxidised form of the methylthioninium (MT) moiety as methylthioninium chloride (MTC) (Wischik, 2015). The stable reduced form of MT (as LMTM) was developed to overcome the absorption limitations observed for MTC. LMTM has 20-fold better red cell uptake than MTC *in vivo* and also better brain uptake. Recent studies in rodents dosed orally with LMTM to simulate the 4 mg twice a day dose in humans found brain levels of MT to be on 0.1 - 0.2 μM, similar to the steady state concentration estimated for the minimum effective dose of MTC. A concentration of approximately 0.05 μM appears to be adequate for a range of reported potentially beneficial effects of the MT moiety such as enhancement of autophagy and enhancement of mitochondrial function. The concentration required for dissolution of PHFs and

oligomers *in vitro* is approximately 1/10th that of aggregated tau, implying that a concentration of 0.05 μM may be adequate *in vivo*, given the brain concentrations of aggregated tau that have been reported. There is no dose-response for oligomer disaggregation *in vitro*, and higher doses of LMTM did not result in greater reduction in tau pathology in transgenic mouse models, at least in the range tested (Melis, 2015) Effects of oxidized and reduced forms of methylthioninium in two transgenic mouse tauopathy models. *Behav Pharmacol* **26**, 353-368). This suggests that there may be a critical threshold for activity at the tau aggregation inhibitor target, and the effect of higher doses may plateau or may even become negative at brain concentrations above 1 μM (Melis, 2015). Several results suggest that 4 mg twice a day may serve better than 100 mg twice a day. The clinical differences in favour of 4 mg twice a day were seen at both CDR 0.5 and 1.0, but only at CDR 0.5 at the higher dose, and the glucose uptake difference in temporal cortex occurred earlier at the lower dose.

The lower dose of 4 mg twice a day had a better overall clinical profile than 100 mg twice a day. The withdrawal rate over 18 months for the 4 mg twice a day dose was less (25%, 94/296) than at 100 mg twice a day (46%, 182/399), and the adverse event profile was more benign with respect to the diarrhoea, dysuria and decreased haemoglobin. There is no increased risk of cerebral microhaemorrhages or oedema with LMTM even at the higher dose, since the ARIA rates observed in both Phase 3 studies reported herein were similar to those previously reported for placebo controls (Doody, 2014; Salloway, 2014).

The differences in favour of LMTM as monotherapy are based on observational cohort analyses, albeit defined *a priori* as statistically primary outcomes for the modified analysis we report here. This pattern of results has been seen now in both separate Phase 3 studies, implying that the effects are consistent across studies. The differences favouring monotherapy are also internally consistent across a range of clinical outcomes, and the clinical outcomes are consistent with the neuroimaging outcomes in both studies.

Allowing for differences in absorption between LMTM and MTC, the results are also consistent with the earlier Phase 2 placebo-controlled study supporting potential efficacy of the MT moiety as monotherapy, and underline the potential beneficial clinical and biological effects of LMTM as monotherapy at the safe and well-tolerated dose of around 4 mg twice a day.

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