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Short Communication

Tau Aggregation Inhibitor Therapy: An Exploratory Phase 2 Study in Mild or Moderate Alzheimer's Disease

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Abstract.
Background: As tau aggregation pathology correlates with clinical dementia in Alzheimer's disease (AD), a tau aggregation inhibitor (TAI) could have therapeutic utility. Methylthioninium (MT) acts as a selective TAI *in vitro* and reduces tau pathology in transgenic mouse models.
Objective: To determine the minimum safe and effective dose of MT required to prevent disease progression on clinical and functional molecular imaging outcomes.
Methods: An exploratory double-blind, randomized, placebo-controlled, dose-finding trial of MT (69, 138, and 228 mg/day) was conducted in 321 mild/moderate AD subjects. The primary outcome was change on the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) at 24 weeks relative to baseline severity. Effect of treatment on regional cerebral blood flow decline was determined in a sub-study in 135 subjects. After 24 weeks, subjects were re-consented to enter sequential 6- and 12-month blinded extension phases. Registered with ClinicalTrials.gov (NCT00515333).
Results: At 24 weeks, there were significant treatment benefits in two independent populations at the 138 mg/day dose: in moderate subjects on the ADAS-cog scale (treatment effect: −5.42 units, corrected $p = 0.047$) and two other clinical scales; in mild subjects on the more sensitive regional cerebral blood flow measure (treatment effect: 1.97%, corrected $p < 0.001$). With continued treatment for 50 weeks, benefit was seen on the ADAS-cog scale in both mild and moderate subjects. The delivery of the highest dose was impaired due to dose-dependent dissolution and absorption limitations.
Conclusion: The minimum safe and effective daily MT dose is 138 mg and suggests that further study of MT is warranted in AD.

Keywords: Alzheimer's disease, controlled clinical trial, intervention studies, methylthioninium, safety, tau protein

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INTRODUCTION

Neurofibrillary tangles discovered by Alois Alzheimer [1] are made up of paired helical filaments (PHFs), composed predominantly of a 12 kDa repeat-domain fragment of the microtubule-associated protein tau [2–4]. 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 (AD) [5–8]. In light of the repeated failures of trials targeting the amyloid-β pathway in mild or moderate AD [9], there is increasing interest in the possibility that a tau aggregation inhibitor (TAI) could have therapeutic utility in AD [9–11].

Methylthioninium (MT) is a diaminophenothiazine and, as the chloride salt of oxidized MT^+ (MTC, methylthioninium chloride, "methylene blue"), has a long history of safe use, both as an approved treatment for methemoglobinemia [12], and experimentally in urolithiasis and bipolar disorder among others [13–15]. MT inhibits tau aggregation *in vitro* and dissolves PHFs from AD brain tissue [16]. We have recently reported that the estimated steady state trough brain concentration of MT and its pharmacologically active desmethyl metabolites in the human brain at the 138 mg/day dose is 0.18 μM [17]. This is in the same range as the IC_{50} value for dissolution of PHFs (0.16 μM) and the calculated intracellular Ki for TAI activity (0.12 μM) (Harrington et al., unpublished results). We have also reported elsewhere that MT produces a monotonic ascending dose-response in the brain concentration range 0.13–1.38 μM in tau transgenic mouse models (Melis et al., Behav. Pharmacol., in press).

We report the results of the first phase 2 clinical trial of a TAI in 321 subjects with mild/moderate AD. The trial was designed as an exploratory double-blind, randomized, placebo-controlled, 24 week dose-finding study of MT as monotherapy in AD (Fig. 1). Treatment could be extended in a blinded fashion in two sequential extension phases. In addition, a nested hexamethyl-propyl-amine-oxime single photon emission computed tomography (HMPAO-SPECT) sub-study was conducted in 135 of the participating subjects to explore whether treatment could prevent progression of molecular imaging deficits. This paper reports the primary and secondary outcomes at 24 weeks and a *post hoc* exploratory analysis at 50 weeks after the first extension of treatment. Although safety data are reported from the second extension phase, there were too few subjects remaining on treatment through to 102 weeks to permit meaningful comparison of treatment groups.

METHODS

Subjects

English-speaking subjects were enrolled at 17 centers (UK, 16; Singapore, 1) between 21 September 2004 and 21 December 2007. Subjects had a diagnosis of probable AD by DSM-IV and NINCDS-ADRDA criteria, a Mini-Mental State Examination (MMSE) score between 10 and 26 inclusive and a Clinical Dementia Rating (CDR) score of 1 or 2 at entry. To be included and retained in the trial, subjects were not to be currently treated with cholinesterase inhibitors or memantine. The full inclusion and exclusion criteria are provided in Supplementary Material S1 and S2, including competency requirements for consent to participate. Apolipoprotein E4 status was not determined as earlier work had shown that it does not impact on levels of aggregated tau in the AD brain [18].

Trial design

Subjects were randomized at baseline to placebo or one of three doses of MT (69 mg, 138 mg, and 228 mg/day, administered as 30 mg, 60 mg, and 100 mg MTC capsules taken three times per day with food) using blocks of size 10 (two per dose level). A separate randomization schedule was used for sites able to undertake HMPAO-SPECT scans (limiting randomization after 31 January 2005 to placebo, 138 mg/day, or 228 mg/day). The dose range was based on a theoretical pharmacokinetic model developed from published human and rat data [19, 20], and recommended dosing [21]. Following completion of 24 weeks, subjects were re-consented to enter sequential 6-month and 12-month extension phases ("E1" and "E2") with dosing changes indicated in Fig. 1. Dosing frequency remained the same throughout. In order to maintain blinding to the subject and raters, capsules and treatment packs were identical. Each MTC dose or placebo was a single dark-blue hard gelatin capsule and safety raters were not permitted to be efficacy raters. Alzheimer's Disease Cooperative Study Clinical Global Impression of Change (ADCS-CGIC) was independently assessed by a third rater blinded to the findings of the other two raters. The study remained double-blind throughout 2 years.

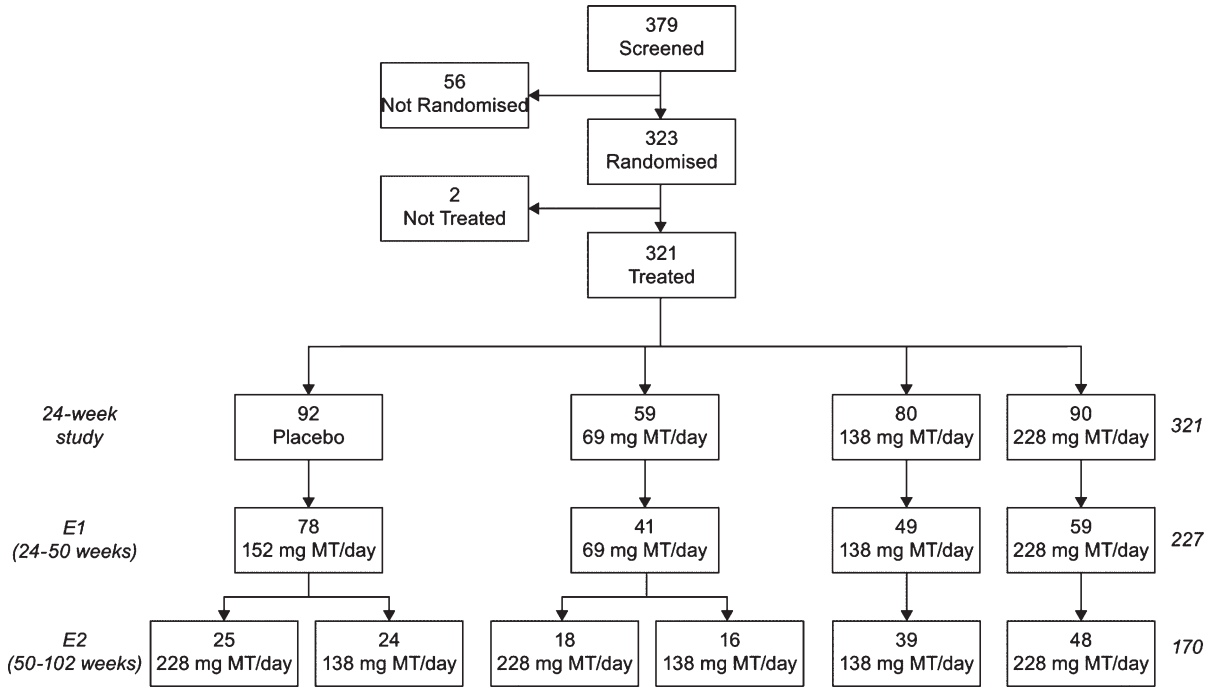

Fig. 1. Trial design.

The study was conducted in accordance with the Declaration of Helsinki and in compliance with the NHS Research Governance Framework, the International Conference on Harmonisation (ICH) Guidelines for Good Clinical Practice (GCP; CPMP/ICH/135/95, July 1996), and the European Directive on Clinical Trials (2004). The trial is registered at http://www.clinicaltrials.gov (NCT00515333).

Clinical and imaging assessments

The primary efficacy outcome was change in Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) from baseline at 24 weeks. Secondary efficacy outcomes included ADCS-CGIC and change in MMSE score. Other secondary rating instruments and their assessment times are listed in Supplementary Material S3.

Change in regional cerebral blood flow (rCBF) was assessed by HMPAO-SPECT scan (see Supplementary Material S4) in all subjects enrolled at nine participating UK study sites with this capability. Baseline SPECT scans (or scans available within the previous 3 months) were performed before randomization. A follow-up scan was performed at the end of the initial 24 week study between 18 and 30 weeks after the first scan. The images were assessed by an expert neuroradiologist (ADM) and classified according to presence or absence of temporoparietal lobe reduction in rCBF.

Subjects were monitored throughout for adverse events (AEs) and clinical laboratory testing was undertaken after 6, 12, and 24 weeks. Monitoring and laboratory testing continued during the extension phases.

Statistical methods

The statistical analysis plan was finalized 5 February 2007, and final data lock was on 20 February 2008. Since the study anticipated the theoretical possibility of non-response in moderate subjects due to more advanced pathology in medial temporal lobes [22], the statistical analysis plan specified that the primary analysis would assess the interaction between treatment and baseline severity as defined by baseline CDR (mild or moderate). The primary efficacy analysis of ADAS-cog change at 24 weeks was modelled as pre-specified using analysis of covariance, including covariates for treatment, baseline severity and a term for the interaction between treatment and severity. Additional terms for study center category (four levels), baseline ADAS-Cog score, age, gender, and previous exposure to acetylcholinesterase inhibitors or memantine were included. In the absence of prior

dose-response information, Westfall's method, which does not require an *a priori* dose-response assumption, was pre-specified to correct for multiple comparisons [23]. This was applied separately for subjects with mild and moderate disease, and the resultant p values were then further adjusted using the Bonferroni procedure (multiplied by 2), to provide correction for 6 treatment/severity levels, referred to in tables as "adjusted". The analysis was conducted using the modified intent-to-treat (mITT) population (ITT population who had at least one post-baseline, on-treatment efficacy assessment) with "last on-treatment observation carried forward" imputation for withdrawn subjects.

The clinical secondary outcome measures at 24 weeks were analyzed in the same way. ADCS-CGIC score was analyzed both as a dichotomous variable (declined versus same/improved) and also as a continuous variable. Analysis of MMSE was the same as for ADAS-cog. Mean change from baseline rCBF on HMPAO-SPECT was analyzed using region of interest (ROI) and statistical parametric mapping approaches [24]. For the ROI analysis, rCBF averaged across all brain regions was analyzed using linear modelling, with MT dose, a term for the interaction between treatment and severity, and temporoparietal lobe reduction pattern as fixed factors, and age and interval between scans as covariates. In addition, a statistical parametric mapping (SPM) approach was used to define the regions in which treatment response was seen without *a priori* brain mapping assumptions.

An exploratory analysis of ADAS-cog at 50 weeks was conducted using the same primary analysis model as for 24 weeks. Subjects randomized to placebo and then receiving a nominal dose of 152 mg/day during weeks 24–50 served as the control arm for assessment of treatment effect at 50 weeks.

Analysis of available dose

The experimental procedures and results of stability/dissolution studies of the capsules used in the trial undertaken at the time the trial was initiated are reported in Baddeley et al. [17]. Likewise, the procedures and results of single-dose fed/fasting studies of MTC and a novel stable reduced form of MT (LMTX®), and a repeated-dose study of MTC, are reported in Baddeley et al. [17]. The calculation of total available dose and dose according to release within or after 60 min and the corresponding dose-response analyses of ADAS-cog effect size at 24 weeks and effect on reduction in red cell count have been reported previously [17].

Role of the funding source

The study was financed entirely by TauRx Therapeutics Ltd. TauRx took the lead in study design and study conduct.

RESULTS

Subjects

Subject disposition is summarized in Fig. 1. Of 321 subjects randomized, 294 were in the mITT population; of these 238, 176, and 62 (81%, 60%, and 21%) remained on treatment at 24, 50, and 102 weeks, respectively. Baseline demographics and clinical characteristics are provided in Table 1. Overall, subjects were balanced with respect to severity of baseline disease based on MMSE. Of the 238 participants who completed the 24 week study, 135 (57%) were imaged twice (after a mean of 20.3 weeks of treatment) and had images suitable for analysis.

Primary analysis of ADAS-cog outcome at 24 weeks

Table 2 reports change in ADAS-cog score and difference with respect to placebo by treatment arm and baseline CDR-severity. The changes in ADAS-cog from baseline to 24 weeks in all subjects are plotted in Fig. 2A. The lower dose of 69 mg/day did not differ statistically from placebo. Dose-dependent formulation and absorption factors affecting the highest strength capsules [17] confound interpretation of the 228 mg/day nominal dose group on this and all other outcome measures and are discussed further below. In the absence of placebo decline in subjects with mild disease over 24 weeks, no treatment benefit with MT could be observed. Subjects with moderate disease declined by an average of 4.3 ADAS-cog units over 24 weeks. The 138 mg MT/day dose was effective in preventing clinical decline and the treatment effect was statistically significant (Table 2; $p = 0.008$, corrected $p = 0.047$). Therefore, the conclusion of the pre-specified primary efficacy analysis in the mITT population is that treatment at 138 mg/day is effective.

Analyses of secondary clinical outcomes at 24 weeks

The plots of change in ADCS-CGIC and MMSE from baseline to 24 weeks are provided in Fig. 2B and C. Both analyses showed a statistically significant treatment

Table 1
Subject baseline demographics and clinical characteristics

Characteristic	Placebo ($n=92$)	69 mg/day ($n=59$)	138 mg/day ($n=80$)	228 mg/day ($n=90$)	Total ($n=321$)
Age (years):					
Mean (SD)	74.6 (8.1)	73.4 (8.7)	73.8 (9.9)	73.3 (9.4)	73.8 (9.0)
Gender:					
Male, *n* (%)	47 (51%)	26 (44%)	33 (41%)	43 (48%)	149 (46%)
Female, *n* (%)	45 (49%)	33 (56%)	47 (59%)	47 (52%)	172 (54%)
Race:					
Afro-Caribbean, *n* (%)	1 (1%)	0 (0%)	0 (0%)	0 (0%)	1 (0%)
Asian, *n* (%)	8 (9%)	1 (2%)	8 (10%)	6 (7%)	23 (7%)
Caucasian, *n* (%)	83 (90%)	58 (98%)	72 (90%)	84 (93%)	297 (93%)
Years since diagnosis:					
Mean (SD)	0.86 (1.18)	1.00 (1.47)	0.89 (1.46)	0.85 (1.11)	0.89 (1.29)
Dementia severity:					
CDR mild, *n* (%)	72 (78%)	41 (69%)	63 (79%)	76 (84%)	252 (79%)
CDR moderate, *n* (%)	20 (22%)	18 (31%)	17 (21%)	14 (16%)	69 (21%)
MMSE:					
Mean (SD)	19.6 (4.6)	18.5 (4.7)	19.7 (4.6)	19.4 (4.6)	19.4 (4.6)
MMSE severity:					
MMSE<20, *n* (%)	44 (47.8)	28 (47.5)	36 (45.1)	42 (46.7)	150 (46.7)
MMSE $\geq$ 20, *n* (%)	48 (52.2)	31 (52.5)	44 (54.9)	48 (53.3)	171 (53.3)
ADAS-Cog:					
Mean (SD)	24.5 (10.4)	26.9 (10.5)	23.3 (8.9)	24.7 (11.0)	24.6 (10.3)

ADAS-Cog, Alzheimer's Disease Assessment Scale – Cognitive subscale; CDR, Clinical Dementia Rating; MMSE, Mini-Mental State Examination; SD, standard deviation.

Table 2
Change from baseline in ADAS-Cog score and MT treatment effect for subjects with mild and moderate disease severity at 24 weeks

	Change from baseline		Treatment effect		
	Estimate (95% CI)	*p*	Estimate (95% CI)	*p*	*p*-adj
MILD					
Placebo	−0.14 (−1.40, 1.11)	0.826			
69 mg/day	0.91 (−0.82, 2.63)	0.301	1.05 (−1.08, 3.17)	0·333	1
138 mg/day	−0.32 (−1.74, 1.10)	0.654	−0.18 (−2.06,1.69)	0.846	1
228 mg/day	−0.87 (−2.15, 0.40)	0.347	−0.73 (−2.51, 1.05)	0.419	1
MODERATE					
Placebo	4.35 (1.78, 6.91)	0.001			
69 mg/day	1.35 (−1.32, 4.01)	0.32	−3.00 (−6.47, 0.47)	0.09	0.325
138 mg/day	−1.08 (−4.36, 2.21)	0.52	−5.42 (−9.44, −1.41)	0.008	0.047
228 mg/day	3.95 (−0.64, 7.26)	0.019	−0.39 (−4.28, 3.50)	0.843	1

The column labelled "*p*-adj" provides the adjusted *p*-values after Westfall/Bonferroni correction for multiple comparisons with 6 dose/severity levels. Also shown are the change from baseline in each of the four study arms at each of two levels of baseline CDR-severity, and the *p*-value for testing if the change from baseline differs significantly from zero. CI, confidence interval.

effect at the 138 mg/day dose in moderate subjects. For the continuous analysis of ADCS-CGIC, the effect size was 1.29 units (95% CI = [0.43, 2.16]; $p = 0.0036$; corrected $p = 0.021$); in the categorical analysis, the effect size was 2.17 log-odds units (95% CI = [0.40, 4.32]; $p = 0.025$; corrected $p = 0.14$; odds-ratio=8.8). Similar results were found for change in MMSE score from baseline. The effect size was 3.79 units (95% CI = [1.16, 6.41]; $p = 0.0048$; corrected $p = 0.028$). No other secondary clinical outcome measures were significant. No change from baseline was observed on any scale in mild subjects receiving placebo.

Imaging outcomes at 24 weeks

Change in rCBF over approximately 24 weeks was analyzed as a secondary outcome in a subset of 135 subjects randomized to sites with SPECT imaging capability. Plots of change in mean rCBF for mild and moderate subjects are provided in Fig. 2D. The analysis of mean change in rCBF averaged across all ROIs is provided in Table 3. SPECT imaging was sufficiently sensitive to show significant placebo decline in mild subjects. There was significantly less decline in rCBF following treatment with MT 138 mg and

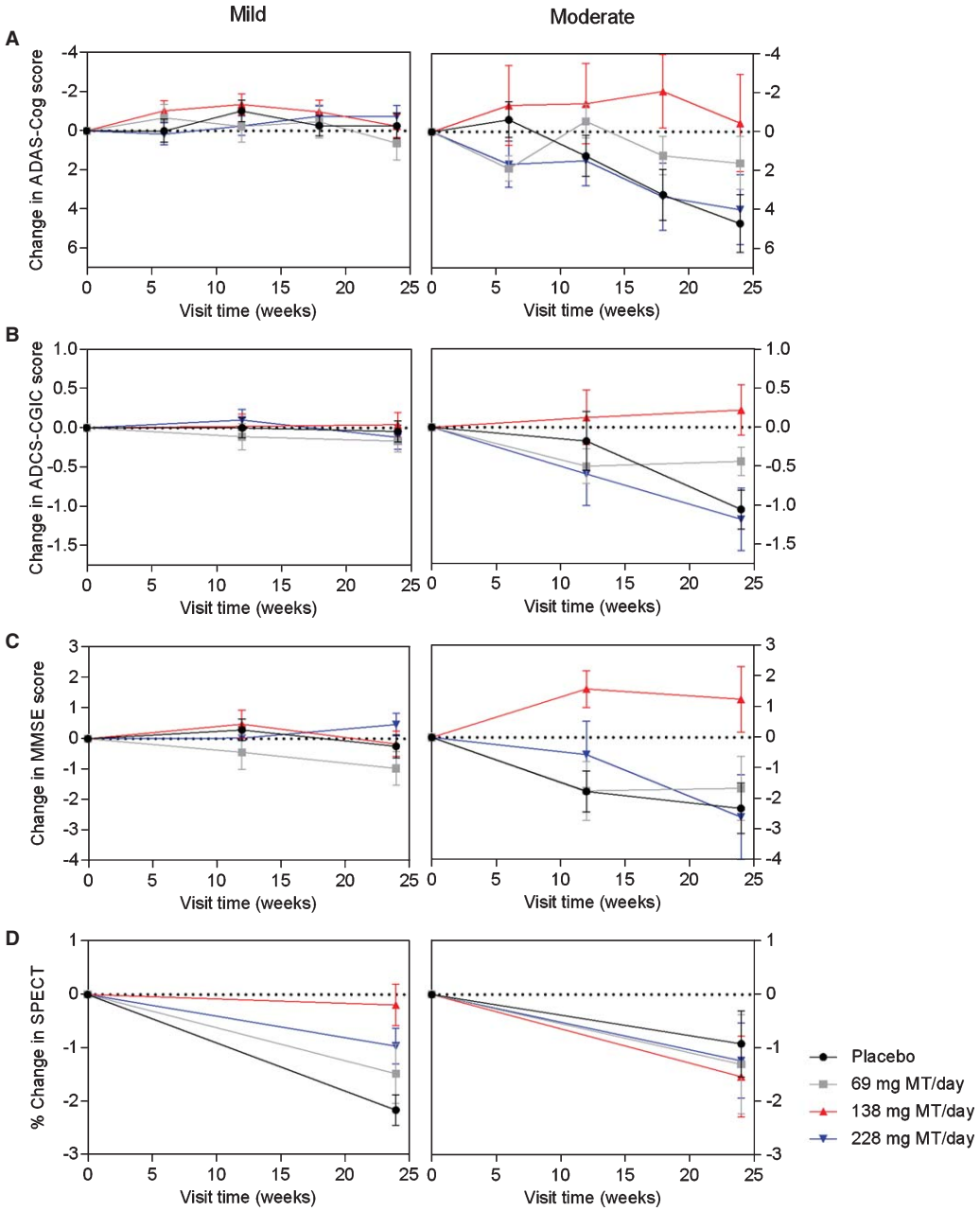

Fig. 2. Clinical and imaging outcomes at 24 weeks for mild and moderate AD.

228 mg/day nominal doses (corrected $p < 0.001$ and 0.031, respectively). The effect size at 138 mg/day was 91% of the decline in the corresponding placebo group. In moderate subjects, known to have more advanced perfusion deficits [25], the further placebo decline was non-significant and there was no evidence of treatment benefit.

In the analysis of rCBF change in individual ROIs (Fig. 3A), mild subjects receiving placebo had significant rCBF decline in all regions. At the 138 mg/day dose, all regions other than the left frontal lobe were significantly different from placebo at the $p < 0.05$ level. In the SPM analysis, the regions with statistically significant differences relative to placebo in subjects receiving any does of MT are illustrated in Fig. 3B, the differences being greatest bilaterally in the medial temporal and temporoparietal regions.

Table 3
Percentage change from baseline in relative cerebral blood flow (rCBF, normalized to cerebellum) and MTC treatment effect for subjects with mild and moderate disease severity after mean 20 weeks of treatment

	Change from baseline		Treatment effect		
	Estimate (95% CI)	*p*	Estimate (95% CI)	*p*	*p*-adj
MILD					
Placebo	−2.16 (−2.72, −1.61)	<0.001			
69 mg/day	−1.48 (−2.57, −0.39)	0.008	0.62 (−0.54, 1.91)	0.275	0.697
138 mg/day	−0.19 (−0.96, 0.57)	0.621	1.97 (1.02, 2.92)	<0.001	<0.001
228 mg/day	−0.96 (−1.62, 0.30)	0.004	1.12 (0.34, 2.06)	0.006	0.031
MODERATE					
Placebo	−0.92 (−2.14, 0.30)	0.138			
69 mg/day	−1.30 (−3.12, 0.52)	0.16	−0.38 (−2.59,1.82)	0.733	0.923
138 mg/day	−1.53 (−3.01, −0.05)	0.043	−0.61 (−2.51,1.29)	0.529	0.877
228 mg/day	−0.012 (−2.62, 0.14)	0.079	−0.32 (−2.17, 0.15)	0.737	0.923

p-adj, adjusted *p*-value after Westfall/Bonferroni correction for multiple comparisons with 6 dose/severity levels. CI, confidence interval.

Exploratory post hoc analysis of ADAS-cog change during first extension phase

With continued treatment for up to 50 weeks, decline was evident in subjects who entered the study with mild disease and were randomized to placebo (and then converted after 24 weeks to capsules with dose-dependent delivery limitations [17]). Patients randomized to MT 138 mg/day, whether mild or moderate at baseline, did not decline and there was a statistically significant treatment benefit of 2.8 to 5.2 ADAS-cog units in mild and moderate subjects, respectively (see Table 4). These effect sizes represent 96% and 91%, respectively, of the decline seen in the corresponding control arms.

The ADAS-cog effect sizes in mild subjects at 50 weeks reported in Table 4 were compared with imaging effect sizes observed in mild subjects at approximately 24 weeks reported in Table 3. As can be seen from Fig. 4, the 138 mg/day dose was the most effective dose in both modalities. The mean treatment benefit seen by SPECT scan at approximately 24 weeks in mild subjects was highly correlated with the mean clinical effect size measured on the ADAS-cog scale in mild subjects at 50 weeks ($r = 0.989$).

Safety

Table 5 provides an overview of treatment emergent AEs, during the placebo-controlled and E1/E2 periods, most of which were considered moderate or mild. The most commonly reported AEs (incidence $\geq 5\%$) in MTC-treated subjects included gastrointestinal disorders (primarily diarrhea), renal and urinary disorders (primarily dysuria and frequency), and falls (Table 6 and Supplementary Material S5). A larger proportion of subjects randomized to MTC experienced at least one AE leading to interruption of study drug or permanent discontinuation compared with those randomized to placebo by 24 weeks. The overall incidence of AEs and AEs requiring a change in dosing was greatest at the 138 mg/day dose.

Eight deaths were reported while participating in the study or shortly after discontinuing. Of the six deaths which occurred in MTC-treated subjects, one was from cancer and five were of a cardiovascular or hemorrhagic etiology in subjects with known risk factors; none were attributed to MTC.

No changes of clinical significance were observed in any routine clinical chemistry parameters in any treatment group. Treatment with MTC produced dose-dependent decreases in red cell count and hemoglobin and increases in methemoglobin (Fig. 5; Supplementary Material S6). There were 4 cases (of 307 exposed to MTC) with methemoglobin greater than 3.5% (a threshold set for withdrawal of treatment) which resolved on cessation of treatment. There were no cases of clinically significant anemia. Initial dose-dependent decreases in white-blood cell counts were also observed which did not progress beyond 24 weeks (Supplementary Material S7).

Post-hoc dose-response analyses

The response profiles with respect to nominal dose for the ADAS-cog effect at 24 weeks (Fig. 6A), the SPECT scan effect at approximately 24 weeks (Fig. 6B), the ADAS-cog effect at 50 weeks (Fig. 6 C), and the percentage of subjects with at least one AE leading to discontinuation or dose interruption at 24 weeks (Fig. 6D) show that the effects at the 228 mg/day nominal dose were consistently less than those at the 138 mg/day dose on all outcomes.

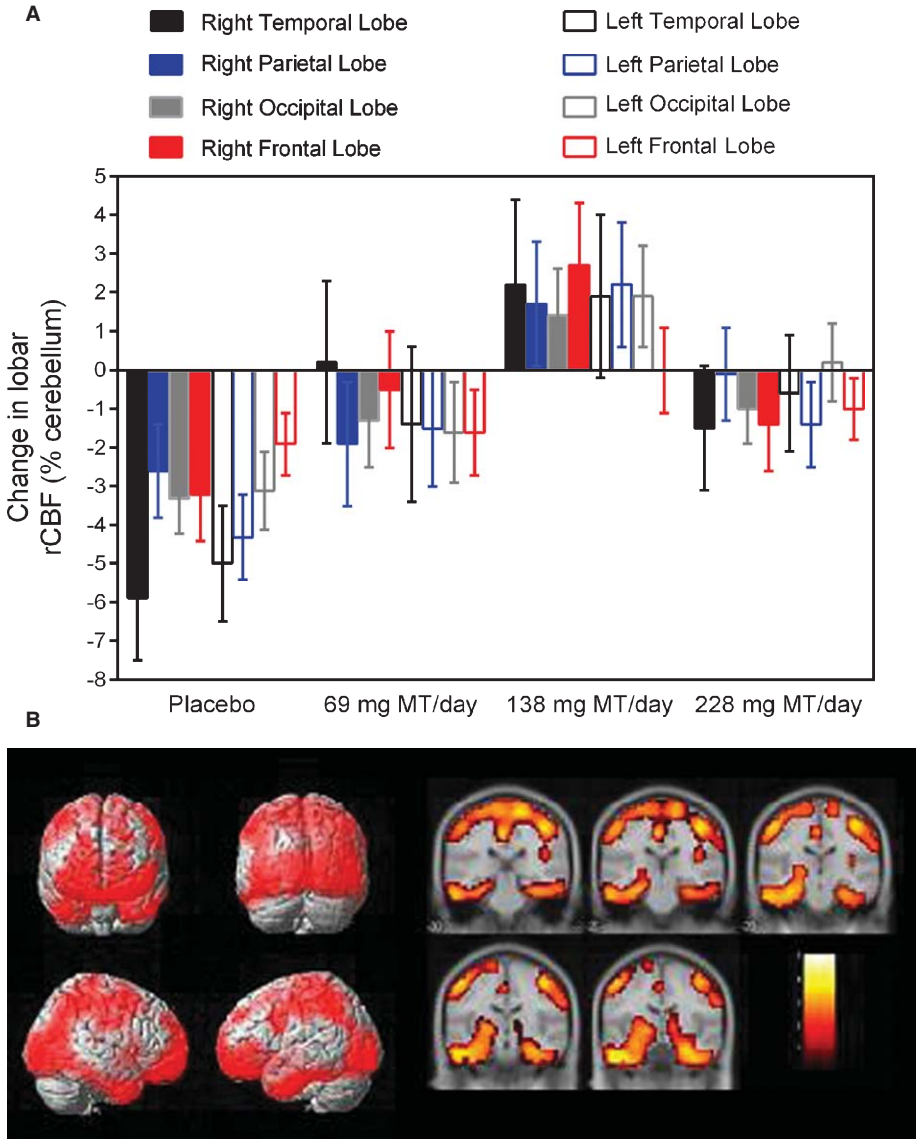

Fig. 3. Percentage change in rCBF SPECT adjusted mean values (normalized to the cerebellum) between baseline and follow-up in mild subjects. A) Differences with respect to placebo were significant at $p < 0.05$ for the 69 mg/day dose only in the right temporal lobe, for the 138 mg/day dose in all lobes except the left frontal lobe, and for the 228 mg/day dose in right and left temporal lobes and in left occipital lobe. B) Results of statistical parametric mapping comparison of change in blood flow between those receiving MTC versus placebo in mild AD subjects. The significant differences are in areas of the brain typically involved with neurofibrillary tangle pathology, i.e., treatment with MT has resulted in significantly less blood flow decline (or in some preservation of blood flow) in colored regions.

The 100 mg MTC gelatin capsule formulation used to deliver the 152 mg and 228 mg MT/day nominal doses was known, at the time that the study was initiated, to suffer from a dose-dependent dissolution limitation in water and simulated gastric fluid, although not in simulated intestinal fluid. In addition, a subsequent fed/fasting study showed that there was a dose-dependent limitation in the absorption of MT given as MTC with food, affecting particularly the 100 mg MTC unit dose that was independent of the formulation defect. Taking account of both of these factors, the calculated total dose of MT available for absorption is shown in Fig. 7A, comprising MT released within 60 min in water or simulated gastric fluid, or after 60 min and released in simulated intestinal fluid. The total available dose is highly correlated with the SPECT scan effect size at 24 weeks (Fig. 7B, $r = 0.974$), the ADAS-cog effect size at 50 weeks (Fig. 7 C, $r = 0.983$) and the percentage of subjects with at least one AE lead-

Table 4
Change from baseline in ADAS-Cog score and MT treatment effect for subjects with mild and moderate disease severity at 50 weeks

	Change from baseline		Treatment effect	
	Estimate (95% CI)	*p*	Estimate (95% CI)	*p*
MILD				
Placebo 152 mg/day	2.94 (1.53, 4.35)	<0.001		
69 mg/day	3.06 (1.11, 5.01)	0.002	0.12 (−2.28, 2.52)	0.921
138 mg/day	0.13 (−1.48, 1.73)	0.877	−2.81 (−4.93, −0.70)	0.009
228 mg/day	1.50 (0.06, 2.94)	0.041	−1.44 (−3.45, 0.57)	0.16
MODERATE				
Placebo 152 mg/day	5.51 (2.61, 8.40)	0.002		
69 mg/day	4.16 (1.15, 7.17)	0.007	−1.35 (−5.27, 2.57)	0.498
138 mg/day	0.32 (−3.39, 4.04)	0.864	−5.18 (−9.72, −0.65)	0.025
228 mg/day	4.08 (0.35, 7.82)	0.032	−1.42 (−5.81, 2.97)	0.524

CI, confidence interval.

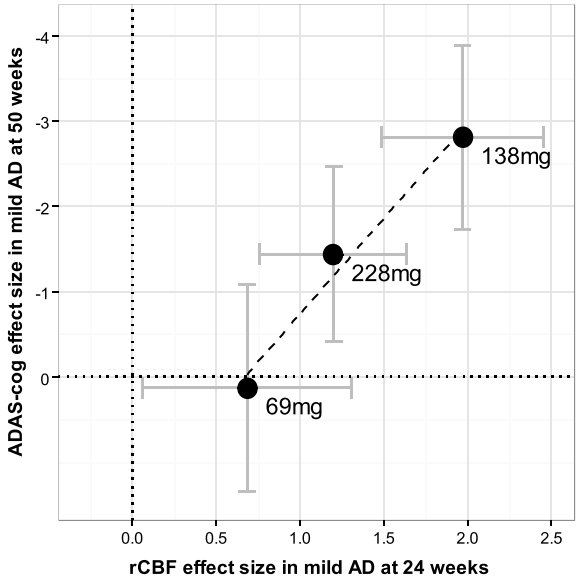

Fig. 4. Comparison of ADAS-cog effect size in mild AD at 50 weeks with the imaging effect sizes at approximately 24 weeks in mild AD.

Table 5
Summary of treatment-emergent adverse events (AEs)

	24 week study				E1	E2
	Placebo *n* = 92	69 mg *n* = 59	138 mg *n* = 80	228 mg *n* = 90	All MT *n* = 227	All MT *n* = 170
Total number of AEs	161	142	198	248	322	289
At least one AE, *n* (%)	61 (66.3)	43 (72.9)	67 (83.7)	70 (77.8)	139 (61.2)	96 (56.5)
At least one AE related to study drug, *n* (%)	22 (23.9)	30 (50.8)	48 (60.0)	53 (58.9)	65 (28.6)	36 (21.2)
At least one severe AE, *n* (%)	4 (4.3)	2 (3.4)	8 (10.0)	8 (8.9)	10 (4.4)	14 (8.2)
At least one AE leading to discontinuation/interruption of study drug, *n* (%)	7 (7.6)	12 (20.3)	34 (42.5)	26 (28.9)	36 (15.9)	23 (13.5)
AEs resulting in permanent discontinuation, *n* (%)	4 (4.3)	6 (10.2)	28 (35.0)	21 (23.3)	20 (8.8)	16 (9.4)
Treatment emergent severe AE, *n* (%)	12 (13.0)	2 (3.4)	13 (16.3)	11 (12.2)	21 (9.3)	26 (15.3)

ing to discontinuation or dose interruption at 24 weeks (Fig. 7D, $r = 0.976$). The correlation between ADAS-cog effect at 24 weeks and total available dose was weaker ($r = 0.664$), but was better correlated with dose avail-

Table 6
Number (%) of subjects with at least one treatment-emergent adverse event (AE) with an incidence of ≥5% of subjects in any treatment group (placebo-controlled safety population)

	All MT ($n=307$)		MT Exposed ≥ 365 days
	All AE	Treatment-related AE	($n=151$)
Number of subjects with at least one AE	268 (87.3%)	184 (59.9%)	80 (52.9%)
Gastrointestinal disorders:			
Constipation	15 (4.9%)	4 (1.3%)	1 (0.7%)
Diarrhea	89 (29.0%)	79 (25.7%)	7 (4.6%)
Dyspepsia	14 (4.6%)	9 (2.9%)	1 (0.7%)
Nausea	20 (6.5%)	14 (4.6%)	4 (2.6%)
Vomiting	19 (6.2%)	14 (4.6%)	2 (1.3%)
Peripheral edema	14 (4.6%)	3 (1.0%)	2 (1.3%)
Infections and infestations:			
Lower respiratory tract infection	27 (8.8%)	1 (0.3%)	6 (4.0%)
Urinary tract infection	27 (8.8%)	3 (1.0%)	9 (6.0%)
Fall	29 (9.4%)	6 (2.0%)	5 (3.3%)
Dizziness	22 (7.2%)	8 (2.6%)	4 (2.6%)
Depression	13 (4.2%)	3 (1.0%)	2 (1.3%)
Renal and urinary disorders:			
Dysuria	21 (6.8%)	20 (6.5%)	1 (0.7%)
Micturition urgency	20 (6.5%)	20 (6.5%)	1 (0.7%)
Pollakiuria	40 (13.0%)	39 (12.7%)	3 (1.0%)
Urinary incontinence	18 (5.9%)	15 (4.9%)	1 (0.7%)
Rash	16 (5.2%)	6 (2.0%)	5 (3.3%)

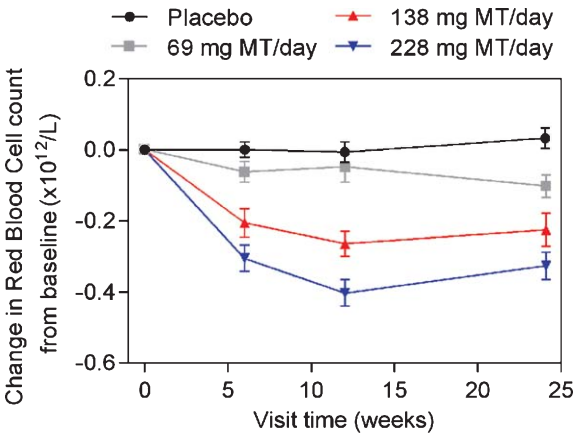

Fig. 5. Change in red blood cell count from baseline to 24 weeks by dose.

able for absorption within 60 min (Fig. 8A, $r=0.835$). Reduction in red blood cell count at 24 weeks was highly correlated with dose available for absorption after 60 min (Fig. 8B, $r=0.955$).

DISCUSSION

The primary aim of this study was to determine whether treatment with a TAI has potential utility in the treatment of AD and, if so, to determine the minimum safe and effective dose and likely treatment effect size for further investigation. The primary efficacy analysis at 24 weeks showed that treatment with MT 138 mg/day is the minimum effective dose required to prevent disease progression in subjects with baseline disease of moderate severity. Efficacy was confirmed in an independent population using more sensitive imaging measures in subjects with baseline mild disease. Specifically, in the pre-specified primary efficacy analysis model, treatment with 138 mg/day produced statistically significant benefit with respect to placebo on the ADAS-cog scale (corrected $p=0.047$) at 24 weeks in patients with moderate AD at baseline, even after correcting conservatively for multiple comparisons. Significant effects were also seen on the ADCS-CGIC and MMSE secondary outcome scales. A significant treatment effect was seen at the same dose on the overall rCBF imaging outcome in a separate population (mild subjects) at approximately 24 weeks after applying similar corrections. Similar conclusions are drawn if one uses a linear mixed effects analysis with an unstructured correlation matrix without imputation. In these analyses, used in current phase 3 clinical trials, the treatment effect at the 138 mg/day dose was significant in moderate subjects at 24 weeks ($p=0.023$) and in the combined mild/moderate population at 50 weeks ($p=0.0073$).

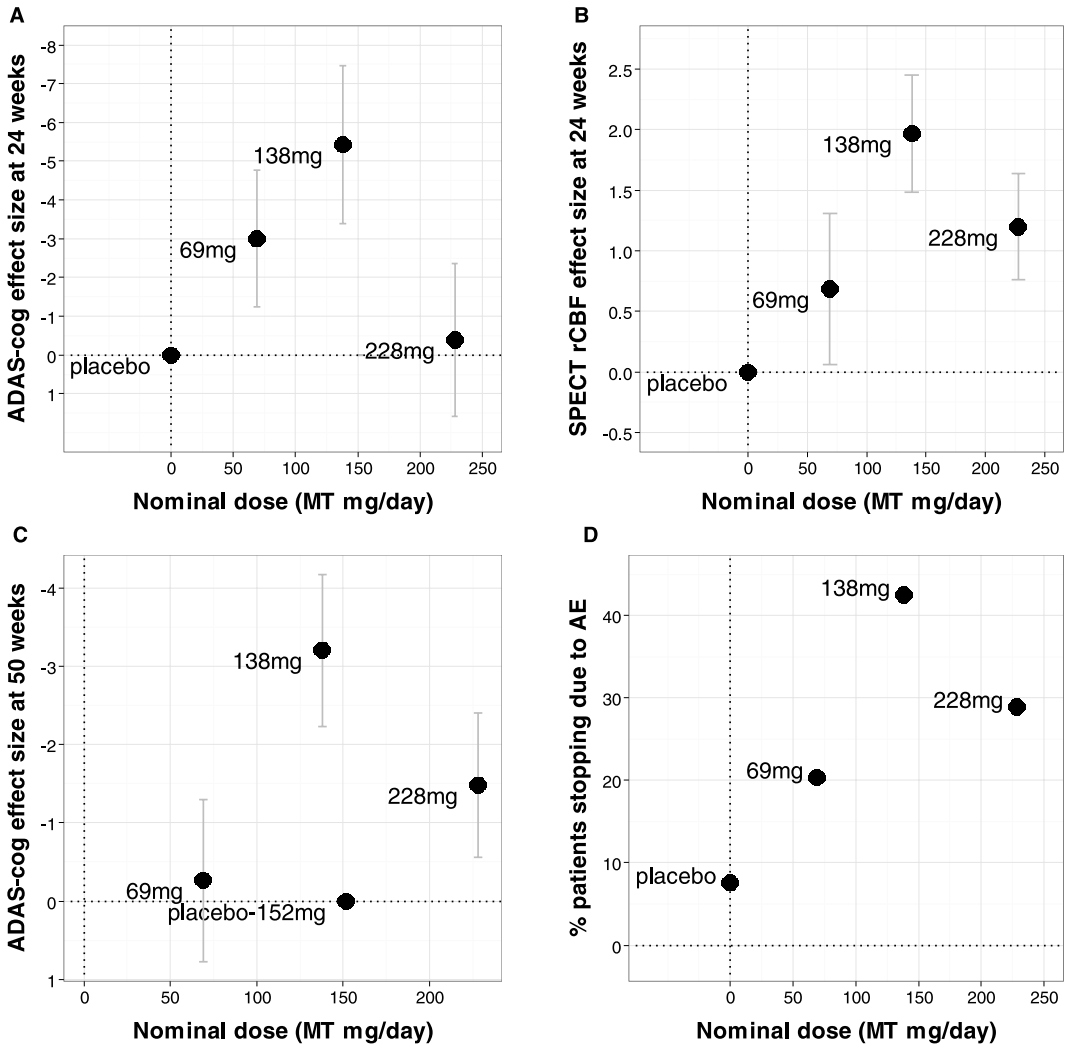

Fig. 6. There is no dose-response relationship for the 228 mg/day or the 152 mg/day doses of MT delivered via 100 mg MTC capsules for cognitive effects, SPECT rCBF effects or frequency of adverse events. A) ADAS-cog effect size at 24 weeks in moderate subjects versus nominal dose. B) SPECT rCBF effect size at 24 weeks in mild subjects versus nominal dose. C) ADAS-cog effect size at 50 weeks in all subjects versus nominal dose. D) Percentage of subjects with adverse events resulting in dose interruption or discontinuation versus nominal dose.

None of the clinical, imaging, or overall safety effects increased monotonically with respect to the nominal dose. In the absence of any prior knowledge of dose-effect, the Westfall correction for multiple comparisons was specified *a priori* because it does not assume monotonic dose-response and permits independent statements to be made about effectiveness at any dose/severity level compared to 5% for a test with family-wise type 1 error probability of 5%.

The failure of the 228 mg/day nominal dose of MT was entirely unexpected, and has taken a considerable body of work to understand (reported in [17]). In summary, MT is a redox molecule and, depending on environmental conditions (e.g., pH, oxygen, reducing agents), exists in equilibrium between a reduced (leuco-methylthioninium, LMT) and oxidized form (MT^+). As the chloride salt (MTC) used in the phase 2 trial, MT exists entirely in the MT^+ form in an oxygen atmosphere. Active conversion to the LMT form is required to permit absorption by passive diffusion [17, 26]. This conversion occurs optimally in the stomach, likely due to the pH dependence of the MT redox potential [27]. MTC is therefore a pro-drug, and the absorption and disposition of MT depend both on the fed/fasting status and on dissolution time of the formulation administered [17].

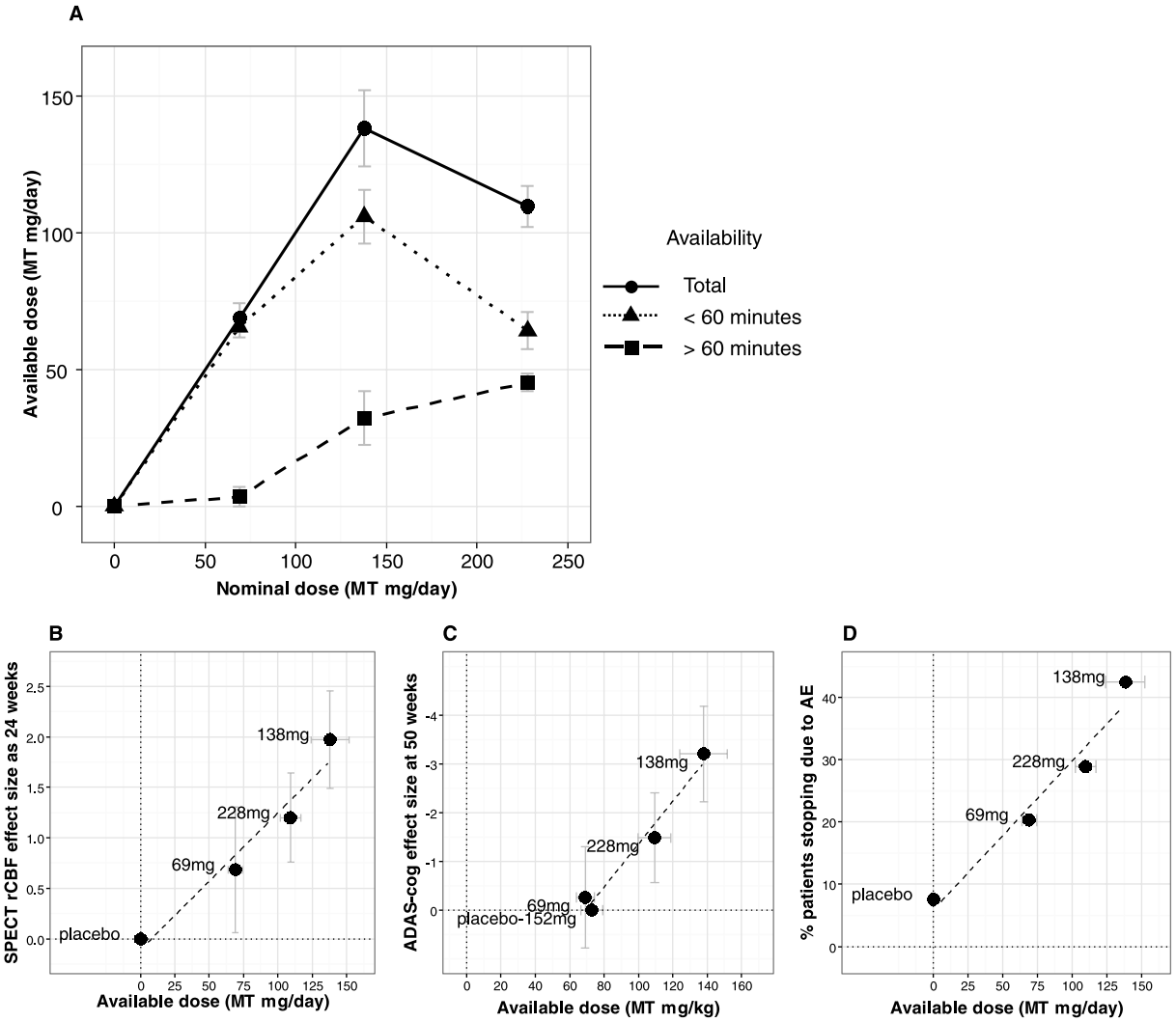

Fig. 7. There are simple dose-response relationships for calculated total dose which can be absorbed in the presence of food. A) Total available dose and dose available for absorption within 60 min or after 60 min. B) SPECT rCBF effect size at 24 weeks in mild subjects versus total available dose ($r = 0.974$). C) ADAS-cog effect size at 50 weeks in all subjects versus total available dose ($r = 0.983$). D) Percentage of subjects with at least one adverse event resulting in dose interruption or discontinuation versus total available dose ($r = 0.976$). [(A) is reproduced, with permission, from Baddeley et al. [17]].

MTC is poorly tolerated in the absence of food and is subject to dose-dependent absorption interference when administered with food. This effect proved to be the predominant determinant of dose-response for the SPECT scan effect at 24 weeks, the ADAS-cog effect at 50 weeks, and the frequency of AEs leading to dose interruption/discontinuation. The dose-response relationships with respect to available dose are monotonic and linear with correlation coefficients for mean effects all greater than 0.97. Dissolution time within or after 60 min is a further contributory factor accounting for the ADAS-cog effect at 24 weeks ($r = 0.835$) and the hematological effect of MT ($r = 0.955$). This suggests a partial dissociation between cognitive and hematological effects that depends on where in the gut MT is available for release, presumably reflecting differential redox processing. It should be noted that there are also strong correlations between the dose available for absorption within 60 min and the SPECT scan effect at 24 weeks ($r = 0.947$), the ADAS-cog effect at 50 weeks ($r = 0.927$), and the frequency of AEs leading to dose interruption/discontinuation ($r = 0.953$). Of the nominal dose of 228 mg/day, only 109 mg/day was available for absorption with food. Therefore, the most effective dose of 138 mg/day simply represents the highest available dose tested in this study. In summary, the clinical,

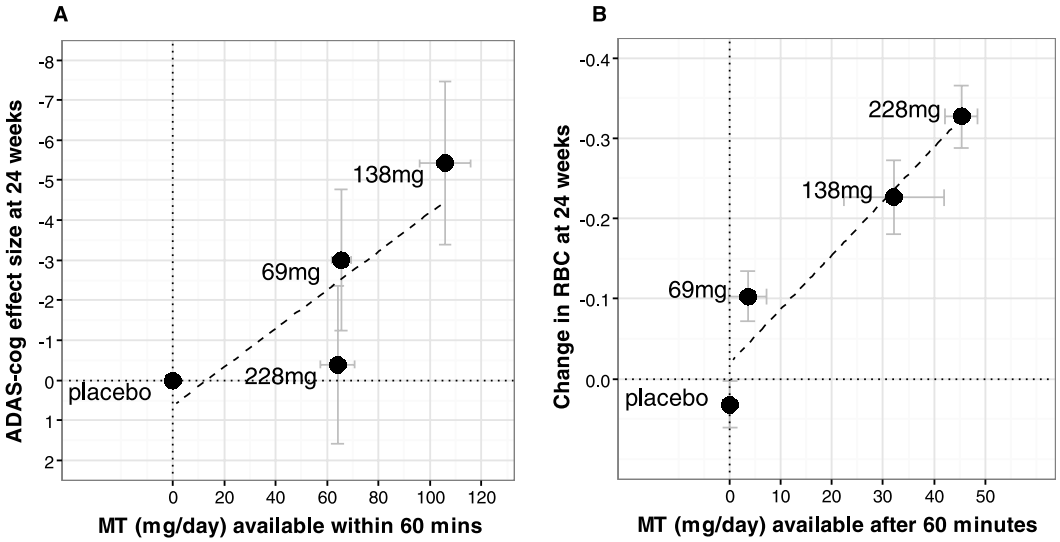

Fig. 8. Partial dissociation of cognitive and hematological effects at 24 weeks depends on dissolution time *in vitro*. A) ADAS-cog effect size at 24 weeks versus dose available for absorption within 60 min ($r = 0.835$). B) Decline in RBC count ($x10^{12}$ / L) from baseline versus dose available for absorption after 60 min ($r = 0.955$).

imaging, and safety effects of MT are highly determined by a combination of redox processing in the gut and ability to absorb MT in the presence of food when given in the oxidized MT^{+} form as MTC.

The complex factors affecting absorption and disposition of MT redox forms were entirely unknown at the time the study was designed, or for the last 100 years that MTC has been in clinical use. However, the unexpected dose-dependent limitations in the absorption of the 100 mg MTC capsule made the blinded extension phase of the study to 52 weeks more informative than would otherwise have been the case. During weeks 24–50, subjects originally randomized to placebo received a nominal dose of 152 mg MT/day administered as 100 mg MTC capsules given twice daily and an additional placebo capsule to maintain the blind. The intention was to obtain preliminary information regarding twice daily dosing using the highest dose capsules. Since these were particularly affected by absorption and dissolution limitations, only 73 mg/day of the 152 mg/day nominal dose administered during weeks 24–50 was available for absorption with food. Therefore, subjects originally randomized to placebo during the first 24 weeks and progressing to the 152 mg/day nominal dose during weeks 24–50 provided a relatively inactive control arm through to 50 weeks which permitted informative *post-hoc* comparisons to be made with respect to subjects randomized to active doses who continued with their original randomized doses for 50 weeks.

The placebo decline not seen in mild subjects at 24 weeks was 2.9 ADAS-cog units at 50 weeks in the control arm, comparable to the mean placebo decline (3.5 ± 1.8 units [mean $\pm$ S.E.]) seen in a meta-analysis of studies in mild AD subjects at 12 months [28–35] performed by two of the authors (CMW, DJW). By contrast, the mean decline seen in mild subjects receiving the 138 mg/day dose was close to zero, implying an effect size equivalent to 96% of the decline seen in the control arm and statistically significant ($p = 0.009$). Similarly, the effect size at this dose in moderate subjects was 94% of the decline seen in the control arm at 50 weeks ($p = 0.025$). This indicates that notwithstanding the differences in decline relative to severity seen in the control arm, the corresponding treatment effect sizes appear to represent a constant fraction of decline in the control arm, as would be expected for a disease-modifying treatment.

It is intriguing that the mean effect size seen by SPECT scan in the absence of apparent clinical benefit at 24 weeks in mild subjects was highly correlated with the mean clinical effect in mild subjects seen on the ADAS-cog scale at 50 weeks ($r = 0.989$). This suggests that the benefit seen early in the more sensitive functional molecular imaging modality could have potential utility as an early predictor of later clinical benefit. Although FDG-PET is more widely used for functional molecular imaging of the brain in the US, both HMPAO-SPECT and FDG-PET are non-specific tracers that show uptake proportionate to

cerebral blood flow and glucose uptake, respectively. As such, they are both indirect measures of normal brain metabolism. Their contribution to an imaging diagnosis in AD is the pattern of reduction in blood flow or glucose uptake in areas of the brain typically affected by tau aggregation pathology [36]. While both provide indirect measures of normal brain metabolic activity (i.e., oxygen and glucose consumption respectively), their ability to demonstrate deficits due to neuropathology is similar [36, 37]. The spatial resolution of PET is higher, but by using voxel-based image analysis methods (as was the case in the present study), both have comparable accuracy [38].

Deficits shown by HMPAO-SPECT in brain regions affected by tau aggregation pathology have been shown to correlate with tau aggregation pathology as measured by Braak stage [36]. The ROI and SPM analyses of rCBF decline provide a mapping of the regions of treatment benefit to those known to be particularly rich in tangles, notably medial temporal lobe structures and temporoparietal regions [39]. This supports the possibility that the clinical and imaging benefits of MT treatment at 138 mg/day may be due to MT activity as a TAI. Activity as a TAI is consistent with the estimated steady state trough brain concentration of MT at the 138 mg/day dose (0.18 μM) [17], the IC_{50} value for dissolution of PHFs (0.16 μM), the calculated intracellular Ki for TAI activity (0.12 μM) (Harrington et al., unpublished results), and the brain concentration range over which MT reverses tau pathology and behavioral deficits in two tau transgenic mouse models (Melis et al., Behav. Pharmacol., in press). We have recently reviewed alternative mechanisms of action of MT that have been proposed in the literature [9]. Most of these, however, are inconsistent with the concentrations of MT that could plausibly be achieved in the brain following oral dosing in humans.

A secondary aim of the study was to explore the safety and tolerability of MT in AD. The safety profile observed in this study is in keeping with previous reports of oral MTC use [13–15, 21]. Gastrointestinal and urological effects are the most common; these were also the most common reason for discontinuation. MTC is known to be associated with dose-related anemia. Effects seen in this study generally were not clinically significant.

There are other important limitations to this study in addition to the formulation and absorption limitations of the highest dose capsules. Although large by phase 2 standards, this exploratory study was designed as dose-finding, with the result that the number of subjects was small in the treatment arm of greatest interest in retrospect. The treatment benefit at 138 mg/day was seen in only 17 moderate subjects in the analysis of ADAS-cog at 24 weeks and in 25 mild subjects by HMPAO-SPECT, although there were 68 subjects in the *post-hoc* 50-week analysis. Nevertheless, the prespecified primary analysis at 24 weeks has taken these small numbers into account, and statistical significance was achieved only because of the large effect sizes observed. The effect sizes of −5.4 ADAS-cog units and 3.8 MMSE units are clinically meaningful at 24 weeks.

Another potential limitation is the lack of urinary discoloration in the placebo arm at 24 weeks. However, discoloration is known not to be proportional to MTC dose [19], and clinical procedures were implemented to minimize its potential impact on efficacy rating. It is unlikely that this consideration undermines the validity of the efficacy signals observed, as the 228 mg/day and 152 mg/day nominal doses delivered via 100 mg MTC capsules proved to be ineffective despite being absorbed and producing discoloration, hematological changes, and clinical AEs at 24 and 50 weeks. In particular, the treatment effect of the 138 mg/day dose at 50 weeks was determined relative to an available dose of 73 mg/day in the control arm. We have shown that a dose of 8 mg/day is sufficient to cause adequate discoloration of excreta to ensure blinding. Therefore the treatment effect observed at 50 weeks in the 138 mg/day group was not confounded by absence of urinary discoloration in the control arm. Most importantly, the molecular imaging findings provide independent biological confirmation at 24 weeks that the 138 mg/day dose is the most effective.

The benefits seen at 24 weeks were sustained to 50 weeks such that the overall decline in subjects randomized to this dose was not significantly different from zero (0.27 ± 0.74 ADAS-cog units [mean ± S.E.], $p = 0.714$). From our meta-analysis of previously reported studies, the decline expected in clinical trial populations of mild or moderate AD at 12 months is 4.4 ± 1.8 (mean ± S.E.). While the efficacy data at 50 weeks, being as they are exploratory and *post hoc*, cannot support an inferential claim in the same way that the 24 week data can, they are nevertheless informative as a basis for planning confirmatory phase 3 trials. The primary purpose of a phase 2 trial in a new treatment paradigm is to inform the design of confirmatory phase 3 trials by providing an estimate of the expected effect size. Notwithstanding the limitations of the 50-week data, they provide an empirical estimate of effect size at the minimal effective dose expected in a mild/moderate population at 12 months in a linear mixed effects unstructured correction matrix analy-

sis without imputatoin as approximately 90% ± 35% (mean ± S.E.) of the expected placebo decline as a basis for planning further studies.

The present study identified a dose of 138 mg/day as the minimum effective dose required to prevent decline on three clinical outcome measures and on rCBF decline at 24 weeks. The AEs seen at the 138 mg MT/day dose, which were predominantly mild and restricted to the gastrointestinal system and micturition, do not preclude further clinical development. While the results support further confirmatory studies with MT, these cannot be conducted using MTC because of absorption and longer term tolerability limitations. The absorption limitations at the highest nominal dose of 228 mg/day limited delivery to an available dose of 109 mg/day. Therefore, the present study did not permit determination of whether greater efficacy than seen at the 138 mg/day dose is achievable at a higher available dose without significant loss of tolerability or safety. Global phase 3 studies in mild and moderate AD are now fully recruited and will report in 2016. They investigate MT doses in the range 150–250 mg/day using a stable reduced form (LMTX®) that directly delivers LMT without need for active conversion from MT^+, is better tolerated, and does not suffer from the dose-dependent absorption limitations of MTC [17].

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SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-142874.

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