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Complex Disposition of Methylthioninium Redox Forms Determines Efficacy in Tau Aggregation Inhibitor Therapy for Alzheimer's Disease

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ABSTRACT

Methylthioninium (MT) is a tau aggregation inhibitor with therapeutic potential in Alzheimer's disease (AD). MT exists in equilibrium between reduced [leucomethylthioninium (LMT)] and oxidized (MT^+) forms; as a chloride salt [methylthioninium chloride (MTC), "methylene blue"], it is stabilized in its MT^+ form. Although the results of a phase 2 study of MTC in 321 mild/moderate AD subjects identified a 138-mg MT/day dose as the minimum effective dose on cognitive and imaging end points, further clinical development of MT was delayed pending resolution of the unexpected lack of efficacy of the 228-mg MT/day dose. We hypothesized that the failure of dose response may depend on differences known at the time in dissolution in simulated gastric and intestinal fluids of the 100-mg MTC capsules used to deliver the 228-mg dose and reflect previously unsuspected differences in redox processing of MT at different levels in the gut. The synthesis of a novel chemical entity, LMTX (providing LMT in a stable anhydrous crystalline form), has enabled a systematic comparison of the pharmacokinetic properties of MTC and LMTX in preclinical and clinical studies. The quantity of MT released in water or gastric fluid within 60 minutes proved in retrospect to be an important determinant of clinical efficacy. A further factor was a dose-dependent limitation in the ability to absorb MT in the presence of food when delivered in the MT^+ form as MTC. A model is presented to account for the complexity of MT absorption, which may have relevance for other similar redox molecules.

Introduction

Alzheimer's disease (AD) is an irreversible, neurodegenerative brain disease characterized by the formation of neurofibrillary tangles that were discovered by Alzheimer (1907). These are made up of pathologic paired helical filaments composed predominantly of a truncated 100-amino-acid fragment of the microtubule-associated protein tau (Wischik et al., 1988; Novak et al., 1993). Numerous studies have demonstrated correlations between tau pathology and the extent of clinical dementia and functional molecular imaging deficits (Wilcock et al., 1982; Arriagada et al., 1992; Bancher et al., 1993, 1996; Duyckaerts et al., 1997; Grober et al., 1999; Mukaetova-Ladinska et al., 2000; Chien et al., 2013; Maruyama et al., 2013; Okamura et al., 2014). There is increasing interest in the possibility of developing a tau-based approach to treatment of AD. Methylthioninium (MT) is one of a class of diaminophenothiazines that have activity as tau aggregation inhibitors (TAIs) in vitro without disrupting normal tau-tubulin interactions (Wischik et al., 1996).

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^+). As a chloride salt (commonly known as "methylene blue"), methylthioninium chloride (MTC) exists entirely in its oxidized form (MT^+) in an oxygen atmosphere. We recently reported a phase 2 clinical trial in 321 subjects in which potential efficacy of three doses of MT (69, 138, and 228 mg MT/day), given in the form of hard gelatin capsules suspended in Gelucire and dosed three times per day with food, was tested (C. Wischik et al., submitted manuscript). The 138-mg MT/day dose was identified as the minimum effective dose on cognitive and functional molecular imaging end points. Although these results were encouraging, further clinical development of MT was delayed pending resolution of the unexpected finding that the 228-mg MT/day dose had no or limited efficacy on the same end points.

The 100-mg MTC gelatin capsules used to deliver the 228-mg MT/day dose were known, at the time that the study was initiated, to suffer from a dose-dependent dissolution limitation in water and simulated gastric fluid. However, dissolution in simulated intestinal fluid (SIF) was achieved and met acceptance specifications over the 18-month shelf-life

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ABBREVIATIONS: AD, Alzheimer's disease; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; CNS, central nervous system; $LCMS^n$, liquid chromatography/ion trap mass spectrometry; LMT, leucomethylthioninium; MT, methylthioninium; MT^+, methylthioninium (oxidized); MTC, methylthioninium chloride; RBC, red blood cell; rCBF, regional cerebral blood flow; SIF, simulated intestinal fluid; SPECT, single photon emission computed tomography; TAI, tau aggregation inhibitor.

of the capsules. The study proceeded on the assumption that the implied difference in site of dissolution within the gut would not materially affect efficacy. We now report a post hoc analysis of the phase 2 efficacy data taking into account both the earlier dissolution data and a subsequent fed/fasting study. This showed that the calculated dose that was both released in solution in water within 60 minutes and able to be absorbed in the presence of food is predictive of clinical and imaging outcomes. Conversely, the remaining dose available for absorption after 60 minutes and able to be released in SIF is a predictor of the hematologic effects of MTC. These data suggest that there is a functional dissociation between the effects of MT in the central nervous system (CNS) and the periphery that depends on where in the gut MT is available for absorption.

In light of prior evidence using red cells as a model system that cellular absorption of MT delivered in the oxidized MT^+ form requires conversion to the reduced LMT form via a thiazine dye reductase activity (May et al., 2004), we hypothesized that the functional dissociation identified clinically could depend on previously unsuspected differences in redox processing of MT at different levels in the gut. We recently reported the synthesis of a novel chemical entity (LMTX) in which LMT is available in an anhydrous crystalline form as the dihydromesylate or the dihydrobromide that is stable in an oxygen atmosphere (Fig. 1; C. Harrington et al., submitted manuscript). This has made possible a systematic comparison of the pharmacokinetic properties of MTC and LMTX in preclinical and clinical study contexts using both native and ^{14}C-labeled versions of these molecules. We summarize the results of these studies and show how they support a general model that could explain the complexity of MT absorption and may have relevance for other similar redox molecules.

Materials and Methods

Phase 2 Study

The study design and results from an exploratory phase 2 study of MTC as a potential treatment of AD were reported elsewhere (C. Wischik et al., submitted manuscript). In summary, the study was conducted in 321 subjects in 16 centers in the United Kingdom and 1 in Singapore. The potential efficacy of three doses of MT was tested: 69 mg MT/day, 138 mg MT/day, and 228 mg MT/day, given in the form of hard gelatin capsules containing 30 mg, 60 mg, and 100 mg MTC, respectively, suspended in Gelucire dosed three times per day with food. The primary efficacy end point was Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) change at 24 weeks. In addition, change in regional cerebral blood flow (rCBF) was determined by hexa-methyl-propyl-amine-oxime single photon emission computed tomography (SPECT) scanning in a nested substudy in 135 subjects. After completion of the initial phase of the study, subjects were reconsented to enroll in blinded extension phases for up to 2 years. During the first 6-month extension (weeks 24–50), subjects originally randomized to placebo received 152 mg MT/day administered as 100-mg MTC capsules given twice daily and an additional placebo capsule to maintain the blind. Subjects randomized to active doses continued with their original randomized doses.

Dissolution of MTC Capsules

Capsules were formulated as a 25% MTC suspension with 75% Gelucire 44/14 manufactured by Encap Drug Delivery (Livingston, UK). Capsule strength was controlled by fill-weight of the final suspension injected at 55°C into gelatin capsules, which set hard upon cooling after capsule capping. The experiments reported here were performed at Encap Drug Delivery in 2004/2005. Dissolution was measured using the PhEur rotating paddle method (paddle speed 75 rpm, metal sinker) at 37°C ± 0.5°C. The amount of MTC dose dissolved over time for each capsule formulation (30 and 100 mg) was investigated using a spectrophotometric assay. Values for 60-mg capsules in water were calculated by interpolation. Capsules were placed in 1000 ml deionized water or SIF (1% pancreatin mix USP in water with potassium dihydrogen orthophosphate and adjusted to pH 6.8 ± 0.1 with 0.2 N sodium hydroxide or 0.2 N hydrochloric acid) with rotation, and 5-ml aliquots were collected for assay at 15, 30, 45, and 60 minutes. The absorbance of the sample aliquots, together with an MTC standard reference, was measured at 665 nm, and the amount of MT^+ in solution at each time point was calculated using the standard curve. Six replicates at each time point up to 60 minutes after storage for 0, 1, 3, 6, and 9 months at 25°C ± 2°C/60% ± 5% relative humidity were assayed for dissolution in water and SIF. Similar data were available for simulated gastric fluid at 0 and 1 month.

Red Blood Cells: In Vitro MT Absorption

Experiments were conducted at Quotient Bioresearch (Rushden, UK). [^{14}C]MTC or [^{14}C]LMTX (7 μM; 1988 ng/ml) was spiked into minipig or human whole blood. Samples ($n = 2$) were incubated at 37°C for 0, 5, 15, 30, or 60 minutes on a rotary mixer. After incubation, aliquots of blood were analyzed for radioactivity with liquid scintillation counting and hematocrit was measured. The remaining blood sample was centrifuged to generate plasma. Aliquots of plasma were analyzed for radioactivity and the blood/plasma ratio was determined. The radioactivity associated with red blood cells (RBCs) (%) was calculated as follows:

$$\text{Radioactivity}(\%) = 100 - \text{partition coefficient},$$

where the partition coefficient = $(C_p \times ([1 - H])/C_b) \times 100$; C_p = plasma radioactivity concentration; C_b = blood radioactivity concentration; and H = hematocrit (packed cell volume).

Red Blood Cells: In Vivo MT Absorption

Göttingen female minipigs were dosed intravenously at Covance Laboratories (Harrogate, UK) with [^{14}C]MTC (5 mg MT/kg; 0.634 MBq/mg; $n = 2$) or [^{14}C]LMTX (5 mg MT/kg; 0.0191 MBq/mg; $n = 2$) via a surgically inserted central catheter. At 5, 15, 30, and 120 minutes, blood samples were collected and whole blood radioactivity levels were determined by liquid scintillation counting, and hematocrit was measured. Plasma was prepared from the remaining blood

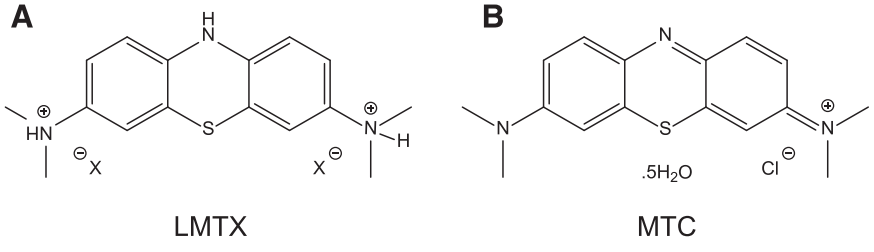

Fig. 1. Chemical structures of MTC and LMTX. For LMTX, X was bromide or methane sulfonate.

sample via centrifugation at 4°C for 10 minutes and radioactivity levels were determined. RBCs were retained and washed twice with 0.9% NaCl, and radioactivity levels were measured. No lysing of RBCs was noted due to this washing. Experimental analysis was conducted at Covance Laboratories.

Göttingen minipigs were treated according to established guidelines, and all experiments were approved by the institutional ethical committee.

Plasma: In Vivo MT Absorption

Dosing and experimental analyses were conducted at Covance Laboratories. Göttingen female minipigs received single oral doses of [^{14}C]MTC or [^{14}C]LMTX (30 mg MT/kg; n = 2/group) on two separate occasions, separated by a washout period of 3 days. After the first oral dose (0.567 and 0.115 MBq/mg, respectively), plasma samples were pooled by time point and added directly to liquid scintillant. After the second oral dose (1.36 and 0.116 MBq/mg, respectively), blood samples were collected after 2 hours only. Plasma was pooled, extracted with acetonitrile, and analyzed for both parent drug (MT) and metabolites by radio-liquid chromatography/ion trap mass spectrometry (LCMS^n) using a hybrid high-performance liquid chromatograph ion trap and time-of-flight mass spectrometer.

Brain Concentrations: In Vivo

Mice. Dosing and experimental analyses were conducted at TauRx Therapeutics Ltd. (Aberdeen, UK). Female Line 66 mice aged 8 to 9 months were orally administered MTC or LMTX once a day for 19 days. Doses were 3.7, 11, and 33 mg MT/kg (n = 12 to 13/group). The Line 66 mice are transgenic for human tau based on an NMRI background (V. Melis et al., submitted manuscript). Animals were euthanized 1 hour postdose on day 19, and brains were removed and frozen immediately. Brain samples were homogenized followed by addition of hydrochloric acid (1 M). Sodium hexanesulfonate and dichloroethane were then added and samples were mixed for 15 minutes. The dichloroethane layer was collected after centrifugation and evaporated under nitrogen. The resulting sample was dissolved in mobile phase and analyzed for total MT concentration by high-performance liquid chromatography with diode array detection at 660 nm. As a result of the acid treatment (Peter et al., 2000), this method measures total MT levels and does not differentiate between parent MT and acid-labile conjugate metabolite(s).

Experiments were carried out in accordance with the European Communities Council Directive (63/2010/EU) with local ethical approval, and a project license under the UK Scientific Procedures Act (1986).

Humans. An open-label, single- and multiple-dose MTC study was conducted at Cetero Research (Fargo, ND) in 12 healthy male and female Caucasian subjects (aged ≥55 years) who received a 69-mg MT dose three times a day (approximately every 8 hours) for a total daily dose of 207 mg MT for 14 consecutive days, followed by a final 69-mg MT dose on the morning of day 15. On days 1 and 15, blood samples were collected (after a 10-hour overnight fast) predose and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 8 hours postdose. Blood samples were also obtained prior to dosing on days 3, 7, 10, 12, 13, and 14 to determine plasma trough levels. Experimental analyses were conducted at Covance Laboratories (Madison, WI). Total MT levels [combined parent MT and conjugate metabolite(s)] in plasma were determined using a protein precipitation extraction method that used $CaCl_2$ followed by 1% trifluoroacetic acid in acetonitrile (Burhenne et al., 2008). To provide a robust analysis, samples were then heated to 70°C for 40 minutes, centrifuged to remove precipitated protein, and a solution of 2.5% formic acid in water was added to the supernatant prior to injection for analysis by liquid chromatography coupled to tandem mass spectrometry.

Food Effect Studies

A food effect MTC study was conducted in 12 healthy volunteers (aged 18–30 years) at Shandon Clinical Trials (Cork, Ireland), while the LMTX food study was conducted in 24 healthy elderly volunteers (aged 55–73 years) at Quotient Bioresearch (Ruddington, UK). A single dose of MTC or LMTX was administered. For the fasted cohorts, individuals were fasted prior to dosing for at least 10 hours in the MTC study and for at least 8 hours in the LMTX study. In the fed phase of the MTC study, subjects received a standardized breakfast (10% protein, 10% fat, 55% carbohydrate, approximately 382 kcal) 30 minutes prior to dosing. In the fed phase of the LMTX study, subjects received a high-fat (500–600 calories), high-calorie (1000 kcal) breakfast 30 minutes prior to dosing. In both studies, blood samples were collected predose and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, and 72 hours postdose. Plasma was prepared and stored at –20°C in the MTC study and at –70°C in the LMTX study until analysis. Experimental analyses were conducted at BioClin Laboratories (Athlone, Ireland).Total MT levels were determined as described for the multiple-dose MTC study above except for the heating step. Because of the acid treatment (Burhenne et al., 2008), this method does not differentiate between parent MT and acid-labile conjugate metabolite(s).

Both studies were conducted in accordance with local laws, the European Clinical Trials Directive (2001/20/EC), the ICH Guideline for Good Clinical Practice (CPMP/ICH/135/95, January 17, 1997), and the current Declaration of Helsinki.

Drugs

[^{14}C]MTC and [^{14}C]LMTX were synthesized at Almac (Craigavon, UK). MTC and LMTX were supplied by TauRx Therapeutics. All drugs in mice were dosed p.o. and administered at 5 ml/kg volume. All doses were administered as free base.

Results

Uptake of MT into RBCs Is Higher When Given as LMTX than as MTC In Vitro and In Vivo. We first confirmed whether redox state affects cellular uptake of MT into RBCs using whole blood from Göttingen minipigs or humans spiked with [^{14}C]LMTX or [^{14}C]MTC for 15 minutes at 37°C as a model system. RBC uptake of MT, as measured by radioactivity associated with RBCs, was greater after LMTX (mean ± S.E.M., 81% ± 2.2% and 95.3% ± 0.4% in minipig and human samples, respectively) than MTC (70% ± 0.5% and 59% ± 1.2% in minipig and human samples, respectively), with the difference already apparent at the nominal zero time point.

A much more marked differentiation between redox forms was observed in vivo after intravenous administration of [^{14}C]MTC and [^{14}C]LMTX to Göttingen minipigs. The uptake of

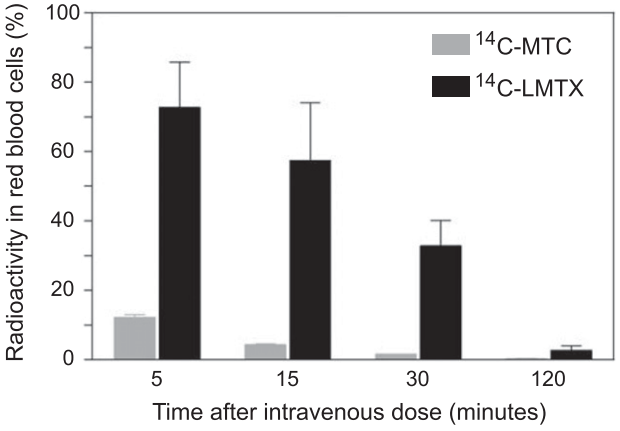

Fig. 2. Uptake of MT-related moieties into RBCs after intravenous administration of [^{14}C]MTC (5 mg MT/kg; n = 2) or [^{14}C]LMTX (5 mg MT/kg; n = 2).

MT-related moieties in RBCs was 6- to 21-fold higher in animals dosed with [^{14}C]LMTX versus [^{14}C]MTC over the first 2 hours of measurement, with the greatest relative difference measured at 30 minutes (21.4-fold ± 7.7-fold difference; Fig. 2). Over the first 30 minutes, the predominant form of radioactivity extracted from the RBCs was parent MT. By 2 hours postdose, MT was distributed into other body compartments, and increased amounts of metabolites had been formed.

In Vivo Plasma Parent MT Levels in Minipigs Are Three Times Greater for Oral LMTX than for MTC. After oral administration of [^{14}C]MTC and [^{14}C]LMTX to Gottingen minipigs, the total radioactivity levels in plasma were comparable regardless of redox form dosed (Fig. 3A). However, when plasma was extracted and profiled by radio-LCMS^n, the amount of this radioactivity that was in the form of parent MT was 3-fold greater at 2 hours for [^{14}C]LMTX (18.1%) than for [^{14}C]MTC (5.5%) (Fig. 3B). Importantly, the proportion of radioactivity extracted and profiled was similar between the [^{14}C]MTC and [^{14}C]LMTX groups (approximately 92 to 93%). The remaining non-MT radioactivity was composed of metabolites of MT. These results suggest that MT is more extensively metabolized when administered as oral MTC than after oral LMTX.

In Vivo Brain Levels of Total MT in Mouse Are Four Times Greater for Oral LMTX than for MTC at Low Doses. Brain concentrations of total MT in mouse brain were 3.9× greater for oral LMTX than for MTC dosed at 3.7 or 11 mg MT/day (Fig. 3C), although at the highest dose (33 mg MT/day) producing brain concentrations >4 μM, the brain concentrations became equivalent. The methodology used for extraction of total MT from mouse brain did not permit separate measurement of acid-labile MT metabolites.

In Vivo Oral MTC Achieves Minipig MT Brain Levels Sufficient to Inhibit Tau Aggregation In Vitro. Mean total radioactivity in brain homogenates obtained 2 hours after oral administration of [^{14}C]MTC (30 mg/kg) to Göttingen minipigs was 0.595 μg Eq/g, a level comparable to that found in pooled RBCs (0.618 μg Eq/g) at the same time. However, when samples were extracted and profiled by LCMS^n for identification of MT and its metabolites, twice as much radioactivity was in an extractable form in brain tissues compared with RBCs. A mean of 0.214 μg Eq/g was identified as MT and a further 0.073 μg Eq/g as desmethyl derivatives of MT. The latter have lost either one or two methyl groups but retain TAI pharmacologic activity similar to MT (Taniguchi et al., 2005). The dose administered in this study (21.4 mg/kg), after adjusting for body surface, is approximately 5-fold greater than the maximum human dose currently being tested in ongoing studies (250 mg MT/day, 4.2 mg/kg).

Similarities between the pharmacokinetics of MT in minipigs and humans (T. Baddeley et al., manuscript in preparation) allow estimates based on the minipig data to be made of the expected brain concentration of MT and its desmethyl derivatives at steady state in humans at the nominal dose of 138 mg MT/day in the phase 2 trial. This dose, given as 60-mg MTC capsules administered three times per day, is associated with an accumulation factor of 1.65 [based on comparison of day 1 and day 15 peak total MT plasma concentrations (C_{max}); Fig. 4A]. At this dose, the calculated steady-state trough concentration of parent MT and its active metabolites in the human brain is estimated to be 0.051 μg Eq/g (0.18 μM).

In Clinical Studies, Food Produces Dose-Dependent Reduction in Absorption of MT Delivered as MTC but not LMTX. A food effect study was conducted in healthy adult volunteers using rapid-release MTC and LMTX tablets to eliminate the dissolution delay identified with aged gelatin capsules. Food was found to impair absorption of the 100-mg MTC (75 mg MT) tablet dose (n = 6; reduced to 48% of the fasted level), but not the comparable 60-mg MTC (46 mg MT) tablet dose (n = 6). For a 100-mg dose of MT given as LMTX (n = 24) in healthy elderly volunteers, no effect of food on absorption was observed (Fig. 4B). Therefore, MT absorption is subject to dose-dependent food interference when dosed as MTC but not when dosed as LMTX.

In the same food effect studies, the median time to peak total plasma MT (T_{max}) under fasting conditions was rapid (1.5–1.75 hours) for both salt forms. For LMTX tablets administered with food, median T_{max} was delayed 1.5 hours (Table 1). For MTC tablets, the effect of food on T_{max} was less, delaying it by 0.5–0.75 hours.

Clinical Efficacy of MTC at 24 and 50 Weeks as a Function of Dissolution Impairment and Food Effect. In a capsule stability study undertaken at the commencement of the phase 2 study, drug dissolution was determined in water and SIF (Fig. 5). The data for water were similar to those for simulated gastric fluid up to 1 month, but the latter analyses were ceased after that time. A dose-dependent impairment of dissolution in water was observed and this deteriorated over storage time. This was found to be due in part to cross-linking of the gelatin capsule shells shown by

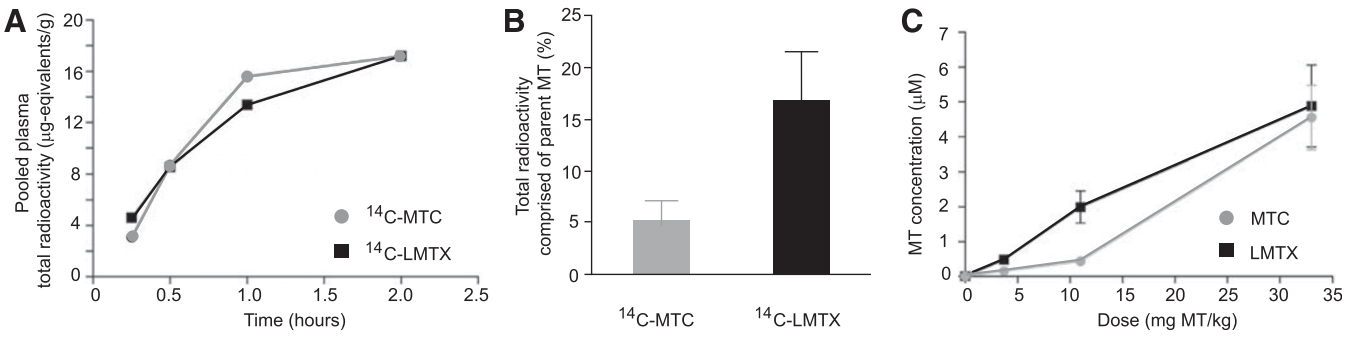

Fig. 3. (A) Pooled plasma total radioactivity after oral dosing of MT (30 mg/kg) as either [^{14}C]MTC or [^{14}C]LMTX in minipigs (plasma samples from two minipigs pooled prior to analysis). (B) Percentage of total plasma radioactivity composed of parent MT after oral dosing (30 mg/kg) of MT as either [^{14}C]MTC or [^{14}C]LMTX in minipigs (mean ± S.E.M.; n = 2). (C) Total MT concentration in brain after daily oral dosing of either MTC or LMTX in tau transgenic mice (mean ± S.E.M.; n = 12 to 13).

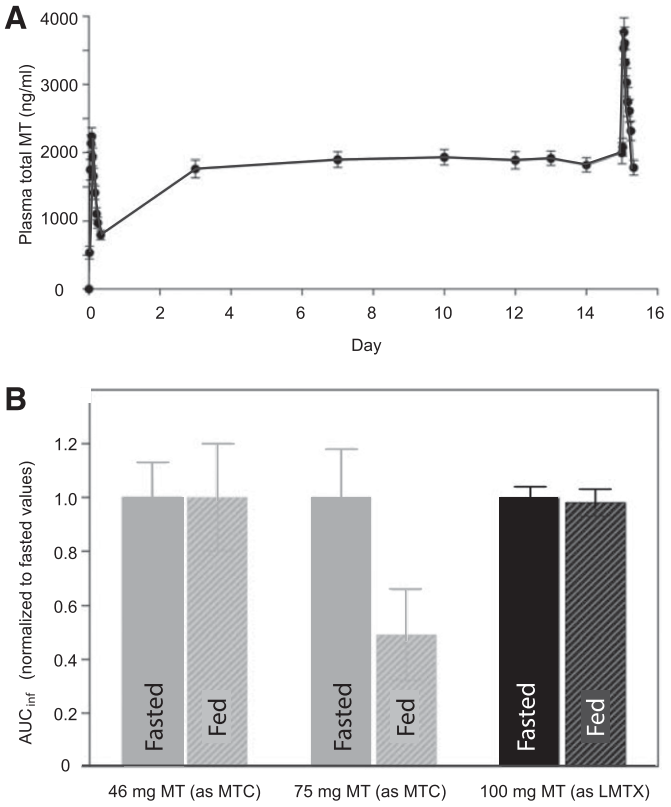

Fig. 4. (A) Plasma total MT levels in 12 healthy human volunteers after a single dose of 90 mg MTC (69 mg MT) on days 1 and 15, and morning predose plasma levels on intervening days (mean ± S.E.M.). On days 2–14, subjects received the 90-mg MTC (69 mg MT) dose three times per day for a total of 207 mg MT/day. Each MTC dose consisted of 3 × 30-mg MTC freshly manufactured gelatin capsules. (B) Plasma AUC_{inf} values for total MT calculated in food effect studies of single doses of either MTC (n = 6/dose) or LMTX (n = 12) in healthy human volunteers given 60 mg or 100 mg MTC (46 mg or 75 mg MT, respectively) or LMTX (100 mg MT) as rapid-release tablets either after fasting for 8–10 hours or after standard meals (mean ± S.E.M. normalized to fasting AUC_{inf}). AUC_{inf}, area under the concentration time-curves from time zero to infinity.

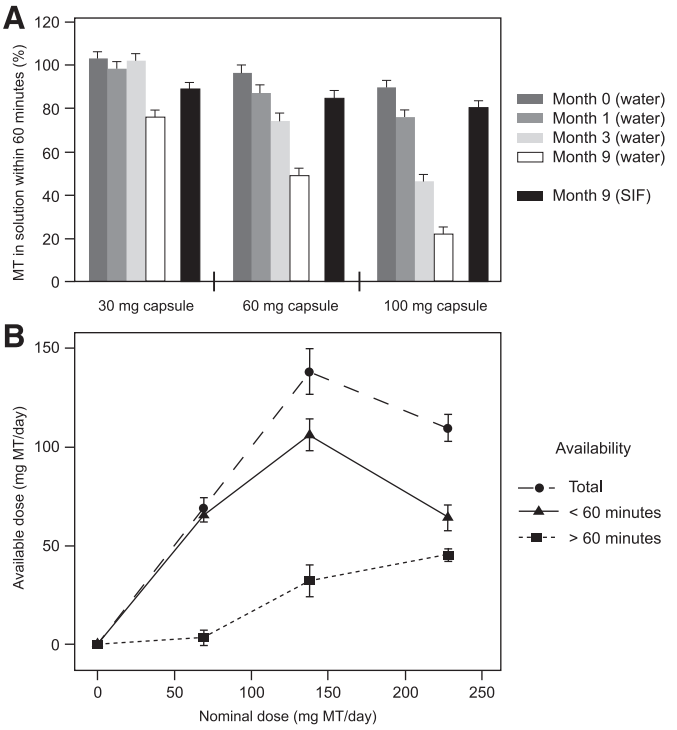

Fig. 5. (A) Dissolution of capsules is indicated as the proportion of MT^+ released during the course of 60 minutes of stirring in either water or SIF. Gelatin capsules containing 30 and 100 mg MTC (water, with values for 60 mg calculated by interpolation) or 30, 60, and 100 mg (SIF) were tested either immediately after manufacture or after storage in standard temperature/humidity conditions for 1–9 months (mean ± S.E.M. from four storage time points and six samples per time point). (B) The dose available for absorption is calculated from the amount dissolved in water (or gastric fluid) within 60 minutes (▲), the remainder available after 60 minutes and capable of dissolution in SIF (■), and the total (●); total absorption for the 100-mg MTC capsule is reduced to 48% of nominal dose due to the presence of food (Fig. 4B).

diffuse reflectance infrared Fourier transform spectroscopy (data not shown), due to trace concentrations of free radicals in the MTC hard Gelucire suspension contacting with the gelatin shell, combined with the increasing insolubility of the Gelucire suspension (Damian et al., 2002). Dissolution in SIF at 9 months was acceptable for all capsule strengths (Fig. 5A).

Although a significant treatment benefit was seen on the ADAS-cog scale at a nominal dose of 138 mg MT/day in moderate subjects at 24 weeks and in both mild and moderate subjects at 50 weeks, the effect in subjects receiving 228 mg MT/day was less than for 138 mg MT/day at the same time points. A similar profile was seen on the reduction in decline in rCBF in mild subjects after 20 weeks of treatment. There was therefore no dose response relative to nominal dose for any of these outcomes (correlation coefficients 0.117, 0.714, and 0.253; Fig. 6, A–C).

The dose available for absorption was calculated on the basis of dissolution in water within 60 minutes or the dissolution after 60 minutes. This alone was not sufficient to predict treatment response. However, when the effect of food interference on absorption (i.e., absorption of MT was reduced to 48% of the fasted level with the 100-mg unit dose of MTC, see above) was also taken into account, we found a set of dose-response relationships that accounted well for the efficacy data seen in the phase 2 trial. The mean dose of MT available for absorption within 60 minutes and able to be absorbed in the presence of food (i.e., as administered in the trial; Fig. 5B) was found to predict the mean effect of treatment on ADAS-cog at 24 weeks (r = 0.835; Fig. 6D) and 50 weeks (r = 0.927; Fig. 6F), and the mean effect on preventing decline in rCBF seen by SPECT scan after 20 weeks of treatment (r = 0.947; Fig. 6E). The effective dose delivered at the nominal 228-mg MT/day dose was approximately equivalent to that delivered by the 69-mg MT/day dose for all three outcomes.

Although there were strong correlations between decline in RBC count from baseline at 24 and 50 weeks (Fig. 7, A and B, respectively) and nominal dose, this could be accounted for entirely by the remaining dose available for absorption after 60

TABLE 1

Food effect studies: influence of food on time to peak total plasma MT for MTC and LMTX

Data are presented as the median (range) unless otherwise stated.

Salt Form	MT Dose	T_{max}	
		Fasted	Fed
	mg	*h*	
MTC	46	1.75 (1.00–2.50)	2.25 (2.00–4.00)
MTC	75	1.50 (1.00–3.00)	2.25 (1.00–3.00)
LMTX	100	1.50 (1.00–2.50)	3.00 (1.00–5.00)

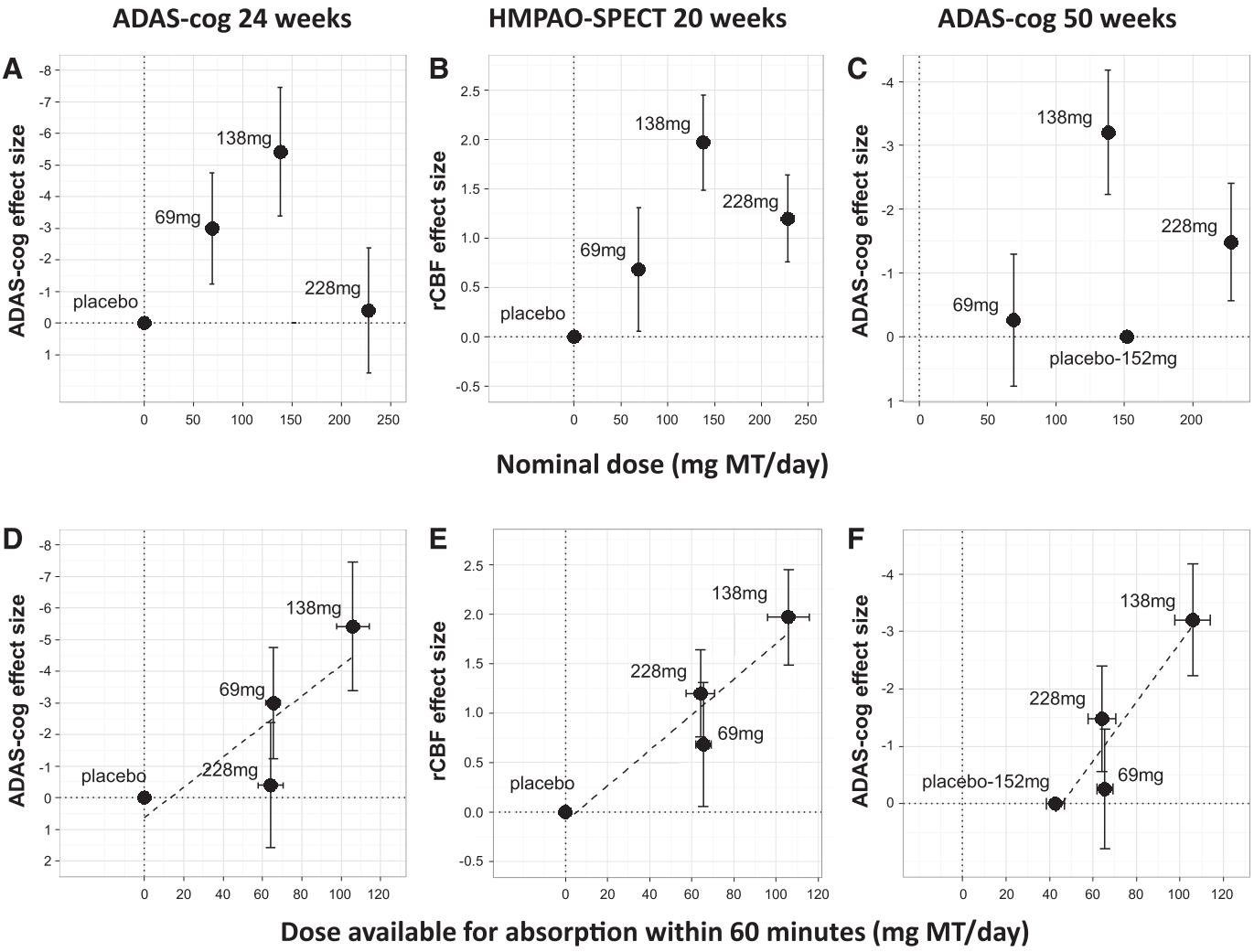

Fig. 6. Cognitive and imaging changes observed as a function of nominal capsule dose (A–C) and as a function of the dose available for absorption within 60 minutes (D–F) calculated from data shown in Fig. 5B. In panel (A), ADAS-cog effect size in moderate AD subjects at 24 weeks ($r = 0.117$). (B) Effect size as reduction in decline in rCBF relative to placebo on SPECT scanning in mild AD subjects after mean 20 weeks of treatment ($r = 0.714$). (C) ADAS-cog effect size in mild AD subjects at 50 weeks, using the arm in which subjects, originally randomized to placebo during weeks 1–24, received 152 mg MT/day during weeks 24–50 administered as 100-mg MTC capsules given twice daily ($r = 0.252$) as the comparator. (D) ADAS-cog effect size in moderate AD subjects at 24 weeks ($r = 0.835$). (E) Effect size as the reduction in decline in rCBF relative to placebo on SPECT scan in mild AD subjects after mean 20 weeks of treatment ($r = 0.947$). (F) ADAS-cog effect size in mild AD subjects at 50 weeks, using the arm as described in (C) as the comparator ($r = 0.927$). Values given as mean ± S.E.M. HMPAO, hexa-methyl-propyl-amine-oxime.

minutes and able to be released from capsules in SIF ($r = 0.955$ and 0.896 at 24 and 50 weeks, respectively; Fig. 7, C and D).

Discussion

MT dosed in the oxidized MT^+ form as MTC was found to have beneficial effects in the CNS as observed on clinical outcome scales and functional molecular imaging at 138 mg MT/day but not 228 mg MT/day. The anomalous behavior of the highest dose could not be explained by a simple failure of absorption, since there was a monotonic dose-response effect for a number of hematologic parameters. Likewise, preclinical studies in vitro (C. Harrington et al., submitted manuscript) and in transgenic mouse models of tauopathy (V. Melis et al., submitted manuscript) do not support an inverted-U dose response.

Based on stability studies undertaken at the time the trial was initiated, we show that the quantity of MT released from the capsules within 60 minutes in water or gastric fluid has proved in retrospect to be an important determinant of clinical efficacy. A further factor identified in subsequent routine fed/fasting studies was a dose-dependent limitation in the ability to absorb MT in the presence of food when it is delivered in the oxidized MT^+ form as MTC. This limitation is independent of the formulation problem, as it was observed using MTC tablets formulated to achieve >80% dissolution within 10 minutes. When both of these factors are taken into account, a simple dose-response profile for CNS activity is revealed, whereby the effective dose delivered via 100-mg capsules proves to be approximately equivalent to that delivered via 30-mg capsules. This equivalence applies both to the calculated dose available for absorption and the corresponding CNS effects. The fact that these relationships could be demonstrated in independent populations (i.e., in mild and moderate AD subgroups), and were seen by different observation modalities (i.e., SPECT scan and clinical scales), implies that they reflect real effects in the CNS caused by MT.

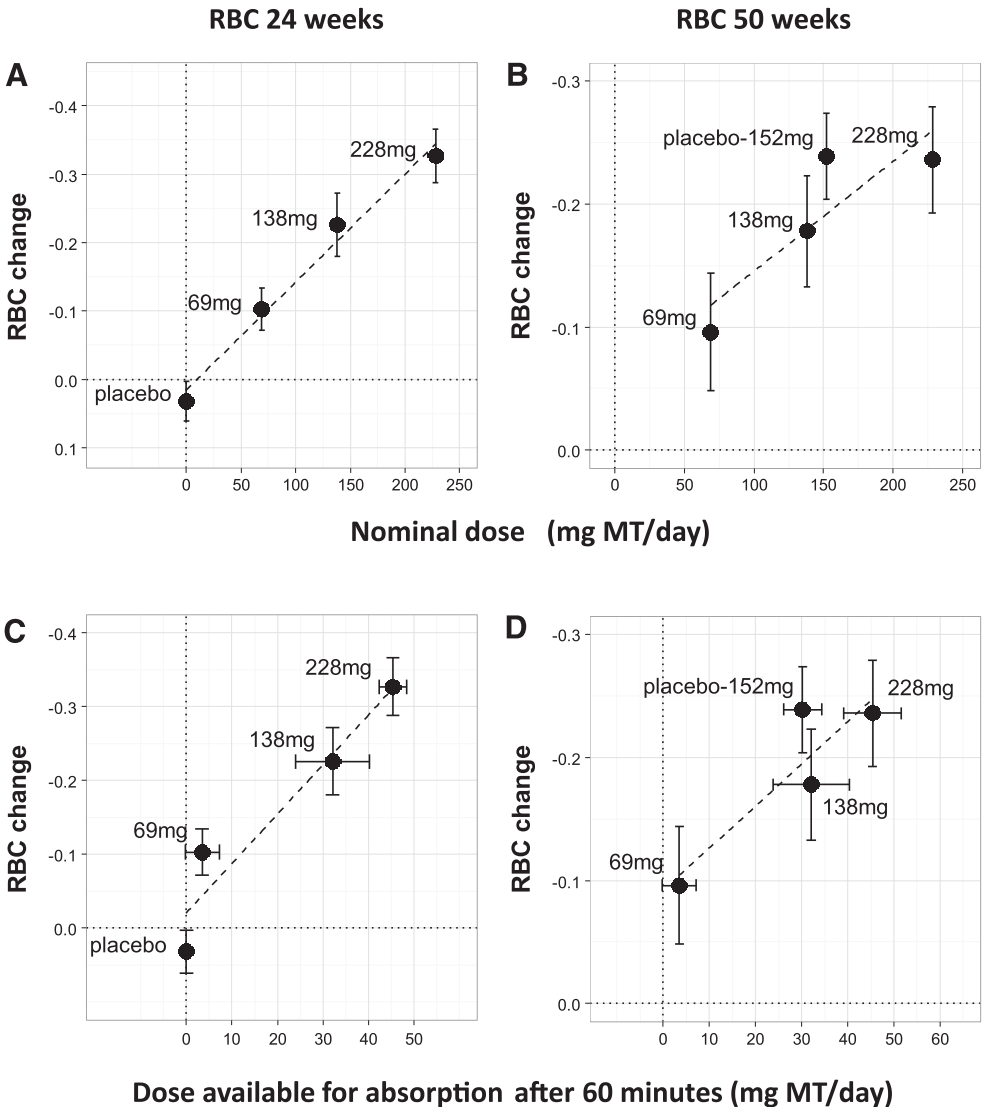

Fig. 7. Change in RBC counts ($\times 10^{12}$ per liter) from baseline as a function of nominal dose (A and B) and dose available for absorption after 60 minutes (C and D). For nominal dose, there were correlations with change in RBC count from baseline over 24 weeks (r = 0.991) (A) and 50 weeks (r = 0.871) (B). For absorption after 60 minutes, calculated from data shown in Fig. 5B, there were correlations for the changes in RBC count from baseline over 24 weeks (r = 0.955) (C) and 50 weeks (r = 0.896) (D). Values given as mean ± S.E.M.

The dependence on early release could be due either to an effect on absorption in the stomach or a chemical processing step that needs to occur in the stomach. The fed/fasting study shows that food delays the time to plasma C_{max} for both MTC and LMTX, implying that the most likely site for absorption of MT is the small intestine. The fact that food interferes with the absorption of MT only when it is provided as MTC but not LMTX implies that the processing that occurs in the stomach is related to a redox conversion step.

The absorption of MT, provided as MT^+, requires conversion at the RBC surface to the LMT form, which is mediated by an energy-dependent thiazine dye reductase activity (May et al., 2004). After conversion to LMT, absorption is by passive diffusion. This model is confirmed in several respects by the present data. Both in isolated RBCs, and more particularly after intravenous dosing, the uptake of MT into RBCs was substantially greater when provided in the LMT form. After oral dosing, the delivery of MT to the brain is greater at lower doses when MT is dosed as LMTX rather than MTC. We conclude that an important step in the absorption and distribution of MT is the conversion of MT^+ to the LMT form, and that the requirement for this to occur in the stomach most likely reflects an effect of pH. It is known from cyclic voltammetry studies of MTC that redox potential varies with pH (Murthy and Reddy, 1984). Our data suggest that the reductase activity required to generate LMT is favored at the low pH of the stomach. Conversely, late dissolution favors absorption of the MT^+ form from the small intestine, presumably via a capacity-limited active transport system distinct from the reductase mechanism. MT absorbed in this way as MT^+ appears to have a greater propensity to oxidize hemoglobin.

A general model (Fig. 8) that collates all of the available data suggests an important role for RBCs in MT metabolism and distribution. Although MT is subject to first-pass metabolism, converting it to labile conjugate(s) with no TAI activity (T. Baddeley et al., manuscript in preparation), early uptake into RBCs has a protective effect. The predominant form of MT found in RBCs is parent MT and its desmethyl derivatives, but not the labile conjugate metabolite(s). Thus, the quantity of MT accumulating within RBCs is inversely proportional to the extent of conjugation, and the quantity of MT in RBCs reflects the quantity in brain as shown by total radioactivity. This results in greater levels of parent MT in plasma after oral dosing with LMTX compared with MTC and also higher levels in brain. We are therefore led to the surprising conclusion that RBCs appear to provide the primary

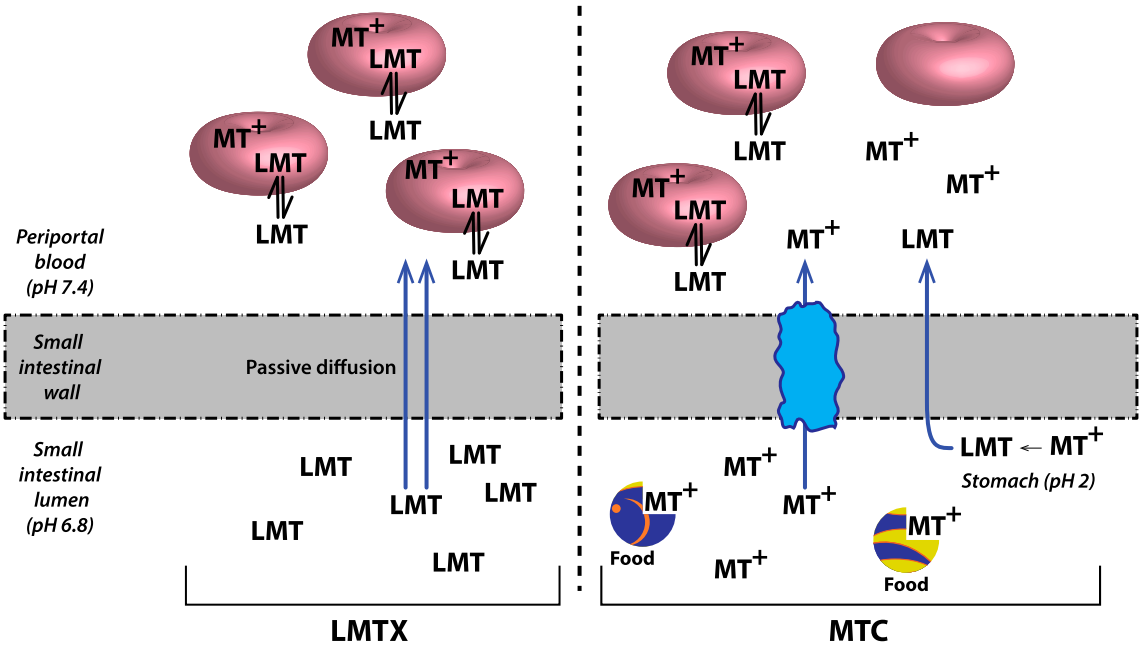

Fig. 8. Postulated absorption and transport pathways for LMTX and MTC. The results reported can be summarized as a single overall model to explain the complex absorption characteristics of MT leading to differential disposition of LMTX and MTC. LMT (dosed as LMTX) is not ionized at intestinal pH of 6.8 and diffuses passively across the small intestinal wall along a concentration gradient with no food interference. In the splanchnic circulation draining the small intestine, some (but not all) LMT is taken up rapidly into RBCs and escapes first-pass hepatic metabolism. Within the RBC, LMT is oxidized to MT^+ until an active equilibrium is reached. MT^+ is trapped inside the cell but LMT is able to diffuse out along a concentration gradient and distribute into the brain and other tissues. RBCs, therefore, serve as the primary reservoir for transporting parent MT throughout the body. By contrast, when MT is dosed in the MT^+ form as MTC, less is absorbed and more is metabolized, the amount of MT in RBCs or circulating in plasma is less, and the amount available to distribute into the brain is lower than with LMTX. Due to the lower pK_a of MTC, MT^+ remains largely ionized in both the stomach and intestine, except for that proportion that is converted to LMT via a presumptive active thiazine dye reductase mechanism (May et al., 2003). It is postulated that this conversion is dependent on pH and therefore formulation release time. If MT^+ is delivered to the small intestine (and accentuated if release is delayed longer than 60 minutes), then it is postulated that the reductase is either unavailable or inactive, and that absorption of the MT^+ species requires an alternative active carrier-mediated process that is capacity limited. Any of the steps (reductase step in the stomach, active transport step in the small intestine, or association of MT^+ with food/fats in the gut lumen) may be responsible for dose-dependent food interference in absorption of MT^+.

pharmacokinetic reservoir for distribution of MT to deeper compartments. To our knowledge, this is the first drug for which RBCs and not plasma serve this function, likely due to the unique redox properties of MT.

Notwithstanding the limitations of MTC as a means of delivering MT to the brain, we show that the brain levels of MT and its pharmacologically active desmethyl derivatives at the 138-mg MT/day dose are very close to the level required for TAI activity. Thus, the IC_{50} for dissolution by MT of paired helical filaments isolated from AD brain tissues is 0.16 μM and the intracellular K_i for inhibition of tau aggregation is 0.12 μM in a cell model of tau aggregation (C. Harrington et al., submitted manuscript). In two transgenic mouse models of tauopathy, the relationship between the effects of MT on behavior and tau pathology was monotonic ascending over a 10-fold concentration range (0.13–1.38 μM) of MT in the brain. We estimate that the steady-state trough brain concentration of MT and its pharmacologically active desmethyl metabolites during three-times daily dosing is 0.18 μm. We recently reviewed alternative mechanisms of action of MT that have been proposed in the literature (Wischik et al., 2014). Most of these are inconsistent with the concentrations of MT that could plausibly be achieved in the brain after oral dosing in humans.

Although we have demonstrated that MTC has potential therapeutic utility at the minimum effective dose, it is clear that MTC has significant limitations relative to LMTX, which make it an inferior candidate for further clinical development. MTC is poorly tolerated in the absence of food and is subject to dose-dependent absorption interference when administered with food. Eliminating the inadvertent delayed-release property of the MTC capsules did not protect against food interference. Therefore, as found in the phase 2 study, MTC cannot be used to explore the potential benefit of higher doses of MT. Nevertheless, the delayed-release property of the MTC capsules permitted the surprising discovery that it is possible to partially dissociate the cognitive and hematologic effects of the MT moiety. Whether the use of LMTX avoids or reduces the undesirable hematologic effects remains to be determined. Longer-term phase 3 trials are currently being conducted to confirm whether treatment with a TAI, such as MT, produces benefit in mild to moderate AD administered as LMTX with doses in the range from 150 to 250 mg MT/day.

Authorship Contributions

Participated in research design: Baddeley, McCaffrey, Storey, Cheung, Harrington, Wischik.

Conducted experiments: Baddeley, Melis, Horsley.

Performed data analysis: Baddeley, McCaffrey, Wischik.

Wrote or contributed to the writing of the manuscript: Baddeley, McCaffrey, Storey, Harrington, Wischik.

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