PBT2 Rapidly Improves Cognition in Alzheimer's Disease: Additional Phase II Analyses

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Abstract. PBT2 is a copper/zinc ionophore that rapidly restores cognition in mouse models of Alzheimer's disease (AD). A recent Phase IIa double-blind, randomized, placebo-controlled trial found that the 250 mg dose of PBT2 was well-tolerated, significantly lowered cerebrospinal fluid (CSF) levels of amyloid-β_{42}, and significantly improved executive function on a Neuro-psychological Test Battery (NTB) within 12 weeks of treatment in patients with AD. In the post-hoc analysis reported here, the cognitive, blood marker, and CSF neurochemistry outcomes from the trial were subjected to further analysis. Ranking the responses to treatment after 12 weeks with placebo, PBT2 50 mg, and PBT2 250 mg revealed that the proportions of patients showing improvement on NTB Composite or Executive Factor z-scores were significantly greater in the PBT2 250 mg group than in the placebo group. Receiver-operator characteristic analyses revealed that the probability of an improver at any level coming from the PBT2 250 mg group was significantly greater, compared to placebo, for Composite z-scores (Area Under the Curve [AUC] = 0.76, $p = 0.0007$), Executive Factor z-scores (AUC = 0.93, $p = 1.3 \times 10^{-9}$), and near-significant for the ADAS-cog (AUC = 0.72, $p = 0.056$). There were no correlations between changes in CSF amyloid-β or tau species and cognitive changes. These findings further encourage larger-scale testing of PBT2 for AD.

Keywords: Alzheimer's disease, clinical trials Randomized controlled, cognition, PBT2

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INTRODUCTION

PBT2 is potentially a disease-modifying drug for Alzheimer's disease (AD). As a zinc/copper ionophore, PBT2 has been shown in animal models to facilitate the clearance of amyloid-β (Aβ) aggregates in the cortex by targeting the zinc and copper ions that mediate the assembly of these aggregates in amyloid and diffuse deposits, effectively detoxifying the Aβ. Copper and zinc ions are released during glutamatergic neurotransmission and glial activation and promote the formation of Aβ oligomers and amyloid deposits [1]. The ionophoric properties of PBT2 liberate copper and zinc ions trapped by amyloid, facilitating the reuptake of these essential metal ions into cells which is believed to promote memory functions such as long-term potentiation [2,3]. Oral PBT2 treatment is strikingly effective in amyloid-β protein precursor transgenic mouse models for AD, markedly improving learning and memory within days, accompanied by a reduction in interstitial Aβ [2].

PBT2 was tested at 50 mg and 250 mg daily oral doses in patients with early AD in a double-blind, placebo-controlled Phase IIa randomized clinical trial over 12 weeks of treatment. The drug was well-tolerated, induced a 12% drop in cerebrospinal fluid (CSF) Aβ_{42} levels, and produced a significant improvement in Executive Factor z-score on a Neuropsychological Test Battery (NTB) [4].

Here, we report the results of a post-unblinding analysis of the cognitive data that was not included in the original paper. We re-analyzed the data to rank the members of the treatment cohorts for cognitive benefits of PBT2, and also determined whether there were associations with biochemical outcomes.

MATERIALS AND METHODS

Cohort and trial description

The cohort and the treatment protocol have been previously reported [4]. Briefly, this was a 12-week, double blind, randomized, parallel group, placebo-controlled trial of two doses of PBT2 (50 mg and 250 mg oral capsules daily). The study was approved by the Medicines Control Agency and the central ethics committees (Sweden) or the human research ethics committees at each center, with notification to the Therapeutic Goods Administration (Australia). Written informed consent was obtained from all patients. All patients met NINCDS-ADRDA criteria for probable AD [5], had early AD (Mini-Mental Status Examination [MMSE] Score 20–26, and/or Alzheimer's Disease Assessment Scale-cognitive subscale [ADAS-cog] score 10–25), and had been taking an acetylcholinesterase inhibitor for at least 4 months before entry. Primary outcomes were safety and tolerability. Key secondary outcomes were plasma and CSF biomarkers (including CSF tau, phospho-tau Thr181 [pTau], Aβ_{40}, and Aβ_{42}) and cognition (NTB, ADAS-cog, and MMSE). 78 patients were recruited and randomized between December 6, 2006 and December 28, 2007 at 8 sites in Sweden and 7 sites in Australia. The study was registered at ClinicalTrials.gov, number NCT00471211.

Statistical analysis

All statistical analysis was performed using R version 2.90 [6]. Linear regression was performed to investigate the correlation between cognitive changes, from pretreatment to week 12, and concomitant changes CSF levels of Aβ_{40}, Aβ_{42}, total tau (tTau), and pTau. Tests of the biochemical changes were corrected for multiple testing by controlling the false discovery rate using the Benjamin-Hochberg method [7] to generate adjusted p-values.

Receiver-operator characteristic (ROC) curves were analyzed according to previously published protocols for adapting clinical trial data [8]. In this approach, True positives are scores from subjects who improved while on PBT2 250 mg, while False positives are subjects who improved while on placebo.

NTB Composite z-scores

The NTB composite z-score is composed of the following tests: Wechsler Memory Scale (WMS) Verbal Paired Associates test (immediate and delayed), Rey Auditory Verbal Learning Test (RAVLT) test (immediate and delayed), WMS Digit Span, Category Fluency Test, Controlled Oral Word Association Test (COWAT), and Trail Making Test parts A and B. The Memory Factor z-score is composed of the following tests: WMS Verbal Paired associates test (immediate and delayed) and RAVLT test (immediate and delayed). The Executive Factor z-score is composed of the following tests: WMS Digit Span, COWAT, Category Fluency test, and Trail Making Test parts A and B.

Aβ and tau

Aβ_{40}, Aβ_{42}, tau, and pTau levels in CSF were assayed by ELISA as previously described [4].

Plasma oxidase activity

The assay was based upon that of Schosinsky and colleagues [9]. Plasma samples were incubated at 37°C after being mixed with 75 mM sodium acetate buffer pH 5.0. 7.88 mM o-dianisidine dihydrochloride substrate (Sigma) was added, mixed and then incubated. Sulfuric acid (9 M) was added to stop the reaction at 5 and 60 min. Oxidized product was monitored by absorbance at 540nm.

Western blot

Samples were separated by polyacrylamide gel electrophoresis (Invitrogen) under non-reducing, denaturing conditions and transferred to a nitrocellulose membrane. Non-specific binding was blocked with 5% fat-free dry milk in TBS-T. The membrane was washed and incubated (0.5 μg/mL, 4°C, overnight) with polyclonal anti-ceruloplasmin (Dako), or mouse monoclonal anti-SOD1 (Ab20926, Abcam). The washed membrane was then incubated with peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (1:10,000, Dako, 1 h, RT). Immunoreactive proteins were visualized by chemiluminescence (ECL, Amersham), captured (LAS-3000 Imaging suite) and analyzed (Multi Gauge, Fuji).

SOD1 analysis

Erythrocytes extracts were prepared as described [10], with minor modification. Briefly, haemolysates were prepared by adding washed erythrocytes (200 μL) to ddH$_2$O (1400 μL) and incubating on ice for 10 min. Aliquots (250 μL) of haemolysate were added to an equal volume of ice-cold ethanol, vortexed (5 s), then ice-cold chloroform added (100 μL), and vortexed again (30 s). The mixtures were centrifuged (3000 g, 10 min, 4°C), and the upper (aqueous) phase removed and stored at −80°C until used. For SOD1 activity, extracts were assayed using a microtiter-plate kit (SOD Assay Kit Cat# S311, Dojindo Molecular Technologies). For SOD1 quantitation, neat extracts were added to sample buffer (NuPAGE LDS, Invitrogen) containing TCEP (50 mM), and analyzed by Western Blotting, compared to standards of purified human erythrocyte SOD1 (Cat# S9636, Sigma).

Plasma free copper assay

The presence of exchangeable or free copper ions in plasma was measured by detection of copper-phenanthroline-dependent DNA degradation with minor modifications. To a 1.5 mL tube the following were added in order: thymus DNA (80 μL, 2 mg/ml), 1,10-phenanthroline (20 μL, 1 mM), NaN$_3$ (10 μL, 100 mM), CuCl$_2$ or neat plasma (10 μL), phosphate buffer (40 μL, 0.25 M, pH 6.4), and 2-β-mercaptoethanol (15 μL, 0.4%, v/v). The samples were then incubated for 1 h at 37°C. EDTA (20 μL, 0.1 M) was then added to stop the reaction, followed by thiobarbituric acid (100 μL 1% w/v in 50 mM NaOH) and trichloroacetic acid (100 μL, 28% w/v), and boiling for 10 min. An additional blank was prepared for each sample containing water instead of the 1,10-phenanthroline. A standard curve of CuCl$_2$ concentrations (1 to 10 μM) was prepared. After cooling, each sample was extracted into butan-1-ol (600 μL). Phases were separated by centrifugation (2000 g, 5 min), and the fluorescence of the butan-1-ol phase measured (em 553nm, ex 532nm).

RESULTS

Distribution of NTB changes

The least squares (ls) mean differences provide only a rudimentary description of treatment effects. We therefore analyzed the NTB z-score changes in greater depth by ranking the ls mean change from pretreatment at 12 weeks for each member of the treatment cohorts, as has been used previously to support the efficacy of donepezil for AD in early Phase II studies [11]. Most subjects showed improved performance at 12 weeks on each of the z-scores (Fig. 1), indicating a considerable learning effect even in this cohort of patients with mild dementia. The greatest learning effect, as exhibited by placebo results, was for the Memory Factor z-scores, where 100% of the placebo subjects performed better than pre-treatment (Fig. 1B). 93% of the placebo subjects scored better for the Executive Factor and Composite z-scores than at pre-treatment. Importantly for the calculation of the Composite z-scores, the dimension of the learning effect in the placebo group was approximately 3-times greater for the Memory Factor z-scores than for the Executive Factor z-scores (Fig. 1).

Despite the learning effects in the placebo group, the percentage of patients who improved was greater
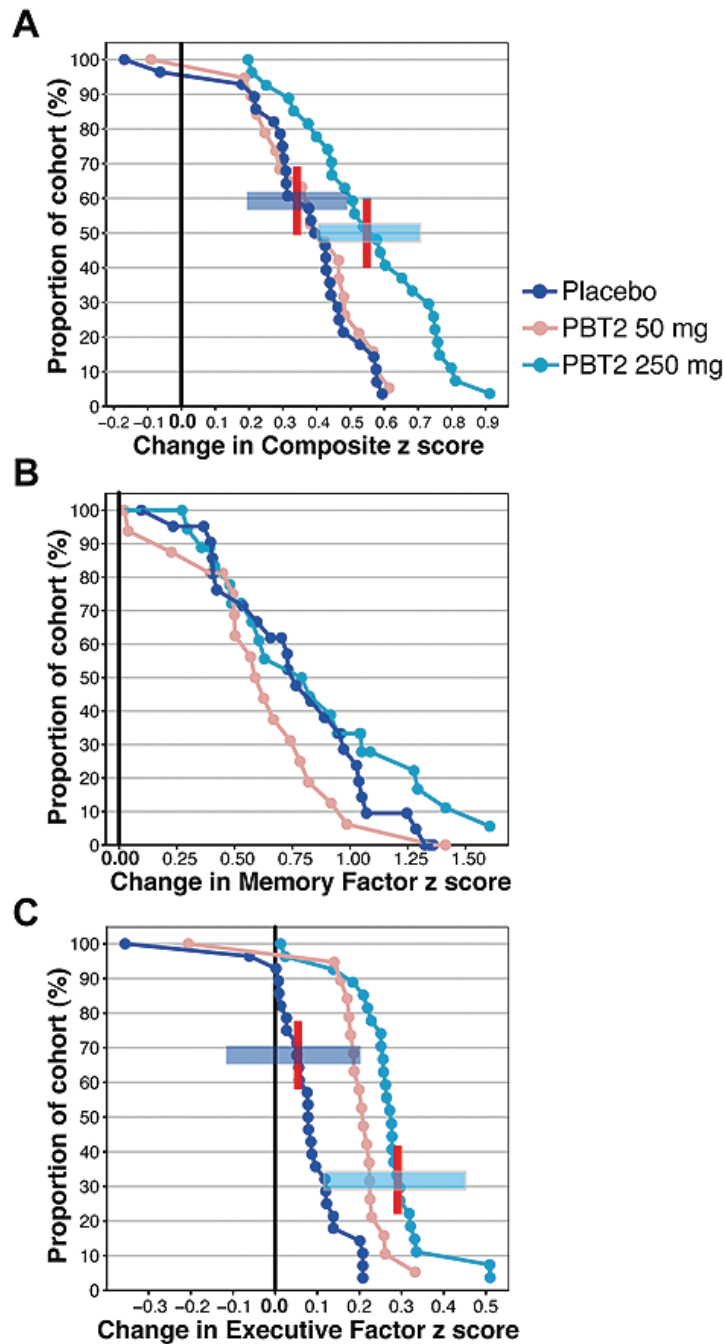
Fig. 1. Response ranking of treatment groups. Plot of the cumulative percentage of patients within each of the three treatment groups who showed an ls-means difference equal to or greater than a given value shown on the x axis. The red vertical lines show the means for the group where a significant difference is noted in later analysis. The shaded areas are the 95% confidence intervals. A) Ranking of NTB Composite z-scores, B) Ranking of NTB Memory Factor z-scores, C) Ranking of NTB Executive Factor z-scores. For the NTB tests a positive change from pretreatment is an improvement.

in the PBT2 250 mg group than the placebo group for all thresholds of improvement in the Composite and Executive Factor z-scores (Fig. 1A and C). 41% of the PBT2 250 mg cohort responded better on the Composite z-score than then best response in the placebo group (Fig. 1A). 81% of the PBT2 250 mg cohort responded

Table 1
Correlations of CSF biomarker readouts. The r^2 and p-value (in parenthesis) of the linear regression analyses of changes in CSF biomarkers for the whole cohort from pre-treatment to week 12 are shown. Those results in bold face showed a significant or near-significant linear relationship between two CSF biochemical markers

	Δ Aβ_{40}	Δ Total Tau	Δ Phospho Tau
Δ Aβ_{42}	**0.444 (2.26×10^{-10})**	0.000 (0.996)	0.018 (0.335)
Δ Aβ_{40}		0.021 (0.335)	**0.071 (0.052)**
Δ Total Tau			**0.084 (0.045)**

better on the Executive Factor z-score than the best response in the placebo group (Fig. 1C). The best 35% of Memory Factor z-score responses for PBT2 250 mg group were uniformly higher than the best 35% of the placebo group (Fig. 1B), indicating that the drug was inducing a greater proportion of larger improvements in memory performance than placebo.

ROC analysis of the likelihood that a cognitive improver was on PBT2 250 mg

To apply a statistical analysis to the distribution of improvements induced by PBT2 250 mg treatment, we performed a ROC analysis adapted to clinical trials [8]. This test analyzed whether treatment with PBT2 250 mg was more likely than placebo to induce an improvement of any dimension in the performance readouts. In this analysis, all the 12-week responses (differences from pretreatment) for a given z-score test were ranked, and then the probability of the response coming from the PBT2 250 mg treatment group (True positive) or the placebo group (False positive) was calculated. This revealed that improvement of any dimension on the NTB Composite z-score was significantly more likely when on PBT2 than on placebo (AUC = 0.76, $p = 0.0007$, Fig. 2A). The curve also revealed that the greatest likelihood of differences between the PBT2 treatment group and the placebo group were in the range of a z-score of 0.49–0.71 above the pretreatment performance score (Fig. 2A). The ROC for the NTB Executive Factor z-scores revealed that improvement of any dimension was also more likely in the PBT2 treatment group than in the placebo group (AUC = 0.93, $p = 1.3 \times 10^{-9}$), with the greatest likelihood of differences between the PBT2 treatment group and the placebo group at a z-score of 0.2 above the pretreatment performance score (Fig. 2C). The ROC of the Memory Factor z-scores found no significant effects of either PBT2 or placebo: there was a trend for improvement in the z-score range of 0.99–1.31, the larger end of the response rank, to be more likely in the PBT2-treated group (Fig. 2B).

Because the ROC analysis appeared to allow us to discriminate better than ls means differences between the PBT2 250 mg and placebo group, we also analyzed the MMSE and ADAS-cog data. MMSE showed no significant difference (not shown) but for ADAS-cog the AUC was 0.72 in favor of PBT2 250 mg, which was a trend to improvement ($p = 0.056$, Fig. 2D).

Effects of PBT2 on blood metalloenzymes and "free" copper

PBT2 and its predecessor clioquinol are sometimes misunderstood to represent pharmacological chelators, although preclinical evidence indicates that these compounds act as ionophores [2,13], and a Phase IIa clinical trial of clioquinol found that plasma zinc levels rose with treatment and copper levels were unaffected [12]. PBT2 treatment in the current trial did not alter total plasma copper or zinc levels [4]. To test whether the drug impacted on major metalloproteins or free copper levels, which have been implicated in AD [13], we assayed ceruloplasmin and copper/zinc superoxide dismutase specific activities in plasma and red cells respectively, as well as plasma "free" (exchangeable) copper levels. These measurements were part of the Statistical Analysis Plan, but were not previously reported. No changes were seen in the placebo or PBT2 treatment groups over the 12 weeks of the study (Supplementary Tables 1 and 2), indicating that PBT2 does not perturb peripheral metal metabolism.

Correlations of CSF Aβ and tau changes

We examined the relationship of the CSF biomarkers (Aβ_{40}, Aβ_{42}, tTau, and pTau) to each other and to the cognitive outcomes. We found that a change in Aβ_{40} significantly correlated with a change in Aβ_{42}, a change in tTau significantly correlated with a change in pTau, and a change in Aβ_{40} near-significantly correlated with a change in pTau (Table 2). These associations were significant only when the combined data from the treatment and placebo cohorts were pooled. There were no significant association of changes in these biomarkers with changes in cognitive outcomes.
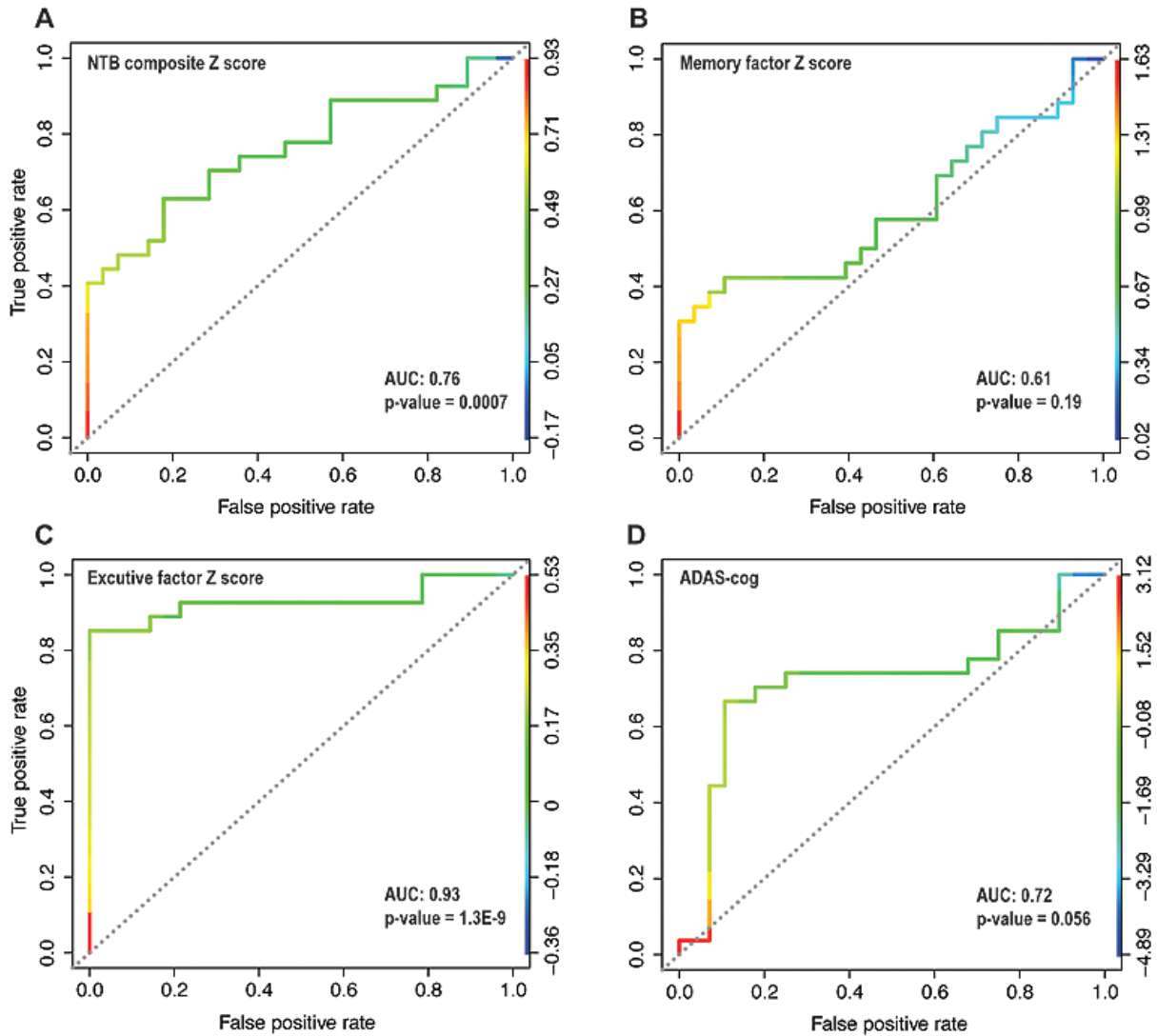
Fig. 2. Receiver-operator characteristics (ROC) curves between ranked placebo and PBT2 250 mg responses. All ls-means difference changes of NTB z-scores between pre-treatment to 12 weeks for the placebo and PBT2 250 mg cohorts were ranked and tested for whether a given threshold of response fell in the placebo or PBT2 group. The left y-axis and the x-axis express the probability of a participant with a given ls-means difference being in the PBT2 250 mg group or placebo group. The right y-axis and color code indicate the threshold of improvement corresponding to the associated point on the ROC. A) ROC curve for the NTB Composite z-scores. B) ROC curve for the NTB Memory Factor z-scores. C) ROC curve for the NTB Executive Factor z-scores. D) ROC curve for the ADAS-cog scores. AUC, area under the curve. The p-value indicates a significant change from 0.5, the dashed line, which indicates random association (AUC = 0.5). For NTB tests a positive value is improvement while for ADAS-cog a negative value is improvement.

Biochemical responders analysis

For the analyses reported here we defined five "biochemical responders" groups, based upon contemporary thinking about the potential of CSF $A\beta$ and tau as predictive markers for AD progression [14], as well as our previous observations that PBT2 lowers interstitial brain $A\beta_{42}$ levels in transgenic mice [2]. A decrease in $A\beta_{40}$ and $A\beta_{42}$, a decrease in tTau and pTau, a de-

crease in all of $A\beta_{40}$, $A\beta_{42}$, tTau, and pTau, an increase in the ratio of $A\beta_{40}/A\beta_{42}$, and a decrease in the ratio of $A\beta_{42}/pTau$ were analyzed. We then tested to determine if there was any difference between the placebo and PBT2 250 mg group within and between the defined biochemical responder groups for NTB Composite z-scores, Executive Factor z-scores and Memory Factor z-scores. After correction for multiple testing there were no significant differences within and be-

tween the biochemical improvers, placebo and PBT2 250 mg group (data not shown).

DISCUSSION

A disease-modifying treatment for AD has proven elusive. Approaches that showed promise in preclinical testing were not authenticated in randomized Phase II and III clinical trials [15–17]. The primary outcomes of our Phase IIa clinical trial were safety and tolerability, with cognitive improvement as a secondary outcome. While there is great interest in determining whether cognitive improvement is evident even in early-phase AD clinical trials, this is especially difficult with a disease-modifying approach, where little cognitive deterioration is expected in short time frames in a placebo group; in our case, 12 weeks. Our initial report of PBT2 treatment over 12 weeks in patients with early AD described that the drug was safe and well-tolerated at 50 mg and 250 mg doses, and that the 250 mg dose induced a significant decrease in CSF Aβ_{42}, and a significant improvement in the NTB Executive Factor z-score [4]. A cognitive battery similar to the NTB has been shown to reliably assess cognitive changes in patients with mild AD [18,19], and may be more sensitive to change in patients with mild AD or early disease, compared to ADAS-cog and MMSE, which are more suited to measurement of patients with more advanced disease. We found the NTB to be more discriminatory than ADAS-cog and MMSE in discerning treatment-associated cognitive improvement in this study.

The ranking analysis reported here has been previously used to demonstrate the efficacy of donepezil for AD in early Phase II studies [11]. Using this approach there was a striking separation, with no overlap, between PBT2 250 mg and placebo cohorts in the ranked responses to treatment for Composite z-scores and Executive Factor z-scores (Fig. 1A and C). This effect could not be appreciated from the primary efficacy analysis that compared the overlapping standard errors of the ls means [4]. To test whether PBT2 250 mg had a significant benefit in this ranked analysis, we utilized ROC curve statistics, which were originally devised to discriminate ambiguous data, and recently validated as an analysis for Phase 2 clinical trials [8]. The ROC analysis confirmed that the treatment effects of PBT2 250 mg compared to placebo over 12 weeks were significant: $p = 0.0007$ for NTB Composite z-score, largely driven by the Executive Factor component ($p = 1.3 \times 10^{-9}$, Fig. 2C), but also driven by the best 35%

of responders on the Memory Factor z-scores (Figs 2B, 3B). ROC analysis also revealed a trend ($p = 0.056$) towards a benefit of PBT2 250 mg treatment over placebo on ADAS-cog (Fig. 2D). Therefore the ranking and ROC analyses provided more confidence in the validity of a PBT2-mediated treatment benefit than the ls means differences for these readouts. Efficacy signals in early Phase II clinical trials are prone to Type II error because of uncertainties about optimal dose and treatment duration, and because it is premature to utilize large numbers of patients. The ranking and ROC analyses may therefore be useful approaches to avoid Type II error at this early phase.

Effects on Executive Function (EF) may prove to be of particular utility in helping to understand drug efficacy in AD clinical studies. Recent data indicate that patients with mild AD, treated with donepezil, showed greater effect-size improvements on the NTB as compared with the ADAS-cog [9]. The same study also found a correlation ($r^2 = 0.6$) between performance on the NTB and the Disability Assessment for Dementia (DAD). These findings suggest that the NTB, which contains well-known measures of EF, can capture important positive effects on cognition that are less readily detected by traditional measures. The correlation between the NTB and DAD suggests that the inclusion of EF measures improves the ability to predict overall function from cognitive abilities.

We conclude that PBT2 250 mg treatment for 12 weeks was highly likely to have induced more cognitive improvement than placebo in this mildly affected AD cohort. In AD animal models, marked cognitive improvement was seen within days of commencing treatment with PBT2 despite the drug lacking neurotransmitter-mediated effects [2]. We hypothesize that improvement was due to rapid clearance of oligomeric Aβ from the brain [2]. Our observations in animals and patients indicate that PBT2's potential disease-modifying effect may be observable early, impacting upon a modifiable disease component where reversible neurotoxicity mediates symptoms, in contrast to later stage irreversible neurodegeneration. As a disease-modifying approach, the effect size of clinical benefit is expected to increase with duration of treatment, which encourages the study of PBT2 in larger scale clinical trials of longer duration.

Another feature of our study was an attempt to determine whether changes from baseline in CSF Aβ or tau species could serve as biomarkers for cognitive change. We found no significant correlative associations, despite significantly decreased CSF Aβ_{42} levels and sig-

nificant EF z-score improvement in the PBT treatment group compared to placebo. This study is probably underpowered to discern such correlations, but could help in the design of future studies.

ACKNOWLEDGMENTS

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