Pharmacodynamics of Cholinesterase Inhibitors Suggests Add-on Therapy with a Low-Dose Carbamylating Inhibitor in Patients on Long-Term Treatment with Rapidly Reversible Inhibitors

Taher Darreh-Shori, Sharokh Makvand Hosseini, and Agneta Nordberg

Department of Neurobiology, Care Sciences and Society, Division of Alzheimer Neurobiology, Karolinska Institute, Stockholm, Sweden

Department of Geriatric Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden

Faculty of Psychology and Educational Sciences, Semnan University, Semnan, Iran

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Abstract. Despite three decades of intensive research in the field of Alzheimer’s disease (AD) and numerous clinical trials of new therapeutic agents, cholinesterase inhibitors (ChEIs) are still the mainstay of therapeutics for AD and dementia with Lewy bodies. Pharmacodynamic analyses of ChEIs provide paradoxical observations. Treatment with the rapidly reversible, non-carbamylating ChEIs (donepezil, galantamine, and tacrine) increases acetylcholinesterase (AChE) protein expression, whereas the carbamylating agent, rivastigmine, produces sustained inhibition with no significant change in AChE protein expression. Still, the symptomatic clinical efficacies of all these agents are similar. We report here for the first time that treatment with phenserine, another carbamylating ChEI, produces a sustained but mild inhibition of AChE in cerebrospinal fluid (CSF) of AD patients. We also show that phenserine treatment reverses donepezil-induced elevation of AChE expression. Further analyses on CSF of another larger patient cohort treated with donepezil revealed that, in addition to its main mode of action, donepezil produced two other pharmacodynamics with potentially contradictory outcomes. Donepezil-induced AChE expression favored an AChE-driven amyloid-β peptide (Aβ) aggregation, whereas donepezil itself concentration-dependently counteracted the AChE-induced Aβ aggregation, most likely by competing with the Aβ peptides for peripheral anionic site on the AChE protein. The reduction of AChE protein expression in the donepezil-treated patients by concomitant administration of the carbamylating agent, phenserine, could allow the donepezil molecule to only prevent interaction between Aβ and AChE. The current study suggests that an add-on therapy with a low-dose formulation of a carbamylating agent in patients on long-term donepezil treatment should be explored as a strategy for enhancing the clinical efficacy of these agents in dementia disorders.

Keywords: Add-on therapy, Alzheimer’s disease, carbamylating ChEIs, cholinesterase inhibitors (ChEIs), donepezil, peripheral anionic site, phenserine, rivastigmine

INTRODUCTION

Sporadic Alzheimer’s disease (AD) is the most common form of dementia and represents 80% of all cases [1]. AD is characterized by memory loss, cognitive dysfunction, behavioral disorders, and
impaired ability to conduct everyday activities. It has been well-documented for the past 30 years that AD selectively affects central cholinergic neurons. This has led to the use of cholinesterase inhibitors (ChEIs) to prevent degradation of acetylcholine (ACh) by acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), and therefore prolonging ACh action. Despite intensive research into agents addressing other targets, ChEIs are still the mainstay of AD therapy. Although they are used as a symptomatic treatment, emerging evidence indicates a disease-modifying effect [2]. So far, no other disease-modifying treatments have been reported, with the possible exception of B-vitamin supplements in early AD patients with hyperhomocysteinemia [3].

Tacrine is a rapidly reversible ChEI with affinity to both AChE and BuChE, but no longer on the market in developed countries. At present, three ChEIs are available for treatment of AD, namely donepezil, rivastigmine, and galantamine. Donepezil is the most selective, rapidly reversible, mixed competitive and noncompetitive AChE inhibitor [4, 5]. Galantamine is a selective, rapidly reversible, competitive AChE inhibitor and may act as an allosteric potentiating ligand of nicotinic acetylcholine receptors [6, 7]. Rivastigmine is a dual inhibitor of AChE and BuChE, with a pseudo-irreversible mode of inhibition [8]. It is the only ChEI that produces sustained inhibition without any significant increase in the expression of the target enzymes [6]. Finally, phenserine, which is not yet on the market, is a carbamylating agent similar to rivastigmine, but is a reversible AChE selective inhibitor (Fig. 1). Currently no AChE expression and inhibition data is available for phenserine in the cerebrospinal fluid (CSF) of patients with AD.

The influence of rivastigmine, donepezil, and galantamine on the activities and protein levels of target enzymes in AD patients has been well-documented [8]. Several longitudinal studies of tacrine, galantamine, and donepezil suggest that these rapidly reversible AChE inhibitors cause a substantial increase in the activity and protein expression of AChE in the CSF of AD patients. The increase seems to be directly related to the potency of the agent, since a stronger inhibition of AChE activity is associated with a larger increase in AChE protein expression [9]. One view is that differences in the binding sites of ChEIs with AChE, and/or the mode of enzyme inhibition (rapidly reversible, irreversible, or slowly reversible) may be involved. This is supported by the lack of strong AChE upregulation with irreversible or slowly reversible inhibitors, such as metrifonate and rivastigmine [10]. This is in contrast to very rapidly reversible inhibitors such as tacrine, donepezil, and galantamine [11]. Finally, there are also suggestion that the BuChE expression can regulate AChE expression [12]. Inhibition of BuChE by rivastigmine (but not donepezil or galantamine, which are AChE-selective) may regulate AChE gene expression.

 Nonetheless, clinically relevant questions include the time course of these increases, the compartments in which elevated AChE activity and protein expression are found, and the pathophysiological consequences of increased AChE activity and expression in the brains of AD patients. Thereby, mechanisms underlying these differential changes in AChE activity and protein levels in the CSF of AD patients following ChEI treatment need more attention. A deeper understanding may lead to improved efficacy of ChEI treatment. The importance of this objective is highlighted by the fact that all other therapeutic strategies tested to date have shown negative results [13, 14].

Postmortem studies on AD brains indicate that the cholinergic enzymes AChE and BuChE are present in
amylloid-β (Aβ) deposits such as Aβ plaques, cerebral amyloid angiopathy, and neurofibrillary tangles of tau protein [15–19]. AChE has been identified as a component of the neurtic plaques and a seeding agent for Aβ aggregation, with more toxicity than aggregates of Aβ alone [20]. Cholinergic neurons are particularly vulnerable to the actions of Aβ [21]. However, recent studies suggest that Aβ may affect a number of other targets, increasing Aβ-mediated toxicity [22–24].

Numerous in vitro reports also suggest that AChE interacts with Aβ, leading to an accelerated rate of Aβ fibrilization [20, 25, 26], whereas BuChE attenuates the fibrilization rate of Aβ peptides [26]. These are confirmed by studies on double transgenic mice expressing both human AChE and AβPPSwe genes, suggesting higher level of plaques in these animals, compared to single transgenic AβPPSwe mice [27, 28] and that the Aβ burden is most tightly correlated with memory impairment in the double transgenics [28]. Studies also indicate increased level of a soluble monomeric form of AChE in the brains of AβPPSwe transgenic mice [29], and in the CSF and brains of rats with intracerebral ventricular injections of the Aβ peptide [30]. Altogether, these observations indicate that increased AChE levels promote plaque accumulation in the brain, with detrimental consequences.

AChE possesses two binding sites for the neurotransmitter acetylcholine: the active center site at the bottom of a 20-Å gorge and the peripheral anionic binding site (PAS) that is rich in hydrophobic residue and located at the rim of the gorge on the enzyme surface [20, 26, 31].

Among AChE inhibitors, only PAS inhibitors block the effect of AChE on Aβ fibril formation [32]. For example, propidium and fasciculin prevent the effect of AChE on the Aβ aggregation process [20]. On the other hand, the Aβ aggregation in the presence of edrophonium, an active site inhibitor of AChE, showed no effect on the role of AChE to accelerate Aβ assembly into fibrils [20]. A peptide identified in PAS region mimics the effect of the full length of AChE on the Aβ aggregation [33].

Donepezil and galantamine, but not rivastigmine and phenserine, interact with PAS [34, 35]. An in vitro study has shown that donepezil at 100 μM concentration attenuates AChE-induced Aβ aggregation by 22% [36], most likely due to donepezil interaction with PAS and blocking the promoting activity of AChE on Aβ aggregation [26].

Given the apparent consequences of increased levels of AChE expression on the Aβ burden and toxicity in the brain, and the long-term clinical efficacy of current ChEIs in AD, the aim of this study was to provide insights that may improve the long-term effect of these drugs. We here showed for the first time that phenserine, a carbamylating agent similar to rivastigmine, produced a mild, sustained AChE inhibition. We also showed that switching drug treatment from donepezil to phenserine led to a strong regression of donepezil-induced AChE elevation in the CSF of AD patients. In addition, we used a large number of CSF samples collected prior to, and after, one and two years of treatment with donepezil to examine the effect of AChE elevation on kinetic parameters of Aβ aggregation. Overall observations provide the rational basis to propose that combination therapy with low-dose of a carbamylating inhibitor with recommended dose of donepezil may improve long-term efficacy of monotherapy with each of these agents.

**MATERIAL AND METHODS**

**Phenserine study design**

This study has been described previously [37]. This was a 12-month study involving 20 patients with mild AD (Mini-Mental State Examination [MMSE] score >21). Patients were recruited from the Department of Geriatric Medicine, Karolinska University Hospital Huddinge, and Stockholm, Sweden. Patients underwent a thorough clinical investigation including neuropsychological assessment, lumbar puncture, and magnetic resonance imaging/computed tomography scans. None of the patients were on treatment with any of ChEIs prior to the start of the study.

Schematic layout of the phenserine study is illustrated in Fig. 2a. Briefly, the first three months were the double-blind, placebo-controlled, randomized phase of the study (Fig. 2a). During this phase, the patients either received phenserine (the phenserine group, n = 10) or placebo (the placebo group, n = 10). The second phase of the study was an open-label extension phase, during which patients in the placebo arm of the study, switched from placebo to 5 mg/day of donepezil for three months (the placebo-donepezil group), and patients in the phenserine arm continued on phenserine. At the six months follow-up, the donepezil-treated patients after a one-week washout period switched to phenserine treatment and was followed-up for an additional six months (and titrated up to a 15-mg bid dose of phenserine) [37]. Thus all patients received phenserine treatment from 6 to 12 months. The phenserine-treated patients received 15 mg bid from week nine to the end of the study.
Fig. 2. Phenserine produces a sustained, mild AChE inhibition in CSF of patients with AD. a) Schematic illustration of the phenserine study. b) Changes in the AChE activity in the CSF after three to 12 months of the study. Phenserine reduced AChE activity, while donepezil caused an increase. Switching from donepezil to phenserine resulted in regression of increased AChE activity to CSF levels of patients on phenserine. c) Changes in the protein levels of the synaptic AChE-S variant in CSF. In contrast to phenserine or placebo treatments, donepezil greatly elevated the AChE-S protein in CSF. This increase was abolished by switching to phenserine. d) Estimated in vivo AChE inhibition based on the procedure described for reversible cholinesterase inhibitors [9, 44]. Thus the actual in vivo AChE inhibition for phenserine is between 15–25%. In addition, this analysis suggests that donepezil, despite the increased AChE protein, produces about 40% in vivo AChE inhibition.

CSF samples were collected by lumbar puncture at baseline and after 3, 6, and 12 months of treatment. All samplings were performed in the morning or 2 to 4 hours after intake of morning medication [37].

Diagnosis of AD

The diagnosis of probable AD was made by excluding other dementias, in accordance with NINCDS-ADRDA criteria (National Institute of Neurological and Communication Disorders and Stroke-Alzheimer’s disease and Related Disorders Association) [38]. The patients underwent a thorough clinical examination, including MMSE [39], apolipoprotein E (ApoE) genotyping, and lumbar puncture for CSF sampling [40]. Informed consent was obtained from each patient or the responsible caregivers. This study was conducted according to the Declaration of Helsinki and subsequent revisions. The Ethics Committee of Umeå, Malmo and Huddinge University Hospital, Sweden approved the study.

Overall CSF AChE and BuChE activity assays

AChE and BuChE activities in the CSF samples were determined by Ellman’s colorimetric assay, as described before [44]. Briefly, 50 μL of samples (in triplicate) was applied to the wells of a microtiter 96-well plate. Then, 150 μL of a master mix was applied to each well, using a multichannel pipette. The absorbance was read at 412 nm at room temperature for 15 minutes and at 30-second intervals. For the measurement of BuChE activity, the master mix (MMB) contained butyrylthiocholine iodide (to get 5.0 mM final concentration, Sigma) as substrate, the selective AChE inhibitor
(1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dihydrobromide) BW226C50 (1.0 μM final concentration, Sigma), DTNB (0.4 μM final concentration, Sigma), and sodium potassium phosphate buffer (50 mM, pH 7.4).

For AChE activity, the master mix (MMA) consisted of acetylthiocholine iodide as the substrate (0.5 mM, final concentration, Sigma), and the selective BuChE inhibitor, ethopropazine (0.1 μM final concentration, Sigma), and DTNB (0.4 μM, final concentration, Sigma). The overall dilution factor of the CSF samples was four times.

Aβ fibrillation experiment on CSF samples

CSF samples

The number of patients in the phenserine study were too small, and the duration of donepezil treatment too short (just 3 months) to provide reliable information with regard to ChEI-derived changes in the inherent CSF properties on kinetics of Aβ fibrillization. Thus, CSF samples from 50 patients who participated in the SATS study (Swedish Alzheimer Treatment Study) were used to evaluate changes in aggregation properties of Aβ peptides. The SATS study has been previously reported [9]. It was an open-label, multicenter study with a naturalistic setting at three geriatric clinics [40]. The patients were examined at baseline, after two to three months for possible dose titration, and then every six months. The day after the baseline visit, the patients received 5 mg donepezil hydrochloride (Aricept®, Pfizer Inc) once daily with food in the evening. After two to three months, the dose was increased to 10 mg/day (maximum recommended dose). Patients having difficulty tolerating the higher dose were maintained on a lower dose.

CSF samples were available from baseline, and after 12 and 24 months of treatment with donepezil. Changes in AChE activity and protein levels in the CSF of these patients were previously reported in detail [9]. Briefly, protein levels of the synaptic AChE-S have shown 112 ± 19% (mean ± S.E.M.) and 196 ± 17% increases in CSF samples collected at the 12 months from patients on 5 mg/day (n = 18) and 10 mg/day (n = 32) donepezil, respectively. The corresponding increases in the 24 months CSF samples have been 118 ± 22% and 187 ± 19%, respectively [9].

Thioflavin-T (ThT) fluorescence assay

The ThT method was used to monitor Aβ fibrilization [42, 43]. CSF samples from 48 AD patients in the SATS study were used. Samples were collected prior to treatment with donepezil (baseline), and after one and two years of treatment [9]. CSF samples were applied in triplicate (25 μL/well) in the wells of a microtitrering plate (NUN96fb, Lam/Nunc FluororoNunc) – Nunclon 96 Flat Black, DK). The final volume was adjusted to 100 μL per well with an Aβ-ThT master mix solution, which was freshly prepared by mixing recombinant Aβ42, Aβ40, and thioflavin-T (cat nr T5316, Sigma) in phosphate-buffered saline buffer (PBS, pH = 7.4). Ultrapure, HFIP-treated, recombinant amyloid β 1-40 (Aβ40, cat nr A-1153 from rPeptide, USA) and 1-42 peptides (Aβ42, cat nr A-1002, rPeptide) was used.

The Aβ peptides stock solutions were prepared in DMSO (dimethyl sulfoxide, D2650, Sigma) by rigorous vortexing, and sonication 10 times for three seconds on ice, and finally filtering the solution through a 0.2 μm filter unit (Spartan 3/0.2PA, Whatman Group, Germany). The stock solutions were freshly prepared at 850 μM (of Aβ42) and 400 μM (Aβ40) stock concentrations, based on the MW of the peptides provided by the manufacturer.

The final concentrations of Aβ42 and Aβ40 peptides in the wells were 2.17 μM and 20 μM, respectively (molar ratio Aβ42/Aβ40 of 0.11, based on physiological proportions observed in CSF). The final concentration of ThT was 1.0 μM.

The plates were then sealed with tape (Nunc, cat nr 236366, DK) with the lock on, and covered carefully with Parafilm to avoid evaporation. Incubation and reading took place with a Tecan spectrophotometer (Infinite 1000) at 37°C for 87 hours, using automatic bottom-reading function at 15-minute intervals at 450 nm excitation wavelength and 490 nm as the emission wavelength. Modification of the method was also made by using different CSF percentages (25%, 50%, and 75%).

AChE and BuChE activity measurements following ThT assay

At the end of ThT fluorescence measurements, each plate was copied twice by transferring 40 μL from
each well of the original plate using a multichannel pipette to the new plates. The volume was adjusted to 100 μl/well of sodium potassium phosphate buffer (50 mM, pH 7.4). These plates were immediately used to measure AChE and BuChE activities as described above [41]. Briefly, 100 μL of the MM or MMA solutions was applied to each well to measure BuChE and AChE activity. Wells containing either the buffer or just the Aβ peptides were the negative controls.

Statistical analysis

Data are given as mean and the standard errors of the mean (Mean ± S.E.M.). Repeated-measure ANOVA (RM-ANOVA) was used for the longitudinal effect of the treatment at 3-, 6-, and 12-month intervals, compared to baseline levels. RM-ANOVA was also used for three distinct measures of Aβ fibrillization (lag time, maximum, and rate) in the CSF samples from one and two years of donepezil treatment, compared to CSF collected at baseline. Finally, Pearson correlation analysis was used for the relationships between three measures of Aβ fibrillization and donepezil concentration in CSF of AD patients at two years. All results were visualized graphically using simple regression plots.

RESULTS

Donepezil but not phenserine treatment increases the CSF AChE activity

The schematic layout of the study is shown in Fig. 2a. None of the included patients were previously on treatment with any of the ChEIs.

The phenserine group showed a 14% significant decrease in CSF AChE activity, compared to baseline (p < 0.02, n = 10, Fig. 2b). This reduction in CSF AChE activity persisted throughout the study, although it only was statistically significant at three-month follow-up, compared to the baseline (Fig. 2b).

Patients who received placebo during the first three months showed no changes in the CSF AChE activity, compared to the baseline (Fig. 2b).

At the end of the first three months of the study, the placebo patients started receiving donepezil (Fig. 2a). These patients were on donepezil for 3 months (from month three to month six) of the study. There was a mild 9% increase in CSF AChE activity. This was statistically significant, compared to both the initial baseline (n = 10, p < 0.007) and to the end of the first three months when they were on placebo (the placebo group, n = 10, p < 0.0007).

At the end of the six-month follow up of the study, the initial placebo patients switched medication. After a 1-week washout period, donepezil was replaced by phenserine for the six- to 12-month period of the study. There was a significant 21% reduction of CSF AChE activity, as measured at the 12-month follow-up (p < 0.0003, n = 7).

Phenserine treatment does not elicit an increased AChE expression common to donepezil treatment

We also measured the protein expression of the synaptic AChE-S variant in CSF (Fig. 2c). At three-month follow-up, the expression of AChE-S protein showed a 17% increase in the CSF of the phenserine group and remained stable throughout the study (Fig. 2c). However, this mild increase was only statistically significant compared to baseline (p < 0.04, n = 10), but not to the placebo group (Fig. 2c).

Between three and six months, the expression of AChE-S in CSF of placebo patients switched to donepezil showed a 75% increase, compared to baseline (p < 0.0003, n = 10), and a 67% increase compared to the placebo period of the study (p < 0.0002, n = 10, Fig. 2c).

Between months six and 12, the medication switch from donepezil to phenserine was accompanied by 87% significant reduction of the CSF AChE-S protein level, which was measured at the end of the study (p < 0.0002, n = 7, Fig. 2c). Hence, the decreased CSF AChE-S expression is consistent with the observed reduction in the CSF AChE activity in this group (compare Fig. 2b with 2c).

These observations are clinically important as they suggest that: 1) Phenserine, despite being like donepezil a highly selective and reversible AChE inhibitor, did not induce AChE overexpression as donepezil treatment did in the same AD patients; 2) Rather, the changes in the CSF AChE levels following treatment with phenserine resemble that of rivastigmine, another carbamylating agent. Thus conceivably treatment with a carbamylating agent seems to normalize donepezil-induced increases in CSF AChE levels back to initial baseline levels.

Estimation of CSF AChE inhibition suggests that the dosage of phenserine may have not been optimal

Then we estimated the CSF AChE inhibition (Fig. 2d), based on the method described previously for the reversible ChEIs, such as donepezil and
galantamine [9, 44]. This procedure integrates the information on the measured changes in the overall CSF AChE activity and protein and has been shown to accurately estimate the in vivo AChE inhibition assessed by positron emission tomography [44].

This estimation suggested that phenserine treatment caused in average 20% (ranging between 15–30%) inhibition at the 3 and 6 months treatment follow-ups compared to the baseline (all p < 0.005, Fig 2d) or compared to the placebo-group (all p < 0.05). The estimated AChE inhibition with donepezil was about 43% (all p < 0.001, Fig 2d).

Thus although the in vivo AChE inhibition by phenserine is close to the reported 30–40% for galantamine [44], this finding may suggest that the dosage of phenserine may have not been optimal in the phenserine trial study.

Interaction between Aβ peptides and AChE leads to deactivation of AChE

Numerous reports indicate that AChE accelerates fibrillization of Aβ peptides [20, 25, 36, 45]. Other reports have shown that the interaction of Aβ peptides with AChE also leads to deactivation of this enzyme after prolong exposure to Aβ [20, 46–49].

We repeated and confirmed previous observations that were based on recombinant AChE protein (Fig. 3a). This experiment showed that prolong incubation (>72 hours) of AChE with recombinant Aβ peptides led to deactivation of approximately 80% of the AChE molecules (Fig. 3a).

However, the in vivo composition of extracellular fluid is much more complex than the buffer system of this experiment. Therefore, we used a series of pooled CSF samples and measured AChE activity with and without addition of Aβ peptides (Fig. 3b). This experiment strongly supported the findings with recombinant AChE (Fig. 3a). In addition, this experiment provided evidence that the interaction between Aβ peptides and AChE was highly specific and readily occurred in vivo, as almost identical results were achieved despite the complexity of the micro-environment in the CSF samples.

In another experiment, we examined if endogenous CSF Aβ peptides interacted with AChE in the CSF samples (Fig. 3c). Two major peaks were observed. One peak showed high AChE activity (Peak H) and the other peak showed approximately 10 times less activity (Peak L). We then performed immunoblotting analyses on some of the fractions from the peak H (lines 19–22, Blot-I) and some from the peak L with low AChE activity (lines 26–31). Immunostaining with an antibody against human AChE revealed that the peak L, in the SDG diagram had much more intense immunostained signals than those in the peak H. This indicated that the peak L contained largely inactivated AChE protein. The Blot-I was then stripped to remove the anti-AChE antibody, and then reprobed with an anti-Aβ antibody (Blot-II). We also used a replicate of Blot-I, and directly probed it with the anti-Aβ antibody (Fig. 3d). The findings indicated that the AChE protein bands in lines 26–31, were in complex with Aβ peptides (Fig. 3d, compare blots II and III with blot I). The CSF Aβ peptides mainly comigrated with the CSF fractions containing inactive/latent AChE (in other words, the peak L). These findings strongly support the observations in Fig. 3a and b that Aβ interact with AChE and that prolonged exposure of AChE to Aβ peptides leads to accumulation of inactive AChE in the CSF of AD patients.

Inherent properties of CSF on kinetics of Aβ fibrillization

Due to the native complexity of the microenviron-
ment in the parenchymal fluids, it is difficult to reliably interpret the in vitro data on kinetics of the fibrillization of Aβ peptides in the commonly used simple buffer systems, such as PBS. We hence decided to use CSF as a surrogate of the parenchymal fluids.

We found that Aβ fibrillization was generally extremely slow in AD CSF. For instance, no fibril-
\[Aβ_{42}\] lization occurred for up to 120 hours, even though we reduced the amount of CSF in the wells to 75% or 50% (data not shown). Arguably, AD brains contain fibrillar Aβ deposits which may act as seed for further and faster fibrillization and deposition of Aβ peptides. Thus, we prepared a fibrillar Aβ suspension in PBS, and included various volume of this prepara-
tion into the ThT assay setup. Even after the seeding, no increase in ThT fluorescence intensity occurred for up to 120 hours in the wells containing 75% or 50% CSF (data not shown). Rather, the fluorescence intensity was decreased, perhaps indicating disintegration of Aβ peptides from the added fibrillar Aβ seeds (data not shown).

We also found that merely mixing Aβ40 and Aβ42 peptides rather than using each peptide alone (another common feature for in vitro studies) caused dramatic changes in the kinetic properties of the recombinant Aβ peptides in PBS (Fig. 4a, the main ThT kinetic graphs). For instance, the lag-time of $Aβ_{42}$ fibrilliza-
tion was increased from ~4 to over 10 hours, as did its...
Fig. 3. Interaction of Aβ peptides with human AChE. a) Recombinant human AChE activity decreased by about 80% when incubated with a mixture of Aβ42 and Aβ40 peptides for 72 hours. b) Similarly, incubation of pooled CSF samples with the Aβ mixture caused a significant reduction in AChE activity. The final concentrations of Aβ42 and Aβ40 in the wells were 2.14 μM and 20 μM, respectively. c) Separation of contents of pooled CSF samples by sucrose-density gradient (SDG) technique showed two peaks of AChE activities, with 10-fold differences in AChE activity. In this particular experiment no recombinant Aβ peptides was added to the pooled CSF samples. The results show interaction of native Aβ and AChE at endogenous CSF levels in patients with AD. d) Western blot analysis of some of the fractions of peak H and peak L. Blot-I shows immunostained bands by the N19 antibody, which recognized the core domain that is common to all AChE splice variants. Comparison between relative amount of AChE protein detected in the peak L fractions (26–31) with that detected in the peak H fractions (19–22, Blot-I) and relative AChE activities in the SDG graph (c) suggest that AChE molecules in the peak L are mainly inactive enzyme. Reprobing the same blot (Blot-II) or a replicate of the blot (Blot-III) with the anti-Aβ 6E10 antibody reveals co-migration of the Aβ peptides with AChE protein in the peak L fractions. These, together with the observations in (a) and (b), strongly suggest that AChE proteins in the peak L fractions are deactivated by their interaction with the Aβ peptides.

The fibrillation rate (Fig. 4a). In addition, even after 87 hours the fibrillation did not reach to its maximum, compared to Aβ42 alone (Fig. 4a).

These observations highlight the importance of studying Aβ fibrillation or testing effect of candidate molecules with anti-Aβ fibrillation properties in microenvironments with similar complexity, which is expected in the brain parenchyma. In addition, they explain why Aβ deposition takes at least 20–25 years before reaching its plateau in AD brain [50].

Nonetheless, we optimized the ThT assay by reducing the amount of CSF to 25% of the final volume in the wells, setting the temperature at 37°C (37 ± 0.014°C), and using the final concentrations of 20.0 μM Aβ40 and 2.2 μM Aβ42, calculated based on the molecular weights provided by the manufacturer (Material and Methods). Due to the noted methodological limitations, we could not reduce the concentration of Aβ peptides further to the more in vivo-representative levels of low nano-molar concentrations (based on a range of 4–8 ng/mL Aβ40 levels in AD CSF). However, the ratio of Aβ42/A40 was kept physiological and representative of endogenous CSF level (~0.11).

Inherent properties of CSF on kinetics of Aβ fibrillation are changed by donepezil

We have previously reported ~110% to ~200% increases in the protein levels of the synaptic AChE-S in these CSF samples collected after 12 and 24 months from the patients on 5 and 10 mg/day of donepezil treatment [9]. These are consistent with observations in Fig. 2, indicating that donepezil treatment produces two main changes in the CSF samples [9]. First, there is a dose-dependent strong increase in AChE protein levels that is most pronounced after two years of treatment. There is also a time-dependent increase in donepezil concentration in CSF [9].
Fig. 4. Longitudinal changes of CSF properties on kinetics parameters of Aβ fibrillization with donepezil. (a) The average kinetic graphs of the Aβ fibrillization by thioflavine-T (ThT) fluorescence assay for recombinant Aβ42 and Aβ40 alone, or Aβ42/40 mixture (Aβmix) in PBS, as indicated in the graphs. The average kinetic readings for the Aβmix are also shown in CSF collected from AD patients at baseline and after 12 and 24 months of donepezil treatment. The amount of CSF was 25% of the final volume (100 μL) in the wells. The inset illustrates the assessed kinetic parameters of the Aβ fibrillization in CSF. b–d) Changes in the lag time (FLT), rate (FRate), and maximum (FMax) fibrillization of Aβ peptides in 12 to 24 months CSF samples, compared to baseline CSF in all patients, regardless of treatment dosage. ∗∗p < 0.01. The CSF properties display donepezil dosage-dependent changes after 24 months of the treatment. The CSF of the patients treated with 5 mg/day donepezil show a significantly higher anti-Aβ fibrillization property than those on 10 mg/day of donepezil. The final concentrations of Aβ42 and Aβ40 peptides in the wells were 2.17 μM and 20 μM, respectively (molar ratio Aβ42/40 of 0.11, based on physiological proportions observed in CSF). The final concentration of ThT was 1.0 μM. ∗∗∗p < 0.001 signifies differences compared to the baseline. **p < 0.01 signifies differences between the groups. ∗p < 0.09 shows trends compared to baseline.
Increases in the protein expression of AChE arguably may augment the fibrillization rate of Aβ peptides in vivo. We hence investigated the kinetics of Aβ fibrillization in the CSF (Fig. 4). The average kinetic profiles of Aβ1-42 fibril formation in these CSF samples are shown in the Fig. 4a. Three kinetic measures (lag time, rate, and maximum, see the inset in Fig. 4a) of the Aβ fibrillization process were determined after addition of Aβ peptide to the CSF samples collected at baseline, and after 12 and 24 months of treatment with 5–10 mg/day of donepezil.

RM-ANOVA revealed a significant effect for the fibrillization rate (FRate) of Aβ in the CSF samples taken two years after donepezil treatment, as compared to the baseline state (Fig. 4c, F = 5.7, p < 0.005, n = 32). No significant effects were found in the CSF collected one year after donepezil treatment (p < 0.082, n = 38).

Similar analyses on the maximum level of Aβ fibrillization (F Max) indicated that the F Max was significantly increased in CSF collected after the first (F = 5.5, p < 0.005, n = 38) and second year of treatment with donepezil, compared to the F Max measured at baseline (Fig. 4d, F = 4.9, p < 0.01, n = 32).

No significant effects were found for fibrillization lag time (FLT) in the CSF of AD patients after the first and second years of donepezil treatment (Fig. 4b).

Complementary between-group analyses examined the effect of donepezil dosages (the 5-mg and the 10-mg groups). ANCOVA analysis revealed a significant effect of the drug dosage (F = 7.3, p < 0.01) and the interaction between dosage and treatment length (F = 5.3, p < 0.03) on changes in the FLT of Aβ after 12 months. This analysis indicated that the FLT of Aβ was 40% shorter in the CSF of the 10-mg group (n = 25), compared to the 5-mg group (n = 11, p < 0.01, Fig. 4c).

Consequently, the FMax of Aβ in the CSF collected after 12 months of donepezil treatment showed about 2.5-fold increase in the 10-mg group (p < 0.01, Fig. 4d) but only about one-fold increase in the 5-mg group (p < 0.008), compared to baseline.

Similarly, a higher F Max of Aβ was observed in the CSF of patients in the 10-mg group, compared to the 5-mg group after two years of donepezil treatment (F = 8.31, p < 0.007, Fig. 4g). No significant difference was seen in the first year for any of the parameters (Fig. 4e–g).

These findings suggest that chronic donepezil treatment intensified the kinetics of Aβ fibrillization in the CSF of AD patients, in a dosage-dependent manner.

Changes in the kinetics of Aβ fibrillization correlate with CSF donepezil concentration

We then examined the effect of the reported time-dependent increase in the concentration of donepezil in the CSF [9], because donepezil and Aβ peptides interact with PAS of AChE [45].

Correlation analysis revealed significant positive linear relationships between FLT and donepezil concentration in CSF after two years of treatment (r = 0.46, p < 0.0008, n = 32, Fig. 5a). Consistently, donepezil concentration negatively correlated with FMax (r = −0.46, p < 0.008, n = 32, Fig. 5b) and FMax of Aβ fibrillization in the CSF (r = −0.59, p < 0.0003, n = 32, Fig. 5c). These observations indicate that high donepezil concentration delayed the onset, and reduced
Donepezil prevents interaction of Aβ with AChE. a) Addition of Aβ40/42 peptide mixture leads to inactivation of 50% of the activity of CSF AChE protein in the absence of donepezil in the CSF samples. However, the presence of donepezil in the samples effectively prevents the Aβ-induced inactivation of the AChE activity, particularly at the highest donepezil concentration. b) In contrast to AChE, the interaction of Aβ peptides boosts BuChE activity in CSF, consistent with previous reports [46, 47, 49]. Donepezil, due to its low selectivity toward BuChE, does not prevent the Aβ-induced boost of BuChE activity. This suggests that donepezil competitively displaces or prevents interaction of Aβ for the binding sites on AChE protein. Data are mean ± SEM. ∗∗∗ or ### or ## p < 0.0001 signify differences, as specified in the graphs. The donepezil concentrations are the average values determined in the undiluted CSF samples reported previously [9].

Donepezil disrupts the interaction of Aβ with AChE, but not with BuChE

To get more insight about these dual and apparently counteracting effects, we examined whether the latter was due to donepezil molecule-preventing interaction of Aβ with AChE, putatively by competing for PAS on AChE protein [45]. Therefore, we measured changes in the AChE activity in the CSF samples, before and after addition and incubation (>72 hours) with recombinant Aβ peptides. The rational is that if Aβ interacts with AChE, and donepezil prevents this, AChE activity will then show a strong reduction in the samples that did not contain donepezil, as was observed when recombinant AChE or pooled CSF samples was used (Fig. 4a, b). The AChE activity would not be affected by Aβ peptide to the same extent as in those samples that contained donepezil.

These analyses showed that the incubation of the baseline CSF samples with the Aβ peptides significantly decreased AChE activity, compared to AChE activity in the same samples incubated with the buffer (Fig. 6a). In contrast, addition of Aβ peptides to the CSF samples collected after donepezil treatment did not show a significant reduction in AChE activity, particularly in the two-year follow-up samples with higher donepezil concentrations (Fig. 6a).

Measurements of BuChE activity in the samples supported this observation (Fig. 6b). Donepezil binds with high selectivity to AChE, but negligibly to BuChE. Thereby it should not interfere with the interaction of Aβ with BuChE. Similar to our previous report, we found that adding Aβ to the CSF samples resulted in sustained hyperactivation of BuChE in all samples, regardless of whether they were collected before or after donepezil treatment (Fig. 6b and [49]).

These findings suggest that differential changes in AChE activity in the baseline samples, compared to those collected after one to two years of donepezil treatment, was most likely due to the presence of donepezil in the latter samples.

Donepezil concentration-dependently prevents Aβ-driven reduction in AChE activity

We then included donepezil dosage (Fig. 7a, c) and stratification based on the actual donepezil concentration measured in the undiluted CSF samples (Fig. 7b, d). These analyses indicated that Aβ-driven inhibition of the AChE activity was prevented in a donepezil dosage- and concentration-dependent manner (Fig. 7a and b, respectively). In contrast, Aβ-induced hyperactivation of BuChE showed no dependency on donepezil.

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### Table 1

<table>
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<th>Aβ40/42 (ng/mL)</th>
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Fig. 6. Donepezil prevents interaction of Aβ with AChE. a) Addition of Aβ40/42 peptide mixture leads to inactivation of 50% of the activity of CSF AChE protein in the absence of donepezil in the CSF samples. However, the presence of donepezil in the samples effectively prevents the Aβ-induced inactivation of the AChE activity, particularly at the highest donepezil concentration. b) In contrast to AChE, the interaction of Aβ peptides boosts BuChE activity in CSF, consistent with previous reports [46, 47, 49]. Donepezil, due to its low selectivity toward BuChE, does not prevent the Aβ-induced boost of BuChE activity. This suggests that donepezil competitively displaces or prevents interaction of Aβ for the binding sites on AChE protein. Data are mean ± SEM. ∗∗∗ or ### or ## p < 0.0001 signify differences, as specified in the graphs. The donepezil concentrations are the average values determined in the undiluted CSF samples reported previously [9].
Fig. 7. Donepezil competes concentration-dependently with Aβ peptides for PAS of AChE. a) CSF samples from AD patients treated with 5 mg/day donepezil showed highest Aβ-driven AChE inhibition. b) Stratification based on measured donepezil concentration indicated that samples containing ≤ 4 ng/ml exhibited the highest Aβ-driven AChE inhibition. c, d) Donepezil has very low affinity for BuChE. The Aβ-driven boost of BuChE activity [46, 47, 49] is not affected by donepezil. These observations provide additional evidence for a competitive displacement reaction between Aβ and donepezil for the putative, peripheral anionic-binding site (PAS) on AChE protein. Data are mean ± SEM. ∗∗∗ or # or p < 0.0001 signify differences, as specified in the graphs. ∗ or # or p < 0.05 signify differences between the ≤ 4 ng/ml group and the other two groups. The stratification based on donepezil concentrations are done on the actual values determined in the undiluted CSF samples reported previously [9].

dosage (Fig. 7c) or concentration (Fig. 7d). This was expected due to the negligible affinity of donepezil for BuChE. These analyses provide strong evidence that donepezil (and/or some of its metabolites) is responsible for disruption of the interaction between Aβ and AChE protein.

Overall, the observations describe two main parallel outcomes of chronic donepezil treatment. One outcome may be clinically detrimental, namely induction/accumulation of AChE protein in the brain. This might accelerate Aβ fibrillation and deposition in the brains of AD patients. The other outcome, which is beneficial, is caused by a direct competing interaction between donepezil molecules (or its metabolites) and Aβ peptides, putatively for the PAS on AChE. This counteracted the AChE-accelerated Aβ fibrillation. Another plausible outcome that is secondary to donepezil interfering with the interaction between Aβ and AChE, and preventing the potent Aβ-mediated deactivation of AChE, which may be involved in regulation of functional status of cholinceptive astroglial cells [51, 52].

**DISCUSSION**

We showed for the first time the effect of phenserine treatment on CSF AChE activity and protein levels in patients with AD. Phenserine produced sustained, but mild AChE inhibition in the CSF of AD patients. This is similar to the inhibition levels observed after treatment with low-dose rivastigmine [6], which is another carbamylating agent. However, phenserine is a selective AChE inhibitor with a reversible mode of action, whereas rivastigmine is a dual, pseudo-irreversible AChE and BuChE inhibitor [8]. Sustained AChE inhibition by phenserine also contrasted with the strong donepezil-induced elevation in AChE activity and protein levels in the placebo-donepezil arm of the study. This was consistent with studies of patients with AD.
after tacrine, donepezil, and galantamine treatment [9, 43, 54]. It is therefore tempting to conclude that carbamylation of the active site of AChE may result in inhibition of this enzyme without elevation of AChE protein expression. The rationale is that in contrast to rapidly reversible inhibitors, the pharmacodynamic half-life of carbamylating agents like phenserine and rivastigmine is directly related to the spontaneous reactivation of the carbamylated-AChE and in vivo turnover rate for synthesis of new enzymes. The biological turnover rate of AChE recovery has a half-life of 6–17 days in the brain (or even longer at neuromuscular junctions) [55, 56]. The reported pharmacodynamic half-life of AChE inhibition after a single oral dose of phenserine is 11 hours [57], which should become much longer after repeated dosages, while the spontaneous reactivation of AChE is practically negligible after rivastigmine [58]. Thus, the cells may sense that upregulation of AChE protein expression is ineffective against such carbamylating agents, and instead adopt another pathway to balance an excessive synaptic AChE inhibition. For instance, by a mild but specific increase in the secretory diffusion read-through AChE-R variant [10], as a decoy for the membrane-anchored synaptic AChE-S, and by changing the density of nicotinic ACh receptors in the synapses. This is supported by an observation based on PET estimation of in vivo brain nicotine binding sites in patients treated with rivastigmine, where we have shown an inverse association between enzyme inhibition and the in vivo nicotine binding in the brain [59]. In contrast, AChE inhibition by galantamine which like donepezil increases the expression of AChE in CSF did not alter the in vivo brain nicotine binding [44, 60].

Overall, the most important finding was the results from the placebo-donepezil-phenserine switching paradigm. It suggested that switching donepezil to phenserine led to significant regression of the elevated AChE protein expression in the CSF of the donepezil-treated patients. To the best of our knowledge, this is the first study in patients with AD showing that donepezil-induced elevation in the AChE protein expression is reversible, and is unlikely to be re-induced by switching to a carbamylating agent such as phenserine (and plausibly also rivastigmine [6]).

This observation may have important clinical implications. First, numerous in vitro and in vivo animal studies strongly suggest that elevation of AChE protein expression in the brains of AD patients may be harmful. It may be accompanied by significant increases in the Aβ deposition and pathology [25, 33, 34, 36, 61]. Nevertheless, although differences have been suggested in sub-populations [62–67], the long-term clinical efficacy of all three current ChEIs, rivastigmine, galantamine, and donepezil, is fairly similar in overall populations of AD patients despite their very different pharmacodynamic effect on AChE activity and protein expression [8, 54].

Understanding the underlying mechanism may therefore be essential to improve efficacy of the current treatment with ChEIs. We initiated a series of experimental paradigms in the current study with a collection of well-defined CSF samples collected prior to, and after one and two years of treatment with donepezil [9]. We used CSF samples from AD patients because changes in the CSF closely reflect the microenvironment of the brain parenchymal fluid at least with regards to matrix complexity, conceivably since no barrier exists between CSF, interstitial fluid, and brain tissues. In this context, CSF and brain levels of Aβ [50] and enzymes responsible for biodegradation and biosynthesis of ACh are also strongly related, although the relationship of the changes in CSF and interstitial fluid is not always straightforward [44, 50, 52, 68]. For instance, a decrease in CSF Aβ is related to an increase Aβ deposition in the AD brain [50], but most likely not in control brain, whereas changes in cholinergic enzymes directly reflect the overall in vivo changes in the brain, induced by aging, AD [10, 69], or treatment with ChEIs [44, 69]. Thus, at least as concerns levels of cholinergic biomarkers and Aβ, solid evidence suggests that CSF is a highly suitable surrogate of the ongoing events in the brain.

Nonetheless, the results indicated that chronic donepezil treatment produces three parallel pharmacodynamic effects in opposite directions. First, donepezil-induced elevation of AChE protein expression accelerated the kinetics of Aβ fibrilization in a dose-dependent manner. This was highly consistent with in vitro and in vivo animal studies. Second, donepezil concentration also correlated with reduction in the fibrilization of Aβ peptides, most likely by competing with Aβ peptides for binding to PAS on AChE [20, 36]. This partially counteracted AChE-accelerated Aβ fibrilization. Finally, donepezil is a potent inhibitor of AChE, which is its main therapeutic function. This prolongs the action of synaptic acetylcholine in the remaining functional cholinergic/cholinceptive neuronal interfaces in the brains of AD patients. In this regard, we have shown that the AD patients on 10 mg/day donepezil had 45–55% inhibition of synaptic AChE in CSF and this was related to stabilized MMSE test results after up to two years [9]. Using the same approach, we also estimated here
~40% in vivo AChE-S inhibition for the AD patients in the placebo-donepezil arm of the phenserine study, which were on 5 mg/day of donepezil for 3 months.

Thus, all inhibitors of synaptic AChE may produce similar dose-dependent enhancement of cholinergic neurotransmission in damaged but functional synapses of ascending cholinergic pathways where the quantity of synaptic ACHe may be controlled by the number of anchoring proteins available to bind ACHe to the membrane. In contrast, the ability to upregulate soluble ACHe (i.e., that is not membrane bound and can diffuse out of the synapse into the extracellular fluid) and/or inhibit BuChE, may induce markedly different effects on extracellular ACh levels, Aβ fibrilization, glial activation, and longer term effects on underlying disease mechanisms [51, 52, 62, 70].

Thus, given that the current and previous report suggest that a greater inhibition of the ACHe with higher doses of donepezil is also directly coupled to a larger elevation of ACHe expression in CSF (~200%, [9]), it is apparent that overall clinical efficacy of donepezil treatment may be significantly improved by preventing elevation of ACHe protein. In this regard, we showed that switching from donepezil to phenserine may at least partially normalize donepezil-induced ACHe elevation back to initial baseline levels. This provides a clinically testable approach.

Therefore, we propose that an add-on therapy with low-dose of the currently approved carbamylating drug formulation, i.e., small rivastigmine patch, for patients on long-term donepezil treatment may increase the therapeutic efficacy of rapidly reversible ChEIs. The small rivastigmine patch shows a superior adverse events profile, compared to its oral formulation [71]. The low-dose patch may be well-tolerated in combination with donepezil. We have shown that low-dose oral rivastigmine capsule produces a mild 10–15% suppression of synaptic ACHe activity [6]. This is essentially identical with observations following 12 months of phenserine treatment in the current study. Thus, concomitant donepezil and low-dose rivastigmine patch treatment may offer superior pharmacodynamic profiles and clinical efficacies compared to monotherapy.

Rivastigmine may prevent induction of ACHe protein expression by donepezil and thereby slow ACHe-accelerated Aβ fibrilization. Donepezil molecules will then more effectively compete with Aβ for the interaction sites on ACHe, which may prove to have a disease modifying impact with regard to Aβ deposition in the brain [72].

The combination may in addition increase penetration of both rivastigmine and donepezil to the brain. Donepezil may by inhibiting for instance the red blood cells (RBC-) ACHe activity in the circulation, reduce ACHe-mediated metabolism of rivastigmine, thereby prolonging the short pharmacokinetic half-life of rivastigmine in peripheral circulation, allowing more rivastigmine molecules to reach CNS compartment. Rivastigmine in turn by pseudo-irreversible inhibition of RBC-ACHe activity (and thereby by displacing donepezil from one of its binding sites on ACHe) may increase the unbound fraction of donepezil molecules in circulation. These mutual effects may be substantial given that RBC-ACHe activity is over two-fold higher than BuChE activity in circulation [52], and that ~96% of donepezil is found bounded to the plasma proteins (and by definition to RBC-ACHe). We have shown that plasma donepezil concentration is 10-fold higher than in the brain [9]. Increased donepezil penetration in the brain may hence enhance prevention of ACHeE-accelerated Aβ fibrilization.

Rivastigmine also inhibits BuChE, thereby adding pharmacodynamic benefits related to preventing extra synaptic ACHe breakdown by BuChE [70], which is not affected by donepezil monotherapy. Analysis of the kinetics of Aβ fibrilization in the CSF samples collected at baseline, compared with those collected after one and two years of donepezil treatment, revealed how outcome of interaction between Aβ and BuChE, differed, compared to interaction with ACHe. Prolonged exposure of ACHe to Aβ led to accumulation of a pool of inactivated ACHe. This increased significantly by adding recombinant Aβ peptides to the CSF samples. In contrast, CSF BuChE activity was strongly boosted by the excess Aβ peptides.

These observations were consistent with our previous reports [47–49], which further suggest that Aβ peptides, together with high ApoE protein interact physically with BuChE and ACHe, leading to formation of highly stable and soluble BuChE/ACHe-Aβ-ApoE complexes (BAβ-AcEβ) in CSF [47–49]. In AD CSF, the BAβ-AcEβ appear dormant but gain ultrafast ACHe hydrolyzing activity with addition of Aβ peptides [46–49, 52], as was also shown in the current study. This indicates that Aβ allosterically modulate the intrinsic ACHe-hydrolyzing capacities of ACHe and BuChE in two opposite directions. Evidence suggest that Aβ also modulate the activity of the ACHe-synthesizing enzyme, choline acetyltransferase, either by oxidative pathway [73] or by changing the phosphorylation state of choline acetyltransferase [74]. Thereby Aβ may act as a physiological modulator of cholinergic function at concentrations, which are insufficient to induce neurotoxicity but that can alter both
SYNAPTIC AND Extrasynaptic Ach Levels, Resulting in Both Cholinergic Hypo- and Hyper-Function Related to Neuronal Signaling, to Functional Status of Non-Neuronal Non-Excitable Cholinopoietic Cells, Such as Microglia, Astrocytes, Oligodendrocytes, Endothelia, and Vascular Smooth Muscles, or Both in the Brain [51, 52]. In a Reciprocal Manner, Dereguulated Cholinergic Activity May Result in Increased Aβ Production or Reduced Clearance due to Dysfunctional Cholinopoietic Cells [51, 52].

These Findings May Be Clinically Important for New Therapeutic Strategies, Such as Immunization or Beta-Sheet Breaker Therapy, That Involve Preventing Aβ Filibrization or Resolving the Aβ Deposit to Its Monomeric Aβ Peptides. For Example, Solubilizing Aβ Deposits of the Cerebral Amyloid Angiopathies, Which by Definition Surround the Micro-Blood Vessels in the AD Brain, Can Theoretically Lead to a Strong Increase of BuChE Activity in the Vicinity of These Blood Vessels. This May Strongly Deplete Extrasynaptic Ach Levels and Disturb the Proper Functional Status of Cholinopoietic Microglia and/or Astrocytes, Endothelial, and Smooth Muscle Cells Comprising the Main Component of Micro-Blood Vessels in the Brain [75, 76]. This May Lead to Micro-Hemorrhages in the Brain, a Phenomenon That Has Happened in Some of the Immunization Trials in AD Patients [77].

Our Proposed Strategy to Combine Low-Dose Patch Rivastigmine with the Recommended Dose of Donepezil Fairly Relies on the Underlying Rationale for Developing and Synthesizing Huprine-Tacrine [78] or Donepezil-Based Hybrid Multi-Targeted Molecules [72, 79]. One Moiety Would Be an Active-Site ChE Inhibitor, While the Other Moiety Would Have High Affinity to PAS. This Would Prevent AchE-Induced Aβ Aggregation [72, 78, 79].

However, a Clinically Important Advantage of Our Add-On Strategy Is the Pharmacological and Toxicological Data for Both Rivastigmine and Donepezil Is Already Well-Known in a Large Population of Patients With AD and MCI. In Addition, Both Drugs Are Available in the Appropriate Formulations.

We Did Not Perform Any Donepezil-Anti-AchE-Aβ Experiment in PBS, Because Such Analyses Have Already Been Done by Others [36], Although Admittedly at Much Higher Donepezil Concentration (100 μM in PBS) Than We Have Reported in the Current CSF Samples (10-20 nM in CSF = 4-9 ng/mL, Based on Mw = 433.97). Given That the Amount of CSF Used in the Current Aβ Filibrization Experiment Was 25%, the Actual Donepezil Concentration in the Wells Was 2.5-5.0 nM (i.e., One-Fourth of the Concentrations in Undiluted Samples). The Observed AchE-Aβ Antagonizing Potency of Donepezil Relative to These Concentrations Compared to That Used in PBS [36] Therefore Suggests That Some of Donepezil’s Metabolites May Also Be Involved, Which Warrants Further Investigation on AchE-Aβ Antagonizing Properties of the Metabolites of Donepezil.

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