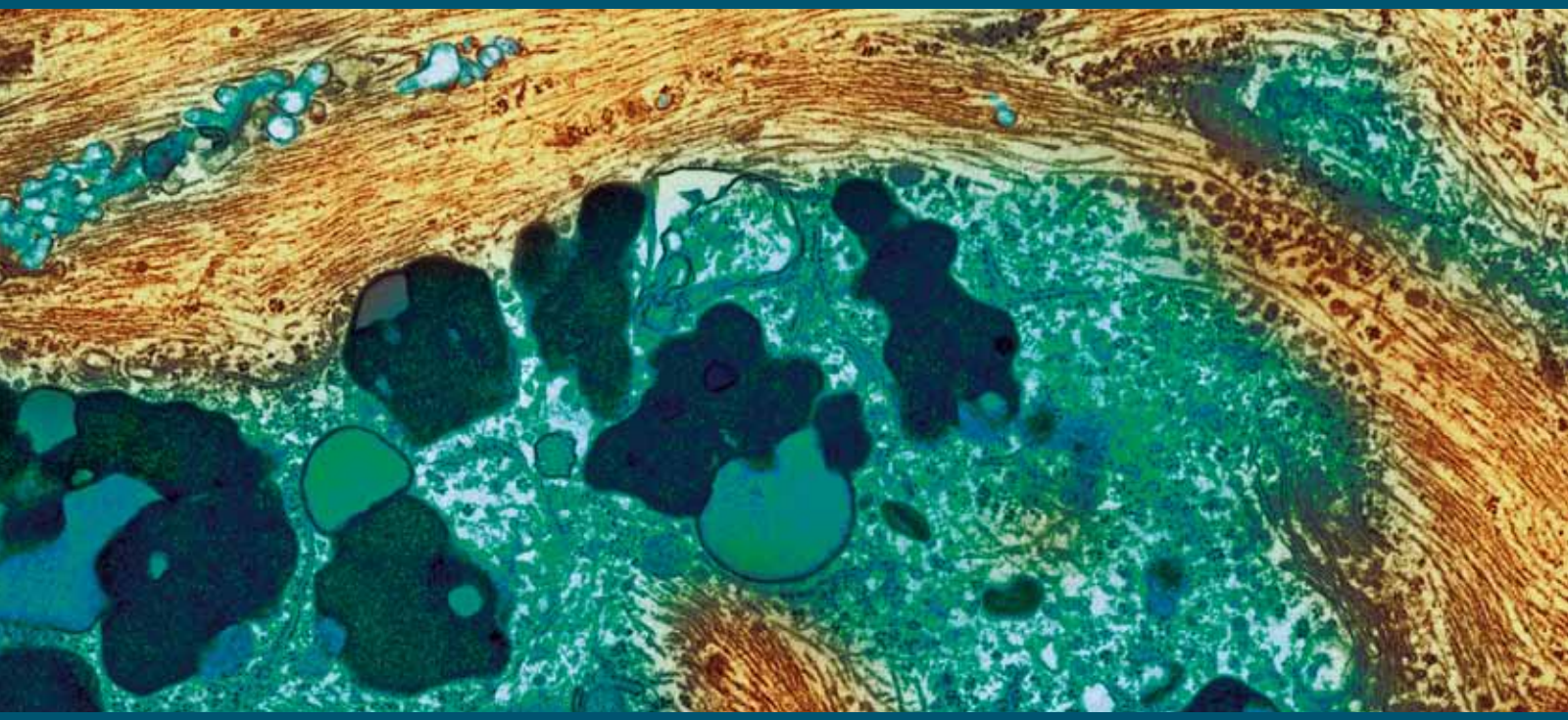
International Journal of Alzheimer's Disease

Novel Therapeutics in Alzheimer's Disease

Guest Editors: Marwan Sabbagh, Anton P. Porsteinsson, Anil Nair, and Abdu Adem



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Editorial Board

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Editorial

Novel Therapeutics in Alzheimer's Disease

Marwan Sabbagh,[1] Anton P. Porsteinsson,[2] Anil Nair,[3] and Abdu Adem[4]

[1] *Banner Sun Health Research Institute, 10515 W. Santa Fe Dr, Sun City, AZ 85351, USA*
[2] *Alzheimer's Disease Care, Research and Education Program (AD-CARE) and Memory Disorders Clinic, University of Rochester School of Medicine and Dentistry, 435 East Henrietta Road, Rochester, NY 14620, USA*
[3] *Quincy Medical Center and Alzheimer's Disease Center/Clinical Trials, 114 Whitwell St, Quincy, MA 02169, USA*
[4] *Department of Pharmacology & Therapeutics, Faculty of Medicine & Health Sciences, UAE University, Al Ain, UAE*

Correspondence should be addressed to Marwan Sabbagh, marwan.sabbagh@bannerhealth.com

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Alzheimer's disease is a progressive disorder characterized by significant cognitive, functional, and behavioral dysfunction. Progressive symptomatic decline appears inevitable at present even with the available therapies, and therefore additional treatment options are urgently needed for a growing epidemic that affects 27 million people worldwide. Currently there are 75 drugs in clinical trials and other 200 or more in development. The drugs being developed are targeting different intra- and extra-cellular targets as well as different mechanisms of action. They include both symptomatic and disease modifying approaches. Nevertheless, the development of drugs that: cross the blood brain barrier; exert an identifiable mechanism of action; have good safety and toxicity profiles; have a robust clinical benefit is 1 becoming increasingly daunting.

In this special edition of the International Journal of Alzheimer's Disease, we have three original submissions and five reviews accepted. The paper by A. J. Bishop et al. examines Centella asiatica extract in an AD mouse model. Centella asiatica (CA), common name gotu kola, is an Ayurvedic herb used to enhance memory and nerve function. Orally administered centella asiatica attenuated beta-amyloid-associated behavioral abnormalities in TG 2576 mice. Another original submission is by April and colleagues. In their study, they engineered specific SOFA-HDV ribozymes, a new generation of catalytic RNA tools, to decrease APP mRNA levels. Additionally, they demonstrated that APP-ribozymes are effective at decreasing APP mRNA and protein levels as well as Aβ levels in neuronal cells. Also there was an original submission by Ma et al. In their study, New Zealand rabbits were fed 2% cholesterol-enriched diet for 6 weeks as a model of BBB disruption in vivo and then were fed chow supplemented with 2% cholesterol with or without 5 mg/kg/d simvastatin for an additional 4 weeks to assess BBB integrity with and without simvastatin. They find that simvastatin improves disturbed BBB function both in vivo and in vitro.

In addition to the original submission, five reviews were accepted. The review by Ryan and colleagues provides a timely and critical review of immunotherapies being developed for the treatment of AD. This is of great interest because the clinical trials of passive immunotherapies are nearing conclusion of phase III studies. The review by Ladurner and colleagues reviews the literature on transcranial magnetic stimulation (TMS) for the treatment of AD. TMS is entering phase II studies in the US in 2012 as a symptomatic treatment for AD. Early data suggest a demonstrable benefit but long-term benefit is not established. The review by Beccano-Kelly summarizes the body of knowledge around leptin as a potential treatment for AD since dysfunctions in the leptin system have recently been linked to neurodegenerative disorders such as Alzheimer's disease. This is based on the observation that leptin has widespread action in the CNS, and evidence is growing that leptin has the capacity to modulate higher brain functions. Leptin appears to have an effect on hippocampal-dependent function and in particular learning and memory processes. The review by Mehta et al. reviews new and developing acetylcholinesterase inhibitors as symptomatic treatments for AD. It turns out that there are dozens of synthetic analogues and hybrid compounds far

beyond the approved donepezil, tacrine, rivastigmine, and galantamine. The review by Berk and Sabbagh that looks critically at the justification of using the new higher doses of donepezil in the treatment of moderate to severe Alzheimer's disease.

Marwan Sabbagh
Anton P. Porsteinsson
Anil Nair
Abdu Adem

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Research Article

Centella asiatica Extract Improves Behavioral Deficits in a Mouse Model of Alzheimer's Disease: Investigation of a Possible Mechanism of Action

Amala Soumyanath,[1] Yong-Ping Zhong,[1] Edward Henson,[1] Teri Wadsworth,[1] James Bishop,[1] Bruce G. Gold,[1] and Joseph F. Quinn[1,2]

[1] *Department of Neurology, Oregon Health & Science University, Portland, OR 97239, USA*
[2] *Department of Neurology, Portland VA Medical Center, Portland, OR 97239, USA*

Correspondence should be addressed to Amala Soumyanath, soumyana@ohsu.edu

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Centella asiatica (CA), commonly named gotu kola, is an Ayurvedic herb used to enhance memory and nerve function. To investigate the potential use of CA in Alzheimer's disease (AD), we examined the effects of a water extract of CA (GKW) in the Tg2576 mouse, a murine model of AD with high β-amyloid burden. Orally administered GKW attenuated β-amyloid-associated behavioral abnormalities in these mice. *In vitro*, GKW protected SH-SY5Y cells and MC65 human neuroblastoma cells from toxicity induced by exogenously added and endogenously generated β-amyloid, respectively. GKW prevented intracellular β-amyloid aggregate formation in MC65 cells. GKW did not show anticholinesterase activity or protect neurons from oxidative damage and glutamate toxicity, mechanisms of current AD therapies. GKW is rich in phenolic compounds and does not contain asiatic acid, a known CA neuroprotective triterpene. CA thus offers a unique therapeutic mechanism and novel active compounds of potential relevance to the treatment of AD.

1. Introduction

Centella asiatica (L.) Urban, family Apiaceae (CA), is known as *Mandookaparni* or *Brahmi* in Ayurvedic medicine. It is highly regarded as a "rasayana" or rejuvenating herb [1] and is reputed to increase intelligence and memory [1]. The dried herb has enjoyed growing popularity in the USA and other Western countries, where it is sold as the dietary supplement "gotu kola" [2].

Cognitive effects of the aqueous extract of CA (100–300 mg/kg/day) have been evaluated in several rodent studies using standard tests including shuttle box, step-through paradigm, elevated plus maze, and passive avoidance tests. CA extract markedly improved learning and memory of wild-type rats [3], rats subjected to CNS toxicity (intracerebroventricular streptozotocin) [4], and pentylenetetrazole (PTZ) kindled rats [5]. When administered to neonatal mice from day 15 to 30 postpartum, the extract caused significant enhancement in learning efficiency and spatial memory with no effects on locomotor function [6]. Direct neurotropic effects of CA have also been reported. CA aqueous extract caused significant increases in dendritic arborization of apical and basal dendrites in hippocampal neurons of neonatal mice [6] and both adult [7] and neonatal [8] rats. These studies, performed in diverse settings, show that CA water extract has biological effects of relevance to memory, learning, and aging, and potentially to disease progression in Alzheimer's disease (AD).

The present study examines the effect of a water extract of CA on behavioral deficits in the Tg2576 transgenic mouse, a murine model of AD. The Tg2576 or "Hsiao" transgenic mouse has been described in detail [9, 10] and is one of the most widely used animal models of AD. A mutant human amyloid precursor protein (APP) gene inserted into the genome gives rise to age-dependent hippocampal and cortical β-amyloid (Aβ) plaques similar to AD pathology.

Plaques are not histologically evident until 10–12 months of age, and plaque pathology is confined to the hippocampus and cerebral cortex. In other words, the age and region dependence of pathology in AD are nicely recapitulated in this strain. Other features of AD reproduced in this strain are astrocytic and microglial activation surrounding the Aβ plaques and dystrophic changes in neurites in the vicinity of plaques [11]. Importantly, these mice display abnormalities in standard behavioral tests. Open-field behavior has been shown to distinguish Tg2576 from wild-type mice, with Tg2576 mice more active in the open field than their wild-type littermates [12]. Hippocampal dysfunction, resulting in impaired spatial memory, is evident in the Morris water maze and has been repeatedly shown to distinguish aged, Aβ plaque-bearing Tg2576 from wild-type mice [9, 13]. These behavioral tests were utilized in the present study.

In addition to *in vivo* studies, the present work examined possible mechanisms underlying CA effects in the Tg2576 mouse, using *in vitro* models. The mechanisms examined include cholinesterase inhibition and neuroprotectant effects against oxidative damage, glutamate toxicity, and beta amyloid toxicity. The ingredient compounds in CA aqueous extract were also investigated.

2. Materials and Methods

2.1. Aqueous Extraction of CA. Dried CA was purchased from Oregon's Wild Harvest, Sandy, OR (Batch no. GOT-10072C-OGA). The identity of the herb was verified by means of visual examination and by comparing its thin layer chromatographic profile with that reported in the literature [14]. A dried water extract (GKW) was prepared by refluxing CA (120 g) with water (1.5 L) for 2 hr, filtering to remove plant debris and freeze-drying to yield a residue (11.5 g).

2.2. Chemical Analysis of CA Extracts. Water and ethanolic extracts of CA were compared by high-performance liquid chromatography coupled to UV detection (LC-UV) and mass spectrometry (LC-MS). The extracts were chromatographed alongside commercial reference standards of asiatic acid, madecassic acid, asiaticoside, and madecassoside (ChromaDex, Irvine, CA). Analysis was performed using LC-MS in negative ion mode on an LCQ Advantage ion trap mass spectrometer (Thermo Electron, San Jose, CA) with an in-line Surveyor autosampler and HPLC (Thermo Electron) coupled to a Surveyor Photodiode array detector (Thermo Electron). HPLC used an Aquasil 5 μm C18 150 $\times$ 2.1 mm column eluting with a gradient of acetonitrile in water both with 0.01% formic acid (acetonitrile 5% to 25% in 20 min, to 40% at 35 min, 60% at 40 min, 75% at 45 min and then returning to starting conditions).

2.3. Administration of GKW to Mice. Fifteen Tg2576 and 20 wild-type 20-month-old female mice were committed to this experiment. Approximately half of each genotype group was administered GKW in the drinking water at a dose of 2 mg/mL of water, with water bottles changed every other day, calculated to yield 200 mg/kg/day, a dose previously shown to improve memory in wild-type rats [3]. The GKW-treated group included 8 Tg2576 and 10 wild-type mice; the untreated group included 7 Tg2576 and 10 wild-type mice. Treatment was continued for 2 weeks, the duration of treatment in published reports showing behavioral effects in wild-type animals [3]. Open-field behavior and Morris water maze testing were performed at the end of this period.

2.4. Open-Field Testing of Mice. Mice were placed in the center of a square arena (38 $\times$ 38 $\times$ 64 cm high) constructed of white acrylonitrile butadiene styrene for two 5-minute open-field sessions on each of three consecutive days. Distance moved and velocity of each mouse were continuously tracked with a digital camera and "ANY-maze" software (ANY-Maze, Inc., Greensburg, PA). After a 5-minute epoch was recorded, the mouse was returned to the cage for 5 minutes and then retested. For each mouse, two tests were conducted on each of 3 days.

2.5. Morris Water Maze Testing of Mice. A subset of the treated animals (5 untreated wild-type, 5 GKW-treated Tg2576, and 5 untreated Tg2576) was also tested in the Morris water maze, a well established assay of hippocampal spatial memory [15]. This paradigm tests the animal ability to learn and remember the spatial location of a platform submerged 1 cm in a 109 cm circular pool of opaque water. The mice were habituated to the examination room and holding cages for two days and then received two days of "nonspatial training" before commencement of hidden platform testing. Each nonspatial training trial comprised placing the mouse on the submerged platform for 60 sec and then placing the mouse in close proximity (within 2-3 cm) to the platform and allowing it to climb onto the platform from the water. Training on the hidden platform water maze task began 24 hr after the last habituation trial. At this stage, curtains were opened to permit the mice visual access to extra maze cues in the room surrounding the maze. Hidden platform training was conducted over 16 trials on 4 consecutive days (4 trials/day). The platform remains in a fixed position throughout hidden platform training. During a given trial, the mouse is introduced into the pool at one of four randomly chosen start points (N, S, W, and E) and allowed 60 sec to find the platform. All trials were monitored by a video camera positioned above the pool and the behavior of each mouse was acquired by a computerized video tracking system (ANY-Maze software). Dependent measures acquired in each trial include the escape latency (i.e., time to find the platform, in sec), the cumulative distance (in cm) of the mouse from the platform, and swim speed (in cm/sec). No mice in this group needed to be excluded based on difficulty in swimming, in climbing onto the platform, or exhibiting abnormal swimming patterns or persistent floating.

2.6. Brain Levels of Soluble and Insoluble Aβ. Brain levels of Aβ were measured in experimental mice at the conclusion of behavioral testing described above. Tg2576 mice were sacrificed and brains rapidly harvested, divided, and frozen until analysis. Cortical tissue was then homogenized in buffered saline and protease inhibitors and ultracentrifuged to yield

a "soluble" fraction, as we have reported previously [16–18]. The remaining pellet was rehomogenized and incubated in guanidine and buffer and subsequently ultracentrifuged to yield a "fibrillar" fraction. Concentrations of $A\beta_{1-40}$ and $A\beta_{1-42}$ were determined in each supernatant using commercial ELISA kits, which distinguish the two isoforms (BioSource international, Camarillo, CA). Total protein in each fraction was determined by the Bradford method.

2.7. Neurotoxicity Induced by Extracellular Aβ. SHSY5Y neuroblastoma cells were grown in DMEM/F12 medium (from Gibco) containing fetal calf serum (FCS; 10%), streptomycin sulphate (100 μg/mL), and penicillin G (1000 U/mL) in a humidified air/5% CO_2 chamber at 37°C. On day 1, the cells were plated in 24-well plates (100,000 cells/well).

On day 4, cells were washed with FCS-free medium and further incubated in FCS-free DMEM/F12 containing neuroblastoma growth supplement N-2 (1%; Gibco). GKW (0–200 μg/mL) was added and incubated overnight. On day 5, cells were exposed to $A\beta_{25-35}$ (American Peptide Company) at 20 or 50 μM for 48 hrs in the presence of GKW. For this assay, fibrillar Aβ solution was freshly prepared by sonicating a solution of $A\beta_{25-35}$ in medium for 1 minute prior to addition to cell cultures. On day 7, the supernatant was harvested for LDH cell integrity assay; LDH release is a marker of cell damage [19]. Fresh medium was added and cell number assessed using CellTiter-Blue reagent (resazurin, Promega), incubating for 2 hours and reading fluorescence at 560 nm excitation and 590 nm emission.

2.8. Intracellular Aβ-Induced Neurotoxicity in MC65 Cells. MC65 cells are an established neuroblastoma cell line that conditionally express the C-terminal fragment of amyloid precursor protein (APP CTF) [20]. Following withdrawal of tetracycline from the media, the cells generate endogenous Aβ and die within 3 days. Expression of APP CTF in MC65 cells leads to the formation of intracellular Aβ aggregates. Previous studies have shown a strong correlation between these aggregates and subsequent cytotoxicity that is associated with oxidative stress [21]. MC65 cells were cultured and maintained in MEMEα supplemented with 10% FBS (Gibco-BRL, Carlsbad, CA) and 1 μg/mL tetracycline (Sigma-Aldrich, St. Louis, MO) as previously described [21, 22]. Confluent cells were trypsinized, washed with PBS, resuspended in OptiMEM without phenol red (Gibco/BRL, Carlsbad, CA), and plated at 25,000 cells/well in 48-well plates in fresh medium containing vehicle or desired concentrations of GKW, in the absence of tetracycline. Cell viability was measured at 2.75 days using the CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI) according to manufacturer's instructions. Experiments were carried out in triplicate wells for each condition and repeated 1-2 times.

2.9. APP CTF Aggregation in MC65 Cells. Cells were plated in culture dishes without tetracycline in the presence or absence of 100 μg/mL GKW. Cells were harvested at 2 days and lysates prepared by sonication and heating in Laemmli sample buffer. Samples were reduced then separated on tricine gels, transferred, and western blotted using mouse monoclonal antibody 6E10 that recognizes $A\beta_{1-17}$.

2.10. Aβ-Induced Nitric Oxide in Macrophages. RAW 264.7 cells were cultured in complete phenol red-free DMEM containing 50 units/mL of penicillin, 50 mg/mL of streptomycin, 44 mM sodium bicarbonate, and 10% fetal bovine serum (complete medium) at 37°C in humidified air containing 5% CO_2. Cells were plated in 24-well plates (approximately 1×10^5 cells/well) and cultured for 2 days until cells reached 80% confluency. Cells were washed, treated with fresh complete media containing various concentrations of GKW for 1 hour, and then induced with LPS (100 ng/mL) or $A\beta_{1-42}$ (20 μM) for an additional 24 hours. Aβ fibril formation was induced by incubating Aβ at 37°C overnight prior to addition to cell cultures. Nitrite was determined in supernatants from treated macrophages using Griess reagent as described previously [23, 24]. Nitrite concentrations were determined using dilutions of sodium nitrite in complete medium as a standard.

2.11. Anticholinesterase Activity. Cholinesterase activity was measured by the method of Ellman et al. [25], which is based on the formation of a yellow reaction product when thiocholine is liberated from acetylthiocholine and combines with the test reagent, dithiobisnitrobenzoic acid (DTNB). Potential cholinesterase inhibitory effect of GKW was tested directly in an assay using mouse plasma cholinesterase. DTNB buffer (80 μL) was added to a 96-well plate, followed by mouse plasma (5 μL), and then test agent (5 μL), consisting of GKW solution (2.5, 25, and 250 μg/mL), neostigmine (2.5 μg/mL; positive control), or buffer (negative control) and the solution was warmed to 37°C. The substrate, acetylthiocholine, was then added and absorbance recorded at 450 nm for 5 minutes.

2.12. Glutamate-Induced Neurotoxicity. Potential protection against glutamate-induced toxicity was examined in cortical neurons obtained from neonatal Sprague-Dawley rats. On day 1, cells were plated (200,000 cells per well) in 48-well plates using neurobasal medium supplemented with B27 (2%) containing antioxidants, 2 mM L-glutamine (Gibco). On day 4, the cells were treated with Ara-C (5 μM) to remove glial cells. On day 7, cells were washed with prewarmed CSF buffer (2x, 0.5 mL/well) then incubated with neurobasal medium, plus L-glutamine (2 mM) and B27 (2%) minus antioxidants, overnight. On day 8, sodium glutamate (0–1000 μM) with or without GKW (0–200 μg/mL) was added to the medium and the cells incubated overnight. On day 9, fresh medium was added and cell number evaluated using CellTiter Blue reagent (resazurin, Promega), incubating for 2 hours and reading fluorescence in a fluorimeter at 560 nm excitation and 590 nm emission.

2.13. Antioxidant Activity. Potential antioxidant effects of GKW were assessed *in vitro* in SH-SY5Y cells treated with hydrogen peroxide as an oxidative stressor. SHSY5Y neuroblastoma cells were grown in DMEM/F12 medium (from Gibco) containing fetal calf serum (FCS; 10%), streptomycin sulphate (100 μg/mL), and penicillin G (1000 U/mL) in

a humidified air/5% CO_2 chamber at 37°C. On day 1, the cells were plated in 24-well plates (100,000 cells/well). On day 4, medium was replenished with fresh medium containing NGF (10 ng/mL). After 24 hours (day 5), GKW (0, 50, 100, and 200 μg/mL) was added and the cells incubated overnight. On day 6, medium containing GKW was replaced with fresh medium containing less FCS (2%) and NGF (10 ng/mL). Cells were treated with a range of concentrations of H_2O_2 (0–500 μM) for 2-3 hours. Medium containing peroxide was removed and cells incubated overnight in fresh medium containing FCS (2%) and NGF (10 ng/mL). On day 7, supernatant was harvested for lactate dehydrogenase (LDH) cell integrity assay (102). Fresh medium was added and cell number evaluated using CellTiter Blue reagent (resazurin, Promega), incubating for 2 hours and reading fluorescence at 560 nm excitation and 590 nm emission.

3. Results and Discussion

The water extractable compounds (GKW) represented approximately 10% of the dry weight of CA herb. There was no difference in water consumption between control animals and animals receiving water containing GKW.

Open-field testing (Figure 1) showed that GKW treatment caused an improvement in behavioral abnormalities seen in Tg2576 mice. Wild-type mice, both GKW treated and untreated, were less active on the second trial of each day, presumably due to habituation. Untreated Tg2576 mice, in contrast, failed to habituate to the surroundings. However, GKW-treated Tg2576 mice explored in a manner similar to wild-type mice, with their data overlapping the two wild-type groups ($P = 0.02$ for difference between untreated and GKW-treated Tg2576 mice by ANOVA). "Normalization" of open-field behavior in Tg2576 mice has also been reported with an intervention that suppresses soluble Aβ levels [12].

In the Morris water maze paradigm (Figure 2), GKW treatment improved the impaired learning ability evident in Tg2576 mice. Wild-type animals exhibit a "learning curve" requiring less time and less distance to find the hidden platform with repeated trials, while untreated Tg2576 mice require equivalent time and distance despite repeated exposures. In contrast, the GKW-treated Tg2576 mice learn in a manner similar to the wild-type animals, with latency and distance traveled to find the platform declining with repeated exposures. On day 4, time to find the platform was significantly greater for untreated Tg2576 mice than for wild-type ($P = 0.002$) or GKW-treated Tg2576 mice ($P = 0.004$). Distance traveled to find the platform was also significantly greater on day 4 for untreated Tg2576 mice compared to wild-type ($P = 0.006$) or GKW-treated Tg2576 mice ($P = 0.003$). There was no significant difference between groups in the "visible platform" control for sensorimotor function (data not shown).

Since treatment with GKW ameliorated a spatial memory impairment in Tg2576 mice, which is specifically associated with the appearance of Aβ plaques, without producing any change in wild-type mouse memory, it would appear that the observed effect of GKW is specific to Aβ. However, Figure 3 shows that there were no significant differences

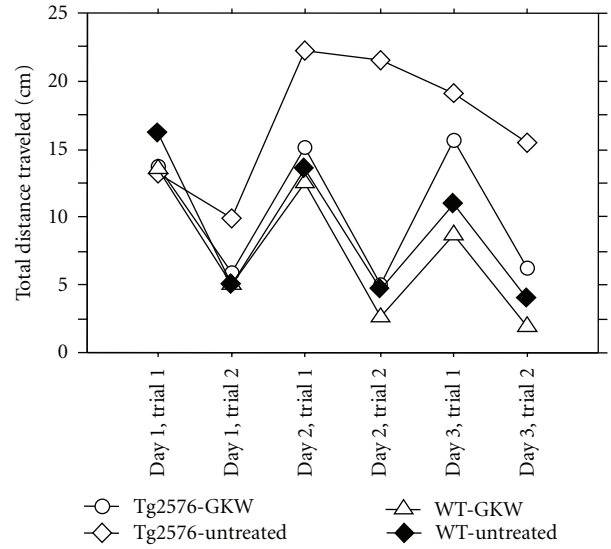

Figure 1: Open-field assay: effect of GKW on total distance travelled. Wild-type mice, both treated (triangle) and untreated (filled diamond), are less active on the second trial of each day, presumably due to habituation. Untreated Tg2576 mice (open diamond), in contrast, fail to habituate to the surroundings. GKW-treated Tg2576 mice (circles) explore in a manner similar to wild-type mice, with their data overlapping the two wild-type groups. From statistical analysis (ANOVA), $P = 0.02$ for difference between untreated ($n = 7$) and GKW-treated ($n = 8$) Tg2576 mice; error bars omitted for clarity.

between levels of any of the forms of Aβ in treated and untreated Tg2576 mice. This is in contrast to results obtained in PSAPP mice, a model for Alzheimer's disease (AD) where mice express both amyloid precursor protein and presenilin 1 mutations, in the long term (8 months). In these mice, administration of CA extract displayed *in vitro* antioxidant effects and also reduced beta amyloid plaque burden [26]. The PSAPP mice develop amyloid plaque pathology at an earlier age than Tg2576 [27], permitting more rapid completion of antiamyloid experiments. However, loss of the age- and region-dependence of pathology diminishes the fidelity of this strain to some extent. Since GKW treatment attenuated the neurologic consequences of abnormal Aβ deposition in Tg 2576 mice without changing Aβ levels *per se*, the ability of GKW to modulate the toxic effects of Aβ were pursued *in vitro*, with an emphasis on mechanisms which are either independent of or "downstream" from Aβ.

In preliminary experiments, GKW showed a moderate protective effect against toxicity due to exogenously added Aβ in SH-SY5Y human neuroblastoma cells *in vitro* (Figure 4(a)). Lactate dehydrogenase (LDH) release from these cells, which is inversely related to cell viability, was reduced in the presence of GKW (Figure 4(b)). The effect of GKW on toxicity due to endogenously generated Aβ was investigated in MC65 human neuroblastoma cells. GKW added to the cell culture medium prevented MC65 cell death following tetracycline withdrawal, in a dose-dependent manner (Figure 5). Evidence from Western blots indicated that GKW may prevent the aggregation of Aβ in these cells.

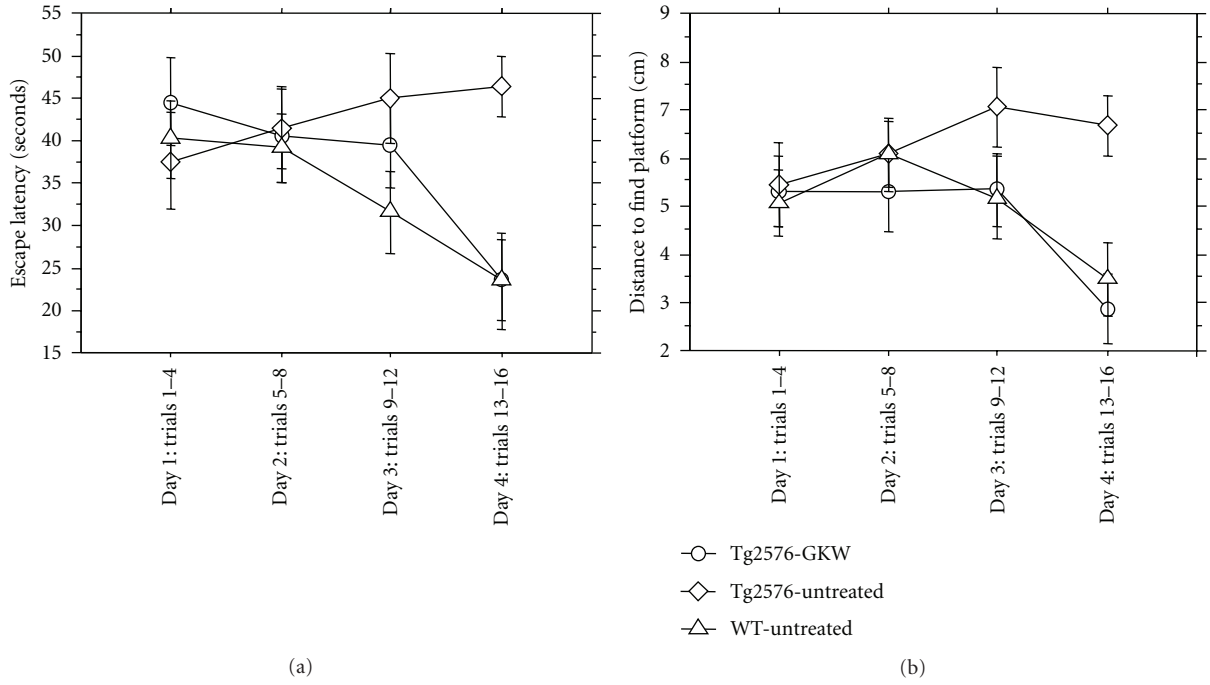

Figure 2: GKW effects in the Morris water maze. Mean ± SEM of (a) escape latency and (b) distance traveled to find platform is shown for each day of testing. Wild-type animals exhibit a "learning curve" requiring less time and less distance to find the hidden platform with repeated trials, while untreated Tg2576 mice require equivalent time and distance despite repeated exposures. In contrast, the GKW-treated mice learn in a manner similar to the wild-type animals, with latency and distance traveled to find the platform declining with repeated exposures. On day 4, time to find the platform was significantly greater for untreated Tg2576 mice than for wild-type ($P = 0.002$) or GKW-treated Tg2576 mice ($P = 0.004$). Distance traveled to find the platform was also significantly greater on day 4 for untreated Tg2576 mice compared to wild-type ($P = 0.006$) or GKW-treated Tg2576 mice ($P = 0.003$).

In a related study (data not shown), GKW inhibited Aβ-induced nitric oxide (NO) production in the RAW 264.7 macrophage cell line. Interestingly, GKW inhibited NO production induced by Aβ but did not influence LPS-induced NO levels in these cells. Taken together, these data indicated that components in GKW are able to modulate the toxic effects of Aβ.

Other potential mechanisms by which GKW may have improved cognitive function in the Tg2576 mice were also investigated but yielded negative results. No direct inhibitory effect of GKW (2.5 to 250 μg/mL) on cholinesterase activity *in vitro* was observed, whereas robust inhibition was observed using the positive control neostigmine. Effects of GKW on glutamate toxicity to rat cortical neurons were investigated. GKW (100 or 200 μg/mL) was not directly toxic to rat cortical neurons nor did it protect the cells from toxicity induced by 250 and 1000 μM glutamate (cell viability 30% and 25% of control, resp.). To examine potential antioxidant effects of GKW, SHSY5Y neuronal cells were exposed to H_2O_2, which showed dose-dependent toxicity to SH-SY5Y cells over the range 125–500 μM. GKW (50–200 μg/mL), while not toxic to the cells, did not protect against toxicity at any of the peroxide concentrations tested. Thus, GKW does not appear to possess antioxidant effects.

Five drugs are currently FDA-approved for the symptomatic treatment of AD, targeting mechanisms of unclear relationship to the primary neurodegenerative process. The first four drugs (tacrine, donepezil, rivastigmine, and galantamine) are acetylcholinesterase inhibitors, which act by augmenting cholinergic neurotransmission [28]. Each of these drugs has shown improved cognitive outcomes in treated AD patients compared to placebo-treated subjects, and the efficacy across drugs makes the case that cholinesterase inhibition is a viable treatment strategy for AD [28]. The fifth and most recent antidementia drug to receive FDA approval is memantine. Memantine is a noncholinergic drug, acting instead at the NMDA class of glutamate receptor. In addition to showing clinical efficacy in human subjects with AD [29, 30], memantine has also been shown to improve cognition in murine models of cerebral amyloidosis. NMDA antagonism should therefore be considered among the possible mechanisms of action of treatments producing cognitive improvement in murine models of AD. Our *in vitro* experiments showed no evidence that CA acts by way of these established therapeutic targets since there was no effect on cholinesterase activity or glutamate neurotoxicity.

In addition to the established therapies just described, strategies aimed at preventing the accumulation of, or promoting the clearance of, Aβ are under study. Inhibitors of amyloid synthesis and immunization against Aβ have diminished brain pathology and yielded cognitive and behavioral improvements in murine models of AD. Although amyloid synthesis inhibitors such as Lilly semagacestat have yielded negative clinical results [31], antiamyloid immunotherapy

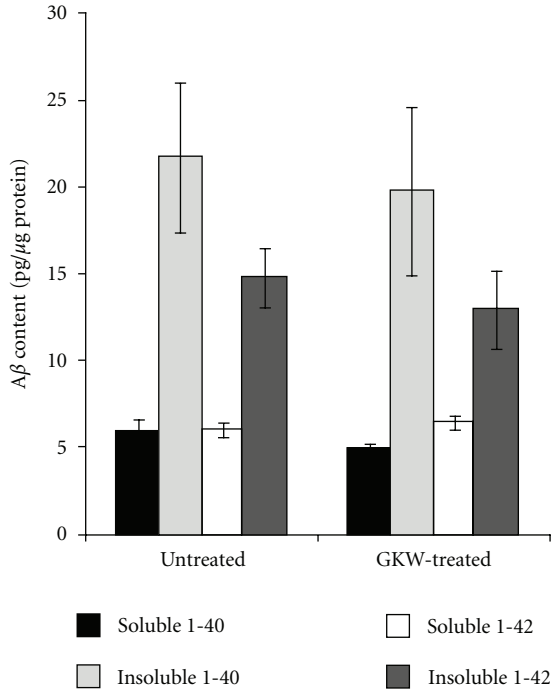

Figure 3: Soluble and insoluble Aβ in cortical tissue from treated and untreated mice. Mean values ± SEM are shown. Treated and untreated Tg2576 mice did not differ significantly in levels of any of the measured isoforms of Aβ.

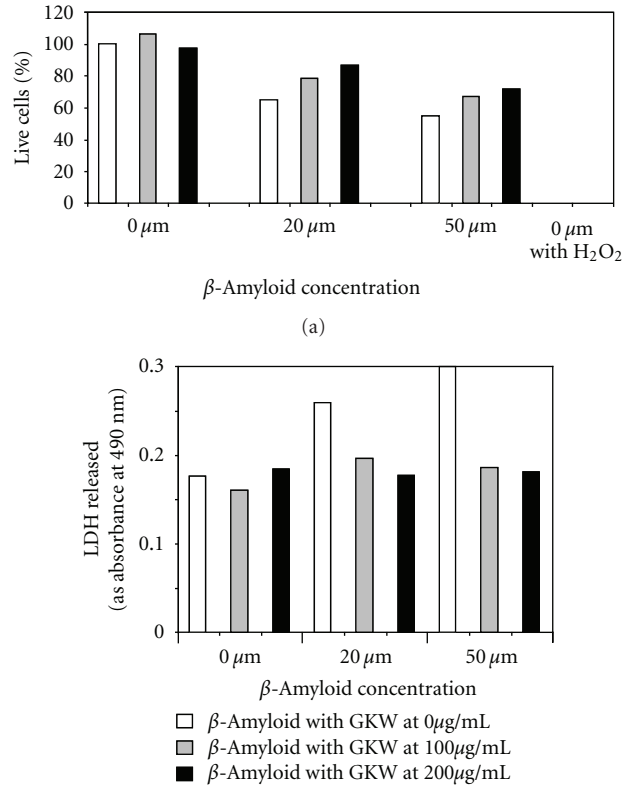

Figure 4: (a) GKW at 100 and 200 μg/mL shows a modest protective effect on SH-SY5Y cells from Aβ toxicity (beta amyloid 25–35) *in vitro*. The % of live cells is decreased on treatment with Aβ, an effect which is attenuated by GKW. Hydrogen peroxide 500 μM is used as a 0 cell viability control. (b) GKW at 100 and 200 μg/mL protects SH-SY5Y cells from Aβ toxicity (beta amyloid 25–35) *in vitro*—LDH release. The amount of LDH (lactate dehydrogenase) released is increased on treatment with Aβ, an effect which is attenuated by GKW. LDH release is a marker of cell damage.

(bapineuzumab) remains under development with some promising initial results [32]. "Antiamyloid" strategies, therefore, represent another potential mechanism of cognition-enhancing therapies in AD. Our results do not show an effect of CA on Aβ levels *per se* but suggest that CA may protect neurons from Aβ-induced neurotoxicity without actually changing brain levels of Aβ. Most current clinical trials are focused on suppression of Aβ levels, thus the neuroprotectant effect of CA described here represents a novel mechanism, potentially complementary to the drugs in development.

CA may also be a source of a novel chemical class for the treatment of AD. HPLC analysis of GKW revealed a complex mixture of substances (Figure 6). This did not include asiatic acid or madecassic acid, well-known triterpene components of CA [33, 34], which were, however, extractable from the same plant material using ethanol (Figure 6). The absence of asiatic acid in GKW is notable since asiatic acid has been previously associated with neuroprotective and neurotropic effects [35–38]. However, an aqueous extract lacking asiatic acid produced robust behavioral effects in this study. Table 1 lists the spectral characteristics of the major peaks found in GKW using LC-UV and LC-MS. UV spectra with maxima over 300 nm are indicative of a highly conjugated system, characteristic of flavonoids. CA is reported to be a rich source of quercetin [39]. Flavonoids isolated to date in CA include 3-glucosylkaempferol, 3-glucosylquercetin and diosmin [40, 41]. The molecular weights listed in Table 1 did not correspond to any of these 3 compounds, nor any other compounds isolated hitherto from CA (online chemical database, SciFinder). These findings imply the presence of potentially novel neuroactive ingredients in GKW which are yet to be fully characterized.

Compared to the wealth of animal data described earlier, there have been fewer studies on cognitive effects of CA in humans. In one study, 30 mentally retarded children aged 7–18 years showed improvement in their general abilities after receiving 500 mg daily of dried CA herb for 3 months [42]. A more recent study [43] showed that an extract of CA (250–750 mg daily for 2 months) improved cognitive performance in healthy, elderly volunteers. In a placebo-controlled study, administration of CA herb (0.5 g/kg body weight) to healthy, middle-aged volunteers for 2 months resulted in improvements in several tests of cognitive function [44]. A study in elderly subjects with mild cognitive impairment found improvements in their cognitive test results, including the mini mental state examination, following administration of 500 mg dried CA twice a day for a 6-month period [45].

The traditional use of CA as an enhancer of cognitive function is therefore well supported by *in vitro*, *in vivo*, and small-scale human studies conducted so far. The ultimate

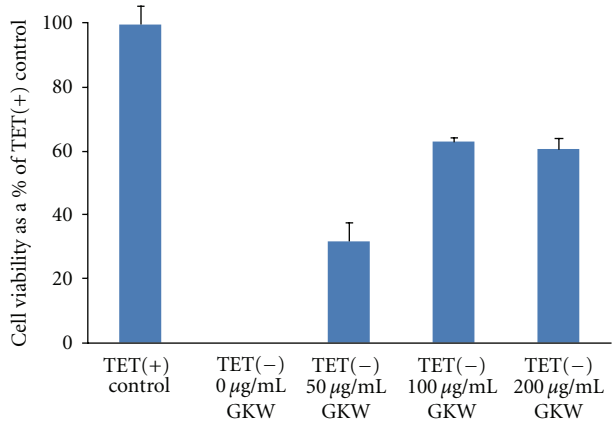

Figure 5: Effect of GKW on survival of MC65 cells following tetracycline withdrawal. Cell viability is expressed as a % of the cell growth obtained in control cultures containing tetracycline, TET(+). On withdrawal of tetracycline from the media, TET(−), the cells generate endogenous Aβ and die within 3 days. In the absence of GKW, cell survival in TET(−) cultures is zero. GKW dose-dependently protects MC65 cells from cell death in TET(−) cultures ($P < 0.01$ at 50, 100, and 200 μg/mL compared to 0 μg/mL GKW; Student's t-test). Mean values of cell viability ± SD are shown.

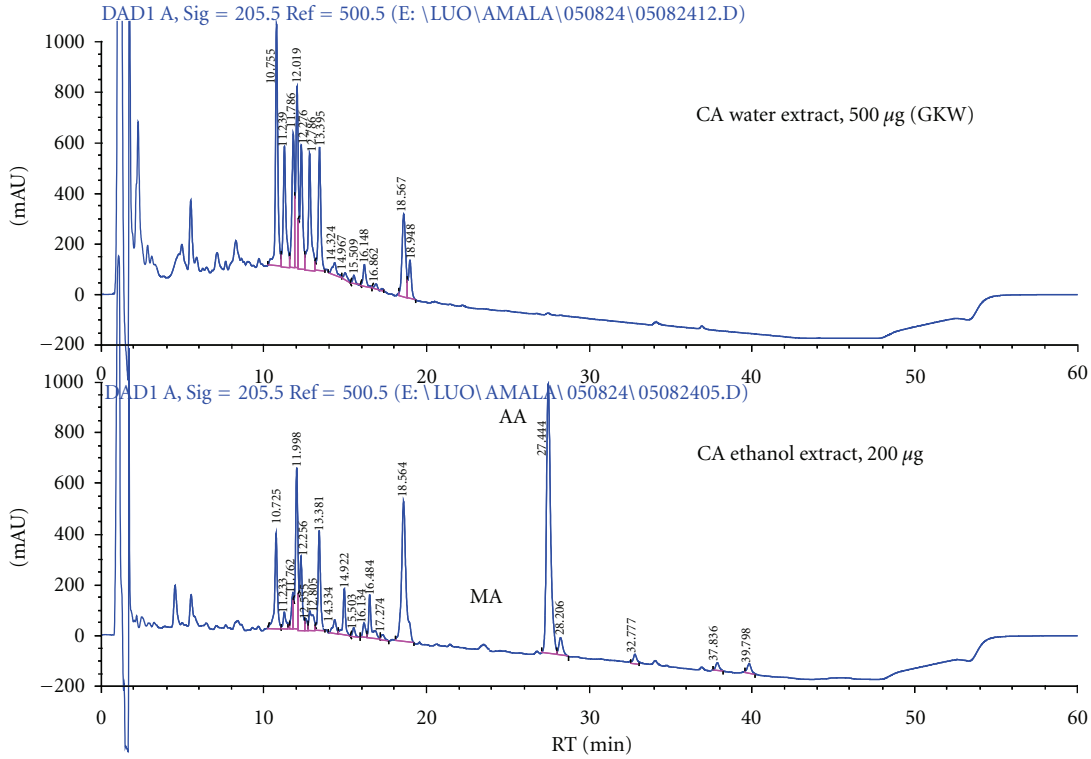

Figure 6: HPLC comparison of CA water and ethanol extracts made from the same batch of plant material. Asiatic acid (AA) and madecassic acid (MA) are detected in the ethanolic extract, but not in the water extract (GKW). The water extract GKW contains mostly very polar compounds as shown by their earlier elution than the triterpenes AA and MA. HPLC was conducted on an Aquasil 150 mm × 2.1 mm C18 column with acetonitrile:water gradient with 0.1% acetic acid. Acetonitrile concentration 10% at 0 min, 50% at 25 min, 90% at 40 min, then returning to starting conditions, detector wavelength 205 nm. AA and MA were identified by comparison of retention times to those of commercial standards.

goal of these studies is to develop evidence for the clinical use of CA, or compounds derived from CA, in the treatment or prevention of AD. The combination of data from *in vitro* and animal studies in the present work supports the impression that CA has the potential for clinical benefit in AD by way of a novel mechanism of action. Well-designed, controlled clinical trials of CA in AD and other forms of cognitive impairment are clearly warranted. The characterization of the active components of CA and elucidation of their mechanism of action would support these clinical studies.

Table 1: Molecular weight and UV data obtained for GKW components using LC-MS and LC-UV. Reversed phase gradient HPLC chromatography with UV and negative ion mass spectral detection was performed as described in Section 2.

Retention time	Most abundant ions (m/z); (Mol Wt−1)	UV λ max (nm)	Possible structure, based on flavonoid handbook [46]
12.33	399, 353	215, 325	Prenylated flavone
16.57	531	215, 265, 310	Malonyl, butyryl, or diacetyl flavone glycoside
21.05	477	205, 255, 355	Glycosyl or glucuronyl methylated flavones
22.64	561, 515	215, 325	Diglycosyl flavonoid or a catechin
25.21	601	215, 325	No matches found
33.49	577	220, 295, 320	Diglycosyl flavonoid or a proanthocyanidin

4. Conclusions

A water extract of CA (GKW) attenuated Aβ-associated behavioral abnormalities in the Tg2576 mouse, a murine model of AD. *In vitro*, GKW protected SH-SY5Y cells and MC65 human neuroblastoma cells from toxicity induced by exogenously added and endogenously generated Aβ, respectively. GKW did not show anticholinesterase activity or protect neurons from oxidative damage and glutamate toxicity, mechanisms of current AD therapies. The combination of data from *in vitro* and animal studies in the present work supports the CA potential for conferring clinical benefit in AD, possibly by way of a novel mechanism of action. GKW does not contain asiatic acid, a known CA neuroprotective triterpene, but is rich in phenolic compounds. CA may therefore contain novel active compounds of relevance to the treatment of AD.

Conflict of Interests

At the time of conducting this research, A. Soumyanath was an employee of Oregon's Wild Harvest, the supplier of *Centella asiatica* used in this study. This potential conflict of interests has been reviewed and managed by OHSU.

Acknowledgments

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Research Article

Silencing of Amyloid Precursor Protein Expression Using a New Engineered Delta Ribozyme

Manel Ben Aissa,[1] Marie-Claude April,[2] Lucien-Junior Bergeron,[2] Jean-Pierre Perreault,[2] and Georges Levesque[1]

[1] *Département de Psychiatrie-Neurosciences, Faculté de Médecine, Unviersité Laval et Neurosciences CHUL, 2705 Laurier, Québec, QC, Canada G1V 4G2*
[2] *RNA Group/Groupe ARN, Département de Biochimie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, 3001 12th Avenue, Sherbrooke, QC, Canada J1H 5N4*

Correspondence should be addressed to Georges Levesque, georges.levesque@crchul.ulaval.ca

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Alzheimer's disease (AD) etiological studies suggest that an elevation in amyloid-β peptides (Aβ) level contributes to aggregations of the peptide and subsequent development of the disease. The major constituent of these amyloid peptides is the 1 to 40–42 residue peptide ($A\beta_{40-42}$) derived from amyloid protein precursor (APP). Most likely, reducing Aβ levels in the brain may block both its aggregation and neurotoxicity and would be beneficial for patients with AD. Among the several possible ways to lower Aβ accumulation in the cells, we have selectively chosen to target the primary step in the Aβ cascade, namely, to reduce APP gene expression. Toward this end, we engineered specific SOFA-HDV ribozymes, a new generation of catalytic RNA tools, to decrease APP mRNA levels. Additionally, we demonstrated that APP-ribozymes are effective at decreasing APP mRNA and protein levels as well as Aβ levels in neuronal cells. Our results could lay the groundwork for a new protective treatment for AD.

1. Introduction

Alzheimer's disease (AD) is a degenerative disorder of the human central nervous system (CNS). Its clinical and neuropathological features are defined by a progressive loss of cognitive function and by the onset of a slowly progressive impairment of memory during mid- to late-adult life. The neuropathological hallmarks of AD include the accumulation and aggregation of amyloid-β peptide (Aβ), neurofibrillary tangles, astrocytic gliosis, and reductions in the numbers of both neurons and synapses in many areas of the brain, particularly in the cerebral cortex and hippocampus [1]. Strong evidence from multiple studies suggests that defects in Aβ regulation are one of the central biochemical events leading to the development of AD [2]. The neurotoxic Aβ fragment originates from the amyloid protein precursor (APP) following sequential cleavages by β (BACE) and γ-secretases (presenilin complex). Observations on the physiological processing of APP and on the effects of pathogenic mutations in the APP and/or the presenilin genes have led to the hypothesis that aberrant processing of APP into Aβ peptides is linked to AD [3]. We have previously reported strong evidence indicating that the amyloid cascade is an early and critical event in the neurodegeneration associated with AD. For example, cell lines and/or transgenic mice expressing mutant presenilin 1 (PS1), presenilin 2 (PS2), or APP exhibit an accelerated rate of neurotoxic Aβ formation [4]. Thus, the three known genetic causes of familial AD affect Aβ metabolism. Moreover, the ε4 allele of apolipoprotein E, a strong genetic risk factor for the development of AD, has been linked to either enhancing Aβ aggregation or decreasing its clearance in brain tissue [5, 6]. Altogether, these observations strongly suggest that targeting Aβ metabolism is a *worthwhile therapeutic approach* and that reducing its level in the brain may block both the neurodegenerative process and cognitive decline. Most likely, an approach that reduces either the level of Aβ or the rate of its aggregation and deposition in the brain would

be beneficial for patients with AD. Targeting the secretases may be risky because they appear to have multiple roles in cells. We have decided to address the problem with a new generation of ribozymes (Rz) targeting the first step in the amyloid cascade, specifically, the APP mRNA.

Hepatitis Delta Virus ribozyme (HDV Rz) is an interesting potential tool for the development of a gene-inactivation system because it is well adapted to the human cell environment [7]. In fact, this ribozyme offers several unique properties, including the natural ability to function in the presence of human proteins and at physiological magnesium concentrations as well as outstanding molecular stability (i.e., it has a long half-life) [8]. Recently, a novel target-dependent ribozyme that increases HDV Rz fidelity was engineered [9]. This new ribozyme possesses a module (the SOFA, for Specific On/Off Adaptor) that switches the cleavage activity from *Off* to *On* when in the presence of the appropriate substrate (Figure 1). Specifically, this module is composed of three domains: a blocker, a biosensor, and a stabilizer. The blocker sequence inhibits the cleavage activity of the ribozyme by intramolecularly binding the recognition domain of the Rz, which was limited to only 7 nucleotides before the addition of the module. Binding of the blocker switches the ribozyme domain to an inactive state, namely, the *Off* conformation. Upon addition of the substrate, the biosensor binds its complementary sequence on the substrate and unlocks the SOFA module, thereby permitting a switch of the ribozyme into the active fold, namely, the *On* conformation. The sequences of the substrate binding of both the ribozyme recognition and biosensor domains are not contiguous, but rather are separated by a small region called the spacer that varies from 4 to 7 nucleotides for optimal design [10]. Finally, the presence of a stem (namely, a stabilizer) that brings together both the 5′ and 3′ extremities has no effect on the cleavage activity but stabilizes the SOFA-HDV Rz *in vivo* against ribonucleases. A proof of concept of this man-made ribozyme has been demonstrated both *in vitro* and *in vivo* using ribozymes that cleaved various mRNA and viral RNA [11–13]. The fact that the SOFA-HDV Rz is activated by its mRNA substrate greatly diminishes its nonspecific effects; consequently, it displays significant potential for applications in both functional genomics and gene therapy.

In this study, we evaluated the potential of the new SOFA-HDV ribozymes as an RNA silencing tool in mammalian cells. In cell culture, we demonstrated the effects of SOFA-HDV Rz targeting APP mRNA on Aβ production.

2. Experimental Procedures

2.1. SOFA-HDV Ribozyme DNA Constructs. SOFA-HDV ribozymes were constructed using a PCR-based strategy that included two complementary and overlapping oligonucleotides. Briefly, two DNA oligonucleotides were synthesized and annealed with the reverse primer (5′-CCAGCTAGAAAGGGTCCCTTAGCCATCCGCGAACGGATGCCCA$(N)_{6(P1)}$ACCGCGAGGAGGTGGACCCTG$(N)_{4(BL)}$) and the sense primer (5′-TTAATACGACTCACTATAGGGCCAGCTAGTTT$(N)_{12(BS)}(N)_{4(BL)}$CAGGGTCCACC), where N is A, C, G, or T, and P1, BS, and BL indicate the P1, biosensor, and blocker sequences, respectively. It is important to note that both the P1 and BS segments were varied to correspond to specific APP mRNA sequences and that the BL was complementary to the first 4 nucleotides on the 5′ end of the Rz's recognition domain. For *in vitro* synthesis of the ribozymes, the sense primer also included the sequence of the T7 RNA polymerase promoter at the 5′ end. The filling reaction was performed in a 100-μL volume containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 2 μM of each dNTP, 1 μM of each DNA oligo, and 5 U of Pwo DNA polymerase (Roche Diagnostics). The reactions were ethanol precipitated and washed and the DNA pellets resuspended in 56 μL of deionized water. The resulting PCR products were directly used for *in vitro* transcription reactions (see below). For the *in cellulo* experiments, the PCR products were inserted into the *Eco*RV site of pCDNA3 (Invitrogen). The SOFA-HDV-Rz cassettes were removed by digestion with *Bam*HI and subcloned into pRNAT-U6.1/lentivector (GenScript) under control of the U6 snRNA promoter. The resulting plasmids were named pRNAT-SOFA-HDV-Rz-APPX, where X represents the APP cleavage position.

2.2. In Vitro Transcription of SOFA-HDV Rz and APP mRNA. RNA transcriptions were performed as previously described [14]. In the case of the SOFA-HDV ribozymes, the resuspended DNA pellets were used in 100-μL transcription reactions containing 80 mM HEPES-KOH (pH 7.5), 24 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 5 mM of each rNTP, 0.01 U of pyrophosphatase (Roche Diagnostics), 24 U of RNAGuard (Amersham Biosciences), and 10 μg of purified T7 RNA polymerase and allowed to proceed for 4 h at 37°C. The reactions were then treated with 4 U of RQ1 DNase (Promega), phenol-chloroform extracted, ethanol precipitated, and washed. Following these steps, the RNA pellets were resuspended in 40 μL of deionized water. One volume of loading buffer (97.5% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 10 mM EDTA) was added, and the samples were fractionated by 8% denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE, 19 : 1 ratio of acrylamide to bisacrylamide), using 45 mM Tris-borate (pH 7.5) and 1 mM EDTA. The RNA bands were visualized by UV shadowing, and the gel slices were cut out and eluted overnight using 500 mM ammonium acetate, 1 mM EDTA, and 0.1% SDS. After ethanol precipitation, the RNA transcripts were resuspended in deionized water and quantified by UV absorbance at 260 nm. The plasmid pAPP12 (containing a full-length copy of the APP mRNA) was used as a template. After linearization by the *Stu*I restriction enzyme, mRNA was synthesized as described above and purified using 5% PAGE. After purification, the transcripts (40 pmol) were dephosphorylated in a final volume of 50 μL containing 200 mM Tris-HCl (pH 8.0), 10 U RNAGuard, and 0.2 U of calf intestinal alkaline phosphatase (Amersham BioSciences) at 37°C for 30 min. The reactions were purified by extracting twice with phenol : chloroform, and the mRNA was then precipitated with ethanol, washed with 70% ethanol, and dried. Dephosphorylated RNA

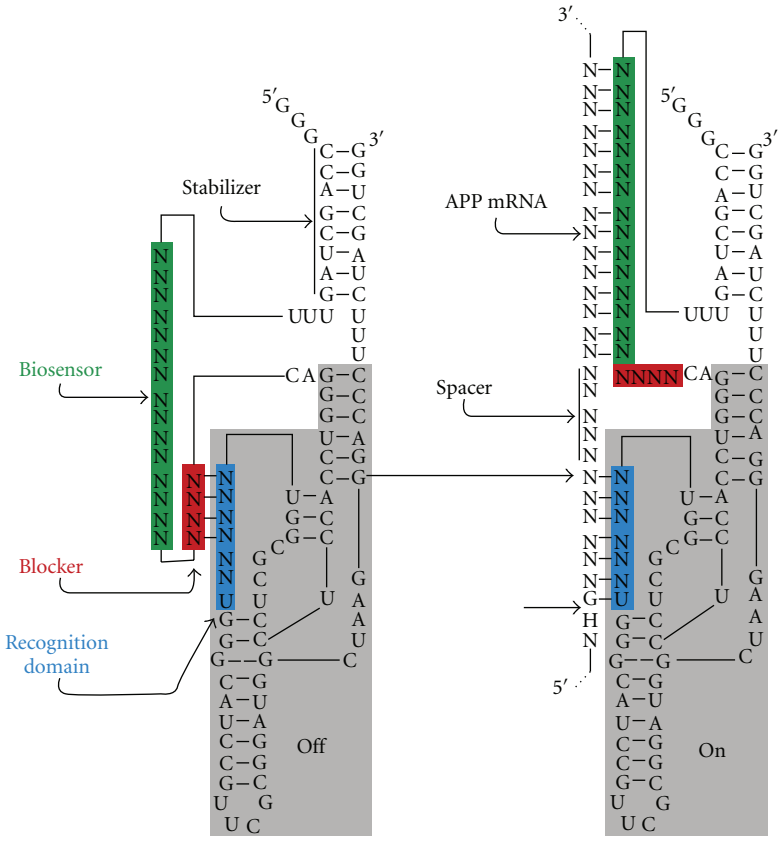

FIGURE 1: Secondary structure of both the *Off* and *On* conformations of the SOFA-HDV ribozyme. The original HDV ribozymes in the grey shaded boxes with its recognition domain (in blue) indicated. The SOFA is composed of three motifs : the biosensor (in green), blocker (in red), and stabilizer stem. Upon the addition of APP mRNA, sequence-specific hybridization to the ribozyme occurs, and the substrate is subsequently cleaved.

(~6 pmol) was 5′-end-labeled in a final volume of 10 μL containing 3.2 pmol of [γ-^{32}P]ATP (6000 Ci/mmol, New England Nuclear), 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, and 3 U of T4 polynucleotide kinase (United States Biochemicals) at 37°C for 90 min. The reaction was stopped by the addition of formamide dye buffer (5 μL), and the reaction mixtures were fractionated through denaturing 5% PAGE gels and recovered as described above.

2.3. Ribonuclease H Probing and Primer Extension Assays. Ribonuclease H (RNase H) reactions were performed with a library of randomized oligonucleotides (5′-N_6CD-3′, where N is for any A, C, G, or T residue and D is for any A, T, or G residue). Specifically, nonradioactive APP mRNA (0.5 μM) and randomized oligonucleotides (5 μM) were preincubated for 10 min at 25°C in a final volume of 8 μL containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT. RNase H (0.5 U, United States Biochemicals) was then added, and the samples were incubated at 37°C for 30 min. After the incubation, 90 μL of water was added, and the mixture was phenol : chloroform extracted. The nucleic acids were then precipitated with ethanol, washed, and dried. Four DNA oligonucleotides complementary to the APP RNA were purchased from Invitrogen (5′-GTTCCTCAGCCTCTTCCT-3′ (position 928-911), 5′-TCAGCCAGTGGGCAACAC-3′ (position 719-702), 5′-GTCAGGAACGAGAAGGGC-3′(position 540-523), and 5′-CTGAATCCCACTTCCCAT-3′ (position 310-293)). The oligonucleotides (10 pmol) were 5′-end-labeled as described above. The ^{32}P-end-labeled oligonucleotides were purified with denaturing 20% PAGE, and the relevant bands were excised from the gel and eluted overnight at 25°C, passed through a G-25 spun column, ethanol precipitated, washed, dried, and dissolved in deionized water (60 μL). 5′-^{32}P-labeled primer (6 μL) and 10X reverse transcriptase buffer (0.6 μL of 500 mM Tris-HCl (pH 8.3), 800 mM KCl, and 100 mM $MgCl_2$) were used to resuspend the pellets resulting from the RNase H hydrolysis. The primer annealing step was performed by successively incubating the samples at 65°C for 2 min followed by 2 min on ice. The reactions were initiated by adding 0.8 mM of each dNTP, 3.3 mM DTT, and 100 U of Superscript II Reverse transcriptase (Invitrogen) in a final volume of 12 μL. The samples were incubated at 45°C for 30 min and then ethanol precipitated and analyzed by 5% sequencing PAGE. DNA sequencing reactions using the same primer were migrated on the same gels to allow for identification of the primer extension stops. The results were visualized with a PhosphorImager.

2.4. Ribozyme Cleavage In Vitro. Cleavage reactions were carried out under single turnover conditions ([Rz] ≫ [S]), as previously described [15]. Specifically, ^{32}P-end-labeled APP mRNA (50 nM) was mixed with SOFA-HDV ribozymes (1 μM) in a 10-μL mixture containing 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ and then incubated at 37°C for 1 h. The reactions were stopped by the addition of loading buffer, RNA fractionated with denaturing 5% PAGE, and analyzed with a PhosphorImager.

2.5. Cell Culture and Transfection. A subclone of the human embryonic kidney cell line HEK-293 (tsA-201 cells, which were kindly provided by Dr. Mohamed Chahine, Laval University) and human neuroblastoma SH-SY5Y cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (Biomedia). Stock cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. The HEK-293 cells were transiently transfected with pRNAT-SOFA-HDV-Rz-APPX (Rz-APP-X) plasmid using the calcium phosphate procedure. The empty pRNAT-U6.1 vector (GenScript) was used as a control. The SH-SY5Y cells were transduced using a lentiviral system. This system consists of the multiply deleted packaging construct pCMVΔR8.91 (which encodes Gag, Pol, Tat, and Rev), the pMD.G expressing vesicular stomatitis virus (VSV-G) surface glycoprotein (G), and pRNAT-U6 (either with or without Rz-APPX). To produce the infectious virions, HEK-293 cells (2×10^6) were plated on 5 dishes (10 cm) and transfected the next day with 20 μg of Rz-APPX, 15 μg pCMVΔR8.91, and 5 μg pMDG using the calcium phosphate procedure. Conditioned medium was harvested at 48 hr after transfection, cleared of debris by low-speed centrifugation, and filtered through 0.45 μm filters (Sarstedt). The filtrate containing the virions was concentrated by ultracentrifugation at 71,000 × g for 90 minutes at 16°C using a SW-40 Beckman rotor, followed by a second cycle of centrifugation for the collected and resuspended pellets at 84,000 × g for 90 min (using a 4-mL centrifuge tube; SW60 Beckman rotor). Virions pellets were then resuspended in 0.5 mL of phosphate buffered saline (PBS). SHSY-5Y cells were infected with 0.2 mL of the virions expressing SOFA-HDV-Rz-APPX in the presence of 6 μg/mL polybrene (hexadimethrine bromide, Sigma). Three days postinfection, the medium was replaced with medium containing 600 μg/mL G418 for selection. The transduced cells were maintained as a stable population. The culture medium was changed every 3-4 days for the duration of the experiment.

2.6. SOFA-HDV Ribozyme Expression. To test the expression of the Rz-APPX, total RNA was extracted from transduced cells using the TRIzol reagent according to the manufacturer's recommendations (Invitrogen). Total RNA extracts were then used in primer extension experiments for ribozyme detection. Briefly, the primers, corresponding to the 3′ complementary sequence of either SOFA-HDV-RzX (5′-GGGTCCCTTAGCCATGCGCGAACG-3′) or U6 RNA (5′-GGCCATGCTAATCTTCTCTG-3′), were 5′-end-labeled with [γ-^{32}P]ATP (6000 Ci/mmol; New England Nuclear), as previously published [13], annealed to 10 μg of total RNA by a 5 min incubation at 65°C and immediately chilled on ice. The reactions were initiated with the addition of 0.4 mM of dNTPs, 10 mM DTT, and 200 units of Superscript II reverse transcriptase (Invitrogen) in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$ in a final volume of 50 μL. The samples were incubated at 42°C for 50 min; the reactions were stopped by heating the samples to 70°C for 15 min and then fractionated through 10% denaturing polyacrylamide gel electrophoresis.

2.7. Real-Time RT-PCR. First-strand cDNA synthesis was performed using 2 μg of total RNA in the presence of poly dT primers and 200 units of SuperScript II reverse transcriptase. Aliquots of 2 μL from the resulting single-stranded cDNA products were used along with the appropriate primers (see below) for APP and GAPDH. Amplifications were performed for each sample from each separate well in a total volume of 25 μL containing 1X SYBR Green Universal PCR Master Mix and 400 nM of specific forward and reverse primers. The primers were designed to overlap the boundaries of two exons (to avoid amplification of genomic DNA), using the Primer Express software v2.0 (Applied Biosystems). Specifically, two pairs of primers were designed to amplify the APP mRNA. The first pair (sense primer 5′-GGCGGTGTTGTCATAGCGA-3′ and antisense primer 5′-TGCATCTTGGACAGGTGGC-3′) provided an amplicon of 136 base pairs (bp), whereas the second pair (sense primers 5′-AACGAAGTTGAGCCTGTTGATG-3′ and antisense primer 5′-AACGAAGGCTGGCACAAC-3′) amplified a 67-bp fragment. Amplification of GAPDH mRNA using the sense primer 5′-CGACACTTCCAGCTCTTTGCT-3′ and antisense primer 5′-GAATCAGGGTTATCTGGTCATCG-3′, which produces an amplicon of 131 bp, was also performed. The PCR amplifications were performed on an ABI Prism 7000 Sequence Detector System (Applied Biosystems), according to the manufacturer's instructions and using the following conditions: 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 58°C for 10 s, 72°C for 20 s, and a final step at 60°C for 60 s. The control samples were amplified without the reverse transcription step.

2.8. Preparation of Cell Lysates. Native and transfected cells were rinsed twice with ice-cold PBS and then lysed for 30 min on ice in cell lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 20 mM PMSF, and minicomplete protease inhibitors (Roche-Diagnostic). Insoluble material was removed by centrifugation at 13,000 × g for 15 min at 4°C. Finally, the proteins were quantified using a standard Bradford assay (Bio-Rad).

2.9. Western Blot Analysis of APP Processing. Western blot analysis was performed as previously described [16]. Briefly, 20 μg of total protein from each sample was mixed with Novex 2X reducing sample buffer containing 500 mM Tris-HCl (pH 6.8), 20% glycerol, 10% SDS, 0.1% bromophenol blue, and 5%β-mercaptoethanol. The samples were then boiled for 5 min and subjected to SDS-PAGE. Following the migration, proteins were transferred onto a PVDF

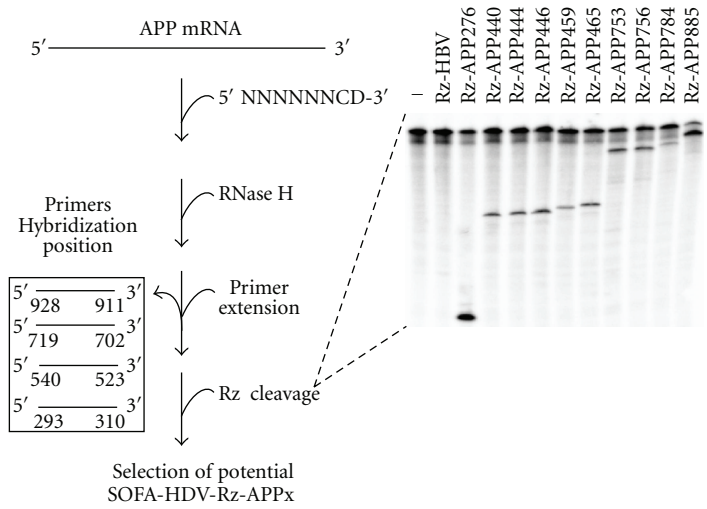

Figure 2: Selection of APP-SOFA-HDV ribozyme with the greatest potential cleavage. The left portion illustrates the strategy that was used to identify potential cleavage sites. APP mRNA was preincubated in the presence of a 7 nt long randomized DNA oligonucleotide, and RNA/DNA heteroduplexes were hydrolyzed by RNase H. Accessible regions were then visualized by primer extension using one of the four 5′-end-labeled primers (in box) complementary to sequences retrieved in the first ~900 nucleotides of the APP mRNA. Once the most accessible sites were identified, the appropriate SOFA-HDV ribozymes were synthesized and the cleavage activity was tested *in vitro* using 5′-end-labeled APP mRNA. A typical autoradiogram of a resulting PAGE is indicated in the right panel. The number of each Rz indicates the cleavage position within the APP mRNA. The Rz-HBV, previously used for HBV RNA cleavage [15], served as an irrelevant Rz. "-" indicates a reaction without Rz.

membrane (Millipore) according to the manufacturer's protocol. The membranes were probed with an anti-APP C-terminal antibody (A8717, Sigma-Aldrich) and β-tubulin specific antibody (antibody E7 for β-tubulin, Developmental Studies Hybridoma Bank). The blots were revealed using a chemiluminescence detection system (Immobilon Western, Millipore) according to the manufacturer's recommendations. The intensity of the signals was analyzed using image densitometry software (Imaging Densitometry, Bio-Rad). The level of β-tubulin was used to normalize the levels of APP (i.e., the ratio of APP versus β-tubulin) to control for differences in the loading of total proteins. Modulations of the APP levels in the cells treated with Rz-APP-X were expressed as a percentage of the level in the control cells (empty vector).

2.10. Quantitation of Aβ Using the Sandwich ELISA Method. Following SH-SY5Y transduction and during the selection, media was collected, preserved, and frozen at −80°C. Following, secreted Aβ was measured by sandwich ELISA, according the manufacturer's protocol (Human Amyloid β (Aβ 1-x) Assay Kit, IBL).

2.11. Statistical Analysis. For the *in vitro* data, the results from several experiments were analyzed using Student's *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Design and Selection of APP-Specific SOFA-HDV Ribozymes. The first step of this study consisted of designing a collection of ribozymes that produced *in vitro* cleavage of the APP mRNA. Due to unfavorable competition with intramolecular base pairing, the target sequences located in single-stranded regions of an mRNA are potentially more accessible for Rz binding than those in double-stranded regions. It has been demonstrated that both target site accessibility and the ability to form an active ribozyme-substrate complex constitute interdependent factors that can be addressed using a combinatorial library of oligonucleotides or ribozymes [17]. To identify the cleavage sites with the greatest potential for targeting, we adopted a procedure based on the use of a library of partially randomized oligonucleotides mimicking the interaction with the recognition domain of the target [15] (Figure 2). In principle, all of the accessible sites within the APP mRNA should be specifically bound by an oligonucleotide and the resulting RNA-DNA heteroduplex subsequently hydrolyzed by RNase H. The resulting cleavage sites were identified by primer extension reactions using 5′-end-labeled primers, and the most potent SOFA-HDV ribozymes were tested for cleavage activity. The library was composed of oligonucleotides that were 8 nucleotides in length corresponding to one residue before the cleavage site (i.e., position -1), which had to be single stranded for cleavage to occur, and the 7 residues of the recognition domain of the ribozyme. It is important to note that this experiment considered only the binding domain of the ribozyme and not the SOFA module. It would be irrelevant to perform RNase H assays using long oligonucleotides that included the complementary sequence of the ribozyme's recognition domain, spacer and biosensor sequences. In that case, the spacer would also be bound, leading to significant formation of the duplexes and the introduction of an important bias. The library was designed

Table 1: Determination of the most potential cleavage sites within APP mRNA.

Cleavage position	mRNA sequence[1] recognition domain/biosensor domain	Accessibility[2]	SOFA-HDv Rz cleavage activity (%)[3]
276	**5′-G**CACAUG/CCAGAAUGGGAA-3′	++	62
440	**5′-G**CAAGCG/GCAAGCAGUGCA-3′	+++	18
444	**5′-G**CGGGGC/GCAGUGCAAGAC-3′	+++	20
446	**5′-G**GGGCCG/AGUGCAAGACCC-3′	+++	26
459	**5′-G**UGCAAG/UCCCCACUUUGU-3′	+++	14
465	**5′-G**ACCCAU/CUUUGUGAUUCC-3′	++	26
753	**5′-G**GAGGAU/GGAUGUCUGGUG-3′	++	36
756	**5′-G**GAUGAC/UGUCUGGUGGGG-3′	+	36
784	**5′-G**CAGACA/UAUGCAGAUGGG-3′	+	25
885	**5′-G**GACGAU/UGGUGAUGAGGU-3′	+	71

[1] The sequences of the mRNA bound by both the ribozyme' recognition and biosensor domains. [2] Accessibility of potential cleavage sites based on RNase H hydrolysis. [3] Percentage of cleavage activity of the various SOFA-HDV ribozymes targeting the APP mRNA.

while taking into consideration the sequence specificities of the HDV ribozyme. Specifically, the nucleotide in position 1 cannot be a guanosine; therefore, the 3′ end residue of the oligonucleotide cannot be a cytosine. Moreover, the first base between the ribozyme's recognition domain and the target must be a GU wobble base pair. Consequently, the oligonucleotide included a cytosine at the corresponding position. This constraint led to a library of 12,288 different variants corresponding to the 5′-N6CD-3′. The action of a ribozyme within the 5′-end of an mRNA region should enhance the probability that the cleavage product results in an RNA that cannot encode an active protein. Because each primer produced a readable sequence of 200 to 300 bases, 4 different oligonucleotides were designed for the reverse transcriptase reaction to analyze the first ∼900 nucleotides of the APP transcripts corresponding to the 1040 nucleotides of the 5′ end (see Section 2). The relative level of accessibility in function of the intensity of the primer extension products is compiled in Table 1. This analysis led to the identification of 10 potential sites, located from positions 276 to 885, of the APP transcript. Seven of these sites appeared to be highly accessible, including 5 that were located near position 450. A high concentration of such sites in the same area is indicative of a single-stranded region, although it may also result from a synergetic effect of several oligonucleotides binding the same RNA transcript, resulting in unfolding of that region and increasing the possibility that additional oligonucleotides can also bind.

Subsequently, HDV-Rz with the appropriate recognition sequences was designed. To increase specificity, the ribozyme was further extended with the addition of a SOFA module. The resulting ribozymes were named SOFA-HDV-Rz-APPX, where X represents the APP cleavage position. The ability of these ribozymes to cleave the 5′-end-labeled APP transcripts was tested under single-turnover conditions ([Rz] ≫ [S]) and analyzed via PAGE (Figure 2 inset). Clearly, all of the SOFA-HDV ribozymes exhibited cleavage activity, although at different levels. Specifically, the cleavage level varied from 4% to 71%. Moreover, all of the ribozymes exhibited a specific cleavage at only the expected site. The 4 SOFA-HDV ribozymes that exhibited a cleavage level higher than 30% were conserved for the subsequent step. These ribozymes included SOFA-HDV-Rz-APP276, -APP753, -APP756, and -APP885, with cleavage activities of 62%, 36%, 36%, and 71%, respectively. These 4 potential SOFA-HDV ribozymes targeting the APP mRNA were tested with the ribosubstrates online software (http://www.riboclub.org/ribosubstrates). This integrated software searches selected cDNA databases for all of the potential substrates for a given SOFA-HDV ribozyme [18]. These potential substrates include not only mRNAs with perfect matches with the catalytic RNA tested, but also the wobble bp and mismatches. Interestingly, none of these 4 potential SOFA-HDV ribozymes seemed to have the potential for off-target effects (data not shown). Moreover, this analysis indicated that no other cleavage could occur within the APP gene family. Therefore, the chosen sequences were specific to APP mRNA. In other words, the SOFA-HDV ribozymes that exhibited significant cleavage activity *in vitro* against a derived APP transcript appeared to be specific to the APP mRNA.

3.2. Expression of APP-Specific SOFA-HDV Rz in Human Cells. In an attempt to achieve a high level of expression of SOFA-HDV ribozymes that maintain their affinity for the targeted mRNA, we adopted the pRNAT/U6 (which employs the U6 RNA polymerase III promoter) for a high level of small RNA expression. The advantage of this promoter is that RNA transcription terminates with the addition of 4 or 5 uridines (U) at the 3′-end, and this change has only a minimal effect on SOFA-HDV ribozyme folding based on RNA structure predictions. This approach also avoids nonspecific effects that might be caused by the transcription of additional regions of the vector sequence.

To determine whether the pRNAT/U6 SOFA-HDV-Rz-APPX vector could express the anti-APP SOFA-HDV ribozymes, these constructs were transfected into HEK-293 cells. Two days after transfection, total RNA from transfected cells was subjected to primer extension analysis. Endogenously

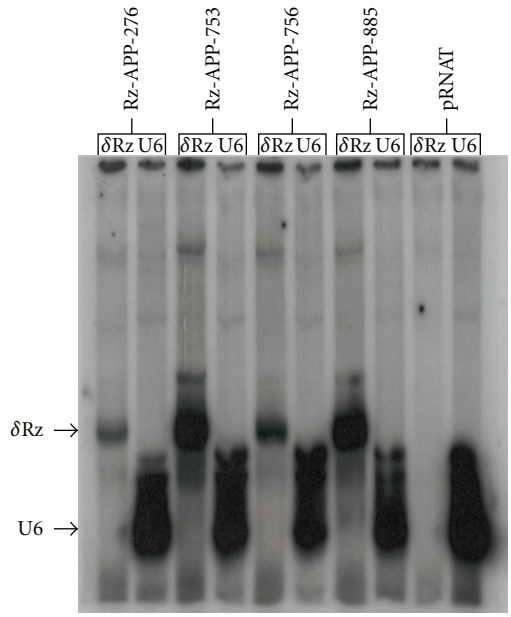

Figure 3: Expression of APP-SOFA-HDV ribozymes in the HEK cell line. Primer extension analysis of total cellular RNA from cells transfected with four selected APP-SOFA-HDV ribozymes from the ribozyme collection (APP-SOFA-HDV-Rz276, APP-SOFA-HDV-Rz753, APP-SOFA-HDV-Rz756, and APP-SOFA-HDV-Rz885). Transcripts corresponding to HDV-ribozymes were detected with HDV-Rz primers (5′-GGGTCCCTTAGCCATGCGCGAACG-3′). U6 primer (5′-GGCCATGCTAATCTTCTCTG-3′) was also used as a positive control, which yielded signals corresponding to endogenous U6 snRNA. Note that all of the 4 selected SOFA-HDV ribozymes were expressed (lanes 1 to 4). A pRNAT empty vector, was used as a negative control.

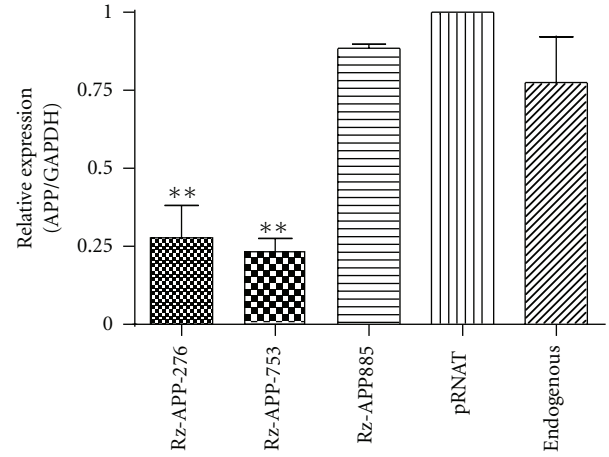

Figure 4: Relative APP mRNA level in HEK-293 cells expressing APP-SOFA-HDV Rz. The expression profile of APP in SOFA-HDV-Rz276-, -Rz753-, -Rz756-, and -Rz885-transfected HEK-293 cells. To assess APP knockdown efficiency at the mRNA level, quantitative real-time PCR was performed with each of the forward and reverse APP or GAPDH primers using the ABI Prism 7000 Sequence Detection System and SYBR Green DNA binding dye (Invitrogen). The specific amplification was assessed based on the dissociation curve profile. The APP gene expression profile was normalized against that of GAPDH. The quantitative PCR procedure was performed in duplicate in three independent reactions for each sample. $^{**}P < 0.01$. An approximately 70–80% decrease in GAPDH-normalized APP mRNA levels was observed with cells expressing active SOFA-HDV Rz (the most potent Rz). The GAPDH-normalized levels of endogenous APP were not significantly altered in the untreated or empty vector-transfected control cells.

synthesized U6 snRNA and SOFA-HDV ribozymes transcribed from the U6 promoter were detected, respectively, by U6- and Drz ^{32}P-labeled primers (see Section 2). As indicated by Figure 3 (lanes 1 to 8), the specific extension products corresponding to U6 SOFA-HDV-Rz-APP276, -APP753, -APP756, and -APP885 were detectable in the transfected cells. An expression vector lacking a SOFA-HDV ribozyme coding sequence (pRNAT/U6) was used as a negative control. No detectable band of Drz ^{32}P-labeled primers was observed with the empty vector (Figure 3, lane 9). The expression levels for SOFA-HDV-Rz-APP753 and -APP885 were among the highest, while two other ribozymes (SOFA-HDV-Rz-APP276, and -APP756) exhibited weaker expression.

3.3. Effect of Selected SOFA-HDV Ribozymes on APP mRNA Expression Level. Considering the close and positive correlation between the level of APP mRNA, protein, and Aβ deposition in AD [19], APP mRNA expression levels following APP SOFA-HDV ribozyme expression were initially monitored. Previously, it has been shown that the SOFA-HDV Rz expressed in HEK-293 cells could be a powerful and specific gene silencing tool [9]. Therefore, SOFA-HDV-Rz-APP was transiently transfected into HEK-293 cells, which are well known for the expression of endogenous APP mRNA. pRNAT-U6 empty vector was used as a control. The total RNA was extracted from cells 48 h after transfection, and APP mRNA levels were quantified by real-time quantitative PCR (qPCR). The GAPDH mRNA was used as a control for the qPCR to normalize the APP mRNA levels. A significant effect of SOFA-HDV-Rz-APP ribozyme expression on cellular APP mRNA levels was observed (Figure 4). SOFA-HDV-Rz-APP276 and -APP753 transfection led to a highly significant ($P < 0.001$; Student's t-test) decrease in APP mRNA steady-state levels (~70% and 80%, resp., relative to the control cells). Conversely, both SOFA-HDV-Rz-APP756 and -APP885 expression did not show a significant decrease at the APP mRNA level, suggesting that cleavage sites at positions 756 and 885 may not be as accessible *in cellulo* as they were in the *in vitro* assays on partial mRNA transcripts. The transfection of cells with an empty vector resulted in a faint increase in APP mRNA compared with untransfected cells (Figure 4, lanes 5 and 6), but this effect was not significant ($P > 0.05$; Student's t-test). More importantly, this experiment provided physical evidence that the expression of both SOFA-HDV-Rz-APP276 and -APP753 in HEK-293 cells resulted in an important decrease of the targeted APP mRNA levels and that nonspecific effects of vector transfection could not account for this decrease.

3.4. Effect of the SOFA-HDV Ribozyme on APP Protein Levels. Because the correlation between the level of mRNA

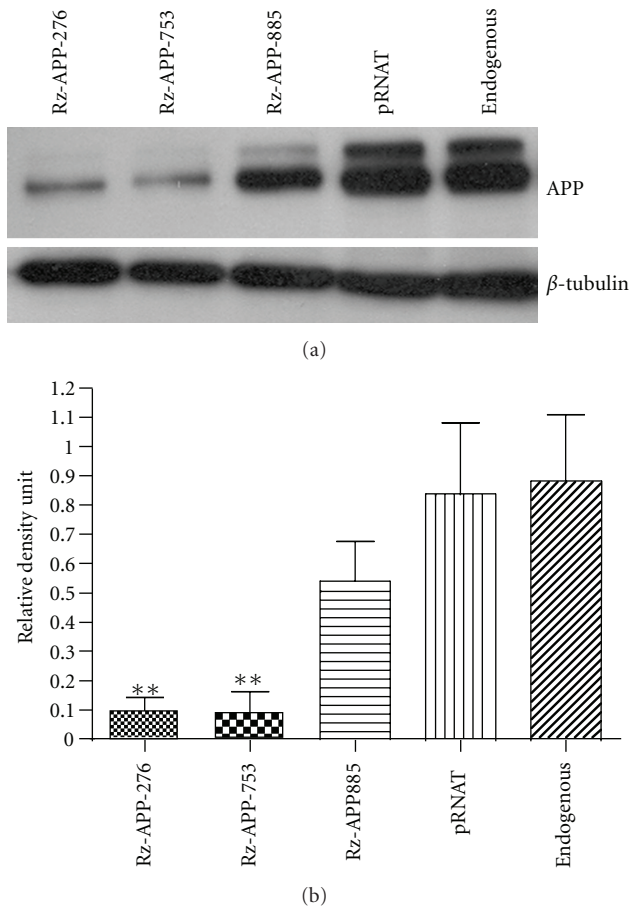

Figure 5: Rz-APP-X ribozymes expression reduces APP levels. (a) Western blotting analysis performed using equal amounts of 20 μg of extracted protein. APP was immunodetected using a polyclonal antibody (Sigma) recognizing the C-terminus of human APP. The tubulin (β-tubulin) controlled the amount of sample loaded in each lane. The highest APP reduction (approximately 85%) was obtained with Rz-APP-276 and Rz-APP-753. (b) Densitometric quantification of the APP lanes in the blot from (a). Relative density unit values were obtained by standardization with the corresponding β-tubulin protein band in each lane. The results in (b) are presented as the means ± SEM from 3 independent experiments. $^{**}P < 0.01$.

and its concomitant protein is not always linear, the effect of APP's directed SOFA-HDV ribozymes on APP protein levels was then investigated to verify whether the decrease in APP mRNA level results in a reduction at the protein level. SOFA-HDV-Rz-APP ribozymes were transfected into HEK-293 cells. As a control, cells were transfected with a pRNAT-U6 vector expressing GFP protein. At 48 h after transfection, the cells were lysed and total proteins were extracted. Subsequently, a Western blot was performed with a specific anti-APP C-terminal antibody as a probe. The level of APP was estimated by densitometry and normalized using endogenous β-tubulin (Figure 5). As expected, cells expressing both SOFA-HDV-Rz-APP276 and -APP753 showed a drastic decrease in APP levels compared with un transfected cells, those transfected with empty vector or those transfected with SOFA-HDV-Rz-APP885 (Figure 5(a)). The last construct exhibited a decrease estimated to be less than 20%, whereas the two other ribozymes led to reductions of over 70% relative to the controls. All of the changes were highly significant ($P < 0.001$) when compared with either untransfected cells or cells transfected with pRNAT/U6. Thus, the decrease in APP mRNA resulting from the expression of SOFA-HDV ribozyme is correlated with the change observed at the protein level. Moreover, these data are strongly consistent with the hypothesis that the expression level of APP mRNA is closely and positively correlated with its concomitant protein level [20].

3.5. Assessment of Aβ Secretion Levels in SOFA-HDV Ribozyme-Treated Cells. From the perspective of AD therapy, any attempts to decrease APP mRNA levels should also result in a decrease in Aβ levels. To assess whether a decline of APP in ribozyme-treated cells leads to a decline in total Aβ levels, the level of secreted Aβ following SOFA-HDV-Rz-APP expression was determined by ELISA. For this experiment, the SOFA-HDV-Rz-APP276 and SOFA-HDV-Rz-APP753 were selected as the two more active and powerful ribozymes. Because neurons will be the target of the ribozymes in the context of AD, this ribozyme was tested on a neuronal cell type, SHSY-5Y, using a lentiviral system of expression. This system is essential for transducing neurons because post-mitotic cells cannot be efficiently transfected by other vectors. Following SHSY-5Y transduction, the SOFA-HDV-Rz-APP753 expression was tested for its effect on the reduction of APP at both the mRNA and protein levels (data not shown). To evaluate the Aβ level, media samples were collected and analyzed for Aβ_{1-x} species, as both Aβ_{40} and Aβ_{42} are associated with AD pathogenesis. Knocking down APP with lentiviral SOFA-HDV-RzAPP276 or OFA-HDV-RzAPP753 transduction of SHSY-5Y cells reduced the total level of Aβ by more than 30% (Figure 6). This result indicates that a SOFA-HDV ribozyme could be a potential means of targeting APP.

4. Discussion

In this study, we designed a new molecular tool to target the top of the amyloid cascade, namely, the APP mRNA. The SOFA-HDV ribozyme is based on a new synthetic HDV ribozyme harboring a biosensor module that activates mRNA cleavage only in the presence of the specific RNA target substrate [9]. This specific *On/Off* adapter (SOFA module) provides not only a higher specificity to the HDV Rz toward its target but also a higher cleavage capacity [10]. An initial experiment to identify the most susceptible site within the 5′ end region of the APP mRNA was performed based on the use of a randomized library of short oligonucleotides mimicking the recognition domain of the ribozyme. The hydrolysis of the formed RNA/DNA heteroduplexes by the RNase H led to the identification of 10 potential sites (Table 1). *In vitro* cleavage of a partial APP transcript by the corresponding appropriate SOFA-HDV ribozymes revealed that 4 of these sites could be cleaved at a significant level (Figure 2). Interestingly, an analysis of the sequence and secondary structure of the SOFA-HDV ribozymes that

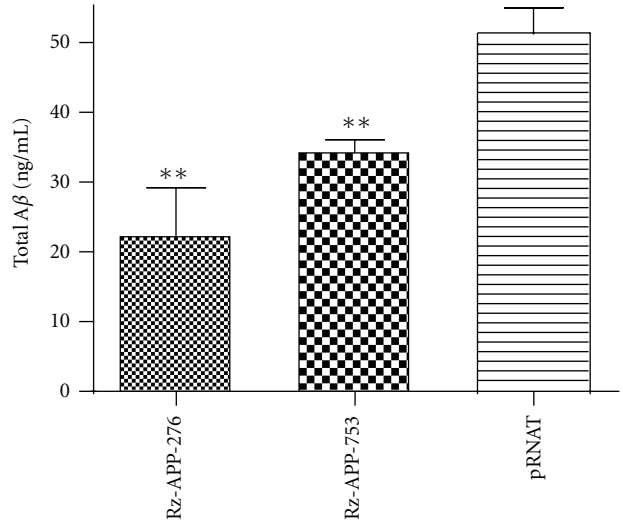

Figure 6: Effect of the most active ribozyme on the level of secreted total amyloid. The secreted levels of $A\beta_{1-x}$ were detected by sandwich ELISA, as described in Section 2. Media were collected following SH-SY5Y infection with the Rz-APP-286, Rz-APP-753, or pRNAT empty vector. The results are presented as the means ± SEM from 3 independent experiments. $^{**}P < 0.001$. Control cells were infected with the empty pRNAT vector.

exhibited only moderate cleavage activity indicated that misfolding of 5 out of 6 of these ribozymes may explain their limited potential (data not shown). Therefore, only one of the SOFA-HDV ribozymes did not cleave efficiently for any specific reason. This result is excellent, considering that the initial analysis was based on the hybridization of small oligonucleotides and that the SOFA-HDV ribozyme is almost a magnitude larger in size but possesses a complex tertiary structure and two binding domains that interact with the substrate (i.e., the recognition and biosensor domains) and undergoes conformation transition [21].

The SOFA-HDV ribozymes exhibiting the highest cleavage activity *in vitro* were further studied *in cellulo*. These ribozymes were expressed from a U6 promoter for the RNA pol III because it allows efficient transcription of small RNA molecules [22]. According to the primer extension assays performed for these four SOFA-HDV ribozymes, they all exhibited good expression, although variable, in transfected HEK293 cells (Figure 3). Therefore, neither their ability to be expressed nor their stability accounted for their variable cleavage activity. Two of the SOFA-HDV ribozymes exhibited equivalent and drastic reductions in APP at both the mRNA and protein levels (Figures 4 and 5; SOFA-HDV Rz-APP276 and -APP753). It is not surprising that only some of the ribozymes that showed excellent cleavage activity *in vitro* exhibited significant cleavage activity *in cellulo*. Several different factors in cells compared with the *in vitro* analysis may account for this result. The *in cellulo* target is the full-length mRNA, which may adopt a different structure, and cellular proteins may be bound to it and create steric hindrances that reduce the accessibility to some of the identified cleavage sites.

To our knowledge, this study is the first reporting acute silencing of APP in human cells using an HDV ribozyme-based approach. SOFA-HDV ribozyme-based gene silencing constitutes an alternative to using small interfering RNA, a method that faces several limitations. One of the largest hurdles in RNAi-based therapy is toxicity. In this context, independent off-target or nonspecific effects of siRNA are a concern [23, 24]. Side effects can result from unintended interactions between an siRNA compound and an unrelated host gene. This nonspecific interaction with host genes may cause adverse effects in the host. Moreover, shRNA expression in neurons has been shown to interfere with dendritic spine structure and function, resulting in a decrease of synapses [25]. Interferon response is the best known adverse effect in the viral-mediated transfection of siRNAs. Similarly, synthetic siRNAs formulated in nonviral delivery vehicles can also be potent inducers of interferons and inflammatory cytokines, both *in vivo* in mice and *in vitro* in human blood [26]. The most important difference between ribozyme technology and RNAi technology is that RNAi requires the recruitment of endogenous proteins, which are responsible for the high intracellular activity. Thus, problems of potency, specificity, and/or cell-type-dependent responses illustrate a lack of understanding of the intracellular mechanisms involved [27]. By contrast, the HDV Rz, which derives from the hepatitis *delta* virus, possesses several unique features that are all related to the fact that it is the only naturally occurring catalytic RNA discovered in humans [7, 28, 29] and that its action does not depend on intracellular factors [30]. In addition, it exhibits an outstanding stability (i.e., a half-life > 100 hr) in cell culture [8]. Moreover, a genome-wide search for innate ribozyme entities revealed the presence of HDV-like sequences in the human CPEB3 gene [31]. Consequently, the HDV Rz should not be recognized by the immune system as an external, invading RNA.

Several studies in human genetic and animal models support the notion that amyloid production or accumulation in the brain plays a central role in the pathogenesis of AD. Lowering amyloid levels in different mouse models has demonstrated therapeutic value [32, 33]. Multiple approaches aimed at interfering with Aβ metabolism have been proposed as a therapy for AD. First, compounds that aim to decrease the aggregation of Aβ by blocking its oligomerization have been tested [34]. Although successful in mice models of AD, they failed in human trials. Second, lowering Aβ levels by increasing its clearance using a vaccine was successful in animal models [35, 36], but the results from human clinical studies indicated important side effects, and there were concerns about safety in humans [37]. Finally, blocking the activity of the secretases (β and γ) is attractive because both of these enzymes participate in Aβ production by cleaving APP. However, because these secretases have numerous substrates essential for cellular functions, blocking their activity raises additional concerns. The data from β-secretase (BACE1) knockout animals have suggested potential liabilities with BACE1 inhibition [38–40]. BACE1 is also known to participate in myelination [41–43] and the processing of sodium channels [43]. Nonetheless, BACE1 inhibitors have been developed, but blood-brain-barrier

penetration and limited access to cellular BACE1 due to its major location in the endosome pose significant challenges that have yet to be overcome [44, 45]. Another potential therapeutic target is γ-secretase, although it has numerous essential cellular substrates. However, recent clinical trials testing a very promising γ-secretase inhibitor have raised major safety issues about this route [46, 47]. This failure does not question the amyloid hypothesis, but instead the nonspecific targeting of an enzyme complex with so many cellular functions [48].

Because there have been many failures in targeting amyloid peptide metabolism, we believe that targeting the top of the cascade by decreasing APP mRNA would be a better way to decrease the overall amyloid level. We do not expect a complete knock down of APP mRNA and Aβ, but we are confident that we can significantly reduce APP mRNA levels (and subsequently, Aβ levels). We believe that there is a threshold effect and that a modest reduction in Aβ levels could shift the balance between toxicity and nontoxicity. Another advantage of specifically targeting APP mRNA is that the level of all forms of the peptide derived from APP will also be decreased. To achieve this end, we engineered specific SOFA HDV ribozymes, a new generation of catalytic RNA tools, to decrease the APP mRNA level. We demonstrated that a SOFA-HDV ribozyme targeting APP mRNA is clearly effective for the reduction of Aβ in neuron-like cells. Further analysis using an expression system based on the lentivirus indicated a significant decrease of ~30% in total Aβ levels (Figure 6). Therefore, this action could possibly affect downstream amyloid-related pathology. Because only a 12% decrease in Aβ levels in mice resulted in a dramatic reduction in Aβ build-up and synaptic deficits [49], we are optimistic that the results obtained in our cellular model will allow for the development of an efficacious form of SOFA-HDV ribozyme-based therapy. The exact role of *Aβ* as a trigger of sporadic Alzheimer's disease is still a question of debate. Moreover it is actually not clear which *Aβ* species is associated with the disease. Over production of *Aβ* is probably not the cause of amyloid accumulation in sporadic AD. A defective clearance of amyloid may trigger its aggregation. This is supported by the fact that the ApoE4 allele, which is the major genetic risk factor for sporadic AD, slows down *Aβ* clearance [50]. Whatever the cause of *Aβ* accumulation, we believe that decreasing *Aβ* production by specifically targeting the APP mRNA will contribute to a decrease in the amyloid load to a non-toxic level. Actually, all therapies targeting *Aβ* (secretase inhibitors, vaccines, etc.) aim to reach this nontoxic level.

One limitation of our gene silencing approach is the delivery of the ribozyme to the nervous system. The presence of the blood-brain barrier limits the penetration of particle as large as a lentivirus into the central nervous system (CNS). To avoid this limitation local stereotaxic injections of lentiviruses could be used. Although this method is invasive, robust long-term and nontoxic lentiviral gene transfer is feasible in the rodent and nonhuman primate brains [51, 52]. Expression over 3 to 8 months can be achieved and it has been demonstrated that up to 90% of cells from the central nervous system transduced by a lentiviral vector under the control of the NSE promoter are neurons [53, 54]. However the method of choice for lentiviral delivery is the i.v. or i.p. route. This could be achieved by the fusion of the low-density lipoprotein receptor-binding domain of the apolipoprotein B to the therapeutic molecule. Successful application of this approach as a general method for the delivery of therapeutic molecules to the CNS has been demonstrated [55]. Moreover it remains possible that systemic presence of SOFA HDV ribozymes will decrease *Aβ* level in the periphery and concomitantly brain *Aβ* levels due to the "sink hypothesis." Evaluation of these delivery methods will involve extending our study to animal models of Alzheimer's disease, thus the exciting potential of this new treatment will be revealed in the future.

This development will involve extending our study to animal models of Alzheimer's disease, so the exciting potential of this new treatment will be revealed in the future.

We have presented an original and unambiguous demonstration that a SOFA-HDV ribozyme can serve as an efficient gene silencing tool. Moreover, our results open the door to further evaluation of SOFA-HDV ribozymes as potential therapeutic molecules, or at least to a study demonstrating whether a reduction in Aβ levels is a viable therapy against Alzheimer's disease.

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Research Article

Simvastatin Blocks Blood-Brain Barrier Disruptions Induced by Elevated Cholesterol Both In Vivo and In Vitro

Xijuan Jiang,[1] Maojuan Guo,[2] Jinling Su,[1] Bin Lu,[2] Dongming Ma,[2] Ruifeng Zhang,[1] Lin Yang,[2] Qiang Wang,[1] Yiwen Ma,[1] and Yingchang Fan[1]

[1] *Department of Pathology, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China*
[2] *Department of Experimental Teaching, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China*

Correspondence should be addressed to Yingchang Fan, jxj2668@sina.com.cn

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Background. Hypercholesterolemia and disruptions of the blood brain barrier (BBB) have been implicated as underlying mechanisms in the pathogenesis of Alzheimer's disease (AD). Simvastatin therapy may be of benefit in treating AD; however, its mechanism has not been yet fully understood. *Objective*. To explore whether simvastatin could block disruption of BBB induced by cholesterol both in vivo and in vitro. *Methods*. New Zealand rabbits were fed cholesterol-enriched diet with or without simvastatin. Total cholesterol of serum and brain was measured. BBB dysfunction was evaluated. To further test the results in vivo, rat brain microvascular endothelial cells (RBMECs) were stimulated with cholesterol in the presence/absence of simvastatin in vitro. BBB disruption was evaluated. *Results*. Simvastatin blocked cholesterol-rich diet induced leakage of Evan's blue dye. Cholesterol content in the serum was affected by simvastatin, but not brain cholesterol. Simvastatin blocked high-cholesterol medium-induced decrease in TEER and increase in transendothelial FITC-labeled BSA Passage in RBMECs. *Conclusions*. The present study firstly shows that simvastatin improves disturbed BBB function both in vivo and in vitro. Our data provide that simvastatin may be useful for attenuating disturbed BBB mediated by hypercholesterolemia.

1. Introduction

The blood-brain barrier (BBB), a barrier between the central nervous system (CNS) and the systemic circulation, maintains homeostasis within the brain microenvironment. The anatomical substrate of the BBB is the cerebral microvascular endothelium, which, together with astrocytes, pericytes, neurons, and the extracellular matrix, constitutes a "neurovascular unit" that is essential for the health and function of the CNS. Failure of the BBB is a critical event in the progression of several diseases, such as Alzheimer's disease (AD) [1–4]. BBB breakdown is accompanied by an increase in the transendothelial permeability to substances, which can damage the microenvironment of the brain and affect the structure and function of the CNS. Therefore, it is not surprising that strategies have been developed to "repair" the BBB in order to restore normal brain homeostasis and prevent the infiltration of pharmacologically active (noxious) substances into the brain. Cholesterol is one of the most notorious natural risk factors for arteriosclerotic cerebrovascular disease [5, 6]. Mounting evidence suggests that cholesterol also plays a critical role in the early stage of AD [7–9]. Hypercholesterolemia leads to increased BBB leakage, an effect that may contribute to AD pathogenesis [10, 11]. Thus, blocking BBB disruption may have beneficial effects against AD caused by hypercholesterolemia. Several epidemiological studies have revealed that cholesterol-lowering statins, which are used for the treatment of coronary arterial disease, are associated with a decreased risk of developing AD. However, the mechanisms underlying this effect remain unclear [8, 12]. Statins have been shown to ameliorate BBB dysfunction resulting from a number of conditions, including diabetes, transient focal cerebral ischemia, and HIV-1 [13–15]. However, the effects of statins on BBB disruptions induced by hypercholesterolemia have not been reported. To address this question, the permeability of the BBB was studied in vivo using rabbits fed a cholesterol-enriched diet and treated with simvastatin, a widely used natural statin derived from

fermentation. The effect of simvastatin on BBB was also performed using rat brain microvascular endothelial cells (RBMECs) cultured under high-cholesterol conditions in vitro.

2. Materials and Methods

2.1. Chemicals. Simvastatin capsules used in vivo were purchased from Nantong Hua Pharmaceutical Co., Ltd (Jiangsu, China). The simvastatin used in vitro and Evans blue dye were obtained from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin, DNase I, collagenase, collagenase/dispase, and fluorescein-isothiocyanate-conjugated bovine serum albumin (FITC-BSA) were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies to ZO-1 and occludin were purchased from Zymed (San Francisco, CA, USA).

2.2. Animals and Treatment. Twenty-four adult male New Zealand White rabbits (2356 ± 212 g) were used in this study. Rabbits were housed individually in the rabbit facility and were kept on a 12 h light/12 h dark cycle at 28 ± 2°C and 37–48% humidity. Animals were randomly assigned to three groups. The *control diet group* ($n = 8$) consisted of rabbits that received normal rabbit chow for 10 weeks. The other rabbits were fed chow supplemented with 2% cholesterol for 6 weeks and were then randomly assigned to either the *cholesterol group* ($n = 8$), which was fed chow supplemented with 2% cholesterol or the *cholesterol + simvastatin group* ($n = 8$), which was fed chow supplemented with 2% cholesterol and 5 mg/kg/d simvastatin for an additional 4 weeks. The olfactory bulbs, brain regions with an intact BBB under normal physiological conditions, have been implicated in Alzheimer's disease by studies reporting that AD patients experience olfactory dysfunction [16]. Other research has shown that the hippocampus is characterized by a selective fragility of the BBB and is often affected by AD pathology early in the disease process [17]. Accordingly, the olfactory bulbs and hippocampus were two primary brain areas examined in this study. All experiments were approved by the Committee for Animal Care and Use at Tianjin University of Traditional Chinese Medicine.

2.3. Evan's Blue Leakage Assay. To assay BBB permeability, Evans blue dye (4%; 25 mg/kg) was injected into rabbits through the ear vein and allowed to circulate for 3 h before the animals were anesthetized with pentobarbital sodium salt (30 mg/kg). While deeply anesthetized, animals were perfused with 37°C saline via the left cardiac ventricle to wash out any vascular Evans blue. Following perfusion, brains were quickly removed, and the olfactory bulbs and hippocampi were isolated, weighed, and incubated for 72 h with formamide in the dark at room temperature (25°C). After incubation, samples were centrifuged at 10,000 ×g for 10 min, supernatants were collected, and the absorbance was determined (Ex 620 nm and Em 680 nm) with a Jasco FP-777 spectrofluorimeter (Jasco UK Ltd, Essex, UK). Evan's blue concentrations were calculated from standard curves, and results were expressed as Evan's blue/specimen wet weight [18].

2.4. Immunohistochemistry. To evaluate the expression of the tight junction proteins, occludin and zonula occluden-1 (ZO-1), cryostat brain sections (10 μm) of the olfactory bulbs and hippocampi were fixed with acetone and then rinsed 3 times in 0.01 M PBS for 5 min each. Fluorescently labeled primary antibodies against ZO-1 (Zymed, clone ZO1-1A12, dilution 1 : 200) and occludin (Zymed, clone OC-3F10, dilution 1 : 200) were diluted in PBS containing 1% Triton X-100, applied to the brain sections and incubated overnight at 4°C in a dark humidified chamber. Sections were rinsed 3 times in 0.01 M PBS for 5 min each and were subsequently examined by Leica microscopy. Images were analyzed with Image J software.

2.5. Serum and Brain Cholesterol Measurements. Total serum cholesterol was measured in venous blood collected from rabbit ear veins using standard enzymatic techniques with a Fully Automatic Biochemistry Analyzer. Following perfusion, brains were removed quickly and the cortex and hippocampus were removed and weighed. Cholesterol levels in the cortex and hippocampus were measured using reverse-phase HPLC (ZORBAX XDB C18 4.6 × 250 mm Spheri-5 RP C18 column, 5 μm, Agilent, flow rate, 0.8 mL/min) with Varian Prostar 325 ultraviolet detection at 208 nm and LC Workstation V6.2 Chromatographic Data System.

2.6. Primary Culture and Treatment of Rat Brain Microvessel Endothelial Cells. Primary cultures of rat brain microvessel endothelial cells (RBMECs) were isolated from Wistar rat (approximately 100 g) brains using a combination of enzyme digestion and ultracentrifugation approaches as described previously [19], with minor modifications. In brief, fresh rat brains were obtained, and the surface vessels and meninges of the brains were removed. The grey matter was cut into 1 mm^3 pieces, digested with 39 U/mL DNase I and 0.7 mg/mL collagenase at 37°C for 1 h, and centrifuged at 1000 ×g for 10 min. After centrifugation, the supernatant was discarded, 20% BSA was added, and the resulting mixture was centrifuged at 1000 ×g for 20 min. The dark red pellet was collected and subjected to further enzymatic digestion in 39 U/mL DNase I and 1 mg/mL collagenase/dispase for 1 h. After centrifugation, the supernatant was discarded, and brain capillary fragments were seeded onto matrigel-coated Transwell filters (a pore size of 0.4 μm Transwell, Corning Life Sciences, USA) in Dulbecco's modified Eagle's medium supplemented with 20% FBS, 100 μg/mL heparin, 30 μg/mL ECGF, and 2 mM glutamine. The purity of RBMECs was >95% as determined by factor-VIII-related antigen and immunocytochemical staining (data not shown). These cultures, which were used as an in vitro model of the BBB, were maintained for 8–10 days to obtain confluence prior to starting experiments. When a stable transendothelial

electrical resistance (TEER) value was obtained, the cultures were washed twice with serum-free DMEM and treated with water-soluble cholesterol (10 μM) for 24 h with or without simvastatin (5 μM) to evaluate disruption transendothelial permeability [20].

2.7. Transendothelial Electrical Resistance (TEER) Measurement with RBMECs. TEER was measured using a Millicell-ERS electrical resistance apparatus (Millipore, Eschborn, Germany) 24 h after treatment with simvastatin. To study the effects of simvastatin on TEER, the Transwell inserts with the RBMECs cultures mentioned above were placed into the chamber electrode filled with HEPES-buffered (25 mM) serum-free DMEM [21]. The symmetrically apposing electrodes were situated above and beneath the membrane, allowing a uniform current density to flow across the membrane. The resistance was recorded when the meter indicated a stable resistance. Resistance values of multiple transwell inserts from each experimental group were expressed in common units (Ω/cm^2) after subtracting the value of a blank cell-free filter.

2.8. Measurement of Transendothelial Protein Passage. Transendothelial permeability to macromolecules was assessed by passage of fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA) across the monolayer as described previously [22]. In brief, primary RBMECs were treated with cholesterol in the presence/absence of simvastatin as mentioned above. FITC-BSA (50 μg/mL) was added to the upper chambers and its diffusion across the model BBB was monitored using an FL600 microplate fluorescent reader (Biotek, Winooski, VT) (Ex 488 nm, Em 525 nm) 3 h after the addition of FITC-BSA. BBB permeability was calculated using the following formula: (BSA lower chamber) $\times$ 100/(BSA upper chamber).

2.9. Statistical Analyses. All data were expressed as mean $\pm$ SD. Statistical significance for multiple comparisons was determined using one-way ANOVAs and Tukey's post hoc tests with SPSS software (SPSS, Chicago, IL, USA). The significance threshold was set at $P < 0.05$.

3. Results

3.1. Simvastatin Attenuates High Cholesterol Diet-Induced Leakage of Evan's Blue Dye In Vivo. BBB integrity was assessed by Evans blue extravasation (Figure 1). Evans blue is normally excluded from the brain parenchyma by the BBB and is only detectable when the integrity of the BBB is compromised. Increased BBB permeability was observed in rabbits fed a high-cholesterol diet (supplemented with 2% cholesterol for 10 weeks), as evidenced by the increased Evans blue content in their olfactory bulbs and hippocampi when compared to those of rabbits fed a normal diet. Oral administration of simvastatin at a dose of 5 mg/kg/day for 4 weeks (weeks 7–10) attenuated the high cholesterol diet-induced leakage of Evan's blue dye in both the olfactory bulb and the hippocampus.

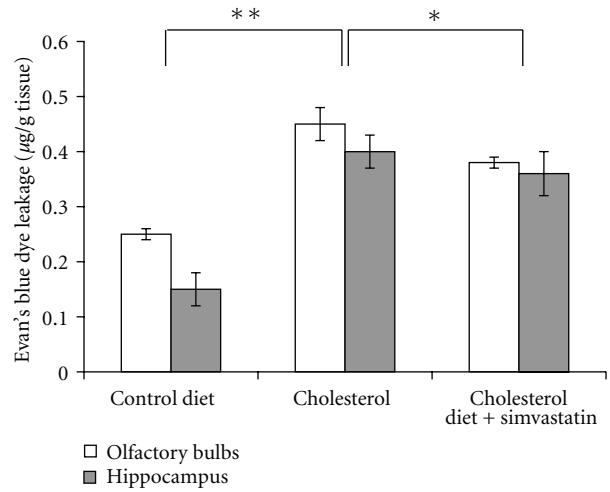

Figure 1: Simvastatin blocks high-cholesterol diet-induced leakage of Evan's blue dye. New Zealand White rabbits were fed a normal diet (10 weeks) or a high-cholesterol diet (10 weeks) with or without simvasatin (over the final 4 weeks). After treatment, BBB permeability was evaluated by measuring Evan's blue dye leakage. High-cholesterol diet significantly increased the leakage of Evan's blue dye into the olfactory bulbs and hippocampus, and these effects were attenuated by treatment with simvastatin (5 mg/kg/day during weeks 7–10) ($n = 8$, $^*P < 0.05$; $^{**}P < 0.01$).

3.2. Simvastatin Had No Effect on the High-Cholesterol Diet-Induced Downregulation of Tight Junction Proteins In Vivo. The tight junctions (TJs) between the endothelial cells serve to restrict blood-borne substances from entering the brain. ZO-1 and occluding were the major interendothelial junctional proteins. Thus, to determine if the effects of simvastatin on BBB permeability resulted from alterations in the major interendothelial junctional proteins, the expression of ZO-1 and occludin was examined in rabbits fed a high-cholesterol diet treated with simvastatin. The results revealed decreased immunostaining for ZO-1 (Figures 2(a) and 3(a)) and occludin (Figures 2(b) and 3(b)) in the olfactory bulbs and hippocampi of rabbits fed a high-cholesterol diet for 10 weeks when compared to rabbits fed a normal diet. However, simvastatin (5 mg/kg/day for 4 weeks) had no effects on either the expression of occludin or ZO-1.

3.3. Simvastatin Affects Plasma Levels of Cholesterol but Not Levels of Cholesterol in the Brain. Cholesterol seems to play an important role in the development of AD. Therefore, it is of interest to evaluate the effects of high doses of simvastatin on levels of cholesterol in serum and in brain. As expected, rabbits fed a cholesterol-enriched diet for 10 weeks exhibited over a 20-fold increase in total serum cholesterol concentration. Treatment of high-cholesterol-diet-fed rabbits with simvastatin (5 mg/kg/day for 4 weeks) significantly reduced plasma cholesterol levels. However, cholesterol levels in the hippocampus and cortex were not affected by a cholesterol-enriched diet in the presence or absence of simvastatin (Table 1).

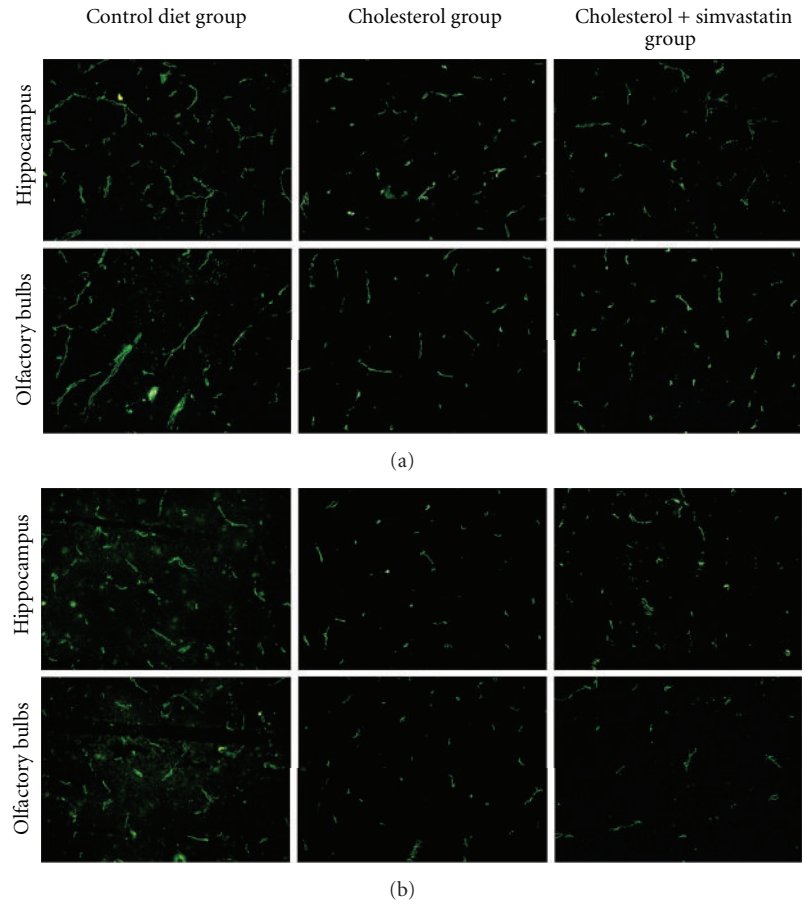

Figure 2: Simvastatin has no effect on high-cholesterol diet-induced downregulation of tight junction proteins. Cryostat sections of the olfactory bulbs and hippocampus were incubated with fluorescently labeled antibodies against occludin and ZO-1, and representative images for each treatment group are shown. (a) Decreased ZO-1 immunostaining was observed in the olfactory bulbs and hippocampus of cholesterol-fed rabbits (supplemented with 2% cholesterol for 10 weeks). This effect was not blocked by treatment with simvastatin (5 mg/kg/day for 4 weeks). (b) Decreased occludin immunostaining was observed both in the olfactory bulbs and hippocampus of cholesterol-fed rabbits, and this effect was not blocked by simvastatin (200×).

Table 1: Concentration of total cholesterol (TC) in the serum, cortex, and hippocampus of rabbits fed a control diet, high-cholesterol diet, and high-cholesterol diet plus simvastatin.

Group	Serum TC	Cortex TC	Hippocampus TC
Control diet	51.4 ± 9.96[b]	1.29 ± 0.16	1.36 ± 0.14
Cholesterol diet	1449.6 ± 677.1	1.25 ± 0.13	1.31 ± 0.23
Cholesterol diet + simvastatin	866.1 ± 147.46[a]	1.27 ± 0.11	1.38 ± 0.10

The concentration of total serum cholesterol is expressed as g/mL and the concentration of total cortex or hippocampus cholesterol is expressed as g/100 g wet weight of tissue. Data are expressed as means ± S.D. ($n = 8$; [a]$P < 0.05$; [b]$P < 0.01$; compared with Cholesterol diet).

3.4. Simvastatin Blocks the TEER Decline and Increased Transendothelial Protein Permeability Induced by High Levels of Cholesterol In Vitro. To further verify the observed effects of simvastatin, primary cultures of brain endothelial cells, the most important component of the BBB, were examined. Primary RBMECs were stimulated with cholesterol (10 μM) in the presence/absence of simvastatin (5 μM) for 24 h. Potential changes in the integrity of the BBB were assessed by measuring TEER and the permeability of an RBMEC monolayer to FITC-BSA. Figure 4(a) shows that cholesterol markedly retarded the development of TEER of RBMEC monolayers during postconfluent growth. A significant effect of simvastatin (5 μM) became visible after a 24 h treatment of RBMECs. Consistent with the results of TEER, cholesterol was shown to induce increased permeability to the large molecular weight tracer FITC-BSA, and this effect was significantly blocked by simvastatin (Figure 4(b)).

4. Discussion

Our results showed that simvastatin significantly reduces leakage of Evan's blue dye across the BBB but does not

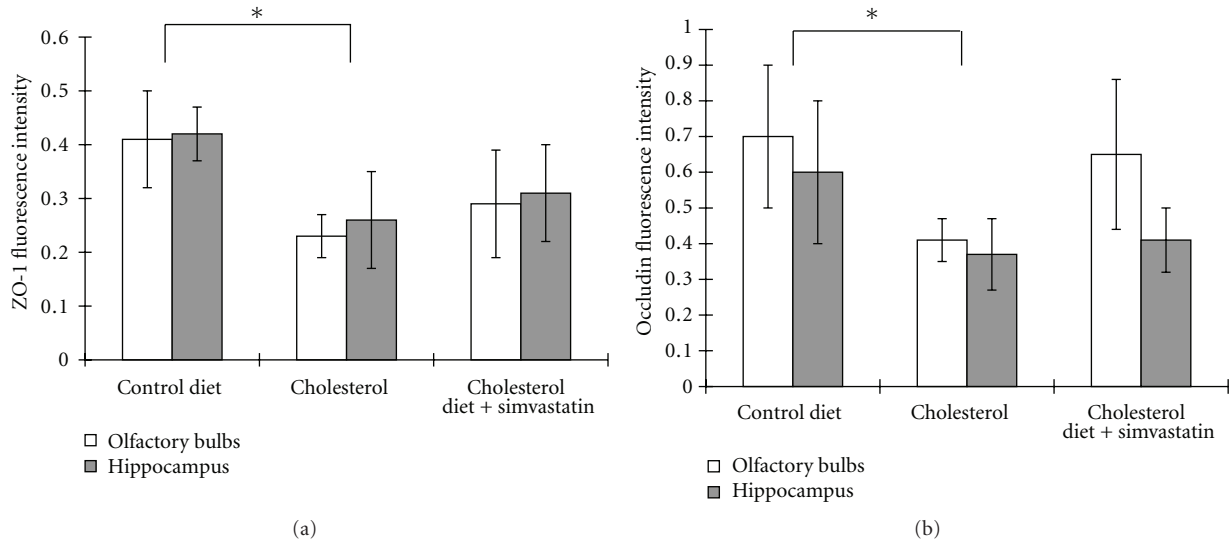

Figure 3: Simvastatin has no effect on high-cholesterol diet-induced downregulation of tight junction proteins. Images from Figure 2 were analyzed with Image J software. (a) Quantitative data from Figure 2(a) demonstrates that a high-cholesterol diet significantly decreases ZO-1 immunoreactivity in the olfactory bulbs and hippocampus, an effect that was not blocked by simvastatin. (b) Quantitative data from Figure 2(b) demonstrates that a high-cholesterol diet significantly decreased occludin immunoreactivity in the olfactory bulbs and hippocampus. However, this effect was not blocked by simvastatin treatment (5 mg/kg/day for 4 weeks) ($n = 8$, $^*P < 0.05$).

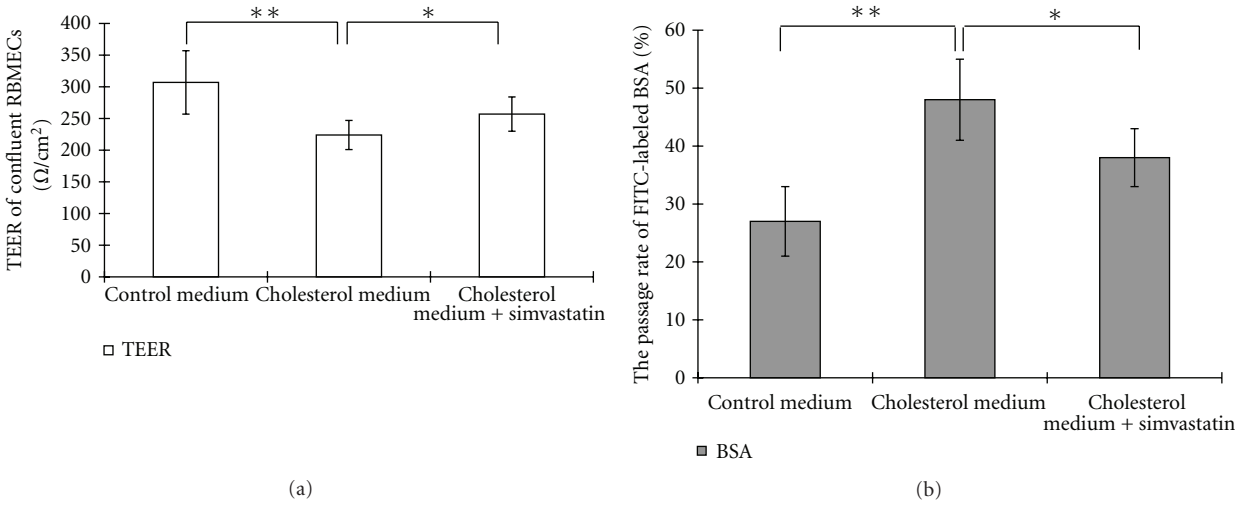

Figure 4: Simvastatin blocks cholesterol-induced TEER decline and transendothelial permeability in vitro. Rat primary brain endothelial cells were stimulated for 24 h with 10 μM cholesterol in the presence or absence of simvastatin (5 μM). TEER of confluent RBMECs (a) and permeability of FITC-BSA (b) were measured. Simvastatin significantly blocks both the TEER decline and the increase in FITC-BSA permeability induced by high cholesterol (a, b). TEER was expressed in ohms per square centimeter for electrical resistance ($n = 8$, $^*P < 0.05$; $^{**}P < 0.01$).

affect the expressions of the tight junction proteins, occlud, and ZO-1, in rabbits fed a cholesterol-rich diet. In addition, our data indicate that simvastatin alters plasma levels of cholesterol but does not affect levels of cholesterol in the brain. We also observed that cotreatment with simvastatin blocked the cholesterol-induced increase in transendothelial permeability and the cholesterol-induced decrease in TEER observed in primary cultured RBMECs. The semipermeable BBB restricts the diffusion of blood-borne substances into the brain parenchyma. Disruptions in the BBB can cause chemical imbalances in the neuronal "milieu," ultimately resulting in synaptic and neuronal dysfunction. Disruption of the BBB is a hallmark of Alzheimer's disease [10, 11]. Furthermore, elevated plasma cholesterol has been shown to increase BBB permeability and is a possible risk factor for AD [2, 23–26]. Consistent with the above findings, the present

study demonstrated that high levels of cholesterol can induce leakage of the BBB in vivo and disrupt the integrity of in vitro models of the BBB.

AD, a type of dementia that affects millions of elderly individuals, is the fourth leading cause of death among the elderly in developed countries [27]. Current therapeutic interventions for AD are largely ineffective. Epidemiological studies reveal a lower prevalence of AD in patients with hypercholesterolemia who are taking statins, a frequently prescribed class of lipid-lowering drugs [28, 29]. Several experiments have shown that statins may be potentially beneficial in the treatment of AD via their direct effects on brain cholesterol metabolism [30, 31]. However, other studies have failed to demonstrate a reduction in the ratio between 24S-hydroxycholesterol and cholesterol, in response to statin treatment [32]. At the same time, recent retrospective epidemiological studies have reported that the use of statins, but not nonstatin lipid-lowering agents, may reduce the risk of developing AD [8, 12]. Consistent with previous findings [33], our results also demonstrate that short-term simvasatin treatment does not affect brain total cholesterol; however, it has an effect on BBB integrity. This result suggests that the mechanisms by which statins protect against AD may extend beyond their lipid-lowering effects. Amyloid deposition is seen as the core pathological aspects of AD. Cholesterol has been mainly considered for dementia through promoting the generation of amyloid. However, immunization trials targeting the removal of amyloid-β plaques in Alzheimer's disease have so far failed to stop the progression of dementia. Recent data show that to reduce oxidative stress and inflammation-related factors in the brain by interfering with the permeability of the BBB may enhance the therapeutic effect mentioned above [34]. Therefore, to maintain the integrity of the BBB maybe more closely associated with the action of simvastatin on preventing AD instead of lipid-lowering effects.

The current study provides the first evidence for the protective effects of simvastatin against BBB disruptions both in rabbits with hypercholesterolemia and in RBMECs exposed to cholesterol-enriched media. In rabbits fed a high-cholesterol diet, simvastatin reduced the leakage of Evan's blue dye into the olfactory bulb and hippocampus. To follow up these findings, we subsequently carried out an in vitro study using brain microvascular endothelial cells exposed to 10 μM cholesterol as a model of BBB disruption. We found that simvastatin blocks the TEER decline and increased transendothelial permeability induced by high cholesterol, a result that corresponded well with our observed results in vivo. Because tight junctions between cerebral microvascular endothelial cells form the basis of the BBB [35], we examined the expression of two tight junction proteins, occludin and ZO-1, after simvastatin treatment. However, we found that simvasatin had no effect on the expression of tight junction proteins. These results suggest that simvastatin blocks BBB leakage induced by elevated cholesterol, independent of changes in the expression of the tight junction molecules, occludin and zonula occluden-1. This finding is in agreement with at least one previous report in the literature [22], which showed similar results in an in vitro model of multiple sclerosis.

In the present study, we did not explore the detailed molecular mechanisms whereby simvastatin protects against BBB disruption. However, we did demonstrate that simvastatin is effective in ameliorating BBB disruption induced by high-cholesterol diet in rabbits and in reducing cholesterol-induced endothelial permeability in an in vitro model of BBB disruption.

5. Conclusion

To the best of our knowledge, the present study is the first to show that simvastatin improves BBB integrity both in rabbits fed a high-cholesterol diet and in a cholesterol-induced in vitro model of BBB disruption using primary cultured RBMECs. Our data provide a new mechanism underlying the neuroprotective activity of simvastatin and suggest that simvastatin may be useful in the treatment of BBB disruptions induced by hypercholesterolemia.

Authors' Contributions

X. Jiang and M. Guo contributed equally to this work.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Targeting Beta Amyloid: A Clinical Review of Immunotherapeutic Approaches in Alzheimer's Disease

Kasia Lobello,[1] J. Michael Ryan,[1] Enchi Liu,[2] Gregory Rippon,[1] and Ronald Black[1]

[1] *Department of Clinical Sciences, Pfizer Inc., Collegeville, PA 19426, USA*
[2] *Janssen Alzheimer Immunotherapy Research & Development, LLC., South San Francisco, CA 94080, USA*

Correspondence should be addressed to Kasia Lobello, kasia.lobello@pfizer.com

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As the societal and economic burdens of Alzheimer's disease (AD) continue to mount, so does the need for therapies that slow the progression of the illness. Beta amyloid has long been recognized as the pathologic hallmark of AD, and the past decade has seen significant progress in the development of various immunotherapeutic approaches targeting beta amyloid. This paper reviews active and passive approaches aimed at beta amyloid, with a focus on clinical trial data.

1. Introduction

Alzheimer's disease (AD) is by far the most common form of dementia, and the social and economic burdens of AD continue to mount. In 2010, an estimated 36 million people worldwide were living with dementia—a number that is projected to increase to 66 million in 2030, and 115 million in 2050 [1]. According to the World Alzheimer Report, the worldwide cost of dementia is estimated at USD $604 billion for 2010 [1], and according to one model, this cost has increased by 34% between 2005 and 2009 [2]. These statistics must be considered in parallel with the immeasurable emotional and psychological burdens that AD places on patients and families.

Significant progress in the treatment of AD has been made since the initial description of the disease by Alois Alzheimer in 1907 [3]. Cholinesterase inhibitors and memantine are potential therapies for the management of many cognitive symptoms of AD, but these neurotransmitter-based approaches do not address the underlying pathology of the illness, and ultimately fail to prevent its progression. The pathologic triad of AD—the accumulation of toxic beta amyloid with the formation of extracellular beta-amyloid-containing plaques, the development of intracellular neurofibrillary tangles, and the degeneration of cerebral neurons—provides numerous potential targets for disease-modifying therapies. Multiple lines of evidence now suggest, however, that it is the production and/or deposition of toxic forms of beta amyloid, along with the slowing of beta-amyloid clearance, that act as the central and primary events in AD pathogenesis, while neurofibrillary tangle formation and neuronal cell death occur downstream in this amyloid cascade [4–6]. Recent *in vitro* work has demonstrated that beta-amyloid dimers (the major form of soluble oligomers in the human brain) isolated from patients with AD induce both the abnormal phosphorylation of tau that is characteristic of AD and the degeneration of neurites, providing further confirmation of the pivotal role of beta amyloid in the pathogenesis of AD [7]. The search for a disease modifying therapy—one that affects underlying pathology and has a measurable and long-lasting effect on the progression of disability—has thus been aimed primarily at the study of beta amyloid.

The demonstration of disease modification is best supported by both clinical and biomarker endpoints. A biomarker is an objectively measured characteristic that can be evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [8]. Several potential biomarkers have been identified in AD and are currently under investigation in interventional clinical trials. These biomarkers should be reflective of changes in the pathology of the AD brain, such as cerebral beta-amyloid deposition, abnormal phosphorylation of tau, or neurodegeneration.

Recent advances in positron emission tomography (PET) imaging have made possible the *in vivo* detection and quantification of beta amyloid using amyloid-specific ligands, such as the ^{11}C Pittsburgh Compound B (PiB) [9, 10]. Elevated levels of tau protein in the cerebrospinal fluid (CSF) are markers of active neuronal degeneration [11], while levels of abnormally phosphorylated tau (P-tau) appear to correlate with the quantity of neurofibrillary tangles in the brain, suggesting that CSF P-tau may serve as an *in vivo* biomarker of the neurofibrillary pathology of AD [12]. Magnetic resonance imaging (MRI)- based measures of cerebral atrophy, most likely the result of excessive neuronal death, correlate closely with the rate of neuropsychological decline in patients with AD [13]. These and other biomarkers will likely play an important role in demonstrating the effect of any therapy on cerebral amyloid and the downstream processes that are affected through beta-amyloid removal.

2. Active and Passive Immunotherapeutic Approaches to Beta-Amyloid Clearance

While numerous strategies have been developed to limit cerebral beta-amyloid deposition and/or facilitate beta-amyloid clearance, the most extensive preclinical and clinical experience to date has come from immunotherapeutic approaches, which can be broadly classified as either active or passive (Figure 1).

Passive immunotherapy refers to the direct administration of anti-beta-amyloid antibodies, obviating the need for patients to mount an antibody response. Passive immunotherapy in the form of specifically designed monoclonal antibodies allows for the precise targeting of beta-amyloid epitopes. In contrast, active immunotherapy involves the administration of either full-length beta-amyloid peptides or peptide fragments to activate the patient's immune system in order to produce anti-beta-amyloid antibodies. The beta-amyloid peptides or peptide fragments can be conjugated to a carrier protein and may be administered with an adjuvant in order to help stimulate the immune response. As active immunotherapy relies on the patient's own immune response, the extent and nature of anti-beta-amyloid antibody production is likely to vary among individuals, and some patients may not be able to mount a meaningful antibody titer. Active immunotherapy can induce an oligoclonal (as opposed to monoclonal) response with antibodies that differ with respect to their binding affinity for a number of toxic beta-amyloid species. Unlike passive immunotherapy, which has to be readministered at frequent intervals, active immunotherapy has the potential to produce persistent levels of anti-beta-amyloid antibody titers with less-frequent administration.

3. Insights from Preclinical Studies

An extensive body of preclinical work (summarized briefly here and reviewed more extensively elsewhere) [14] provides support for an immunotherapeutic approach to beta-amyloid lowering in AD. In 1999, Schenk et al. published a seminal study demonstrating that the administration of beta amyloid$_{42}$ prevented beta-amyloid plaque formation in platelet-derived growth factor promoter (PDAPP) transgenic mice, a mouse model which overexpresses human amyloid precursor protein [15]. Animals treated with this active immunotherapy also demonstrated a marked attenuation in neuritic dystrophy and astrogliosis [15]. Older mice that had already developed some neuropathologic changes at the time of treatment showed a reduction in AD-like neuropathology as compared with older nontreated controls [15]. Schenk's pathology-focused work was followed by the demonstration that beta-amyloid vaccination protected "double transgenic" (APP + PS1) mice from developing the learning and memory deficits that normally occurred in this animal model [16]. Vaccinated double transgenic mice performed as well as non-transgenic controls on the radial-arm water-maze test, suggesting that vaccination may have the potential to restore the wild type phenotype. The ability of beta-amyloid vaccination to attenuate beta-amyloid pathology and behavioral deficits has also been demonstrated in other transgenic models [17].

In parallel with the active immunotherapeutic approaches described above, preclinical studies utilizing passive immunotherapy spearheaded by Bard et al. established that peripherally administered anti-beta-amyloid antibodies enter the central nervous system and bind to amyloid plaques in PDAPP mice, resulting in a plaque reduction of up to 86% as compared with untreated controls [18]. Plaque clearance was shown to occur through fragment crystallizable (Fc) receptor-mediated phagocytosis by microglial cells, with no evidence of T-cell response activation [18]. Additional work by Wilcock et al. confirmed that administration of anti-beta-amyloid antibodies resulted in the activation of brain microglia (as evidenced by microglial expression of CD45 and the Fc$_\gamma$ receptor), reduced brain beta-amyloid deposits, and improved performance on the Y-maze behavior task in APP transgenic mice [19]. Recent *in vitro* findings demonstrate that antibodies directed at the N-terminal of beta amyloid neutralize the cytoskeletal alterations that are induced by beta-amyloid dimers [7] and that the murine form of bapineuzumab (3D6) interacts with soluble beta-amyloid species. The murine form of bapineuzumab was also effective at neutralizing several *in vitro* and *in vivo* measures of synaptotoxicity in preclinical models [20].

Although Fc receptor-mediated phagocytosis is believed to play a role in immunotherapy-induced beta-amyloid clearance, other studies have demonstrated that Fc receptor interactions are not necessarily required for beta-amyloid removal [21, 22]. These experiments suggest that other mechanisms may also be involved in the antibody-mediated clearance of beta amyloid with active and passive immunotherapy. One of these proposed mechanisms speculates that anti-beta-amyloid antibodies exert their effect not in the brain but rather in the periphery, where they bind to circulating beta-amyloid molecules and reduce the free concentration of beta amyloid in the blood. According to this "peripheral sink" hypothesis, the equilibrium across the blood-brain barrier is then altered to favor the next efflux of beta amyloid from the brain [23]. Another hypothesis proposes that the binding of anti-beta-amyloid antibodies to the beta-amyloid molecule alters its conformation so that it

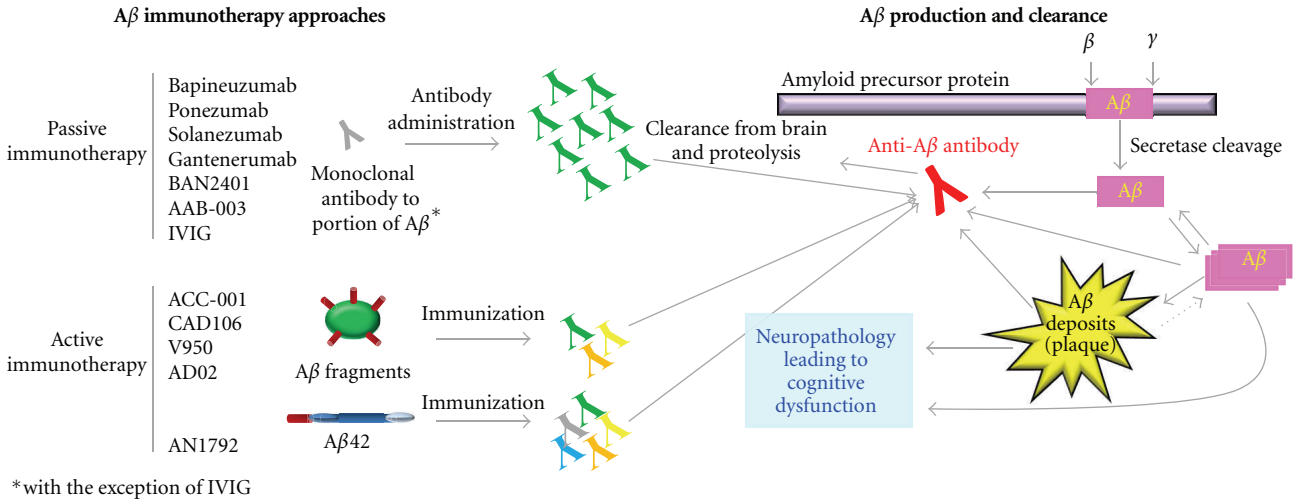

Figure 1: Passive and active immunotherapeutic approaches to beta-amyloid clearance. Beta-amyloid immunotherapeutic compounds currently in clinical trials utilize anti-beta-amyloid antibodies, generated through either passive or active immunotherapy approaches (left), to target beta amyloid and promote its clearance from the brain and proteolysis (right), potentially reversing the neuropathology that leads to cognitive dysfunction. Aβ: beta amyloid.

is less likely to form the fibrillar aggregates associated with AD pathology [24].

Preclinical data is also available for human intravenous immunoglobulin (IVIG). Magga et al. demonstrated that peripherally administered IVIG penetrated the blood-brain barrier and bound to beta-amyloid deposits in mouse brain [25]. In addition, IVIG obtained from the plasma of healthy human volunteers protected mouse hippocampal neurons from beta-amyloid toxicity *in vitro* [25]. The effects of IVIG may be due in large part to the presence of naturally occurring anti-beta-amyloid antibodies, which are abundant in human plasma but decline with age and advancing AD [26]. Numerous other mechanisms of action have been proposed for IVIG, including complement binding, interference with B-cell differentiation, and cytokine modulation [27].

4. Clinical Trials with Active Immunotherapy

4.1. AN1792

4.1.1. Phase 1 Trial. Following on the promising preclinical results described above, AN1792, a synthetic beta-amyloid peptide, was the first active amyloid immunotherapy tested in clinical trials [28]. The initial study randomized 80 subjects with mild-to-moderate AD; 64 subjects received AN1792 with QS-21 (adjuvant) and 16 received QS-21 alone [28]. Injections were administered 4 times over a 24-week period, with an optional extension phase that allowed subjects to receive up to 4 additional injections over a total follow-up time of 84 weeks [28].

Of the 64 subjects who received AN1792, 53% developed a positive anti-AN1792 antibody response (defined as an antibody titer $\geq 1:1{,}000$) at one or more points during the trial [28]. Exploratory efficacy analyses showed no difference between rates of cognitive decline in treated and control groups as measured by the Alzheimer's Disease Assessment Scale-Cognitive (ADAS-cog) and Mini-Mental State Examination (MMSE) [28]. At week 84, however, patients who had received AN1792 showed less functional decline (as measured by the Disability Assessment for Dementia [DAD]) than those treated with QS-21 alone (adjusted mean values -14.15 versus -36.42, $P < 0.002$) [28].

Although the vast majority of adverse events (AEs) reported during the initial study were either mild or moderate in nature, one patient treated with AN1792 developed severe dizziness, disorientation, and functional deterioration [28]. This patient died approximately 1 year after her fifth and final injection of AN1792, and a few weeks after dose administration in the phase 2a trial was halted due to cases of meningoencephalitis (see below) [28]. The patient's postmortem examination revealed changes consistent with T-lymphocytic meningoencephalitis and was also significant for extensive cortical areas devoid of beta-amyloid plaques [29].

4.1.2. Phase 2a Trial and Meningoencephalitis. The phase 2a study of AN1792 in mild-to-moderate AD randomized 300 patients to receive AN1792 (QS-21) and 72 patients to receive placebo [30]. Dosing was halted after 18 AN1792 (QS-21)-treated patients (6% of all patients who received active therapy) developed aseptic meningoencephalitis [30]. AN1792-associated meningoencephalitis was variable with respect to clinical presentation, severity, and resolution. Most patients developed progressive confusion, lethargy, and headache [31]. Other reported signs and symptoms included fever, nausea, vomiting, seizures, and focal neurologic signs [31]. Meningoencephalitis developed from 5 to 168 days after the last injection of AN1792, with a median latency of 40 days [31]. While most patients experienced a monophasic illness, 4 patients developed a relapse following the initial resolution of meningoencephalitis, and 2 of these relapses were severe [31]. Recovery was reported in 12 of the 18

patients, while 6 patients were noted to have persistent sequelae at the conclusion of the trial [31]. No additional cases of meningoencephalitis were reported over a 4.6-year follow-up study of subjects previously enrolled in the 2a trial [32]. No cases of meningoencephalitis occurred in the placebo-treated patients [31, 32].

Geometric mean serum anti-AN1792 antibody titers were not significantly higher in those patients who developed meningoencephalitis than in those who did not [30]. Five of the 18 patients with meningoencephalitis did not meet the antibody responder criterion (defined here as a serum anti-AN1792 IgG [total] titer $\geq 1:2{,}200$ at any time after injection), and one patient with meningoencephalitis had serum IgG titers $<1:50$ over the entire study course [30]. No correlation was established between antibody titers and time-to-symptom onset, severity of illness, or relapse [31].

MRI findings in patients with meningoencephalitis were also variable, ranging from subtle meningeal enhancement to cerebral edema and extensive parenchymal signal abnormalities with a predominantly posterior distribution [31]. No hemorrhagic findings (such as microhemorrhages or larger hemosiderin deposits) were reported [31]. CSF analysis was performed in 17 of the 18 cases; 16 of these revealed a lymphocytic pleocytosis, with WBC counts ranging from 15 to 130 cells/mL, and CSF protein from 0.33 to 3.1 g/L (33–310 mg/dL) [31]. Glucose levels were within normal limits [31]. IgG levels were elevated in 3 of 4 patients tested, and oligoclonal bands were reported in 2 of the 18 cases [31].

One possible factor in the development of meningoencephalitis (which had not occurred in preclinical studies) in this subset of AN1792 (QS-21)-treated patients stems from the introduction of polysorbate 80 into the formulation [30]. At the late stages of the phase 1 trial described above, polysorbate 80 was added to prevent AN1792 (QS-21) precipitation [29]. The single subject who developed meningoencephalitis in the phase 1 study did so approximately 36 days after receiving the fifth dose of AN1792 (QS-21), which was also the first dose using the altered formulation [29]. It is possible that the addition of polysorbate 80 resulted in an increased exposure of amyloid-beta$_{1-42}$ amino acids to epitopes capable of mounting an inflammatory T-cell response [30, 33]. Peripheral blood mononuclear cell isolates from patients who received AN1792 were analyzed for cytokine response to beta-amyloid-derived peptides using enzyme-linked immunosorbent spot (ELIspot) assays. These assays showed a difference in the quality of the T-cell response induced by the two different formulations of AN1792, with isolates from patients who received the polysorbate 80-containing formulation more likely to exhibit a beta-amyloid-specific proinflammatory Th1 response. The specificity of the antibody response did not differ between the two formulations and was directed almost exclusively to the N-terminus [33]. Neuropathologic examination of one case of meningoencephalitis revealed a perivascular T-cell infiltrate with a lack of B lymphocytes, as well as microglial activation and multinucleated giant cells [34]. Lymphocyte distribution was most prominent in the temporal cortex, hippocampus, and amygdale and did not match the observed distribution of beta-amyloid clearance. Lymphocyte distribution did appear to correspond with the finding of collapsed plaques, which were characterized by abnormal morphology and composed of dense amyloid cores surrounded by activated microglial cells. This colocalization of lymphocytic infiltrates and abnormal plaques may suggest that meningoencephalitis was related to abnormal beta-amyloid processing and not to beta-amyloid clearance [34].

4.1.3. Immunologic and Clinical Outcomes. At the time of discontinuation of dosing due to meningoencephalitis, over 90% of patients in the phase 2a trial had received 2 of the planned 6 injections of AN1792 (QS-21)/placebo [30]. The study was amended to allow safety follow-up for at least 9 months after the last injection [30]. Despite the limited number of administered injections, approximately 20% of the subjects treated with AN1792 (QS-21) were classified as antibody responders [30].

As expected, the AN1792 phase 2a study demonstrated no differences between treatment groups in the majority of the cognitive, functional, and global change scores, which became exploratory measures when dosing was discontinued [30]. However, the composite Neuropsychological Test Battery (NTB) z-score, as well as other NTB component scores including the memory scores, showed less worsening in the antibody responder group compared with the placebo group ($P = 0.020$) at month 12 [30]. Moreover, there was a direct relationship between mean antibody titers and the overall composite NTB z-score, as well as NTB z-scores for all memory, immediate memory, and delayed memory, indicating greater improvements from baseline in patients with higher antibody titers [30].

The short-term results of the abbreviated phase 2a trial must be viewed in the context of the long-term follow-up study, which enrolled 159 of the patients who had originally participated in the AN1792 phase 2a trial, including 25 antibody responders, 104 low/nonresponders, and 30 placebo-treated patients [32]. Of the 19 antibody responders who submitted a blood sample for testing, 17 (89.5%) demonstrated a persistently positive anti-AN1792 antibody titer approximately 4.6 years after their last injection of AN1792 [32]. Although these titers were low (geometric mean of $1:331.5$), they seemed sufficient to provide meaningful long-term benefits in some of the efficacy findings that were examined in this population [32]. As compared with placebo-treated patients, antibody responders had a 25% lower decline in activities of daily living as assessed by the DAD, a reduction in decline on the Rey Auditory Verbal Learning component of the NTB, and 20% less decline on the Clinical Dementia Rating Scale Sum of Boxes (CDR-SOB) [32]. The differences between placebo-treated patients and antibody responders were statistically significant for the first 2 of these assessments ($P = 0.015$ and $P = 0.046$, resp.) [32]. After approximately 4.6 years of follow-up, 76% of antibody responders were living in their own home and 16% were living in long-term care institutions; the percentages for placebo-treated patients were 53% and 30%, respectively [32]. On the Dependence Scale, antibody responders had a 17.6% lower mean score in caregiver dependence compared

with placebo-treated patients ($P = 0.033$). No significant differences between antibody responders and placebo-treated patients were noted on the composite NTB z-score, the MMSE, or the ADAS-Cog [32].

It is important to note that while the dependence measures (such as DAD and institutional status) could be assessed based on caregiver input, cognitive measures had to be obtained from patients directly [32]. Many patients in the long-term follow-up study were unable or unwilling to provide responses to these cognitive assessments, resulting in a proportionally higher percentage of missing data for these endpoints, as compared with the functional scales [32]. The extent of missing functional data may have contributed to the lack of clear placebo/treatment differences on cognitive endpoints [32].

4.1.4. Biomarkers. Along with the potential clinical efficacy signals described above, several important biomarker changes were observed in the AN1792 phase 2a trial. Ten antibody responders and 11 placebo-treated patients underwent pre- and postbaseline CSF analysis [30]. A statistically significant reduction in CSF tau was seen in the antibody responder group, but there was no treatment effect on CSF beta-amyloid levels [30].

Baseline and post-treatment brain MRI scans (obtained either at month 12 or at early termination) were available for 288 of the 372 patients who participated in the study, and were used to determine whole-brain, ventricular, and hippocampal volumes [35]. The change in whole-brain boundary shift interval (BSI) over the treatment period was greater in the antibody responders than in the placebo group ($P = 0.007$), indicating a greater loss of brain volume in the antibody responder group [35]. Antibody responders also had a significantly greater increase in ventricular volume than placebo-treated patients ($P < 0.001$) [35]. Anti-AN1792 IgG serum titers correlated with the percent change in whole-brain BSI (Pearson correlation coefficient $r = 0.293$; $P = 0.003$) and ventricular BSI ($r = 0.472$; $P < 0.0001$) in treated patients who had titers $\geq 1:100$ [35].

Both placebo-treated patients and antibody responders exhibited a decrease in hippocampal volumes over the study period, with no significant difference between the 2 groups [35]. Whole-brain, ventricular, and hippocampal volumes in nonresponders did not differ from those of placebo-treated patients [35]. The AN1792 follow-up study [32] showed no significant difference in whole-brain or hippocampal volume changes from baseline between antibody responders and placebo-treated patients at a mean follow-up of 4.6 years, but the number of patients for whom MRI data was available was small. The antibody responders, however, showed a greater increase in ventricular volume than that seen in placebo-treated patients ($P = 0.021$) [32].

The etiology and clinical significance of the above-described changes in MRI-measured brain and ventricular volumes remains unclear. The suggestion that AN1792 caused an accelerated rate of neurodegeneration in antibody responders is unsupported, given the lack of worsened clinical decline (and in light of the potential signals of clinical benefit) in patients who developed positive titers [35]. An alternative explanation is that at least some of the cerebral volume loss can be accounted for by the removal of beta-amyloid plaques in antibody responders and/or by parenchymal/CSF fluid shifts that may have occurred in parallel with shifts in beta amyloid [35]. The latter two hypotheses are now supported by multiple autopsy cases that have been performed on AN1792 responders, which clearly demonstrate effective beta-amyloid clearance (see below).

4.1.5. Pathologic Findings. In 2006, Nicoll et al. published the neuropathologic findings of 3 patients who had received between 2 and 5 doses of AN1792 (QS-21) [36]. Two of the 3 cases were known to have developed anti-beta-amyloid antibodies over the course of the study, and both of these patients also developed meningoencephalitis [36]. The causes of death in the autopsied cases, however, were nonneurologic in nature (pulmonary embolism, bronchoaspiration, and abdominal aortic aneurysm) [36]. Nicoll et al. compared the findings in the AN1792 (QS-21)-treated cases to 7 untreated cases that met neuropathologic criteria for AD [36].

A marked reduction in beta-amyloid plaque deposition was noted in the temporal cortex of the 2 AN1792 (QS-21) cases that had developed anti-beta-amyloid antibodies: 69% and 89% of the temporal cortex was classified as plaque-free, as compared with <1% in control cases [36]. Plaque removal appeared patchy, with a relatively higher plaque density in the frontal lobes [36]. The antibody nonresponder case demonstrated no plaque-free areas in the temporal and medial frontal cortex but showed some patchy areas of plaque removal elsewhere [36]. Morphologic studies revealed further evidence of plaque clearance in all 3 immunized cases and demonstrated the presence of beta-amyloid granules within lysosomes and activated microglia [36]. Beta amyloid with an intact N-terminus had been cleared effectively, while beta-amyloid species truncated at the N-terminus persisted [36].

Although both antibody-positive subjects in this series also had a history of meningoencephalitis [36], other neuropathologic examinations have demonstrated plaque clearance in subjects without meningoencephalitis [37]. In contrast to cortical amyloid, vascular amyloid was not removed by active immunization, as all 3 autopsy cases had severe cerebral amyloid angiopathy (CAA) at autopsy [36]. This finding has been confirmed by Patton et al., who examined 2 AN1792-immunized patients and noted that while both compact core and diffuse amyloid deposits were diminished, vascular deposits were relatively preserved or even increased [38].

Importantly, the work of Nicoll et al. showed that plaque-free cortical regions also exhibited a decrease in the density of dystrophic neurons, although there was no clear evidence of an impact on neuronal tau or neurophil threads [36]. The downstream effects of beta-amyloid immunization were further elucidated by Serrano-Pozo et al., who performed detailed quantitative analyses of hippocampal sections from AN1792-treated patients and compared these with samples from nondemented controls and untreated patients with AD [39]. In addition to the expected clearance of beta-amyloid plaques in immunized patients, Serrano-Pozo et al.

demonstrated the normalization of neurite morphology and a significant reduction in the hyperphosphorylation of tau [39]. The ability of AN1792 to reduce tau hyperphosphorylation has also been reported elsewhere [34].

While the neuropathologic findings discussed above provide compelling evidence for the beneficial effects of active beta-amyloid therapy on both beta-amyloid plaque burden and the downstream effects of beta-amyloid pathology, the clinical significance of beta-amyloid clearance has been challenged by other autopsy studies. Holmes et al. published long-term findings of AN1792-treated subjects up to 5 years after the last injection of AN1792 [40]. When compared with placebo-treated subjects who had also consented to long-term follow-up, immunization had no effect on long-term survival or clinical outcomes, although the number of subjects was small [40]. Eight AN1792-treated patients with AD consented to autopsy, which demonstrated a long-term reduction in mean beta-amyloid load as compared with untreated controls [40]. Seven of these 8 patients, 2 of whom had nearly complete beta-amyloid removal at autopsy, also had severe end-stage dementia at the time of death, leading the authors to conclude that progressive neurodegeneration had occurred despite effective clearance of beta amyloid [40]. It is important to point out, however, that the neuropathologic examinations performed in this sample included only those patients who died, and the findings are therefore not generalizable to those treated patients who survived. As such, the results of this study are in direct conflict with those reported by Vellas et al., who demonstrated a long-term clinical benefit with AN1792 [32].

4.2. Active Immunotherapies Currently in Clinical Trials. While studies with AN1792 were discontinued due to the occurrence of meningoencephalitis, the trials paved the way for the many active immunotherapeutic clinical trials currently in progress (Table 1 and Figure 1). In a study of serum samples from patients immunized with AN1792, Lee et al. established that the predominant antibody response in these patients was against the free N-terminus of beta amyloid; specifically, against beta amyloid$_{1-8}$ [41]. Vanutide cridificar (ACC-001) is a conjugate of multiple copies of beta-amyloid$_{1-7}$ peptide linked to a nontoxic variant of diphtheria toxin [42]. Preclinical data indicate that vanutide cridificar generates N-terminal anti-beta-amyloid antibodies without inducing a beta-amyloid-directed T-cell response, and that it reverses cognitive impairment in murine models of AD [42]. Vanutide cridificar is currently in phase 2 clinical trials in mild-to-moderate AD and early AD (NCT01284387; NCT01227564; NCT00479557; NCT00955409; NCT00498602; NCT00752232; NCT01238991; NCT00960531; NCT00959192). Other active immunotherapies currently under study include CAD106 (Novartis, Inc.), V950 (Merck & Co.), and AD02 (AFFiRiS AG/ GlaxoSmithKline plc).

4.2.1. CAD106. CAD106 is composed of the beta-amyloid$_{1-6}$ peptide coupled with a Qβ carrier [43, 44]. A 52-week study with CAD106 included 58 patients with mild-to-moderate AD in 2 cohorts: 50 μg CAD106 or placebo administered at weeks 0, 6, and 18 (cohort 1); or 150 μg CAD106 or placebo at weeks 0, 2, and 6 (cohort 2) [43, 44]. Injection-site erythema was the most frequent AE observed with CAD106 (4% in cohort I; 64% in cohort II); most AEs were mild, and serious AEs were considered unrelated to study medication [43, 44]. CAD106 was associated with an antibody response in 16/24 treated patients in cohort 1 and 18/22 patients in cohort 2 [43, 44]. In 2 52-week, phase 2a studies in 58 patients with mild AD, 150 μg CAD106 was administered subcutaneously at weeks 0, 6, and 12 (study 1), or either subcutaneously or intramuscularly at weeks 0, 2, and 6 (study 2) [45]. Results of study 1 showed antibody response in 20/22 patients. Because the results indicated that week 2 injection did not enhance antibody response, a 0/6/12 week regimen was selected for further study [45]. Two phase 2 studies currently in progress are investigating repeated administration of CAD106 intramuscularly (NCT01097096) or subcutaneously (NCT00956410; NCT01023685).

4.2.2. V950. V950 is a multivalent beta-amyloid vaccine [46]. To date, no clinical data have been presented. Preclinical studies have shown that administration of V950 results in the production of anti-beta-amyloid antibodies in the serum and CSF that recognize pyroglutamate-modified and other N-terminally truncated beta-amyloid fragments [46]. A phase 1 study of V950 in patients with AD is currently underway (NCT00464334).

4.2.3. AFFITOPE AD02. AFFITOPE AD02 is composed of a 6-amino acid peptide that mimics part of the N-terminus of beta amyloid [47]. It is hypothesized that AD02 and other active immunotherapeutic approaches using this technology may have a favorable safety profile because they are nonself and thus do not need to overcome tolerance—their small size prevents autoreactive T-cell activation, and their controlled specificity prevents cross-reactivity with amyloid precursor protein [48]. Phase 1 data showed a favorable safety profile with AD02 and AD01, another AFFITOPE compound [48]. A randomized, multicenter, phase 2 trial with AD02 in patients with early AD is currently recruiting participants (NCT01117818).

5. Clinical Trials with Passive Immunotherapy

Passive immunotherapeutic approaches to AD are being investigated in parallel with the active therapies described above. To date, the largest quantity of published data on passive immunotherapy pertains to bapineuzumab, which is being codeveloped by Pfizer Inc. and Janssen Alzheimer Immunotherapy Research & Development, LLC.

5.1. Bapineuzumab

5.1.1. Phase 1 Trial. Bapineuzumab is a humanized monoclonal antibody that targets the N-terminal region of beta amyloid [49] (Figure 1). Bapineuzumab at doses of 0.5, 1.5, or 5 mg/kg was first tested in patients with mild-to-moderate AD in a 12-month, single ascending-dose study [49].

Table 1: Active immunotherapy agents.

Drug name	Sponsor(s)	Mechanism of action	Status	Key clinical data	Source of key clinical data
ACC-001 (vanutide cridificar)	JANSSEN Alzheimer Immunotherapy Research & Development, LLC.; Pfizer Inc.	Multiple copies of Aβ_{1-7} peptide linked to a nontoxic variant of diphtheria toxin	Phase 2	No clinical data have been presented to date	—
AD02	AFFiRiS AG	Short (6 aa) peptide mimicking parts of the native Aβ N-terminus sequence	Phase 2	Phase 1 safety data support proof-of-concept for improved safety profile using AFFITOPE technology	[48]
CAD106	Novartis, Inc.	Aβ_{1-6} peptide coupled with Qβ carrier	Phase 2	In a phase 2a study, CAD106 showed a favorable safety profile and antibody response in 20/22 patients with mild AD	[45]
V950	Merck & Co.	Multivalent Aβ vaccine	Phase 1	No clinical data have been presented to date	—

Aβ: beta amyloid; AD: Alzheimer's disease.

The majority of treatment-emergent AEs were mild to moderate in severity and were not considered related to treatment by study investigators [49]. The phase 1 study included protocol-specified periodic MRI monitoring, and MRI abnormalities consistent with vasogenic edema were reported in 3 of the 10 patients randomized to bapineuzumab 5.0 mg/kg [49]. Recently published recommendations from the Alzheimer's Association Research Roundtable Workgroup include the use of the term amyloid-related imaging abnormalities (ARIA) in reference to the spectrum of imaging findings associated with amyloid-lowering therapies and ARIA-edema/effusions (ARIA-E) to refer to findings previously referred to as "vasogenic edema" [50]. The authors have chosen to adopt this terminology for the purposes of this paper. The MRI findings in the bapineuzumab study consisted of hyperintensities on fluid-attenuated inversion recovery (FLAIR) sequences [49]. In one of the 3 cases, the FLAIR abnormality was accompanied by the development of a new microhemorrhage [49]. The MRI abnormalities, with the exception of the microhemorrhage, resolved in all 3 cases over a period of weeks to months [49]. Two of the 3 patients with ARIA-E were asymptomatic, and one patient experienced mild and transient confusion [49]. Two of the 3 patients with ARIA-E underwent CSF analysis. In contrast to the meningoencephalitis cases reported with AN1792, CSF was acellular in both cases, with minor elevations in CSF protein (58.5 and 59.8 mg/dL) [49].

Plasma levels of bapineuzumab increased over 1-2 hours following an infusion, and plasma half-lives ranged from 21 to 26 days [49]. MMSE was performed as an exploratory efficacy measure, and mean MMSE increased from baseline over the course of the trial at the 0.5 and 1.5 mg/kg bapineuzumab doses (except for month 6 at the 1.5 mg/kg dose) [49]. Mean MMSE decreased in patients on placebo except at month 6 and also decreased in patients who received 5.0 mg/kg of bapineuzumab [49]. At the primary time point (week 16), at the 1.5 mg/kg dose, the treatment versus placebo difference in MMSE (2.6) was statistically significant ($P = 0.047$) in favor of bapineuzumab [49].

5.1.2. Phase 2 Trial. The phase 1 study was followed by a multiple ascending dose trial in which 124 patients with mild-to-moderate AD were randomized to 1 of 4 doses (0.15, 0.5, 1, or 2 mg/kg) of bapineuzumab, and 110 patients received placebo [51]. Study assessments consisted of numerous clinical evaluations (including the ADAS-Cog, NTB, and DAD), safety, tolerability, and biomarkers, including CSF and brain volume [51]. Bapineuzumab or placebo infusions were given every 13 weeks for up to 78 weeks [51]. In the prespecified efficacy analyses (within-dose-cohort differences between bapineuzumab and placebo from baseline to week 78), no significant difference was seen in any of the cohorts on either of the pre-specified primary outcomes (ADAS-Cog or DAD). Exploratory analyses on the overall treatment groups (pooled bapineuzumab versus placebo) revealed trends on the ADAS-Cog and NTB, but not on the DAD or other outcomes [51]. Treatment differences in ADAS-Cog, NTB, and DAD became more apparent when analyses were carried out on the "completer" population [51].

Post-hoc exploratory efficacy analyses were also carried out by apolipoprotein E ε4 (ApoE4) carrier status, following the observation that ARIA-E was more common in ApoE4 carriers (see below) [51]. In the 79 ApoE4 noncarriers, bapineuzumab/placebo treatment differences were observed in several outcomes, including the ADAS-Cog and the NTB, although there was no difference on the DAD [51]. No

treatment differences were observed on any of the endpoints in the 146 ApoE4 carriers although potential efficacy signals became apparent in analyses limited to those ApoE4-positive patients who completed the trial [51]. In general, treatment differences began to emerge at approximately month 9 of the trial [51].

The bapineuzumab phase 2 trial included CSF biomarkers and MRI volumetric endpoints. CSF samples were obtained in 35 study subjects. There were no observed treatment differences in either CSF beta amyloid or total tau levels, but there was a trend towards greater reduction in P-tau in bapineuzumab-treated patients when compared with placebo ($\delta = -9.1$ pg/mL; 95% CI, 18.5–0.3; $P = 0.056$) [51]. In a subsequently conducted exploratory pooled analysis including patients from the PET PiB study (described below), when comparing the change from baseline to end-of-study CSF P-tau values, a significant treatment reduction was observed in the bapineuzumab-treated patients compared with patients who received placebo (-7.26 pg/mL, $P = 0.0270$) [52].

No overall differences between combined bapineuzumab and placebo-treated patients were observed with respect to whole-brain and ventricular volumes as measured by MRI volumetric analyses over 18 months [51]. However, ApoE4 noncarriers treated with bapineuzumab showed less brain volume loss than those on placebo ($\delta = -10.7$ mL; 95% CI, 3.4–18.0; $P = 0.004$) [51]. No differences in brain volume were noted in ApoE4 carriers, but the bapineuzumab group had a greater increase in ventricular enlargement than the placebo-treated subjects ($\delta = 2.6$ mL; 95% CI, 0.2–5.0; $P = 0.037$) [51].

The most common AEs (reported in >5% of bapineuzumab patients and occurring at a rate of at least twice that of placebo) included ARIA-E, back pain, anxiety, and paranoia. Other AEs which also occurred more frequently in the bapineuzumab group included deep vein thrombosis, syncope, seizures, vomiting, hypertension, weight loss, skin laceration, gait disturbance, muscle spasm, and pulmonary embolism [51].

ARIA-E, which was noted in the phase 1 trial, was detected in 12 of the 124 bapineuzumab-treated subjects (9.7%) and none of the placebo-treated subjects in the phase 2 study [51]. As was true in the earlier study, ARIA-E was more likely to occur at higher bapineuzumab doses, with rates of 3.2%, 0%, 10%, and 26.7% for the 0.15 mg/kg, 0.5 mg/kg, 1 mg/kg, and 2 mg/kg doses, respectively [51]. Eleven of the 12 cases of ARIA-E were detected following either the first or second bapineuzumab infusion [51]. Six of the patients with ARIA-E had no clinical symptoms, while 6 patients experienced symptoms such as headache, confusion, vomiting, and gait disturbance [51]. These symptoms were transient although one patient required treatment with steroids [51]. The MRI findings in these ARIA-E cases were consistent with those described in the earlier trial and resolved over a period of several months [51]. The clinical and MRI characteristics of bapineuzumab-associated ARIA-E, along with the lack of evidence of inflammation as illustrated by the CSF findings described above, differentiate bapineuzumab-related ARIA-E from the severe cases of meningoencephalitis that occurred in association with AN1792.

One of the most unexpected findings in the bapineuzumab phase 2 study was the increased rate of ARIA-E in ApoE4 carriers. Ten of the 12 ARIA-E cases occurred in ApoE4 carriers, and the ARIA-E rates in ApoE4 carriers and noncarriers were 13.5% and 4.3%, respectively [51]. Moreover, among ApoE4 carriers, the rate of ARIA-E increased with the gene dose, with rates of 7.1% in ApoE4 heterozygotes and 33.3% in ApoE4 homozygotes [51]. These findings are particularly intriguing in light of the potential ApoE4-dependent efficacy differences discussed above, and may be due at least in part to the increased load of beta amyloid in ApoE4 carriers, including a higher vascular beta-amyloid burden [51, 53]. It should be noted that the prevalence of the ApoE4 allele appears to vary by geographic location. An estimated 37–43% of Asian and southern European/Mediterranean AD patients are ApoE4 carriers, compared with 58% of patients in North America and 64% in northern Europe [54].

Although its mechanism is unknown, ARIA-E may result from transient increases in cerebral vascular permeability secondary to vascular amyloid clearance [51]. This theory is supported by reports of spontaneously occurring amyloid-related imaging abnormalities similar to those seen in the bapineuzumab trials [55, 56].

5.1.3. Positron Emission Tomography (PET) Carbon-11-Labelled Pittsburgh Compound B (^{11}C-PiB) Study. The ability of bapineuzumab to clear cerebral beta amyloid was demonstrated *in vivo* in a trial of patients with mild-to-moderate AD who underwent serial PET scans with carbon-11-labelled Pittsburgh compound B (^{11}C-PiB) [57]. PiB is known to bind to aggregated fibrillar beta-amyloid deposits and is therefore a marker of fibrillar beta-amyloid load [9, 58]. PiB also binds to cerebrovascular amyloid [59]. In the trial, 20 patients were randomized to bapineuzumab at 1 of 3 doses (0.5, 1.0, or 2.0 mg/kg) for a total of up to 6 infusions, 13 weeks apart; 8 patients received placebo [57]. The primary outcome measure for the trial was the difference between the pooled bapineuzumab groups and the placebo group in the mean change (from screening to week 78) in the ^{11}C-PiB cortical to cerebellar retention ratio [57]. The cerebellum was used as a reference region because it exhibits a relatively low beta-amyloid load in AD [57].

By week 78, the estimated mean ^{11}C-PiB retention ratio decreased by 0.09 in the bapineuzumab group and increased by 0.15 in the placebo group, with an estimated treatment difference of -0.24 (95% CI,0.39 to -0.09; $P = 0.003$). This finding correlates to an approximately 25% reduction in cortical beta amyloid in bapineuzumab-treated patients [57] (Figure 2). The extent of beta-amyloid reduction was not clearly dose dependent [57]. Bapineuzumab/placebo differences in ^{11}C-PiB retention were statistically significant in all prespecified cortical regions (anterior and posterior cingulate, frontal, temporal, parietal, and occipital cortex) [57]. After adjustment for imbalances in baseline clinical and ^{11}C-PiB-binding characteristics, there were no treatment

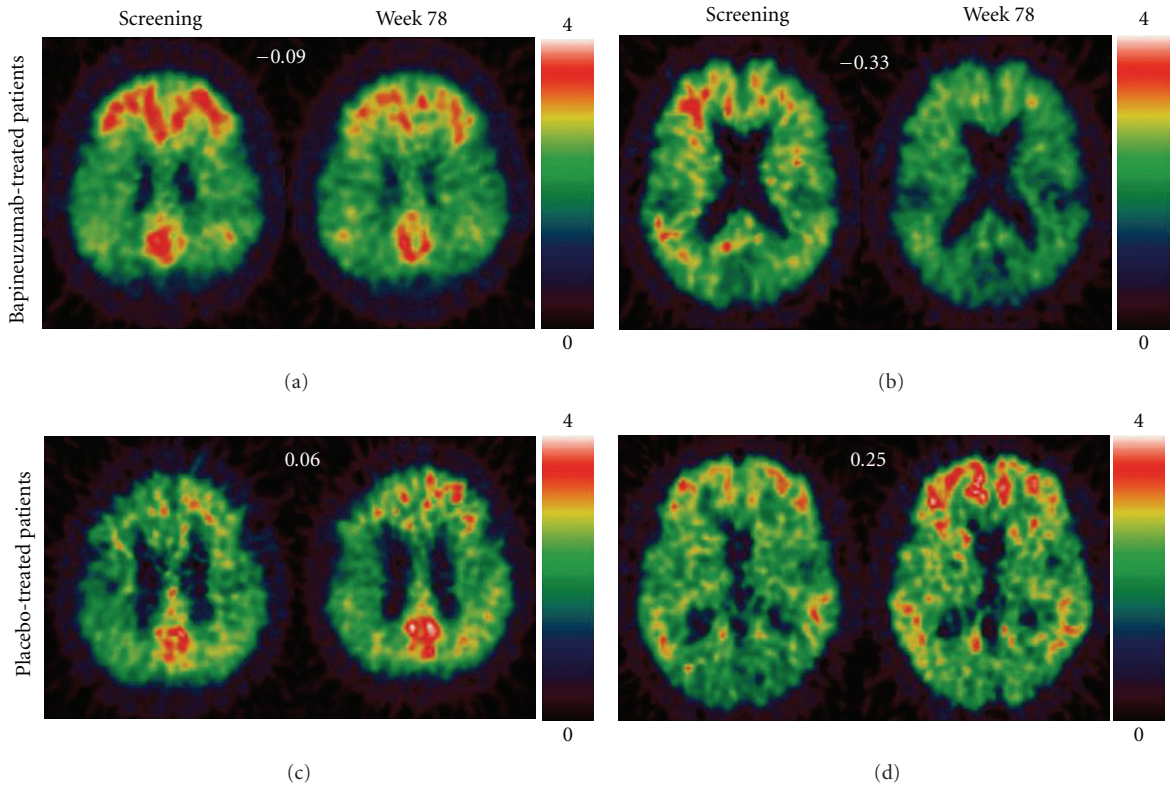

Figure 2: Positron emission tomography (PET) carbon-11-labelled Pittsburgh compound B (^{11}C-PiB) images from patients treated with bapineuzumab and those given placebo [57]. *Reprinted from* [57] *with permission from Elsevier.* Changes from screening to week 78 in patients treated with bapineuzumab (a, b) and in patients treated with placebo (c, d). Mean ^{11}C-PiB PET changes are shown at the top center of each panel for each patient. The scale bar shows the PiB uptake ratios relative to cerebellum by color. The scans before and after treatment are from MRI coregistered images in the same plane.

differences noted on clinical or biomarker endpoints between the bapineuzumab and placebo groups [57].

5.2. Other Passive Immunotherapies Currently in Clinical Trials. Building on the data presented above, a phase 3 program for bapineuzumab is currently in progress, comprised of 4 trials: 2 studies in ApoE4 carriers (NCT00575055; NCT00676143) and 2 trials in ApoE4 noncarriers (NCT00574132; NCT00667810). There is considerably less published data available on other passive immunotherapeutic approaches, which include solanezumab (Eli Lilly and Company), ponezumab (Pfizer Inc.), gantenerumab (Hoffmann-La Roche, Ltd.), BAN2401 (Eisai Co., Ltd.), and intravenous immunoglobulin (Baxter International Inc. and Octapharma AG). These compounds are described briefly below and in Table 2 (see also Figure 1).

5.2.1. Solanezumab. Solanezumab (LY2062430) is a humanized monoclonal antibody against the mid-domain of beta amyloid [60]. In a study to assess the safety and tolerability of single-dose solanezumab (0.5, 1.5, 4.0, or 10.0 mg/kg) in patients with mild-to-moderate AD, solanezumab was associated with infusion reactions in 2 out of 4 patients receiving the highest dose (10.0 mg/kg) [60]. Serious AEs were not considered to be related to the study medication [60]. There was no evidence of inflammation based on MRI or CSF white blood cells counts, and analysis of C-reactive protein (CRP) in blood samples showed only isolated elevations [60]. In a multicenter, multiple-dose, open-label study in Japan, 33 patients with mild-to-moderate AD received a 400 mg dose of solanezumab intravenously every week, every 4 weeks, or every 8 weeks [61]. Most AEs were mild to moderate; one severe event was reported but was considered unrelated to the study medication [61]. There were no reports of infusion reactions or meningoencephalitis [61]. Studies examining biochemical biomarkers found that plasma and CSF-amyloid$_{1-40}$ and beta-amyloid$_{1-42}$, plasma pyro-Glu 3–42 beta-amyloid (N3pGluAβ), and plasma and CSF N-terminally truncated beta-amyloid peptide (fragment 2), but not CSF total tau and phosphorylated tau (P-tau$_{181}$), exhibited significant changes in patients receiving solanezumab, indicating the utility of these biomarkers for evaluating the pharmacodynamic effects of solanezumab [60, 62–66]. In addition, there was a correlation between plasma beta-amyloid$_{1-42}$ and assessment of amyloid burden using single photon emission tomography with IMPY [64]. Three phase 3 studies—2 evaluating the effects of solanezumab on disease progression, and one extension study monitoring safety for participants in those studies—are in progress (NCT00904683; NCT00905372; NCT01127633).

Table 2: Passive immunotherapy agents.

Drug name	Sponsor(s)	Mechanism of action	Status	Key clinical data	Source of key clinical data
Bapineuzumab	JANSSEN Alzheimer Immunotherapy Research & Development, LLC.; Pfizer Inc.	Humanized mAb that targets the N-terminal region of Aβ	Phase 3	No significant differences compared with placebo in primary outcomes (ADAS-Cog or DAD); potential treatment differences based on ApoE4 carrier status	[51]
IVIG (Gammagard)	Baxter International Inc.	Intravenous Ig; contains antibodies against Aβ	Phase 3	Significant differences compared with placebo in primary outcome measures (ADAS-Cog and ADCS-CGIC)	[78]
Solanezumab	Eli Lilly and Company	Humanized monoclonal antibody against the mid-domain of Aβ	Phase 3	Favorable safety profile: no evidence of meningoencephalitis, microhemorrhage, or ARIA-E	[60]
Gantenerumab	Hoffmann-La Roche, Inc.	Monoclonal antibody that targets Aβ	Phase 2	No clinical data have been presented to date	—
IVIG (Octagam)	Octapharma AG	Intravenous Ig; contains antibodies against Aβ	Phase 2	No clinical data have been presented to date	—
IVIG (Newgam)	Sutter Health	Intravenous Ig; contains antibodies against Aβ	Phase 2	No clinical data have been presented to date	—
Ponezumab	Pfizer Inc.	Humanized IgG2deltaA monoclonal antibody that binds to amino acids 33–40 of the Aβ_{1-40} peptide	Phase 2	2 phase 1 studies showed favorable safety profiles, with no microhemorrhage, ARIA-E, or encephalitis	[67, 68]
BAN2401	Eisai Co., Ltd.	Humanized monoclonal antibody that selectively recognizes and eliminates Aβ protofibrils	Phase I	No clinical data have been presented to date	—

Aβ: beta amyloid; ApoE4: apolipoprotein E4A; DAS-Cog: Alzheimer's Disease Assessment Scale-Cognitive Subscale; DAD: Disability Assessment for Dementia; Ig: immunoglobulin.

In addition, a phase 2 study evaluating biomarkers with solanezumab in individuals with or without AD is currently in the recruitment phase (NCT01148498).

5.2.2. Ponezumab. Ponezumab (PF-04360365) is a humanized IgG2deltaA monoclonal antibody that binds to amino acids 33–40 of the beta-amyloid$_{1-40}$ peptide [67]. Two 1-year, phase 1 studies were performed to assess the safety, pharmacokinetics, and pharmacodynamics of ponezumab in patients with mild-to-moderate AD [67–69]. In the first study, which was randomized and double-blinded, patients received placebo or ponezumab 0.1, 0.3, 1, 3, or 10 mg/kg via 2-hour infusion; in the second study, an open-label, parallel-group study, patients received ponezumab 1, 3, 5, or 10 mg/kg via 10-minute infusion [69]. All AEs were mild or moderate, with no serious AEs considered to be related to study drug [67, 68]. In the 2-hour infusion study, one patient receiving ponezumab 10 mg/kg had a mild hypersensitivity reaction, and a preexisting brain lesion showed a slight increase in size in a patient receiving ponezumab 0.1 mg/kg [68]. No new microhemorrhage, ARIA-E, or encephalitis was found in either study [67, 68]. Ponezumab showed linear pharmacokinetics in both studies [67–70]. Low ponezumab concentrations were detected in CSF in 2 out of 8 patients receiving the highest dose (10 mg/kg) in the 2-hour infusion study, but ponezumab was not detected in the CSF in the 10-minute infusion study [67–69]. No antidrug antibodies were detected in either study [69]. In the 2-hour infusion study, there were dose-dependent increases in CSF beta amyloid$_{1-x}$ and increases from baseline to day 29 in CSF beta amyloid$_{1-x}$ and CSF beta-amyloid$_{1-42}$ with the 10 mg/kg dose [68, 69]. Mass spectrometry following immunoprecipitation identified elevated levels of beta-amyloid$_{1-40}$ and beta-amyloid$_{11-40}$ in CSF following a single dose of 10 mg/kg ponezumab in patients with mild-to-moderate AD [71]. In Japanese patients with mild-to-moderate AD, single-dose ponezumab (0.1–10 mg/kg) showed similar safety and pharmacokinetic profiles as in Western patients [72, 73]. Studies of multiple-dose ponezumab in patients with mild-to-moderate AD are currently in progress (NCT00722046; NCT01125631), and a phase 1 study investigating the effects on single-dose ponezumab on beta amyloid in AD patients

and in healthy volunteers is currently recruiting patients (NCT01005862).

5.2.3. Gantenerumab. Gantenerumab (RO4909832/R1450/RG1450), another monoclonal antibody that targets beta amyloid, is currently in clinical development. A phase 1, multiple ascending dose study of gantenerumab in patients with AD has been completed (NCT00531804), while a phase 2 study in patients with prodromal AD is currently recruiting patients (NCT01224106).

5.2.4. BAN2401. BAN2401 is a humanized monoclonal antibody that targets beta-amyloid protofibrils [74]. A phase 1 single- and multiple-ascending dose study of BAN2401 in patients with mild-to-moderate AD is currently recruiting patients (NCT01230853).

5.2.5. Intravenous Immunoglobulin. Putative clinical efficacy data for intravenous immunoglobulin (IVIG) have been reported in 2 small open-label studies. Dodel et al. administered monthly IVIG to 5 AD patients over a 6-month period and demonstrated a decrease in total CSF beta-amyloid levels and an increase in total beta-amyloid serum levels, with no change in beta amyloid$_{1-42}$ in either compartment [75]. The authors reported a slight improvement in mean ADAS-cog and MMSE scores [75]. The second study involved the administration of IVIG to 8 patients with mild AD and demonstrated a dose-proportional increase in serum anti-beta-amyloid antibodies and a decrease in CSF beta amyloid [76]. The CSF beta-amyloid changes were transient, reverting to baseline levels after the discontinuation of IVIG infusions and decreasing again when infusions were restarted [76]. Mean MMSE scores increased during the first 6 months of IVIG, declined when infusions were withheld, and stabilized when infusions were restarted [76]. There was no placebo group in either of these 2 studies, sample sizes were very small, and no brain MRI scans were performed [75, 76]. No serious treatment-emergent AEs were reported [75, 76]. Another study employed a retrospective case-control analysis to demonstrate that previous treatment with IVIG was associated with a reduced risk of AD development [77]. The possible benefits of IVIG, if any, would likely be attributed to the presence of naturally occurring anti-beta-amyloid antibodies in human plasma [26]. Two IVIG clinical trials have been completed (NCT00299988; NCT00812565), and 2 are currently recruiting patients: one phase 2 study (NCT01300728) and one phase 3 trial (NCT00818662).

6. Conclusions and Future Directions

More than 100 years after the initial description of AD and the identification of beta amyloid as a key pathologic component, the search for effective anti-beta-amyloid therapies continues, and immunotherapeutic approaches are poised at the front lines of the anti-beta-amyloid battle. Although the preclinical literature is resplendent with examples of effective beta-amyloid clearance, initial attempts to translate these early successes into safe and effective AD therapies were marred by the development of serious and severe side effects in some patients. The next generation of immunotherapies, both active and passive, must demonstrate an acceptable safety profile and the ability to clear beta amyloid, ultimately slowing or halting clinical disease progression. As several pivotal clinical trials in patients with mild-to-moderate AD near completion, studies in patients with mild cognitive impairment/prodromal AD are just beginning, with the hope that targeting beta amyloid earlier in the disease process will provide better clinical outcomes.

Conflicts of Interest

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Review Article

Leptin: A Novel Therapeutic Target in Alzheimer's Disease?

Dayne Beccano-Kelly and Jenni Harvey

Division of Neuroscience, Medical Research Institute, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, UK

Correspondence should be addressed to Jenni Harvey, j.z.harvey@dundee.ac.uk

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It is well established that the hormone leptin circulates in the plasma in amounts proportional to body fat content and it regulates food intake and body weight via its actions in the hypothalamus. However, numerous studies have shown that leptin receptors are widely expressed throughout the CNS and evidence is growing that leptin plays a role in modulating a variety of neuronal processes. In particular, recent studies have highlighted a potential cognitive enhancing role for leptin as it regulates diverse aspects of hippocampal synaptic function that are thought to underlie learning and memory processes including glutamate receptor trafficking, dendritic morphology, and activity-dependent synaptic plasticity. Characterisation of the novel actions of leptin in limbic brain regions is providing valuable insights into leptin's role in higher cognitive functions in health and disease.

1. Introduction

The hormone leptin plays a pivotal role in regulating a number of hypothalamic driven functions including energy homeostasis, reproductive function, and bone formation. However, recent studies have demonstrated that leptin has widespread action in the CNS, and evidence is growing that leptin has the capacity to modulate higher brain functions. Indeed, leptin has a marked effect on hippocampal-dependent function and in particular learning and memory processes. In addition, dysfunctions in the leptin system have recently been linked to neurodegenerative disorders such as Alzheimer's disease. Here we review the evidence that leptin is a potential cognitive enhancer and also examine the possibility of utilising leptin replacement therapy in the treatment of Alzheimer's disease.

2. Leptin

The endocrine hormone leptin is principally, but not exclusively, derived from white adipose tissue. The circulating levels of this hormone vary during the day, but are mainly determined by body fat content and also feeding status [1, 2]. Leptin enters the brain via saturable transport across the blood brain barrier [3]. Additionally, leptin may be released locally within the CNS as there is evidence for expression of leptin mRNA and protein in specific neuronal populations [4]. It is well documented that leptin plays a pivotal role in the regulation of food intake and body weight via signaling information about the status of fat stores to leptin receptors expressed on specific hypothalamic nuclei. However, leptin receptors are also widely expressed throughout the CNS with high levels detected in many brain regions involved in higher cognitive processes including the hippocampus, cortex, and amygdala. Moreover, growing evidence indicates that leptin is a pleiotropic hormone that exhibits diverse central actions including its ability to regulate hippocampal synaptic plasticity [5] and to play a role in mood disorders such as depression [6].

2.1. Leptin Receptors. Leptin mediates its biological effects via activation of the leptin receptor (ObR) which is encoded by the diabetes (*db*) gene [7]. Alternative splicing of the *db* gene results in the generation of six leptin receptor isoforms (ObRa-f) with identical N-terminal binding domains but distinct C-terminal regions and signaling capacity. ObRbis, the long form of the receptor, and the main signaling competent isoform as key motifs required for signaling are contained within its extended C-terminal domain. ObRs

display the greatest homology with the class I cytokine receptor superfamily [7]: receptors that lack intrinsic tyrosine kinase activity but signal via association with janus tyrosine kinases (JAKs). Indeed ObR activation results in the phosphorylation of JAK2 which subsequently promotes the association with and activation of various signaling molecules including PI 3-kinase (phosphoinositide 3-kinase), Ras-Raf-MAPK (mitogen activated protein kinase), and STAT3(signal transducer and activator of transcription).

2.2. Leptin Regulation of Hippocampal Synaptic Plasticity. The hippocampal formation is an area of the brain that plays a pivotal role in learning and memory. Indeed, both long-term potentiation (LTP) and long-term depression (LTD), which are activity-dependent forms of synaptic plasticity that result in persistent alterations in excitatory synaptic strength, and which are thought to underlie certain aspects of learning and memory, are evident in this brain region. Moreover, N-methyl-D-aspartate (NMDA) receptor-dependent LTP induced in the hippocampal CA1 region has been implicated in spatial learning and memory. Several lines of evidence indicate that many growth factors and hormones, including insulin [8, 9] and leptin [10]; have the ability to modulate hippocampal synaptic plasticity. Indeed, leptin insensitive obese rodents (*fa/fa* rats and *db/db* mice) display deficits in hippocampal LTP and long-term depression (LTD; [11]). Leptin insensitivity also results in impairments in spatial learning and memory tasks performed in the Morris water maze [11, 12]. Furthermore, rodent performance in spatial memory tasks is significantly enhanced after direct administration of leptin into the CA1 region of the hippocampus, whereas leptin administration into the dentate gyrus increases the magnitude of LTP [13]. At the cellular level, leptin promotes conversion of short-term potentiation (STP) into LTP, and it facilitates the induction of LTP in acute hippocampal slices [13, 14].

2.3. Leptin Regulates NMDA Receptor Function. It is well established that the synaptic activation of NMDA receptors coupled with a postsynaptic rise in intracellular Ca^{2+} is crucial for the induction of LTP at hippocampal CA1 synapses [15]. Moreover, the ability of hormones to influence the magnitude of LTP predominantly results from modification of NMDA receptor function. Indeed, leptin facilitation of NMDA responses underlies its effects on hippocampal LTP as this hormone enhances both NMDA receptor-dependent synaptic currents in slices and Ca^{2+} influx via NMDA receptor channels in cultured neurons [14]. Studies in *Xenopus* oocytes expressing recombinant NMDA receptors indicate that leptin receptor-driven signalling is required for enhancement of NMDA receptor-mediated currents by leptin. Furthermore, leptin increased maximal NMDA receptor-mediated currents without altering channel kinetics, suggesting that leptin increases the number of functional NMDA receptor channels by boosting NMDA receptor trafficking to the cell membrane.

In contrast to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors that readily move to and away from synapses as well as laterally within the plasma membrane [16, 17], NMDA receptors were viewed, until fairly recently, as static entities. However, there is growing evidence that the molecular identity and number of synaptic NMDA receptors can be modulated in an activity-dependent manner and in response to sensory experience [18]. For instance, the induction of LTP at adult hippocampal CA1 synapses is accompanied by an increase in NMDA receptor surface expression [19]. In addition, hormones such as insulin and the extracellular matrix protein reelin have been shown to modify NMDA receptor trafficking processes [20, 21]. NMDA receptors are heteromeric assemblies of NR1 and NR2 subunits, with or without an NR3 subunit, and the NR2 subunits determine the biophysical and pharmacological properties of NMDA receptors [22]. The expression and localization of NR2 subunits changes during development. Moreover the polarity of hippocampal synaptic plasticity depends on NMDAR subunit composition at different developmental stages. In adult hippocampus NR2A subunits underlie LTP whereas NR2B subunits are implicated in LTD [23]. In contrast, NR2A and NR2B underlie LTP, and LTD is NR2B independent in juvenile hippocampus [24]. Recent studies indicate that the intracellular C-terminal region of NR2 subunits plays a central role in controlling trafficking of NMDA receptors. Moreover, phosphorylation and palmitoylation of NR2C-terminal domains are also key events regulating NMDA receptor trafficking [25, 26]. Previous studies have shown that leptin has the ability to increase NR1/NR2A-mediated currents in *Xenopus* oocytes; an effect that is likely to be due to increased trafficking of NMDA receptors to the plasma membrane [5]. However no studies to date have examined if leptin alters the trafficking of other NR2 subunits, and thus it remains to be established if this hormone regulates NMDA receptors in an NR2-dependent manner.

2.4. Leptin Promotes Trafficking of GluR2-Lacking AMPA Receptors to Synapses. Previous studies indicate that application of leptin to juvenile (2-3 weeks old) hippocampal slices results in a transient depression of excitatory synaptic transmission [14, 27]. Conversely, leptin evokes a robust enhancement of excitatory synaptic transmission in adult hippocampus, and this effect persists after leptin washout [28]. Activation of leptin receptors is necessary for the leptin-driven increase in excitatory synaptic strength as leptin was without effect in slices from leptin-insensitive Zucker *fa/fa* rats, but a robust effect of leptin was observed in age-matched Zucker lean animals. In addition, the leptin increase in synaptic transmission was not associated with any significant change in PPR or CV indicating a likely postsynaptic expression mechanism. It is well documented that NMDA receptor activation is pivotal for LTP induction [29], and activation of NMDA receptors underlies the ability of leptin to facilitate LTP induction, reverse established LTP, and promote changes in dendritic morphology [14, 29, 30]. Similarly, leptin failed to increase excitatory synaptic strength in slices perfused with the NMDA receptor antagonist D-AP5, indicating the involvement of an NMDA receptor-dependent process. Moreover, in two pathway experiments leptin had no effect when stimulation was stopped whereas

leptin significantly increased synaptic transmission in the control pathway indicating that the synaptic activation of NMDA receptors was required.

It is known that NMDA receptor activation underlies the trafficking of AMPA receptors to hippocampal synapses during LTP [31], and that changes in the subunit composition of synaptic AMPA receptors are linked to activity-dependent changes in synaptic efficacy [3]. AMPA receptors are heterotetrameric ion channels composed of GluR1-4 subunits. GluR2-lacking AMPA receptors are more important for hippocampal LTP than GluR2-containing AMPA receptors, due to their permeability to Ca^{2+} which in turn allows for the activation of specific intracellular signalling pathways required for long-term changes in synaptic efficacy [32, 33]. Neurons within the hippocampus predominantly express functional GluR2-containing AMPA receptors until times of increased synaptic activity, when the membrane localized complement alters to include more GluR2-lacking AMPA receptors. Recent studies indicate that alterations in AMPA receptor trafficking processes also contribute to the increase in synaptic efficacy induced by leptin. Indeed, in studies examining the rectification properties of synaptic currents, the leptin-driven increase in synaptic transmission was accompanied by an increase in AMPA receptor rectification indicating that an increase in the synaptic density of GluR2-lacking AMPA receptors underlies this effect. Moreover, application of philanthotoxin resulted in reversal of the leptin-driven increase in synaptic transmission which is also consistent with an increase in the GluR2-lacking AMPA receptors underlying the increase in synaptic efficacy induced by leptin. In parallel studies, the effects of leptin on the cell surface expression of GluR1 and GluR2 in acute hippocampal slices and hippocampal cultures were examined using biotinylation assays and immunocytochemistry, respectively [28]. In adult hippocampal slices leptin enhanced GluR1, but not GluR2, surface expression. Conversely leptin failed to alter the surface expression of either GluR1 or GluR2 in slices from younger animals (3-4 week old). Furthermore, in dual immunolabeling experiments leptin was circa 50fold more potent at increasing GluR1 relative to GluR2 surface expression in hippocampal cultures. The ability of leptin to increase GluR1 surface expression involves promotion of GluR1 exocytosis as the effects of leptin were blocked by inhibitors of exocytosis, namely, NEM (N-ethylmaleimide-sensitive fusion protein) and brefeldin A. Consistent with this, the leptin-dependent enhancement of excitatory synaptic transmission in adult hippocampal slices was prevented following whole cell dialysis with exocytotic (NEM and brefeldin A), but not endocytotic (bafilomycin), inhibitors.

2.5. Role of PTEN in Leptin-Driven AMPA Receptor Trafficking to Synapses. Previous studies have demonstrated that PI 3-kinase, an enzyme that phosphorylates PtdIns(4,5)P_2 into PtdIns(3,4,5,)P_3, plays a pivotal role in leptin-driven signaling in the hippocampus [10]. PI 3-kinase is also implicated in NMDA receptor-dependent AMPA receptor trafficking to synapses during hippocampal LTP [31]. Similarly a PI 3-kinase-driven process underlies the effects of leptin as the increase in GluR1 surface expression was correlated with enhanced levels of PtdIns(3,4,5,)P_3 immunostaining, suggesting that an increase in PtdIns(3,4,5,)P_3 levels underlies leptin-driven alterations in AMPA receptor trafficking. Additionally blockade of PI 3-kinase signaling with either wortmannin or LY294002 prevented both the increase in excitatory synaptic transmission and GluR1 surface expression induced by leptin. In support of a possible role for PtdIns(3,4,5,)P_3, enhanced PtdIns(3,4,5,)P_3 synthesis results in significant enhancement of AMPA, but not NMDA, receptor-mediated synaptic transmission [34]. Although these findings suggest the involvement of a PI 3-kinase-dependent process, PtdIns(3,4,5,)P_3 levels are also regulated by the phosphatase PTEN as it antagonizes PI 3-kinase activity by catalysing the conversion of PtdIns(3,4,5,)P_3 to PtdIns(4,5)P_2 [35]. Moreover PTEN has been identified as a key signaling pathway activated by hypothalamic leptin receptors, and leptin receptor activation of K_{ATP} channels involves phosphorylation and subsequent inhibition of PTEN [36, 37]. In support of a role for PTEN, exposure of hippocampal slices to leptin increased the phosphorylation of PTEN and this effect was absent in slices from Zucker *fa/fa* animals indicating the involvement of a leptin receptor-driven process. Furthermore, the increase in GluR1 surface expression induced by leptin was coupled with an increase in P366-PTEN immunostaining in hippocampal cultures [28]. Previous studies have shown that CK2 phosphorylates PTEN at the threonine 366 site [38]. In agreement with these studies, the ability of leptin to increase GluR1 surface expression, P366-PTEN phosphorylation and excitatory synaptic transmission were all blocked by casein kinase2 (CK2) inhibition. This in turn supports the notion that CK2 phosphorylation and subsequent inhibition of PTEN underlies leptin-driven alterations in AMPA receptor trafficking and excitatory synaptic strength.

GluR1 surface expression was also significantly increased in neurons transfected with dominant-negative PTEN mutants (C124S or G129E). However, the ability of leptin to increase GluR1 surface expression was occluded in cells expressing the PTEN mutants, suggesting that analogous mechanisms underlie both processes. Leptin also increased the amplitude but not the frequency of mEPSCs, an effect attributable to insertion of GluR2-lacking AMPA receptors as it was reversed by addition of philanthotoxin. In contrast leptin failed to alter mEPSC amplitude in neurons transfected with the PTEN mutants. Thus inhibition of PTEN not only increases the functional expression of GluR1 subunits at hippocampal synapses but it also prevents the trafficking of GluR1 subunits by leptin. Similarly, pharmacological inhibition of PTEN with the phosphatase inhibitor bisperoxovanadium (bpV; [39]) resulted in a persistent increase in excitatory synaptic transmission in hippocampal slices, and it increased trafficking of GluR1 to hippocampal synapses. In addition, leptin failed to enhance synaptic transmission or alter AMPA receptor trafficking in the presence of bpV, which further supports the notion that leptin increases the synaptic expression of GluR1 via inhibition of PTEN and subsequent increase in PtdIns(3,4,5,)P_3 levels. However it is not exactly known how elevations in PtdIns(3,4,5,)P_3

levels modify AMPA receptor trafficking processes. Recent studies have shown that the availability of PtdIns(3,4,5,)P_3 is pivotal for sustaining AMPA receptor clustering and synaptic function at hippocampal synapses [34]. As inositol lipids are important regulators of the actin cytoskeleton, PtdIns(3,4,5,)P_3 may influence AMPA receptor trafficking by rearranging the actin cytoskeleton [40]. Alternatively, PtdIns(3,4,5,)P_3 may stimulate the activation of the protein kinase, Akt, which in turn may phosphorylate and subsequently inhibit glycogen synthase kinase 3 (GSK-3). In support of this possibility, Akt-driven inhibition of GSK-3 underlies AMPA receptor insertion after hippocampal LTP [41]. The ability of leptin to rapidly alter AMPA and NMDA receptor trafficking processes and evoke persistent changes in excitatory strength provides further evidence to support a role for this hormone as a potential cognitive enhancer. The leptin receptor-driven alterations in hippocampal synaptic function are likely to play an important role not only in normal brain function, but also in CNS-driven diseases associated with leptin dysfunction.

3. Leptin and Aging

Several lines of evidence support the notion that the effectiveness of metabolic hormonal systems declines with age and that impaired energy metabolism not only accelerates the aging process but also increases the susceptibility to neuronal degeneration [42]. Numerous studies have examined how insulin signaling in the CNS is altered during aging, but our understanding of how the leptin system changes with age is limited. A recent study comparing the effects of leptin on excitatory synaptic transmission in hippocampal slices from 3-4 month- and 12–14 month-old animals found that the responsiveness of hippocampal CA1 neurons to leptin declines with age [43]. In accordance with previous studies [28], leptin resulted in a persistent increase in the efficacy of hippocampal excitatory synaptic transmission (leptin-induced LTP) at 12–14 months, however, the magnitude of increase was significantly less at this age compared to 3-4 months. It is known that the magnitude of hippocampal LTP attenuates with age and this has been linked to reduced activation of NMDA receptors during the induction of LTP [44, 45]. It is feasible that a reduction in NMDA receptor activation contributes to the fall in the magnitude of leptin-induced LTP with age, as the ability of leptin to induce LTP also requires the synaptic activation of NMDA receptors and leptin-induced LTP and synaptically induced LTP share similar expression mechanisms [43]. Indeed, the magnitudes of LTP induced by leptin and high frequency stimulation were analogous in both adult and aged hippocampus, and synaptically induced LTP occluded the persistent increase in synaptic transmission induced by leptin and vice versa [43]. Although this study provides good evidence for a decline in hippocampal leptin function with age, it is not yet clear if the ability of leptin to modulate other CNS functions is altered during the aging process.

4. Leptin and Alzheimer's Disease

As life expectancy is increasing steadily, the prevalence of age-related neurodegenerative disorders such as AD is also increasing. Our understanding of the cellular changes that occur in the early stages of AD has advanced significantly in recent years, but it is still extremely difficult to uncover these early aberrant changes in a clinical setting. It is known that various factors can enhance the risk of developing AD including lifestyle and diet. Moreover, several studies have highlighted an association between midlife obesity and the incidence of AD, however, the mechanisms underlying this association are unclear. A number of studies have proposed that leptin dysfunction provides a link between obesity and AD [46]. Indeed, it is known that obesity is triggered by elevated leptin levels and the subsequent development of leptin resistance. In support of a possible link between obesity and AD, the circulating levels of leptin are significantly lower than normal in AD patients [47]. In addition, recent studies have shown that individuals with higher leptin levels have a much lower risk of developing AD [48, 49]. Moreover, leptin levels are also significantly reduced in murine models (APP_{Swe}; PSI_{M146V}) of AD [50].

Previous studies have indicated that leptin has neuroprotective and antiapoptotic properties as it protects neurons from a variety of neurotoxic agents including TNFα, ferrous iron (Fe^{2+}), and 6-OHDA [51–53]. Recent studies also support a neuroprotective role for leptin against ischaemic episodes [54]. These findings have important implications for the role of leptin in neurodegenerative disorders, as ischaemic incidents (such as cerebral thrombosis or stroke) have been shown to increase the incidence of sporadic AD by as much as 10-fold [55, 56]. Leptin has also been shown to have proliferative effects on neurones [57] increasing hippocampal volume and neuronal progenitor number, as well as reducing neurodegeneration caused by AD-related mutations [57]. Several studies have shown that leptin has neurotrophic actions in the CNS, however, this may be restricted to specific neuronal populations as leptin promotes neurite outgrowth in cerebellar purkinje, but not granule cells [58]. Further support for a neurotrophic role comes from a study by Yamada et al. [59] that demonstrated that leptin could alter cognitive state by reducing depression measured as a function of despair response in mice. Furthermore, leptin increased the levels of BDNF in the hippocampus, resulting in direct and inverse effects on the depressed state of the mice [59]. These findings lend additional support to the notion that leptin treatment could be beneficial in AD which is associated with neurodegeneration and cognitive impairments such as depression and dementia.

It is well established that two key pathological hallmarks of AD are the formation of amyloid plaques, due to the build-up and accumulation of β-amyloid (Aβ) and neurofibrillary tangles resulting from hyperphosphorylation of tau. Thus it is feasible that leptin limits the toxic effects of Aβ in neurons. Indeed, leptin is reported to attenuate Aβ levels in neurons by inhibiting β-secretase activity and thereby reducing Aβ production [60]. Furthermore, leptin promotes ApoE-driven uptake of Aβ into neurons [60]. Leptin also has the capacity

to alter the levels of hyperphosphorylated tau as leptin reduces the accumulation of phosphorylated tau in neuronal cells [61] and it limits phosphorylation of tau by inhibiting GSK3β [61]. Treatment of murine models of AD with leptin also resulted in significant reductions in the levels of both Aβ and phosphorylated tau compared to vehicle-treated littermates [62]. In addition to ameliorating AD pathology, treatment of CRND8 transgenic mice with leptin resulted in enhanced performance in novel object recognition tests as well as contextual and cued fear conditioning [63]. In SAMP8 mice, with elevated Aβ levels, administration of leptin improved memory processing in the T-maze foot-shock avoidance and step-down inhibitory avoidance tests [63]. Thus together these findings indicate that leptin has the ability to not only reduce the toxic accumulation of Aβ and phosphorylated tau but it also improves memory in murine models of AD.

Although numerous studies indicate that leptin markedly influences CNS function in rodent models, to be an effective therapeutic agent in CNS-driven disease leptin must have the capacity to modulate human brain function. Recent clinical studies have shown that treatment of three adults, with congenital leptin deficiency, with physiological doses of leptin resulted in significant and persistent elevations in grey matter volume in specific regions of the brain including the cerebellum and anterior cingulated gyrus [64]. Another study found evidence that leptin replacement therapy influences cognitive function, as treatment of a five-year-old boy with congenital leptin deficiency not only restored normal body weight and glycemic control, but also significantly improved neurocognitive skills [65].

5. Conclusions

Evidence is accumulating that the hormone leptin has widespread actions in the brain and it has the ability to regulate numerous CNS functions. In particular, evidence is accumulating that leptin plays a pivotal role in modulating higher cognitive functions such as learning and memory. Indeed, recent studies indicate that leptin is a potential cognitive enhancer as it rapidly alters glutamate receptor trafficking processes and in turn the efficacy of hippocampal excitatory synaptic transmission. However, the effects of leptin on hippocampal synaptic function markedly decline with age. Obesity in humans is closely associated with development of type II diabetes, and it is well documented that cognitive deficits are prevalent in diabetics. As obesity and obesity-linked disorders such as type II diabetes are associated with resistance to leptin, it is feasible that leptin dysfunction plays a role in cognitive impairments in these individuals. In addition, recent studies have linked dysfunctions in the leptin system with the development of neurodegenerative disorders such as Alzheimer's disease. Moreover, growing evidence indicates that leptin prevents the toxic accumulation of Aβ and phosphorylated tau in neurons, and it has the ability to improve performance in various memory tasks in murine AD models. These findings, coupled with the already established safety of leptin in humans, make this hormone or related leptin-mimetics novel therapeutic candidates for the treatment of neurodegenerative disorders like AD.

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Research Article

New Acetylcholinesterase Inhibitors for Alzheimer's Disease

Mona Mehta,[1] Abdu Adem,[2] and Marwan Sabbagh[1]

[1] *The Cleo Roberts Center for Clinical Research, Banner Sun Health Research Institute, 10515 W Santa Fe Dr, Sun City, AZ 85351, USA*
[2] *Department of Pharmacology and Therapeutics, United Arab Emirates University, Al Ain, UAE*

Correspondence should be addressed to Marwan Sabbagh, marwan.sabbagh@bannerhealth.com

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Acetylcholinesterase (AChE) remains a highly viable target for the symptomatic improvement in Alzheimer's disease (AD) because cholinergic deficit is a consistent and early finding in AD. The treatment approach of inhibiting peripheral AchE for myasthenia gravis had effectively proven that AchE inhibition was a reachable therapeutic target. Subsequently tacrine, donepezil, rivastigmine, and galantamine were developed and approved for the symptomatic treatment of AD. Since then, multiple cholinesterase inhibitors (ChEI) continue to be developed. These include newer ChEIs, naturally derived ChEIs, hybrids, and synthetic analogues. In this paper, we summarize the different types of ChEIs in development and their respective mechanisms of actions. This pharmacological approach continues to be active with many promising compounds.

1. Introduction

Acetylcholinesterase (AChE) has proven to be the most viable therapeutic target for symptomatic improvement in Alzheimer's disease (AD) because cholinergic deficit is a consistent and early finding in AD. Inhibition of AChE was considered to be achievable as a therapeutic target because of proven efficacy of inhibition of peripheral AChE as a treatment for myasthenia gravis (MG) proving that the approach was feasible. However, selective inhibition of the central nervous system (CNS) AChE initially proved to be daunting. Before tacrine, physostigmine, the classic AChE inhibitor (AChEI) was investigated as a treatment for AD. Physostigmine was subsequently abandoned because of poor tolerability. Four drugs are currently available for AD treatment: galantamine, rivastigmine, donepezil, and memantine. The first three are AChE inhibitors and memantine is not.

There are two types of cholinesterase, AChE and butyrylcholinesterase (BuChE). AChE is found primarily in the blood and neural synapses. BuChE is found primarily in the liver. The biggest difference between the two is the substrates. AChE hydrolyzes acetylcholine (ACh) more quickly, and BuChE hydrolyzes butyrylcholine (BuCh) more quickly. BuCh is a synthetic compound used to distinguish AChE receptors from BuChE receptors. Many of the drugs that are available for treatment of AD target both AChE and BuChE, but some are more selective than others. In this paper, we will be focusing on older acetyl cholinesterase inhibitors (ChEIs), current ChEI, naturally derived ChEI, hybrid ChEI, and synthetic analogues.

2. Older Cholinesterase Inhibitors

2.1. Physostigmine (Eserine). Physostigmine was the first ChEI investigated for the treatment of AD. It is isolated from the seeds of *Physostigma venenosum* as a parasympathomimetic plant alkaloid. Although it is able to pass through the blood-brain barrier (BBB), it has a short half-life and a narrow therapeutic index. It also has many side effects such as nausea, vomiting, headaches, diarrhea, and dizziness. Physostigmine was in use for MG, glaucoma, and delayed gastric emptying. However, the drug was not approved and was abandoned for AD use due to the disadvantages mentioned above. The newer drugs proved to be more effective with a lower side effect profile [1].

2.2. Tacrine. Tacrine was the first drug approved for treatment of AD in 1993 [2]. It is a potent inhibitor of both AChE and BuChE. Tacrine was approved both because

of efficacy on the ADAS-Cog and on the global measure compared to placebo in phase II and phase III clinical trials of AD subjects [3]. However, widespread use of tacrine was limited as it was poorly tolerated as it caused a number of side effects including nausea, vomiting, dizziness, diarrhea, seizures, and syncope. Also, administration and compliance were challenging due to four times a day dosing regimen because of a short half-life. In addition, patients who used the drug required periodic blood monitoring due to hepatotoxicity [4]. Eventually, tacrine was discontinued due to the aforementioned liver toxicity which was thought to be caused by the affinity for BuChE [2] and because less toxic, better tolerated drugs with easier dosing schedule were approved.

2.3. Donepezil. Donepezil was approved in 1996 for the treatment of mild-to-moderate AD. A twelve-week double-blind study was performed by Rogers et al. A total of 468 AD patients were separated into three groups: placebo, low dose (5 mg/day), and high dose (5 mg/day for week 1 and then 10 mg/day thereafter). Improvements were seen as soon as three weeks, and clinically significant effects were seen at nine weeks. The side effects were comparable with the placebo for the most part. Patients who were on the high dose occasionally experienced transient nausea, diarrhea, and insomnia [5]. Donepezil is also thought to have an additional mechanism of action other than just as a ChEI. It is believed that donepezil does not act only at just the neurotransmitter level but also at a molecular and cellular level in nearly every stage involved with the pathogenesis of AD. These include, but are not limited to, inducing a neuroprotective isoform of AChE, blocking various aspects of the excitotoxic cascade induced by glutamate, mitigating the effects of oxidative stress, and reducing the expression of inflammatory cytokines [6]. Donepezil is approved for use in mild, moderate, and severe AD but not for other forms of dementia. It has shown some benefit in mild cognitive impairment [7, 8] but is not approved for this indication. Recently, a higher dose of 23 mg formulation was approved for use in moderate-to-severe AD subjects. In the US, generic donepezil is now available. Donepezil is well absorbed with a relative oral bioavailability of 100% and reaches peak plasma concentrations in 3 to 4 hours. The elimination half-life of donepezil is about 70 hours and is approximately 96% bound to human plasma proteins. Donepezil is metabolized by CYP 450 isoenzymes 2D6 and 3A4 and undergoes glucuronidation.

2.4. Rivastigmine. Rivastigmine is a small molecule and has easy BBB permeability. Rivastigmine also has BuChE and AChE inhibitory properties. Rivastigmine was approved for the treatment of mild-to-moderate AD in 2000 and has since gained approval for Parkinson's related dementias. Corey-Bloom et al. conducted a trial with 699 patients with AD ranging from mild-to-moderately severe. The patients were split into three groups: a placebo group, a group on 1–4 mg/day, and a group on 6–12 mg/day for 26 weeks. Patients in the 6–12 mg/day group demonstrated significant improvements in cognition (ADAS-cog 4.94 points), activities of daily living, global assessment of change, and the Mini-Mental State Examination (MMSE) [9]. In capsular form, rivastigmine has been frequently associated with side effects of nausea, vomiting, anorexia, and diarrhea. In 2007, rivastigmine was reformulated for delivery through a transdermal patch. This has resulted in significantly lower GI side effects compared to the oral capsule. It is suitable to be converted to a patch delivery system because it is a small molecule (<300 Da in size), and it is both lipophilic and hydrophilic. It is minimally metabolized by CYP450 cytochrome system with weak binding to plasma proteins (approximately 40%). The duration of anticholinesterase activity in cerebrospinal fluid is approximately 10 hours after a 6-mg oral dose.

2.5. Galantamine. The therapeutic action of galantamine has been reported to be mainly produced by its sensitizing action on nAChRs rather than by general cholinergic enhancement due to cholinesterase inhibition. Galantamine was approved for the treatment of mild-to-moderate AD in February 2001. The trial conducted by Tariot et al. in 2000 consisted of 978 patients randomized to galantamine or matching placebo for five months. Administration of galantamine was associated with a significant improvement on the ADAS-cog score with 3.3 points for the 16 mg/day group and 3.6 points for the group on 24 mg/day when compared to placebo. Discontinuation of treatment due to adverse effects was minimal in both groups and equal to the placebo group. Both of the treatment groups showed significant improvement in the behavioral, cognitive, and functional symptoms of AD. Having a slow escalation of the dosage increased the tolerability of the drug [10]. Absorption of galantamine is rapid and complete and shows linear pharmacokinetics. It is well absorbed with absolute oral bioavailability between 80 and 100%. It has a half-life of seven hours. Peak effect of inhibiting acetylcholinesterase was achieved about one hour after a single oral dose of 8 mg in some healthy volunteers. Plasma protein binding of galantamine is about 18%.

2.6. Metrifonate. Metrifonate is a long-acting irreversible ChEI that was originally used to treat schistosomiasis. Although there was a low risk of side effects with short-term use, long-term use caused respiratory paralysis and neuromuscular transmission dysfunction similar to a myasthenic crisis. As such, the FDA submission was halted and the clinical trials were discontinued during phase III. However, it did demonstrate a robust therapeutic effect on ADAS-cog and other measures. But, until the relationship between metrifonate and neuromuscular dysfunction is further explored, metrifonate is not an option for AD treatment at this time [11]. Metrifonate is not an approved AD treatment but shows efficacy that was outweighed by safety risks.

2.7. Older ChEIs Summary. Dozens of short-term clinical trials, open-label extension studies, and long-term clinical cohort observational studies generally point to the following (a) current ChEIs (donepezil, rivastigmine, and

galantamine) reduce cognitive, functional, and behavioral decline in AD, (b) their efficacies appear similar (there is no level 1 grade data that shows otherwise), (c) their benefits are sustained with treatment persistence, (d) their benefits are generally dose-related (until limited by side-effects at very high doses), and (e) they appear to be relatively safe and well-tolerated (see Table 1).

3. New Cholinesterase Inhibitors

3.1. Physostigmine Derivatives

3.1.1. Phenserine. Phenserine is the phenylcarbamate derivation of physostigmine that has a dual effect: decreasing beta-amyloid precursor protein and has a reversible AChE inhibition. It has a quick absorption rate and is less toxic than physostigmine and tacrine. It has also shown improved learning and memory in aging rats and dogs. Another study involving dogs showed that phenserine improved learning and memory compared to dogs receiving placebo group [1]. Phase III clinical trials were conducted during 2003-2004 in which 384 AD patients recruited. The trials were conducted using 10 mg and 15 mg twice daily doses. However, phenserine did not achieve significant improvement on the ADAS-cog scores compared to placebo. Subsequently, further clinical trials for AD have been abandoned [1]. Post hoc analysis of all three Phase III clinical trials identified that the group of subjects receiving the highest tested dose (15 mg per day) for more than 12 weeks demonstrated a statistically significant benefit of phenserine over placebo in ADAS-cog, but only a trend toward improvement in the CIBIC+ measure.

3.1.2. Tolserine. Tolserine only differs from phenserine at the 2′-methyl substitution on its phenylcarbamoyl moiety. Preclinical studies were initiated in 2000, and it was shown to be 200-fold more selective against hAChE versus BuChE [1]. Tolserine proved to be a highly potent inhibitor of human AChE compared to its structural analogues physostigmine and phenserine. However, there are no published reports on Tolserine in humans so it is difficult to identify potential risks and benefits in preclinical or clinical models.

3.1.3. Esolerine. Zhan et al. conducted a study in which a series of physostigmine analogues were prepared and assessed for ChEI. Compound 17, a cyclic alkyl carbamate of esolerine, a very strong AChE inhibitor and significantly favored AChE over BuChE [12]. Esolerine is a tacrine derivative, but there are no published reports in humans so it is difficult to identify potential risks and benefits in preclinical and clinical models.

3.2. NS2330 (Tesofensine). Both *in vitro* and *in vivo* studies have shown that tesofensine inhibits the presynaptic uptake of the neurotransmitters serotonin, norepinephrine, and dopamine. The compound enhances the function of the neurotransmitters acetylcholine, noradrenaline, and dopamine, which are all impaired in Alzheimer's patients. A decrease in beta-amyloid concentration was found in mice after use of tesofensine which was thought to be a neuroprotective effect. Phase IIA trials showed significant cognitive improvement in those with mild AD. However, Phase IIB trials showed limited activity. The trials were discontinued in 2008. Currently, tesofensine is being marketed as a drug against obesity [13, 14].

4. Naturally Derived

4.1. Huperzine A. Huperzine A (HupA) is a Lycopodium alkaloid isolated from the Chinese medicinal herb *Huperzia serrata* used for memory deficiency. *Huperzia serrata* is widely grown in China and Chinese medical tradition emphasize herbal remedies. It has attracted much attention because it is a highly selective, reversible, and potent AChE inhibitor [15]. The synthetic racemic mixture of HupA has less AChE inhibitory effects than the natural kind. Many of the initial derivates demonstrated lower potency than the natural HupA. HupA has also been hybridized with tacrine and donepezil. While the donepezil hybrid proved to be less effective, the HupA-tacrine hybrids called huprines Y and X have shown to be more effective in amplifying AChE *in vitro* than tacrine. Huprines Y and Z were shown to have better inhibitory activity than either of the parent drugs as well [1]. HupA has a higher oral bioavailability compared to tacrine and donepezil. The improvement is more noticeable on working memory than reference memory as compared to tacrine and donepezil as well [16]. HupA is also shown to be more potent than tacrine, rivastigmine, and galantamine in terms of inhibition activities, and it had the least amount of activity against BuChE [15].

HupA is considered to be the drug of choice in China for the treatment of memory disorders. It is also in Phase II clinical trials in Europe [16]. Phase II trials have also been conducted in over 30 sites in the US. A trial using a lower dose of 200 mcg twice daily showed no improvement on the ADAS-cog scores, but a different trial using a higher dose of 400mcg twice daily showed statistically significant improvement in the ADAS-cog scores compared to placebo [15, 17] with GI side effects reported. Since it is marketed in the US as a nutraceutical supplement because it lacks a proprietary patent for the treatment of AD, FDA approval is not being pursued. It is widely available as a supplement marketed to enhance memory but with no labeling to treat specific diseases.

4.2. Huperzine B. Natural Huperzine B (HupB) is a Lycopodium alkaloid isolated from the Chinese medicinal herb *Huperzia serrata* which has been demonstrated as an effective and reversible inhibitor of AChE. HupB is less potent and selective than HupA, but it has higher therapeutic index and other positive benefits. HupB derivatives were created to be more potent than natural HupB. A novel series of 16-substituted derivatives were synthesized. 9i has the highest potency *in vitro*. The efficacy of 9i was more potent *in vitro* than HupA, galantamine, and rivastigmine, and it was equivalent to donepezil. 9c and 9i showed moderate

Table 1: Summary of ChEIs in preclinical and clinical development.

Drug	Disposition
Physostigmine	First drug investigated; however, it is no longer used due to side effects, short half-life, and better treatment options.
Donepezil	Highly selective AChEI. Approved for mild, moderate, and severe AD.
Rivastigmine	Has dual bChEI and AChEI properties**.** Approved for mild-to-moderate AD. Patch formulation has reduced cholinergic-related side effects.
Galantamine	A lower potency AChEI with allosteric nicotinic receptor modulation properties.
Metrifonate	A highly selective AChEI that demonstrated a robust and significant clinical effect, but was abandoned after Phase III RCTs because of risk of neuromuscular dysfunction.
Phenserine	A derivative of physostigmine with a dual mechanism of action including anti-Aβ properties as well as AChEI. Despite a good safety profile, it did not achieve significant efficacy during Phase II trials.
Tolserine	A physostigmine derivative, has shown promise in the preclinical stages.
Esolerine	Another physostigmine derivative, is also a strong AChEI and favors AChE over BuChE greatly. It has not entered clinical trials.
NS2330 (tesofensine)	Robust preclinical efficacy and safety data. Early Phase II studies showed a positive signal on cognition. Follow-up RCTs for AD were discontinued in 2008 because of lack of clinical efficacy signal. It is currently being investigated as a treatment for obesity treatment.
Huperzine A	HupA is natural herb that is marketed as a supplement in the US. It acts as a ChEI. It is drug of choice for AD in China. Phase II trials in the US showed a modest but clinical significant effect on cognition in AD.
Huperzine B	HupB is less potent and selective than HupA, but it has a higher therapeutic index.
Nelumbo nucifera	The stamens of *Nelumbo nucifera* has demonstrated an improvement in memory in rats and favors AChE over BuChE.
Himatanthus lancifolius	A shrub with multiple medicinal purposes. The uliene in *Himatanthus lancifolius* is what is likely responsible for the significant AChE inhibitory effects.
Galangin	Galangin is a flavonoid that demonstrated significant inhibition of AChE. It has not been tested in human trials.
Donepezil hybrids	(1) Of the series of hybrids derived from Donepezil and AP2238, compounds 15, 21, and 22 demonstrated the most potential. Human studies have not been undertaken yet. (2) The entire series of donepezil-tacrine hybrids showed more significant benefits than either parent drug alone. Human studies are planned.
Tacrine hybrids	(1) The beta-carboline derivatives (2A, 2B, 2C) and tacrine/ferulic acid hybrids (1A, 1B) were shown to have no efficacy *in vivo,* and 1B actually worsened the impairment in an scopolamine-induced *in vivo* model. Clinical development has not been pursued further. (2) The tacrine-8-hdroxyquinoline hybrids showed potential *in vitro*, but the effects have yet to be shown *in vivo*.
Synthetic analogues	(1) The majority of the phenyl-5,6-dimethoxy-1-oxo-2,3-dihydro-1H-2-indenylmethanone analogues demonstrated significant AChEI in *in vitro* and *in vivo models*. (2) N-alkyl-7-methoxytacrine hydrochlorides are also an area of interest as compounds 5–7 have more efficacy than THA and 7-MEOTA.

neuroprotection against H_2O_2-induced neurotoxicity [18]. However, given the ubiquitous availability of HupA and its higher potency, clinical development of HupB has been reportedly limited. Side effects would be expected to be similar in nature (GI) to HupA and other ChEIs.

4.3. Nelumbo nucifera. Nelumbo nucifera is an aquatic plant with numerous medicinal properties. It has recently demonstrated that the stamens fed to rats performing maze learning tasks improved memory. The MOA is felt to be AChE inhibition. One new compound and four known compounds were isolated from the n-butanol fraction of the *N. nucifera* for the first time. The new compound is a beta-cyclogeraniol diglycoside, nuciferoside (**5**), and the four known compounds are cycloartenol (**1**), *p*-hydroxybenzoic acid (**2**), vanilloloside (**3**), and 5′-*O*-methyladenosine (**4**). Compounds **5** and **1–3** demonstrated good and noncompetitive AChE inhibition and compounds **1, 2,** and **5** showed exhibited BuChE inhibition. Compounds **1–3** and **5** have possible ChE inhibitory effects with the potential to be used for AD treatment. The primary effect would be as an AChE inhibitor rather than as BACE1 inhibitors [19]. There are no reports of human studies. Preclinical and clinical safety and toxicity data are not reported.

4.4. Himatanthus lancifolius. Himatanthus lancifolius is a shrub that contains several indole alkaloids with a number of medicinal properties such as antimicrobial effects, gastroprotection, and the ability to affect the vascular and nonvascular smooth muscle responsiveness. Seidl et al. [20] conducted a study to determine if there were any AChE inhibiting properties from the *Himatanthus lancifolius* extract and uliene *in vitro*. The dichloromethane (DCM), and ethyl acetate (EtOAc), fractions showed significant AChE inhibitory effects. Uliene was the significant compound present in both fractions [20]. There are no reports of human

studies specific to cognition. Preclinical and clinical safety and toxicity data are not reported.

4.5. Galangin. Guo et al. studied 21 different flavonoids for potential AChE inhibition properties in the brain *in vitro*. Flavonoids have been of great interest in AD research and treatment because of their free radical scavenging properties. Epidemiological evidence suggests that higher consumption of flavonoids is associated with lower incidences of AD. A flavonol isolated from Rhizoma Alpiniae Officinarum called galangin demonstrated the highest inhibitory effects on AChE activity. However, it is unknown if the galangin binds to the AChE directly or has the same binding site as the ATCh substrate [21]. Nevertheless, this suggests that galangin could be developed as a potential treatment for AD because of the dual MOA of ChEI and free radical scavenging properties. There are no human studies reported to date. Clinical and preclinical toxicities have not been established.

4.6. Cardanol Derivatives. de Paula et al. designed new AChEI from nonisoprenoid phenolic lipids (NIPLs) of *Anacardium occidentale*. Cardols, cardanols, anacardic acids, and methylcardols are the primary NIPL components of cashew nut-shell liquid (CNSL) and have been used to generate potential bioactive compounds. The derivates have structural, electrical, and hydrophobic properties that are relevant to recognition of AChE molecules. The study concluded that the most promising candidates to the development of AChEI for AD treatment were derived from cardanol [22]. Development of cardanol is appealing because of the abundancy of the raw material source. There are no human studies reported to date. Clinical and preclinical toxicities have not been established.

5. Hybrids

Since AD is a multifactorial disease, the innovative model is of the "one molecule, multiple targets" approach. Hybrids combine BBB permeability with drugs targeting multiple receptors or epitopes. Further, hybrids offer the promise of dual MOAs and added potency. The multipotent approach includes novel tacrine-melatonin hybrids, dual inhibitors of AChE and MAO or serotonin transporters, potent cholinesterase inhibitors with antioxidant and neuroprotective properties, gallamine-tacrine hybrids binding at cholinesterases and M_2 muscarinic receptors, NO-donor, tacrine hybrids as hepatoprotective drugs focusing on AD or fluorescent tacrine, coumarin hybrids [23]. The side effects profiles are unknown in humans at present.

5.1. 5-(N-Methyl-N-propargyl-aminomethyl) Quinolin-8-yl Dimethyl Carbamate (2) and 5-(N-Methyl-N-propargylaminomethyl) Quinolin-8-yl Ethylmethyl Carbamate (3). One type of new drug is a site-activated chelator that inhibits both AChE and MAO A/B. Although the original chelators (clioquinol and desferrioxamine) had success in clinical trials, the drugs did not selectively target the biometals in the brain. Therefore, the therapeutic use was limited due to toxicity (in the case of clioquinol) or poor selectivity and permeability. Now, chelators are designed with MAO A/B inhibitory activity to enhance efficacy and as prodrugs to enhance targeting. This new prochelator would act as HLA 20A in that it would selectively inhibit AChE with minimal metal ion binding affinity, possess increased activity against MAO A/B, and it would be activated by AChE, which is located primarily in the brain, to release the active chelator M30. The two compounds synthesized are compounds 2 and 3. Compounds 2 and 3 are like rivastigmine in that the AChE inhibited progressively increases as incubation time increases. Compound 2 is more potent and 3 is less potent than rivastigmine in blocking AChE at 1 μM. Both compounds are more potent at inhibiting MAO-A than M30, but compound 2 is less potent than M30 at inhibiting MAO-B and compound 3 has minimal inhibitory effects. The biggest novelty is that M30 is combined with these compounds into prochelators. Compound 2 is more promising of the two [24].

5.2. Donepezil and AP2238. AP2238 is the first published compound to bind both anionic sites of AChE. The potency against AChE is comparable to donepezil, while its ability to contrast beta-amyloid aggregation is higher. Rizzo et al. reports on a series of hybrids developed from donepezil and AP2238 in which the idanone core from donepezil is linked to the phenyl-N-methylbenzylamino moiety from AP2238. A derivate in which the phenyl-N-methylbenzylamino moiety from AP2238 was replaced by phenyl-N-ethylbenzylamino moiety from AP2243 and the idanone ring was replaced by a tetralone scaffold. A total of 22 compounds were synthesized. Derivates 21 and 22 were the most active of all the compounds, and the potency was equal to the reference compounds. Compounds 15, 21, and 22 had a 5-carbon alkyl chain with an amino moiety at one end which gave the compounds better contact at the peripheral anionic site (PAS). This improved the inhibition of AChE-induced aggregation vastly when compared to the reference compounds. Compound 21 was a part of the tetralone series, and the methoxy substituent in position 6 was replaced by a pentyl chain. Compound 22 was a piperidine derivative of the tetralone series. In compound 15, the second amino moiety was replaced by piperidine with $n = 5$. Overall, compounds 15, 21, and 22 showed the most promise [25]. There are no reports of human studies. Preclinical and clinical safety and toxicity have not been established.

5.3. Donepezil-Tacrine Hybrids. Camps et al. designed a novel series of donepezil-tacrine hybrids, which interact simultaneously at the peripheral, active, and midgorge binding sites of AChE. They are desirable because both compounds have known efficacy and BBB permeability with slightly different MOAs. They were tested for the ability to inhibit BuChE, AChE, and beta-amyloid aggregation induced by AChE. The compounds are combination of the 5,6-dimethoxy-2-[(4-piperidinyl)methyl]-1-idanone moiety of donepezil and 6-chlorotacrine. All of the new hybrids have proven to be highly potent hAChE inhibitors. All of the new

compounds also demonstrated significant inhibition of beta-amyloid aggregation and were shown to be more potent than parent compounds [26].

5.4. Tacrine/Ferulic Acid Hybrids and Beta-Carboline Derivatives. Three beta-carboline derivatives (referred to as without chemical names (2A, 2B, 2C)) and two tacrine/ferulic acid hybrids (1A, 1B) were tested *in vivo* in three-month-old female rats. The tacrine/ferulic acid hybrids showed higher AChE inhibition and comparable BuChE inhibition when compared to tacrine *in vitro*. However, neither compounds 1A nor 1B showed any therapeutic effects against scopolamine-induced cognitive deficits *in vivo*. Compound 1B actually worsened the impairment. Compound 2C was shown to be the most effective of the three *in vitro*. Compound 2B did not show any significant activity, and compound 2A was comparable to galantamine. However, none of these three compounds were shown to be effective *in vivo*. Despite the promising *in vitro* information, none of the five compounds are suitable for AChE inhibition against AD [27].

5.5. Tacrine-8-hydroxyquinoline Hybrids. Both tacrine and PBT2 (an 8-hydroxyquinoline derivative) are known for ChE inhibition and decreasing beta-amyloid concentrations, respectively. Fernandez-Bachiller et al. designed, synthesized, and evaluated novel tacrine-8-hdroxyquinoline hybrids as possible therapeutic drugs for AD treatment. The hybrids were found to be more effective than tacrine against AChE and BuChE. They also show low cell level toxicity and could possibly penetrate the CNS as demonstrated by an *in vitro* BBB. The drug has also demonstrated antioxidant and copper-complexing properties. With all the properties that these hybrids possess, it could be a possible therapeutic drug treatment *in vivo* as well [3].

5.6. Other Hybrids Combinations. Samadi et al. studied tacripyrines and developed a new compound called p-methoxytacripyrine (RL2/101). RL2/101 impedes the proaggregation beta-amyloid effect of hAChE and has mild inhibitory effects on the self-aggregation of Aβ 42. RL2/101 is a potent Ca2+ antagonist that passes through the blood-brain barrier (BBB). It also displays antioxidant and neuroprotective properties. Samadi et al. carried out with the synthesis of a number of series of simple, and readily available 2-aminopyridine-3,5-dicarbonitriles (3–22), and 2-chloropyridine-3,5-dicarbonitriles (21–28) derived from 2-amino-6-chloropyridine-3,5-dicarbonitrile (1) and 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile (2). Compounds 3, 4, 21–23, 25, 26 were shown to be highly selective for hAChE. Compounds bearing small groups such as the N, N^0-dimethylamino or the pyrrolidino preferentially inhibit AChE. Compounds 3, 17, 22, 24, and 26 showed the highest neuroprotection values. These compounds could be further developed to have multifaceted approach against cholinergic dysfunction and oxidative stress [23].

Camps et al. designed two new series of hybrid AChEIs and tested them for AChE inhibition, butyrylcholinesterase, BACE-1, and self-induced and AChE-induced beta-amyloid aggregation and BBB permeability. The hybrids are composed of a 6-unit chlorotacrine and a multicomponent reaction-derived pyrano [3,2-c]-quinoline scaffold as the active and peripheral sites interacting moieties, respectively. Due to the dual binding site activity, both hybrids demonstrate the ability to inhibit AChE-induced beta-amyloid$_{1-40}$ aggregation and self-induced beta-amyloid$_{1-42}$ aggregation. Some of compounds can also inhibit BACE-1. These hybrids also have the ability to permeate the BBB. Hybrid 27 emerged as the most promising therapeutic as it was able to target both AChE and beta-amyloid production and aggregation [28].

6. Synthetic Analogues

6.1. Phenyl-5,6-dimethoxy-1-oxo-2,3-dihydro-1H-2-indenylmethanone Analogues. Synthetic analogues are under development because, with targeted pharmacological development, class-specific hepatotoxicity and known gastrointestinal side effects may be avoided. The risk of developing synthetic analogs is that they might not have the potency and BBB permeability as naturally derived ChEIs or they may have unanticipated pharmacological properties. Ali et al. synthesized phenyl-5,6-dimethoxy-1-oxo-2,3-dihydro-1H-2-indenylmethanone analogues labeled **5a–5n**. Their data showed that the majority of the compounds had moderate AChE inhibitory effects. **5f** had the most significant effects followed by **5e** and then **5a**. The study suggests that having an OCH_3 group substituted phenyl ring at diketone derivatives creates a vast improvement in AChE inhibition [29].

6.2. N-Alkyl-7-methoxytacrine Hydrochlorides. The first AChEI developed was THA, but using the drug caused dose-dependent but reversible liver toxicity. 7-methoxytacrine (7-MEOTA) is an analogue of THA that has far less toxicity and is pharmacologically equivalent to THA. In a study by Korabecny, fourteen 7-MEOTA analogues were synthesized. Models of human recombinant AChE (hAChE) and human plasmatic BuChE (hBuChE) were used to evaluate these new analogues *in vitro* and were compared to THA and 7-MEOTA. Compounds 5–7 showed better inhibition of hAChE than compared to 7-MEOTA and THA, especially compound 5 as it was found to be five times more potent than THA. Compounds 9, 10, and 14 were found to be ineffective against hAChE. The structure-activity relationship findings point at the C6-C7 N-alkyl chains for cholinesterase inhibition [30].

6.3. Ladostigil. Ladostigil is a novel anti-Alzheimer's disease drug, with neuroprotective, multimodal brain-selective monoamine oxidase, and cholinesterase inhibitor properties [31]. It has neuroprotective and antioxidant activities in cellular models at much lower concentrations than those inhibiting MAO or AChE. When ladostigil (1 mg/kg/day) was given for 6 months to 16-month-old rats, it prevented the age-related increase in activated astrocytes and microglia in several hippocampal and white matter regions and increased

proNGF immunoreactivity in the hippocampus towards the levels in young rats.

Ladostigil also prevented the age-related reduction in cortical AChE activity and the increase in butyrylcholinesterase activity in the hippocampus, in association with the reduction in gliosis. The immunological and enzymatic changes in aged rats were associated with improved spatial memory. Ladostigil treatment had no effect on memory, glial, or proNGF immunoreactivity in young rats [32]. Ladostigil ((N-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate) is, presently in a Phase IIb clinical trial and intended for the treatment of Alzheimer's disease and dementia comorbid with extrapyramidal disorders and depression [31].

7. Discussion

A great deal of research has been done regarding AChEI as a therapeutic drug for AD. All of the current first-line treatments in the US are AChEIs. Physostigmine was the first ChEI investigated for AD, but it was discarded due to a number of disadvantages. Although phenserine, a physostigmine derivative, has failed the Phase II clinical trials, there are other derivatives in development. In addition, metrifonate also has a profound effect on those with AD. A metrifonate derivative with the same or better efficacy and a lesser side effect profile would be a promising therapeutic drug. Naturally derived AChEIs are also a promising area of interest. HupA is the drug of choice in China [16]. Many hybrids are also being created. Some hybrids have completely new materials, but other hybrids are using the older AChEI and are trying to improve upon them. The synthetic analogues have only been tested *in vitro* at this time, but it is an area that can help direct future treatment options for AD. Although the majority of the drugs discussed in this paper have not yet been tested in animals or humans, each of these areas will continue to develop because this class of drugs has demonstrated its value in symptomatic therapy. This class of drugs will continue to be developed because it is a proven symptomatic therapy with a recognized target. Drugs in the class have a proven track of CNS permeability, known side effect profile, and demonstrated efficacy. It is logical to consider further development of novel agents in this class. Challenges to development include potency, safety, and side effects as well as comparison to current ChEIs, many of which are generic. Many compounds developed to date have no human or animal data. Thus, safety, efficacy, and toxicity have not been established. Further, this class of medications has not been established to possess disease-modifying properties. The risk of developing newer ChEIs is that they will need to be more effective than donepezil, rivastigmine, and galantamine to garner approval since these three drugs are FDA approved. Future research in this class will need to focus on whether ChEIs directly affect the pathophysiology of AD.

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Review Article

Broader Considerations of Higher Doses of Donepezil in the Treatment of Mild, Moderate, and Severe Alzheimer's Disease

Camryn Berk and Marwan Sabbagh

The Cleo Roberts Center for Clinical Research, Banner Sun Health Research Institute, 10515 W. Santa Fe Dr, Sun City, AZ 85351, USA

Correspondence should be addressed to Marwan Sabbagh, marwan.sabbagh@bannerhealth.com

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Donepezil, a highly selective acetylcholinesterase inhibitor (AChEI), is approved as a symptomatic treatment mild, moderate, and severe Alzheimer's disease (AD). Donepezil exerts its treatment effect through multiple mechanisms of action including nicotinic receptor stimulation, mitigation of excitotoxicity, and influencing APP processing. The use of donepezil at higher doses is justified given the worsening cholinergic deficit as the disease advances. Donepezil has been investigated in several clinical trials of subjects with moderate-to-severe AD. While the side effects are class specific (cholinergically driven), demonstrable benefit has been shown at the 10 mg dose and the 23 mg doses. Here, we review the clinical justification, efficacy, safety, and tolerability of use of donepezil in the treatment of moderate-to-severe AD.

1. Introduction

Alzheimer's disease is an age-related progressive neurological disorder that ultimately results in cognitive and behavioral dysfunction and functional loss. These later stages of disease are impacting an increasingly large number of people each year. At present, the treatment of Alzheimer's disease (AD) is limited to symptomatic therapy. As symptoms worsen and the effects of late-stage Alzheimer's disease become more severe, there is an urgent need for new and novel treatment options. Of the 5.3 million people estimated to be afflicted with AD in the United States, more than half are suspected to have moderate-or-severe disease. Because these stages of AD can last for several years, the difficulty presented to both patients and caregivers increases dramatically in advanced stages [1].

Donepezil, a highly selective acetylcholinesterase inhibitor (AChEI), is one of only two treatments approved in the United States for use beyond the mild-to-moderate stage. While it can slow progression modestly and treat symptoms, it cannot halt progression of the actual disease or ultimately stop the decline in cognitive and functional abilities. Agents such as donepezil create notable symptomatic improvements with modest but debatable impact on disease progression from mild to moderate is concerned, decreasing speed of progression from the earliest signs of cognitive impairment to severe dementia and finally death [2, 3].

An important component of Alzheimer's disease management is the group of caregivers who support Alzheimer's patients. As Alzheimer's disease progresses, the demands on the caregiver increase; often this takes a toll on the care provider in the form of health problems of their own, stress, anxiety, depression, fatigue, and other physical and emotional problems [4]. Donepezil is a valuable treatment option not just for its capacity as a selective inhibitor of acetylcholinesterase for the patients but also for its capacity to ease the burden of the caregiver by prolonging the AD patient's ability to perform self-care tasks and delaying the progression of symptoms that would impact a caregiver [5].

2. Overview

Donepezil has a mode of action based on inhibiting acetylcholinesterase selectively. Cholinergic function is improved when there are greater concentrations of acetylcholine in the brain, and the depletion of acetylcholine that is common

with AD patients is positively affected with a donepezil regimen. Additionally, cholinesterase inhibitors hypothetically could operate within the scope of the "amyloid hypothesis" of AD pathogenesis. Donepezil in particular has been shown to attenuate neurotoxicity of amyloid beta peptide Aβ as well as influencing APP processing [6]. In accordance with the cascade concept, therapies that directly target the accumulation, aggregation, and deposition of amyloid beta peptide Aβ_{42} and effectively reduce the inciting event may be more effective than distally targeted treatments, such as those that target neurotransmitter deficits.

In addition to attenuating Aβ neurotoxicity, donepezil also has an effect on cholinergic deafferentation. Cell bodies in the nucleus basalis in the brain are the origin of cholinergic tracts, the axons of which are prolific in the cerebral cortex and in the pedunculopontine nucleus with expansion into the thalamus. Loss of cholinergic neurons in the nucleus basalis are a hallmark of the earliest stages of AD. The functional deafferentation of the cholinergic neurons is thought to induce Aβ production in the cerebral cortex, which is what begins the cascade to neurodegeneration. Donepezil disrupts the mechanism by which Aβ generation occurs following cholinergic deafferentation by interfering with the process that leads to the exposing of the toxic N-terminal region of APP during the preferential processing of the amyloid precursor protein. One way donepezil interupts this process is by cholinergic input to cortical neurons [7].

Donepezil also impacts nicotinic receptors in the cortex through upregulation. At clinically relevant concentrations, donepezil decreases the reduction of nicotinic acetylcholine receptor expression in the cerebral cortex of AD patients and prevents some of the reduction in nicotinic binding that correlates to disease severity. This process reduces glutamate neurotoxicity and also has been shown to inhibit excitotoxic injury, which is significant regarding AD pathogenesis and progression [8].

Other mechanisms of action indicate donepezil could provide a treatment option for affecting the cellular and molecular processes of neurodegeneration rather than symptomatic treatment. Donepezil has been found to influence acetylcholinesterase isoform expression by inhibiting the expression of the AChE-S form and causing an increase in the expression of the AChE-R form through reduction of cholinesterase, generating neuroprotective effects [9]. Donepezil has also been shown to upregulate nicotinic receptors in the cerebral cortex, increasing nicotinic acetylcholine receptor expression and nicotinic binding [10]. Furthermore, under conditions of upregulations donepezil has been proven to maintain neuroprotective actions are lower drug concentrations and reduce glutamate toxicity [8]. Donepezil has been shown to mitigate the effects of oxidative stress in a streptozotocin-induced model of dementia in mice, indicating use against free radicals that may be implicated in AD [11]. Donepezil treatment has also resulted in improved cerebral blood flow in patients with mild cognitive impairment, with no reduction after six months compared to statistically significant reduction in the placebo cohort [12, 13]. Potential mechanisms of action also include enhancing neuroplastic activity through cholinergic modulation. In rodent models of basocortical degeneration, donepezil induced cortical sprouting, mitigating reduction in cholinergic neurotransmission [14].

3. Efficacy

Donepezil has been found to be one of the most effective symptomatic treatments for AD available on the market with additional potential as a disease-modifying therapy. Many but not all studies have shown that both when compared to a placebo, donepezil has proven to have significant efficacy in reducing the severity of neuropsychiatric symptoms in patients with mild-to-moderate AD and patients in more severe stages of the disease [15]. Additionally, donepezil has been found to be effective in symptomatic treatment in a variety of dosing levels giving it variability as a treatment option. In an open-label, multicenter, Phase 3 extension study of the safety and efficacy of donepezil in patients with Alzheimer's disease [15], the primary efficacy measures were the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-Cog) and the CDR-Sum of the Boxes (CDR-SB). These measures were evaluated in a 152-week long extension study on patients aged 50 and older that participated in at least one of the previous phases of donepezil trials, with a total patient number (n) of 763. Among this population, the most common reason for study discontinuation (46% of discontinued patients) was departure due to the commercialization of donepezil after the successful completion of nonextension trials, allowing for prescriptive use of the drug [15], but of those that completed the study declined from baseline significantly less than the placebo arm in the same period with regards to ADAS-Cog scores. After a three-week placebo washout, the mean change for patients using CDR-SB scores actually improved above baseline for the experimental arm and declined in the placebo arm.

In comparison studies with rivastigmine and galantamine, specifically a 52-week trial [16] and a 12-week trial [17] showed that regarding cognitive function, there is no statistically significant difference between patients treated with donepezil versus those treated with galantamine, nor is there a difference regarding behavior when comparing 10 mg/day of donepezil to 24 mg/day of galantamine. Similarly, rivastigmine and donepezil have been found to have comparable effects on cognition and behavior [18, 19] and similar gastrointestinal side effects profiles [20].

4. Safety and Tolerability

In patients with moderate-to-severe Alzheimer's disease, donepezil has been found to be safe and well tolerated at doses up to 23 mg, with an increase in adverse events over lower dosages, most of which dissipate after the first month of treatment [1, 21]. In a randomized, double-blind study, 1371 patients (mean age: 73.8 years, 62.8% female, 73.5% white) from 219 different sites in Asia, Europe, Australia, North America, South America, and South Africa were analyzed for comparative effects of 10 mg/day donepezil and 23 mg/day donepezil. All patients had an MMSE of less than 20 and

assessed for changes in cognition and global functioning as assessed using least square regressions as well as deviation from baseline between the two cohorts. At the highest doses, the most significant adverse events reported in safety trials were nausea, vomiting, and diarrhea; in the event that these symptoms did not dissipate, dosages were reduced to improve tolerability. Nevertheless, a certain percent of participants did not tolerate donepezil even after the dose was reduced. Donepezil has proven to be safe and well tolerated as well as effective in the treatment of moderate-to-severe AD [21].

Donepezil has been found to be an effective option for maintaining a desirable quality of life and prolonging patient performance of activities of daily living (ADL); under study of 290 (mean age: 73.6 years, range 48–92) patients randomized into a 24-week, double-blind, placebo-controlled study, donepezil demonstrated a vastly slower decline trajectory than placebo in ADLS in patients with moderate-to-severe AD [22]. Using the Disability Assessment for Dementia (DAD), the Instrumental Acitvities of Daily living (IADL) scale, and the Physical Self-Maintenance Scale (PSMS), researchers have determined that moderate-to-severe AD patients taking donepezil were improved in tasks such as the use of household appliances, managing personal mail, moving around in and outside of homes, understanding explanations or new situations, and maintaining leisure activities as compared to moderate-to-severe AD patients who took a placebo [22, 23]. In a six-month study, assessing responder rates of moderate-to-severe AD patients taking donepezil in a long-term care facility, greater proportions of patients defined as responders to donepezil showed significant stabilization or improvement compared with placebo on individual efficacy measures including the severe impairment battery (SIB) (≥ 0, ≥ 4 or ≥ 7 points) and Mini-Mental State Examination (≥ 0 or ≥ 3 points), and positive trends on the ADCS-ADL-severe (≥ 3 points) and the neuropsychiatric inventory (NPI) cluster based on mood items. All 3 composite measures of efficacy showed a significantly higher proportion of responders in the donepezil group. The responders had a similar distribution between the 2 subgroups of cognitive and functional disease severity at baseline. The donepezil-treated patients taking psychotropic drugs showed significantly greater improvement on the SIB, less deterioration on the ADCS-ADL, and had higher Clinical Global Impression of Improvement scores and a trend toward lower NPI scores [23].

In addition to assessing patient conditions, caregivers were assessed using the Caregiver Stress Scale (CSS) for the first time in a clinical trial to measure a caregiver's status regarding cognitive status, overload, relational deprivation, job-caregiving conflict, economic strains, role captivity, loss of self, care-giving competence, personal gain, management of distress, and expressive support. Statistical analysis of efficacy was based on change from baseline of MMSE scores. According to the caregiver journal assessments, patients treated with donepezil were more likely to be capable of interacting with caregivers and others, engaging and being interested in conversations with others, and enjoying leisurely activities. Caregivers of donepezil-treated patients remained close to their baseline assessments of stress levels while caregivers for the placebo group reported significant increases in stress over the same period. Treatment of moderate-to-severe AD with donepezil has also been associated with a much more gradual increase in time a caregiver spends caring for a patient with progressing disease compared to patients who received a placebo [24].

As indicated in the aforementioned Doody study, the adverse events experienced when treated with donepezil are transient and mild, often resolving themselves without the need for dose modification but in the rare cases resolution is elusive, lowering the dosage is an effective way to decrease adverse events. 92% of patients in the study experienced at least one treatment-related adverse event, with only 28% (214) of patients experiencing a serious (grade 3 or 4) AE; in the case of 27% (203), the serious nonfatal AEs experienced was determined to be not related to donepezil treatment. Treatment with donepezil also indicated no clinically significant results or changes in clinical laboratory tests, physical examinations, or electrocardiograms. The results of this study indicated that donepezil is a safe and effective agent for long-term treatment of moderate-to-severe disease [15].

5. Conclusions

It is broadly recognized that potent disease modifying agents are needed to prevent the progression of Alzheimer's disease. While the "amyloid hypothesis" of AD pathogenesis is still unproven, there is substantial evidence that suggests that inhibiting Aβ plaque formation will be a valuable approach for achieving this goal. But the foundation of AD management will continue to include a cholinesterase inhibitor such as donepezil. A multitude of well-controlled clinical studies have demonstrated the cognitive benefit of this agent. In addition, by interfering with the process that leads to the exposing of the toxic N-terminal region of APP during the preferential processing of the amyloid precursor, donepezil disrupts the mechanism by which Aβ generation occurs following cholinergic deafferentation. As new therapeutics come on line, the next advance in AD patient management will come from understanding how they synergize with donepezil or other cholinesterase inhibitors.

Going forward, the role donepezil plays in disease management should not be limited solely to cholinestrase inhibition at the level of neurotransmitter. Rather, as preclinical evidence amply demonstrates, the drug affects on cellular and molecular processes should be exploited for their depth and breadth of influence at every stage of disease.

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Review Article

Effect of Transcranial Brain Stimulation for the Treatment of Alzheimer Disease: A Review

Raffaele Nardone,[1,2] Jürgen Bergmann,[3] Monica Christova,[4] Francesca Caleri,[2] Frediano Tezzon,[2] Gunther Ladurner,[3] Eugen Trinka,[1] and Stefan Golaszewski[1,3]

[1] *Department of Neurology, Christian Doppler Clinic, Paracelsus Medical University, 5020 Salzburg, Austria*
[2] *Department of Neurology, Franz Tappeiner Hospital, Via Rossini 5, 39012 Meran/o, Italy*
[3] *Neuroscience Institute, Christian Doppler Clinic, 5020 Salzburg, Austria*
[4] *Department of Physiology, Medical University of Graz, 8010 Graz, Austria*

Correspondence should be addressed to Raffaele Nardone, raffaele.nardone@asbmeran-o.it

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Available pharmacological treatments for Alzheimer disease (AD) have limited effectiveness, are expensive, and sometimes induce side effects. Therefore, alternative or complementary adjuvant therapeutic strategies have gained increasing attention. The development of novel noninvasive methods of brain stimulation has increased the interest in neuromodulatory techniques as potential therapeutic tool for cognitive rehabilitation in AD. In particular, repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tDCS) are noninvasive approaches that induce prolonged functional changes in the cerebral cortex. Several studies have begun to therapeutically use rTMS or tDCS to improve cognitive performances in patients with AD. However, most of them induced short-duration beneficial effects and were not adequately powered to establish evidence for therapeutic efficacy. Therefore, TMS and tDCS approaches, seeking to enhance cognitive function, have to be considered still very preliminary. In future studies, multiple rTMS or tDCS sessions might also interact, and metaplasticity effects could affect the outcome.

1. Introduction

Given the limited efficacy of pharmacological treatments [1], nonpharmacological approaches in AD are of great interest. In recent years, new techniques for studying the human brain that allow for the noninvasive neurostimulation have emerged. In particular, two techniques of noninvasive brain stimulation—repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tDCS)—are capable for modulating cortical excitability and inducing lasting effects [2, 3]; both have been shown to have potential therapeutic efficacy in cognitive neuroscience [4]. By using rTMS, depending on the location and the stimulation parameters as well as on the physiology of the underlying cortical tissue, behavioural changes may also be seen, including enhancement of or interference with cognitive performance [5, 6]. rTMS has been increasingly utilized for various neurological [7–9] and psychiatric conditions [10–12]. tDCS has also been shown to induce cognitive improvements in healthy subjects [13–15] and patients with neuropsychiatric disorders such as depression [16–18], Parkinson disease [19, 20], and stroke [21]. Both neuromodulatory techniques can induce lasting modulation of brain activity in the targeted brain region and across brain networks through transcranial induction of electric currents in the brain [4]. In the last years, these two neuromodulatory techniques have been proposed as a possible treatment to improve cognitive performances, also in subjects affected by dementia in which it may represent a useful tool for cognitive rehabilitation.

2. Therapeutic Interventions

2.1. Neuromodulatory Techniques. rTMS is a technique that delivers single TMS pulses in trains with a constant frequency and intensity for a given time. tDCS is another simple and

powerful tool to modulate brain activity, which delivers low-intensity electrical currents (below the perceptual threshold, 1 to 2 mA) over the scalp using two large saline-soaked sponge electrodes. The resulting constant electrical field penetrates the skull and influences neuronal function.

rTMS can be applied as continuous trains of low-frequency (1 Hz) or bursts of higher-frequency (≥5 Hz) rTMS, while tDCS can be applied as anodal or cathodal stimulation [4]. In general, low-frequency rTMS and cathodal tDCS are thought to reduce, and high-frequency rTMS and anodal tDCS to enhance excitability in the targeted cortical region. However, it is not completely understood by which mechanisms of action rTMS and tDCS can induce lasting effects on the brain. The physiologic impact of both techniques involves synaptic plasticity, specifically long-term potentiation (LTP) and long-term depression (LTD). A link between the aftereffects induced by rTMS and the induction of synaptic plasticity has been recently identified [22]. Similarly, tDCS may modulate synaptic strength within the cortex, with evidence pointing to the involvement of intracortical neurons [23].

2.2. Repetitive Transcranial Magnetic Stimulation. A recent meta-analysis of publications searching for the effects of rTMS on cognitive functions [24] found convincing data supporting improvement in several cognitive functions, including executive functions, learning, and memory.

It has been demonstrated in elderly subjects that rTMS induces a transient improvement in the associative memory task and that it is associated with recruitment of right prefrontal and bilateral posterior cortical regions [25]. Three studies have been carried out to assess the effects of rTMS on naming and language performance in patients with probable AD.

In two crossover, sham-controlled, single-session studies [26, 27] rTMS was applied to the dorsolateral prefrontal cortex (DLPFC) during the execution of naming tasks (on-line rTMs). In the first study, a significantly improved accuracy in action naming, but not in object naming, was found following high-frequency rTMS of either left or right DLPFC in each of the 15 examined patients [26]. In the second study [27], the effect of rTMS applied to the DLPFC on picture naming was assessed in 24 AD patients with different degrees of cognitive decline. The authors found that the results of the previous study were replicated only in mild AD patients (Mini-Mental-State-Examination (MMSE) ≥17/30); in contrast, in patients with moderate to severe AD (MMSE < 17/30), both action and object naming were facilitated after both left and right DLPFC rTMS. The lack of effects of rTMS on object naming in early-stage AD might be related to a "ceiling" effect; when object naming is impaired, such as in moderate to severe AD patients, rTMS to the DLPFC results in an improved performance also for this class of stimuli. The rTMS effect was bilateral both in mild and severe AD patients. The bilateral facilitation effect could be attributed to the presence of compensatory mechanisms based on the recruitment of right hemispheric resources to support the residual naming performance.

In a later study, Cotelli et al. [28] aimed to investigate whether the application of high-frequency rTMS to the left DLPFC may lead to significant facilitation of language production and/or comprehension in patients with moderate AD. Ten patients were randomly assigned to one of two groups. The first group underwent a 4-week real rTMS stimulation protocol, while the second underwent a 2-week placebo treatment, followed by 2-weeks of real rTMS stimulation. rTMS intervention consisted of a total of 4 weeks of daily stimulation. No significant effects were observed on naming performance. However, a significant effect was observed on auditory sentence comprehension after 2 weeks of real rTMS sessions, as compared to sham. Two additional weeks of daily rTMS sessions resulted in no further improvements, but a significant benefit on auditory sentence comprehension was still detected 8 weeks after the end of the rTMS intervention. An important finding was the absence of any effects on memory and executive functions. Therefore, these results were thought to be specific to the language network, and not due to a general, nonspecific effect on cognitive processing.

None of these three studies reports any side effects of the rTMS applications, but it is not clear what safety evaluations (if any) were completed.

In another study, Ahmed et al. [29] aimed to compare the long-term efficacy of high- versus low-frequency rTMS, applied bilaterally over the DLPFC, on cortical excitability and cognitive function of AD patients. The high-frequency rTMS group improved significantly more than the low-frequency and sham groups in all assessed rating scales (MMSE, Instrumental Daily Living Activity Scale and the Geriatric Depression Scale) at all time points after treatment. The improvement was maintained for 3 months. The authors thus concluded that high-frequency rTMS may be a useful addition to therapy for the treatment of patients with mild to moderate degree of AD.

Since cognitive training (COG) may improve cognitive functions in AD, in a recent study Bentwich et al. [30] aimed to obtain a synergistic effect of rTMS interlaced with COG (rTMS-COG) in patients with AD. Eight patients with mild to moderate probable AD were subjected to daily rTMS-COG sessions (5/week) for 6 weeks, followed by maintenance sessions (2/week) for additional 6 months. The following six regions, located individually by MRI, were stimulated: Broca and Wernicke (language functions), right and left DLPFC (judgment, executive functions, and long-tem memory), and right and left parietal somatosensory association cortex (spatial and topographical orientation and praxias). COG tasks were developed to fit these regions. Primary outcome measures were average improvement of Alzheimer Disease Assessment Scale-Cognitive (ADAS-cog) and Clinical Global Impression of Change (CGIC); secondary objectives were average improvement of MMSE, the ADAS-Activities of Daily Living (ADAS-ADL), Hamilton Depression Scale (HAMILTON), and Neuropsychiatric Inventory (NPI). ADAS-cog improved by approximately 4 points after both 6 weeks and 4.5 months of treatment, and CGIC by 1.0 and 1.6 points, respectively. MMSE, ADAS-ADL, and HAMILTON improved, but without statistical significance, while NPI did

not change. These findings provide direct evidence that rTMS is helpful in restoring brain functions and could reflect rTMS potential to recruit compensatory networks that underlie the memory-encoding and the other cognitive functions [31]. Therefore, rTMS-COG seems a promising and safe modality for AD treatment.

2.3. Transcranial Direct Current Stimulation. Two crossover designed studies using tDCS were performed to enhance recognition memory in patients with AD [32, 33]. Because temporoparietal areas are thought to be hypoactive in AD [34], Ferrucci et al. [32] tested in a pilot study whether anodal tDCS applied over temporoparietal cortex can increase cortical function, thus improving recognition memory in AD patients. They were delivered in 10 patients with mild AD anodal tDCS, cathodal tDCS, and sham tDCS over bilateral temporoparietal areas in three separate sessions (15 min at 1.5 mA, at least 1 week apart). Anodal tDCS significantly improved recognition memory, cathodal tDCS significantly decreased accuracy in the word recognition task, sham tDCS did not change it. Moreover, no effects were observed in a visual attention task, suggesting that the effects of tDCS were likely specific for recognition memory. Notably, no safety considerations were reported. Boggio and colleagues exposed patients with mild to moderately severe AD to a session of anodal tDCS to the left DLPFC, anodal tDCS to the left temporal cortex (cathode electrode was placed over the right supraorbital area for these 2 sessions), or a session of sham stimulation [33]. Since declarative memory is the most affected cognitive domain in AD patients, the aim of this study was to investigate the impact of anodal tDCS on recognition memory, working memory, and selective attention in AD. Sessions were 48 h apart, and patients were tested during each of the stimulation sessions, starting 10 min after stimulation onset and lasting until the end (30 min at 2 mA). Stimulation over both prefrontal and temporal areas resulted in a significant improvement of visual recognition memory, which was not attributable to a nonspecific attentional process, as assessed by the Stroop task. On contrary, no effects were obtained on working memory.

This important study has several limitations. As the authors themselves recognized, since a bipolar montage was used, it cannot be excluded that the observed effects may be the result of the stimulation from the reference electrode. Moreover, the study did not measure whether the effects of the study were long lasting, and the authors did not perform any other behavioural assessment to measure whether the effects observed in the study are clinically relevant. It should be also considered that working memory was measured by a digit span task, more indicative of attentional than working memory function.

Interestingly, Scelzo and colleagues [35] found an increased short latency afferent inhibition (SAI) after anodal tDCS in 12 healthy subjects. SAI is a TMS protocol that may give direct information about the function of some cholinergic pathways in the human motor cortex [36]. The enhancement of cortical cholinergic circuits may thus represent an important mechanism explaining anodal tDCS action in AD and several other pathologic conditions.

3. Discussion and Future Perspectives

The possible mechanisms that can account for the effects of rTMS and tDCS on cognitive performance conceivably reflect the potential of these methods to improve the subject's ability to relearn or to acquire new strategies for carrying out behavioural tasks.

The use of rTMS involves the discharge of a transient electromagnetic field through the skull. Electric currents are induced in the brain by means of rapidly changing magnetic fields; in turn, these determine transsynaptic depolarizations of pools of neurons located in the superficial cortical layers. The capability of the rTMS to interact with the intrinsic abilities of the brain to restore or compensate for damaged function is a promise for possible applications in the field of cognitive rehabilitation. Since there is no clear knowledge about spatial resolution of rTMS, it is difficult to interpret the observed functional effects in terms of exact anatomical effects. The neurophysiological mechanisms responsible for rTMS-induced facilitation remain unknown, even if they are most likely related to the activation of impeded pathways or inhibition of maladaptive responses. On the other hand, rhythmic transcranial stimulation can exert positive effects on cognitive performance [37]. The modification of cortical activity through the use of rhythmic stimulation may readjust pathological patterns of brain activity, thus providing an opportunity to induce new, healthier activity patterns within the affected functional networks [38].

While rTMS elicits neuronal firing, tDCS modulates the spontaneous neuronal activity [39]. Though rTMS and tDCS both yield similar effects, tDCS has several practical advantages over rTMS. Therefore, it is simpler, safer [40], and less expensive and would be suitable for use in large series of patients, possibly even at home. In fact, the above illustrated findings prompt further studies using repeated tDCS, in conjunction with other therapeutic interventions for treating patients with AD. The prolonged aftereffects of tDCS are probably due to synaptic [41] and nonsynaptic mechanisms [42].

The effects of tDCS might be related to a facilitation of the corresponding brain area induced by the anodal electrode; this neuronal network may become, consequently, more reactive during the encoding phase of the task. The DCS is thus responsible for priming the area to receive additional behavioural intervention.

These preliminary studies highlight the therapeutic potential of the induction of long-term neuromodulatory effects using brain stimulation. They hold considerable promise, not only for advancing our understanding of brain plasticity mechanisms, but also for designing new rehabilitation strategies in patients with neurodegenerative disease.

Therefore, rTMS and tDCS might become useful in the rehabilitation of AD patients. However, although promising, results of noninvasive stimulation to enhance cognitive function in AD to date have to be considered extremely preliminary. Most applications have been of short duration; the effects seem to be short lived and were not replicated after longer-duration interventions. Conversely, some of the effects obtained after longer-lasting interventions were not

detected after single stimulation session [26–28]. Moreover, both techniques appear safe in patients with AD, even if long-term risks have been insufficiently considered.

On the other hand, the specific intervention that helps in short-term studies may not help in long-term studies. For all future studies a careful experimental design is needed and patient selection aspects, stimulation parameters, and clinical, cognitive, and behavioural assessment tools should be considered. Of great importance is a careful choice of outcome measures, also to enable comparison across studies. It would also be valuable examining therapeutic efficacy of the neuromodulatory techniques in AD to employ the neuropsychological battery of the Uniform Data Set (UDS) or outcome scales commonly used in trials of pharmacological agents for AD, such as the Cognitive subscale of the Alzheimer's Disease Assessment Scale (ADAS-Cog) [43]. Anyway, it is possible that the effects of the plasticity-based interventions with high-frequency or anodal tDCS in the brain of AD patients may differ to those in normal subjects, and studies to physiologically characterize this would be important to guide future therapeutic trials.

It should be considered that most previous studies have failed to be evidence based, and the assumption that cortical plasticity enhancement is needed for the betterment of the cognitive status of AD patients remains conjectural [43].

While TMS studies showed in AD cortical hyperexcitability, the therapeutic attempts are based on techniques aimed at increasing cortical excitability (anodal tDCS, high-frequency rTMS). This hyperexcitability may be the consequence of other underlying pathophysiologic mechanisms, such as decreased synaptic efficiency or hypoplasticity. Therefore, the cortical physiology seems to require more solid investigation and should be appropriately tested before and after therapeutic interventions. On the other hand, the assumption that high-frequency rTMS will enhance cortical excitability in AD patients may be wrong. Indeed, rTMS effects dependent on the state of activity of the brain at the time of stimulation also remain debatable [44]. In addition, the assumption that in AD the mechanisms of plasticity might be abnormally reduced in the brain areas targeted in the previous studies, the DLPFC or the temporoparietal regions, has not been completely demonstrated.

Finally, improving performance in one task actually may not necessarily represent cognitive enhancement. More comprehensive outcome measures are needed to assess the clinical significance of rTMS or tDCS in AD and appropriately powered studies with sound blinding procedures are necessary [43]. It seems unlikely that stimulations over a single brain area will contribute to a significant improvement of the cognitive status of AD patients, particularly those with more advanced stages of disease. Multiple-target stimulation protocols are necessary in order to overcome the multiple cognitive deficits characterizing moderate or severe AD.

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