New Perspectives for the Treatment of Alzheimer's Disease

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Abstract: The hallmark pathologic lesions of Alzheimer's disease (AD) are extracellular senile plaques, composed by Amiloid β (Aβ) peptide and intraneuronal neurofibrillary tangles, made of tau protein. According to the amyloid hypothesis, the increased production or decreased clearance of Aβ peptide initiates a pathological process leading to neurodegeneration, dementia and death. Under normal circumstances, the Amyloid Precursor Protein (APP) is cleaved by α–secretase, but, in pathological conditions, it is cleaved first by β–secretase (BACE) and subsequently by γ-secretase, to form Aβ42 toxic peptide. Accumulation of Aβ42 starts a cascade of events associated with neuronal and synaptic dysfunction, inflammatory responses, hyperphosphorylation of tau protein and neuronal death. This theory identifies biological targets for disease-modifying treatments, including the modulation of APP metabolism, the reduction of Aβ aggregation or the enhancement of Aβ clearance, and the reduction of inflammation. Regarding secretases, different approaches are under evaluation, primarily aiming to decrease β- and γ-secretase. A promising approach is the modulation of Aβ, with either vaccination or antiaggregation agents. Regarding inflammation, despite trials with Rofecoxib, Naproxen or Diclofenac failed to slow progression of cognitive decline in patients with AD, Indomethacin showed positive results in delaying cognitive decline. However, gastrointestinal toxicity was treatment-limiting, therefore other compounds have been developed, including Nitroflurbiprofen. In addition, Rosiglitazone, an oral anti-diabetic agent with anti-inflammatory properties, is currently in phase III study.

A further approach aims to inhibit tau deposition with methyl thioninium chloride.

In this review, the pathogenic steps leading to neurodegeneration will be discussed, together with an update of disease-modifying drugs under testing.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the elderly, with a prevalence of 5% after 65 years of age, increasing to about 30% in people aged 85 years or older. It is characterized clinically by a progressive cognitive impairment, including impaired judgement, decision-making and orientation, often accompanied, in later stages, by psychobehavioural disturbances as well as language impairment. Mutations in genes encoding for Amyloid Precursors Protein or presenilins 1 and presenilin 2 genes (*APP*, *PSEN1* and *PSEN2*, respectively) account for about 5% of cases, characterized by an early onset (before 65 years). So far, 30 different mutations, causing amino acid changes in putative sites for the cleavage of the protein, have been described in the *APP* gene in 83 families, together with 171 mutations in *PSEN1* in 383 families and 12 mutations in *PSEN2* in 20 families (http://molgen-www.uia.ac.be).

The two major neuropathologic hallmarks of AD are extracellular Amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs). The production of Aβ, which represents a crucial step in AD pathogenesis, is the result of cleavage of APP, that is overexpressed in AD [1]. Aβ forms highly insoluble and proteolysis resistant fibrils known as senile plaques (SP). In contrast to the low-fibrillar Aβ plaques (diffuse plaques), highly fibrillar (amyloidogenic) forms of Aβ plaques are associated with glial and neuritic changes of the surrounding tissue (neuritic-plaques) [2]. NFTs are composed of the tau protein. In healthy controls, tau is a component of microtubules, which represent the internal support structures for the transport of nutrients, vesicles, mitochondria and chromosomes within the cell. Microtubules also stabilize growing axons, which are necessary for the development and growth of neurites [1]. In AD, tau protein is abnormally hyperphosphorylated and forms insoluble fibrils, which originate deposits within the cell.

THE AMYLOID HYPOTHESIS

The APP plays a central role in AD pathogenesis and in AD research, as it is the precursor of Aβ, which is the heart of the amyloid cascade hypothesis of AD.

APP Gene Family. The human *APP* gene was first identified in 1987 by several laboratories independently. The two *APP* homologous, *APLP1* and *APLP2*, were discovered several years later. APP is a type I membrane protein. Two predicted cleavages, one in the extracellular domain (β-secretase cleavage) and another in the transmembrane region (γ-secretase cleavage) are necessary to release Aβ from the precursor protein. Notably, *APP* is located on chromosome 21, and this provided an immediate connection to the invariant development of AD pathology in trisomy 21 (Down's syndrome) individuals. The first mutations demonstrated to be causative of inherited forms of familial AD were identi-

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fied in the *APP* gene [3], providing an evidence that APP plays a central role in AD pathogenesis. Importantly, only *APP* but not its homologous *APLP1* and *APLP2* contain sequences encoding the Aβ domain.

APP Processing. Full-length APP undergoes sequential proteolytic processing (Fig. **1**). It is first cleaved by α-secretase (non-amyloidogenic pathway) or β-secretase (amyloidogenic pathway) within the luminal domain, resulting in the shedding of nearly the entire ectodomain and generation of α- or β-C-terminal fragments (CTFs). The major neuronal β-secretase, named BACE (β-site APP cleaving enzyme), is a transmembrane aspartyl protease which cleaves APP within the ectodomain, generating the N-terminus of Aβ [4]. Nevertheless, several zinc metalloproteinases such as TACE/ADAM17, ADAM9, ADAM10 and MDC-9, and the aspartyl protease BACE2, can cleave APP at the α-secretase site [5] located within the Aβ domain, thus precluding the generation of intact Aβ.

The second proteolytic event in APP processing involves intramembranous cleavage of α- and β-CTFs by γ-secretase, which liberates a 3kDa protein (p3) and Aβ peptide into the extracellular milieu. The minimal components of γ-secretase include presenilin (PS)1 or PS2, nicastrin, APH-1 and PEN-2 [6]. Protein subunits of the γ-secretase assemble early during biogenesis and cooperatively mature as they leave the endoplasmic reticulum. Biochemical evidence is consistent with PS1 (or PS2) as the catalytic subunit of the γ-secretase. APH-1 and PEN-2 are thought to stabilize the γ-secretase complex, and nicastrin to mediate the recruitment of APP CTFs to the catalytic site of the γ-secretase. The major sites of γ-secretase cleavage correspond to positions 40 and 42 of Aβ.

Amyloidogenic processing is the favoured pathway of APP metabolism in neurons, due to the greater abundance of BACE1, whereas non-amyloidogenic pathway is predominant in other cell types.

It appears that none of the above mentioned secretase has unique substrate specificity towards APP. Besides APP, a number of other transmembrane proteins undergo ectodomain shedding by enzymes with α-secretase activity. Regarding BACE1, its low affinity for APP lead to the hypothesis that APP is not its sole physiological substrate. Similarly, PS1 and PS2 play a crucial role in intramembranous γ-secretase cleavage of several type I membrane proteins other than APP, including Notch1 receptors and its ligands [7].

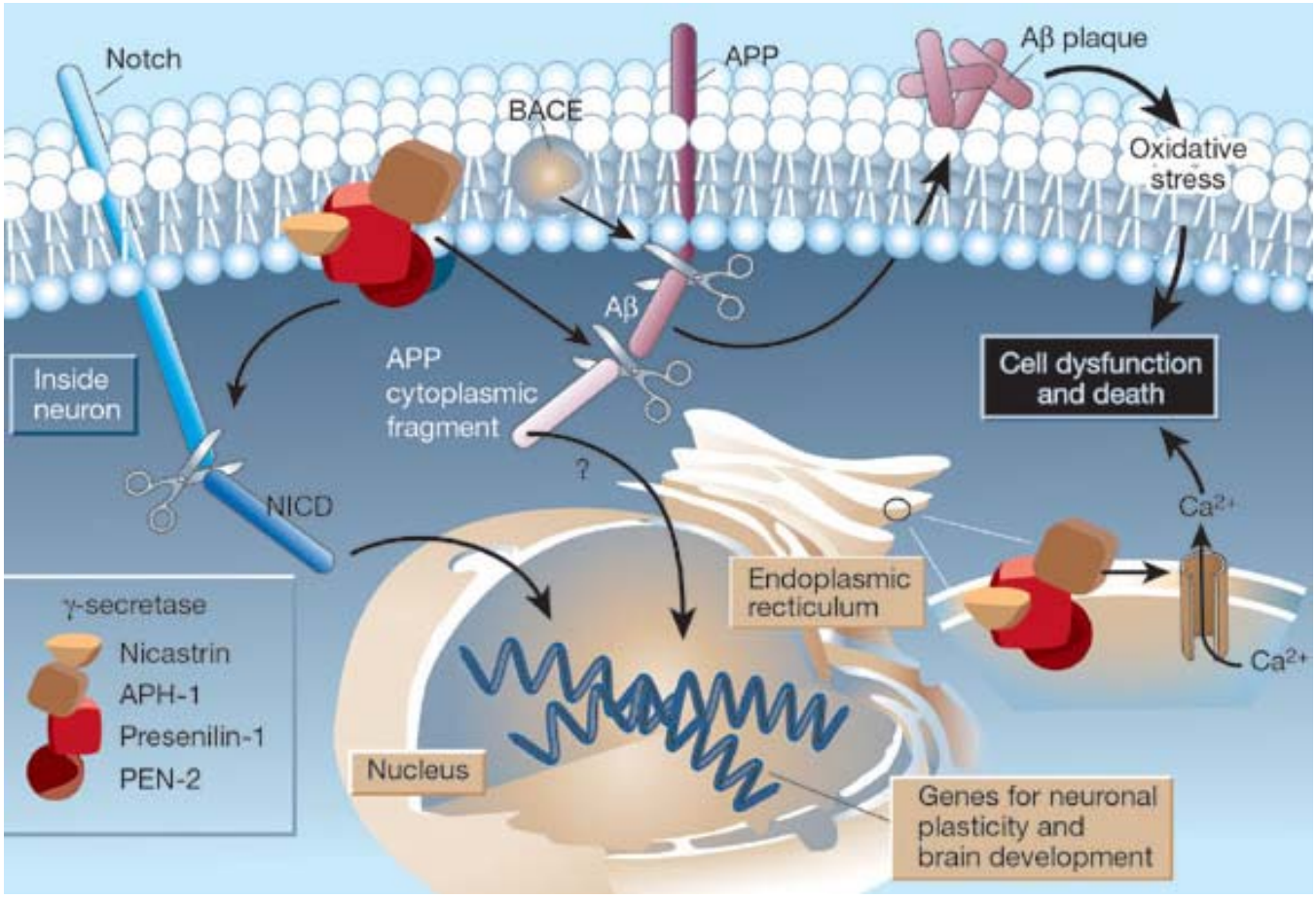

Fig. (1). Amyloid processing. Presenilin-1, nicastrin, APH-1 and PEN-2 form a functional γ-secretase complex, located in the plasma membrane and endoplasmic reticulum of neurons. The complex cleaves Notch (left) to generate a fragment (NICD) that moves to the nucleus and regulates the expression of genes involved in brain development and adult neuronal plasticity. The complex also helps in generating the Aβ (centre). This involves an initial cleavage of the APP by β-secretase. The γ–secretase then liberates Aβ, together with an APP cytoplasmic fragment.

APP Role. A number of functional domains have been mapped to the extra- and intracellular region of APP, including metal (copper and zinc) binding, extracellular matrix components (heparin, collagen and laminin), neurotrophic and adhesion domains. Thus far, a thropic role for APP has been suggested, as it stimulates neurite outgrowth in a variety of experimental settings. The N-terminal heparin-binding domain of APP also stimulates neurite outgrowth and promotes synaptogenesis. In addition, an "RHDS" motif near the extralumenal portion of APP likely promotes cell adhesion, possibly acting in an integrin-like manner. Similarly, APP colocalizes with integrins on the surface of axons at sites of adhesion [8, 9].

Despite APP was initially proposed to act as a cell surface receptor, the evidence supporting this hypothesis has been unconvincing. Only recently, aside of from interactions with extracellular matrix proteins, a candidate ligand has been proposed. In was in fact reported that F-spondin, a neuronally secreted signalling glycoprotein that may function in neuronal development and repair, binds to the extracellular domain of APP as well as of APLP1 and APLP2 [10]. This binding reduces β-secretase cleavage of APP, suggesting therefore that F-spondin binding may regulate APP processing.

APP-deficient animals are a useful model to better understand the role of APP. Deficient APP mice did not show major phenotypic abnormalities [11]. However, *APLP2*$^{-/-}$*/APLP1*$^{-/-}$ and *APP*$^{-/-}$*/APLP2*$^{-/-}$ mutants, but not *APP*$^{-/-}$*/APLP1*$^{-/-}$ animals, showed early postnatal lethality, indicating that members of the APP gene family are essential genes, which exhibit partial overlapping functions. Deficiency of all the *APP* genes lead to death shortly after birth. The majority of animals studied showed cortical dysplasia suggestive of migrational abnormalities of the neuroblasts and partial loss of cortical Cajal Retzius cells [12]. Taken together, these findings presented a convincing picture that members of the *APP* family play essential roles in the development of the nervous system related to synapse structure and function as well as in neuronal migration.

Given the trophyc properties of APP, it would be natural to predict that overexpression of APP would lead to phenotypes related to the enhanced neurite outgrowth and cell growth, which indeed was demonstrated [13]. However, convincing negative phenotypes have been reported as well. Overexpression of APP in cells induced to differentiate into neurons lead to cell death [14]. Genetic in-vivo engineering to overexpress APP carrying various familial AD mutations in transgenic mice resulted in the development of Aβ deposition and Aβ associated changes in the brain, including loss of synaptic markers, thus confirming the pathogenic nature of these mutations. A detailed examination also showed axonal swellings and varicosities, which were observed months before any evidence of Aβ deposition [15].

In this model, tau deposition occurs as a consequence of a disregulation of its phosphorylation induced by Aβ deposition. Nevertheless, according to other studies in transgenic mice, tau deposition represent the first pathogenic step of AD. Therefore, basing on this hypothesis, new drugs aimed to block tau deposition are under development.

Disease-modifying drugs available to date are listed in Table **1**.

DRUGS INFLUENCING Aβ DEPOSITION

Anti-Amyloid Aggregation Agents. A number of anti-Aβ aggregation agents are currently in clinical testing. Despite their biological mechanisms of action are not completely understood, they are believed to prevent fibril formation and to facilitate soluble Aβ clearance. The most studied is named tramiprosate (Alzhemed^{TM}, Neurochem, Inc.), a glycosaminoglycan (GAG) mimetic designed to cross the brain blood barrier. GAGs binds to soluble Aβ, promoting fibril formation and deposition of amyloid plaques. GAG mimetics compete for GAG-binding sites, thus blocking fibril formation and reducing soluble Aβ [16, 17]. In transgenic mice, tramiprosate reduces plaque burden and decreases CSF Aβ levels, but cognitive and behavioural outcomes in this animal model have not been reported [18]. A phase I study in healthy adults demonstrated that the drug is well tolerated. A 3-month phase II study was subsequently conducted in 58 patients with mild to moderate AD, who were randomized to tramiprosate 50 mg, 100 mg or 150 mg twice a day or placebo. Patients who completed the study were eligible for a 21-month open-label extension with 150 mg twice daily. Baseline CSF Aβ levels declined by up to 70% after 3 months for patients randomly assigned to the 100-mg or 150-mg twice-daily group. However, no differences were observed in cognitive functions between the tramiprosate and placebo groups. A phase III study was then carried out in the US in 1052 patients with AD to test tolerability, efficacy and safety of the drug, but according to the Food and Drug Administration (FDA), results of this study are inconclusive (www.alzforum.org). The FDA ruled out that the statistical models used to analyze both the cognitive efficacy as well as the brain volume data were problematic. Therefore, results obtained could not support a claim for clinical efficacy. Multiple factors likely contributed to the failure of the study. Overall, variability among the 67 clinical sites in the trial overwhelmed the observed treatment effects. In particular, changes in people's concomitant treatment with cognitive-enhancing drugs including cholinesterase inhibitors, memantine and antidepressants affected the results for the primary cognitive endpoints based on neuropsychological testing. Unexpected problems also arose in the control group, confounding the interpretation of Alzhemed efficacy. Thirty percent of the control group did not decline in cognition over the 18-month trial period, whereas a portion of this group unexpectedly showed a significant improvement in cognition. Another similar trial conducted in Europe has been discontinued. In addition, recent data suggest that tramiprosate promotes an abnormal aggregation of the tau protein in neuronal cells [19], emphasizing the importance of testing on both types of pathology (amyloid and tau) the potential drugs to be used for the treatment of AD.

Another molecule under testing is named colostrinin. It is a proline-rich polypeptide complex derived from sheep colostrum (O-CLN; ReGen Therapeutics), which inhibites Aβ aggregation and neurotoxicity in cellular assays [20] and improves cognitive performance in animal models [21]. A 3-week phase I study in patients with AD demonstrated it is well tolerated [21]. A subsequent phase II trial demonstrated modest improvements in MMSE scores for patients with

Table 1. Disease-Modifying Drugs for AD

Drugs influencing Aβ deposition	Name	Ref.	Proposed Modes of Actions	Type of Test	Outcome of Test (Definitive or Not)
Anti-aggregants	Tramiprosate (Alzhemed™)	[16-18]	GAG mimetic	Phase III	No efficacy (definitive)
	Colostrinin	[20-23]	Inhibits Aβ aggregation	Phase II	Modest improvement in MMSE (not definitive
	Clioquinol	[24]	Inhibits zinc and copper from binding to Aβ	Phase II	Trial halted due to toxic impurities in the formulation (definitive)
	PBT2	[25]	Metal-protein attenuation	Phase II	Improvement in cognition (not definitive)
Vaccination	AN1792	[26-29]	Aβ removal (active immunisation)	Phase II	Unclear cognitive results-severe adverse events (definitive)
	Bapineuzumab		Aβ removal (passive immunisation)	Phase III	Ongoing (not definitive)
γ-secretase inhibitors	LY450139	[33-35]	Inhibits γ-secretase	Phase II	No changes in cognitive measures (not definitive
SALAs	Tarenflurbil (Flurizan™)	[37-42]	Inhibits γ-secretase	Phase III	No effect on cognition (definitive)
Drugs influencing tau deposition	MTC (Rember™)		Interferes with tau aggregation	Phase II	Improvement in cognition (not definitive)
Anti-inflammatory drugs	Diclofenac	[70]	NSAID	Phase II	No efficacy (definitive)
	Naproxene	[71]	Non-selective NSAID	Phase III	No efficacy (definitive)
	Rofecoxib	[71,72]	Inhibits COX2	Phase III	No efficacy (definitive)
	Indomethacin	[73,74]	NSAID	Phase II	No efficacy-toxicity (definitive)
	Nitroflurbiprofen	[75,76]	Nitric oxide-donating derivative of Flurbiprofen	Phase II	Ongoing (not definitive)
	Rosiglitazione (Avandia™)	[77]	Anti-diabetic and anti-inflammatory	Phase III	Ongoing (not definitive)
	Etanercept	[78]	Inhibits TNF-α	Open study	Improvement in cognition (not definitive)

mild AD over a treatment period of 12 to 16 months, but this beneficial effect was not sustained during 18 to 28 months of continued treatment [22, 23].

Another potential disease-modifying drug for AD is named clioquinol (PBT-1; Prana Biotechnology). It inhibits zinc and copper ions from binding to Aβ, thus promoting the solubilization and clearance of Aβ. Early clinical studies showed a reduction in the rate of cognitive decline [24], but clinical trials were halted due to toxic impurities inherent in the formulation.

A novel metal-protein attenuating compound named PBT2 has been recently tested in a phase II trial. Seventy-eight patients with mild AD were randomly assigned to PBT2 50 mg, PBT2 250 mg or placebo (in addition to acetylcholinesterase inhibitors) for 12 weeks. No serious adverse events were reported by patients on PBT2. Patients treated with PBT2 250 mg had a dose-dependent and significant reduction in CSF Aβ42 concentration compared with those treated with placebo [25]. Cognitive efficacy was however restricted to two measures only, therefore future larger and longer trials are needed to test the efficacy of this drug on cognition.

Amyloid Removal: Vaccination. In 1999 Schenk *et al.* [26] demonstrated that immunization with Aβ as an antigen attenuated AD-like pathology in transgenic mice overexpressing the *APP* gene by removing amyloid from the central nervous system. This transgenic mouse model of AD progressively develop several neuropathological features of the disease in an age-related and brain-region-dependent manner. Immunization of young animals with Aβ prevents the development of plaque formation, neuritic dystrophy and astroglyosis, whereas in older animals, vaccination reduces extent and progression of AD-like pathologies. Given these preclinical results, a multicenter, randomized, placebo-controlled, phase II double-blind clinical trial using active immunization with Aβ42 plus adjuvant was started in 2001 on 300 patients using the pre-aggregated Aβ peptide AN1792. However, following reports of aseptic meningoencephalitis in 6% of treated patients, the trial was halted after 2-3 injections. Of the 300 patients treated, 60% developed antibody response. The final results of the trial were published in 2005 [27].

Double-blind assessment were maintained for 12 months, demonstrating no significant differences in cognition between antibody responders and placebo group for ADAS-Cog, Disability Assessment for Dementia (DAS), Clinical Dementia Rating, Mini Mental State Examination and Clinical Global Impression of Change. In a small subset of pa-

tients, CSF tau levels were decreased in antibody responders but Aβ levels were unchanged.

A quite disappointing observation was the finding of greater brain volume decrease and great ventricular enlargement at MRI in responders than in placebo patients [28]. However, this brain atrophy was not associated with worsening of cognitive performances. A possible explanation is that the brain volume changes observed may result from an association between amyloid removal and intracerebral fluid shifts.

Long-term follow-up of treated patients and further analysis of autopsy data modified and moderated the negative impact of the first results, encouraging additional clinical attempts. Subsequent observations on AN1792 vaccinated patients or transgenic models and on brain tissue derived from mice and humans using a new tissue amyloid immunoreactivity (TAPIR) method suggested that antibodies against Aβ-related epitopes are capable of slowing the progression of neuropathology in AD. Hock and Nitsch [29] followed for four years 30 patients who received a prime and booster immunization over the first year after vaccination, providing further support to continue investigation of antibody treatment in AD.

Therefore, despite the severe adverse events occurred in the first AN1792 trial and the unclear cognitive results, immunization was not abandoned, but the treatment was modified from active into passive in order to avoid excessive activation of the T-cell response and thus prevent complications. The humanized monoclonal anti-Aβ antibody Bapineuzumab (Wyeth and Elan) has been tested in a phase II trial in 200 patients with mild to moderate AD. The 18-month, multidose, one-to-one randomization trial was conducted at about 30 sites in the US. It was designed to assess safety, tolerability and standard efficacy endpoints (ADAS-Cog, Neuropsychological Test Battery, DAS) of multiple ascending doses of Bapineuzumab in patients. The 18-month trial includes an interim analysis, as well as data collection on clinical endpoints and biomarkers. On May 21, 2007, Elan and Wyeth announced their plans to start a phase III clinical trial of Bapineuzumab. The decision to launch phase III studies prior to the conclusion of the ongoing phase II was based on the totality of the accumulated clinical data from phase I, phase II and a 4.5-year follow-up study of those patients involved in the original AN1792 trial.

In 2008 a paper was published describing the relation between $A\beta_{42}$ immune response, degree of plaque removal and long-term clinical outcomes [30]. In June 2003, 80 patients (or their caregivers), who had entered the phase I AN1792 trial in 2000, gave their consent for long-term clinical follow-up and post-mortem neuropathological examination. In patients who received immunisation, mean Aβ load was lower than in the placebo group. Nevertheless, there was no evidence of improved survival or an improvement in time to severe dementia in such patients. Therefore, plaque removal in not enough to halt progressive neurodegeneration in AD, prompting some intriguing challenges to the amyloid hypothesis.

γ-Secretase Inhibition. Several compounds which inhibit γ-secretase activity in the brain have been identified. Nevertheless, γ-secretase has many biologically essential substrates [31]. One of the most physiologically important γ-secretase substrate in the Notch signaling protein, which is involved in the differentiation and proliferation of embryonic cells, T cells, gastrointestinal goblet cells and splenic B cells. Experience with transgenic mice showed that the administration of a γ-secretase inhibitor in doses sufficient to remove Aβ concentrations interferes with lymphocyte differentiation and alters the structure of intestinal goblet cells [32]. Therefore, safety is a very important consideration for this kind of compounds.

A nonselective γ-secretase inhibitor named LY450139 (Eli Lilly) has been evaluated in a phase I placebo-controlled study in 37 healthy adults (at doses ranging from 5 to 50 mg). Aβ CSF levels were reduced in both active treatment and placebo groups, but differences were not statistically significant. Transient gastrointestinal adverse effects (bleeding, abdominal pain) were reported by 2 patients treated with 50 mg [33]. A subsequent phase II randomized, controlled trial was carried out in 70 patients with AD. Patients were given 30 mg for 1 week followed by 40 mg for 5 weeks. Treatment was well tolerated. No significant changes in plasma and CSF Aβ40 and Aβ42 were observed [34].

Subsequently, a multicenter, randomized, double-blind, dose-escalation, placebo-controlled trial was carried out. Fifty-one patients with mild to moderate AD were randomized to receive placebo, or LY450139 (100 mg or 140 mg). The LY450139 groups received 60 mg/day for two weeks, then 100 mg/day for 6 weeks, then either 100 or 140 mg/day for six additional weeks. Primary outcomes included safety, tolerability and CSF/plasma Aβ levels; secondary outcome was neuropsychological testing. LY450139 was generally well tolerated at doses of up to 140 mg/day for 14 weeks. However, adverse events were seen, including 3 possible drug rashes, 3 reports of hair color change and 3 adverse event-related discontinuation, therefore a close clinical monitoring will be needed in future studies. Plasma Aβ, but not CSF, levels were reduced in treated patients, consistent with inhibition of γ-secretase. No differences were seen in cognitive or functional measures [35].

Selective Aβ42-Lowering Agents (SALAs). Tarenflurbil is the first compound in this new class of drugs, which modulate γ-secretase activity without interfering with Notch or other γ-secretase substrates [36]. It binds to a γ-secretase site other than the active/catalytic center of relevance to production of Aβ42, thereby altering the conformation of γ-secretase and shifting production away from Aβ42 without interfering with other physiologically essential γ-secretase substrates.

Tarenflurbil (MPC-7869; Myriad Pharmaceuticals; Flurizan™) is the pure R-enantiomer of flurbiprofen. It shifts cleavage of APP away from Aβ42, leading to the production of shorter non-toxic fragments [37, 38]. In contrast with S-flurbiprofen or other non-steroidal anti-inflammatory drugs (NSAIDs), it does not inhibit cyclo-oxygenase (COX) I or COX 2 and it is not associated with gastrointestinal toxicity [39]. In mice, treatment with tarenflurbil reduces amyloid plaque burden and prevents learning and behavioural deterioration [40].

A 3-week, placebo-controlled, phase I pharmacokinetic study of tarenflurbil (twice-daily doses of 400, 800 or 1600

mg) in 48 healthy, older volunteers, showed that the drug is well tolerated, with no evidence of renal or gastrointestinal toxicity. CSF was collected at baseline and after 3 weeks. The compound penetrated the blood brain barrier in a dose-dependent manner. No significant changes of Aβ42 CSF levels were shown after treatment. However, in plasma, higher drug concentrations were related to statistically significant lower Aβ levels [41].

Myriad conducted a large, placebo-controlled Phase II trial for Flurizan of 12 month-duration in 210 patients with mild to moderate AD (MMSE score: 15-26). Patients were randomly assigned to receive Tarenflurbil twice per day (400 mg or 800 mg or placebo) for 12 months. Primary outcome measures for the trial were the rate of change (slope of decline) of: activities of daily living, quantified by the Alzheimer's Disease Cooperative Study—Activities of Daily Living inventory (ADCS-ADL), global function, measured by the Clinical Dementia Rating-sum of boxes (CDR-sb), cognitive function, measured by the Alzheimer's disease Assessment Scale-cognitive subscale (ADAS-cog). In a 12-month extended treatment phase, patients who had received tarenflurbil continued to receive the same dose, and patients who had received placebo were randomly assigned to tarenflurbil at 800 mg or 400 mg twice a day.

A preliminary analysis revealed that patients with mild AD (MMSE: 20-26) and moderate AD (MMSE: 15-19) responded differently to tarenflurbil in the ADAS-Cog and the ADCS-ADL, therefore these groups were analyzed separately. Patients with mild AD in the 800 mg tarenflurbil group had lower rates of decline than did those in the placebo group in the activities of daily living, whereas slowing of cognitive decline did not differ significantly. In patients with moderate AD, 800 mg tarenflurbil twice per day had no significant effects on ADCS-ADL and ADAS-Cog and had a negative effect on CDR-sb. The most common adverse events included diarrhoea, nausea and dizziness. Patients with mild AD who were in the 800 mg tarenflurbil group for 24 months had lower rates of decline for all three primary outcomes than did patients who were in the placebo group for months 0-12 and a tarenflurbil group for months 12-24 [42].

Given these results, two phase III study were carried out, in US and in Europe. ActEarliAD trial was started in 2007 all over Europe. It is a 18-month, multinational, randomized, double-blind, placebo controlled study in over 800 patients with AD. Patients enrolled in the trial took 800 mg twice a day of either Flurizan or placebo and attended periodic physician visits for analysis of their performance on memory, cognition and behavioral tests. The two primary clinical endpoints of the trial were the change in cognitive decline and function, as measured by the ADAS-cog, and changes in activity of daily living, as measured by the ADCS-ADL. A secondary endpoint of the trial was the change in overall function, measured by the CDR-sb. Additional exploratory outcome measures were designed to assess the psychological, physical and financial impact of this disease on caregivers and medical resources. The trial was designed to meet the requirements of the European Agency for the Evaluation of Medicinal Products (EMEA) for marketing of Flurizan in Europe. The global endpoints in this trial were identical to those in the US trial. As was the case with the phase II trial, all patients in the phase III studies are allowed to take currently standard of care medicines in addition to Flurizan or placebo, provided their dose has been stable for 6 months.

Disappointingly, on July 2, 2008, the sponsor of Flurizan announced that this γ-secretase-modulating agent had fallen flat in its definitive Phase III trial and was finished as a development product (www.alzforum.org). In fact, on both primary efficacy endpoints, the ADAS-Cog and the ADCS activities of daily living scales, the treatment and placebo curves overlapped almost completely, and there was no effect whatsoever in the group as a whole. In addition, while the overall side effect profile was similar between placebo and treatment groups, anemia, infections and gastrointestinal ulcers appeared more often in people on Flurizan than in the placebo group.

DRUGS INFLUENCING TAU DEPOSITION

A phase II trial of a tau-blocking compound named methyl thioninium chloride (MTC) is ongoing (TauRx Therapeutics, Rember^{TM}). This is a reducing agent better known as methylene blue, a deep blue dye used in analytical chemistry as a tissue stain in biology and in various industrial products. MTC interfere with tau aggregation by acting on self-aggregating truncated tau fragments. The company conducted a phase II trial randomizing 321 patients with mild or moderate AD to treatment with either placebo or one of three oral doses of MTC: 30 mg, 60 mg or 100 mg three times a day. Patients were not taking acetylcholinesterase inhibitors or memantine. Primary outcomes were to compare the effect of MTC to placebo on cognitive abilities measured by the ADAS-Cog at 24 weeks. Preliminary results were presented at the International Conference on Alzheimer's disease (Chicago, July 26-31, 2008). No differences among groups were observed for patients with mild AD at six months. Conversely, for patients with moderate AD, a roughly 5.5-point decline on ADAS-Cog was reported for placebo patients *vs* a 1.5-point decline in the treated groups, resulting in an approximately four-point treatment effect. Notably, if this holds up, it would be larger than currently approved drugs typically achieve. However, it is not clear why the treatment was not effective in mild AD.

INFLAMMATION IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE

The fibrillar deposition of extracellular Aβ is closely associated with a neuroinflammatory response, which includes a local upregulation of acute-phase proteins, complement fragments, cytokines and other inflammatory mediators [43]. So far, epidemiological studies suggested that an inflammatory process contributes to AD pathology. Perspective case-cohort studies showed that higher serum levels of certain acute-phase proteins are a risk factor for the development of AD [44-46]. Moreover, epidemiological studies indicate that longstanding use of non-steroidal anti-inflammatory drugs can prevent or delay the development of AD [47, 48]. Despite these studies, however, treatment of AD patients with anti-inflammatory drugs failed to demonstrate clinical benefits [49].

Microglial cells are the major producers of inflammatory factors. Recent studies demonstrated that, during the early stages of AD pathogenesis, activated microglia were clus-

tered within classic (dense-cored) plaques in the AD neocortex [50]. These plaques showed strong immunostaining for complement factor C1q and serum amyloid P component (SAP). Plaque-associated factors C1q and SAP may trigger microglia to secrete high levels of proinflammatory cytokines [2].

Cytokines and Alzheimer's Disease. Activated microglial cells colocalize with Aβ, and *in vitro* studies demonstrated that Aβ induces the production of Tumor Necrosis Factor (TNF)α in such cells [51]. This cytokine is a pleiotropic factor acting as an important mediator of inflammatory responses in a variety of tissues. Levels of TNFα in CSF from AD patients are 25-fold higher than in CSF from age-matched controls [52], suggesting a role for inflammation in neurodegeneration. Nevertheless, other findings demonstrated a protective effect of TNFα, as it likely protects neurons against Aβ triggered cytotoxicity [53]. Recently, the tumor necrosis factor type 1 death receptor (TNFR1), which contributes to apoptosis, has been deleted in APP23 transgenic mice, which overexpress βAPP, demonstrating an inhibitory effect on Aβ generation and plaque formation in the brain. Moreover, the deletion of TNFR1 leads to reduced β-secretase levels and activity [54].

Interleukin-1 (IL-1) exerts pleiotropic actions by binding to its receptor IL-1R and has been identified as a mediator in several forms of neurodegeneration. In AD, an increased production of IL-1 has been demonstrated by immunohistochemistry. In particular, it is expressed by microglia localized around amyloid deposits, possibly participating to plaque formation [55].

Interleukin-6 (IL-6) binds to a specific receptor, activating an intracellular kinase-based signal pathway. Conflicting results have been reported with regard to IL-6 levels in serum and CSF of AD patients. However, it has been shown that its mRNA levels are increased in the entorhinal cortex and the superior temporal gyrus of AD patients [55]. Interestingly, previous studies demonstrated a large increase in endogenous IL-6 bioactivity in response to ischemia, as well as a marked neuroprotection produced by exogenous IL-6, thus suggesting that this cytokine is an important inhibitor of neuronal death during cerebral ischemia [56].

Additional cytokines of the IL-6 family are IL-11 and leukaemia inhibitory factor (LIF). All IL-6-type cytokines have overlapping bioactivities and share a common helical framework [57]. Intracellular signalling activated by these cytokines requires either homodimerization of their common signal transducing receptor subunit gp130 or heterodimerization of gp130 with a further β-receptor component, such as LIF (LIFR). Interleukin-11 mean levels were significantly increased in AD and Frontotemporal Lobar Degeneration (FTLD) as compared with controls, whereas CSF LIF levels were not detectable either in patients or controls [58]. In accordance with previous results [59], in AD patients, a significantly positive correlation between MMSE scores and IL-11 CSF concentration was observed [58].

In contrast with the previously described cytokines, Transforming Growth Factor beta (TGF-β) has mainly an anti-inflammatory action. Several data show that its levels are increased in the brain of AD patients, as well as in plasma and CSF. Moreover, TGF-β was also found both in amyloid plaques and tangles [55].

Microglial-produced inflammatory cytokines have neurophatic as well as neuroprotective actions. For instance, whereas excess levels of TNFα might cause neurotoxicity, low-dose TNFα could, alternatively, trigger the neuroprotective and/or anti-apoptotic genes [60]. The role of glial cells is to support and sustain proper neuronal function and microglia are no exception to this general principle. In acutely injured central nervous system (CNS) microglia have a neuroprotective and pro-regenerative role [61]. Therefore, the primary mode of action of microglia seems to be CNS protection. However, upon excessive or sustained activation, microglia could significantly contribute to chronic neuropathologies, leading to neurotoxicity [2].

Chemokines and Alzheimer's Disease. Chemokines are low molecular weight chemotactic cytokines that have been shown to play a crucial role in early inflammatory events. Based on the arrangement of cysteine residues, they are divided into two main groups: CXC or α-chemokines, i.e. Interferon-γ-inducible Protein-10 (IP-10) and Interleukin-8 (IL-8), responsible for attracting neutrophils, and CC or β-chemokines, i.e. Monocyte Chemotactic Protein-1 (MCP-1), and Macrophage Inflammatory Protein-1α and β (MIP-1α and β), which act basically on monocytes, but also stimulate the recruitment of basophils, eosinophils, T-cells and NK-cells [62]. In addition to their chemotactic effect in the immune system, they modulate a number of biological responses, including enzyme secretion, cellular adhesion, cytotoxicity, tumor cell growth and T-cell activation [63].

Upregulation of a number of chemokines has been associated with AD pathological changes [64]. IP-10 immunoreactivity was markedly increased in reactive astrocytes in AD brains, as well as the level of its expression. Astrocytes positive for IP-10 were found to be associated with senile plaques and showed an apparently coordinated upregulation of MIP-1β [65,66]. Significant increased IP-10 levels were observed in CSF from patients with mild AD as compared with severe AD. Similarly to mildly impaired AD, IP-10 increased levels were also found in subjects with amnestic MCI [59]. Regarding MCP-1 and IL-8, significantly higher levels were found in all AD patients as compared with healthy subjects, and highest peaks were observed in mild AD and MCI [59].

With regard to a possible use of chemokine to easily predict evolution from MCI to AD, few investigation in serum have been so far carried out, despite a growing body of evidence supporting the hypothesis that some peripheral biochemical modifications also occur very early during AD pathogenesis. For instance, serum MCP-1 levels have been demonstrated to be increased in MCI subjects, similarly to findings described in the CSF [67]. Notably, CSF MCP-1 levels in patients studied were higher than the correspondent serum levels, and the Brain Blood Barrier (BBB) was intact, as shown by normal IgG indices. Thus, these data strongly suggest that peripheral modifications of MCP-1 levels occurs early during AD development, when the clinical manifestation of the disease is not already clearly defined. Conversely, IP-10 serum levels were not increased in AD patients, but were found to correlate with aging [68].

ANTI-INFLAMMATORY DRUGS

A large body of epidemiologic evidence suggested that long-term use of NSAIDs protects against the development of AD [47, 48, 69]. Nevertheless, prospective studies of Rofecoxib, Naproxen or Diclofenac failed to slow progression of cognitive decline in patients with mild to moderate AD [70-72]. In contrast, Indomethacin may delay cognitive decline in this subset of patients, but gastrointestinal toxicity is treatment-limiting [73, 74]. Because of general concerns about lack of efficacy, gastrointestinal toxicity, myocardial infarction and stroke, NSAIDs are not considered to be viable treatment options for patients with AD.

Nitroflurbiprofen (HCT-1026; NicOx) is a nitric oxide-donating derivative of the NSAID flurbiprofen. It improves cognitive functions in rat following chronic lypopolisaccharide infusions [75] and reduces plaque burden in mice [76]. In humans, this compound was shown to penetrate the BBB. In addition, it reduces the rate of gastrointestinal ulcers by 60 to 80% compared with flurbiprofen [17]. Nitroflurbiprofen is currently being evaluated in a phase II study in patients with AD.

Another promising compound is Rosiglitazone (AVAN DIA®), an anti-diabetic agent with anti-inflammatory properties, which was tested in two small clinical trials. Rosiglitazone treatment for 24 weeks resulted in a modest but significant improvement in cognition in non-ApoE *ε4* carriers, but no improvement and rather a decline in cognition in *ε4* carriers was demonstrated [77]. A phase III trial is currently ongoing.

Lastly, a rapid improvement in verbal fluency and aphasia following perispinal etanercept administration was described [78]. Etanercept is a TNFα inhibitor, which acts by blocking the binding of this cytokine to its receptor. It was tested in 12 patients with mild to severe AD, at a dose of 25-50 mg weekly for six months, showing improvement in a number of neuropsychological testing, particularly in verbal fluency [78].

CONCLUSIONS

From data presented in this review, two main considerations emerged, to be carefully considered for planning future clinical trials. First, treatments for AD are likely effective only in certain phases of the disease. A few disease-modifying compounds showed some benefits in mild, moderated but not severe AD. The same was observed for anti-inflammatory drugs, according to recent studies demonstrating a high degree of inflammation in very mild but not in severe AD. Thus, therapeutic trials should be carried out as early as possible during the course of the disease, implying the need to identify more accurate tools for early diagnosis. The reliability of CSF biomarkers as well as imaging techniques to anticipate the time of clinical diagnosis were replicated by several research groups independently. New research diagnostic criteria have been proposed in 2007 [79], introducing the use of CSF analysis, structural and functional imaging, and genetics, in addition to classical neuropsychological testing, for early diagnosis. These new research criteria should be applied in the setting of future clinical trials to select more homogeneous study groups. Second, mechanisms at the basis of the pathogenesis of AD need to be investigated further before developing novel disease-modifying compounds. Despite promising premises related to the so-called "amyloid hypothesis", larger phase III trials with drugs influencing Aβ deposition failed to demonstrate an effect on cognition. Moreover, the neuropathological analysis of brains from patients who received immunisation demonstrated that although mean Aβ load was lower than in the placebo group, no evidence of improved survival or improvement in time to severe dementia was observed. Therefore, plaque removal seems to be not sufficient to halt progressive neurodegeneration in AD, prompting some intriguing challenges to the amyloid hypothesis. In light of these results, it is of crucial importance to better understand the relationship between tau and Aβ for developing novel disease-modifying drugs.

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