Nitric Oxide: Target for Therapeutic Strategies in Alzheimer's Disease

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Abstract: Alzheimer´s disease (AD) constitutes a progressive neurodegenerative disorder and the main cause of dementia. Numerous studies have focused on the pathogenic mechanism of AD to cure or prevent this devastating disease. But, despite recent advances, our understanding on the pathophysiology of this genetically complex and heterogeneous disorder is rather limited and treatment of the disease consists of medications to control the symptoms. Acetylcholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists are the only available treatments recommended to manage the cognitive deficits caused by the disease. Therefore, the production of new drugs that may be able to cure the underlying cause of this chronic disease, not just the symptoms, is a matter of clinical interest. There is data implicating nitric oxide (NO) in the progression of the disease. The three isoforms of the NO-synthesizing enzyme (NOS) operate as central mediators of amyloid beta-peptide (Aß) action, giving rise to elevated levels of NO that contributes to the maintenance, self-perpetuation and progression of the disease. Reducing Aß production and the cholinergic deficit is a goal in the treatment of AD. In addition, a possible way to delay the progression of the illness must include a rationale design of enzyme inhibitors, subtype selective, targeting NOS isoforms implicated in damage to brain cells in AD.

We are now presenting an overview regarding approved drugs for AD treatment and substances that although are not in use for the treatment of AD, including NOS inhibitors, may represent useful tools to unravel the pathophysiologic enigma of AD.

Keywords: Alzheimer´s disease, Nitric oxide, Nitric oxide synthase inhibitors, Acetylcholinesterase, Amyloid beta-peptide, Neuroinflammation, Neurodegeneration, Immunotherapy.

INTRODUCTION

Alzheimer´s Disease: General Topics

Alzheimer´s disease (AD), first described by Alois Alzheimer in 1907 [1], constitutes the most common progressive neurodegenerative disorder and the main cause of dementia [2]. This chronic disorder syndrome evolves with loss of neurons and their synapses and with cognitive deficits: memory, intellectual and emotional dysfunctions [3]. The disease appears usually associated with characteristics risks factors including old age, family history, apolipoprotein E epsilon 4 genotype, angiopathy, etc. [4]. Environmental factors and/or epigenetic phenomena may also contribute to AD pathology and phenotypic expression of dementia [5, 6].

Numerous studies have focused on the pathogenic mechanism of AD but, despite recent advances, our understanding on the pathophysiology of this genetically complex and heterogeneous disorder is rather limited and the currently available treatments approved by the US Food and Drug Administration and the European Medicines Agency do not halt progression or cure the illness. Histopathologically, the brains of AD patients share specific characteristics, namely amyloid plaques and neurofibrillary tangles (NFT), which are insoluble deposits made of an abnormally folded, fibrillar form of the amyloid beta-peptide (Aβ), as shown in Figs. (**1-2**), and hyperphosphorylated Tau, respectively. Aβ peptides are composed of 39–43 amino acids derived from the proteolytic processing of the amyloid precursor protein (APP) by β and γ secretases that give rise two structural variants, Aβ 1-40 and Aβ 1-42 [7]. Characteristically, the ratio of these structural variants differed by 10-fold between brains from nondemented controls and those with sporadic AD [8]. High ratio of the structural variants Aβ 1-40 and Aβ 1-42 is considered a biomarker of AD

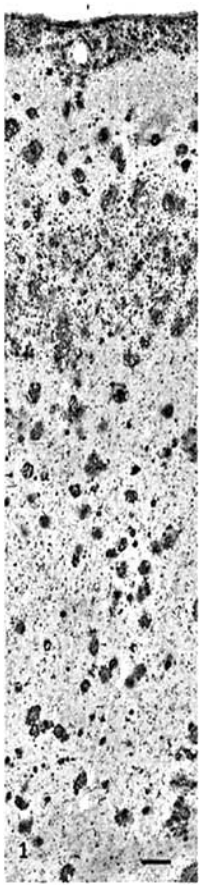

Fig. (1). Aβ expression and distribution in the cerebral temporal cortex from postmortem brain of CDR3 Alzheimer´s disease patient showing immunoreactive plaques in all cortical layers. Scale bar: 100 μm.

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[9]. Although it is not clear whether abnormal processing of the APP is an initial cause or rather a late event in the pathophysiology of AD [10], intraneuronal NFT together with accumulation of Aβ and neurotoxicity constitutes the major neuropathological hallmark of AD [11, 12]. Overwhelming evidence indicates that the progressive abnormal deposits of insoluble aggregates of Aβ within the neuropil and in the cerebrovascular walls (amyloid angiopathy) is the basis of the formation of senile plaques (amyloids) containing massive quantities of Aβ structural variants [8, 13] that causes neuronal injury and loss, inflammation and characteristic activation of microglia and astrocytic cells that occurs in AD [14-17], as shown in Figs. (**3-5**).

A growing body of evidence has suggested a critical role for APP metabolism and oxidative stress in the pathogenesis and advancement of the disease [18-22]. Together with aberrant Aβ-deposits in the neuropil originated in an anomalous proteolytic processing of APP [23], a genetic basis is essential in influencing to onset and/or modifying the progression of the disease [24, 25]. Early-onset dominant/familial AD (FAD) and the late-onset/sporadic AD (LAD) share identical pathology, thus suggesting common pathogenic pathways for both forms of AD. Mutations of three genes that influence the accumulation of the Aß have been recognized tightly linked to FAD [10], which include the APP and the catalytic components of the γ secretase complex, presenilins 1 (PS1) and 2 (PS2) that are responsible for the intramembranous proteolysis of APP. The e4 allele of apolipoprotein E (ApoE), contains a polymorphism which increases the risk to develop sporadic AD [26]. In addition, genomic factors induced by environmental factors and epigenetic phenomena, might be responsible for AD pathogenesis leading to premature neuronal death [5, 6]. Extracellular insoluble aggregates of long Aß 1-42, known to induce neurotoxicity [27], are exhibited by both FAD and LAD patients together with intracellular NFT [28] triggered by Aβ through the action of protein kinases [29-31]. APP was the first gene associated with FAD and the recent description of an extra copy of the APP gene in FAD [32] provided further support that increased Aß production can primarily drive the disease. Abnormal APP and presenilins linked to AD lead invariably to the production of insoluble amyloidogenic form Aß 1-42 peptide in a pathological manner [33-36], which strongly supports the amyloid hypothesis of AD [37]. Aβ also mediates synaptic dysfunction leading to cognitive deficits [38-40], which correlates very well with synapse loss [41, 42]. As a result of anomalous deposits accumulation of Aß, neuronal damage and dysfunction occur through the activation of microglia and astrocytic cells [43], synaptic failure [44] and associated amyloid angiopathy [45, 46]. The current Aß hypothesis suggests that the soluble oligomers can disrupt synaptic connections between neurons and, simultaneously, may aggregate in amyloid plaques which can trigger a local inflammatory response [47] essential in the progression of the disease [48]. It has been reported an increased expression of the pro-apoptosis gene products p53 and Bax associated with Aβ deposits in senile plaques [49-51]. We have also found elevated expression of a mutated form of p53 in older AD mice, suggesting an alternative mechanism for the predisposition of AD brains to develop brain neoplasms [52]. An established mechanism of cell loss in both neurons and glial cells in AD involves Bax and p53-mediated apoptosis [53, 54]. Both p53 and Bax inhibit the expression of Bcl-2, a cell survival gene product, thereby permitting Bax homodimers to enter the nucleus, promote cytochrome c release into the cytoplasm, and activate caspases. Caspases cause proteolytic damage, cytoskeletal collapse, and nuclease release followed by DNA fragmentation. Therefore, Aβ depletes the neurons of one of its anti-apoptotic mechanisms dowregulating Bcl-2 [55]. It has been also proved that p53 upregulation in AD induces tau-phosphorilation [56]. It remains to be determined as to whether Aß-deposits directly induces apoptotic cell death or triggers an alternative pathway, *via* nitric oxide (NO) systems, that then leads to degeneration [57, 58].

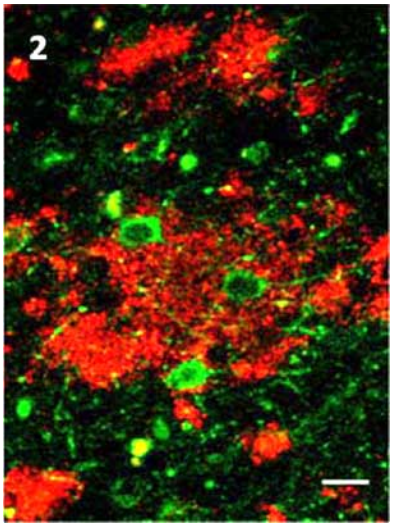

Fig. (2). Confocal microscopy microphotograph of a histological section through the frontal cerebral cortex of a postmortem brain of an Alzheimer´s disease patient, CDR3 case, showing double immunostaining for Aβ (red colour), and for nNOS (green colour). Notice that nNOS immunopositive neurons are in close relation with senile plaques. Scale bar: 20 μm.

As stated above, the generation of Aβ and other fragments requires sequential intramembrane cleavages of the APP by β- and γ-secretases. The γ-secretase complex operates as a cleaving multiprotein complex that cleaves APP and a large number of other type I integral transmembrane proteins including Notch-1, ErbB4, neuregulin, E- and N-cadherines, CD44, Low-density lipoprotein (LDL)-receptor related protein (LRP), nectin-1 and growth hormone receptor [59]. The Notch receptor undergoes a series of proteolytic events, in a manner similar to APP, ultimately releasing the intracellular domain that mediated the expression of genes controlling many types of cellular differentiation [60]. The first step in the production of Aβ peptides from APP is initiated by the β-site APP-cleaving enzyme 1 (BACE-1) [47] that catalyzes the rate-limiting reaction in the generation of Aβ [61]. β-Secretase cleavage of APP occurs predominantly in endosomes, and endocytosis of APP and β-secretase is essential for β cleavage and Aβ production [62-64]. Genetic dysregulation of APP near the ß-secretase cleavage site make APP a better substrate for this enzyme and lead to increased production of all variants of Aβ. However, mutations near γ-secretase cleavage site lead to increase in the more aggregation-prone Aß 1-42 relative to Aß 1-40. Mutations in the Aß itself change the biophysical properties of the peptide to render it more likely to aggregate [65]. The intramembrane γ-secretase complex includes presenilins together with Nicastrin, Aph-1 and presenilin enhancer 2 [66-74]. Alterations in the presenilin genes appear to be the major cause of FAD [75]. Presenilins are involved not only in the processing of APP but also in a number of pathways for cell death and survival modulating various cell signal transduction pathways as well [74, 76, 77], including the intracellular calcium signalling [78-80]. In addition, presenilins regulate phospholipase C and consequently Protein kinase C activity [81]. Therefore, binding of presenilins to APP also plays a central role inducing intracellular signalling [82]. In this regard, genetic mutation of presenilins have been associated with calcium signaling abnormalities that might play an early proximal and perhaps central role in AD pathogenesis [83, 84].

It has been also proposed that PS1 has a role for NO synthase (NOS) activation in neurons and confers oxidative stress-resistance on neurons in a calcium/NO-dependent manner [67]. Thus, oxida-

tive stress plays a critical role in the pathogenesis of AD related with APP proteolytic dysfunction.

Reducing Aß production is a goal in the treatment of AD. Both β- and γ-secretase are thus propitious therapeutic targets [85, 86] but may be detrimental for AD therapy. It has been shown that nonselective γ-secretase inhibitors exert deleterious effects on embryogenesis in zebrafish and on lymphoid and gastrointestinal tissues in mammals [87, 88], and also may impair neurogenesis. Numerous neurogenic players, such as Notch-1, are cleaved by PS1/γsecretase. In this context, PS1 familial AD mutation leads to defective associative learning and impairs adult neurogenesis in PS1M146V knockin mice [89]. In view of the multiple functions of γ-secretase, β-secretase might then be considered as the preferred therapeutic target [88]. Aß plaques induce BACE-1 in neighbouring neurons at early stages of disease, suggesting that BACE-1 induction triggered by the amyloid pathway may drive a positive-feedback loop in AD [90-92]. In this regard, morphine that down-regulates the expression of BACE-1 *via* NO could be a protective instrument for AD [93]. In addition, compounds like pioglitazone and ibuprofen that decrease BACE-1 mRNA and protein levels in APPV717I transgenic mice [94] might be of potential use. Therefore, identification and characterization of secretases is a focus on interest in AD basic research and therapeutic use in clinic.

A number of neurotransmitter specific systems are implicated in AD, including the glutamatergic, cholinergic and nitrergic systems [95-104]. Typically, the cholinergic deficit is a trait of the AD. During the evolution of the disease, there is a conspicuous decrease in the cholinergic innervation of the brain, particularly in the hippocampus and neocortex, where the number of cholinergic elements and cholinergic activity dramatically decrease [105]. Based on the latter, drugs have been developed to repair the cholinergic deficit with the aim to obtain symptomatic benefits based on improving cholinergic function by increasing acetylcholine levels. In this field of research, many efforts are being performed to develop acetylcholinesterase (AChE) inhibitors (AChEIs) with clinical applications [106]. However, a recent meta-analysis on the efficacy of AChEIs indicates that these treatments can result in statistically significant but clinically marginal improvement [107]. Therefore, a "causative therapy" should be promoted. The discovery of new drugs to modify the pathological steps leading to AD by acting on the evolution of the disease, represents a paradigm shift away from symptomatic treatment. By interfering with the pathogenic steps of AD, these products collectively termed as "disease-modifying" agents [108] might be able to interact with different therapeutic targets, thus blocking the progression and evolution of the disease, even preclinical. An update on disease-modifying drugs has been recently published [108]. Multifunctional disease-modifying agents that simultaneously inhibit the human AChE and the βA aggregation and that also display neuroprotective properties against the mitochondrial oxidative stress are now being tested [109]. In this regard, this review provides information concerning AChEIs targeting NO metabolism.

The Nitrergic System in Alzheimer´s Disease

The short-lived free radical messenger NO functions as an atypical diffusible neurotransmitter in an autocrine/paracrine manner in different tissues [110, 111]. NO is involved in a multitude of inter- and intra-cellular signalling pathways participating in considerable physiological and pathophysiological scenarios such as blood pressure control, neurotransmission, learning and memory, and as a defensive cytotoxin [99]. This highly diffusible gas, that crosses the biological membranes without any difficulty, plays a central role in neurodegenerative diseases, including Parkinson´s and Alzheimer´s diseases [112]. NO is synthesized by a specific enzyme, NOS (NOS; EC 1.14.13.39), present in the mammalian brain in three different isoforms, two constitutive enzymes (i.e., neuronal, type-I, nNOS, and endothelial, type III, eNOS) and one inducible enzyme (type II, iNOS) which is the catalyst of high-output pathway of NO production [113]. From the discovery by Vodovotz in 1996 that iNOS is localized in AD lesions, other studies confirmed this observation identifying iNOS protein in neurons, astrocytes, and microglia that are related with the anomalous Aβ deposits [12, 17, 94, 114-117], but at far lower incidence, extent, and intensity in brains from age-matched controls. Whereas nNOS and eNOS are expressed constitutively in normal brain, a widespread expression of iNOS in the central nervous system is considered pathologic. The oxidative stress caused by NO in the brain has been proposed as a pathogenic mechanism in AD [118, 119], being suggested that NO plays a central role in the cascade of events leading from enhanced intracellular Aβ accumulation to enhanced vulnerability to apoptosis and cell death [120]. NO can be scavenged in a rapid reaction with superoxide (O^{2-}) to generate peroxynitrite ($ONOO^-$) [121], that may act as a potent source for oxidative stress in AD [12, 122, 123] and the production of nitrotyrosine. At the cellular level, the mitochondrion appears to be of importance when considering the deleterious effects of both Aβ and induced NO [124]. Generation of highly reactive nitrogen species secondary to high concentrations of NO causes irreversible inhibition of mitochondrial respiration and damage to various mitochondrial components *via* oxidizing reactions leading to cell death. Nitrotyrosine, that derives from peroxynitrite-mediated nitration of protein tyrosine residues [125, 126], is considered as an indicator of cell damage and inflammation secondary to overproduction of NO [122]. Nitration may impair protein function, thus leading to neuron degeneration [12, 123]. A progressive elevation of NO markers and protein nitration in reactive astrocytes and microglia, as shown in Figs (**2**-**5**), was reported in Tg2576 transgenic mice [123] and in clinically evaluated cases of AD in human [12, 127, 128]. This is in agreement with previous reports that Aβ can stimulate NO production from astrocytes [129], and that activated astrocytes are able to produce NO levels that are detrimental to neurons [130]. *In vitro* studies in rat glioma C6 cells support this conclusion showing that Aβ treatment leads to increased production of NO through iNOS activation, yielding cytotoxicity in these cells [131]. In this context, a primary possibility to explain how Aß accumulation leads to neurotoxicity is that Aß may triggers oxidative and/or nitrosative injury in both neurons and glial cells.

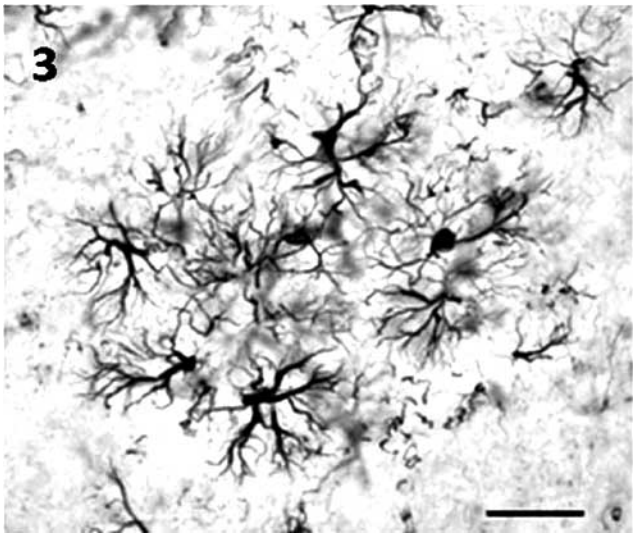

Fig. (3). Inflammation in AD. Microphotograph showing immunostaining for glial fibrillary acid protein (GFAP) in the cerebral cortex of Tg2576 transgenic mice. An amyloid plaque is surrounded by reactive astrocytes showing immunoreactions product. Scale bar: 25 μm.

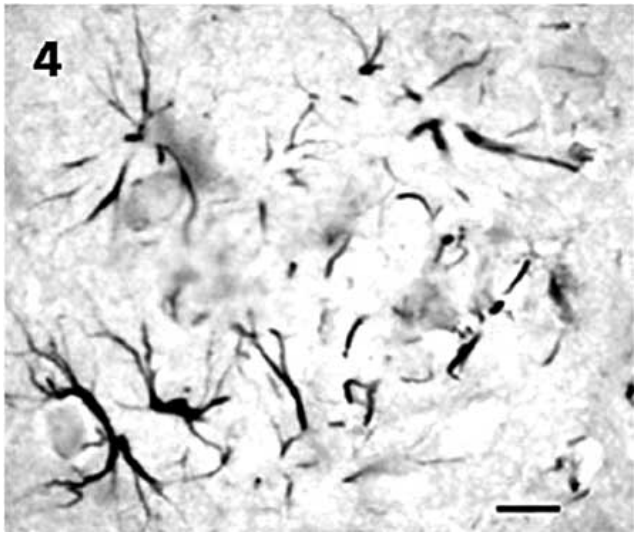

Fig. (4). Microphotograph showing immunostaining for nitrotyrosine in the cerebral cortex of Tg2576 transgenic mice. Nitrotyrosine immunoreactivity appears in stellate shaped cells, resembling reactive astrocytes, surrounding a senile plaque. Scale bar: 10 μm.

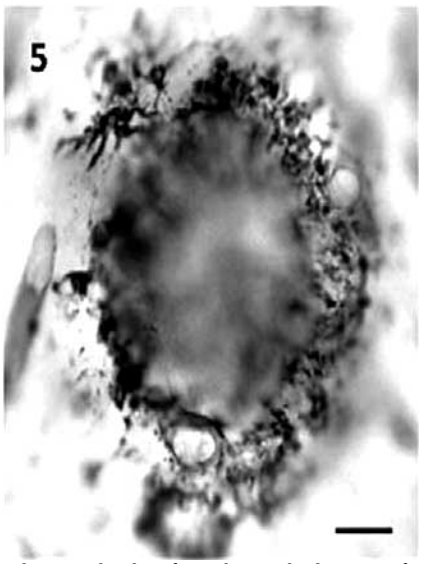

Fig. (5). Microphotograph taken from the cerebral cortex of Tg2576 transgenic mice showing immunostaining for iNOS. Notice that the immunoreaction product is distributed outside the core of the senile plaque labelling numerous round shaped small cells resembling microglia. Scale bar: 10 μm.

There is evidence implicating NO as a mediator of glutamate-mediated neuronal death (glutamatergic excitotoxicity) *via* N-methyl-D-aspartate (NMDA) receptors. Influx of calcium followed by activation of nNOS may conducts to the generation of peroxynitrite, which has been implicated in the mechanism of neuron loss in neurodegenerative diseases. All the three isoforms of the NO-synthesizing enzyme are bizarrely expressed in AD models, giving rise to elevated levels of NO. However, it is controversial as to whether aberrant expression of NOS isoforms constitutes a primary event in the pathogenesis cascade of AD, or merely reflects a secondary effect shown at more advanced stages of the disease process [132]. In both AD patients and transgenic mice models of the disease, eNOS-expressing astrocytes exceeded those expressing iNOS in number [17]. Double immunolabeling studies revealed that in astrocytes iNOS and eNOS are co-localized with nitrotyrosine [122]. In some cases of AD, genetics defects in PS1 on microglial cells resulted in enhanced NO through superinduction of iNOS and eNOS [17, 133, 134].

Astrocytes with elevated levels of iNOS or eNOS were persistently seen in direct association with clusters of Aβ-deposits in AD patients and transgenic mice. The expression of both iNOS and eNOS is increased in activated astrocytes under experimental conditions in association with elevated expression of APP or bulk of Aβ-deposits in APP23 transgenic mice [17]. Whereas it is clear from the data in the literature that NO systems are involved in the pathophysiology of AD [98, 135] acting iNOS and eNOS as central mediators of Aß action [49, 124, 136, 137], it has been also reported that changes in the activity of nNOS by the action of Aβ is responsible for the increased NO production in neurons [98]. Thus, reducing oxidative stress should be of potential value for limiting the incidence and progression of AD [138].

Taken together, studies on NO system in AD suggest that NO contributes to the maintenance, self-perpetuation and progression of the neurodegenerative process, and also that the altered expression of NOS isoforms might be secondary to the amyloid pathology. In view of the wide range of isoform-specific NOS inhibitors, the determination of the most responsible isoform of NOS for the formation of peroxynitrite in AD could be of therapeutic importance in the treatment of AD. It therefore appears important to gain a better understanding of the pathogenic mechanisms involving the NO systems, since they may represent an interesting therapeutic avenue for patients.

NITRIC OXIDE AND ITS RELATIONSHIP WITH CONSIDERED ALZHEIMER´S DISEASE TREATMENTS

Cholinergic Therapy and Nitric Oxide

Amongst AChEIs, four products: tacrine, donepezil, rivastigmine, and galantamine are approved for clinical treatment, and the latter three are widely recommended for clinical use [139]. These products together with *N*-methyl-D-aspartate receptor antagonist (memantine) represent the available treatments for Alzheimer's disease [140]. Other cholinergic drugs such as muscarinic agonists have been explored, and although they are not approved, there is robust preclinical evidence for a beneficial, perhaps disease-modifying effect [141].

Acetylcholine (ACh) plays a role regulating the information flow and processing during learning and memory. There is certainly a link between cholinergic neurotransmission and cyclic guanosine monophosphate (cGMP) synthesis. The NO–signaling cascade has a role in learning and memory processes as well, which is also regulated by cGMP. Therefore, NO chimeras, for example GT 1061 and hybrid nitrates, may be a potential therapy for AD [142]. The nNOS isoform is ubiquitous in the brain and involved in glutamate neurotoxicity, learning and memory processes.

As stated above, AChEIs are the main recommended drugs for AD treatment. However, sometimes their efficacy has been questioned. Neurogenic vasodilation mediated by alpha 7-neuronal ACh nicotinic receptor has been described to be NO mediated and was blocked by AChEIs. Statins can prevent this blockage at the receptor level [143]. Statins have been found alter APP metabolism by lowering cholesterol levels (FDA Phase: Phase II/IIa/IIb).

Donezepil, galanthamine, and tacrine are AChEIs used widely for the treatment of AD. All these compounds have neuroprotective properties which have been related with glutamate neurotoxicity. Donezepil and galanthamine protect cortical neurons steps before NO formation and tacrine after [144]. In addition, Dimebon (3,6-dimethyl-9-(2-methyl-pyridyl-5)-ethyl-1,2,3,4-tetrahydro-γ-carboline dihydrochloride), shows activity as an inhibitor of cholinesterase and NMDA receptors. Through mitochondrial-mediated inhibition of apoptosis restrain neuronal death (FDA Phase III).

The cholinergic deficit that occurs in AD affects the cerebral vasoregulation. In fact, in AD there is a vasoregulative deficit that could be related with a lower eNOS expression. It has been shown

that the use of AchEIs, such as Donezepil, improves cerebrovascular regulation in Alzheimer´s patients [106].

More recently, a new promising dimeric AChEI: bis(7)-Cognitin [145] has been reported. At neuroprotective concentrations, it reverses the over-activation of nNOS caused by glutamate without interfering with the basal activity of NOS. Through inhibition of AChE, NMDA receptor, nNOS, and APP/ βA cascade concurrently, it possesses remarkable neuroprotective activities. The synergism between these targets might serve as one of the most effective therapeutic strategies to arrest/modify AD pathology.

Lipoic acid function has been related as a mediator in acetylcholine production by activating of choline acetyltransferase. In fact, lipoic acid has been shown to ameliorate AD symptoms in patients with mild dementia and also in animal models [146].

Anti-Inflammatory Therapy and Nitric Oxide

Neuroinflammation is an essential component of many neurodegenerative diseases, including AD. This has been supported largely by observational studies that anti-inflammatory approaches may be protective against the development of AD [147, 148]. In the pathogenesis of neuroinflammation, free radicals are playing a relevant role, particularly NO produced through the activation of microglia and astrocytic cells. Thus, the increased understanding of the effects of NO in AD will lead to the development of novel anti-inflammatory therapeutic strategies.

Anomalous deposition of Aß peptide has been widely described responsible for AD pathogenic process [13]. As a result of such anomalous deposits accumulation, neuronal damage and dysfunction occur through the activation of microglia and astrocytic cells [44]. Detrimental neuroinflammatory factors are characteristically expressed by activated glia in such a process, including pro-inflammatory cytokines and chemokines, oxidative stress-related enzymes, acute phase proteins, and components of the immunologic and complement cascades [40, 92]. Amyloid plaques are responsible for a local inflammatory response [48] essential in the progression of the disease [149, 150]. Particularly, astrocytes and microglial cells in close apposition to the extracellular accumulation of Aß account for the synthesis and pathological release of variety of cytokines, chemokines and reactive oxygen species which may all lead to degeneration of neurotransmitter specific neuronal populations. It is discussed whether overproduction of cytokines (IL-1, IL-6, TGF-ß, and TNF-α) secondary to cerebral inflammatory processes, may be involved in the development and progression of the disease [151]. Aß deposits influence NO system leading to degeneration [123]. Because of the pathogenic inflammatory response that takes place in AD, it is possible that therapeutic intervention in such an inflammatory process hold great promise for the amelioration of AD sympthoms. The compounds listed down here are described to exert a pharmacological therapeutic in AD through affecting inflammatory mechanism in which NO appears to be concerned.

The nonsteroidal anti-inflammatory drugs (NSAIDs) may lessen the inflammatory response acting on Aβ peptide deposition and therefore protecting against AD. NAIDs antiinflammatoy action results by inhibiting cyclooxygenase-1 and cyclooxygenase-2 and by activating the peroxisome proliferator g nuclear transcription factor (PPARα). These drugs have been proposed for the treatment of AD [155]. Cyclooxygenase-2 upregulation, an enzyme that plays a crucial role in mediating inflammatory response, seems to be associated with Aβ plaque formation in AD. This well described pro-inflammatory mediator acts close together with iNOS [152]. NSAIDs also possess inhibitory effects in the generation of NO radicals [153]. Ibuprofen, the most commonly used NSAIDs, is a cyclooxigenase-2 (COX-2) inhibitor that decreases the production of NO. This compound protects neurons against glutamate toxicity, decreases the production of proinflammatory cytokines and suppresses neuritic plaques pathology and inflammation in AD [154]. This compound is clinical trial fase III. Flurbiprofen is and additional NSAIDs that influences Aβ peptide deposition and metabolism. Flurbiprofen racemate, its *R*-enantiomer and its NO-releasing derivatives, HCT 1026 and NCX 2216, are effective on AD amyloid pathology [155] (FDA Phase: discontinued).

Resveratrol (3,5,4'-trihydroxy-trans-stilbene), a naturally occurring phytoalexin found in grapes and wine, has a neuroprotective action against Aβ induced toxicity in C6 glioma cells by inhibiting iNOS and COX-2 expression [130]. (FDA Phase III).

Retinoic acid has an inhibitory effect on TNF-α and iNOS expression *in vitro* preparations of activated rat microglia by Aβ peptide [156].

Lipoic acid acts downregulating iNOS activity and pro-inflammatory proteins [146]. Thanks to its neuroprotective action, lipoic acid has been proposed as an alternative for AD treatment.

Catalpol, an iridoid glycoside isolated from the root of Rehmannia glutinosa, protects neuronal cells by deacreasing the Aβ 1-42 and inhibiting the to some extent glial activation [151].

Gemfibrozil, a member of the fibrate class of hypolipidaemic drugs, inhibits LPS-induced expression of iNOS as well as proinflammatory cytokines in mouse BV-2 microglial cells and primary microglia [157].

The pharmacological blockage with the iNOS inhibitor aminoguanidine reduced the cognitive deficit evoked by intracerebroventricular injection of Aβ 1-40 in mice. Similar results were obtained in iNOS knock-out mice [89].

Vitamin E (alpha-tocopherol) inhibits iNOS expression and therefore NO production in astrocytes stimulated with Aβ [158]. Therefore, prevents brain cell damage by destroying toxic free radicals. (FDA Phase III).

Poly(ADP-ribose) polymerase-1 (PARP-1) has been shown to play a key role in NF-κβ driven expression of inflammatory mediators by glia. Inhibitors of PARP-1 enzymatic activity reduce the expression of inflammatory mediators such as iNOS and APP among others, and the neurotoxic potential of activated glia *in vitro* [159].

Induced cholinergic degeneration produces increased expression of inflammatory mediators, among others iNOS. The nonsteroideal anti-inflammatory drug, Nimesulide, attenuate iNOS activity [160].

Phospholipases: a Target for Therapeutic Intervention

Phospholipases hydrolyse fatty acids of membrane phospholipids. Activation of phospholipase A_2 (PLA_2) mediates arachidonic acid (AA) release from phospholipids sn-2 position. NO affects the intracellular level of AA through alteration of PLA_2 activity. Excess of activation of PLA_2 produces damage of cell membrane integrity. PLA_2 acts by altering membrane permeability and calcium homeostasis increasing the levels of intracellular Ca^{2+}, that leads an excitotoxic phenomena involved in a lot of pathologies [161]. The interaction of Aβ with cell surface receptors results in the stimulation of phospholipases [162]. Particularly, a marked stimulation of PLA_2 activity has been shown to occur in AD [163, 164]. The nNOS isoform is one of the target enzymes of the PLA_2 activation and responsible for excitotoxicity-induced neurodegenerative diseases, including AD [165]. Therefore, phospholipases may represent a therapeutic target for AD. However, using the selective inhibitiors of PLA_2 activity, indomethacin and nor-dihydroguaiaretic acid, the Aβ peptide continues inducing cell death *in vitro* as well as with the NOS inhibitors NG-monomethyl-L-arginine and N omega-nitro-L-arginine [166]. DP-55, a lipid compound which is cleaved by PLA_2, has been described as a compound of potential therapeutic action for AD. This compound is efficacious in re-

ducing levels of Aβ in transgenic AD mouse (Tg2576) brains as well as in reducing neurodegeneration, and inflammation in an *in vitro* AD model [167]. It has been described that Resveratrol prevents NO dependent AA action [168].

Phosphodiesterases (PDE) Inhibitors

The cyclic nucleotide phosphodiesterase (PDE) comprise a group of enzymes that degrade phosphodiester bond in the second messenger molecules cyclic adenosine monophosphate (cAMP) and cGMP. They regulate the localization, duration, and amplitude of cyclic nucleotide signaling within subcellular domains. Phosphodiesterases (PDEs) are therefore important regulators of signal transduction mediated by these second messenger molecules. The PDE superfamily of enzymes is classified into 11 families in mammals, namely PDE1-PDE11. PDEs have different substrate specificities. Some are cAMP selective hydrolases (PDE4, 7 and 8), others are cGMP selective (PDE5, 6 and 9). Others can hydrolyse both cAMP and cGMP (PDE1, 2, 3, 10 and 11). The potential for selective PDE inhibitors to be used as therapeutic agents was predicted as early as 1977 by Weiss and Hait [169, 170].

NO, memory enhancement, and PDE are correlated *via* cGMP signaling. Activation of NMDA glutamate receptors causes increased intracellular calcium that binds to calmodulin activating nNOS and the production of NO. NO in turn activates soluble guanylyl cyclase followed by an increased formation of cGMP. Increased formation of cGMP leads to protein synthesis, synaptogenesis and memory enhancement [171].

Phosphodiesterase-5 (PDE5) belongs to an important family of proteins that regulates the intracellular level of cGMP. Recent studies have shown that PDE-5 inhibitors can counteract memory deficits in an AD model [172]. Sildenafil, a PDE5 inhibitor, increases the level of cGMP leading to beneficial effects in some targeting organs [173]. Sildenafil is the first oral drug approved by the United States Food and Drug Administration for the therapeutic treatment of erectile dysfunction. Besides, it may offer a novel strategy in the therapeutic treatment of a lot of pathologies: age-related memory impairment, pain, pulmonary hypertension, multiple sclerosis and progression, prevention and, eventually, AD [174]. This drug attenuates memory impairment induced by NOS inhibition, among others [175]. Propentofylline, another PDE5 inhibitor, prevents the neuronal damage NO-related in dementing processes [176]. However, in a clinical trial, this compound has shown a very limited capacity for improve cognition and global function in AD [177]. (FDA Phase: Discontinued). Phosphodiesterase-4 (PDE4) belongs to an important family of proteins that regulates the intracellular level of cAMP. Targeting PDE4 with selective inhibitors may offer a novel therapy aimed at slowing progression and, eventually, therapy of AD. In this sense, Rolipram, a specific PDE4 inhibitor improves memory in young and aged mice [178] as well as in double-transgenic mice APP /PS1 mice [179].

Caffeine, an adenosine receptor antagonist, inhibits phosphodiesterases action thus being a potential candidate for the treatment of AD. Caffeine reduces Aβ deposition and improves cognitive behavior in a mouse model of AD as well as in neuronal cultures from such a model [180].

Besides caffeine, other selective adenosine A (2A) antagonists prevent the toxic effects of Aβ peptide. For example, the compound SCH58261 avoid Aβ induced cognitive deficits following intracerebroventricular administration of this peptide in mouse [181]. Also, the compound ZM 241385 protects rat cerebellar neurons from the toxic effects of Aβ peptide [182].

FUTURE DIRECTIONS IN ALZHEIMER´S DISEASE TREATMENT: DRUGS TO RESTRAIN NITRIC OXIDE SYNTHESIS

Regarding the involvement of NO in the progression of the disease, research is directed towards the production of new drugs that may be able to treat the underlying cause of this chronic disease, not just the symptoms. We are describing the progress being made in the scientific community and pharmaceutical industry on the development of any breakthrough product of potential use.

NOS Inhibitors

Because of the harmful effects of excess NO, selective restrain of NO biosynthesis through a controlled inhibition of the NOS isoforms, particularly iNOS [183], might provide an important therapeutic opportunity. Many efforts have been focused to the design of high affinity drugs, most based on strategies carried out to modify the enzyme`s substrate. The inhibitory action of a putative drug on iNOS and nNOS activities may be beneficial in various forms of shock and inflammation and may protect against neuroinjury, respectively [184]. But since NO plays certain central physiological roles, a complete inhibition of its production may be detrimental**.** Therefore, to design drugs that target a specific NOS isoenzyme in a therapeutic dosage is critical to avoid side effects. These drugs should reach a proper homeostasis of NO metabolism in the tissue where endogenous production of NO is excessively increased. The use of non-specific NOS inhibitors, such as L-nitroarginine methyl ester (L-NAME), impairs long-term memory consolidation [185]. It is also noteworthy the role of eNOS in maintaining vascular tone and spatial memory function [186]. On the other hand, complete removal of iNOS, which is primarily associated with the immune response, from an APP transgenic mouse (APPSwDI/NOS2$^{-/-}$) results in development of a much greater spectrum of AD-like pathology and behavioral impairments [187]. Studies on the Tg2576 APP mouse with an iNOS knock-out background also support the evidence suggesting that NO generated by iNOS under conditions of long-term injury or disease reduces functional loss and mitigates pathological changes in brain [98].

In summary, a possible way to delay the progression of AD must include a rationale design of enzyme inhibitors, subtype selective, targeting NOS isoforms implicated in damage to brain cells [188]. The catalytic activity of NOS can be tone down by a pharmacological attack on different enzyme sites, in example, the arginine, heme, and tetrahydrobiopterin (BH_4) sites.

We are now presenting an overview of enzyme inhibitors targeting NOS isoforms. These products, although are not in use for the treatment of AD, may represent useful tools for investigating the biological functions of NO in AD.

From a chemical point of view, these chemicals are divided into two principal groups: aminoacid-based inhibitors and non-aminoacid-based inhibitors.

1. Aminoacid-Based Inhibitors

This type of derivatives have been grouped, based on their chemical structures, into three categories of compounds [188, 189]: L-arginine analogues, conformationally restricted arginine analogues and dipeptides.

1.1. L-Arginine Analogues

Specific modifications of the enzyme`s substrate became a strategy outlook to produce NOS inhibitors that have become a putative targeted therapy strategy in AD for the pharmaceutical industry. The first NOS inhibitors derived from L-arginine were mono- or di-substituted guanidino analogues, most notable L-N^G monomethyl arginine (L-NMMA) and L-N^G nitroarginine (L-NOARG) and its methyl ester (L-NAME). These products showed a potent inhibitory action but a poor selectivity among the NOS isoforms.

Arginine analogues encompass L-NAME [190] and its derivates; N- methyl-L-arginine (L-NMA) [191], N^{δ}-(1-iminoethyl)-L-ornithine (L-NIO) , N-nitro-L-arginine (L-NNA) [190], N-alkyl-L-arginines , N-(1-imino-3-butenyl)-L-ornithine (L-VNIO) , L-thiocitrulline [192]; , L-homothiocitrulline [193], N-(1-iminoethyl)-

L-lysine (L-NIL) [194], and S-alkyl-L-thiocitrullines [195]. The development of N^{ω}-2-nitroarylaminoacid derivatives and heterocyclic analogues of L-citrulline [196] were obtained by means of modifications of the guanidine moiety of L-arginine . N^{ω}-2-nitroarylaminoacid showed a good inhibitory potency against nNOS, and less active against eNOS and iNOS, maintaining the same order of the potency and selectivity of the prototypic NOS inhibitors L-NMA and L-NNA.

1.2. Conformationally Restricted Arginine Analogues of Endogenous NOS Substrate L-Arginine

Guanidine, N^{G}-methylguanidine and amidine L-arginine analogues have been synthesized. With some exceptions, these structurally related analogues were, generally, weak inhibitors with moderate selectivity, if compared to the parent molecules. Conformational restriction appears to prevent the molecules from assuming the appropriate discriminatory binding orientations needed for high selectivity of the NOS isoenzymes [197]. Thus, restricted conformations do not seem to be a proper approach for the increase of inhibitors selectivity.

1.3. Dipeptides

This group includes L-NNA-containing dipeptides. Because of the selectivity of L-NNA for nNOS vs iNOS, in order to improve selectivity in favour of nNOS, [197] designed L-NNA (Arg^{NO2})- and phenylalanine (PHE)-containing dipeptides and dipeptide esters.

A library of 152 dipeptides amides containing nitroarginine and aminoacids different from PHE were synthesized and screened for activity [198]. Excellent inhibitory potency and selectivity for nNOS over eNOS and iNOS is achieved with the dipeptide amides containing a basic amine side chain. Among these compounds, the most potent nNOS inhibitor is L-Arg^{NO2}-L-Dbu-NH_2, which also exhibits the highest selectivity over eNOS (>1500-fold) with a 192-fold selectivity over iNOS. These compounds do not exhibit time-dependent inhibition. This compound can be considered the most selective nNOS inhibitor known. Structure Activity Relationship (SAR) studies demonstrated the importance of the α-amino group and the NH moiety of the peptide bond for binding at the active site.

Several L-arginine analogs are known as potent inhibitors of NOS. Since dipeptide containing are potent and isozyme-selective NOS inhibitors, following the dipeptide approach, it has been describe additional dipeptides containing arginine-analogues [199]. S-methyl-L-isothiocitrullinyl-L-phenylalanine showed 66-fold selectivity for iNOS over nNOS, while S-methyl-L-isothiocitrullinyl-L-leucine and N^{G}-nitro-L-argininyl-L-phenylalanine showed 20- and 14-fold selectivity, respectively. It is suggested that each NOS isozyme has a cavity of different size near the C-terminal of the L-arginine binding site, and that the selectivity of inhibitors is due to the differences in the size of the cavity.

Recently, a new cyclic dipeptide product, cyclo(dehydrohistidyl-l-tryptophyl) (CDHT), was found to inhibit NO in lypopolysaccharide (LPS)-treated BV-2 cells by preventing iNOS dimerization [200]. Characteristically, this product has no effect on iNOS expression or enzyme activity. In contrast, CDHT did not inhibit eNOS activity. These results reveal that CDHT could be a useful therapeutic agent for inflammation-mediated diseases.

2. Non-Aminoacid-Based Inhibitors

In order to improve the selectivity and therapeutic profile of L-arginine derivatives, non-aminoacid-based inhibitors of NOS have been studied; these include a continually growing list of compounds that, from a chemical point of view, may be divided into two principal groups: amidinic and heterocyclic compounds.

2.1. Amidinic Compounds

2.1.1. Guanidines

Despite the fact that simple guanidines are only weak inhibitors [201], guanidine moiety can mimic L-Arg for recognition at the binding site so that it can be considered a potential pharmacophore for NOS inhibition. Aminoguanidine is a selective inhibitor of mouse inducible NOS and numerous *in vivo* studies have demonstrated that this inhibition could be useful in the treatment of disease states characterized by the pathological overproduction of NO from iNOS [202].

2.1.2. Isothioureas

Inhibition by non-amino acid isothioureas has provided compounds which are potent and selective inhibitors of human iNOS vs eNOS [203]. The inhibitory effect of these compounds is caused not only by their competition with the substrate for the L-arginine-binding site and/or oxidizing center of the enzyme (heme) but also by interaction with peptide motifs of the enzyme that influence its dimerization, affinity for cofactors, and interaction with associated proteins. The clinical use of these isothiourea-based compounds is limited owing to their poor cellular penetration and high toxicity.

2.1.3. Amidines

There are a great number of derivatives of amidines, but the most active compounds are: 2-iminopiperidine, 2-iminohomopiperidine and (1*S*,5*S*,6*R*,7*R*)-7-Chloro-3-imino-5-methyl-2-azabicyclo [4.1.0]heptane hydrochloride (ONO-1714). These are more potent inhibitors of human iNOS than L-NMA with little preference for iNOS vs eNOS when compared with L-NMA.

ONO-1714 constitutes a novel cyclic amidine analogue that inhibits human iNOS with a K_i of 1.88 nM and rodent iNOS with similar potency *in vitro*. ONO-1714 was found to be 10-fold selective for human iNOS over human eNOS. When the inhibitory activity of ONO-1714 was compared for iNOS, it was found to be 451-fold and >20,000-fold more potent than L-NMMA and aminoguanidine, respectively [204].

2.2. Heterocyclic Compounds

This group of non-aminoacid-based NOS inhibitors includes a range of heterocyclic compounds among which the most represented are: indazoles, imidazoles and analogues of BH_4.

2.2.1. Indazoles

Despite the absence of *in vitro* selectivity in enzymatic or functional assays [205]; 7-nitroindazole named 7-NI, may be considered as the first selective inhibitor of nNOS *in vivo*; in fact, several studies have demonstrated its protective effects in pain , experimental stroke [206] and in mouse model of Parkinson`s disease [207] with minimal systemic pressure effects.

2.2.2. Imidazoles

Since it has been demonstrated that NOS contains a very similar heme site to that of cytochrome-P_{450} [208], imidazole, 1-phenyl, 2-phenyl and 4-phenyllimidazole and antimycotic drugs (myconazole, ketoconazole and clotrimazole) were tested as inhibitors [209]. In fact, it is well known that imidazoles inhibit the activity of various heme-containing proteins by binding to the heme group [210].

2.2.3. Analogues of Tetrahydrobiopterine

Owing to the structural relation with the enzymatic cofactor BH_4, pteridine based compounds have been described as NOS inhibitors. This approach appears promising because of the much lower affinity and selectivity of BH_4 binding to other pteridine dependent enzymes [211]. Among a series of tested compounds, the 4-amino analogue of BH_4, 5,6,7,8-tetrahydro-6-(D-theo-1,2-dihydroxypropyl)pterin, was a potent inhibitor of the recombinant rat brain

NOS both *in vitro* and *in vivo* and, although to a lesser extent, also of the other isoform.

Other pteridine-based compounds, named anti-pterines, structurally related to 5,6,7,8-tetrahydro-6-(D-theo-1,2-dihydroxypropyl) pterin, were described as NOS inhibitors . Some of these compounds specifically interacted with the BH_4 binding site of NOS without interference with any other known cofactors or substrate binding sites [212].

Most recently, a novel antipterin NOS-inhibitor, 4-amino-(6R,S)-5,6,7,8-tetrahydro-L-biopterin (VAS203) has been described [23] to inhibit NOS working over the BH_4 cofactor binding site. Characteristically, much lower concentrations of VAS203 are required to inhibit iNOS as compared to the constitutive NOS isoforms.

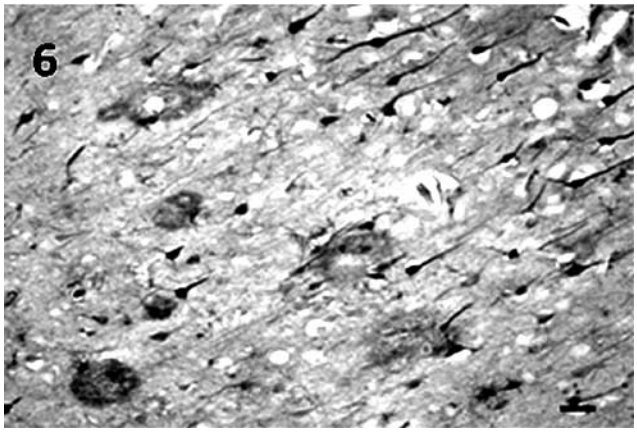

Fig. (6). Represents a low-power magnification micrograph taken from a histological section through the temporal cortex of a postmortem brain of AD patient, CDR3 case, showing the distribution of anti-human IgG immunolabeling. Notice immunoreactivity in neurons, senile plaques and surrounding glial cells. Scale bar: 100 μm.

IMMUNOTHERAPY

Although little is known on the etiology and pathogenesis of AD, several reports indicate the involvement of immunological mechanisms playing an important role in the disease process. In the temporal cortex from postmortem brain of CDR3 AD patients, we have found cortical neurons (both interneurons and pyramidal cells), senile plaques and its surrounding glial cells exhibiting autoantibodies (human IgGs), as shown in Fig. (**6**) (unpublished results), which might represent an autoimmune aggression. In addition, a prominent innate immune response occurs in the brain in association with Aß deposition and plaque formation. This response includes the activation of complement, secretion of proinflammatory cytokines and NO, and the expression of the chemokines [213]. These observations support a close relationship between both the immune system and the pathophysiology of AD. There are data that support the capacity of anti-Aβ antibodies to reduce Aβ aggregation and neurotoxicity. Based on the latter, it has been suggested that immunotherapy with a vaccine that avoid toxic side effects might generate specific and effective immune response against Aβ, the causative agent of synaptic loss and cognitive decline, providing a powerful tool for the treatment of AD [214]. This is particularly consistent given findings that microglia-mediated release of NO by toxic Aβ contributes to AD neurotoxicity.

Aβ stimulates inflammation and the production of neurotoxic reactive oxigen species by inducing the pathological over-expression of iNOS [215, 218]. Therefore, patients might benefit with immunotherapy which enhances clearing of this neurotoxic peptide from the brain [215-217]. Since only a small percentage of patients with AD show symptomatic benefits from current drug therapy and for only a relatively short time, immunotherapy is among the leading therapeutic directions for the disease. The use of intravenous Immunoglobulin (Gammagard, IVIg) is in FDA Phase III.

CONCLUSIONS

Presently, there is no cure for AD and no proven treatment has been developed to slow or halt its progression. Strictly, five drugs have been approved by the US Food and Drug Administration and the European Medicines Agency that improve the mental function of people with AD, these include: tacrine (Cognex), rivastigmine (Exelon), galantamine (Razadyne), donepezil (Aricept), and memantine (Namenda). All except memantine are approved for the treatment of mild to moderate AD. Memantine is approved for the treatment of moderate to severe AD only. All of these drugs, except memantine, act by increasing brain levels of acetylcholine, a neurotransmitter that is abnormally low in patients with AD. Memantine works differently interacting with the NMDA receptor.

The production of new specific drugs that cure the underlying cause of this chronic disease, not just the symptoms, is a matter of clinical interest. The three isoforms of the NOS operate as central mediators of Aß action, giving rise to elevated levels of NO that contributes to the maintenance, self-perpetuation and progression of the disease. Indeed, NO links mitochondrial disfunction to AD. At the cellular level, the mitochondrion appears to be of importance when considering the deleterious effects of both Aβ and induced NO. Generation of highly reactive nitrogen species secondary to high concentrations of NO causes irreversible inhibition of mitochondrial respiration and damage to various mitochondrial components *via* oxidizing reactions leading to cell death. Attacks on the mitochondrial protein dynamin-related protein 1 (Drp1) by NO, which causes a chemical reaction called S-nitrosylation, mediates neurodegeneration associated with AD [219]. Prior to this study, the mechanism by which Aβ caused synaptic damage to neurons in AD was unknown. These findings suggest that preventing S-nitrosylation of Drp1 may reduce or even prevent neurodegeneration in Alzheimer's patients. Therefore, a possible way to delay the progression of AD must include a rationale design of enzyme inhibitors, subtype selective, targeting NOS isoforms implicated in damage to brain cells in AD.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

Aβ	=	Amyloid beta-peptide
AA	=	Arachidonic Acid
ACh	=	Acetylcholine
AChE	=	Acetylcholinesterase
AchEIs	=	Acetylcholinesterase Inhibitors
AD	=	Alzheimer Disease
ApoE	=	Apolipoprotein E
APP	=	Amyloid precursor protein
BACE-1	=	β-site APP-cleaving enzyme 1
BH_4	=	Tetrahydrobiopterin
cAMP	=	cyclic adenosine MonoPhosphate
CDHT	=	Cyclo (dehydrohistidyl-1-tryptophyl

CDR3	=	Clinical dementia rating 3 (severe dementia)
cGMP	=	cyclic Guanosine MonoPhosphate
COX-2	=	Cyclooxigenase-2
eNOS	=	endothelial Nitric Oxide Synthase
FAD	=	Familial Alzheimer Disease
GFAP	=	Glial fibrillary acid protein
iNOS	=	inducible Nitric Oxide Synthase
LAD	=	Late-onset/sporadic Alzheimer Disease
LDL	=	Low-Density Lipoprotein
L-NAME	=	L-nitroarginine methyl ester
L-NIL	=	N-(1-iminoethyl)-L-lysine
L-NIO	=	N^{δ}-(1-iminoethyl)-L-ornithine
L-NMA	=	N-methyl-L-arginine
L-NMMA	=	L-N^{G} monomethyl arginine
L-NNA	=	N-nitro-L-arginine
L-NOARG	=	L-N^{G} nitroarginine
LPS	=	Lipopolysaccharide
LRP	=	LDL-receptor related protein
L-VNIO	=	N-(1-imino-3-3-butenyl)-L-ornithine
NFT	=	Neurofibrillary tangles
NMDA	=	N-methyl-D-aspartate
nNOS	=	neuronal Nitric Oxide Synthase
NO	=	Nitric Oxide
NOS	=	Nitric Oxide Synthase
NSAIDs	=	Nonsteroidal anti-inflammatory drugs
ONO-1714	=	(1S,5S,6R,7R)-7-Chloro-3-imino-5-methyl-2-azabicyclo[4.1.0] heptane hydrochloride
$ONOO^{-}$	=	Peroxinitrite
PARP-1	=	Poly(ADP-ribose) polymerase-1
PDE	=	Phosphodiesterase
PDE-4	=	Phosphodiesterase-4
PDE-5	=	Phosphodiesterase-5
PHE	=	Phenylalanine
PKC	=	Protein Kinase C
PLA_2	=	Phospholipase A_2
PS1	=	Presenilins 1
PS2	=	Presenilins 2
SAR	=	Structure Activity Relationship
TNF-α	=	Tumor Necrosis Factor-alpha
VAS203	=	4-amino-(6R,S)-5,6,7,8 tetrahydro-L-biopterin

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