

# A Direct Interaction Between Mitochondrial Proteins and Amyloid- $\beta$ Peptide and its Significance for the Progression and Treatment of Alzheimer's Disease

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**Abstract:** The amyloid- $\beta$  peptide (A $\beta$ ) has been associated with Alzheimer's disease (AD) for decades. The original amyloid cascade hypothesis declared that the insoluble extracellular plaques were responsible for A $\beta$  toxicity. Later, this hypothesis has been updated and soluble intracellular A $\beta$  forms and their effects within the cell have come into focus. Mitochondrial dysfunction plays an important role in the pathophysiology of AD. A $\beta$  was detected inside mitochondria and several mitochondrial proteins were found to interact directly with A $\beta$ . Such interactions can affect a protein's function and cause damage to the mitochondria and finally to the whole cell. This review summarizes the current knowledge of mitochondrial proteins directly interacting with A $\beta$  and discusses their significance for the development of therapeutics in the treatment of AD.

**Keywords:** Alzheimer's disease (AD), amyloid- $\beta$  peptide (A $\beta$ ), drug target, enzyme inhibitor, mitochondria, pharmacotherapy.

## INTRODUCTION

Alzheimer's disease (AD) is the most common cause of senile dementia and about 20 million people worldwide currently suffer from this devastating illness, with the number continuing to steadily rise due to an aging population [1]. Despite years of intensive research, the pathogenic mechanisms of AD are still not fully understood and consequently an effective treatment is yet to be developed. AD is characterized by the progressive decline of cognitive function and memory caused by the extensive death of neurons, which starts in the entorhinal cortex and hippocampus and proceeds to other parts of the brain cortex and subcortical grey matter. The main pathological hallmarks found in affected parts of the AD brain are represented by extracellular amyloid- $\beta$  (A $\beta$ ) deposits also called senile plaques, intracellular deposits of phosphorylated tau-protein called neurofibrillary tangles and diffuse loss of neurons [2, 3].

Although the aetiology of AD is still unknown, a build-up of A $\beta$  is considered to play an important role in disease progression. A $\beta$  peptide is generated from APP (amyloid precursor protein) via its sequential cleavage by  $\beta$ -secretase and  $\gamma$ -secretase. This action takes place at several intracellular

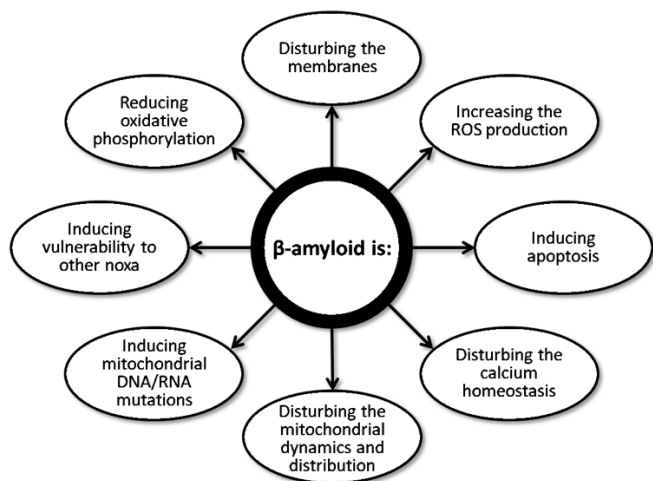
sites, including within Golgi apparatus, endoplasmic reticulum (ER), endosomal-lysosomal systems, and multivesicular bodies. Mutations in either *APP* or in the *presenilin* genes have been linked to familial, early onset forms of AD. However, these early onset cases represent only a small minority of AD patients (~5%), whereas the vast majority of AD cases have developed sporadically [4, 5]. The original amyloid cascade hypothesis defined by Hardy *et al.* in 1992, proposed that insoluble extracellular plaques were responsible for the majority of A $\beta$  toxicity. This hypothesis has since been refined, as recent data indicates that soluble intracellular oligomers are now responsible for the majority of A $\beta$  induced toxic effects [6-9].

A link between mitochondrial dysfunction and AD has long been suggested to exist. Mitochondria are central to many processes including: cellular energetic metabolism, regulation of intracellular Ca<sup>2+</sup> levels, the regulation of cell death and they are also the main source of reactive oxygen species (ROS). All these functions are found to be disturbed in AD, as well as in the presence of increased intracellular concentrations of A $\beta$  in different *in vitro* and *in vivo* studies. These findings are summarized in recent review articles, therefore suggesting that A $\beta$  is responsible for the induction of mitochondrial dysfunction typically observed in AD (Fig. 1) [10-12].

Although A $\beta$  is known to be present in mitochondria, its precise location within this compartment is a contentious issue. In most studies A $\beta$  was found associated with the in-

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ner mitochondrial membrane (IMM) and not in the mitochondrial matrix, which is in contrast with findings that A $\beta$  interacts with several proteins residing in the mitochondrial matrix. This discrepancy can be explained by the rapid A $\beta$  degradation occurring in the matrix by mitochondrial proteases (e.g. PreP or IDE) [13-15].



**Fig. (1).** Possible mitotoxic mechanisms induced by  $\beta$ -amyloid in AD. Reproduced with permission, from Tillement *et al. Mitochondrion* **2011**, *11*, 13-21 [10]. © Elsevier.

Also the origin of mitochondrial A $\beta$  is still a matter of debate. There is experimental evidence for both local production (described later in the mitochondrial  $\gamma$ -secretase section of this review) and direct import either from ER or from cytosol. The strongest evidence supports the hypothesis that A $\beta$  can be transported into the mitochondria from the cytosol via mitochondrial TOM/TIM (translocase of the outer membrane/ translocase of the inner membrane) protein-import machinery. In accordance with this theory, TOM40 complex was shown to transport A $\beta$  across the outer mitochondrial membrane (OMM). However, the mechanistic details of how A $\beta$  gains access to the different mitochondrial sub-compartments are currently unestablished, since there are several pathways for protein translocation across (TIM23 complex;) and into the IMM (TIM23 or TIM22 complexes). Another theory assumes mitochondria-associated membranes (MAMs) of the ER to be responsible for the import of A $\beta$  from this compartment. MAMs are a physical connection between the ER membrane and the mitochondrial outer membrane, where lipids and membrane proteins are thought to be exchanged directly between the organelles (Fig. 2) [13-15].

The particular molecular mechanism, through which A $\beta$  exerts its toxicity in mitochondria, has not yet been determined. However, several mitochondrial proteins were ascertained to, or are thought to, directly interact with A $\beta$ . Such interactions could lead to the disruption of their physiological functions and consequential mitochondrial dysfunction finally resulting in the development or progression of AD. In this article, we bring together an overview of proteins that have been suggested to directly interact with A $\beta$ . We discuss their implications for AD pathogenesis and the potential of their pharmacological targeting with regards to therapeutics

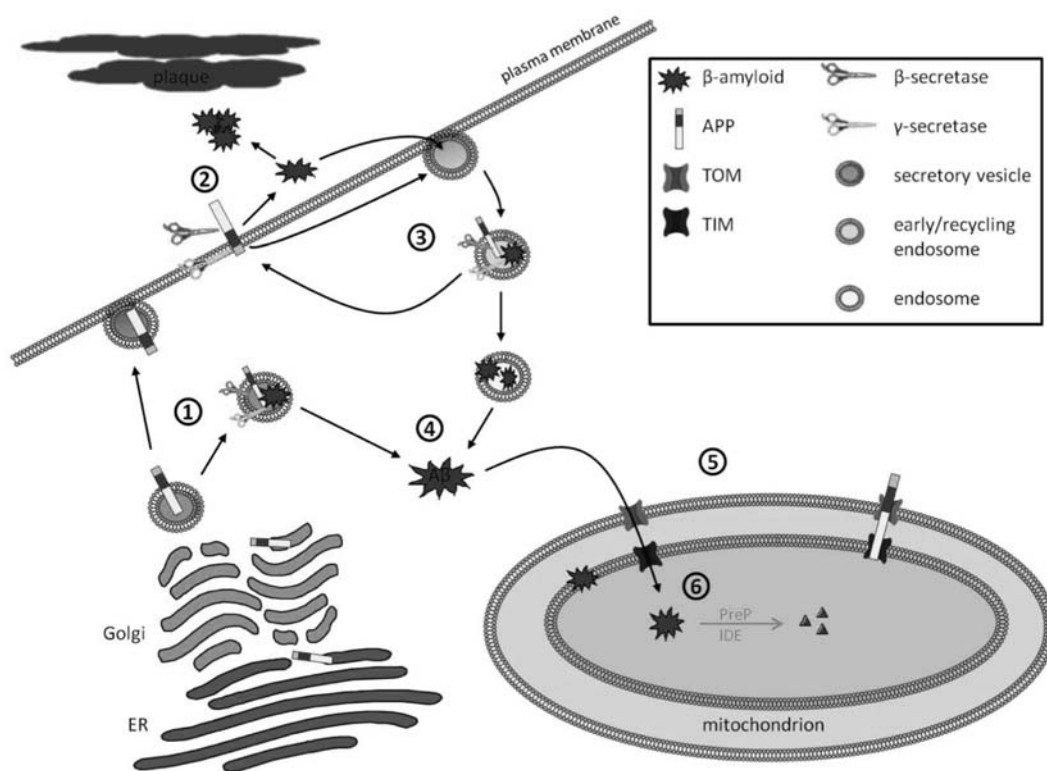
for AD. For each of these proteins we have also tried to present validated compounds that may be useful in AD treatment. In terms of druggability, the AD targeted compounds should be designed to fulfil the criteria of being able to cross the blood-brain barrier (BBB), therefore at the end of each chapter the reader can find a table of calculated physical-chemical properties (ACDLabs PhysChemSuite 12.0 [16]) for the presented compounds, compared with optimal properties for CNS targeted drugs according to the updated Lipinski rule of five [17].

## AMYLOID-BINDING ALCOHOL DEHYDROGENASE (ABAD)

Amyloid-binding alcohol dehydrogenase (ABAD) is a 27 kDa tetrameric, NAD-dependent oxidoreductase, member of the short chain dehydrogenase reductase (SDR) family, residing in the mitochondrial matrix. ABAD is a multifunctional enzyme catalysing the reduction of aldehydes and ketones and oxidation of alcohols and as such it is known to act on a broad range of structurally diverse substrates and consequently to participate in several metabolic pathways. Its main physiological function is its involvement in the third step of  $\beta$ -oxidation of fatty acids with short branched side chains. ABAD's ability to utilise the ketone body  $\beta$ -hydroxybutyrate makes ABAD play an important role for the cell's capacity to overcome periods of metabolic stress. The enzyme is also required in isoleucine catabolism. Furthermore, a role in sex steroid and neurosteroid metabolism was also attributed to ABAD due to its 3 $\alpha$ - and 17  $\beta$ -hydroxysteroid dehydrogenase activities [14, 18, 19].

ABAD is currently the most characterized A $\beta$ -binding intracellular protein and the interaction between ABAD and A $\beta$  (both 1-40 and 1-42) has been demonstrated by many different approaches. The finding that ABAD's expression levels are increased in the brains of AD patients further supports the connection between ABAD and AD. A $\beta$  was shown to bind to ABAD on a region of the enzyme called loop D, changing its conformation and preventing the binding of the enzyme's cofactor NAD<sup>+</sup> in a competitive manner, thus inhibiting enzyme activity at micromolar concentrations (Fig. 3). This binding also occurs in nanomolar levels, which is also indicative of the cytotoxic effect of A $\beta$ . Furthermore, only catalytically active ABAD was shown to enhance A $\beta$  toxicity, as cell culture studies found that the expression of a catalytically inactive form of ABAD in the presence of A $\beta$  does not enhance cytotoxicity. Similar conclusions were also made for *in vivo* studies with transgenic mice. Although, the precise mechanism of ABAD-mediated A $\beta$  toxicity is currently unknown, it is independent of enzyme's inactivation. Combining the evidence from these studies reveals that the binding of A $\beta$  to ABAD triggers a cascade of events leading to mitochondrial dysfunction a characteristic of AD [14, 18, 19].

Lustbader *et al.* established that the ABAD-A $\beta$  interaction is a suitable therapeutic target by using the ABAD-decoy peptide (ABAD-DP)[20]. ABAD-DP mimics the binding region of ABAD for A $\beta$  (loop D). *In vitro* studies found ABAD-DP bound to A $\beta$ 1-40 and A $\beta$ 1-42 making it unable to interact with ABAD. When tested in cell culture experiments, ABAD-DP ameliorated the mitochondrial impairment



**Fig. (2).** APP and A $\beta$  inside the cell. During protein synthesis, the APP is targeted to the ER and transported to the plasma membrane by vesicular transport through the Golgi apparatus. Amyloidogenic processing of APP by the  $\beta$ - and  $\gamma$ -secretases at the plasma membrane produces the A $\beta$ . This cleavage has also been found to take place prior to exocytosis in the trans-Golgi network (1). A $\beta$  can aggregate extracellularly forming extracellular plaques, which are one of the hallmarks of AD (2). APP undergoes endocytosis and is normally recycled to the plasma membrane via recycling endosomes. A $\beta$  peptides can also enter the cell by endocytosis, but can also be produced from APP by  $\beta$ - and  $\gamma$ -secretase cleavage in endosomes (3). A $\beta$  can also compromise the integrity of endosomes and secretory vesicles and can be found in the cytosol, probably due to leakage out of these compartments (4). A $\beta$  can be imported into the mitochondria via the TOM/TIM mitochondrial import machinery. Owing to its chimaeric targeting sequence, APP can also be transported to mitochondria where it gets stuck within TOM and TIM proteins, disturbing mitochondrial protein import (5). Mitochondrial peptidases PreP and IDE are capable of degrading A $\beta$  in the mitochondrial matrix (6). Reproduced with permission, from Muirhead *et al.*, 2010, *Biochemical Journal*, **426**(3), 255-270. © The Biochemical Society [14].

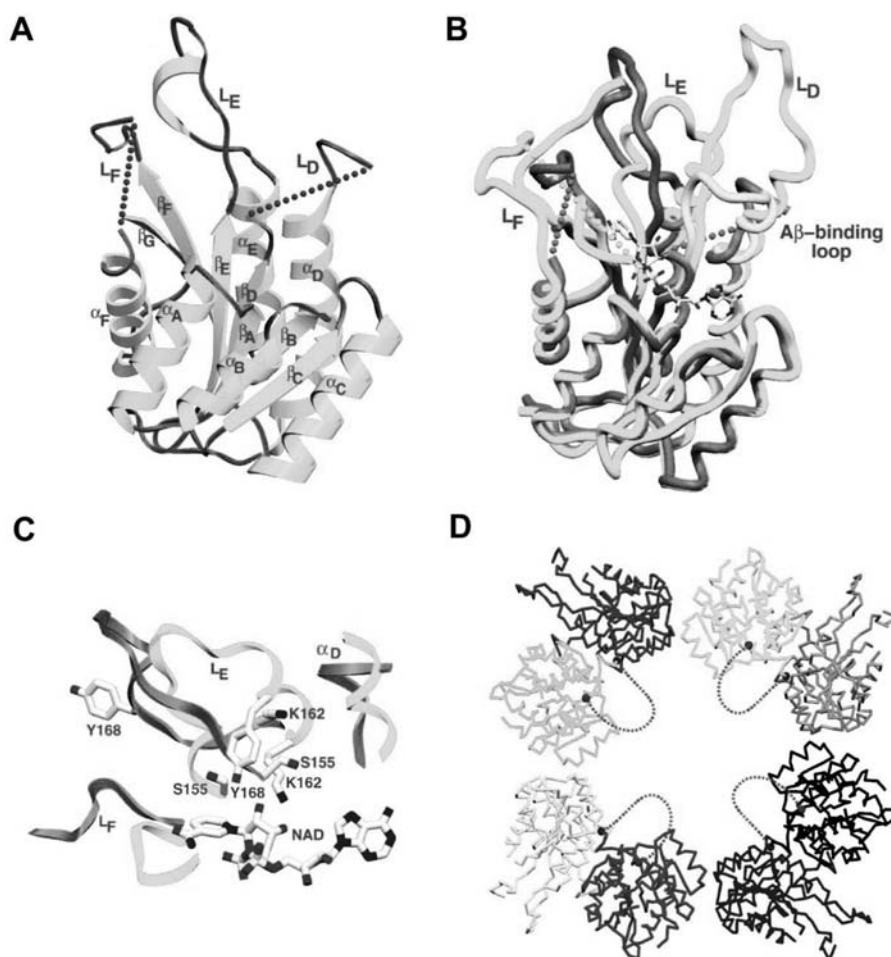
caused by A $\beta$  and its cytotoxicity. Transgenic AD mice treated with ABAD-DP displayed improved cognitive function [20-22]. In an *in vitro* screen searching for small molecule inhibitors of the ABAD-A $\beta$  interaction, frentizole (an FDA approved immunosuppressant; Fig. 4; Table 1) was identified. Consequently, several analogue series of frentizole compounds have been generated and two of these compounds (4a, 4b; Fig. 4; Table 1) were tested (*in vitro*) for their ability to ameliorate A $\beta$ 1-42 cytotoxicity with promising results [23, 24].

As ABAD activity is necessary for enhancing A $\beta$  toxicity, ABAD inhibitors could also be used as a therapeutic for AD. However, some side effects may be encountered by the use of such treatments as this may potentially cause the inhibition of the enzyme's physiological functions. Currently only two specific small molecule ABAD inhibitors are known; AG18051 (Fig. 4; Table 1) an irreversible inhibitor creating a covalent adduct with the cofactor NAD<sup>+</sup> inside the active site. AG18051 has been shown in several studies to decrease A $\beta$ 1-42 toxicity at the cellular level [25, 26]. The second compound, named RM-532-46 (Fig. 4; Table 1), is a reversible ABAD inhibitor with a steroid structure [27].

## CYCLOPHILIN D (CypD)

Cyclophilin D (CypD), also termed peptidyl-prolyl *cis-trans* isomerase F, is a 22 kDa protein residing in the mitochondrial matrix. CypD has several enzymatic functions including roles in protein folding (*cis-trans* isomerisation of peptide bonds) and as a chaperone protein. However, it was later shown that CypD (and other cyclophilins) do not play an essential role in protein folding and it was proposed that each cyclophilin could regulate a restricted number of unique partner proteins. CypD was found to play a role in the regulation of the mitochondrial permeability transition (mPT) and within the regulation of ATP synthase activity. Recent studies suggest that these two effects may be closely related [28-30].

Mitochondrial permeability transition could be defined as the collapse of the chemiosmotic gradient across the IMM mediated by the opening of a large conductance pore called the mitochondrial permeability transition pore (mPTP), whose molecular composition remains unknown. The mPTP plays a key role in cell death and has proved to be a viable target for therapeutic intervention in a variety of diseases including neurodegeneration. CypD is a key regulator and component of mPTP. In times of cellular stress CypD



**Fig. (3).** Crystal structure of A $\beta$ -bound human ABAD. **A**) A ribbon diagram with labeled secondary structures and the loop D (L<sub>D</sub>), L<sub>E</sub>, and L<sub>F</sub> loops. Helices are shown in green,  $\beta$  strands are shown in blue, and loops are shown in pink. Disordered regions are shown by dotted lines. **B**) Superposition of A $\beta$ -bound human ABAD (pink) and rat ABAD in complex with NAD (blue). The L<sub>D</sub> of 3 $\alpha$ -hydroxysteroid dehydrogenase is shown in yellow to demonstrate that L<sub>D</sub> of ABAD contains a unique insertion absent in all other SDR family members. NAD is shown as a stick model with gray for carbon atoms, red for oxygen atoms, blue for nitrogen atoms, and yellow for phosphate atoms. The proposed A $\beta$ -binding loop is indicated. **C**) Superposition of the active sites of A $\beta$ -bound human ABAD (pink) and rat ABAD (blue), showing distortion of the NAD binding site and the catalytic triad S<sup>155</sup>, K<sup>162</sup>, and Y<sup>168</sup>. Colors are the same as in B). **D**) A section of the crystal packing interactions, showing the large solvent channels. Each ABAD molecule is shown in a different color. The ordered ends of the L<sub>D</sub>, residues 94 and 114, are marked as red and blue balls, respectively, and the hypothetical loops are shown in pink as dotted lines. Reproduced with permission, from Lustbader *et al. Science* **2004**, 304, 448-452 [20]. © The American Association for the Advancement of Science.

translocates from the mitochondrial matrix to the IMM, where it interacts with components of the mPTP inducing pore opening [28-30]. CypD mediated mPTP opening was shown to be involved in AD pathophysiology by several different approaches both *in vitro* and *in vivo*. Moreover, A $\beta$  was shown to induce mPTP opening and CypD deficiency/inhibition was shown to reduce A $\beta$  cytotoxicity. A more detailed account of these findings are presented in several recent review articles [30-33].

CypD was also found to directly interact with A $\beta$  showing a higher affinity to oligomeric rather than to monomeric A $\beta$  and to A $\beta$ 1-42 over A $\beta$ 1-40 [34]. The binding between CypD and A $\beta$  was also studied using computational simulations [35]. It was suggested that CypD mediated A $\beta$  toxicity could be driven in two ways, indirectly by elevated ROS levels caused by A $\beta$  accumulation inside mitochondria and directly, specifically via the CypD-A $\beta$  interaction [31].

CypD inhibitors were shown to suppress mPTP opening and therefore represent potential drugs against diseases, in which increased mPT and cell death takes place including AD [28]. Cyclic undecapeptide cyclosporine A (CsA; Fig. 5; Table 2), which is used clinically as an immunosuppressant, is the best-known CypD inhibitor. CsA lacks clinical significance because of its immunosuppressive effect mediated by calcineurin inhibition and its inability to pass through the blood-brain barrier. However, several non-immunosuppressive CsA derivatives have been developed, including alisporivir (Debio 025, UNIL-025), NIM 811 and SCY-635 [36, 37]. Macrolide sangliferin A (SfA; Fig. 5; Table 2) and other sangliferins present another class of CypD inhibitors with similar mechanisms of action to CsA. Although SfA does not inhibit calcineurin activity, it still exerts an immunosuppressive effect by an unknown mechanism different to CsA [38]. Interestingly, synthetic SfA analogues devoid of immunosuppressive properties and with improved

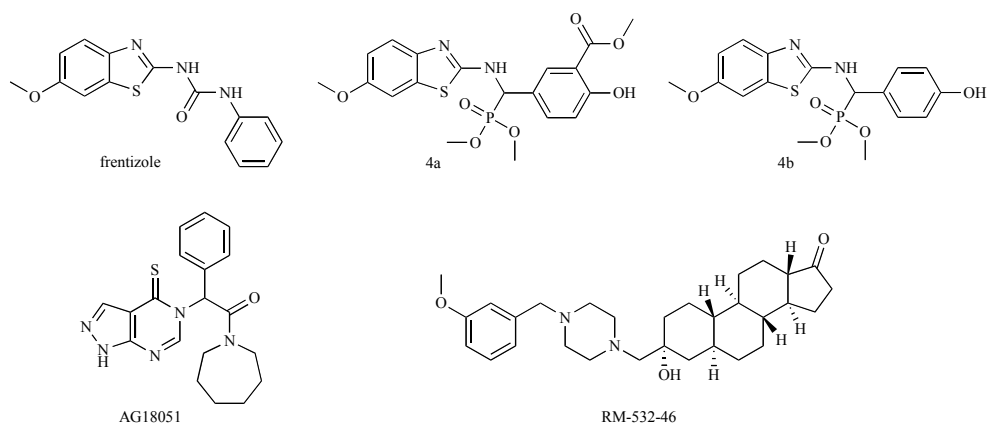


Fig. (4). Structure of selected ABAD modulators.

Table 1. Predicted Physical-chemical Properties of Selected ABAD Modulators Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
Frentizole	299.35	5/2	91	3.2	2.5	7.0
4a	452.42	9/2	154	4.0	4.0	9.3
4b	394.38	7/2	128	3.0	3.0	9.4
AG18051	367.47	6/1	97	2.0	1.9	8.8
RM-532-46	480.68	5/1	53	4.4	4.1	14.7

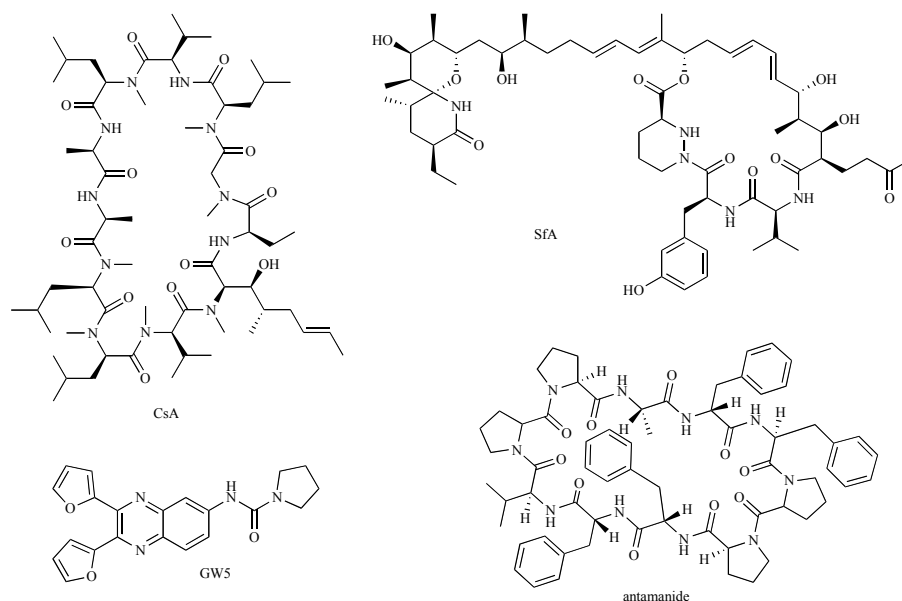


Fig. (5). Structure of selected CypD inhibitors.

pharmacokinetics were prepared [38, 39]. Recently a cyclic decapeptide antamanide (Fig. 5; Table 2) was identified as a CypD inhibitor. Unfortunately, it seems to have the same therapeutic disadvantages as the previously described CsA [40].

Collectively, currently available CypD inhibitors lack clinical significance in AD, because they are large molecules with high molecular weights, resulting in poor cell permeability and an inability to cross the blood-brain barrier [36, 37]. Moreover, these inhibitors are not specific for CypD

**Table 2. Predicted Physical-chemical Properties of Selected CypD Inhibitors Compared to Optimal Properties for CNS Targeted Drugs [16, 17]**

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
CsA	1202.61	23/5	279	2.8	2.8	13.3
SfA	1090.39	18/9	273	6.3	6.3	9.9
GW5	374.39	7/1	84	3.6	3.6	12.7
antamanide	1147.37	20/6	256	4.0	4.0	13.4

over the other cyclophilin subtypes. Hence, there is a need for developing new small molecules that can overcome any potential side effect and specificity issues. In search for small molecule CypD inhibitors, Gue *et al.* synthesized a series of novel quinoxaline analogues. Remarkably, one of these compounds (GW5; Fig. 5; Table 2) exhibited some selectivity for binding CypD over CypA [41]. Other small molecule inhibitors could be found from the results of crystallographic studies, which identified the molecular differences between CypD and other cyclophilins [42].

#### ADENINE NUCLEOTIDE TRANSLOCASE (ANT)

Adenine nucleotide translocase (ANT), a mitochondrial protein that facilitates the exchange of ADP and ATP across the IMM, plays an essential role in cellular energy metabolism. Human ANT presents four isoforms (ANT1-4), each with a specific expression depending on the nature of the tissue, cell type, developmental stage and status of cell proliferation. Thus, ANT1 is specific to muscle and brain tissues, ANT2 occurs mainly in proliferative undifferentiated cells, ANT3 is expressed ubiquitously and ANT4 is found in germ cells. ANT1 and ANT3 export the ATP produced by oxidative phosphorylation (OxPhos) from mitochondria into the cytosol while importing ADP. In contrast, ANT2 imports glycolytically produced ATP into the mitochondria, when OxPhos activity is impaired [43]. ANT1 and ANT3 seem to act proapoptotically, possibly due to their involvement in mPT, whereas ANT2 and ANT4 seem to act antiapoptotically [44].

Early studies suggested that ANT is a critical component of mPTP, along with voltage-dependent anion channel (VDAC) in the OMM and CypD in the matrix. In contrast with this theory, Kokoszka *et al.* inactivated the two ANT isoforms (ANT1 and ANT3) relevant for mPT in mice and they still detected the opening of mPTP triggered by  $\text{Ca}^{2+}$ , suggesting that ANT is not an essential part of the mPTP. However, ANT still seems to play a role in the regulation of mPTP since more  $\text{Ca}^{2+}$  was required to activate the mPTP and ANT ligands no longer regulated the mPTP in this mouse model [45]. Recent studies defined the composition of mPTP as the dimer of the  $\text{F}_0\text{F}_1$ -ATP synthase that is regulated by CypD. In view of the fact that adenine nucleotides are the substrates of the ATP synthase, ANT may contribute to mPTP regulation by affecting nucleotide levels in the matrix, where the  $\text{F}_1$ -ATPase sector of the ATP synthase is located [46].

The connection between AD and ANT is mostly based on its involvement in mPTP formation and cell death. A direct interaction between ANT and  $\text{A}\beta$  was firstly predicted in a computational simulation and later described using an *in vitro* assay. In that study  $\text{A}\beta$  1-42 fibrils were shown to inhibit ANT1 mediated ADP/ATP exchange in a non-competitive manner [35, 47]. However, the consequences of such an interaction remain elusive as well as characterising the role of ANT in mPT and cell death.

Due to its regulatory role in mPTP opening, ANT (or more precisely isoforms 1 and 3) presents a potential pharmacological target for the treatment of AD and other diseases, in which pronounced cell death takes place. Interestingly, ANT inhibitors can have different effects on mPT and cell death regulation. Carboxylatractyloside (CAT) and bongkreikic acid (BKA; Fig. 6; Table 3), both inhibitors of ANT, were shown to exert opposite effects on mPTP opening. CAT binds to the intermembrane space (IMS) side of ANT, from the cytosolic surface and induces the termed “c” conformation of ANT and sensitizes pore opening by  $\text{Ca}^{2+}$ . Conversely, BKA binds to the matrix side of ANT, promoting the “m” conformation and inhibiting pore opening. The transition between CAT and BKA conformations is suggested to be the structural switch involved in ADP/ATP transport [46, 48]. Based upon the above mentioned data, only ANT inhibitors with effects similar to BKA can be considered for AD treatment.

Several synthetic analogues which focused on the structure of BKA (Fig. 6; Table 3) were designed and synthesized in an attempt to find new mPT inhibitors [49, 50]. Nelfinavir (Fig. 6; Table 3), an inhibitor of HIV protease, was also shown to inhibit mPT perhaps via an interaction with ANT [51, 52]. Additionally, *N*-ethylmaleimide and nitro-linoleate (LNO<sub>2</sub>; Fig. 6; Table 3) were found to modify ANT's critical cysteine residues leading to the inhibition of both ANT and mPT [53, 54].

#### VOLTAGE-DEPENDENT ANION CHANNEL (VDAC)

Voltage-dependent anion channel (VDAC) is a small (31 kDa) highly conserved protein residing primarily in the outer mitochondrial membrane of all eukaryotes. Furthermore, VDAC can also be found inside the plasma membrane and ER. VDAC's principal function as a membrane channel in the OMM is to facilitate and regulate the flow of ions, nu-

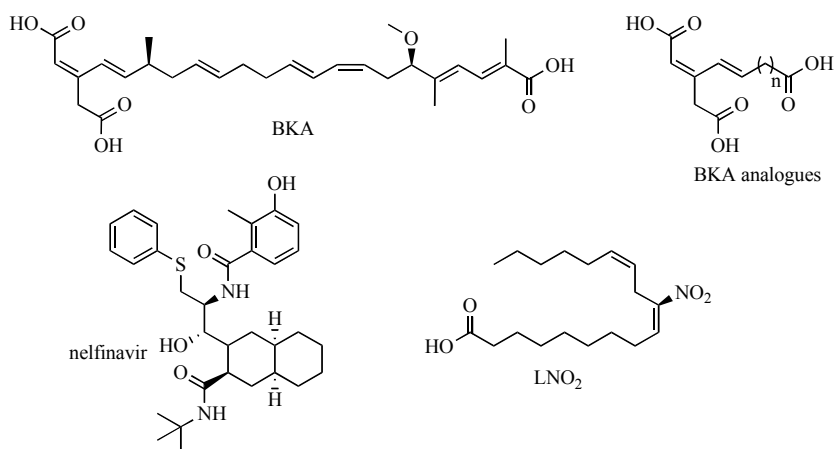


Fig. (6). Structure of selected ANT inhibitors.

Table 3. Predicted Physical-chemical Properties of Selected ANT Inhibitors Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
BKA	486.6	7/3	121	4.6	-1.1	4.2
nelfinavir	552.77	6/4	124	7.0	7.0	9.6
LNO <sub>2</sub>	325.44	5/1	83	4.1	1.5	4.8

cleotides and other metabolites between the cytosol and the mitochondrial intermembrane space. Therefore, it plays an important part in the regulation of energetic metabolism,  $\text{Ca}^{2+}$  signalling and cell death [55, 56]. VDAC functions can be affected by posttranslational modifications occurred via oxidation, phosphorylation, carbonylation or nitration. Subsequently, several VDAC binding partners have also been identified, e.g. members of Bcl-2 family, hexokinase and many others. Hence, from these findings VDAC seems to be involved in many different signalling pathways inside the cell [55, 57].

Three eukaryotic VDAC isoforms, encoded by three separate genes, VDAC1, VDAC2 and VDAC3, have been identified in mitochondria. The specific role of each isoform remains unclear, although evidence indicates that the three isoforms may serve different physiologic functions in different tissues. It seems apparent that VDAC1 has a proapoptotic function, VDAC2 an anti-apoptotic function and VDAC3 has no influence on apoptosis. As a consequence of this VDAC1 and 2 are involved in the control of ROS. It should also be mentioned, that before the three isoforms were discovered, the majority of studies conducted on VDAC were in fact conducted on the VDAC1 isoform [58].

The connection between VDAC and AD was originally based upon the assumption, that VDAC is a structural component of the mPTP. This theory has recently been challenged, although VDAC still seems to participate in the regu-

lation of cell death and apoptosis in AD [59, 60]. Elevated VDAC1 levels were found in AD brain samples and VDAC1 levels were also shown to increase in an age-dependent manner [55, 60]. However, its specific contribution to the development of AD is unknown.

The direct interaction of VDAC1 with monomeric and oligomeric  $\text{A}\beta$  as well as full length APP and phosphorylated tau was described by Manczak and Reddy. In this work they suggest that such interactions may in turn block mitochondrial pores and interrupt crosstalk between mitochondria and cytosol leading to mitochondrial dysfunction or in the rupture of the OMM. In relation to the interaction between  $\text{A}\beta$  and VDAC1, the C-terminus of the  $\text{A}\beta$  peptide motif 'GSNKG' (at positions 25-29) has been proposed to interact with the N-terminal motif of VDAC1 'GYGFG' [61].

However, there are some other theories considering the role of VDAC in AD or cell death. Thinnes suggested, that the interaction of  $\text{A}\beta$  and VDAC1 (within the plasma membrane), and its consequent opening could present the basic model for an extrinsic apoptotic pathway and the cause of AD [56]. Moreover, VDAC1 found localized in this region, was suggested to mediate the neuroprotective effect of estradiol against  $\text{A}\beta$  induced toxicity [62]. Other theories consider the formation of a mitochondrial pore based upon VDAC oligomerization or VDAC hetero-oligomerization together with Bax, which could lead to the release of cytochrome *c* and cell death [57].

Because of its ambiguous role in AD pathophysiology, exploiting VDAC as a drug target for AD therapeutics is a contentious issue. However, there are some drugs that have been shown to exert cytoprotective effects via an interaction with VDAC, namely olesoxime (TRO-19622), a cholesterol-like neuroprotectant for the potential treatment of amyotrophic lateral sclerosis (ALS), or fluoxetine, a clinically used antidepressant (Fig. 7; Table 4) [63, 64].

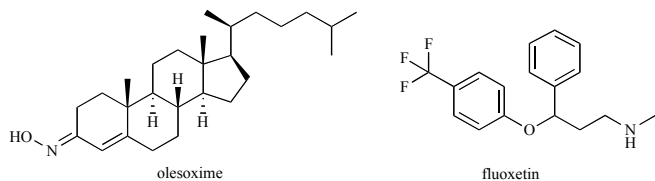


Fig. (7). Structure of selected VDAC interacting compounds.

### ELECTRON TRANSPORT CHAIN (ETC)

The mitochondrial ETC (electron transport chain) is composed of five enzyme complexes embedded in the inner membrane of the mitochondria. The reduced equivalents of NADH and FADH<sub>2</sub> derived from the oxidation of carbohydrates and fatty acids flow from Complex I through Complex IV via a series of oxidation-reduction events. The energy, released during the process, charges the inner mitochondrial membrane and drives ATPase (Complex V) to synthesize ATP from ADP and inorganic phosphate [65].

The impairment of the respiratory chain can lead to decreased electron transfer efficiency, reduced ATP production, and increased reactive oxygen species (ROS) production by the mitochondria, which are typical hallmarks of AD [10]. The most reported defect in the ETC, in connection with AD, is the decreased activity of Complex IV. Changes in activity of the remaining ETC complexes were also reported, but the data is inconsistent, varying from inhibition to activation. Aβ was reported to directly interact with Complex I, IV and V, which in all cases lead to their inhibition. Generally, enzyme complexes of ETC do not appear to be feasible drug targets for the treatment of neurodegenerative disorders.

### COMPLEX I

In Complex I (NADH: ubiquinone oxidoreductase; EC 1.6.5.3), two electrons are removed from NADH and transferred to a lipid-soluble carrier, ubiquinone. The reduced product, ubiquinol, freely diffuses within the membrane and

Complex I translocates four protons across the membrane, thus producing a proton gradient. Complex I is one of the main sites at which premature electron leakage to oxygen occurs, thus making it one of the main sites of superoxide production [66].

The direct interaction between Aβ and Complex I was described in two independent studies and the C-terminal sequence of the ND3 subunit was identified as the binding site for Aβ1-42 [65, 67]. Aβ was shown to inhibit Complex I activity and to increase Complex I-dependent production of ROS, which in turn lead to mitochondrial dysfunction. However, even when a mitochondrial defect can be attributed to Complex I, it is hard to determine whether the effects observed arise from a loss of Complex I activity, an increase in its ROS production, or both [65].

Interestingly, MPTP and rotenone, classical Complex I inhibitors, are used for creating a pharmacological model of Parkinson's disease (PD), which further supports the role of Complex I in neurodegeneration [68]. The natural compound bilobalide (Fig. 9; Table 5) and its two synthetic analogues were shown to possess protective effects towards the ETC [69]. Their studies revealed that they were able to prevent the inhibition of complex I by amobarbital [69], unfortunately the precise mechanism of action is unknown, so we can only speculate whether this effect is mediated via a direct interaction with Complex I.

### COMPLEX IV

In Complex IV (cytochrome *c* oxidase; EC 1.9.3.1), four electrons are removed from four molecules of cytochrome *c* and transferred to molecular oxygen, producing two molecules of water. At the same time, four protons are removed from the mitochondrial matrix (although only two are translocated across the membrane), contributing to the proton gradient [66].

Decreased activity of Complex IV was reported in AD patient brain samples [70, 71], transgenic animal models [72, 73] and *in vitro* studies using Aβ [74, 75]. Recently, a direct interaction of Aβ1-42 with cytochrome *c* oxidase subunit I was also reported (Fig. 8) [76]. Another study showed that the apparent *K<sub>m</sub>* of cytochrome *c* oxidase for reduced cytochrome *c* was raised, indicating that Aβ 1-42 and Aβ25-35 may act as an inhibitors for cytochrome *c* binding [75].

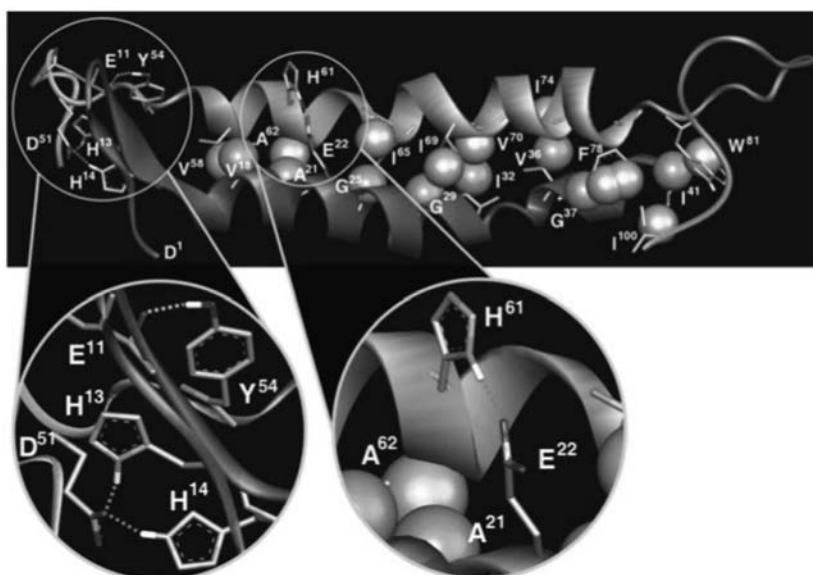
In regard to AD treatment, caprospinol (Fig. 9; Table 5), a naturally occurring, stable steroid molecule devoid of steroid activity demonstrates neuroprotective properties, which,

Table 4. Predicted Physical-chemical Properties of Selected VDAC Interacting Compounds Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [Å <sup>2</sup> ]	ClogP ± SD	ClogD	CpKa ± SD
		acc./don.			(pH 7.4)	
Optimal properties	≤400	≤7/≤3	≤60-70	1-5	0-3	4-10
olesoxime	399.65	2/1	33	9.7	9.7	12.3
fluoxetine	309.33	2/1	21	3.9	1.4	NA*

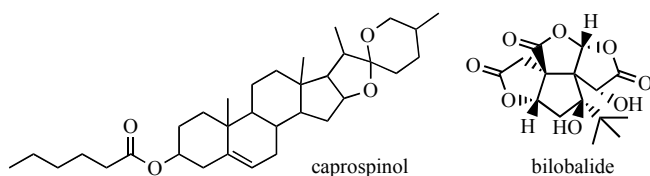
\* Not available.





**Fig. (8).** Molecular Dynamics complex of A $\beta$ 1-42 (blue) and cytochrome *c* oxidase subunit 1 (gold). Upper panel: nine pairs of carbons (spheres) interacting through van der Waals forces forming hydrophobic contacts between both peptides are shown. Insets show the hydrogen-bond (green dots) between E11-Y54 (left); the salt bridge (red dots) of H13 and H14 with D51 (left) and the salt bridge between E22-H61 (right). Reproduced from Hernandez-Zimbron *et al. PLoS One* **2012**, 7, e42344 [76]. © Hernandez-Zimbron *et al.*

amongst its other roles, is attributed to affecting complex IV and V of respiratory chain [77, 78].



**Fig. (9).** Structure of selected neuroprotectants that affecting ETC.

### ATP SYNTHASE (COMPLEX V)

The electrochemical gradient created by proton pumps (Complex I, III and IV) is used by the F<sub>0</sub>F<sub>1</sub> ATP synthase complex to generate ATP via oxidative phosphorylation. The F<sub>0</sub> component of ATP synthase acts as an ion channel that provides a proton flux into the mitochondrial matrix. This reflux releases free energy, which is used to drive ATP synthesis catalyzed by the F<sub>1</sub> component of the complex [66]. This complex was also found on the cell surface, where it is capable of synthesizing ATP extracellularly [79, 80].

The direct binding of A $\beta$ 1-40 to the  $\alpha$ -subunit (a member of the F<sub>1</sub> component) of cell surface ATP synthase and its consequent inhibition has been previously described [80, 81]. Furthermore, it was proposed that the inhibition of cell surface ATP synthase by A $\beta$  may consequently affect synaptic plasticity leading to memory deficits in AD [80-82]. Whether this interaction also takes place in mitochondria has not yet been confirmed.

### $\alpha$ -KETOGLUTARATE DEHYDROGENASE COMPLEX (KGDHC)

$\alpha$ -ketoglutarate dehydrogenase complex (KGDHC) catalyzes the oxidative decarboxylation of  $\alpha$ -ketoglutarate to succinyl-CoA, a key step in the tricarboxylic acid cycle. Its

function is therefore important for energy metabolism, production of reducing equivalents (NADH), glutamate neurotransmission and protein synthesis. KGDHC consists of three protein subunits E1k ( $\alpha$ -ketoglutarate dehydrogenase, E.C. 1.2.4.2), E2k (dihydrolipoamide succinyltransferase, EC 2.3.1.61) and E3 (dihydrolipoamide dehydrogenase, EC 1.8.1.4) and is located within the IMM [83, 84].

Diminished KGDHC activity occurs in brain samples of AD patients and it appears that decreased KGDHC activity in the AD brain is part of a neurodegenerative cascade [83, 85]. Oxidative stress is most often mentioned as a link between KGDHC deficiency and AD, because KGDHC is particularly vulnerable to free radicals and elevated ROS levels [83, 86]. On the other hand, there is also evidence suggesting that KGDHC could be inhibited by a direct interaction with A $\beta$ . A $\beta$ 25-35 was shown to inhibit KGDHC activity in a dose dependent manner for both purified enzyme and isolated mitochondria [75, 87]. However no binding studies, which would confirm the direct interaction have been reported to date.

### PYRUVATE DEHYDROGENASE COMPLEX (PDHC)

Pyruvate dehydrogenase complex (PDHC) is distributed heterogeneously within the mitochondrial matrix, where it catalyzes the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA. This reaction links the glycolysis in the cytoplasm to the oxidative phosphorylation in the mitochondria and is rate-limiting (under aerobic conditions) for the oxidative removal of glucose and pyruvate [88, 89]. PDHC is composed of multiple copies of three catalytic, one binding and two regulatory components, namely PDH (pyruvate dehydrogenase, EC 1.2.4.1), E2 (dihydrolipoamide acetyltransferase, EC 2.3.1.12) and E3 (dihydrolipoamide dehydrogenase, EC 1.8.1.4) plus E3BP (E3-binding protein), PDHK (PDH kinase) and PDP (PDH phosphatase) [88].

**Table 5. Predicted Physical-chemical Properties of Selected Neuroprotectants Affecting ETC Compared to Optimal Properties for CNS Targeted Drugs [16, 17]**

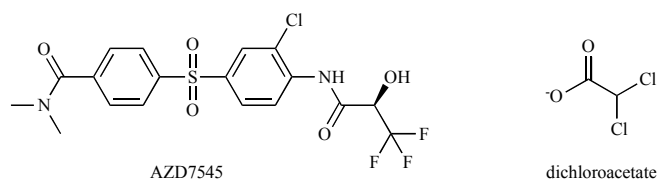
Compound	Mw	H-bond	PSA [Å <sup>2</sup> ]	ClogP ± SD	ClogD	CpKa ± SD
		acc./don.			(pH 7.4)	
Optimal properties	≤400	≤7/≤3	≤60-70	1-5	0-3	4-10
caprospinol	512.76	4/0	45	9.6	9.6	NA*
bilobalide	326.30	8/2	119	4.5	4.5	11.7

\* Not available.

Postmortem analyses of brains from patients with AD have repeatedly demonstrated significant decreases in the activity of the PDHC and similar results were obtained using an AD animal model [85, 90]. The mechanism behind PDHC deficiency in AD has not yet been clarified, although several hypotheses exist including enzyme oxidation due to increased oxidative stress [91] or increased phosphorylation by TPPI/GSK-3β [92, 93].

Another possibility is that PDHC could be inhibited by a direct interaction with Aβ. Aβ25-35 was shown to inhibit PDHC activity in a dose dependent manner for both purified enzyme and isolated mitochondria [75, 87]. Similarly to KGDHC, no binding studies that would further confirm this hypothesis have been reported as yet.

Activation of PDHC is possible through the inhibition of its regulatory kinase PDHK. Synthetic PDHK inhibitors (e.g. sodium dichloroacetate or AZD7545; Fig. 10; Table 6) were developed for the treatment of several diseases such as diabetes, heart disease and cancer. Though their application for the treatment of neurodegenerative diseases has also been suggested, no such studies have been performed as yet [89, 94, 95].

**Fig. (10).** Structure of selected PDHK inhibitors.

### CREATINE KINASE (CK)

Creatine kinase (CK; EC 2.7.3.2) catalyzes the reversible conversion of creatine and ATP to phosphocreatine and ADP. Therefore, CK is an important mediator of cellular homeostasis since it can create a pool of phosphocreatine for temporal and spatial ATP buffering. Four CK isoforms are distinguished based on their tissue expression (muscle or brain) and subcellular distribution (cytosolic or mitochondrial). Therefore, there are two tissue specific mitochondrial CK (mtCK) isoenzymes termed as sarcomeric mtCK, which are found in striated muscle, and ubiquitous mtCK, which is found in most other tissue types including neural tissue. Both mtCKs are located in the mitochondrial IMS [96, 97].

Changes in creatine metabolism, CK activity and CK expression were observed in AD [98-101]. CK activity in brains of AD patients was decreased whereas elevated CK levels were found in AD mouse model. The diminished CK activity may be related to oxidative stress, considering that CK is very sensitive to oxidative insult. This was supported by proteomic studies, which found CK to be significantly oxidatively modified in AD brains [98-101].

The direct interaction of CK with Aβ is a contentious issue as there is only one published study reporting indirect evidence [102]. In this study, it was shown that Aβ25-35 inhibits CK activity in a dose-dependent manner. The proposed mechanism suggested that Aβ derived radicals react non-specifically with the enzyme to oxidize it or to create covalent CK-Aβ adducts [102]. Interestingly, ubiquitous mtCK was found to directly interact with APP. This interaction stabilizes the CK pre-protein, which could play a protective role against cell stress and axonal injury [103].

### γ-SECRETASE

γ-secretase is a protein complex of approximately 500 kDa, made up of enzymatically-active PS1 or PS2, nicastrin (NCT), presenilin enhancer 2 (PEN2), and anterior pharynx-defective phenotype (APH-1) [104]. It is most often mentioned in connection with AD, because γ-secretase together with β-secretase (BACE) gives rise to Aβ by the sequential cleavage of APP. In addition to APP cleavage, the γ-secretase complex is responsible for the processing of Notch, E-cadherin, ErbB4, and p75NTR. γ-secretase activity and components of the γ-secretase complex have been found in the ER, the Golgi apparatus, the plasma membrane, the endosomal/lysosomal system and recently, the mitochondria [105, 106]. Mutations in *PS1* are responsible for more than 50% of familial AD cases. These *PS1* mutations tend to shift the γ-secretase cleavage specificity of C99 to increase the production of the toxic Aβ1-42 over Aβ1-40. *PS2* mutations are less abundant, but also lead to increased Aβ1-42 production [106].

PS1 was found to be located in mitochondria and preferentially in the IMM by Ankarcrone and Hultenby and later the whole functional γ-secretase complex was established to be present inside mitochondria by Hansson *et al.* [104, 107]. From these findings the question of whether Aβ could be produced locally in mitochondria arose. Although, APP is known to be imported to mitochondria, it was shown to be trapped inside the mitochondrial importers TOM40 and

**Table 6. Predicted Physical-chemical Properties of Selected PDHK Inhibitors Compared to Optimal Properties for CNS Targeted Drugs [16, 17]**

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
AZD7545	464.84	7/2	112	0.6	0.6	10.3
dichloroacetate	128.94	2/1	37	0.7	-3.1	1.4

TIM23 in an orientation which is incompatible for cleavage by mitochondrial  $\gamma$ -secretase and therefore the possibility of local A $\beta$  production seemed to be unlikely [108]. Later, an interaction between mitochondrial  $\gamma$ -secretase and HtrA2/Omi protease was described (the role of HtrA2 in AD is described in a later section of this review) and also within this study, amyloid precursor protein intracellular domain (AICD) was found to be produced from APP inside the mitochondria potentially due to the cooperation of  $\gamma$ -secretase and HtrA2 [109].

In 2011 Pavlov *et al.* described for the first time, that C83 (product of APP cleavage by  $\alpha$ -secretase) is present in the OMM and can be cleaved by the mitochondrial  $\gamma$ -secretase to produce AICD which also remains attached to the IMS side of the OMM. However, the proposed mechanism of  $\gamma$ -secretase action would lead to the release of A $\beta$  into the cytoplasm [110]. Interestingly, Devi and Ohno found C99 (a product of APP cleavage by BACE) and APP to be located in the mitoplast [111]. But it is unclear, whether there is a functional  $\gamma$ -secretase in IMM capable of C99 cleavage. Therefore the possibility of local A $\beta$  production in mitochondria remains obscure as well as the physiological function of mitochondrial  $\gamma$ -secretase.

Recently, HIG1 (hypoxia-inducible gene 1, domain member 1A), a novel regulator of  $\gamma$ -secretase activity, has been described [112]. HIG1 was found to be localized on mitochondrial membrane where it interacts with proteins of the  $\gamma$ -secretase complex. HIG1 overexpression was able to decrease mitochondrial  $\gamma$ -secretase activity and A $\beta$  intracellular production and consequently to reduce hypoxia-induced mitochondrial dysfunction. Conversely, HIG1-knockdown also resulted in mitochondrial dysfunction [112]. Based on these results, mitochondrial  $\gamma$ -secretase seems to be a potential drug target for AD therapy.

In an effort to find a therapy for AD, many  $\gamma$ -secretase inhibitors/modulators have been discovered during the past 20 years. Initial compounds were non-selective  $\gamma$ -secretase inhibitors (GSIs). These compounds actually proved to be toxic, because they inhibited not only APP processing, but also the processing of other  $\gamma$ -secretase substrates, particularly Notch 1. Therefore, currently developed GSIs are designed as APP selective or "Notch sparing". Another class of potential AD therapeutics targeting  $\gamma$ -secretase are  $\gamma$ -secretase modulators (GSMs). These compounds do not inhibit the overall enzyme activity but shift  $\gamma$ -secretase activity towards the increased production of the less toxic shorter A $\beta$  species and decreased production of the toxic longer A $\beta$  spe-

cies, principally A $\beta$ 1-42. A large amount of work has been done in the field of GSIs/GSMs and further information can be found in several other recent review articles [113, 114].

### NITRIC OXIDE SYNTHASE (NOS)

Nitric oxide synthase (NOS; EC 1.14.13.39) is presented by a family of three NO $\cdot$  synthase enzymes, neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). Each NOS isoform is encoded by a unique gene. NOS catalyzes the degradation of L-arginine to equal amounts of NO $\cdot$  and L-citrulline, and requires Ca $^{2+}$ -calmodulin for its activity [115].

NO $\cdot$  exerts a broad spectrum of functions in several systems, including the cardiovascular system, PNS, CNS and immune system. These functions are mediated through the reactions of NO $\cdot$  with targets that include hemoproteins, thiols and superoxide anions. Mitochondria possess several such targets and they are also major cellular source of superoxide anion. Therefore, mitochondria contribute to many biological functions exerted by NO $\cdot$  [115].

The presence of NOS inside mitochondria had not been conclusively validated until recently. All three NOS isoforms have been previously proposed to be located within mitochondria of different cells [116]. Furthermore, the existence of a novel isoform, specific to mitochondria, cannot be excluded [117, 118]. On the contrary, some studies suggest that NOS is not present in mitochondria at all [119]. Another theory is that mtNOS is tissue specific and/or that in different tissues the function of mtNOS could be exerted by a different NOS isoform. However, recent research conducted in this field indicated that most often nNOS $\alpha$  are identical to mtNOS [116]. mtNOS was found to be localized at the matrix side of the IMM, where it interacts with complex I and IV of the mitochondrial respiratory chain [120] and the role of mtNOS in the regulation of mitochondrial respiration and mPT was proposed [116, 117, 120].

The data regarding the role of NO $\cdot$  and changes in NOS expression levels in AD are quite inconsistent, differing from protective [121, 122] to deleterious [123, 124] and from decreased [125] to elevated [123, 124]. A possible explanation for this discrepancy could be explained by the finding that physiological NO $\cdot$  levels are neuroprotective whereas increased levels are cytotoxic [126].

Several studies suggested a connection between NOS activity and the development of AD, whereby the activity of the iNOS and eNOS isoforms were found to be protective in

AD as their gene deletion led to an enhancement of A $\beta$  toxicity and an exacerbation of AD symptoms [122, 127, 128]. Conversely, nNOS was found to be toxic in AD, where the deleterious effect could potentially be mediated by an nNOS interaction with Cdk5 and A $\beta$  [129]. Therefore, it is not surprising that inhibitors of NOS (particularly nNOS) are considered as potential AD treatment [130, 131]. However, it should be mentioned that there are also studies bringing contrary evidence for the role of particular NOS isoforms in AD and there is still a lot of questions to be answered.

Recently, the direct interaction of A $\beta$  with nNOS was described by Padayachee *et al.* [132]. Different A $\beta$  species were found to inhibit nNOS activity in a non-competitive manner and hydrophobic regions of A $\beta$  (pentapeptide patch and three glycine zipper regions) were found to be crucial for this interaction. Moreover, nNOS was found to act as a catalyst of A $\beta$  fibrillogenesis and aggregation [133]. Whether this interaction is universal for other NOS isoforms or mtNOS is yet to be determined.

As far as we are aware, there have been no published studies depicting mtNOS in AD pathology. However, mtNOS has been reported to be involved in the pathophysiology of ALS, another neurodegenerative disease, where mtNOS activity was observed to decrease in an age-dependent manner. This finding could play a part in the development of age-related disease including AD [134, 135].

Many NOS inhibitors are known, both non-specific (e.g. L-NAME; Fig. 11; Table 7) and isoform specific (e.g. nNOS-specific 7-nitroindazole and iNOS-specific aminoguanidine; Fig. 11; Table 7). However, we can still only speculate, which type of pharmacological modulation of mtNOS or other NOS isoforms would be beneficial for AD treatment [130, 131].

## PEPTIDYLARGININE DEIMINASES (PAD)

Peptidylarginine deiminases (PAD; EC 3.5.3.15), also called as protein-arginine deiminases, are a family of calcium dependent enzymes that mediate post-translational modifications of proteins' on arginine residues by deimination or demethylimination producing citrulline. There are five PAD family members (PAD1-4 and 6), each with a unique tissue distribution and functional roles [136]. PAD2 and PAD4 have been previously connected with AD pathophysiology as they can be both found in CNS. Therefore we will only further describe these two isoenzymes in this review.

PAD2 is widely expressed in multiple tissues, including secretory glands, brain, uterus, spleen, pancreas and skeletal muscle and is mainly a cytoplasmic protein, but a fraction of PAD2 may become nuclear [136]. Recent evidence suggests that PAD2 is also found in mitochondria. Moreover, levels of PAD2 expression and levels of citrullinated proteins in mitochondria were found increased in mice infected with scrapie, a fatal neurodegenerative prion disease [137]. However, there are no studies reported regarding the role of mitochondrial PAD2 in AD. PAD4 is expressed mainly in white blood cells including granulocytes and monocytes under physiological conditions and in a wide range of tumors in various tissues. Recently, PAD4 activity was also detected in neurons. Inside the cell, PAD4 is primarily located in the nucleus [136, 138].

Increased activity of PAD2 and elevated levels of citrullinated proteins were found in AD brain samples, however, no PAD2 activity was found in healthy controls. Therefore, it is assumed in a healthy individual that PAD2 normally remains inactive, but becomes activated and citrullinates cellular proteins only when the intracellular calcium balance is disrupted during neurodegenerative processes [139]. Moreover, PAD2 expression increases during life, which supports

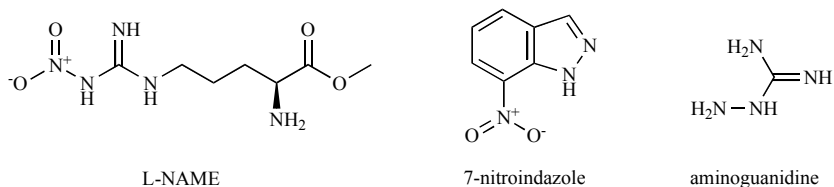


Fig. (11). Structure of selected NOS inhibitors.

Table 7. Predicted Physical-chemical Properties of Selected NOS Inhibitors Compared to Optimal Properties for CNS Targeted Drugs

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
L-NAME	233.23	9/5	146	-1.3	-3.4	5.0
7-nitroindazole	163.13	5/1	75	1.4	1.4	10.0
aminoguanidine	74.09	4/6	88	-1.5	-2.6	NA*

\* Not available.

its possible role in development of age-related diseases [140]. The toxic mechanism of citrullinated proteins in AD has not yet been elucidated. However, several theories exist for instance the autoimmunity against citrullinated proteins or negative impact on enolase activity [138, 141]. Therefore, inhibitors of PAD2/PAD4 were suggested as potential AD treatment [138, 141].

Different A $\beta$  species were shown to bind to PAD2, inhibiting its activity. The nature of the interaction is very similar to a previously described mechanism for another arginine metabolising enzyme, nNOS (see NOS section). Similarly, PAD2 was found to catalyze A $\beta$  fibrillogenesis and aggregation, although at a slower rate in comparison to nNOS [133, 142].

Many non-selective PAD inhibitors (e.g. Cl-amidine; Fig. 12; Table 8) or isoform selective (especially towards PAD4; e.g. TDFA, streptonigrin; Fig. 12; Table 8) [143, 144] have been discovered, however, a specific inhibitor of PAD2 has not yet been identified.

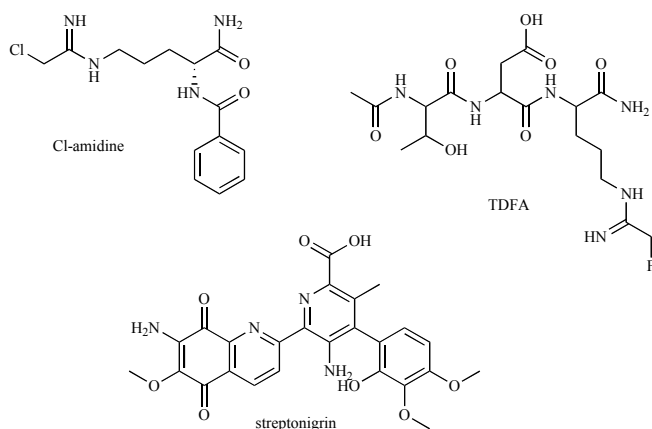


Fig. (12). Structure of selected PAD inhibitors.

### CU/ZN SUPEROXIDE DISMUTASE (SOD1)

Cu/Zn superoxide dismutase (SOD1; EC 1.15.1.1) is a ubiquitously expressed homodimeric metalloenzyme acting as a free radical scavenger. It catalyzes the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. SOD1 is predominantly localized in the cytosol, but it can also be found in other cellular compartments including the nucleus, ER, lysosomes and mitochondria. In mitochondria, SOD1 is concentrated in the IMS, but it is also found in the matrix and on the OMM [145-147].

Some mutations in the *SOD1* gene lead in the development of a familial form of the neurodegenerative disease ALS, which shares some similarities with AD and PD [148]. In regard to AD, impaired SOD1 function is connected with increased oxidative stress, which is one of AD hallmarks. Impaired function of SOD1 was also observed in AD patients and the animal models. Choi *et al.* found that SOD1 is heavily oxidized in AD and PD brains by carbonylation and that the important Cys146 thiol group is irreversibly oxidized to sulfonic acid. They also found that the total level of SOD1 is significantly increased in both AD and PD. Finally, they observed and characterized SOD1 aggregates in AD and PD brains concluding that oxidative damage to SOD1 and its subsequent aggregation may contribute to the neurodegeneration associated with AD and PD [149]. This is in agreement with other study showing that A $\beta$  accelerates SOD1 aggregation [150]. Later, Murakami *et al.* showed that SOD1 deficiency in an APP-overexpressing AD mouse model accelerated A $\beta$  oligomerization and memory impairment, when compared to control AD mice. However, unlike Choi *et al.*, they found that levels of SOD1 were significantly decreased in AD patients rather than non-AD age-matched individuals [151]. Therefore, changes in SOD1 expression and activity in regard to AD remain obscure as several studies showed inconsistent results [149, 151, 152]. In several studies increased SOD1 activity was shown to protect against A $\beta$  toxicity [153-155]. However and in contrast with these studies, Harris-Cerruti *et al.* concluded that increased expression of SOD1 has deleterious effects in APP-overexpressing mice [156].

In 2009 a direct interaction between SOD1 and various species of A $\beta$  and its consequent inhibition was observed for the first time by Yoon *et al.* and later their results were confirmed by Oyatsi and Whiteley. The glycine zipper region of A $\beta$  was identified as being responsible for SOD1 binding and inhibition, particularly the pentapeptide A $\beta$ 29-33 [157, 158]. Alternatively, A $\beta$  was suggested to inhibit SOD1 activity through the sequestration of Cu<sup>2+</sup> cations, since Cu<sup>2+</sup> is fundamental for the redox reaction involving the dismutation of superoxide [159]. The activators of SOD1 might be beneficial in this case for AD treatment, but to date there is no published literature data on specific SOD1 activators.

### CATALASE

Catalase (EC 1.11.1.6) is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes

Table 8. Predicted Physical-chemical Properties of Selected PAD Inhibitors Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
Cl-amidine	310.78	6/5	108	0.8	-1.1	13.3
TDFA	448.45	13/9	224	-2.7	-5.2	4.2
streptonigrin	506.46	12/6	197	0.1	-3.2	3.6

the decomposition of two hydrogen peroxide molecules to water and molecular oxygen. Continuous and effective degradation of  $H_2O_2$  is essential for aerobic life for two main reasons, the removal of excessive  $H_2O_2$  and the strict regulation of its concentration in signaling pathways. Catalase can be found predominantly in peroxisomes and in small amounts within the cytosol [160]. Little is known about catalase localized in the mitochondrial matrix, however it was first discovered in rat heart mitochondria and later catalase was found in rat liver mitochondria [161, 162]. Still, it remains to be established whether there are other tissues containing mitochondrial catalase and what is its physiological function [163].

Increased ROS levels have been connected with AD for a long time, either as a cause or as a consequence. Therefore, it is no surprise, that catalase, an enzyme with antioxidant properties, is considered to be implicated in AD pathology [164, 165]. In mice, the overexpression of mitochondria-targeted catalase was shown to ameliorate  $A\beta$  toxicity and contrary, the inhibition of catalase was shown to enhance  $A\beta$  toxicity [166, 167].

$A\beta$  was found to directly interact with catalase and to inhibit its enzymatic activity [168, 169]. Aminoacids 400-409 of the human catalase protein were identified as binding site for the  $A\beta_{25-35}$  peptide. Catalase derived peptide (CA $\beta$ BD) comprising these critical residues was shown to bind and scavenge  $A\beta$ , which consequently led to decreased  $A\beta$  binding to catalase and neuroprotection [170]. These findings are very similar to the ones obtained using ABAD-DP as described in the ABAD section of this review. Interestingly, kisspeptin, a physiological peptide sharing some structural similarities with CA $\beta$ BD, was shown to ameliorate  $A\beta$  toxicity in the same way. This finding raises a question, whether kisspeptin plays a part in  $A\beta$  detoxification *in vivo* [171].

Moreover, small molecule inhibitors of catalase- $A\beta$  interaction have also been discovered (BTA-EG<sub>4</sub> and BTA-EG<sub>6</sub>; Fig. 13; Table 9). They were shown to significantly reduce the co-localization of  $A\beta$  and catalase inside the cell, protect catalase enzymatic function and reduce  $A\beta$  toxicity. However, these inhibitors are derivatives of the  $A\beta$ -binding dye thioflavin T and their effects are based on the interaction with  $A\beta$  and not with catalase. Therefore, they are able to prevent the interaction of  $A\beta$  with other binding partners (e.g. ABAD), which could also contribute to their cytoprotective properties [169, 172]. Conversely in another study, an inhibitor of the catalase- $A\beta$  interaction (BTA-EG<sub>4</sub>) was

shown to decrease some of the neuroprotective effects of catalase overexpression. A possible explanation for this observation is that catalase ameliorates  $A\beta$  toxicity via two independent ways, firstly it decreases elevated ROS levels and secondly that it acts as a scavenger of  $A\beta$  and the latter function is blocked by BTA-EG<sub>4</sub> [173]. However, the latest study demonstrated that BTA-EG<sub>4</sub> produces an age-specific improvement in synaptic density and cognitive function in an AD mouse model, when administered daily for two weeks and an associated change in dendritic spine density with increased Ras activity [174]. Additionally, polyphenol phloroglucinol (Fig. 13; Table 9) was found to increase catalase activity and expression, which could be applicable for an AD treatment. However, the mechanism for catalase activation has not yet been elucidated [175, 176].

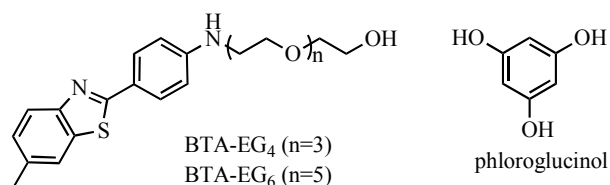


Fig. (13). Structure of selected catalase acting agents.

#### DYNAMIN-RELATED PROTEIN 1 (DRP1)

The balance between fission and fusion is important for maintaining normal mitochondrial size, shape, and distribution, and to supply energy to high-demand sites, such as nerve terminals.  $A\beta$  causes changes to the expression and distribution of mitochondrial fission and fusion proteins leading to impaired mitochondrial dynamics and neuronal dysfunction [177].

Dynammin-related protein 1 (Drp1), also referred to as dynamin-like protein 1 (DLP1), is one of the dynamin-related GTPase, enzymes regulating mitochondrial fission and fusion. Most of Drp1 is localized to the cytoplasm, but a small part is localized in the OMM, where it punctuates and promotes mitochondrial fission [178]. Drp1 also plays a part in mitochondrial fragmentation, mitochondrial outer membrane permeability (MOMP) and cytochrome *c* release during apoptosis [179]. However, it remains to be established, whether its role in apoptosis is mediated by the same signal pathway as mitochondrial fission, or whether this function is mediated through an independent mechanism [180].

Table 9. Predicted Physical-chemical Properties of Selected Catalase Acting Agents Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
BTA-EG <sub>4</sub>	416.53	6/2	101	3.0	3.0	14.4
BTA-EG <sub>6</sub>	504.64	8/2	120	2.5	2.5	14.4
phloroglucinol	126.11	3/3	61	0.1	-0.1	9.1

Both monomeric and oligomeric forms of A $\beta$  were identified to interact with Drp1 using co-immunoprecipitation and immunofluorescence analyses, where such an interaction could lead to disruption of mitochondrial dynamics, shape and size observed in AD [178].

P110 is a selective peptide inhibitor of Drp1 GTPase activity preventing its interaction with Fis1 (binding site for Drp1 on OMM) and consequently inhibiting the translocation of Drp1 to the mitochondria. P110 was originally designed as an inhibitor of the protein-protein interaction (PPI) between Drp1 and Fis1. The peptide comprises residues of Drp1, which were supposed to be involved in PPI of Drp1 with Fis1, but surprisingly it was shown to specifically inhibit Drp1 GTPase activity. It also prevents the binding of Drp1 to Fis1, but it is not known whether this is caused by the same interaction of P110 with Drp1 or whether P110 also binds Fis1 as proposed during its design. P110 was demonstrated to have a cytoprotective effect under stress conditions in cultured neuronal cells, exhibiting protection from mitochondrial dysfunction, excessive fission and cell death [181].

Dynasore (Fig. 14; Table 10) is a small molecule inhibitor of the GTPase activity of dynamin-1, dynamin-2 and Drp1, which was discovered in a chemical screen for inhibitors of dynamin-1 GTPase activity. Dynasore inhibits GTP hydrolysis in these enzymes in non-competitive manner by binding to their GTPase domain. Dynasore was found to decrease cell damage in heart reperfusion injury through maintaining mitochondrial morphology and ATP production [182].

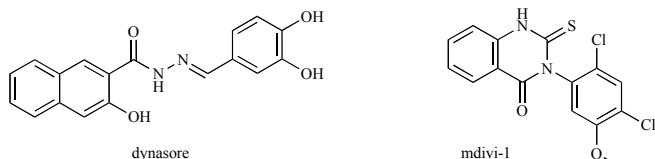


Fig. (14). Structure of selected Drp1 inhibitors.

Mdivi-1 (Fig. 14; Table 10) was the first selective Drp1 inhibitor. The mechanism of mdivi-1 inhibition is distinct in comparison to the more general Drp1 inhibitor dynasore, whereby mdivi-1 binds to an allosteric site that does not exclusively act through the GTPase domain. Upon binding, mdivi-1 creates or stabilizes a conformational form of unassembled, likely dimeric Drp1 that can bind GTP, but at a significantly lower affinity. This mdivi-1-dependent conformational state is not able to assemble into a Drp1 filament/spiral, indicating that mdivi-1 inhibits mitochondrial fission by blocking the Drp1 polymerization. Mdivi-1 was

also found to exert a cytoprotective effect in brain or heart reperfusion injury and to ameliorate A $\beta$  induced neurotoxicity. These cytoprotective effects are probably mediated via inhibiting mitochondrial outer membrane permeabilization and cytochrome *c* release [183, 184].

### GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC. 1.2.1.12) is a soluble 148 kDa homotetrameric, intracellular protein that exerts multiple functions. Originally known for its role in glucose metabolism GAPDH was recently found to participate in many other cellular processes such as DNA repair, tRNA export, membrane fusion and transport, cytoskeletal dynamics, Ca<sup>2+</sup> release or cell death and it also possesses kinase/phosphotransferase activity. Moreover, GAPDH binds numerous endogenous small molecules (e.g. TNF- $\alpha$  ribozymes, glutathione, p53, and nitric oxide) and was found to interact with disease-associated proteins like huntingtin, A $\beta$  and APP. In addition to the cytoplasm where the majority of GAPDH is located under basal conditions, GAPDH is also found in other cell fractions such as nuclear, mitochondrial, and the small vesicular fractions [185, 186]. Although GAPDH was found in mitochondrial subcellular fractions, it remains to be elucidated, whether it is capable of entering the mitochondrial lumen or if it is just absorbed through the OMM.

There is strong evidence that GAPDH participates in AD development and progression. The recently performed meta-analyses of genetic association studies found 13 genes that play a significant role in AD and among them GAPDH [187]. On the biochemical level, increased expression together with decreased activity of GAPDH was observed in AD and denaturated GAPDH was found to be incorporated in A $\beta$  and tau aggregates [188]. In connection with AD, another interesting protein-protein interaction was observed between GAPDH and VDAC, a protein residing in the OMM and participating in mPTP opening (described earlier in the review). GAPDH overexpression was found to cause mitochondrial swelling and cell death probably due to its interaction with VDAC [189].

The direct interaction between GAPDH and A $\beta$ 1-40 and A $\beta$ 1-42, both monomeric and fibrillary, was observed in several studies [190-192], although in one particular study this interaction was observed only for a denaturated form of GAPDH and soluble A $\beta$  [188]. However, the consequences of this interaction on the enzyme's functions remain unclear.

Table 10. Predicted Physical-chemical Properties of Selected Drp1 Inhibitors Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
dynasore	322.31	6/4	102	3.3	3.2	8.8
mdivi-1	353.22	4/1	74	4.5	4.5	9.9

Deprenyl (Fig. 15; Table 11) and other irreversible monoamine oxidase B (MAO B) inhibitors comprising a propargylamine moiety were found to possess neuroprotective properties that are independent of MAO B inhibition. Studies on one such molecule, CGP 3466 (Fig. 15; Table 11), a neuroprotective propargylamine that does not inhibit MAO B, identified GAPDH as the target structure for its neuroprotective effects. CGP 3466 was shown to interact with the binding site of its cofactor,  $\text{NAD}^+$ , which resulted in apoptosis inhibition [193-195]. A similar mechanism of action was also proposed for the aliphatic propargylamines (e.g. *R*-2HMP; Fig. 15; Table 11) with anti-apoptotic activity [196]. Accordingly, isatin (Fig. 15; Table 11), an endogenous reversible MAO B inhibitor, was identified to bind to GAPDH without significant influence on its activity, where it binds to the same site as deprenyl and it was also proposed to possess similar neuroprotective properties [197].

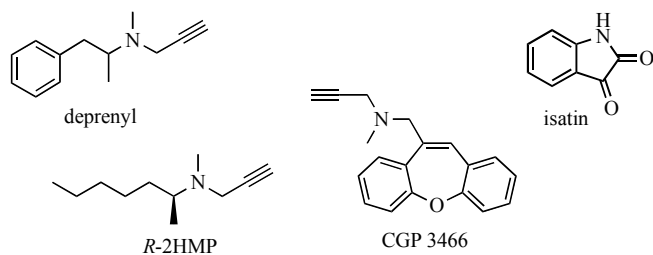


Fig. (15). Structure of selected GAPDH neuroprotectants.

### HIGH TEMPERATURE REQUIREMENT PROTEASE A2 (HTRA2)

High temperature requirement protease A2 (HtrA2), also called Omi or PARK13, is a mitochondrial serine protease with chaperone activity residing in the mitochondrial IMS. A fraction of the endogenous HtrA2/Omi pool has been also detected in the nucleus and associated with the cytosolic side of ER [198, 199]. Its main physiological function is proposed to be the maintenance of mitochondrial function by handling misfolded proteins in the IMS. In addition, HtrA2 has been implicated as a pro-apoptotic factor upon release into the cytosol during the cell death cascade [199, 200]. However, according to genetic studies, the normal physiological function of HtrA2 appears to be anti-apoptotic where decreased HtrA2 activity leads to a neurodegenerative disease phenotype [201]. Therefore, HtrA2 seems to have two

distinct roles, under normal physiological conditions it is required within the mitochondria for the maintenance of mitochondrial homeostasis, and under apoptotic conditions where it is released from the mitochondria and has a pro-apoptotic role [202].

To date, studies of HtrA2 have predominantly focused on PD. The first association of HtrA2 with AD was a study in which HtrA2 was identified as a presenilin-1-interacting factor in a yeast two-hybrid screen [203]. The c-terminus of presenilin-1 was later shown to interact with the PDZ domain of HtrA2, which led to the stimulation of HtrA2 protease activity [204]. Recently, HtrA2 was shown to modulate mitochondrial  $\gamma$ -secretase activity and to interact with presenilin in an active  $\gamma$ -secretase complex located within the mitochondria [109]. Another connection to AD is corroborated by the observation that APP is a direct substrate for HtrA2 and it was suggested that HtrA2 prevents the accumulation of APP in mitochondria through APP cleavage by its serine protease activity [205]. Westerlund *et al.* observed changes to the expression and activity of HtrA2 in AD patient brains, where the total levels of processed HtrA2 were decreased, although the levels of mRNA were unchanged and the proteolytic activity was even increased [206].

HtrA2 directly interacts with  $\text{A}\beta$  through its PDZ domain which was described for the first time by Park *et al.* in 2004 [207]. Another study elucidated that HtrA2 preferentially binds to oligomeric  $\text{A}\beta_{1-42}$  in comparison to monomeric  $\text{A}\beta$  or APP [201]. Additionally, HtrA2 was shown to prevent  $\text{A}\beta$  aggregation and also to be capable of disaggregating oligomeric  $\text{A}\beta$ , which consequently led to a decrease in  $\text{A}\beta$  toxicity [201]. Furthermore, the interaction with  $\text{A}\beta$  inhibited the pro-apoptotic activity of HtrA2 [201]. Kooistra *et al.* have found that HtrA2 is capable of  $\text{A}\beta_{1-42}$  cleavage, which could contribute to the inhibition of its aggregation. However, they also observed that HtrA2 retains its anti-aggregation properties even when devoid of either its proteolytic activity or its PDZ domain [208]. In contrast, another study revealed that the overexpression of HtrA2 decreases  $\text{A}\beta_{1-40}$ / $\text{A}\beta_{1-42}$  generation, although HtrA2 was not found to be directly involved in  $\text{A}\beta$  production or its cleavage [209].

Combining these findings it becomes apparent that HtrA2 appears to be involved in AD pathophysiology, although the

Table 11. Predicted Physical-chemical Properties of Selected GAPDH Neuroprotectants Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
deprenyl	187.28	1/0	3	2.7	2.3	NA*
<i>R</i> -2HMP	167.29	1/0	3	3.4	2.9	NA*
CGP 3466	275.34	2/0	12	3.5	3.5	NA*
isatin	147.13	3/1	46	1.4	1.4	10.3

\* Not available.



precise connection between HtrA2 and AD remains unclear and it is not yet known, what type of HtrA2 pharmacological modulation would be beneficial in regards to an AD treatment. The inhibition of HtrA2 enzymatic activity was shown to suppress apoptotic cell death. Hence, HtrA2 inhibition could present a novel therapeutical strategy for the treatment of many diseases where apoptosis is one of the key pathological components [210, 211]. Ucf-101 (Fig. 16; Table 12) is currently the only known specific HtrA2 inhibitor, where it acts in a reversible and competitive manner [212]. Other compounds described to inhibit HtrA2 activity are generally non-specific inhibitors of serine proteases (e.g. phenylmethanesulfonyl fluoride or diisopropylfluorophosphate) [203].

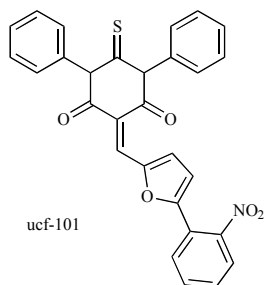


Fig. (16). Structure of ucf-101.

Several proteins and peptides have been shown to activate HtrA2 protease activity through binding to its PDZ domain [200, 204, 213, 214]. This finding presents potential background for the development of specific activators acting in a similar manner. In addition, a novel HtrA2 allosteric binding site called selective binding pocket (SBP) was discovered recently and the binding of substrate peptides to SBP was shown to increase HtrA2 activity [215].

### AMYLOID- $\beta$ DEGRADING ENZYMES

Two enzymes, presequence protease (PreP) and insulin-degrading enzyme (IDE), were found to be capable of A $\beta$  clearance inside mitochondria (and possibly HtrA2; see earlier described HtrA2 chapter) [213]. These enzymes are both members of pitrilysin oligopeptidase family with inverted zinc-binding motif also called inverzincin family [216].

### PRESEQUENCE PROTEASE (PreP)

Presequence protease (PreP), also termed metalloendopeptidase 1 or metallopeptidase 1 [217, 218], was firstly identified and characterized as a mitochondrial matrix and chloroplast stroma metalloendopeptidase in *Arabidopsis thaliana*, but this enzyme can also be found in all species

except among the *Archea*. It is responsible for the degradation of mitochondrial presequences (targeting peptides) that are generated after mitochondrial precursor protein import and processing. PreP has been shown to degrade not only the targeting peptides but also other unstructured peptides and the human variant of PreP was found to be responsible for A $\beta$ 1-40 and A $\beta$ 1-42 degradation inside the matrix of human brain mitochondria (Fig. 17) [219]. In an *in situ* study by Falkevall *et al.*, it was found that the immuno-inactivation of PreP completely inhibits mitochondrial proteolytic activity against A $\beta$ 1-40 suggesting that PreP is the sole enzyme responsible for A $\beta$  cleavage inside mitochondria [220] and the same conclusion was drawn in a later study using both immuno- and chemical PreP inactivation [221]. This finding is in contrast with the mitochondrial localization of another A $\beta$  cleaving enzyme IDE (described later in this article).

Decreased PreP activity was found in AD brains and in the brains of a transgenic mouse model for AD. However, PreP expression levels were not significantly changed suggesting that the lower PreP activity was caused via its inactivation [221], as PreP can be inactivated under oxidizing conditions. Firstly, it was concluded that this inactivation was caused by the formation of a disulfide bridge between the two close cysteine residues locking the enzyme in a closed (inactive) conformation. This was further supported by the finding that mutating one of the cysteine residues to a serine leads to the protection against oxidizing conditions [220]. However, another study attempting to reactivate the oxidatively inactivated PreP enzyme by pre-incubation with DTT (dithiothreitol; a reducing agent) revealed inconsistent results indicating that other factors, such as amino acid modification rather than disulphide bond formation may be responsible for PreP inactivation [221, 222]. These findings connect PreP to AD, because both, elevated ROS levels and PreP inactivation are observed in AD.

Recently, small molecule PreP activators (3c, 4c; Fig. 18; Table 13) have been discovered and their capability to increase A $\beta$  degradation was evaluated *in vitro*. Such compounds potentially present new therapeutics against AD [223].

### INSULIN-DEGRADING ENZYME (IDE)

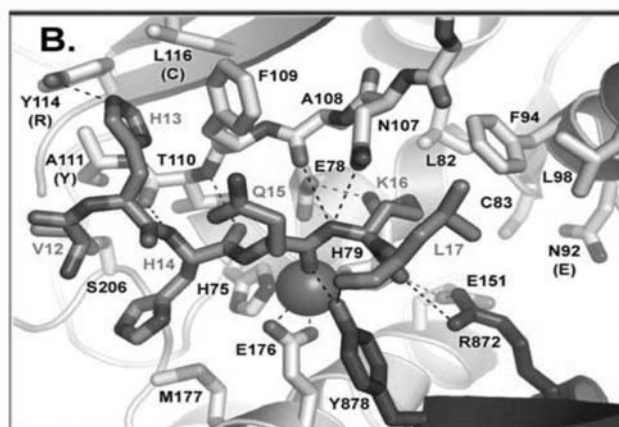
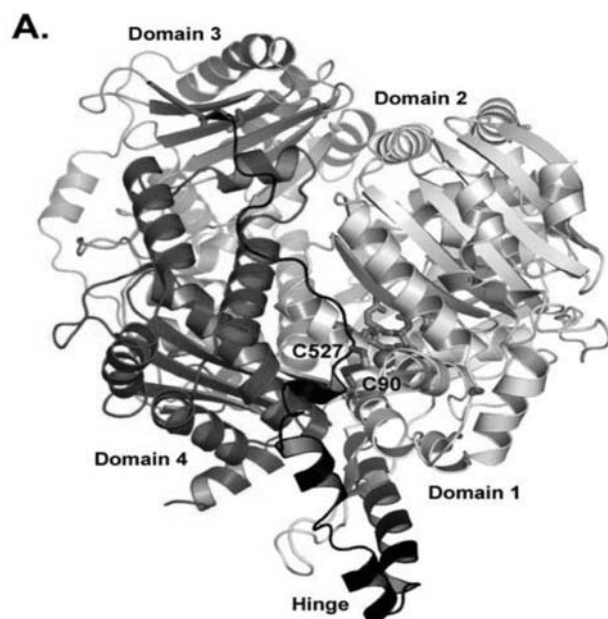
Insulin-degrading enzyme (IDE, EC 3.4.24.56), also termed insulysin or insulinase, is a 110kDa widely expressed zinc metallopeptidase that has been shown to regulate both plasma insulin levels and cerebral A $\beta$  *in vivo*. IDE is predominantly localized in the cytosol, with smaller amounts observed in peroxisomes, rough ER, plasma membranes and mitochondria depending on cell type. A secreted form of

Table 12. Predicted Physical-chemical Properties of ucf-101 Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
ucf-101	493.53	6/0	125	4.9	4.9	NA*

\* Not available.

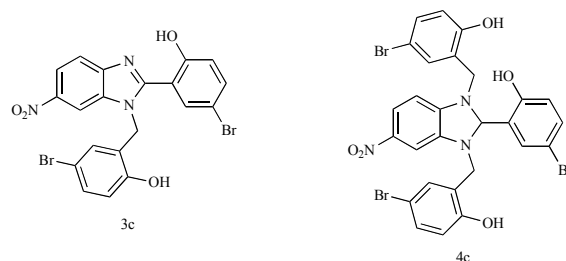
IDE is also present within extracellular compartments such as CSF. IDE has been shown to play a major role in the degradation and clearance of insulin *in vivo*, however its expression in insulin-insensitive tissues suggests that IDE may exert other functions. Among insulin, IDE degrades a wide range of substrates that share a common amyloidogenic secondary protein structure e.g. amylin, insulin-like growth factors I and II and A $\beta$ . Therefore, IDE likely plays a role in catabolic regulation, especially in preventing the formation of amyloid deposits by cleaving the component peptides (Fig. 19) [224-226]. IDE was also shown to possess chaperon like activity as A $\beta$  was found to form a highly stable complex with IDE (in addition to also being IDE's substrate) which consequently blocks its self-assembling into cytotoxic oligomers [227, 228].



**Fig. (17).** Structural homology model of hPreP with the A $\beta$ 12-17 based on the three-dimensional structure of AtPreP. **A)** Structural model of hPreP with A $\beta$ 12-17 bound to the active site. **B)** Close-up of the active site with residues that differ in AtPreP shown in parentheses. Reproduced with permission, from Falkevall *et al. J. Biol. Chem.* **2006**, *281*, 29096-29104 [220]. © The American Society for Biochemistry and Molecular Biology.

A mitochondrial isoform of IDE was identified in 2004 by Leissing *et al.* This isoform is generated by IDE transla-

tion beginning at an alternative initiation codon, upstream of the canonical starting site, thus producing a novel protein targeted to the mitochondria [224]. Recently, the PGC-1 $\alpha$ /NRF-1 signaling pathway was shown to regulate the expression of mitochondrial IDE and consequently to modulate A $\beta$  levels in mitochondria [229]. It was also proposed that inside mitochondria IDE could metabolize mitochondrial presequence [224]. However, it should be noted that the same functions are also executed by the related and mitochondrial enzyme PreP [220, 221].



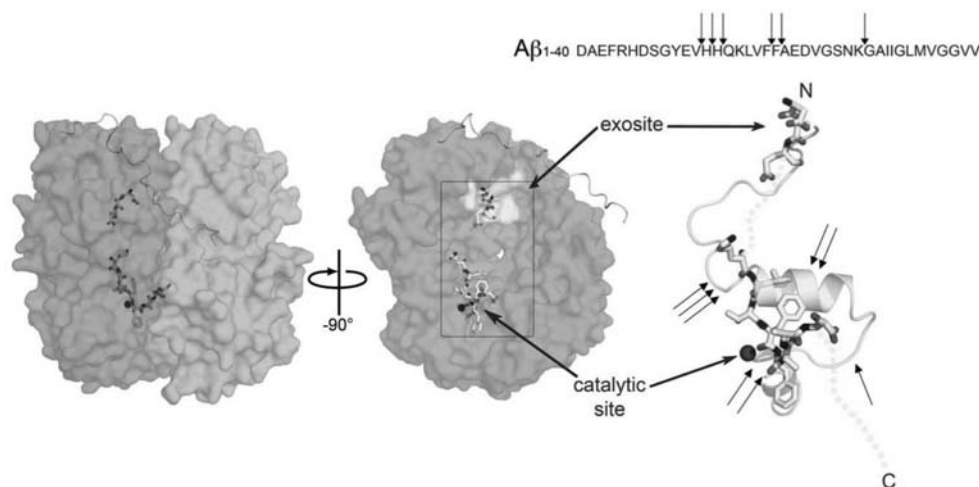
**Fig. (18).** Structure of selected PreP activators.

In relation to AD, IDE activity was found to be decreased in brains of AD patients and negatively correlated with A $\beta$ 1-40 and A $\beta$ 1-42 levels in brain [230, 231]. Furthermore, IDE expression and activity can be regulated by several signaling pathways that have been previously connected with AD development, e.g. estradiol [232], norepinephrin [233], NOTCH [234], ApoE [235], PPAR $\gamma$  [236] or S-nitrosylation [237]. Meta-analysis performed by Zhang *et al.* suggested the association between IDE polymorphism and AD [238]. Interestingly, IDE also presents a possible link between type 2 diabetes and AD as decreased IDE activity is a common feature in both diseases and people with type 2 diabetes are statistically more likely to develop AD [225, 231, 239].

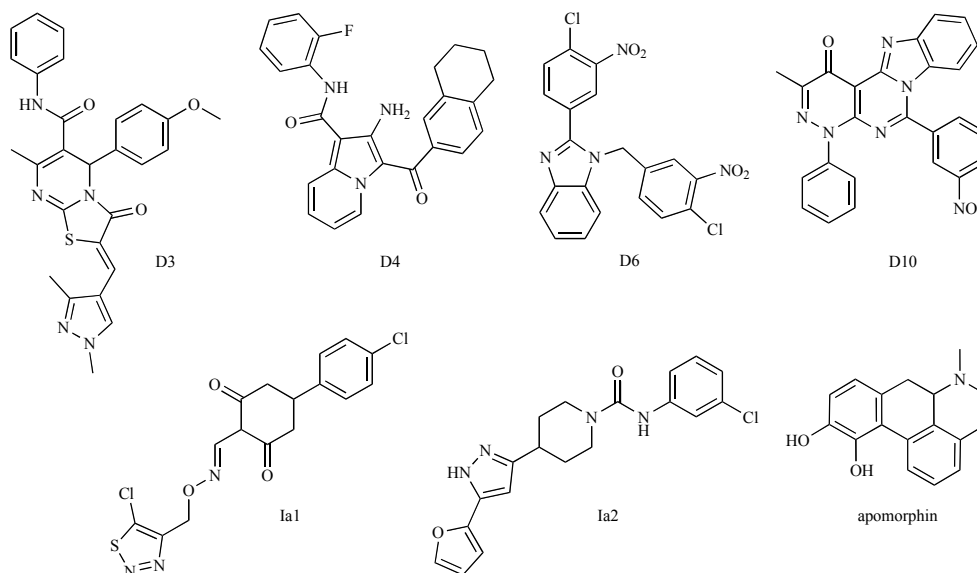
IDE is an allosteric enzyme displaying sigmoidal substrate consumption and possessing two different allosteric binding sites. The first one binds small peptide substrates (e.g. bradykinin,  $\beta$ -endorphin, some dynorphins, insulin B chain or somatostatin), which leads to enzyme activation. Interestingly, such activation of IDE is selective towards A $\beta$ 1-40 cleavage when compared to insulin. This finding suggested the possibility of developing small-molecule peptide analogs that can serve to increase IDE activity towards A $\beta$  cleavage *in vivo*, without affecting the IDE-dependent cleavage of insulin [240-243]. Accordingly, 10 small molecule compounds were recently identified by virtual screening and four of them (D3, D4, D6, D10; Fig. 20; Table 14) were shown to be potent IDE activators [244]. The second allosteric site, called the anion binding site, is activated by polyanions such as ATP and triphosphate [243, 245, 246]. A high throughput screen was performed to detect compounds that interact with the ATP-binding domain leading to the discovery of two small molecule activators (Ia1, Ia2; Fig. 20; Table 14). Despite promising IDE activating properties, shown with the use of fluorescing substrates, these compounds do not enhance A $\beta$  degradation if A $\beta$  is present as the sole substrate, but only if it is present in a mixture with shorter substrate peptides [247]. Additionally, apomorphin (Fig. 20; Table 14) was also shown to induce IDE activity in an *in vitro* assay, however the mechanism of this activation is not yet known [248].

**Table 13.** Predicted Physical-chemical Properties of Selected PreP Activators Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
3c	519.14	7/2	104	5.0	4.9	8.2
4c	706.18	8/3	113	6.7	6.6	8.9



**Fig. (19).** Interactions between A $\beta$  and IDE. **Left panel:** surface representation of IDE in complex with A $\beta$ . IDE is depicted as transparent surface to allow visualization of the bound A $\beta$  in the cavity. IDE-N and IDE-C domains are depicted as transparent blue and gray surfaces, respectively. The hinge region of IDE is depicted as yellow ribbon, and A $\beta$  is depicted as sticks with carbon, nitrogen, and oxygen atoms colored yellow, blue, and red, respectively. **Central panel:** view of IDE-N after a rotation of  $-90$  degree around the y axis separating IDE-N and IDE-C has been applied. Catalytic site and exosite are indicated by the catalytic zinc ion, depicted as magenta sphere, and by the green surface, respectively. **Right panel:** details of A $\beta$ 1-40 conformation bound to IDE (depicted as yellow sticks) superimposed to A $\beta$  alone (depicted as transparent gray cartoon). Yellow dash lines show segments of IDE-bound A $\beta$ 1-40 not revealed in the electron density map. Black arrows show the position of the known cleavage sites of A $\beta$  by IDE, according also to the sequence shown above. Reproduced with permission, from Malito *et al. Cell. Mol. Life Sci. CMLS* **2008**, 65, 2574-2585 [216]. © Springer Science and Business Media.

**Fig. (20).** Structure of selected IDE activators.

**Table 14.** Predicted Physical-chemical Properties of Selected IDE Activators Compared to Optimal Properties for CNS Targeted Drugs [16, 17]

Compound	Mw	H-bond	PSA [Å <sup>2</sup> ]	ClogP ± SD	ClogD	CpKa ± SD
		acc./don.			(pH 7.4)	
Optimal properties	≤400	≤7/≤3	≤60-70	1-5	0-3	4-10
D3	499.58	8/1	114	2.2	2.2	13.9
D4	427.47	5/3	77	5.5	5.5	12.1
D6	443.24	8/0	109	5.4	5.4	NA*
D10	448.43	9/0	109	5.1	5.1	NA*
Ia1	398.26	6/0	110	3.2	0.7	3.9
Ia2	370.83	6/2	74	4.6	4.6	13.5
apomorphin	267.32	3/2	44	1.7	1.2	9.4

\* Not available.

### HEAT SHOCK PROTEINS (Hsps)

Heat shock proteins (Hsps) exert their function as molecular chaperones. Molecular chaperones are a family of proteins that facilitate and regulate proper protein folding. Whereby, molecular chaperones make sure that other proteins arrive safely and are functional at their destination within the cell. They also ensure that proteins deemed to be 'ill-behaved' or abnormal are destroyed [249-251].

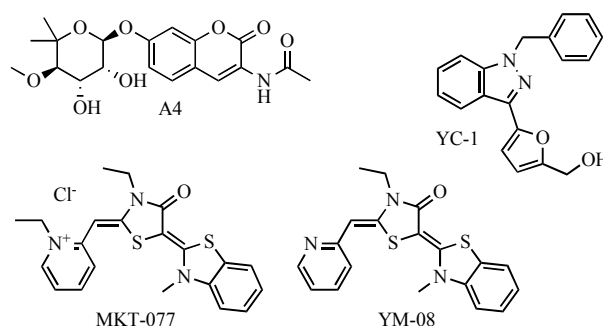
Heat shock proteins can be divided into two different families based on size and function: classic Hsps such as Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsps. Hsps with a molecular weight of 60 kDa or more possess an ATP-binding site and are actively involved in the process of refolding misfolded proteins. Small Hsps, with a molecular weight of 40 kDa or less, lack this ATP-binding site and assist the classic Hsps in their refolding function [249-251]. Several heat shock proteins can be found in mitochondria, namely the classic Hsps Hsp60, mtHsp70 (mortallin), mtHsp90 (TRAP-1) and Hsp78 and small Hsps Hsp10, Hsp27,  $\alpha$ B-crystallin, Hsp22, mtHsp40 [251-255].

It is no surprise that some Hsps are known to directly interact with A $\beta$ , namely Hsp70 and small Hsps; Hsp27 (HSPB1),  $\alpha$ B-crystallin (HSPB5), Hsp22 (HSPB8) and Hsp20 (HSPB6), because the handling of misfolded proteins is part of their natural role [249, 256-258]. More interestingly, all of these Hsps are also proposed to be localized within mitochondria.

Generally, it seems that Hsps are protective against A $\beta$  toxicity in AD. The interaction of Hsps with A $\beta$  was shown to decrease A $\beta$  toxicity through its sequestration, affecting its trafficking, aggregation, clearance and fibril formation [249, 251, 257]. Moreover, a similar mechanism of action could occur in the processing of other pathological proteins involved in AD development, e.g. tau-protein and APP [250, 259, 260]. However, it remains to be elucidated, whether these interactions take place inside the mitochondria and play a significant role in the processing of mitochondrial A $\beta$ .

Many Hsp90 and several Hsp70, Hsp40 and Hsp27 inhibitors were developed primarily for cancer treatment [261-

263], and surprisingly the inhibitors of both Hsp90 and Hsp70 proved to be potentially useful for AD treatment. Inhibition of Hsp90 promotes the proteasomal degradation of tau and also activates heat shock factor-1 (HSF-1) to induce Hsp70 and Hsp40 production as well as other chaperone production, which in turn, promotes the disaggregation and degradation of the disease associated proteins A $\beta$  and tau [264, 265]. Many Hsp90 inhibitors/HSF-1 activators were found to exert neuroprotective properties in AD models or to ameliorate A $\beta$  toxicity. From these compounds the most promising ones (to be used as AD therapeutics) seem to be the analogues of the coumarin antibiotic novobiocin (e.g. A4; Fig. 21; Table 15) targeting the C-terminal of Hsp90 [266, 267]. Inhibition of Hsp70 also promotes proteasomal degradation of tau via a mechanism of action distinct from Hsp90 inhibition. The suitability of this approach was confirmed using the selective Hsp70 inhibitor MKT-077 or its BBB-permeable analogue YM-08 (Fig. 21; Table 15) [265, 268, 269]. Additionally, YC-1 (Fig. 21; Table 15) an activator of guanylyl cyclase, was shown to decrease A $\beta$  toxicity via the induction of Hsp70 expression and activity. However, its precise mechanism of action needs to be clarified [270].

**Fig. (21).** Structure of selected Hsp inhibitors.

### HS-1-ASSOCIATED PROTEIN X-1 (HAX-1)

HS-1-associated protein X-1 (HAX-1) comprises a family of ubiquitously expressed proteins that have been implicated in a wide range of biological functions and particularly

**Table 15. Predicted Physical-chemical Properties of Selected Hsp Inhibitors Compared to Optimal Properties for CNS Targeted Drugs [16, 17]**

Compound	Mw	H-bond	PSA [ $\text{\AA}^2$ ]	ClogP $\pm$ SD	ClogD	CpKa $\pm$ SD
		acc./don.			(pH 7.4)	
Optimal properties	$\leq 400$	$\leq 7/\leq 3$	$\leq 60-70$	1-5	0-3	4-10
A4	393.3	9/3	124	-0.3	-0.3	12.2
YC-1	304.34	4/1	51	3.7	3.7	13.8
MKT-077	396.55	4/0	78	0.8	0.8	NA*
YM-08	367.49	4/0	87	4.1	4.1	NA*

\* Not available.

in the regulation of programmed cell death. At the subcellular level, HAX-1 is predominantly localized in the mitochondria, and to a lesser extent in the ER and nuclear membrane [271, 272]. In a yeast two-hybrid screen HAX-1 was identified as a binding partner of A $\beta$  [209], however, there is no other evidence currently available connecting HAX-1 with AD or A $\beta$  pathophysiology.

## CONCLUSION

Amyloid- $\beta$  peptide is by its nature a very promiscuous fellow. As summarized in this review, about 20 A $\beta$ -binding partners are currently known in the mitochondria alone, and this number is probably not definitive (Table 16). Hence,

effects of elevated A $\beta$  levels on mitochondria (and ultimately the whole organism) are likely to be a consequence of many different protein-peptide interactions. Regarding an AD treatment, the pharmacological targeting of a single A $\beta$  binding partner will probably not be sufficient to cure the whole disease, but it could ameliorate some of the symptoms or at least slow down the disease progression. As presented in (Table 16), at least five such A $\beta$ -binding partners (drug-gability score \*\*\*) seem to be very rational drug targets for AD treatment, namely ABAD, CypD,  $\gamma$ -secretase, GAPDH and IDE. These proteins have all been successfully targeted *in vivo* using neurodegenerative animal models and a change in either their expression or their pharmacological modulation resulted in a decrease of neurodegeneration symptoms.

**Table 16. Summary of Presented Proteins, Their Mitochondrial Localization, Evidence for the Direct Interaction With A $\beta$ , Significance of Their Role in AD Pathophysiology and Their Potential and Suitability As a Drug Target for AD Treatment. The Number of Stars Represents the Degree of Significance With \*\*\* Being the Highest Score, \*\* An Intermediate Score and \* the Lowest Score**

Protein	Localization in Mitochondria	Evidence for Direct Interaction with A $\beta$	Significance in AD Pathophysiology	Potential As a Drug Target
ABAD	matrix	***	***	***
CypD	matrix, IMM	***	***	***
ANT	IMM	**	**	**
VDAC	OMM	**	**	**
ETC - Complex I	IMM	**	**	*
ETC - Complex IV	IMM	**	**	*
ETC - Complex V	IMM	*	**	*
KGDHC	IMM	**	*	*
PDHC	matrix	**	*	**
CK	IMS	*	*	*
$\gamma$ -secretase	OMM	***	***	***
NOS	IMM	**	**	**
PAD	not specified	**	**	**

(Table 16) contd....

Protein	Localization in Mitochondria	Evidence for Direct Interaction with A $\beta$	Significance in AD Pathophysiology	Potential As a Drug Target
SOD1	IMS, matrix, OMM	***	**	*
Catalase	matrix	***	**	**
Drp-1	OMM	**	**	**
GAPDH	OMM	***	***	***
HtrA2	IMS	***	**	**
PreP	matrix	***	**	**
IDE	not specified	***	***	***
Hsps	not specified	***	**	**
HAX-1	not specified	*	*	*

**ABBREVIATIONS**

ABAD = Amyloid-binding alcohol dehydrogenase  
 ABAD-DP = ABAD-decoy peptide  
 AD = Alzheimer's disease  
 AICD = Amyloid precursor protein intracellular domain  
 ALS = Amyotrophic lateral sclerosis  
 ANT = Adenine nucleotide translocase  
 APP = Amyloid precursor protein  
 A $\beta$  = Amyloid- $\beta$  peptide  
 BACE =  $\beta$ -secretase  
 BBB = Blood-brain barrier  
 BKA = Bongkreikic acid  
 CAT = Carboxylatractyloside  
 CK = Creatine kinase  
 CsA = Cyclosporine A  
 CypD = Cyclophilin D  
 Drp1 = Dynamin-related protein 1  
 ER = Endoplasmic reticulum  
 ETC = Electron transport chain  
 GAPDH = Glyceraldehyde-3-phosphate dehydrogenase  
 GSI =  $\gamma$ -secretase inhibitor  
 GSM =  $\gamma$ -secretase modulator  
 HAX-1 = HS-1-associated protein X-1  
 HIG1 = Hypoxia-inducible gene 1 = domain member 1A  
 HSF-1 = Heat shock factor-1  
 Hsp = Heat shock protein  
 HtrA2 = High temperature requirement protease A2

IDE = Insulin-degrading enzyme  
 IMM = Inner mitochondrial membrane  
 IMS = Intermembrane space  
 KGDHC =  $\alpha$ -ketoglutarate dehydrogenase complex  
 MAO B = Monoamine oxidase B  
 MOMP = Mitochondrial outer membrane permeability  
 mPT = Mitochondrial permeability transition  
 MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine  
 mPTP = mPT pore  
 NOS = Nitric oxide synthase  
 OMM = Outer mitochondrial membrane  
 PAD = Peptidylarginine deiminases  
 PD = Parkinson's disease  
 PDHC = Pyruvate dehydrogenase complex  
 PDHK = Pyruvate dehydrogenase kinase  
 PreP = Presequence protease  
 PS = Presenilin  
 ROS = Reactive oxygen species  
 SDR = Short chain dehydrogenase reductase  
 SfA = Sangliferin A  
 SOD1 = Cu/Zn superoxide dismutase  
 TIM = Translocase of the inner membrane  
 TOM = Translocase of the outer membrane  
 VDAC = Voltage-dependent anion channel

**CONFLICT OF INTEREST**

The authors confirm that this article content has no conflicts of interest.

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