HHS Public Access

Author manuscript

Biochem Pharmacol. Author manuscript; available in PMC 2021 June 01.

Published in final edited form as:

Biochem Pharmacol. 2020 June ; 176: 113818. doi:10.1016/j.bcp.2020.113818.

DEVELOPMENT OF NOVEL PHOSPHODIESTERASE 5 INHIBITORS FOR THE THERAPY OF ALZHEIMER'S DISEASE.

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Abstract

Nitric oxide (NO) is a gaseous molecule that plays a multifactorial role in several cellular processes. In the central nervous system, the NO dual nature in neuroprotection and neurotoxicity has been explored to unveil its involvement in Alzheimer's disease (AD). A growing body of research shows that the activation of the NO signaling pathway leading to the phosphorylation of the transcription factor cyclic adenine monophosphate responsive element binding protein (CREB) (so-called NO/cGMP/PKG/CREB signaling pathway) ameliorates altered neuroplasticity and memory deficits in AD animal models. In addition to NO donors, several other pharmacological agents, such as phosphodiesterase 5 (PDE5) inhibitors have been used to activate the pathway and rescue memory disorders. PDE5 inhibitors, including sildenafil, tadalafil and vardenafil, are marketed for the treatment of erectile dysfunction and arterial pulmonary hypertension due to their vasodilatory properties. The ability of PDE5 inhibitors to interfere with the NO/cGMP/PKG/CREB signaling pathway by increasing the levels of cGMP has prompted the hypothesis that PDE5 inhibition might be used as an effective therapeutic strategy for the treatment of AD. To this end, newly designed PDE5 inhibitors belonging to different chemical classes with improved pharmacologic profile (e.g. higher potency, improved selectivity, and blood-brain barrier penetration) have been synthesized and evaluated in several animal models of AD. In addition, recent medicinal chemistry effort has led to the development of agents concurrently acting on the

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PDE5 enzyme and a second target involved in AD. Both marketed and investigational PDE5 inhibitors have shown to reverse cognitive defects in young and aged wild type mice as well as transgenic mouse models of AD and tauopathy using a variety of behavioral tasks. These studies confirmed the therapeutic potential of PDE5 inhibitors as cognitive enhancers. However, clinical studies assessing cognitive functions using marketed PDE5 inhibitors have not been conclusive. Drug discovery efforts by our group and others are currently directed towards the development of novel PDE5 inhibitors tailored to AD with improved pharmacodynamic and pharmacokinetic properties. In summary, the present perspective reports an overview of the correlation between the NO signaling and AD, as well as an outline of the PDE5 inhibitors used as an alternative approach in altering the NO pathway leading to an improvement of learning and memory. The last two sections describe the preclinical and clinical evaluation of PDE5 inhibitors for the treatment of AD, providing a comprehensive analysis of the current status of the AD drug discovery efforts involving PDE5 as a new therapeutic target.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder affecting millions of people worldwide. AD represents the most common and one of the most extensively studied forms of dementia. At the pathological level, it is characterized by intracellular amyloid plaques consisting of amyloid beta (Aβ) aggregates as well as extracellular neurofibrillary tangles (NFTs) formed by hyperphosphorylated tau fibrils that hamper proper neuronal functioning [1]. Currently used drugs, acetylcholinesterase (AChE) inhibitors or N-methyl-D-aspartate (NMDA) antagonists have very limited efficacy. Most of the efforts have been directed towards different therapeutic approaches, such us inhibiting neurofibrillary tangles (NFTs) formation, interfering with tau protein function, combatting inflammation and oxidative damage, decreasing Aβ load in the brain either by the use of agents that inhibit β- and γ-secretases or increase α-secretase (enzymes involved in the Aβ processing), inhibiting Aβ oligomerization [2, 3] or using treatments such as immunization with Aβ that appear to augment the removal of Aβ from the brain [4]. However, the role of tau, the amyloid-precursor protein (APP), Aβ, and the secretases in normal physiological processes [1, 5–19] might present a problem in providing effective and safe approaches to AD therapy. Developing agents that interact with tau and Aβ targets that lead to neuronal dysfunction and death is another approach that might became our sole resource in case all other strategies fail. Within this frame of thinking, the nitric oxide (NO) cascade offers unique possibilities that will be discussed in this review. Particularly, phosphodiesterase 5 (PDE5) looks very appealing due to the widespread use of its inhibitors with limited side effects. This review focuses on the drug discovery efforts to develop PDE5 inhibitors that cross the blood-brain barrier (BBB) and are suitable for chronic treatment of elderly people with comorbidity such as AD patients. Following a discussion of the involvement of the NO cascade in AD, medicinal chemistry efforts to develop novel selective PDE5 inhibitors that are also BBB permeant and efficacious in AD animal models are described. Finally, both preclinical and clinical studies relevant to AD and cognition using the inhibitors are reviewed.

2. Nitric Oxide Signaling and Alzheimer's Disease

NO is a small gaseous molecule that is produced from the metabolism of L-arginine by the enzyme nitric oxide synthase (NOS). In the central nervous system (CNS), NO was characterized as an unconventional neurotransmitter since it is not stored in vesicles, but it might travel, upon production, from the post-synaptic to the pre-synaptic neuron, as a retrograde messenger [20, 21]. Due to its high lipophilicity, NO can easily diffuse through cell membranes, participating in a wide range of physiological functions, including vasodilation, inflammation, neuroprotection, neurotoxicity and synaptic transmission [22].

The action of NO is mediated either after binding to its biological receptor soluble guanylate cyclase (sGC) or via S-nitrosylation, a posttranslational modification that regulates the activity of substrate proteins and involves the formation of S-nitrosothiol (SNO) [23]. Binding of NO to sGC stimulates the production of the second messenger cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) (Fig 1). Subsequently, cGMP activates its downstream effector protein kinase G (PKG) that activates, among other substrates [24], the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding element (CREB), promoting neurotransmission, synaptic plasticity and memory formation [25–27]. PKG also activates the phosphatidylinositol 3-kinase (PI3k)/Akt signaling pathway [28] that mediates neuroprotection via the inhibition of apoptosis [29]. Additionally, NO could act at the presynaptic cell and promote the release of neurotransmitters, like glutamate, via activation of the sGC/cGMP/PKG pathway [30] (Fig. 1). Another mechanism by which NO can facilitate CREB signaling is via S-nitrosylation of nuclear proteins associated with CREB target genes and thereafter promote CREB-DNA binding [31, 32]. Other proteins that exhibit enhanced activity after nitrosylation include cyclic nucleotide-gated ion channels (CNGC) and ryanodine receptor Ca^{2+} release channels (RyR). Importantly, nitrosylation reduces the activity of apoptotic caspase enzymes and N-methyl-D-aspartate receptor (NMDAR) [33, 34]. The inhibition of NMDAR is suggested to be part of the neuroprotective action of NO, since excessive NMDAR activity leads to abnormal elevation in intracellular Ca^{2+} levels resulting in excitotoxicity [35].

Apart from the important role in neurotransmission and synaptic plasticity, unbalanced production of NO induces neurotoxicity. The concentration of NO in the brain is determined by the activity of the 3 isoenzymes that constitute the NOS family: i) the neuronal form (nNOS or NOS1) that is widely expressed in the brain and mainly located in the striatum, nucleus accumbens, hippocampus and amygdala, ii) the inducible form (iNOS or NOS2) that is present in neurons, astrocytes, microglial cells, smooth cells, and macrophages, and is produced in response to inflammation or trauma, and iii) the endothelial form (eNOS or NOS3) expressed in the endothelial cells. nNOS and eNOS are classified as constitutive forms of NO (cNOS) that produce low amounts of NO transiently and their activation requires Ca^{2+} and more precisely phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [36]. On the contrary, activation of iNOS is Ca^{2+}-independent and could lead to the production of high levels of NO that last even for days [37]. At high concentrations, NO reacts with oxygen anion superoxide to form peroxynitrite, a highly reactive species that could cause cellular damage and it is responsible for the so-called nitrosative stress [22, 38].

Due to the multifactorial role of NO during several cellular processes, and especially its biphasic nature leading to both neuroprotection and neurotoxicity, several research lines have focused on the involvement of NO signaling in Alzheimer's disease (AD).

In addition to the well-recognized Aβ and tau pathological hallmarks, AD is characterized by abnormal NO/sGC/cGMP/PKG/CREB signaling in the brain [38–41], an alteration in the expression of the different NOS isoforms [42, 43], and aberrant S-nitrosylation of target proteins [44], providing a strong link between AD and NO signaling. Nevertheless, the role of NO in AD remains controversial, since there are studies suggesting a neuroprotective function, while others support a neurotoxic action.

In agreement with the involvement of iNOS in the production of neurotoxic NO, iNOS expression is significantly increased in postmortem brain tissue from AD patients [45–47]. Of note, augmented immunoreactivity for iNOS was found in astrocytes surrounding amyloid plaques [45, 46]. The study from Lüth *et al.* has further shown that in addition to iNOS, the expression of eNOS was also increased in astrocytes in both postmortem AD brains and APP23 transgenic AD mice. Importantly, the authors suggested that the presence of iNOS-and eNOS-expressing astrocytes close to early, but not late, stage amyloid plaques indicates that elevation of NOS isoforms is a byproduct of the disease and does not participate in its etiopathogenesis [46]. In this regard, it is possible that increased expression of iNOS and eNOS represents an inflammatory response triggered by Aβ deposition. This notion is supported by an *in vitro* study showing that synthetic $A\beta_{1–42}$ peptide increased the expression of iNOS and, in turn, NO production in neuronal-glial cultures from rats [47]. Noticeably, a recent study suggested that the elevation of NOS activity could be a compensatory mechanism to restore the subtle synaptic changes occurring in pre-symptomatic AD, as shown with the 3xTg-AD transgenic mouse model [48]. In these mice, blockage of NO resulted in synaptic depression, suggesting that increased NO signaling is required for maintaining synaptic plasticity that is essential for memory formation. The latter observation could explain the increased levels of nNOS expression in AD brain [49, 50]. However, during the progression of the disease, excessive production of NO could lead to neurotoxicity through formation of peroxynitrate and S-nitrosylated proteins, that will eventually accelerate the pathology of AD [44, 48, 51].

The importance of NO signaling to maintain intact cellular functions in AD was also outlined by studies showing that eNOS deficiency in aged wild-type mice (14–15 months old) led to increased production of Aβ, promoted inflammatory processes, and impaired hippocampus-dependent memory formation [52, 53]. In addition, genetic depletion of iNOS in AD mice overexpressing the amyloid precursor protein Swedish mutation (APP_{SW}), elevated Aβ levels, tau hyperphosphorylation, and neuronal degeneration [54]. Noteworthy, NO signaling appears to have a direct effect on Aβ and tau pathology (Fig. 2). In particular, it was shown that both low and high levels of NO prevent the cleavage of APP by beta-secretase 1 (BACE1), a process involved in the production of Aβ. Lower concentration of NO blocked BACE1 expression, whereas higher concentration prevented its enzymatic activity via S-nitrosylation. The latter finding comes in agreement with the observed decrease in S-nitrosylation in AD brains [55]. With regard to the involvement of NO in tau pathology, a study from Reynolds and coworkers showed that peroxynitrate-mediated

nitrosylation in specific tyrosine residues of the tau protein inhibits its assembly and oligomerization, preventing formation of NFTs. [56]. In addition, NO can regulate tau aggregation via activation of the P13K/Akt pathway. Specifically, Akt inhibits glycogen synthase kinase 3β (GSK-3β) that mediates tau phosphorylation, and therefore the reduction of NO could enhance tau hyperphosphorylation [54, 57, 58].

Considering the neuroprotective role of NO signaling, at least at the early phase of AD, several studies have shown that activation of NO *per se* or other molecules in the NO/sGC/cGMP/PKG/CREB pathway can ameliorate altered neuroplasticity and memory deficits in animal models exhibiting AD-like pathology. Consistent with a plethora of studies reporting decreased phosphorylated CREB (pCREB) in AD brains [59–67], it has been shown that Aβ or tau oligomers lead to a reduction in pCREB during neuronal plasticity and memory formation. To this extent, two studies have provided electrophysiological, behavioral and biochemical evidence showing that the activation of the NO/sGC/cGMP/PKG/CREB pathway could rescue Aβ or tau pathology and restore pCREB levels [39, 40]. This has been shown for several pharmacological agents, including NO donors, sGC stimulators, cGMP analogs, phosphodiesterase 5 (PDE5) inhibitors, and PKG activators.

3. Drug Development of PDE5 Inhibitors against Alzheimer's disease

Among the various components of the NO cascade, PDE5 is currently used as a target for the therapy of male erectile dysfunction (ED) and pulmonary hypertension, strongly supporting the possibility of exploiting the enzyme for the cure of other conditions in which the cascade is involved, including AD. PDE5 is one of the eleven subfamilies of PDE enzymes that are responsible for the degradation of cGMP and/or cAMP. While PDE1, 2, 3, 10, and 11 are dual-substrate PDEs, having affinity for both cyclic nucleotides, PDE5, 6, and 9 are cGMP-specific enzymes, and PDE4, 7, and 8 are cAMP-specific enzymes [68]. PDE5 inhibitors have been historically developed for ED treatment due to their vasodilation and smooth muscle relaxant effects. A challenge in developing PDE5 inhibitors is to attain high selectivity especially versus the PDE6 isozyme, which shares high structural similarity with PDE5 [69]. In addition, the inhibition of PDE isozymes such as PDE1, 6 and PDE11 has been associated with the onset of adverse effects, including tachycardia, vision disturbances and back pain [70]. The first synthesized PDE5 inhibitor was zaprinast (IC_{50} values is 0.76μM). It was an effective bronchodilator in exercise-associated asthma and was able to produce smooth muscles relaxation and a NO/cGMP-dependent relaxation of the corpus cavernosum. Zaprinast was a precursor of the chemically related PDE5 inhibitor cGMP-based derivative, sildenafil (Viagra) [71]. Sildenafil was originally developed as an anti-hypertensive drug, but ultimately was approved by FDA for ED in 1998 [72]. Subsequently, new PDE5 inhibitors were approved by FDA for ED treatment, namely vardenafil, tadalafil (both introduced in 2003), and avanafil (2012). Sildenafil exhibits a PDE5 and PDE6 IC50 of 2.2 and 9.5 nM, respectively. Tadalafil (Cialis), developed by Eli Lilly, is a PDE5 as well as PDE11 inhibitor, with IC50 values of 1.2 and 11nM, respectively, while vardenafil (Levitra) shows an inhibitory profile similar to that one of sildenafil [73]. A new generation of PDE5 inhibitors lodenafil (Helleva), udenafil (Zydena), and mirodenafil (Mvix) are also available in Brazil and Korea for ED treatment, but none of them have been approved by the FDA, yet (Fig. 3) [74].

The involvement of the NO cascade in memory mechanisms led to the hypothesis that PDE5 inhibition might be used in AD therapy, a disorder characterized by subtle mnesic problems since the disease onset [75]. Given that AD is a chronic condition, affecting an elderly population with likely comorbidity, it became evident that should PDE5 inhibitors be used in AD, they should have minimal side effects. Quinoline-based, naphthyridine-based and 1H-pyrroloquinolinone-based PDE5 inhibitors were thought to have potential for chronic treatment of AD patients (Fig. 4 and Table 1). Compounds **1** and **2** bearing the quinoline scaffold showed excellent selectivity against all eleven PDEs [73], thus suggesting that they might have less side effects than classic PDE5 inhibitors. Importantly, both compounds exhibited an improved PDE5/PDE6 potency compared to sildenafil, vardenafil and tadalafil (PDE5/PDE6 IC50 of **1** is 0.27/339 nM, PDE5/PDE6 IC50 of **2** is 0.4/5100 nM) and no inhibitory activity versus the other PDE isozymes [73]. Compound **1** crossed the BBB and increased the level of cGMP in the APP/PS1 mouse model of AD, thus counteracting the synaptic plasticity and memory damage caused by the elevation of Aβ. However, compound **1** showed a low water solubility, which unfortunately prevented the advancement of the compound into preclinical studies. Naphthyridine-based and 1H-pyrroloquinolinone-based PDE5 inhibitors, compounds **3** and **4** (Fig. 4 and Table 1), were designed by locking the rotatable bonds of the hydroxymethyl group of the quinoline-base compounds into a ring [76]. Compound **3** showed improved water solubility with respect to **1** together with excellent PDE5 potency and selectivity (IC_{50} (PDE5)=0.056 nM, IC_{50} (PDE6)=30.1 nM). Compound **3** was tested in the APP/PS1 mouse model of AD and showed to ameliorate learning and memory deficits. These *in vivo* positive pharmacologic effects were correlated to an increased cGMP levels in the mouse hippocampus. 1H-pyrroloquinolinone-based PDE5 inhibitor, compound **4**, showed a potency in picomolar range (IC_{50} (PDE5)=0.059 nM, IC_{50} (PDE6)=6.6 nM) and led to a selectivity for PDE5 of ~100-fold compared to PDE6. An *in silico* docking study was performed with both compounds **3** and **4**, showing their interaction with residues in the cGMP pocket of PDE5A1. The targeted pocket was chosen based on the fact that multiple complex crystal structures show that diverse PDE5 small molecule inhibitors consistently bind in it, showing that it has highly druggable characteristics. However both compounds, **3** and **4**, lacked *in vitro* metabolic stability, which suggests the need of further effort toward the optimization of these drugs property physicochemical and pharmacological properties [76].

An alternative therapeutic strategy that has recently drawn much attention concerns the development of multi target-directed ligands (MTDLs) that are able to act on different molecular targets involved in the pathological processes in AD [77]. To this end, dual inhibitors targeting PDE5 and histone deacetylases (HDACs) have been tested as a potentially novel therapeutic approach against AD. Similar to PDE5 inhibitors, HDAC inhibitors are potential modulators of cognitive impairment in AD [78, 79]. A series of cGMP-based derivatives and β-carboline derivatives were designed and synthesized with the aim to validate this multi target approach (Fig. 5 and Table 1) [80, 81]. Compound **5** shares the 1*H*-pyrazolo[4,3-*d*]pyrimidine scaffold of sildenafil, with an additional hydroxamic acid moiety that typically confers HDAC inhibitory activity. Compound **5** showed a PDE5 IC_{50} value of 60 nM and moderate inhibition activity against HDAC class I (HDAC1: 310 nM, HDAC2: 490 nM, HDAC3: 322 nM, HDAC4: 91 nM). A follow-up study showed that

compound **5** was able to cross the BBB and increase histone acetylation and CREB phosphorylation in the hippocampus, leading to the rescue of LTP in APP/PS1 mice [80]. The next goal in developing dual target ligands with PDE5/HDAC activity focused on reducing the toxicity deriving from the inhibition of other HDAC class I enzymes by improving the HDAC6 selectivity. Compound **6** demonstrated potent activities against PDE5 (IC_{50}=11 nM) and HDAC6 (IC_{50}=15 nM), retaining excellent HDAC6 selectivity over HDAC1 (1.3 log units). Although compound **6** reduced the levels of the AD-related markers APP and phosphorylated tau protein in Tg2576 neurons, however *in vivo* studies did not produce a significant memory improvement [81].

In the last decades, AChE/PDE5 dual target inhibitors have been investigated for the treatment of AD. Such approach is based on the rationale that the currently available AD drugs are AChE inhibitors. Two generations of dual-target AChE/PDE5 inhibitors with β-carboline scaffold were designed. The first generation includes compounds **7** and **8** (Fig. 5 and Table 1), which are derived from modifying the substituent at the nitrogen atom of piperazine-2,5-dione (compound **7**) and the substituent at the phenyl ring (compound **8**) of tadalafil. According to this study, the substituent ethyl-(1-benzylpiperidin-4-yl) can represent the AChE inhibition pharmacophore, which modulates liposolubility and improved BBB permeability [82]. As a result, these two candidate compounds exhibited the expected properties. Both compounds showed good AChE inhibition (IC_{50} **7**: 36 nM; IC_{50} **8**: 32 nM) as well as moderate PDE5 inhibitory activities (IC_{50} **7**: 153 nM; IC_{50} **8**: 1.53 μM). Importantly, in *in vivo* studies compound **7** reversed the cognitive dysfunction of scopolamine-induced AD mice and enhanced CREB phosphorylation. Unfortunately, this compound possessed poor water solubility, which represents a limitation to its further development [82]. To overcome this limitation, a second generation of dual target AChE/PDE5 inhibitors was synthesized with improved water solubility [83]. The structure-activity relationship studies revealed that the stereo-configuration at position 6 of the tadalafil core was vital to AChE inhibitory activity with the favored configuration was 6*R*, while the stereo-configuration at position 12 of the tadalafil nucleus had little influence on AChE inhibitory activity, but it was important for exerting PDE5A1 inhibitory activity. Compound **9** exhibited a dual-target AChE/PDE5 inhibitory activity of 15 nM and 3.23 μM, respectively, and good BBB permeability. Compound **9** was characterized by improved water solubility and good therapeutic effects against scopolamine-induced cognitive impairment, with an inhibition of cortical AChE activities and an enhancement of CREB phosphorylation *ex vivo* [83].

When proposing a new class of drugs as therapeutic agents it is imperative to consider their possible side effects that might be dependent upon interference with other targets or with the activity of the NO/cGMP/CREB axis and the role that it plays in many physiologic processes in the brain and elsewhere in the body. An advantage of using PDE5 inhibitors is that their side effects are known as they have already been utilized for many years, such that FDA has authorized the daily use of PDE5 inhibitors in arterial pulmonary hypertension. Recently, a number of clinical studies have explored the association between PDE5 inhibitors and the risk for melanoma and, interestingly, both positive and negative correlations have been reported [84–88]. However, those studies showing an increased risk

of incident melanoma upon PDE5 inhibitors exposure did not account for confounding risk factors, such as sun exposure, or adjusted their results for different factors (e.g. age, comorbidities, smoking). Thus, it has been concluded that there is no causality between the use of PDE5 inhibitors and melanoma and further evidence to show the association is needed [89, 90]. A few cases of priapism have also been reported following the intake of PDE5 inhibitors. However, it is possible that this side effect is due to a dysregulation of PDE5 function following NO cascade down-regulation [91] - a phenomenon that is also caused by Aβ increase [39] - such that, paradoxically, PDE5 inhibitors have been proposed as therapeutic agents against priapism [92, 93]. Additional adverse events of the PDE5 inhibitors include mild vasodilatory events such as headache, flushing, dyspepsia, and nasal congestion or rhinitis, hypotension (if nitrates are given concurrently), transient visual impairment. However, although Aβ is primarily accumulating in the CNS, Aβ is also present in the blood of patients with AD and related dementia [94, 95]. Interestingly, Aβ potentiates vasoconstriction both in cerebral vasculature and in other districts of the vascular system [96–102]. Moreover, hypertension has been often associated with AD [97–99]. Thus, it is very attractive to hypothesize that PDE5 inhibitors might counteract not only CNS symptoms, but also vascular symptoms that often affect AD patients.

4. Preclinical Studies of PDE5 Inhibitors in Alzheimer's Disease

At the preclinical level, there is a growing body of evidence showing that PDE5 inhibition can improve memory function. The first PDE5 inhibitor exhibiting cognitive enhancing properties in animals was zaprinast, a phosphodiesterase inhibitor that targets PDE5, PDE6, PDE9 and PDE11 [103]. During the training in the object recognition test, zaprinast and 7-nitroindazole, a putative selective inhibitor of nNOS, were administered i.p. to rats, immediately after the exposure to two identical objects [103]. After 1hr, control rats spent more time exploring the new object, indicating that they recognized the previously seen object. However, animals injected with 7-nitroindazole could not discriminate between the two objects. At 4hrs, control rats did not discriminate between the objects, whereas zaprinast facilitated object recognition and reversed the recognition memory deficit induced by 7-nitroindazole [103]. In addition to this original study performed in young rats, it was later confirmed that this effect on improving memory consolidation could be extended to older animals [104]. Moreover, the memory enhancing effect of zaprinast was extended to memory acquisition and consolidation in rats administered with the compound and tested with an object recognition task [104].

Following these initial studies with zaprinast, considering that the compound inhibits several PDEs [105], other studies were performed using inhibitors that are selective towards PDE5 in view of a potential therapeutic use of a strategy including enhancement of cGMP levels. Sildenafil was found to improve acquisition and consolidation of object recognition memory in both mice and rats [106–108], spatial memory in the elevated plus maze in mice [109], and performance in the active avoidance task in mice [110]. Additionally, it was shown that treatment with both sildenafil or zaprinast improves memory in young and age-impaired mice in an adapted version of the elevated plus maze and passive avoidance task [111]. As a matter of fact, the memory improving effect was more pronounced in aged mice than in young mice. Nevertheless, a later study reported that sildenafil had no effect on the memory

performance of young and aged rats tested in the passive avoidance task [112]. Recently, aged mice were also tested after chronic sildenafil treatment [113]. It was found that sildenafil improved age-impaired object and spatial memory performance in the object recognition task and Morris water maze task, respectively. Sildenafil also restored phosphorylation of hippocampal CREB in these aged mice. Altogether, these data suggest the possibility of using PDE5 inhibitors to enhance normal memory and age-related memory decline.

The possibility of using both genetically modified models of AD and pharmacologic models displaying cognitive dysfunction led to experiments assessing the efficacy of PDE5 inhibitors against memory loss associated with the disease [75, 114, 115]. Puzzo *et al.* showed that chronic administration of sildenafil in a 3-month-old transgenic APP/PS1 mouse model could restore AD related cognitive deficits, synaptic dysfunction, decreased activity of the memory related transcription factor CREB, and, interestingly, diminished hippocampal Aβ levels. In addition, administration of sildenafil to hippocampal slices reversed the impairment of LTP in these APP/PS1 mice [75]. Zhang and coworkers also showed that sildenafil reversed memory deficits in APP/PS1 mice in the object recognition task and led to improved cGMP/PKG/pCREB signaling and a decrease in soluble Aβ levels. These findings were in part consistent with a study conducted by Cuadrado-Tejedor et al. demonstrating that chronic administration of sildenafil reverses memory deficits in Tg2576 transgenic mice without changing Aβ load [114]. Moreover these studies were in agreement with the observation that pre-training administration of sildenafil reverses the spatial learning impairment induced by the nonspecific NOS inhibitor Nω-L-nitro-arginine methyl ester (L-NAME) [116, 117]. Finally, consistent with these studies, Rutten et al. extended their earlier findings from rodents to monkeys by showing that sildenafil dose-dependently improved cognitive function in a prefrontal task, i.e., the object retrieval task, in cynomolgus macaques [118].

Later studies have replicated the sildenafil data in the object recognition task in rats using other PDE5 inhibitor, such as vardenafil or tadalafil [75, 119, 120]. For instance, acute treatment with vardenafil improved spatial memory tested with the object location test [121]. Additionally, in a study from Devan *et al.*, repeated daily injections of vardenafil for one week improved long-term memory performance [119]. Tadalafil, in turn, was able to reverse the reduction of LTP when directly administered to slices of the APP/PS1 mouse model of AD but failed to achieve behavioral benefits similar to sildenafil when administered to adult APP/PS1 mice *in vivo*. The lack of *in vivo* efficacy was attributed to the inability of tadalafil to cross the BBB that had been reported by the producer of the compound [75]. Conversely, Garcia-Barroso *et al.* showed data indicating that tadalafil improves the Morris water maze performance in the J20 mouse model of AD, and reduced tau phosphorylation without affecting the Aβ burden [120]. They also reported that the compound crosses the BBB [120]. Nevertheless, in 2017 Fei Mao *et al.* prepared a series of tadalafil analogues with improved BBB penetrability and confirmed that tadalafil possesses poor BBB penetration [82]. Thus, a plausible explanation for the beneficial effect of the compound in the Garcia-Barroso *et al.* manuscript [120] might be related to a possible alteration in the BBB of the AD mice, which facilitated tadalafil access to the CNS. Alternatively, one cannot exclude that the inhibitor might act through vascular mechanisms, as PDE5 inhibitors were initial developed for their

antihypertensive effects due to the resulting vasodilation effects deriving from an increase of cGMP in the endothelial tissue.

Most recently, other PDE5 inhibitors such as icariin, yonkenafil, compound 7a (1 in Fig. 4) and compound 6c (3 in Fig. 4) were developed specifically for exploring their therapeutic potential in learning and memory. Jin *et al.* showed a beneficial effect of icariin against memory loss in APP/PS1 transgenic mice [122]. They found that icariin treatment significantly improves the memory defect in the Y-maze task. Moreover, levels of amyloid precursor protein (APP), amyloid-beta (Aβ1–40/42) and both PDE5 mRNA and protein were increased in the mouse hippocampus and cortex.

The effects of yonkenafil were investigated on cognitive behaviors as well as the pathological hallmarks of AD using transgenic models of the disease. Daily treatment of seven-month-old APP/PS1 mice with yonkenafil for 3 months improved nesting-building ability, working memory deficits in the Y-maze task, and Morris water maze performance. In addition, yonkenafil reduced Aβ plaque area, and inhibited over-activation of microglia and astrocytes. Furthermore, yonkenafil increased neurogenesis in the *dentate gyrus*. These results are consistent with the hypothesis that PDE5 inhibition attenuates cognitive deficits in AD by reducing the amyloid burden, and inhibiting over-activation of microglia and astrocytes as well as restoring neurogenesis [123].

The pharmacological efficacy of compound 7a (1 in Fig. 4) was investigated in several transgenic mouse models of AD [40, 73]. Compound 7a was able to improve contextual memory in both mice pre-treated with Aβ or tau oligomers, and APP/PS1mice. Additionally, an amelioration of spatial memory in tau oligomer treated and APP/PS1 mice was observed. These behavioral findings were corroborated by the biochemical analysis of the hippocampus of mice that revealed an increase of cGMP and pCREB levels. Thus, compound 7a confirmed the therapeutic potential of using PDE5 inhibitors in AD and extended their usefulness to tauopathies [40, 73].

The drug optimization of compound 7a led to the discovery of compound 6c (3 in Fig. 4), which was profiled for its ability to rescue learning and memory in the APP/PS1 transgenic mouse model [76]. LTP studies with compound 6c showed strengthening of synaptic plasticity in hippocampal slices of APP/PS1 mice. *In vivo* studies also demonstrated positive behavioral outcomes when compound 6c was administered to young mice that were assessed with a radial arm water maze task. Remarkably, the beneficial effects of the PDE5 inhibitor were still observed three months after the administration of the therapy, suggesting that the inhibition of PDE5, and therefore the elevation of cGMP, is inducing persisting biochemical changes [76].

The study of PDE5 inhibition as potential cognition enhancement strategy gained particular interest, since the PDE5 protein is highly expressed in the cytoplasm of neuronal cells in human brains [124]. Specifically, in cortex and hippocampus, PDE5 is expressed primarily in large, pyramidal-type neurons, whereas in cerebellum, it is prominent in Purkinje neurons [124]. However, PDE5 inhibition is currently used as a therapeutic approach for treating erectile dysfunction and pulmonary hypertension due to the resulting vasodilation effects

deriving from an increase of cGMP in the endothelial tissue. These cardiovascular effects of PDE5 inhibitors caused a controversy with regard to their mechanism of action as cognitive enhancers. Vasodilation might lead to an increase in blood flow and glucose metabolism in the brain and, as such, could induce improved brain functions without directly affecting neuroplasticity mechanisms. However, it has been preclinically shown that the effective dose of PDE5 inhibitors required for improving cognition is below the dose that induces vascular and metabolic effects. For example, a study has shown that sildenafil could increase the mean arterial blood pressure in rats following 10 mg/kg oral administration [125]. Yet, the memory improvement effect of sildenafil has been observed at a dose of 1–3 mg/kg, as demonstrated by Puzzo *et al.* [75]. Also, sildenafil had no effect in humans on blood flow in the middle cerebral artery, just as there were no changes in radial and temporal artery diameters [126–128]. Another study showed that the cognitive enhancement observed in rats treated with vardenafil was not related to any main effects on blood flow and glucose utilization in the brain [129]. Finally, a recent report by Akkerman *et al.* showed results in support of the hypothesis that PDE5 inhibitors act through central mechanisms in a study in which they administered vardenafil via the intracerebroventricular route [130]. Nevertheless, one cannot exclude that in some circumstances (see for instance the beneficial effect of tadalafil in Garcia-Barroso *et al.* [120]), PDE5 inhibitors might enhance cognition through vascular mechanisms.

5. Clinical Studies of PDE5 Inhibitors as Cognitive Enhancers in Normal Conditions and Disease Status

Despite the robust memory improving action of PDE5 inhibitors in animal studies, these effects have not yet been translated into clinical efficacy to enhance cognition. Several clinical studies have involved health volunteers as well as different populations of patients. However, none of the investigated drugs has reached the market as a cognitive enhancer/anti-AD drug so far.

Data obtained from administration in humans have shown a very wide variety of results ranging from no effect to beneficial effect on normal memory. For instance, in a study sildenafil was tested on various cognitive functions in six male healthy volunteers [131]. A single oral dose of 100 mg sildenafil enhanced performance in a simple reaction time test, but showed no significant effects on short-term memory, divided attention and other psychomotor tasks. Another study conducted on young healthy male volunteers confirmed that the same dose of sildenafil did not significantly improve memory performance in healthy subjects [132]. In this placebo-controlled trial, sildenafil significantly affected patterns of auditory event-related brain potentials consistent with enhanced ability to focus attention and to select relevant target stimuli, although induced no direct cognition enhancing effect on auditory attention and word recognition [132]. Moreover, sildenafil showed an effect on information processing since a reduced negativity in the electroencephalogram was found in the word recognition experiment [132].

The cognition enhancing potential of sildenafil was also investigated in a trial for schizophrenia and another one for Parkinson’s disease. In the first study, pairing of sildenafil

with antipsychotic treatment did not affect cognitive performance [133, 134]. It is possible that the doses of 50 and 100 mg used in this study were not optimal or repeated dosing may be necessary to achieve therapeutic effects. In the latter study, sildenafil was tested for its efficacy in reducing dyskinesia in patients with Parkinson's disease (ClinicalTrials.gov Identifier: NCT02162979). However, this study was terminated due to insufficient participant inclusion. Sildenafil has also been evaluated in a Phase I study for its neuroprotective properties in the treatment of stroke (ClinicalTrials.gov Identifier: NCT00452582). However, in 2011 this study was terminated because of a failure to recruit in the expected time period. Also, a recent study on AD patients demonstrated that a single, 50 mg dose of sildenafil could improve significantly cerebral hemodynamic function and increase cerebral oxygen metabolism [135]. These results together suggest that PDE5 inhibitors might enhance memory via a cerebrovascular mechanism.

Tadalafil is considered to be less efficacious than sildenafil or vardenafil for cognition enhancement because of its poor brain penetration [75]. This inhibitor is currently evaluated in a phase II clinical trial initiated at St George's (University of London) in elderly patients with cerebral small vessel disease. Cognitive performance was measured after the treatment of a single oral dose of 20 mg tadalafil. The overall idea of the clinical trial is that the vasodilatory properties of tadalafil should improve blood flow to these brain areas thus preventing vascular cognitive impairment (ClinicalTrials.gov Identifier: NCT02450253).

Two studies from Shim *et al.* reported that chronic treatment with the PDE5 inhibitor udenafil (100 mg) for 2 months in patients with erectile dysfunction improved cognitive and frontal executive function [136]. Moreover, a follow-up study from the same group showed that even a low dose of udenafil (50 mg) could exert cognitive enhancing action after daily administration for 2 months [137]. The latter findings probably indicate that chronic treatment of PDE5 inhibitors is necessary in order to exert their cognitive enhancing action in humans. Importantly, no severe adverse events were observed in both studies, and no patient discontinued the treatment. The only adverse events were mild dizziness in 2 out of 27 patients in the first study [136] and hot flushing or nasal congestion in 4 out of 24 patients in the second study [137].

6. Conclusions

Altogether, while PDE5 inhibitors have shown clear cognition enhancing potential in preclinical studies, these effects have not yet resulted in a clear demonstration of clinical efficacy. Although PDE5 inhibitors can induce vasodilatory effects when administered at high doses, the cognition enhancing properties observed in the current studies using lower doses were not related to any main effect on blood flow and glucose utilization in the brain. Interestingly, due to the FDA-approval of PDE5 inhibitors for the treatment of non-neurological conditions, their side effects have been extensively documented and consist of mild adverse effects that are generally well-tolerated by patients like headache, flushing, runny nose, stomach pain, back pain, and indigestion [138]. Nevertheless, it is acknowledged that currently available PDE5 inhibitors are characterized by suboptimal specificity, which could limit their clinical application. For instance, sildenafil also inhibits PDE6 that is expressed in the retina and therefore causes visual disturbances after chronic

administration. As a result, development of novel PDE5 inhibitors is focusing on improving specificity of the inhibitor for the enzyme, optimizing pharmacokinetic properties in humans, and increasing BBB permeability. Medicinal chemistry data that have been obtained so far together with a growing body of preclinical and clinical evidence here described strongly support the possibility that PDE5 inhibitors represent a novel class of compounds that might effectively counteract AD progression.

Acknowledgments

Financial Support

This work was supported by the NIH grants R01NS110024 and R01AG049402 to OA, and grant AARF-17-504483 from the Alzheimer's Association to JF.

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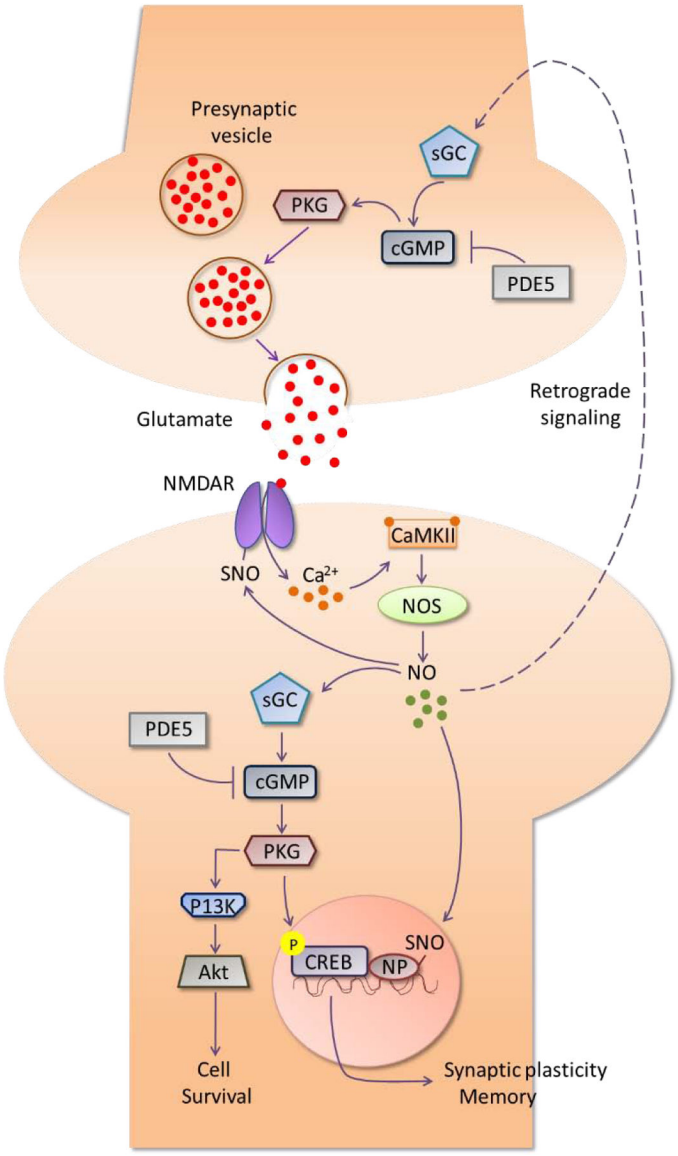

Figure 1.

Nitric oxide signaling pathway in the brain. NO, produced by NOS, binds to and activates sGC, which catalyzes the production of cGMP, a second messenger activator of PKG. Levels of cGMP are regulated by the cGMP-degrading enzyme PDE5. Finally, PKG phosphorylates CREB protein, an important transcription factor responsible for the expression of memory-related genes. An alternative route shows the activation of the PI3k/Akt signaling pathway by PKG, leading to the promotion of cell survival. S-nitrosylation of NP associated with CREB-binding genes facilitates the activation of CREB, while S-nitrosylation of NMDAR inhibits the receptor activity. At the presynaptic neurons, NO is involved in the retrograde signaling and promotes the release of neurotransmitters, like glutamate, after activation of the sGC/cGMP/PKG pathway. CaMKII: Ca2+/calmodulin-dependent protein kinase II; cGMP: cyclic guanosine monophosphate; CREB: cAMP response element-binding element; NMDAR: N-methyl-D-aspartate receptor; NO: nitric oxide; NOS: nitric oxide synthase; NP:

nuclear proteins; PI3k: phosphatidylinositol 3-kinase; PDE: phosphodiesterase; PKG: protein kinase G; SNO: S-nitrosothiol; sGC: soluble guanylate cyclase.

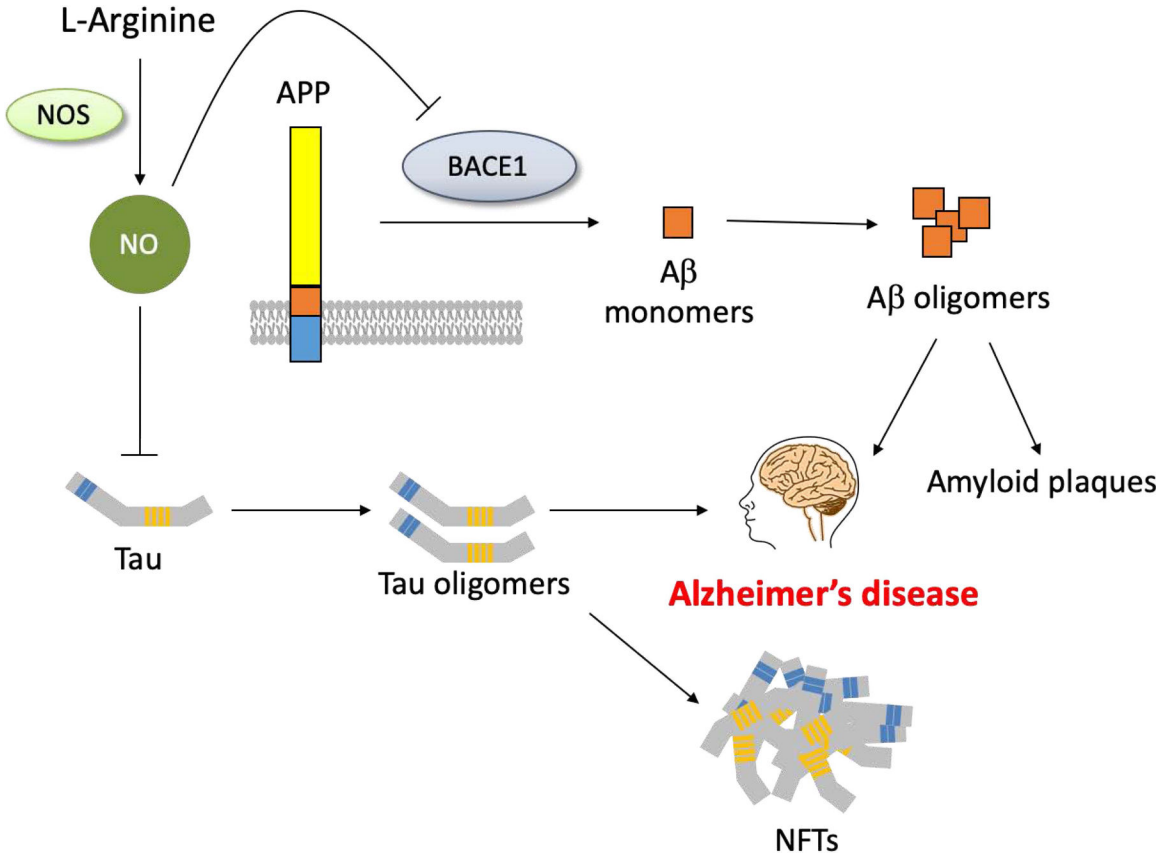

Figure 2.

Effects of the NO signaling on Aβ and tau pathology. Low and high levels of NO prevent the cleavage of amyloid precursor protein (APP) by beta-secretase 1 (BACE1), a process involved in the production of Aβ. Lower concentration of NO block BACE1 expression, whereas higher concentration prevent its enzymatic activity. At the same time, NO inhibits tau assembly and oligomerization, preventing formation of neurofibrillary tangles (NFTs).

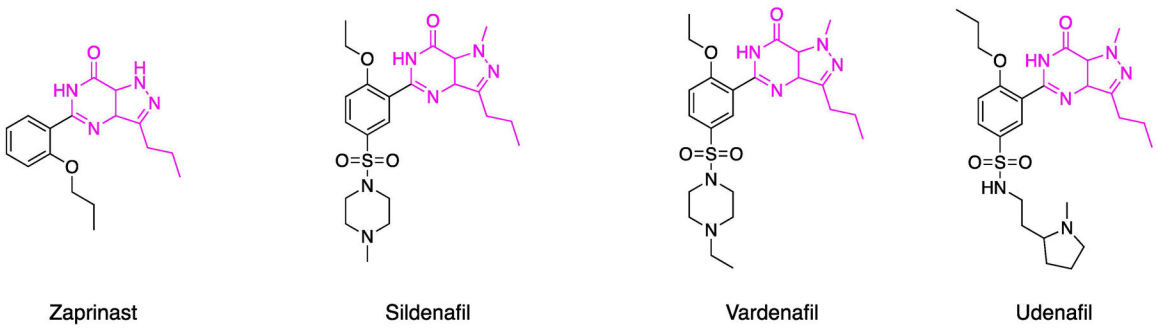

Figure 3.
Chemical structures of commercial PDE5 inhibitors.

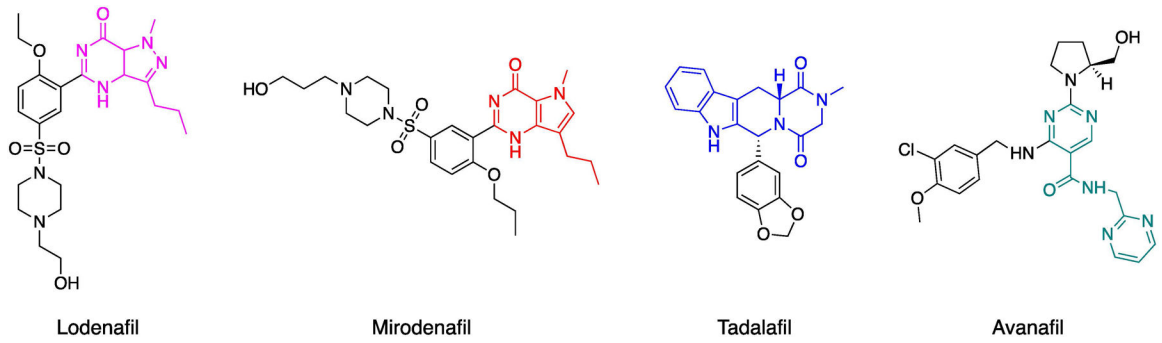

Figure 4.

Chemical structures of quinoline-based (**1** and **2**), naphthyridine-based (**3**) and 1H-pyrroloquinolinone-based (**4**) PDE5 inhibitors.

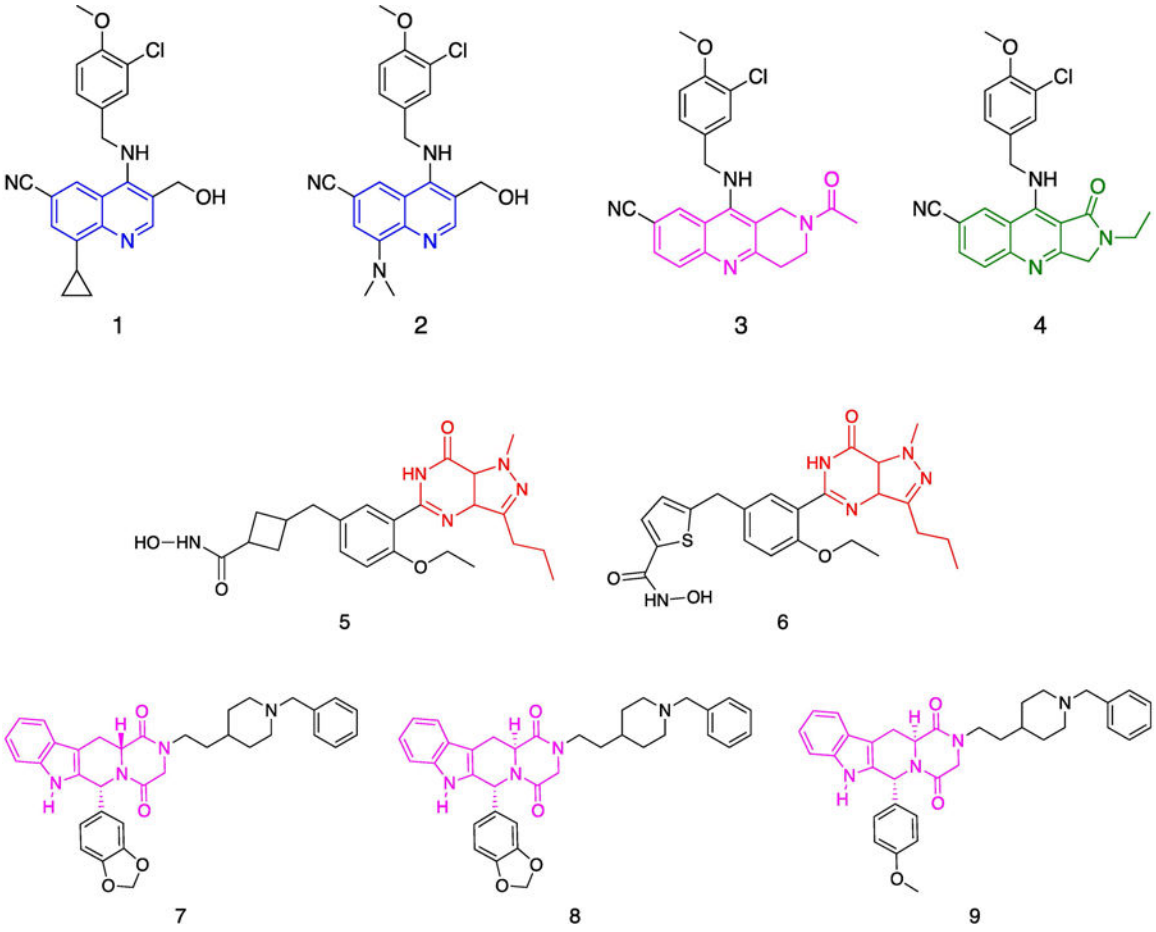

Figure 5.
Chemical structures of PDE5/AChE inhibitors.

Table 1.

Current most relevant PDE5 inhibitors, PDE5/HDAC inhibitors and PDE5/AChE inhibitors.

COMPOUNDS	TARGET	INDICATION	EFFECT	REFERENCE	LIMITATIONS
1	PDE5	Alzheimer's disease	Increased cGMP levels in the hippocampus of mice and improved learning and memory deficits in transgenic mouse model of AD.	Fiorito *et al.* 2013 [57]	Low water solubility.
3	PDE5	Alzheimer's disease	Increased cGMP levels in the hippocampus of mice and improved learning and memory deficits in transgenic mouse model of AD.	Fiorito *et al.* 2017 [58]	Lack of *in vitro* metabolic stability.
5	PDE5-HDAC	Alzheimer's disease	Reduced Aβ levels and tau phosphorylation levels	Cuadrado-Tejedor *et al.* 2017 [62]	Moderate HDAC class I inhibitor and HDAC6 and PDE5 PDE9 inhibitor. not every isoform-selective HDACis target each class I isoform (HDAC1, HDAC2, and HDAC3) is associated with cytotoxicity.
6	PDE5-HDAC	Alzheimer's disease	Reduced tau phosphorylation levels	Rabal *et al.* 2018 [63]	Poor BBB penetration; Lack of in vitro metabolic stability.
7	PDE5-AChE	Alzheimer's disease	Inhibition of cortical AChE; improved CREB phosphorylation	Mao *et al.* 2018 [67]	Nearly insoluble in water. More selective for AChE
8	PDE5-AChE	Alzheimer's disease	Inhibition of cortical AChE; improved CREB phosphorylation	Mao *et al.* 2018 [67]	Nearly insoluble in water. More selective for AChE
9	PDE5-AChE	Alzheimer's disease	Inhibition of cortical AChE; improved CREB phosphorylation	Ni *et al.* 2018 [68]	More selective for AChE and BuChE

Table 2.

PDE5 inhibitors in cognition. EEG, electroencephalogram, i.m. intramuscular, i.p. intraperitoneal, ORT object recognition test, p.o. per os, s.c. subcutaneously.

PDE5 inhibitor	Doses	Route	Test	Animals	Behavioral Results	Reference
Zaprinast	3 or 10 mg/kg	i.p.	ORT (4h interval)	3 months old Tryon–Maze–Bright rats	10 mg/kg improved memory consolidation	Prickaerts *et al.* 1997 [69]
Zaprinast	0.5, 1 or 2 mg/kg	i.p.	Elevate plus-maze and passive avoidance task	3 and 20–22 months old Swiss mice	All doses improved memory in aged animals; 1 and 2 mg/kg improved memory in young animals	Patil *et al.* 2004 [77]
Zaprinast	0.3 mg/kg	s.c.	ORT (2h interval)	3 and 12 months old Wistar rats	Pro-cognitive action only for the young animals. No effect in older animals.	Domek-Łopacińska and Strosznajder 2008 [70]
Sildenafil	1, 3, 10 or 30 mg/kg	i.p.	Active avoidance learning	2 months old Swiss mice	3 mg/kg improved performance	Baratti and Boccia 1999 [76]
Sildenafil	0.25, 0.5 or 1 mg/kg	i.p.	Elevate plus-maze and passive avoidance task	3 and 20–22 months old Swiss mice	All doses improved memory in aged animals-0.5 and 1 mg/kg improved memory in young animals	Patil *et al.* 2004 [77]
Sildenafil	1, 3 or 10 mg/kg	p.o.	ORT (24h interval)	4 months old Wistar rats	3 and 10 mg/kg improved memory consolidation	Prickaerts *et al.* 2005 [73]
Sildenafil	0.3, 1 or 3 mg/kg	p.o.	ORT (24h interval)	6 months old Swiss mice	1 mg/kg improved memory consolidation	Rutten *et al.* 2005 [74]
Sildenafil	1, 3, 10 or 20 mg/kg	i.p.	Passive avoidance task	2 and 12-month old Wistar rats	No memory enhancing effect	Shafiei *et al.* 2006 [78]
Sildenafil	0.3, 1 or 3 mg/kg	i.m.	Object retrieval task	Cynomolgus macaque	1 and 3 mg/kg improved memory performance	Rutten *et al.* 2008 [84]
Sildenafil	3mg/kg daily for 3 weeks	i.p.	ORT and MWM	26–30 months old C57Bl/6J mice	Improved performance in both tests	Palmeri *et al.* 2013 [79]
Vardenafil	0.5 mg/kg	p.o.	14-unit T-maze	3,17, and 24-month-old rats	Improved spatial memory retention	Devan et al. 2014 [85]
Vardenafil	0.3 mg/kg	i.p.	Object location test (24h interval)	4–5 months old mice	Improved spatial memory acquisition and early consolidation	Argyrousi *et al.* 2019 [87]
Tadalafil	15 mg/kg	p.o.	MWM	3-month-old J20 transgenic mice	Improved memory performance	Garcia-Barroso et al. 2013 [86]

Table 3.

PDE5 inhibitors oral single dose in clinical trials.

Drugs	Doses	Field	Phase	Patients	References and ID clinical number
Sildenafil	100 mg	cognition	Completed Phase VI	Healthy males	Grass *et al.* 2001 [97]
Sildenafil	100 mg	cognition	Completed Phase VI	Healthy males	Schultheiss *et al.* 2001 [98]
Sildenafil	50 and 100 mg	schizophrenia	Completed Phase VI	Schizophrenic patients	Goff *et al.* 2009 [99]
Sildenafil	50 mg	Parkinson's disease	Phase II (insufficient participants)	Parkinson's disease patients	Identifier: NCT02162979
Sildenafil	50 mg	hemodynamic function	N/A	AD patients	Sheg *et al.* 2017 [101]
Sildenafil		ischemic stroke	Phase I (terminated due to non-recruitment of patients within the scheduled time period)	Ischemic stroke patients	Identifier: NCT00452582
Tadalafil	20 mg	cognition	Phase II	Elderly with small vessel disease	Identifier: NCT02450253
Vardenafil	10, 20 mg	sensory gating	Completed phase II	Healthy young adults	Reneerkens *et al* 2013 [105]
Vardenafil	10, 20 mg	cognition	Completed phase II	Healthy young adults	Reneerkens *et al* 2013 [106]
Udenafil	100 mg	cognition	N/A	Patients with ED	Shim *et al* 2011 [102]
Udenafil	50 mg	cognition	N/A	Patients with ED	Shim *et al.* 2014 [103]