Discovery of novel PDE9A inhibitors with antioxidant activities for treatment of Alzheimer’s disease

Chen Zhang*a, Qian Zhou*a, Xu-Nian Wu*a, Ya-Dan Huanga, Jie Zhoua, Zengwei Laiab, Yinuo Wua and Hai-Bin Lua,c

aSchool of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou, PR China; bState Key Laboratory for Chemistry and Molecular Engineering of Medicinal Resources, Guangxi Normal University, Guilin, PR China; cCollaborative Innovation Center of High Performance Computing, National University of Defence Technology, Changsha, PR China

ABSTRACT
Phosphodiesterase-9 (PDE9) is a promising target for treatment of Alzheimer’s disease (AD). To discover multifunctional anti-AD agents with capability of PDE9 inhibition and antioxidant activity, a series of novel pyrazolopyrimidinone derivatives, coupling with the pharmacophore of antioxidants such as ferulic and lipolic acids have been designed with the assistance of molecular docking and dynamics simulations. Twelve out of 14 synthesised compounds inhibited PDE9A with IC50 below 200 nM, and showed good antioxidant capacities in the ORAC assay. Compound 1h, the most promising multifunctional anti-AD agent, had IC50 of 56 nM against PDE9A and good antioxidant ability (ORAC (trolox) = 3.3). The selectivity of 1h over other PDEs was acceptable. In addition, 1h showed no cytotoxicity to human neuroblastoma SH-SY5Y cells. The analysis on structure-activity relationship (SAR) and binding modes of the compounds may provide insight into further modification.

Introduction
Alzheimer’s disease (AD) is the most common type of dementia, which is characterised by progressive memory loss, decline in language skills, and other neurodegenerative disorders1. According to Alzheimer’s Association (USA), about 46.8 million people worldwide are suffering from AD. The morbidity and mortality are high, especially for elder persons above 60 years old. The global societal economic cost for AD patients was estimated at $818 billion in 2015 and will rise to $1000 billion in 2018, and $2000 billion in 20302. Currently, AD has been one of the most challenges for public health around the world, severely impacting lives of patients and their families.

The pathogenic mechanism of Alzheimer’s disease is complicated and still has not fully understood yet. Several factors, such as low level of acetylcholine, deposition of β-amyloid (Aβ), aggregation of Tau-protein, and oxidative stress have been regarded as important in the pathophysiology of AD3. Current drugs for the treatment of AD include three acetylcholinesterase (AChE) inhibitors (donepezil, rivastigmine, and galantamine) and one N-methyl-d-aspartate receptor antagonist (memantine)4. However, administration of these drugs only can improve cognition and dementia degree of AD patients, but not reverse the underlying progression. In recent decades, discovery of anti-AD candidate drugs mainly focus on the anti-Aβ pathology. However, most clinical trials using these candidates failed5. One important reason for this failure might be that the single targeted drugs were not able to control the complicated pathogenic progression of AD. Thus, development of multifunctional anti-AD agents which simultaneously hit more than single target in the AD pathophysiology, has become an important research area in recent years6,7, especially those hitting at non-Aβ targets.

Phosphodiesterases (PDEs) are a super-enzyme family in charge of hydrolysing the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)8,9. According to their structural and functional properties, PDEs can be divided into 11 subfamilies (PDE1–PDE11). In brain, the levels of cAMP and cGMP are increased via the inhibition of PDEs, activating AC/cAMP/PKA, or NO/cGMP/PKG signalling pathway, and thus increasing the level of the cAMP response element-binding protein (CREB), enhancing synaptic transmission, and reducing cognitive deficit10. PDEs inhibitors of PDE1, PDE2, PDE4, PDE5, and PDE9 have been demonstrated to be effective in restoring cognitive deficits in some preclinical models and clinical trials of AD11.

Among all the PDE subfamilies, Phosphodiesterase 9 (PDE9) has unique advantages on AD therapy for its highest affinity with cGMP among all the PDE families and its high expression level in the cortex, hippocampus, basal ganglia, and cerebellum of brain12. Currently, the effects of PDE9 inhibitors in the AD animal models have been deeply explored. In the social and object recognition tasks of rodents, PDE9 inhibitor BAY73–6691 enhanced the ability of acquisition, consolidation, and retention of long-term potentiation (LTP), which also improved scopolamine-induced passive avoidance deficit and MK-801-induced short-term memory
In comparison with AChE inhibitor donepezil that only enhanced short memory in the early LTP, BAY73–6691 not only increased both early and late LTP in rat hippocampal slices, but also transformed the early LTP into late LTP. In addition, BAY73–6691 protected Aβ25-35-induced oxidative damage in hippocampus via the activation of the cGMP-related NO-dependent signalling. Besides BAY73–6691, PDE9 inhibitors PF-04447943 also exhibited memory improvement in several AD models. Currently, PF-04447943 has already completed six phase I clinical trials and one phase II trial of AD. Despite good effects on memory improvement obtained by PDE9 inhibitors in the preclinical and clinical trials, the number of PDE9 inhibitors for treatment of AD is very limited, especially those combining the PDE9 inhibition as well as hitting at other pathophysiology of AD simultaneously.

Oxidative stress is an important risk factor in the AD onset and progression. The abnormality of redox state may participate in the neurodegenerative process, resulting in reactive oxygen species (ROS) mediated and reactive nitrogen species (RNS) mediated impairment in AD brain. Furthermore, the levels of oxidative markers of biomolecules including proteins, lipids, carbohydrates, and nucleic acids, as well as the antioxidant enzymes, were observed to be changed. Numbers of antioxidants such as resveratrol, ferulic acid have been proved to be neuroprotectants. However, results from the clinical trials suggested that only antioxidant efficacy was not sufficient to modify AD progression. Antioxidants combining with additional pharmacological effects might combat the complicated pathogenic mechanism of AD more efficiently. Thus, numerous multifunctional anti-AD agents with antioxidant activity and also hitting other targets in the AD pathophysiology were developed. Some of them even went into clinical trials. Vinpocetine, a moderate PDE1 inhibitor with antioxidant activity, significantly improved learning and memory in the streptozotocin infused AD rat models. Further studies demonstrated that both the regulation of cyclic nucleotide signalling and antioxidant mechanism were responsible for the memory improvement obtained by vinpocetine. For unique advantages of PDE9 inhibition over other PDEs, development of PDE9 inhibitors with antioxidant activity, to regulate both cGMP signalling and antioxidant mechanism is of great importance.

With our continuing interest in discovery of PDE9 inhibitors, we reported here the design, synthesis, and biological evaluation of novel pyrazolopyrimidinone derivatives with both PDE9 inhibition and antioxidant activity. Molecular docking and dynamics simulation were applied at the beginning to choose potential lead compounds with appropriate binding modes and binding free energies, in order to reduce the next synthetic work and improve the efficiency of lead discovery.

**Materials and methods**

**Chemistry**

1H NMR and 13C NMR spectra were recorded on a Bruker BioSpin GmbH spectrometer (Bruker, Billerica, MA) at 400.1 and 100.6 MHz, respectively. Coupling constants (J) were reported in Hertz (Hz). Spectra were referenced internally to the residual proton resonance in CDCl3 (δ 7.26 ppm) or DMSO (δ 2.50 ppm) as the internal standard. High-resolution mass spectra (HRMS) were obtained on an IT-TOF mass spectrometer. Flash column chromatography was performed using silica gel (200–300 mesh). Thin-layer chromatography was performed on precoated silica gel F-254 plates (0.25 mm) and was visualised with UV light. Unless otherwise noted, all the starting materials and reagents were purchased from commercial suppliers and used directly without further purification.

**Cyclopropent-4-ocyano-1h-pyrrozol-5-amine (M-3)**

To a solution of cyclopropenthydrazinedihydrochloride M-2 (2.7 g, 20 mmol) in ethanol (20 ml), triethylamine (7.1 g, 70 mmol) was added slowly at 0 °C. After stirring for 2 h, 2-(methoxymethylene)-malononitrile M-1 (2.4 g, 22 mmol) was added to the mixture dropwise. The mixture was then stirred at room temperature for 3 h and refluxed at 80 °C for 3 h. After the reaction was finished, ethanol was evaporated to half of its volume. Water was added and the resulted precipitate was collected as a brown solid. The brown solid was purified by recrystallisation with ethanol as the solvent, providing compound M-3 as a yellow solid (3.0 g, 85%). 1H NMR (400 MHz, DMSO) δ 8.11 (s, 1H), 5.51 (d, J = 6.8 Hz, 1H), 4.50–4.38 (m, 1H), 3.39 (s, 1H), 2.03–1.93 (m, 2H), 1.88–1.79 (m, 2H), 1.76–1.68 (m, 2H), 1.63–1.53 (m, 2H). 13C NMR (101 MHz, DMSO) δ 157.33, 134.01, 114.78, 76.01, 62.18, 31.82, 23.64. HRMS (ESI-TOF) m/z [M + H]+ calcld for C9H13N4 171.1143, found 171.1140.

**S-Amino-1-cyclopropent-1h-pyrrozol-4-carboxamide (M-4)**

To a solution of compound M-3 (1.8 g, 10 mmol) in ethanol (15 ml), 30% hydrogen peroxide (1.5 ml), and 25% ammonium hydroxide (4.0 ml) were added sequentially. The mixture was stirred at room temperature for 1 h. When the reaction was finished, saturated sodium thiosulfate was added and a precipitate was formed. The precipitate was filtered, washed with water three times, and dried under vacuum to give compound M-4 as a white solid. 1H NMR (400 MHz, DMSO) δ 7.63 (s, 1H), 7.16 (br s, 1H), 6.62 (br s, 1H), 6.13 (m, 1H), 4.57–4.45 (m, 1H), 3.39 (s, 1H), 2.00–1.87 (m, 2H), 1.87–1.72 (m, 4H), 1.63–1.50 (m, 2H). 13C NMR (101 MHz, DMSO) δ 165.73, 148.24, 136.27, 96.17, 55.30, 30.65, 23.48. HRMS (ESI-TOF) m/z [M + H]+ calcld for C16H22N4O3 295.1524, found 295.1526.

(R)-benzylo[(1–(1-cyclopropent-4-oxo-4,5-dihydro-1h-pyrrozolo[3,4-d]pyrimidin-6-yl)ethyl)carbamate (M-5)

NaH (60% dispersion in mineral oil, 0.80 g, 30 mmol) was added to the solution of M-4 (1.9 g, 10 mmol) and (R)-ethyl-2-[(benezoxyl)-carbonylamino]propionate (7.5 g, 30 mmol) in anhydrous THF (40 ml). The mixture was stirred at room temperature overnight. Water was added slowly to the mixture. The mixture was then extracted with ethyl acetate three times, washed with brine, dried over Na2SO4, and concentrated. The residue was purified by silica gel chromatography (methanol/dichloromethane 1:100) to afford compound M-5 as a yellow oil (2.0 g, 42%). 1H NMR (400 MHz, CDCl3) δ 11.90 (s, 1H), 8.05 (s, 1H), 7.32–7.28 (m, 5H), 5.98 (d, J = 6.1 Hz, 1H), 5.23–5.05 (m, 3H), 4.98–4.83 (m, 1H), 2.10 (dd, J = 13.6, 6.9 Hz, 4H), 2.02–1.92 (m, 2H), 1.79–1.69 (m, 2H), 1.60 (d, J = 7.1 Hz, 3H). 13C NMR (101 MHz, CDCl3) δ 160.26, 159.79, 155.83, 151.90, 136.12, 134.56, 128.50, 128.18, 104.41, 67.18, 57.89, 50.43, 32.47, 24.78, 20.56. HRMS (ESI-TOF) m/z [M + H]+ calcld for C34H32N6O3 562.2386, found 562.2385.

(R)-6–(1-aminoethyl)-1-cyclopropent-1h-pyrrozolo[3,4-d]pyrimidin-4(5H)-one (M-6)

To a solution of compound M-5 (3.8 g, 10 mmol) in methanol, Pd/C (10%, 0.4 g) was added. The mixture was stirred at room
temperature for 24 h under an atmospheric pressure of hydrogen. The catalyst was filtered and the filtrate was concentrated. The residue was purified by silica gel chromatography (methanol/ dichloromethane 1:50) to afford compound **M-6** as a yellow solid (1.3 g, 52%). \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta 8.05\) (s, 1H), 5.22–5.08 (m, 1H), 4.12 (q, \(J = 6.8\) Hz, 1H), 2.20–2.03 (m, 4H), 2.01–1.91 (m, 2H), 1.76–1.67 (m, 2H), 1.53 (d, \(J = 6.9\) Hz, 3H). \(^1^C\) NMR (101 MHz, CDCl\(_3\)) \(\delta 162.65, 158.53, 152.48, 134.49, 104.72, 57.81, 49.70, 32.44, 32.32, 24.75, 23.15. HRMS (ESI-TOF) \(\text{m/z}\) [M + H]+ calcd for C\(_{12}\)H\(_{17}\)N\(_2\)O\(_4\) 247.1438, found 247.1433.

**General procedures for the preparation of compound 1a-1l, 2, and 3**

A mixture of compound **M-6** (0.25 g, 1.0 mmol), EDC.HCl (0.15 g, 1.5 mmol), DMAP (0.002 g, 0.02 mmol), and the corresponding acid (1.0 mmol) was stirred at room temperature overnight. Water was added to the mixture. The mixture was then extracted with ethyl acetate three times, washed with brine, dried over Na\(_2\)SO\(_4\), and concentrated. The resulting residue was purified by silica gel chromatography and eluted with methanol and dichloromethane (1:100) to afford the final product.

\(\text{(R, E)-N-\{1-(1-cyclopentyl-4-oxo-4, 5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)ethyl\}-3-(4-hydroxy-3-methoxycinnamic acid as a white solid}. \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta 11.81\) (s, 1H), 8.09 (s, 1H), 7.60 (d, \(J = 15.4\) Hz, 1H), 7.00 (d, \(J = 6.7\) Hz, 1H), 6.94 (s, 1H), 6.87 (m, 1H), 6.33 (d, \(J = 15.1\) Hz, 1H), 5.99 (s, 1H), 5.30 (dd, \(J = 15.1, 8.8\) Hz, 1H), 5.20 (dd, \(J = 16.8, 10.1\) Hz, 1H), 3.85 (s, 3H), 2.15 (m, 4H), 1.98 (m, 2H), 1.78 (m, 2H), 1.66 (d, \(J = 4.5\) Hz, 3H). \(^1^C\) NMR (101 MHz, Acetonitrile) \(\delta 166.53, 160.67, 157.50, 151.74, 148.62, 147.75, 141.14, 133.78, 127.00, 121.97, 117.79, 115.28, 110.54, 104.79, 57.67, 55.33, 48.40, 32.11, 24.51, 17.85. HRMS (ESI-TOF) \(\text{m/z}\) [M + H]+ calcd for C\(_{23}\)H\(_{25}\)N\(_5\)O\(_3\) 408.2036, found 408.2038.

**Compound 1a** (0.18 g, 42%) was synthesised from furfural as a white solid. \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta 11.98\) (s, 1H), 8.10 (s, 1H), 7.59 (dd, \(J = 15.6, 2.9\) Hz, 1H), 6.99 (d, \(J = 8.2\) Hz, 1H), 6.94 (t, \(J = 5.3\) Hz, 2H), 6.85 (d, \(J = 8.2\) Hz, 1H), 6.32 (t, \(J = 14.4\) Hz, 1H), 5.99 (d, \(J = 63.7\) Hz, 1H), 5.32–5.86 (m, 1H), 5.26–5.17 (m, 1H), 3.84 (s, 3H), 2.22–2.11 (m, 2H), 2.10–2.01 (m, 2H), 2.00–1.90 (m, 2H), 1.79–1.69 (m, 2H), 1.66 (d, \(J = 7.1\) Hz, 3H). \(^1^C\) NMR (101 MHz, CDCl\(_3\)) \(\delta 162.89, 160.26, 158.37, 152.03, 148.93, 148.29, 140.26, 134.46, 126.71, 122.16, 118.75, 116.15, 111.27, 104.76, 57.62, 55.97, 48.39, 32.66, 32.44, 24.76, 19.78. HRMS (ESI-TOF) \(\text{m/z}\) [M + H]+ calcd for C\(_{22}\)H\(_{25}\)N\(_5\)O\(_3\) 424.1979, found 424.1985.

**Compound 1b** (0.19 g, 45%) was synthesised from acetic anhydride as a white solid. \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta 9.31\) (s, 2H), 8.07 (s, 1H), 7.30 (d, \(J = 15.7\) Hz, 1H), 6.90 (d, \(J = 8.2\) Hz, 1H), 6.80 (d, \(J = 8.1\) Hz, 1H), 6.52 (d, \(J = 15.7\) Hz, 1H), 5.20–5.08 (m, 1H), 4.91 (p, \(J = 6.7\) Hz, 1H), 2.09 (d, \(J = 11.9\) Hz, 2H), 2.03–1.94 (m, 2H), 1.92 (d, \(J = 16.4\) Hz, 2H), 1.69 (s, 2H), 1.48 (t, \(J = 8.9\) Hz, 3H). \(^1^C\) NMR (101 MHz, CDCl\(_3\)) \(\delta 165.89, 162.06, 158.37, 152.03, 148.93, 148.29, 140.26, 134.46, 126.71, 122.16, 118.75, 116.15, 111.27, 104.76, 57.62, 55.97, 48.39, 32.66, 32.44, 24.76, 19.78. HRMS (ESI-TOF) \(\text{m/z}\) [M + H]+ calcd for C\(_{23}\)H\(_{25}\)N\(_5\)O\(_3\) 408.2030, found 408.2032.

**Compound 1c** (0.26 g, 59%) was synthesised from 3,4-dimethoxycinnamic acid as a white solid. \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta 12.08\) (s, 1H), 9.90 (s, 1H), 8.46 (d, \(J = 5.9\) Hz, 1H), 8.02 (s, 1H), 7.50–7.35 (m, 2H), 7.34 (d, \(J = 15.7\) Hz, 1H), 6.80 (d, \(J = 7.7\) Hz, 2H), 6.55 (d, \(J = 15.9\) Hz, 1H), 5.23–4.96 (m, 1H), 4.94–4.68 (m, 1H), 2.03 (s, 2H), 1.91 (d, \(J = 37.4\) Hz, 4H), 1.64 (s, 2H), 1.45 (d, \(J = 6.4\) Hz, 3H). \(^1^C\) NMR (101 MHz, CDCl\(_3\)) \(\delta 165.92, 162.08, 158.49, 158.39, 152.02,
Compound 1h (0.15 g, 39%) was synthesised from 3-hydroxycinnamic acid as a white solid. 1H NMR (400 MHz, DMSO) δ 12.06 (s, 1H), 8.74 (d, J = 19.1 Hz, 2H), 8.34 (d, J = 6.2 Hz, 1H), 8.00 (s, 1H), 6.85–6.56 (m, 2H), 6.53 (d, J = 7.5 Hz, 1H), 5.09–4.84 (m, 1H), 4.84–4.53 (m, 2H), 3.33–3.24 (m, 2H), 2.03 (s, 2H), 1.88 (s, 4H), 1.68 (s, 2H), 1.39 (d, J = 6.7 Hz, 3H). 13C NMR (101 MHz, DMSO) δ 171.07, 161.94, 158.36, 151.92, 145.39, 134.44, 134.12, 127.12, 120.22, 117.10, 115.63, 104.63, 57.39, 49.00, 41.90, 32.81, 32.38, 24.84, 19.86. HRMS (ESI-TOF) m/z [M + H]+ calc.det for C20H29N5O2 398.1823, found 398.1826.

Compound 1i (0.22 g, 56%) was synthesised from 3, 4-dihydroxyphenylacetic acid as a white solid. 1H NMR (400 MHz, DMSO) δ 12.06 (s, 1H), 8.74 (d, J = 19.1 Hz, 2H), 8.34 (d, J = 6.2 Hz, 1H), 8.00 (s, 1H), 6.85–6.56 (m, 2H), 6.53 (d, J = 7.5 Hz, 1H), 5.09–4.84 (m, 1H), 4.84–4.53 (m, 2H), 3.33–3.24 (m, 2H), 2.03 (s, 2H), 1.88 (s, 4H), 1.68 (s, 2H), 1.39 (d, J = 6.7 Hz, 3H). 13C NMR (101 MHz, DMSO) δ 171.07, 161.94, 158.36, 151.92, 145.39, 134.44, 134.12, 127.12, 120.22, 117.10, 115.63, 104.63, 57.39, 48.00, 41.90, 32.81, 32.38, 24.84, 19.86. HRMS (ESI-TOF) m/z [M + H]+ calc.det for C20H29N5O2 398.1823, found 398.1826.

In vitro assay for PDE9 inhibitors

Protein expression and purification

The recombinant pET-PDE9A2 plasmid (catalytic domain, 181–506) was subcloned and purified in the same protocols we reported previously and then transferred into the E. coli strain BL21 (Codonplus, Stratagene, San Diego, CA). The E. coli cells carrying pET-PDE9A2 were grown in LB medium (containing 100 μg/ml ampicillin and 0.4% glucose) at 37°C until OD600 = 0.6–0.8. 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to induce the PDE9A2 protein expression at 15°C for 20 h. PDE9A was purified on the nickel nitriolacetic acid (Ni-NTA) column (Qiagen, Hilden, Germany) and eluted with 0.25 M imidazole. The concentration of PDE9A was estimated by the absorbance at 280 nm (calculated using ProtParam software, http://web.expasy.org/protparam). A typical batch of purification yielded 30–60 mg PDE9A2 protein from a 0.51 cell culture. The PDE9A2 proteins had purity greater than 90% as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

(R, E)-N-[(1-cyclopentyl-4-oxo-4, 5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)ethyl]-3-(4-trifluoromethyl)phenyl)acrylamide (1k)

Compound 1k (0.17 g, 39%) was synthesised from 4-trifluoromethylphenylacetic acid as a white solid. 1H NMR (400 MHz, DMSO) δ 12.13 (s, 1H), 8.71 (d, J = 6.8 Hz, 1H), 8.03 (s, 1H), 7.80 (s, 4H), 7.52 (d, J = 15.9 Hz, 1H), 6.92 (d, J = 15.9 Hz, 1H), 5.18–5.04 (m, 1H), 4.96–4.83 (m, 1H), 2.13–2.00 (m, 2H), 1.95 (dd, J = 17.8, 11.5 Hz, 2H), 1.86 (dd, J = 9.8, 6.9 Hz, 2H), 1.64 (s, 2H), 1.47 (d, J = 7.0 Hz, 3H). 13C NMR (400 MHz, DMSO) δ 166.67, 163.65, 159.42, 156.07, 149.85, 141.77, 135.38, 130.64, 126.91, 121.90, 116.23, 110.17, 61.59, 48.95, 36.17, 25.81, 22.00. HRMS (ESI-TOF) m/z [M + H]+ calc.det for C22H12F3N3O2 446.1794.
Enzymatic assays against PDE9A
The enzymatic activities of the catalytic domain of PDE9A2 were measured by using \(^3\)H-cGMP as the substrate and the assay buffer of 50 mM Tris-\(\text{HCl}\) (\(pH \, 8.0\)), 10 mM \(\text{MgCl}_2\), and 1 mM DTT. Each assay used \(^3\)H-cGMP 20,000–30,000 cpm. The reaction was carried out at room temperature (25 °C) for 15 min and then terminated by addition of 0.2 M \(\text{ZnSO}_4\). The reaction product was precipitated by 0.2 N \(\text{Ba(OH)}_2\), whereas unreacted \(^3\)H-cGMP remained in the supernatant. The radioactivity in the supernatant was measured in 2.5 ml of Ultima Gold liquid scintillation cocktails (PerkinElmer, Waltham, MA) using a PerkinElmer 2910 liquid scintillation counter.

2.5 ml of Ultima Gold liquid scintillation cocktails (PerkinElmer, Waltham, MA) was added to each cell well and the system was further incubated at 570 nm using EnSpire-2300 Multimode Reader (PerkinElmer, Waltham, MA).

Molecular docking
The crystal structure of PDE9A in the complex with a highly selective inhibitor 28 (PDB ID:4GHI) was used in this study.\(^{34}\) Surflex-dock\(^{35}\) embedded in the software Tripos Sybyl version 2.0 (Tripos Software, Inc., El Cerrito, CA) was used for molecular docking. Two metal ions in this crystal structure were kept as they were crucial for the PDE’s catalytic activity. Most of the water molecules in this crystal structure were removed except the ones near these two metal ions. Hydrogen atoms were added and the ionisable residues were protonated at the neutral pH. The protomol was generated using a ligand-based approach and the bound ligand was used for protomol generation. The proto_thresh and proto_bloat parameters represent how much the protomol can be buried in the protein and how far the protomol extends outside the cavity, respectively. The proto_thresh was selected as 0.5 and proto_bloat was selected as 0. After the preparation of the protomol, molecular docking was performed. All molecules designed were docked to the prepared structure of PDE9A and 20 molecules with the highest docking scores and appropriate binding modes went into the following MD simulations and binding free energy calculations.

Result and discussion
The design of novel PDE9A inhibitors with assistance of molecular docking and dynamics simulation
The crystal structures of PDE9A-inhibitors showed that two conserved residues Gln453 and Phe456 play essential roles for binding of the inhibitors\(^{11,18,27,34}\) (Figure 1). Most PDE9A inhibitors contain a pyrazolopyrimidinone scaffold to form hydrogen bond with Gln453 and \(\pi-\pi\) interactions with Phe456. A narrow and long pocket next to pyrazolopyrimidinone is available in the PDE9 structure for introducing an extra fragment with antioxidant activity. In addition, Tyr424 in this narrow pocket, which is unique to PDE8 and PDE9, has been shown to be important for selectivity of PDE9 inhibitors over other PDEs\(^ {27,34}\). Thus, we took pyrazolopyrimidinone as the scaffold of our designed compounds. Functional fragments from common antioxidants such as ferulic acid, caffeic acid, lipolic acid were attached to 6-position of pyrazolopyrimidinone with various linkers for antioxidant activities (compounds structure shown in Figure S1)\(^{28,40,41}\).

In order to improve the efficiency and reduce synthetic work, molecular docking was performed on the designed compounds to predict their binding patterns. This is quite different from the usual drug discovery in which molecular docking was used to...
predict the binding mode after lead compounds were identified. Furthermore, binding free energies between designed compounds and protein in atomic scales, which can improve the hit rate significantly but are not available in the experiments sometimes were obtained by the molecular dynamics simulation for a more precise prediction. According to the calculation results, we found that the compounds with an amide group as the linker had stable MD simulations trajectories and high binding free energies (compounds structure in Figure 1, Binding free energies in Table 1, Binding mode in SP), indicating that they might be potential PDE9A inhibitors.

In order to filter possible false hits, pan-assay interfering compound substructures (PAINS) screening was performed using the online program PAINS-Remover (http://cbligand.org/PAINS)42. All the compounds with an amide group as the linker (Figure 1) passed this test and were then synthesised.

**Chemistry**

As outlined in Scheme 1, condensation of 2-(methoxymethylene)-malononitrile (M-1) with cyclopentylhydrazine (M-2) afforded M-3 in the presence of triethylamine. Hydrolysis of the cyano group of M-3 in the presence of ammonium hydroxide and hydrogen peroxide produced M-4 with an amide group27. M-5 was synthesised by the reaction of M-4 with (R)-ethyl-2-(((benzyloxy)carbonylamino)propanoate under basic condition at room temperature. The benzyol group of M-5 was removed by hydrogenation in hydrogen atmosphere and the presence of the Pd/C catalyst, producing M-6 in high yield20. Compounds 1a-1l were prepared in moderate to high yield by condensation reaction of M-6 with the acids such as ferulic acid28. Compounds 2 and 3 were prepared in the same procedure by using lipoic acid and trolox28.

**Inhibition of designed compounds on PDE9A in vitro**

The inhibitory activities of compounds 1a–1l, 2, and 3 against PDE9A were assayed with the procedure previously reported26. The results are summarised in Table 1. BAY73–6691, a PDE9 inhibitor developed by Bayer company serves as the reference in the assay (Lit: IC50 = 55nM)43. Compounds 1a–1l, which contain the fragment of ferulic acid derivatives, have the IC50 values of 56–190nM against PDE9A. The substitutions on the phenyl ring showed remarkable impacts on the inhibitory activities against PDE9A. Among all the compounds in series 1, compound 1h with the 3-hydroxyl on the phenyl ring gave the best IC50 value of 56 nM, which is as effective as BAY73–6691 (55 nM). Compounds 1b with two hydroxy group and 1c with two methoxy groups gave comparable inhibitory activities. Introduction of an additional methoxy group from compound 1a to 1e makes the IC50 drop to 56 nM.
Table 1. The inhibitory activities against PDE9A, binding free energies, and oxygen radical absorbance capacity of compounds 1a–1l, 2, and 3.

| Compound | R               | IC₅₀±SD (nM)  | GBTOT (kcal·mol⁻¹) | ORAC  |
|----------|-----------------|---------------|-------------------|------|
| 1a       | HO              | 126 ± 15      | -31.09 ± 2.64     | 2.4  |
| 1b       | HO              | 64 ± 2        | -29.78 ± 2.33     | 2.5  |
| 1c       | HO              | 65 ± 4        | -29.53 ± 2.82     | 0.23 |
| 1d       | HO              | 83 ± 11       | -35.41 ± 3.09     | 1.26 |
| 1e       | OMe             | >200          | -35.77 ± 2.64     | 0.50 |
| 1f       | HO              | 190 ± 7       | -27.39 ± 3.05     | 0.12 |
| 1g       | HO              | 63 ± 3        | -35.71 ± 2.81     | 2.9  |
| 1h       | HO              | 56 ± 7        | -38.16 ± 3.08     | 3.3  |
| 1i       | MeO             | 133 ± 22      | -33.80 ± 3.08     | 1.00 |
| 1j       | Cl              | 186 ± 7       | -35.52 ± 2.72     | 0.08 |
| 1k       | F₃C             | 130 ± 6       | -43.39 ± 2.53     | 0.02 |
| 1l       | OMe             | 57 ± 4        | -28.79 ± 2.93     | 2.6  |
| 2        | HO              | 25 ± 4        | -40.01 ± 3.74     | 0.28 |
| 3        | HO              | >500          | -31.98 ± 2.52     | 0.24 |
| BAY73-6691| –              | 46            | –                 | n.a. |
| Ferulic acid | –              | n.d. b        | –                 | 1.6  |

*IC₅₀ values are given as the means of three independent determinations.

b n.d. means not determined.

c n.a. means not active.

>200 nM, suggesting steric hinder in the binding pocket is important. Using other substitution groups such as dimethylamine, trifluoromethyl, and chloro to instead the methoxy or hydroxyl group of ferulic acid formed compounds 1i–1k. The inhibitory activities of these compounds against PDE9A dropped to 130–180 nM. We concluded that the hydroxyl group on the phenyl ring might form interactions with residues in the binding pocket of PDE9A, improving the inhibitory activity against PDE9A. Compared with 1b derived from caffeic acid, compound 1i which does not contain a double bond, gave a comparable IC₅₀ value of 57 nM, suggesting that the double bond may not be essential for the affinity with PDE9A. Compound 2 having lipolic acid linked to 6-position of pyrazolopyrimidinone gave the best IC₅₀ of 25 nM, in comparison with IC₅₀ >500 nM of compound 3 with trolox group. We speculated that the trolox group might be too large to fit into the binding pocket of PDE9A. The linear correlation between docking score values and IC₅₀ values of compound 1a–1l, 2, and 3 was observed with a moderate R² value of 0.58 (Table S1 and Figure S2). Compound 3 and 1e with the lowest docking score values, gave the lowest inhibitory activities against PDE9A. However, for some compounds such as 1i and 1f, there are some deviations between the docking score values and IC₅₀ values.

Antioxidant activity test by the ORAC method

Oxygen radical absorbance capacity assay (ORAC-FL) has been widely used to evaluate antioxidant activities of hydrophilic antioxidants including ferulic acid and phenolic derivatives. Thus, the antioxidant activities of compounds 1a–1l and compound 2 were evaluated by ORAC-FL method. The results were expressed as trolox equivalents and summarised in Table 1. Ferulic acid was used as the positive reference compound, showing an ORAC-FL value of 1.6 trolox equivalents. The ferulic acid derivatives with the hydroxyl group on the phenyl ring (1a, 1b, 1d–1h, 1l) showed good ORAC-FL values in a range of 1.3–3.3 fold of trolox equivalents, while compounds without the hydroxyl group (1c, 1f, 1j, and 1k) showed low ORAC-FL values except for compound 1i with dimethylamino group substituted. These results are consistent with the previous reports that the hydroxyl-phenyl groups of the ferulic acid are the main source of antioxidant activity. To our surprise, compound 2 with the fragment of lipolic acid showed low antioxidant activity.

Selectivity of compounds 1h and 2 over PDE1 and PDE8

The PDE super-family of enzymes are divided into 11 subfamilies according to the differences in structure and distribution. Each PDE subfamily is in charge of different biological process. In order to reduce drug adverse effects, the selectivity over other PDEs is important for potential PDE inhibitors. Since the binding pocket of PDE subfamily is structurally similar, selective PDE inhibitors are not easy to be obtained. For the PDE9A inhibitors, the selectivity over PDE1 and PDE8 is usually difficult to obtain. Thus, compound 1h with PDE9A inhibition and good antioxidant activity, compound 2 with the best inhibition towards PDE9A were chosen for exploration of selectivity over PDE1 and PDE8 (Table 2). Compounds 1h and 2 at 1 μM inhibited 13 and 59% activity of PDE1B, thus estimating 25 and 40-fold selectivity, respectively. The selectivity is better than BAY73-6691 but not PF-0447943. The inhibitory activities of compounds 1h and 2 against PDE8A at 10 μM were 10 and 9%, respectively, implying the 250–400 fold selectivity of these compounds towards PDE8A. Thus, both compounds 1h and 2 are good selective PDE9 inhibitors.

Effects of compounds 1h and 2 on cell viability

The cytotoxicity of compounds 1h and 2 were tested using human neuroblastoma cell line SH-SYSY by the MTT method. As shown in Figure 2, after incubation for 48 h, the cellular viability was about 90% at 40 μM of compound 1h and 70% at 100 μM. Compound 2 at 100 μM showed no significant effect on cell viability. Control experiments were performed, eliminating the
possibility that compounds 1h and 2 interfered with the MTT assay (Figure S8). These results suggested that both 1h and 2 are not toxic to SH-SY5Y cells and potential candidates for further development as therapeutics for AD.

**Analysis of the structure-activity relationship by molecular docking and dynamics simulation**

In order to improve the efficiency and reduce synthetic work in the process of identifying PDE9 inhibitors, the binding mode, and binding free energies of all these compounds were performed by molecular docking and dynamics simulation.

According to the difference in the binding modes, the designed compounds in series 1 can be divided into three classes.

**Table 2. Inhibitory activities of compounds 1h and 2 with PDE1 and PDE8.**

| Compound | IC<sub>50</sub> (nM) of PDE9A | Inhibition of PDE1B at 1000 nM | Inhibition of PDE8A at 10 nM |
|----------|-------------------------------|-------------------------------|-------------------------------|
| 1h       | 56 ± 1                        | 13% (>=25)<sup>a</sup>        | 10% (>250)<sup>a</sup>        |
| 2        | 25 ± 4                        | 59% (40)<sup>b</sup>         | 9% (>400)<sup>a</sup>         |
| BAY73-6691 | 55<sup>c</sup>             | 1400 (25)<sup>b</sup>        | –                            |
| PF-04447943 | 8<sup>c</sup>            | 1394 (174)<sup>c</sup>      | –                            |

<sup>a</sup>The numbers in parentheses are the fold of selectivity of inhibitors against PDE9 over other PDEs (IC<sub>50</sub>, nM).
<sup>b</sup>Data was obtained from the reported literature.<sup>c</sup>
<sup>c</sup>Data was obtained from the reported literature.<sup>d</sup>

According to the difference in the binding modes, the designed compounds in series 1 can be divided into three classes. Compounds 1a, 1c, 1d, and 1e adopted a similar binding mode (Figure S3) and form two hydrogen bonds with invariant Gln453 and p–p interactions with conserved Phe456. Compounds 1b, 1j, and 1k formed an additional hydrogen bond with Ala452 in addition to the interactions with Gln453 and Phe456 (Figure S4). Compounds 1f, 1g, 1h, and 1i formed an additional hydrogen bond with Tyr424 besides the interactions with Gln453 and Phe456 (Figure S5). As mentioned before, the interactions with Gln453 and Phe456 are the main reasons for the affinity of inhibitors against PDE9. Thus, all these compounds should have good inhibitory activities against PDE9A. Further biological tests demonstrated that most compounds exhibited the inhibition activity against PDE9 with IC<sub>50</sub> below 200 nM except compound 1e. Compound 1h, which has IC<sub>50</sub> of 57 nM against PDE9 and the highest ORAC value of 3.3 trolox, belongs to the class II, forming an additional hydrogen bond with Tyr424 (Figure 3). Since Tyr424 is unique to PDE9 and PDE8, the interaction with Tyr424 will render high selectivity of PDE9 inhibitors. This is supported by the good selectivity of compound 1h over PDE1B and PDE8A. Compound 2, which has IC<sub>50</sub> of 25 nM against PDE9 and good selectivity over PDE1 and PDE8 showed no interaction with Tyr424, implying a new way to design selective PDE9 inhibitors (Figure 3).
The binding free energy calculations were performed by the MM-PBSA method. The results of compounds 1h and 2 are shown in Table 3, suggesting that van der Waals ($\Delta E_{\text{vvdw}}$) and electrostatic components ($\Delta E_{\text{ele}}$) are two important factors for the high affinity of both compounds 1h and 2 while polar component to solvation ($\Delta E_{\text{pol,solv}}$) is less important.

**Conclusion**

In summary, a series of novel pyrazolopyrimidinone derivatives have been designed, synthesised and evaluated as multifunctional anti-AD ligands, in combination of PDE9A inhibition and antioxidant activities. With the assistance of molecular docking and dynamics simulation, high selective PDE9 inhibitors with antioxidant activities have been successfully developed as shown by compounds 1h (IC$_{50}$ of 56 and 3.3 trolox of ORAC). These PDE9 inhibitors do not disturb cell viability of SH-SY5Y and thus are worth for further development as therapeutics for treatment of AD. The structure-activity relationship was explained by the binding modes of inhibitors with PDE9 protein, providing evidence for further structural modification.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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![Figure 2. Cell viability of compounds 1h and 2.](image)

![Figure 3. The binding modes of compounds 1h and 2.](image)

![Table 3. Components of the binding free energy (kcal/mol) for PDE9A in complex with 1h or 2 by using the MM-PBSA method.](table)

![Figure 4.](image)
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