Structure-based discovery of orally efficient inhibitors via unique interactions with H-pocket of PDE8 for the treatment of vascular dementia

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**Abstract**
Our previous study demonstrated that phosphodiesterase 8 (PDE8) could work as a potential target for vascular dementia (VaD) using a chemical probe \(3a\). However, compound \(3a\) is a chiral compound which was obtained by chiral resolution on HPLC, restricting its usage in clinic. Herein, a series of non-chiral 9-benzyl-2-chloro-adenine derivatives were discovered as novel PDE8 inhibitors. Lead 15 exhibited potent inhibitory activity against PDE8A (IC\(_{50}\) = 11 nmol/L), high selectivity over other PDEs, and remarkable drug-like properties (worthy to mention is that its bioavailability was up to 100%). Oral administration of 15 significantly improved the cAMP level of the right brain and exhibited dose-dependent effects on cognitive improvement in a VaD mouse model. Notably, the X-ray crystal structure of the PDE8A–15 complex showed that the potent affinity and high selectivity of 15 might come from the distinctive interactions with H-pocket including T-shaped π−π interactions with Phe785 as well as a unique H-bond network, which have never been observed in other PDE–inhibitor complex before, providing new strategies for the further rational design of novel selective inhibitors against PDE8.

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1. Introduction

Vascular dementia (VaD) has been regarded as the second most common type of dementia after Alzheimer’s disease in the worldwide. Cerebrovascular disease, ischemic or hemorrhagic brain injury will trigger the cognitive impairment in VaD patients. Risk factors such as age, diabetes, and hypertension are also involved in the pathogenesis of VaD. Thus, the mechanism of VaD is complicated and still unclear. No specific medicine for VaD has been approved so far.

The cAMP/PKA and NO/cGMP/PKG signaling play a considerable role in the memory consolidation and long-term potentiation (LTP) of synaptic transmission. The phosphodiesterases (PDEs), a superfamily in charge of hydrolyzing cAMP and cGMP, can be divided into eleven isoforms (PDE1–PDE11). The PDE4, PDE7, and PDE8 subtypes specially hydrolyze cAMP whereas the PDE5, PDE6, and PDE9 ones specially hydrolyze cGMP. The other subfamilies hydrolyze cAMP and cGMP, simultaneously. Many PDEs inhibitors could enhance the cognitive abilities in the mouse models of Alzheimer’s disease, Parkinson’s disease, schizophrenia, and etc., identifying PDEs as potential targets for memory improvement. For VaD, PDE3 inhibitors have been developed yet, but are even non-selective, limiting the exploration of the biological functions of PDE8 and the recognition mechanism studies of inhibitors with PDE8. Most recently, our group developed a series of selective PDE8 inhibitors and identified that PDE8 could work as a potential drug target against VaD via a chemical probe. However, a chiral compound was difficultly obtained by chiral resolution on HPLC.

Among all the PDEs, PDE8 showed the highest affinity for the hydrolysis of cAMP and cGMP, indicating that PDE8 may be an efficient target for the cAMP-related diseases. However, only a few PDE8 inhibitors have been developed yet, and most of them are even non-selective, limiting the exploration of the biological functions of PDE8 and the recognition mechanism studies of inhibitors with PDE8. Most recently, our group developed a series of selective PDE8 inhibitors and identified that PDE8 could work as a potential drug target against VaD via a chemical probe. However, a chiral compound was difficultly obtained by chiral resolution on HPLC.

With our continuous interest in the discovery of PDE8 inhibitors, structural optimization of compound 3a was performed in this work (Fig. 1). A non-chiral compound 15 with potent affinity for PDE8 and high selectivity over other PDEs was selected as the hit compound, and the cocrystal of the PDE8–10 complex was obtained and analyzed for the rational design.

The binding pocket of PDE8 can divide into four subpockets like PDE4 and PDE2, including Q-pocket, M-pocket, S-pocket, and H-pocket (Fig. 2A). The 2-chloroadeinone scaffold of 10 bound to Q-pocket, forming a π–π stacking interaction with Phe781 as well as four hydrogen bonds with Gln778 and Asn729. Compared with 3a forming a hydrogen bond with Tyr748, the difluoroethoxy of 10 stretched deeper in H-pocket and formed a hydrogen bond with His673 instead of Tyr748 (Supporting Information Fig. S1). In addition, owing to the strong electron-withdrawing effects of two fluorine atoms, the adjacent C–H tended to perform positive electrostatic potential, and interacted with Asp726 and Thr668 through water-bridged hydrogen bonds. Furthermore, the benzyl of compound 10 formed T-shaped π–π interactions with Phe781 and Phe785. To our knowledge, the hydrogen bond network and T-shaped π–π interactions in H-pocket have never been observed in PDE-inhibitor complex before. We observed that compound 10 mainly occupied Q-pocket and H-pocket, but hardly interacted with S-pocket. Thus, substituents were introduced at the meta-position of benzyl group to occupy S-pocket (Fig. 2B), which might form extra interactions with the adjacent residues Phe785, Met764, Phe767, and Tyr748, enhancing the inhibitory activity of target compounds.

To accelerate the process of optimization, a MM-GB/SA approach was adopted to predict binding free energies (ΔGbind, pred) of designed compounds 11–25 with PDE8A (Table 1). Compared with hit 10 (–33.22 ± 3.46 kcal/mol), most designed compounds showed comparable binding free energies. Figure 1 shows the structural optimization of non-chiral PDE8 inhibitors as anti-VaD agents.
compounds showed more negative $\Delta G_{\text{bind, pred}}$ values, except for compound 12 ($-32.14 \pm 3.05$ kcal/mol) with a comparable $\Delta G_{\text{bind, pred}}$ value.

A PAINS-Remover$^{20}$ (https://www.cbligand.org/PAINS/) screening was performed to avoid the pan assay interference compounds (PAINS), and all compounds satisfied the filter test and were synthesized.

### 2.2. Structure—activity relationships (SARs) of target compounds

Compared with the initial hit 10 (IC$_{50}$ = 117 nmol/L), compound 11 with F atom at the R$_1$ position exhibited a slightly better IC$_{50}$ value of 51 nmol/L while compound 12 with a hydroxyl group showed a comparable IC$_{50}$ value of 193 nmol/L against PDE8A.

Table 1  The prediction of binding free energies ($\Delta G_{\text{bind, pred}}$), IC$_{50}$, and metabolic stability RLM $t_{1/2}$ of target compounds.

| No. | R$_1$ | $\Delta G_{\text{bind, pred}}$ (kcal/mol) | IC$_{50}$ (nmol/L)$^*$ | RLM $t_{1/2}$ (min) |
|-----|------|-------------------|----------------------|-------------------|
| 10  | H    | $-33.22 \pm 3.46$ | 117 ± 6              | —                 |
| 11  | F    | $-35.50 \pm 2.31$ | 51 ± 3               | —                 |
| 12  | OH   | $-32.14 \pm 3.05$ | 193 ± 17             | —                 |
| 13  | $\cdot$ | $-36.52 \pm 2.85$ | 591 ± 11             | —                 |
| 14  | $\cdot$ | $-37.27 \pm 3.01$ | 593 ± 69             | —                 |
| 15  | $\cdot$ | $-37.94 \pm 2.85$ | 11 ± 1               | 169               |
| 16  | $\cdot$ | $-40.93 \pm 2.66$ | 4.6 ± 0.6            | 6                 |
| 17  | $\cdot$ | $-39.68 \pm 2.66$ | 5.0 ± 0.3            | 16                |
| 18  | $\cdot$ | $-43.03 \pm 2.61$ | 20 ± 2               | —                 |
| 19  | $\cdot$ | $-38.80 \pm 2.42$ | 40 ± 1               | —                 |
| 20  | $\cdot$ | $-41.17 \pm 2.33$ | 52 ± 5               | —                 |
| 21  | $\cdot$ | $-37.38 \pm 3.02$ | 597 ± 61             | —                 |
| 22  | $\cdot$ | $-43.04 \pm 2.53$ | 3.1 ± 0.2            | 8                 |
| 23  | $\cdot$ | $-42.34 \pm 3.27$ | 4.8 ± 0.3            | 39                |
| 24  | $\cdot$ | $-41.24 \pm 2.75$ | 5.9 ± 0.6            | 27                |
| 25  | $\cdot$ | $-39.57 \pm 2.78$ | 13 ± 2               | —                 |

$^*$Data are given as mean ± SD ($n \geq 3$).

Figure 2  (A) Surrounding residues of four subpockets represents by four different colors in the PDE8A—10 complex (PDB ID: 7VSL). (B) Rational design of potent PDE8 inhibitors.
Both compound 13 with a methoxyl group and 14 with an ethoxyl group showed similar inhibitory activities against PDE8A. Compared with 13, 15 with a difluoromethoxyl group demonstrated significant increased inhibitory potency, indicating that fluorine atom is in favor of S-pocket.

After changing the methoxyl group of 13 to substituents with larger volume of isopropyl (16) and isopropoxyl (17), the inhibitory activities against PDE8A were significantly improved. However, further increasing the volume of R1 substituents, such as cyclobutoxyl (18), cyclopropylmethoxyl (19), 2-methoxyethoxyl (20) and benzyloxyl (21), resulted in decreased inhibitory activities against PDE8A. Thus, the steric effect of R1 substituents played an important role when binding S-pocket. Isopropyl and isopropoxyl groups, with appropriate volume, were preferred for binding with S-pocket.

In order to enhance the \( \pi-\pi \) interactions with Phe781 and Phe785, compounds 22–25 with aromatic heterocycles at the R1 position were designed. As expected, all these compounds showed significantly increased inhibitory activities against PDE8A compared with compound 10. Compounds 22 with 4-pyridinyl, 23 with 3-pyridinyl, and 24 with 2-fluoropyridin-3-yl groups gave the IC\(_{50}\) values of 3.1, 4.8, and 5.9 nmol/L against PDE8A, Table 2

| PDE subtype   | 15 IC\(_{50}\) (nmol/L) | Selectivity index | 3a IC\(_{50}\) (nmol/L) | Selectivity index |
|---------------|--------------------------|-------------------|--------------------------|-------------------|
| PDE8A1 (480–820) | 11 ± 1                   | /                 | 10 ± 1                   | /                 |
| PDE1C (147–531)  | 6179 ± 429               | 562               | 3095 ± 495               | 310               |
| PDE2A (580–919)  | 4315 ± 171               | 392               | 2177 ± 104               | 218               |
| PDE3A (679–1087) | >10,000                  | >909              | >10,000                  | >1000             |
| PDE4D2 (86–413)  | 5485 ± 486               | 499               | 7148 ± 340               | 715               |
| PDE5A1 (535–860) | 4835 ± 353               | 440               | >10,000                  | >1000             |
| PDE7A1 (130–482) | 4853 ± 359               | 441               | >10,000                  | >1000             |
| PDE9A2 (191–506) | >10,000                  | >909              | >10,000                  | >1000             |
| PDE10A (449–770) | 1224 ± 28                | 111               | 4436 ± 160               | 444               |

\(^a\)Data are given as mean ± SD (n ≥ 3). 
\(^b\)Data reported from our previous report \(^1\), except the data of PDE1C, which was determined in this research.

![Figure 3](image-url)
respectively. Compound 25 with a smaller π-conjugated 2-furyl core afforded a relatively low inhibitory activity with an IC₅₀ value of 13 nmol/L against PDE8A.

The predicted binding free energies (ΔGₜₐₐₜ,ₚₑₙ_dₑₚₑₜₑₚₑₜₑₚₑₙ) of target compounds were outlined in Table 1. The experimental binding free energies (ΔGₜₐₜ,ₑₓₚₑ) were calculated by the IC₅₀ values against PDE8A (ΔGₜₐₜ,ₑₓₚₑ = RT lnIC₅₀). A linear correlation achieved (Supporting Information Fig. S2) a considerable Pearson correlation coefficient (r = 0.68) between ΔGₜₐₜ,ₚₑₙ_dₑₚₑₜₑₚₑₚₑₙ and ΔGₜₐₜ,ₑₓₚₑ, which demonstrated the MM-GB/SA approach could be efficiently applied to predict the ΔGₜₐₜ,ₚₑₙ_dₑₚₑₜₑₚₑₚₑₙ and thus could save the synthesis plus bioassay efforts.

2.3. The evaluation of metabolic stability by the rat liver microsomes (RLM)

The metabolic stability of most potent compounds including 15, 16, 17, 22, 23, and 24 were evaluated by the rat liver microsomes (RLM). Compound 16 with an isopropyl group and compound 17 with an isopropoxyl group gave the t₁/₂ values of 6 min and 16 min, respectively, revealing that aromatic hydrocarbon and alkyl aryl ether are unstable in the present of RLM. Due to the fluorine atom’s blocking effect in metabolic oxidation, compound 15 (R₁ = difluoromethoxy) showed a great metabolic stability (t₁/₂ = 169 min). Compounds 22, 23, and 24 gave the t₁/₂ values of 8, 39, and 27 min, respectively, indicating that 3-pyridinyl derivatives are more stable than 4-pyridinyl derivatives. Thus compound 15 was subsequently subjected to other evaluations.

2.4. Remarkable selectivity index of compound 15

The selectivity profile of compound 15 over other PDEs was evaluated and the results are outlined in Table 2. Similar to 3a, compound 15 also exhibited remarkable selectivity index.

selectivities against PDE3A and PDE9A2 were more than 900-fold. Its values of selectivity index against PDE1C, PDE2A, PDE4D2, PDE5A1, PDE7A1, and PDE10A were 562-, 392-, 500-, 437-, 441-, and 111-fold, respectively. These results demonstrated that compound 15 exhibits high selectivity over other PDEs, thus is suitable for the further development as a lead compound.

2.5. Unique interactions observed in the cocrystal structure of 15 bound to PDE8

The crystal structures of complexes of PDE8–15, PDE8–17, and PDE8–22 (Fig. 3) were obtained, respectively. These three compounds adopted similar binding modes with PDE8 to hit 10.

The 2-chloroadenine core of compound 15 occupied Q-pocket and formed π–π interactions, van der Waals interactions, and H-bond interactions with Q-pocket. Its benzylic methoxy occupied H-pocket. Furthermore, it maintained the T-shaped π–π interaction with Phe785 and H-bond network (a direct H-bond with His673 and water-bridged hydrogen bonds with Asp726 and Thr668) in H-pocket. As mentioned above, these interactions have never been observed in PDE–inhibitor complex before. The difference between lead 15 and hit 10 is that the difluoromethoxyl group of 15 occupied S-pocket as our designed. Some part of compound 15 even stretched toward the edge of Q-pocket, forming van der Waals interactions plus hydrophobic interactions with Phe781, Phe785, Phe767, Tyr748, and Met764 (the side chain of Met764 is missing when analyzing the crystal structure).

In order to validate the importance of unique H-pocket network, we modified the difluoroethoxyl group of 17 (IC₅₀ = 5.0 nmol/L) to an ethoxyl group of 26 (Fig. 4). The IC₅₀ value of 26 dropped to 58 nmol/L, indicating that the H-pocket network is in favor of ligand binding. In addition, we found that Phe785 in PDE8 is a unique residue according to the comparison of residues in H-pocket by sequence alignment (Supporting Information Table S1). Only PDE8 includes the residue with aromatic side chain (phenylalanine) at position 785, while other PDEs with alkyl side chains. The unique H-pocket network with Phe785 and H-pocket network may account for the high selectivity of 15, providing a novel approach for the discovery of potent PDE8 inhibitors with high selectivity index.

2.6. Remarkable drug-like profiles of 15

In terms of the inhibitory activity, selectivity index, and metabolic stability, compound 15 was further subjected to evaluate
pharmacokinetic (PK) profile using SD rats (Table 3). After an oral dose of 5.0 mg/kg, 15 exhibited a moderate half-life of 7.92 h and an excellent bioavailability (F) of 100%. The $C_{\text{max}}$ and $AUC_{0-\infty}$ of compound 15 were 1560 ng/mL and 23,665 h ng/mL, indicating that 15 had a high level of exposure in vivo. The plasma concentration of compound 15 was still up to 275 ng/mL at 24 h after the oral administration, which was 62 times of its IC$_{50}$ value.

We further examined other drug-like profiles of 15, including solubility, human plasma protein binding (PPB), unbound brain concentration, cytochrome P450 (CYP450) inhibition, hERG inhibition, and acute toxicity (Table 4). The human PPB of 15 was 97.6%. The inhibition against CYP450 and hERG were weak, and no acute toxicity was observed for an oral dose of 1.5 g/kg. After an oral dose of 5.0 mg/kg at 4 h in C57BL/6J mice, the brain concentration of 15 in the brain homogenates was 114 ng/g (about 281 nmol/L), which is

Figure 5  The cognitive impairment of UCCAO mice has been improved after orally administration with compound 15 at the doses of 2.5 mg/kg and 5.0 mg/kg. (A) Escape latency time of mice (s). (B) Site crossings (min$^{-1}$). (C) Time in the target quadrant (%). (D) cAMP levels of the right brain in mouse (pmol/g). (E) Image is representative trajectories from each group. (F) Representative images of each group with hematoxylin and eosin staining in hippocampal CA3 region (HE, ×200). Donepezil at a dose of 1.0 mg/kg was probed as a positive control. Scale bar: 100 μm. Data are represented as mean values ± SEM (n = 8–12 in each group). *$P$ < 0.05 vs control; **$P$ < 0.01 vs control; ***$P$ < 0.001 vs control; ****$P$ < 0.0001 vs control, respectively.
much higher than the IC$_{50}$ (11 nmol/L) of 15 and strong enough to activate the cAMP signal for memory improvement.

Compared with the PDE8 inhibitors 24 (IC$_{50}$ = 43 nmol/L) reported by Pfizer$^{15,16}$, lead 15 achieved the excellent and higher inhibitory activity (IC$_{50}$ = 11 nmol/L) against PDE8A and high selectivity over other PDEs. In addition, the pharmacokinetic profiles such as oral bioavailability and metabolic stability have been also improved. Furthermore, lead 15 had considerable brain penetrability and physicochemical properties (tPSA: 84, $c$log$P$: 3.4), which is more suitable to be developed for the CNS diseases.

2.7. Significant therapeutic effects in VaD mice

It is well demonstrated that mice treated with permanent UCCAO would lead to chronic hypoperfusion and ischemic injury including ischemic white matter (WM) lesions and lacunes$^{21,22}$. Thus, a VaD mouse model with the right common carotid artery occlusion was adopted. After treatment with vehicle, compound 15 (2.5 and 5.0 mg/kg, p.o.) and donepezil (1.0 mg/kg, p.o.) for 3 weeks, respectively, the learning and memory ability was evaluated by Morris water maze (MWM) test. After removing the platform on the spatial probe trial day, the trajectories of each mouse were recorded, and the escape latency time (ELT) to find the platform site of each mouse, the number of the platform site crossings, and residence time in the target quadrant were analyzed.

As shown in Fig. 5, the ELT of the model group significantly increased in comparison with the control group. Meanwhile, the number of the platform site crossings was notably decreased compared with the control group and the identical trend was
the level of cAMP.

observed in residence time in the target quadrant, which strongly demonstrated that the cognitive impairment occurred in UCCAO-treated mice and the VaD mouse model was built successfully.

Compared with the model group, oral administration with compound 15 at the dose of 2.5 and 5.0 mg/kg as well as the positive control donepezil at the dose of 1.0 mg/kg markedly decreased the ELT after the platform was removed in the spatial probe trial day. Furthermore, a significant increase was observed in the number of the platform site crossings in 15-treated and donepezil-treated mice and the similar trend came to the residence time in the target quadrant. These results indicated that learning and memory functions had been improved in those mice. Besides, oral administration of 15 at 5.0 mg/kg daily showed a better ELT than that of 2.5 mg/kg. Consistently, the same dose responses in their therapeutic effects were observed on the number of the platform site crossings in all groups (Fig. 5D). Brain tissue cAMP levels of the model group were significantly decreased in comparison with the control group. Compared with the control group with hippocampus and conditions: (a) aromatic boric acids or pinacol vinylboronate, [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II), potassium carbonate, 1,4-dioxane/water, 100 °C, overnight; (b) Pd/C, H2, methanol/tetrahydrofuran, r.t., overnight.

![Scheme 3](image)

The synthetic routes for target compounds are depicted in Schemes 1–4. The key intermediate M4 was synthesized by a process starting from the substitution of 3-bromo-5-methylphenol (M1a) with 2-bromo-1,1-difluoroethane, followed by Wohl–Ziegler bromination and then coupling to 2-chloro-9H-purin-6-amine using cesium carbonate. Intermediate M4 was treated with Pd/C/H2 to give 10. The synthesis of compound 11 was similar to that of M4 using 3-fluoro-5-methylphenol as starting material (Scheme 1).

For the preparation of compounds 12–14 and 17–21 (Scheme 2), methyl 3,5-dihydroxybenzoate (M5) was substituted by 2-bromo-1,1-difluoroethane, followed by substitution with corresponding alkyl bromides or benzyl bromide to give M7a–g. The esters M7a–g were reduced using lithium aluminum hydride to yield alcohols M8a–g. The alcohols M8a–g were brominated using phosphorus tribromide to give benzyl bromide derivatives M9a–g, followed by coupling to 2-chloro-9H-purin-6-amine using cesium carbonate to produce target compounds 13–14 and 17–21. Reduction of compound 21 with H2 and Pd/C as a catalyst gave compound 12.

As shown in Scheme 3, compounds M10 and 22–25 were synthesized by Suzuki coupling of intermediate M4 with aromatic boric acids or pinacol vinylboronate in the presence of potassium carbonate as a base and Pd(dppf)Cl2, CH2Cl2 as a catalyst. Then, reaction of M10 with H2 and Pd/C produced compound 16.

The synthesis of compound 15 is shown in Scheme 4. The starting material 5-methylbenzene-1,3-diol (M11) was subjected to difluoromethylation, difluoroethylation, bromination and coupling to 2-chloro-9H-purin-6-amine to obtain compound 15.

![Scheme 4](image)

3. Conclusions

Herein, we performed structure-based optimization on compound 3a, affording a series of non-chiral PDE8 inhibitors. Lead 15 showed potent inhibitory activity against PDE8A (IC50 = 11 nmol/L) and high selectivity profile against other PDE isoforms. The X-ray structure of PDE8–15 revealed a novel binding pattern including a T-shaped π–π interaction with Phe785 and hydrogen bond network with H-pocket, providing new evidences to design highly selective PDE8 inhibitors. In addition, lead 15 exhibited remarkable drug-like properties like a bioavailability of 100% and its oral administration improved the cAMP level of the right brain, and exhibited dose-dependent effects on the improvement of spatial learning and memory capability in VaD mouse model.

4. Experimental

4.1. Chemistry

The synthetic routes and characterization data of target compounds were provided in Supporting Information.
4.2. Molecular modeling

The cocrystal structure of the PDE8-10 complex (PDB ID: 7VSL) was chosen for molecular modeling. All target compounds were directly constructed on the basis of the conformation of 10 bound to PDE8. The procedures for molecular dynamics (MD) simulation were same as our previous studies36,37. For each protein-ligand system, 8 ns MD simulations were carried out by NPT ensemble with pressure of 1 atm and temperature of 300 K under periodic boundary conditions. The bonds with hydrogen atoms were restrained by SHAKE algorithm so that the time step was set to two fs28,29. The long-range electrostatic interactions was treated by the partial mesh Ewald (PME) method with a cutoff of 8 Å30,31. After 8 ns MD simulation, the binding free energy was predicted by the MM-GB/SA approach32 in Amber 2033 suite using 100 snapshots from 7 to 8 ns trajectory.

4.3. Protein expression and purification

The PDE8A protein was expressed and purified by the previously reported protocols37. E. coli BL21 (Codonplus, DE3) cells harboring the recombinant pET15b-PDE8A1 plasmid (480–820)6,37 were cultured in 2 × YT medium at 37 °C until OD600 = 0.6–0.8, followed by induction with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mmol/L) at 25 °C for one day. The pellet was denatured in guanidine (7.8 mmol/L) and Tris-HCl (0.1 mmol/L, pH 8.0) for 12 h, and then purified by the nickel nitriloacetic acid (Ni-NTA) column (Qiagen). The elution was added dropwise into the refolding buffer and carried out without swinging at 4 °C for 72 h, followed by purification using hydroxyapatite HTP GEL (Bio-Rad), Q-Sepharose column (GE Healthcare) and a gel filtration column Sephacryl S100 (GE Healthcare).

Other PDE isoforms were expressed and purified by similar procedures without denaturing and refolding process as our previous papers9,17,27,36,41.

4.4. Bioassay test and crystallization

3H-cAMP, the substrate for PDE8A, was diluted with the buffer containing Tris-HCl (20 mmol/L, pH 7.5), manganese chloride (10 mmol/L), and diethioctrol (1 mmol/L) to about 20,000 cpm per assay. The mixture was reacted at 25 °C for 15 min and then terminated by adding zinc sulphate, following by barium hydroxide. The radioactivity of unreacted 3H-cAMP in the supernatant was tested by a PerkinElmer 2910 liquid scintillation counter. The IC50 was fitted by nonlinear regression using at least eight different concentrations. Each test was measured at least three times.

Same procedures for crystallization were performed as previous report9,17,36,38–40. And the details of diffraction data and structure refinement statistic were given in Supporting Information Table S2.

4.5. Drug-like profiles determinations

The procedures for the determination of RLM stability, PK properties, human PPB, CYP450 inhibition, hERG inhibition, and acute toxicity were same as our previous studies17,38–40.

4.6. UCCAO mouse model and Morris water maze test

The similar experiments (Supporting Information) have been performed as our previously reported protocols9,17. All animal care and experimental protocols were in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health Publication, revised 1996, No. 86-23, Bethesda, MD) and approved by the Institutional Ethical Committee for Animal Research of Sun Yat-sen University (IACUC number: SYSU-IACUC-2021-000129).

4.7. cAMP concentration assay

The mice’ brains were quickly removed, immediately frozen in liquid nitrogen and then stored at −80 °C before tested. The competitive enzyme immunoassay was used to measure the cAMP levels (ADI-900-163, Enzo Life Sciences, Exeter, UK). Brain tissue homogenized in 0.1 mol/L HCl with 10 volumes. After centrifuge for 10 min, the supernatant was diluted to 5 times with 0.1 mol/L HCl and then tested by non-acetylated protocol.

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Author contributions

Xu-Nian Wu and Qian Zhou contributed equally to this work, lead the research, data analysis, and writing of the manuscript. Ya-Dan Xu-Nian Wu and Qian Zhou contributed equally to this work, lead the research, data analysis, and writing of the manuscript. Yinuo Wu and Hai-Bin Luo supervised the entire research with conceptualization, analysis and resources.

Conflicts of interest

The authors declare no competing financial interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2022.02.012.

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