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Catecholic amides as potential selective phosphodiesterase 4D inhibitors: Design, synthesis, pharmacological evaluation and structure-activity relationships



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ABSTRACT

In this study, a series of catechol-based amides (**8a**–**n**) with different amide linkers linking the catecholic moiety to the terminal phenyl ring was designed and synthesized as potent phosphodiesterase (PDE) 4D inhibitors. The inhibitory activities of these compounds were evaluated against the core catalytic domains of human PDE4 (PDE4CAT), full-length PDE4B1 and PDE4D7 enzymes, and other PDE family members. The results indicated the majority of compounds **8a**–**n** displayed moderate to good inhibitory activities against PDE4CAT. Among these compounds **8a**–**n** displayed moderate to good inhibitory activities against PDE4CAT. Among these compounds **8a**–**n** displayed moderate to good inhibitory interestingly, compound **8g**, a potent and selective PDE4D inhibitor ($IC_{50} = 410 \text{ nM}$) with rolipram. More interestingly, compound **8g**, a potent and selective PDE4D inhibitor ($IC_{50} = 94 \text{ nM}$), exhibited a 10-fold selectivity over the PDE4B subtypes and an over 1000-fold selectivity against other PDE family members. Docking simulations suggested that **8g** forms three extra H-bonds with the N–H of residue Asn487 and two water molecules.

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1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs) comprise a group of enzymes that control the rates of hydrolysis of cAMP and cGMP. Hence, these enzymes are important regulators of the signals mediated by these second messenger molecules. Eleven different PDE families (PDE1–PDE11) are ubiquitously expressed in the body, play specific roles in distinct physiological and pathological processes.¹ Among the large family of PDEs, phosphodiesterase IV (PDE4) which specifically catalyzes the hydrolysis of cAMP is ubiquitous in the body, such as inflammatory and immune cell type's relevant, central nervous system (CNS) tissue^{2–4} (see Fig. 1).

PDE4 is comprised of four genes (PDE4A–D) and each gene has multiple transcripts that can produce three isoforms of the protein termed long, short and super short.⁵ And PDE4 is highly expressed in brain regions involved with regulation of memory, anxiety and depression.^{6,7} Many PDE4 inhibitors, such as rolipram (1), displayed antidepressant activities⁸ followed by memory-enhancing effects.^{9–11} However, these PDE4 inhibitors that can block all the different PDE4 isoforms have not been brought to market because

of undesirable effects.¹² Many studies have suggested that PDE4D may play a pivotal role in the antidepressant-like and cognition enhancing effects of PDE4 inhibitors.^{13–16} Although there are still outstanding issues regarding the relationship between PDE4D, emesis, depression, and cognition, there is clear evidence that the development of selective PDE4D inhibitors without emesis could prove a winning strategy to produces antidepressant-like and cognition-enhancing effects, such as PDE4D inhibitors DF159687 (**2**),¹⁷ chlorbipram (**3**)¹⁸ and GEBR-7b (**4**).^{19,20}

The crystal structure of human PDE4D in complex with rolipram,^{17,21} indicates that the catechol moiety of rolipram is an important pharmacophore, in which conformers interact with the purine-selective glutamine and hydrophobic clamp pocket (Fig. 2), and the side-chain region of rolipram still has much space for structural optimization and exploration of subtype selectivity. On the other hand, many studies have indicated that the linker length and its spatial direction play critical roes, particularly in determining the selectivity toward the different PDE4 isoforms. For example, the replacement of the $-C=NOCH_2CO-$ linker of GEBR-7b with a longer linker ($-C=NOCH_2CH(OH)CH_2NH-$) give compound **5** improved the PDE4D selectivity (85-fold) over PDE4B.²² In addition, the PDE4D inhibitor CPD-B (**6**)²³ with a short CONHCH₂ linker also displayed 142-fold subtype selectivity for PDE4D over PDE4B (Fig. 2).

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Figure 1. General structures of PDE4 inhibitors (1-6).

There is no denying the prevalence of the dialkyl catechol group in PDE4 inhibitors. The catechol motif, which is known to accept a double hydrogen bond from the carboxamide of a glutamine residue at the back of the PDE4 catalytic site,^{24–26} is found in a range of PDE4 inhibitors, from the earliest described compounds such as rolipram through to marketed roflumilast²⁷ and apremilast.²⁸ On the other hand, many amide derivatives^{29,30} as drugs and agrichemicals display wonderful activities. For example, catecholbased N-acylhydrazone 7 showed wonderful inhibitory activities for PDE4B and PDE4D.³¹ It provided us with an opportunity to design selective PDE4D inhibitors by the replacement of acylhydrazone linker (-CH=N-N(Me)CO-) of compound 7 with shorter or longer amide linkers linking the catecholic moiety to the terminal phenyl ring. substituted Although N-(2-chlorobenzyl)-3-(cyclopentyloxy)-4-methoxybenzamide with CONHCH₂ linker has been reported to be inactive towards PDE4,³² 3-(cyclopentyloxy)-4-methoxybenzamides with other substituted benzyl groups were not evaluated in this paper. Thus, in the present study, we report the design and synthesis of a series of catechol-based amides in which the acylhydrazone linker of compound **7** is replaced with a shorter linker (CONHCH₂), or a longer linker (CONHCH₂CH₂O) (**8a–n**, Fig. 2).

It is known that PDE4 enzymes contain unique regulatory domains, called upstream conserved regions 1 and 2 (UCR1 and UCR2), and the UCR2 or C-terminal regulatory domain can interact with the catalytic domains of PDE4 enzymes to regulate enzyme activity.¹⁷ Since the access of PDE4 inhibitors to the catalytic pocket are controlled by these helix-capped conformations, the inhibitory activity and selectivity of full length enzymes were different from those with truncated enzymes. Many studies have confirmed that compounds with truncated enzymes exhibit high selectivities, while full-length enzymes exhibit lower selectivities.³³ Therefore, in this study, we used full length human PDE4B1 and PDE4D7 enzymes for the in vitro assay to explore PDE4D sub-type-selective inhibitors, along with other PDE family members for isoform selectivity. The structure–activity relationships (SARs) of these derivatives are also discussed.

2. Results and discussion

2.1. Chemistry

The synthetic pathways employed for the synthesis of compounds **8a–n** is illustrated in Schemes 1 and 2. 4-Methoxy-3alkoxybenzoic acids **11a–b** were obtained by the alkali hydrolysis of methyl 4-methoxy-3-alkoxybenzoates **10a–b**, which were synthesized by the alkylation at position 3 of methyl 4-methoxy-3hydroxybenzoate **9** with corresponding bromoalkanes (Scheme 1). Selective difluoromethylation at position 4 of compound **13** using sodium chlorodifluoroacetate/NaOH led to the formation of 3-hydroxy-4-difluoromethoxybenzaldehyde **14**. Further alkylation at position 3 with bromoalkane gave 3-alkoxy-4-methoxybenzaldehydes **15a–b**, which were oxidized to corresponding acids **16a–b** by 30% H₂O₂ (Scheme 2). Acids (**11a–b** and **16a–b**) were converted by thionyl chloride to acid chlorides (**12a–b** and **17a–b**), which were subsequently reacted with the corresponding amines to prepare benzamides **8a–n** with good yields.

2.2. Pharmacology

Recent studies have indicated that the catalytic domains of all subtypes of PDE4 (PDE4A–D) exhibit a high degree of sequence conservation with a single active-site amino acid determining the



Figure 2. Design strategy.



Scheme 1. Synthetic route of compounds 8a-f.

nucleotide selectivity.^{24,34} Preliminary tests on PDE4CAT activity were performed according to reported protocols,³⁵ using rolipram as a positive control. All compounds were tested on PDE4CAT at nine concentrations $(10^{-8}-10^{-4} \text{ M})$ and IC₅₀ values were determined by nonlinear regression analysis of their inhibition curves (see Table 1).

It can be seen from Table 1 that the inhibitory activities of compounds with the shorter amide linker (CONHCH₂) were strongly modulated by the substituents on the terminal phenyl ring. For example, all catechol-based amides containing a 3,4-dimethoxybenzyl group (i.e., **8b**, **8d**, **8h**, and **8k**) exhibit no activity against PDE4 (IC₅₀ > 100 μ M). However, catechol-based amides containing either a 4-methoxybenzyl group (**8a**, **8c**, **8g**, and **8j**) or a benzyl group (**8i** and **8l**) displayed moderate to good PDE4CAT inhibitory activities (IC₅₀ = 0.41–2.22 μ M) of rolipram. Moreover, the PDE4 inhibitory activity of compound **8j** was comparable with that of



Scheme 2. Synthetic route of compounds 8g-n.

able 1					
nhibition of PDE4CAT	by	target	com	pounds	8a-n

Compound	R ¹	R ²	$IC_{50}^{a}(\mu M)$
8a	Cyclopentyl	4-MeO	0.96
8b	Cyclopentyl	3,4-MeO	>100
8c	Cyclopropylmethyl	4-MeO	0.91
8d	Cyclopropylmethyl	3,4-MeO	>100
8e	Cyclopentyl	2-MeO	18.30
8f	Cyclopropylmethyl	2-MeO	11.10
8g	Cyclopentyl	4-MeO	1.10
8h	Cyclopentyl	3,4-MeO	>100
8i	Cyclopentyl	Н	2.20
8j	Cyclopropylmethyl	4-MeO	0.41
8k	Cyclopropylmethyl	3,4-MeO	>100
81	Cyclopropylmethyl	Н	2.10
8m	Cyclopentyl	2-MeO	12.40
8n	Cyclopropylmethyl	2-MeO	6.90
Rolipram			0.55

^a Data reported are the mean of two experiments.

rolipram. It's mean that 3,4-dimethoxy group on the terminal phenyl ring of the compounds with the shorter amide linker (CONHCH₂) may be unfavourable for the PDE4 inhibitory activity. In addition, compounds **8e**, **8f**, **8m** and **8n** with the longer amide linker (CONHCH₂CH₂O) showed weaker PDE4 inhibitory activities (IC₅₀ = 6.9–18.3 μ M). Further analysis of the SARs of **8a–n** suggests that the N-substituents have profound effects on inhibitory activity; the activities decrease in the following order: 4-MeOPhCH₂ > PhCH₂ > 2-MeOPhOCH₂CH₂ >> 3,4-MeOPhCH₂.

Subsequently, compounds **8a**, **8c**, **8g**, and **8j**, which exhibited good activities, were screened for their ability to inhibit full-length human PDE4B1 and PDE4D7 enzymes in vitro which were purchased form BPS Bioscience Inc., and using rolipram as a positive control (Table 2). As shown in Table 2, all screened compounds displayed both weaker PDE4B1 inhibition, and more potent PDE4D7 inhibitory activity as compared to rolipram. A careful analysis of the SAR of *N*-(4-methoxybenzyl)benzamides (**8a**, **8c**, **8g**, and **8j**) reveals that compounds **8g** and **8j** with 4-difluoromethoxy groups showed higher inhibitory activities than their corresponding analogs (**8a** and **8c**) with 4-methoxy groups. Moreover, compound **8g** displayed good selectivity (~10 fold) for the PDE4D7 subtype over the PDE4B1 subtype. This suggests that this compound is exploitable as a potential lead compound for the design of PDE4D inhibitors.

PDEs exist as 11 different isozymes involved in various physiological processes; therefore the selective inhibition of PDE4 is very important. Thus, we determined the selectivity of compound **8g** toward the other PDEs isoforms using human PDE1A1, PDE1A, PDE2A, PDE3B, PDE5A, PDE6C, PDE7A, PDE8A1, PDE9A2, PDE10A2 and PDE11A, which were purchased form BPS Bioscience Inc.

Table 2Inhibition (IC50, nM)^a of PDE4B1 and PDE4D7



^a Data reported are the means of two experiments.

Table 3			
Inhibition of various	PDEs by	compound	8g

PDEs	Inhibition (%) ^a at 10 μM	$IC_{50}^{a}(nM)$
PDE1A	11	>1 × 10 ⁵
PDE2A	2	>1 × 10 ⁵
PDE3B	2	>1 × 10 ⁵
PDE5A	16	>1 × 10 ⁵
PDE6C	5	>1 × 10 ⁵
PDE7A	3	>1 × 10 ⁵
PDE8A1	10	>1 × 10 ⁵
PDE9A2	6	>1 × 10 ⁵
PDE10A2	1	>1 × 10 ⁵
PDE11A	2	>1 × 10 ⁵

^a Data reported are the mean of two experiments.

(Table 3). As shown in Table 3, compound **8g** showed the highest PDE4 selectivity (at least \sim 1000-fold) over other PDEs.

2.3. Molecular docking

To support the biological data, we performed a docking study on compounds **8a** and **8g** with the active site of PDE4D. In this investigation, the 3D structure of PDE4D (Protein Data Bank (PDB) entry 3G4K)¹⁷ with rolipram was taken from a PDB entry. The new inhibitors were built and optimized using the Powell method in sybyl 7.3. Hydrogen and Gasteiger–Hückel charges were added to every molecule. Water molecules conserved in all PDE4D structures deposited into the PDB were considered for calculations.

To validate docking reliability, the co-crystalized ligand (rolipram) was removed from the active site and docked back into the known binding pocket. The root mean square deviation (RMSD) between the predicted conformation and the actual conformation from the crystal structure of ligand was 1.29 Å, which is smaller than the resolution of X-ray crystallography 1.95 Å¹⁷). Thus, the parameters set for this Surflex-dock simulation could successfully reproduce the ligand-binding motif from the X-ray structure, and could be extended to predict the binding conformations of the synthesized inhibitors.

The docking studies highlighted the great conformational variability of the inhibitors inside the catalytic site: all the minimum energy conformations of compounds **8a** and **8g** in the active site assumed a V-shaped binding conformation (Fig. 3). In the active site of PDE4D, it is clear that the catechol moieties in **8a** and **8g** were predicted to occupy the pocket of the catechol moiety in rolipram with subtle apparent variances, and to interact in the binding site by hydrogen bonding with NH₂ of the Gln535 residue and π -stacking with Phe538. Meanwhile, the linker regions (CONHCH₂) are in position to form an extra hydrogen bond with a conserved water molecule, which extends to Tyr325.

Compound **8g** is one of the most potent and selective PDE4D inhibitors among the newly synthesized compounds. As observed in the active site of PDE4D (Fig. 3B), this improvement in activity could be attributed to the extra H-bond of 4-difluoromethyl group with the N—H of residue Asn487 and the extra H-bond of the 4-methoxy group in the terminal phenyl ring with a conserved water molecule in contact with residues Ser374 and Ser521. This finding is consistent with earlier studies²⁴ that the 4-difluromethoxy group fits better in the small sub-pocket and generates more hydrophobic interactions than the 4-methoxy group.

3. Conclusion

In conclusion, a series of catechol-based amide derivatives was designed and synthesized as potential PDE4 inhibitors. Among these compounds, *N*-(4-methoxybenzyl)benzamides **8a**, **8c**, **8g**, and **8j** showed higher PDE4 inhibitory activities than their analogs



Figure 3. Potential binding poses of **8a** (A) and **8g** (B) in the active site of PDE4D. The C atoms in **8a** and **8g** are colored in white and green–blue, respectively; total docking scores of **8a** and **8g** are 8.522 and 9.537, respectively. The C atoms in the native ligand (rolipram, total docking score = 9.018) are colored in orange. The H-bonds between each ligand and active-site amino acids are shown in yellow.

bearing other substituted benzyl groups (e.g., benzyl and 3,4methoxybenzyl groups), and other benzamides with longer linkers (CONHCH₂CH₂O) bearing 2-methoxyphenyl groups. The PDE4 inhibitory activity of compound **8j** was comparable with that of rolipram. More interestingly, compound **8g** displayed a preference for PDE4D with a 10-fold selectivity over PDE4B1, and an at least 1000-fold selectivity over other PDEs. In the PDE4D catalytic domain, docking studies revealed that the catechol moiety in **8g** assumes a similar binding profile to PDE4 with rolipram in the hydrophobic clamp pocket. In addition, the 4-difluoromethyl group in the catechol moiety forms an extra H-bond with the N—H of residue Asn487, while the side chain in **8g** forms two extra H-bonds with two water molecules. Further efforts will be aimed at developing more potent PDE4D inhibitors with increased selectivities.

4. Experimental section

4.1. Chemistry

ESI spectra were obtained using a Waters UPLC/Quattro Premier XE mass spectrometer. ¹H and ¹³C NMR spectra were recorded for CDCl₃ or DMSO- d_6 using a Varian Mercury 400 spectrometer and TMS as an internal reference. Elemental analyses were performed using a Vario ELIII CHNSO elemental analyzer. Rolipram was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and used without further purification.

4.1.1. Synthesis of methyl 4-methoxy-3-alkoxybenzoate 10

General procedures: A mixture of 4-*methoxy*-3-hydroxybenzoate **9** (1.82 g, 10 mmol), alkylbromide (20 mmol), and cesium carbonate (6.51 g, 20 mmol) in DMF (15 mL) was stirred at 65 °C for 1 h and monitored by TLC. Then, the reaction solution was poured into water and the solid was collected. The obtained precipitate was chromatographed using a mixture of petroleum ether and acetone (v/v = 20:1) to give corresponding methyl 4-methoxy-3-alkoxybenzoate.

4.1.1.1. Methyl 3-(cyclopentyloxy)-4-methoxybenzoate 10a. Yield 95%. ESI-MS (*m*/*z*): 273.4 ([M+Na]⁺), 251.5 ([M+H]⁺).

4.1.1.2. Methyl 3-(cyclopropylmethoxy)-4-methoxy benzoate 10b. Yield 95%. ESI-MS (*m*/*z*): 259.4 ([M+Na]⁺), 237.6 ([M+H]⁺).

4.1.2. Synthesis of 4-methoxy-3-alkoxybenzoic acid 11

General procedures: A mixture of methyl 4-methoxy-3-alkoxybenzoate (5 mmol), KOH (1.12 g, 20 mmol) in methanol (9 mL) and water (1 mL) was stirred at room temperature for 12 h, and monitored by TLC. Then, the reaction mixture was adjusted to pH 2 with cool hydrochloric acid (2 M). The formed precipitate was collected by filtration to give pure 4-methoxy-3-alkoxybenzoic acid.

4.1.2.1. 3-(Cyclopentyloxy)-4-methoxybenzoic acid 11a²². Total yield: 93%. ¹H NMR (400 MHz, CDCl₃) δ 7.77 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.63 (d, *J* = 2.0 Hz, 1H), 6.93 (d, *J* = 8.4 Hz, 1H), 4.90–4.85 (m, 1H), 3.94 (s, 3H), 2.07–1.83 (m, 6H), 1.71–1.61 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 172.07, 154.73, 147.34, 124.28, 121.58, 115.65, 110.76, 80.59, 56.08, 32.77, 24.10. ESI-MS (*m*/*z*): 235.6 ([M–H]⁻).

4.1.2.2. 3-(Cyclopropylmethoxy)-4-methoxybenzoic acid **11b**³⁶. Total yield: 94%. ¹H NMR (400 MHz, CDCl₃) δ 7.77 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.59 (d, *J* = 2.0 Hz, 1H), 6.92 (d, *J* = 8.4 Hz, 1H), 3.95 (s, 3H), 3.92 (d, *J* = 7.2 Hz, 2H), 1.41–1.33 (m, 1H), 0.69–0.64 (m, 2H), 0.40–0.37 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 171.99, 154.22, 148.15, 124.61, 121.59, 114.20, 110.61, 74.06, 56.10, 10.17, 3.47. ESI-MS (*m*/*z*): 245.9 ([M+Na]⁺), 223.8 ([M+H]⁺).

4.1.3. Synthesis of 4-difluoromethoxy-3-hydroxybenzaldehyde 14³⁷

To a solution of 3,4-dihydroxybenzaldehyde (1.66 g, 12 mmol) and sodium chlorodifluoroacetate (1.83 g, 12 mmol) in DMF

(15 mL) and water (0.3 mL) was added sodium hydroxide (0.48 g, 12 mmol). Then, the mixture was heated at 120 °C and stirred at this temperature for 2 h. The solvent was removed by vacuum distillation, and to the residue was added aqueous HCl (2 mL). The mixture was extracted with Et₂O and washed with brine. The solvent was removed under reduced pressure, and the crude product was purified by chromatography on silica gel (petroleum ether/EtOAc = 80:20) to afford 12 as a white solid (1.05 g, 5.28 mmol, 46%). ESI-MS (m/z): 187.4 ($[M-H]^-$).

4.1.4. Synthesis of 4-difluoromethoxy-3-alkoxybenzoic acid 16

General procedures: A mixture of 4-difluoromethoxy-3-hydroxybenzaldehyde **14** (1.88 g, 10 mmol), alkylbromide (20 mmol), and cesium carbonate (6.51 g, 20 mmol) in DMF (15 mL) was stirred at 65 °C for 1 h and monitored by TLC. Then, the reaction solution was poured into water and the solid was collected. The obtained precipitate **15** was used in all the following reactions without further purification.

A mixture of 4-difluoromethoxy-3-alkoxybenzaldehyde **15** (6.25 mmol), 30% H₂O₂ (1 mL) and KOH (2.8 g, 50 mmol) in methanol (10 ml) was stirred at room temperature for 1 h and monitored by TLC. Then, the reaction mixture was diluted by water (30 mL) and adjusted to pH 2 with concentrated hydrochloric acid. The formed precipitate was collected by filtration to pure 4-difluoromethoxy-3-alkoxybenzoic acid.

4.1.4.1. 3-(Cyclopentyloxy)-4-(difluoromethoxy)benzoic acid 16a. Yield: 98%. ¹H NMR (400 MHz, CDCl₃) δ 7.71–7.68 (m, 2H), 7.22 (d, *J* = 8.8 Hz, 1H), 6.64 (t, *J* = 74.9 Hz, 1H), 4.92–4.87 (m, 1H), 2.02–1.79 (m, 6H), 1.71–1.62 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 171.32, 149.37, 145.34, 145.31, 145.28, 127.24, 123.28, 122.15, 118.42 (-CHF₂), 116.46, 115.83 (-CHF₂), 113.24 (-CHF₂), 81.02, 32.75, 23.92. ESI-MS (*m*/*z*): 271.5 ([M–H]⁻).

4.1.4.2. 3-(Cyclopropylmethoxy)-4-(difluoromethoxy)benzoic acid 16b. Yield: 97%. ¹H NMR (400 MHz, CDCl₃) δ 7.75 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.70 (d, *J* = 2.0 Hz, 1H), 7.26 (d, *J* = 8.4 Hz, 1H), 6.77 (t, *J* = 74.8 Hz, 1H), 3.98 (d, *J* = 6.8 Hz, 2H), 1.39–1.30 (m, 1H), 0.73–0.66 (m, 2H), 0.43–0.39 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 170.98, 150.19, 144.88, 144.85, 144.82, 127.20, 123.72, 121.83, 118.31 (-CHF₂), 115.72 (-CHF₂), 115.67, 113.12 (-CHF₂), 74.15, 10.04, 3.28. ESI-MS (*m*/*z*): 257.6 ([M-H]⁻).

4.1.5. Synthesis of compounds 8a-n

General procedures: To the intermediate alkoxybenzoic acid (0.5 mmol), excess thionyl chloride (5 mL) was added, and the reaction mixture was stirred at 60–80 °C for 3 h. Excess thionyl chloride was then evaporated under reduced pressure to give the corresponding alkoxybenzoyl chloride as a crude yellow oil, which was used in the below mentioned reaction without further purification.

To a solution of amine (0.5 mmol) and anhydrous triethylamine (1 mmol) in dry CH_2CI_2 (5 mL), a solution of previously prepared alkoxybenzoyl chloride (0.5 mmol) in dry CH_2CI_2 (2 mL) was slowly added at 0 °C. The reaction mixture was stirred at room temperature for 3 h, monitored by TLC, and then concentrated under reduced pressure; the obtained residue was purified using flash chromatography (petroleum ether/acetone 20:1) to give the desired amide products as colorless solids.

4.1.5.1. 3-(Cyclopentyloxy)-4-methoxy-*N***-(4-methoxybenzyl) benzamide (8a).** Yield: 66%. ¹H NMR (400 MHz, CDCl₃) δ 7.44 (d, *J* = 2.0 Hz, 1H), 7.30–7.27 (m, 2H), 7.24 (dd, *J* = 8.4, 2.1 Hz, 1H), 6.91–6.86 (m, 2H), 6.83 (d, *J* = 8.4 Hz, 1H), 6.26 (s, 1H, NH), 4.87–4.82 (m, 1H), 4.56 (d, *J* = 5.6 Hz, 2H), 3.87 (s, 3H), 3.80 (s, 3H), 1.99–1.79 (m, 6H), 1.65–1.59 (m, 2H). ¹³C NMR (100 MHz, CDCI3) δ 167.0, 159.1, 152.8, 147.7, 130.5, 129.3, 126.9, 119.2, 114.1, 114.1, 110.8, 80.6, 56.1, 55.3, 43.6, 32.8, 24.0. ESI-MS (*m*/*z*): 378.6 ([M +Na]⁺), 356.6 ([M+H]⁺) and Anal. Calcd for C₂₁H₂₅NO₄: C, 70.96; H, 7.09; N, 3.94. Found: C, 70.77; H, 7.19; N, 4.11.

4.1.5.2. 3-(Cyclopentyloxy)-*N***-(3,4-dimethoxybenzyl)-4-methoxybenzamide (8b).** Yield: 55%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.79 (t, *J* = 6.0 Hz, 1H), 7.51 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.46 (d, *J* = 2.0 Hz, 1H), 7.01 (d, *J* = 8.4 Hz, 1H), 6.94 (d, *J* = 1.5 Hz, 1H), 6.89 (d, *J* = 8.2 Hz, 1H), 6.83 (dd, *J* = 8.2, 1.6 Hz, 1H), 4.89–4.78 (m, 1H), 4.39 (d, *J* = 6.0 Hz, 2H), 3.79 (s, 3H), 3.73 (s, 3H), 3.72 (s, 3H), 1.95–1.81 (m, 2H), 1.77–1.50 (m, 6H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 165.6, 152.1, 148.6, 147.7, 146.4, 132.4, 126.6, 120.5, 119.4, 113.6, 111.8, 111.4, 111.3, 79.6, 55.6, 55.4, 42.3, 32.2, 23.5. ESI-MS (*m*/*z*): 384.5 ([M–H][–]) and Anal. Calcd for C₂₂H₂₇NO₅: C, 68.55; H, 7.06; N, 3.63. Found: C, 68.37; H, 7.14; N, 3.78.

4.1.5.3. 3-(Cyclopropylmethoxy)-4-methoxy-N-(4-methoxyben-zyl)benzamide (8c). Yield: 45%. ¹H NMR (400 MHz, CDCl₃) δ 7.42 (d, *J* = 2.0 Hz, 1H), 7.30–7.27 (m, 2H), 7.25–7.24 (m, 1H), 6.91–6.86 (m, 2H), 6.84 (d, *J* = 8.4 Hz, 1H), 6.26 (s, 1H, NH), 4.56 (d, *J* = 5.6 Hz, 2H), 3.91–3.89 (m, 5H), 3.80 (s, 3H), 1.39–1.30 (m, 1H), 0.67–0.60 (m, 2H), 0.37–0.33 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 166.9, 159.1, 152.2, 148.5, 130.5, 129.3, 127.0, 119.4, 114.1, 112.5, 110.6, 74.1, 56.0, 55.3, 43.6, 10.2, 3.4. ESI-MS (*m*/*z*): 342.7 ([M +H]⁺) and Anal. Calcd for C₂₀H₂₃NO₄: C, 70.36; H, 6.79; N, 4.10. Found: C, 70.07; H, 6.51; N, 4.32.

4.1.5.4. 3-(Cyclopropylmethoxy)-*N***-(3,4-dimethoxybenzyl)-4-methoxybenzamide (8d).** Yield: 57%. ¹H NMR (400 MHz, DMSO- d_6) δ 8.78 (t, *J* = 6.0 Hz, 1H), 7.51 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.44 (d, *J* = 2.0 Hz, 1H), 7.01 (d, *J* = 8.4 Hz, 1H), 6.93 (d, *J* = 1.6 Hz, 1H), 6.89 (d, *J* = 8.0 Hz, 1H), 6.82 (dd, *J* = 8.0, 1.6 Hz, 1H), 4.39 (d, *J* = 5.9 Hz, 2H), 3.87–3.79 (m, 5H), 3.73 (s, 3H), 3.72 (s, 3H), 1.30–1.16 (m, 1H), 0.60–0.54 (m, 2H), 0.34–0.29 (m, 2H). ¹³C NMR (100 MHz, DMSO- d_6) δ 165.6, 151.4, 148.6, 147.7, 147.5, 132.3, 126.6, 120.5, 119.4, 111.8, 111.5, 111.0, 72.8, 55.6, 55.5, 55.4, 42.4, 10.2, 3.2. ESI-MS (*m*/*z*): 370.5 ([M–H]⁻) and Anal. Calcd for C₂₁H₂₅NO₅: C, 67.91; H, 6.78; N, 3.77. Found: C, 67.73; H, 6.59; N, 3.58.

4.1.5.5. 3-(Cyclopentyloxy)-4-(difluoromethoxy)-*N***-(2-(2-methoxyphenoxy)ethyl)benzamide** (8e). Yield: 80%. ¹H NMR (400 MHz, CDCl₃) δ 7.52 (d, *J* = 2.0 Hz, 1H), 7.26–7.22 (m, 1H), 7.16 (d, *J* = 8.4 Hz, 1H), 7.06 (s, 1H, NH), 7.01–6.96 (m, 2H), 6.94–6.90 (m, 2H), 6.58 (t, *J* = 75.2 Hz, 1H, -CHF₂), 4.91–4.87 (m, 1H), 4.20 (t, *J* = 4.8 Hz, 2H), 3.87–3.83 (m, 5H), 1.99–1.75 (m, 6H), 1.68–1.64 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 166.7, 149.8, 149.7, 147.8, 143.3 (t, *J* = 3.1 Hz), 132.9, 122.4, 122.3, 121.2, 118.6 (-CHF₂), 116.0 (-CHF₂), 115.4, 114.7, 113.4 (-CHF₂), 112.0, 80.9, 69.1, 55.8, 39.6, 32.8, 23.9. ESI-MS (*m*/*z*): 444.6. ([M+K]⁺) and Anal. Calcd for C₂₂H₂₅F₂NO₅:C, 62.70; H, 5.98; N, 3.32. Found: C, 62.39; H, 5.72; N, 3.47.

4.1.5.6. 3-(Cyclopropylmethoxy)-4-(difluoromethoxy)-N-(2-(2-methoxyphenoxy)ethyl)benzamide (8f). Yield: 84%. ¹H NMR (400 MHz, CDCl₃) δ 7.51 (d, *J* = 1.6 Hz, 1H), 7.28 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.18 (d, *J* = 8.4 Hz, 1H), 7.04 (s, 1H, NH), 7.01–6.95 (m, 2H), 6.95–6.89 (m, 2H), 6.69 (t, *J* = 75.2 Hz, 1H), 4.20 (t, *J* = 4.8 Hz, 2H), 3.93 (d, *J* = 7.2 Hz, 2H), 3.86–3.82 (m, 5H), 1.33–1.28 (m, 1H), 0.67–0.63 (m, 2H), 0.37–0.33 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 166.6, 150.5, 149.8, 147.8, 142.8 (t, *J* = 3.1 Hz), 132.9, 122.4, 122.0, 121.3, 119.1, 118.5 (–CHF₂), 115.9

 $(-CHF_2)$, 115.4, 113.9 $(-CHF_2)$, 113.3, 112.0, 74.1, 69.1, 55.8, 39.6, 10.1, 3.2. ESI-MS (m/z): 408.4 $([M+H]^+)$,430.4 $([M+Na]^+)$ and Anal. Calcd for $C_{21}H_{23}F_2NO_5$: C, 61.91; H, 5.69; N, 3.44. Found: C, 61.64; H, 5.43; N, 3.57.

4.1.5.7. 3-(Cyclopentyloxy)-4-(difluoromethoxy)-*N***-(4-methoxy-benzyl)benzamide (8g).** Yield: 87%. ¹H NMR (400 MHz, CDCl₃) δ 7.53 (s, 1H), 7.28 (s, 1H), 7.18–7.12 (m, 2H), 6.88 (d, *J* = 8.4 Hz, 2H), 6.57 (t, *J* = 75.2 Hz, 1H, –CHF₂), 6.36 (s, 1H, NH), 4.92–4.84 (m, 1H), 4.56 (d, *J* = 5.2 Hz, 2H), 3.80 (s, 3H), 1.96–1.76 (m, 6H), 1.70–1.64 (m, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 166.3, 159.2, 149.8, 143.3, 132.8, 130.1, 129.3, 122.3, 118.5 (–CHF₂), 118.3, 116.0 (–CHF₂), 114.7, 114.2, 113.4 (–CHF₂), 80.9, 55.34, 43.78, 32.8, 23.9. ESI-MS (*m*/*z*): 390.5 ([M–H][–]) and Anal. Calcd for C₂₁-H₂₃F₂NO₄: C, 64.44; H, 5.92; N, 3.58. Found: C, 64.39; H, 5.88; N, 3.65.

4.1.5.8. 3-(Cyclopentyloxy)-4-(difluoromethoxy)-*N***-(3,4-dimethoxybenzyl)benzamide (8h).** Yield: 78%. ¹H NMR (400 MHz, CDCl₃) δ 7.54 (d, *J* = 1.2 Hz, 1H), 7.19–7.13 (m, 2H), 6.92–6.82 (m, 3H), 6.58 (t, *J* = 75.2 Hz, 1H, –CHF₂), 6.34 (s, 1H, NH), 4.93–4.86 (m, 1H), 4.57 (d, *J* = 5.6 Hz, 2H), 3.87 (s, 6H), 1.99–1.75 (m, 6H), 1.70–1.63 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 166.3, 149.9, 149.3, 148.7, 143.3, 132.8, 130.6, 122.4, 120.3, 118.5 (–CHF₂), 118.3, 115.9 (–CHF₂), 114.7, 113.4 (–CHF₂), 111.4, 111.3, 80.9, 56.0, 56.0, 44.2, 32.8, 23.9. ESI-MS (*m*/*z*): 420.5 ([M–H][–]) and Anal. Calcd for C₂₂H₂₅F₂NO₅: C, 62.70; H, 5.98; N, 3.32. Found: C, 62.82; H, 6.02; N, 3.41.

4.1.5.9. *N*-Beyl-3-(cyclopentyloxy)-4-(difluoromethoxy)benzamide (8i). Yield: 90%. ¹H NMR (400 MHz, CDCl₃) δ 7.54 (d, *J* = 1.6 Hz, 1H), 7.40–7.27 (m, 5H), 7.21–7.12 (m, 2H), 6.79–6.35 (m, 2H), 4.92–4.86 (m, 1H), 4.63 (d, *J* = 5.6 Hz, 2H), 1.99–1.76 (m, 6H), 1.71–1.59 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 166.4, 149.8, 143.3, 138.0, 132.7, 128.8, 127.9, 127.7, 122.4, 118.5 (–CHF₂), 118.3, 116.0 (–CHF₂), 114.7, 113.4 (–CHF₂), 80.9, 44.3, 32.8, 23.9. ESI-MS (*m*/*z*): 362.5 ([M+H]⁺), 384.5 ([M+Na]⁺) and Anal. Calcd for C₂₀H₂₁F₂NO₃: C, 66.47; H, 5.86; N, 3.88. Found: C, 66.25; H, 5.97; N, 3.72.

4.1.5.10. 3-(Cyclopropylmethoxy)-4-(difluoromethoxy)-N-(4-methoxybenzyl)benzamide (8j). Yield: 87%. ¹H NMR (400 MHz, CDCl₃) δ 7.50 (d, *J* = 1.2 Hz, 1H), 7.30–7.27 (m, 1H), 7.21 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.15 (d, *J* = 8.4 Hz, 1H), 6.91–6.86 (m, 2H), 6.58 (t, *J* = 75.2 Hz, 1H), 6.34 (s, 1H, NH), 4.55 (d, *J* = 5.2 Hz, 2H), 3.92 (d, *J* = 6.8 Hz, 2H), 3.80 (s, 3H), 1.34–1.27 (m, 1H), 0.67–0.62 (m, 2H), 0.37–0.33 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 166.2, 159.2, 150.7, 142.8 (t, *J* = 3.6 Hz), 132.8, 130.0, 129.3, 122.1, 118.7, 118.4 (–CHF₂), 115.8 (–CHF₂), 114.2, 113.9 (–CHF₂), 113.3, 74.1, 55.3, 43.8, 10.1, 3.2. ESI-MS (*m*/*z*): 376.5 ([M–H]⁻) and Anal. Calcd for C₂₀H₂₁F₂NO₄: C, 63.65; H, 5.61; N, 3.71. Found: C, 63.65; H, 5.87; N, 4.01.

4.1.5.11. 3-(Cyclopropylmethoxy)-4-(difluoromethoxy)-*N***-(3,4-dimethoxybenzyl)benzamide** (8k). Yield: 76%. ¹H NMR (400 MHz, CDCl₃) δ 7.51 (d, *J* = 2.0 Hz, 1H), 7.22 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.16 (d, *J* = 8.0 Hz, 1H), 6.91–6.84 (m, 3H), 6.49 (t, *J* = 75.2 Hz, 1H), 6.34 (s, 1H, NH), 4.56 (d, *J* = 5.6 Hz, 2H), 3.93 (d, *J* = 7.2 Hz, 2H), 3.87 (s, 6H), 1.35–1.29 (m, 1H), 0.68–0.63 (m, 2H), 0.37–0.34 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 166.2, 150.7, 149.3, 148.7, 142.8 (t, *J* = 3.1 Hz), 132.8, 130.6, 122.1, 120.3, 118.8, 118.4 (–CHF₂), 115.8 (–CHF₂), 113.9, 113.2 (–CHF₂), 111.4, 111.3, 74.1, 56.0, 55.9, 44.2, 10.1, 3.2. ESI-MS (*m*/*z*): 406.5 ([M–H]⁻) and Anal. Calcd for C₂₁H₂₃F₂NO₅: C, 61.91; H, 5.69; N, 3.44. Found: C, 62.10; H, 5.83; N, 3.62.

4.1.5.12. N-benzyl-3-(cyclopropylmethoxy)-4-(difluoromethoxy) benzamide (8l). Yield: 87%. ¹H NMR (400 MHz, CDCl₃) δ 7.51 (d, J = 1.8 Hz, 1H), 7.40–7.28 (m, 5H), 7.22 (dd, J = 8.3, 1.9 Hz, 1H), 7.16 (d, J = 8.2 Hz, 1H), 6.68 (t, J = 75.2 Hz, 1H), 6.37 (s, 1H), 4.63 (d, J = 5.7 Hz, 2H), 3.93 (d, J = 6.9 Hz, 2H), 1.37–1.27 (m, 1H), 0.70–0.60 (m, 2H), 0.39–0.30 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 166.3, 150.6, 142.8, 138.0, 132.7, 128.8, 127.9, 127.7, 122.1, 118.8, 118.4 (–CHF₂), 115.8 (–CHF₂), 113.8, 113.2 (–CHF₂), 74.1, 44.3, 10.0, 3.2. ESI-MS (m/z):370.4 ([M+Na]⁺) and Anal. Calcd for C₃₉H₄₈F₄N₂O₆: C, 65.35; H, 6.75; N, 3.91. Found: C, 65.08; H, 6.57; N, 3.77.

4.1.5.13. 3-(cyclopentyloxy)-4-methoxy-*N***-(2-(2-methoxyphenoxy) ethyl)benzamide (8m).** Yield: 65%. ¹H NMR (400 MHz, CDCl₃) δ 7.42 (d, *J* = 2.0 Hz, 1H), 7.30 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.01–6.97 (m, 2H), 6.93–6.89 (m, 2H), 6.85 (d, *J* = 8.0 Hz, 1H), 6.83 (s, 1H, NH), 4.87–4.82 (m, 1H), 4.21 (t, *J* = 5.1 Hz, 2H), 3.88 (s, 3H), 3.86–3.82 (m, 5H), 1.98–1.79 (m, 6H), 1.66–1.56 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 167.3, 152.8, 149.9, 147.9, 147.6, 127.0, 122.3, 121.2, 119.5, 115.5, 114.1, 112.0, 110.9, 80.6, 69.2, 56.1, 55.8, 39.5, 32.8, 24.1. ESI-MS (*m*/*z*): 408.6. ([M+Na]⁺), 386.7 ([M+H]⁺) and Anal. Calcd for C₂₂H₂₇NO₅: C, 68.55; H, 7.06; N, 3.63. Found: C, 68.75; H, 6.81; N, 3.94.

4.1.5.14. 3-(Cyclopropylmethoxy)-4-methoxy-*N***-(2-(2-methoxy-phenoxy)ethyl) benzamide (8n).** Yield: 6%. ¹H NMR (400 MHz, CDCl₃) δ 7.42 (d, *J* = 2.0 Hz, 1H), 7.32 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.01–6.96 (m, 2H), 6.93–6.89 (m, 2H), 6.87 (d, *J* = 8.4 Hz, 1H), 6.84 (s, 1H, NH), 4.20 (t, *J* = 5.0 Hz, 2H), 3.92–3.89 (m, 5H), 3.86–3.82 (m, 5H), 1.40–1.30 (m, 1H), 0.67–0.62 (m, 2H), 0.37–0.34 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 167.2, 152.3, 150.0, 148.4, 147.9, 127.0, 122.4, 121.2, 119.7, 115.6, 112.6, 112.1, 110.6, 74.1, 69.3, 56.1, 55.9, 39.5, 10.2, 3.4. ESI-MS (*m*/*z*): 394.6. ([M+Na]⁺), 372.6 ([M+H]⁺) and Anal. Calcd for C₂₁H₂₅NO₅: C, 67.91; H, 6.78; N, 3.77. Found: C, 67.67; H, 6.59; N, 3.69.

4.2. Pharmacology

4.2.1. Materials

Compounds **8a–n** were dissolved in DMSO and stored as stock solutions (100 mM) at -20 °C. For experimental use, all assays were prepared using stock solutions, diluted with a growth medium, and used immediately. FAM-Cyclic-3',5'-AMP and IMAP binding reagent was purchased from Molecular Devices Inc. PDE4CAT and Other PDEs were purchased from Sbdrugdiscovery Inc. and BPS Bioscience Inc. respectively.

4.2.2. In vitro assay of compounds for the inhibition of PDEs

Standard PDE assays were performed using substrate concentrations below the Km determined for each enzyme such that $K_i = IC_{50}$, as described previously.³⁸ All enzymatic reactions were performed at 25 °C for 60 min. Each 50 μ L reaction mixture contained 40 mM MOPS (pH 7.5), 0.5 mM EDTA, 15 mM MgCl₂, 0.15 mg/mL BSA, 1 mM DTT, 0.05% Proclin 200, 15 ng/mL PDE 4CAT, and 100 nM FAM-Cyclic-3',5'-AMP. The compounds were diluted in 10% DMSO, and $5\,\mu\text{L}$ of the dilution was added to a $50 \,\mu\text{L}$ reaction such that the final concentration of DMSO was 1% in all reactions. The reaction mixtures were incubated at 25 °C for 1 h. A diluted binding agent (100 µL) was then added to each well, and the reaction mixtures were incubated at 25 °C for another hour with slow shaking. The fluorescence polarization of each sample was then obtained using an excitation filter of 360 nm and an emission filter of 480 nm. The percentage of inhibition was calculated using the formula as follows:

$$\% \text{ activity} = \left[(FP_{drug} - FP_{control}) / (FP_{enzyme} - FP_{control}) \right] \times 100\%.$$

All compounds were tested at nine concentrations $(10^{-8}-10^{-4} \text{ M})$. Prism GraphPad software was used to calculate IC₅₀ values using nonlinear regression and a normalized dose–response fit.

4.3. Molecular docking

Molecular docking was performed using the Surflex-Dock program interfaced with Sybyl 7.3, which uses an empirical scoring function and a patented search engine.^{39,40} Ligands were docked into the corresponding enzyme-binding site guided by ProtoMol, which is an idealized representation of a ligand that considers all potential interactions with its binding site. In this study, the Proto-Mol was established using the known ligand from the crystal structure of PDE4D (PDB entry: 3G4K). Prior to docking, all ligands were removed and random hydrogen atoms were added. The receptor structure was then minimized in 10.000 cycles using the Powell method in Svbvl 7.3. All ligands were constructed using the sketch molecular module. Hydrogen and Gasteiger-Hückel charges were added to each molecule, the geometries of which were optimized using the TRIPOS force field and by employing a conjugate gradient method. The energy convergence criterion was 0.001 kcal/mol. Default values were selected to finish these jobs except for cases in which the threshold was 1 when the ProtoMol was generated.

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Supplementary data

Supplementary data (structural characterization of compounds **8a–n**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.10.033.

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