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Original article

Montmorillonite K-10 mediated green synthesis of cyano pyridines: Their evaluation as potential inhibitors of PDE4

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ABSTRACT

An efficient and green synthesis of functionalized cyano pyridines has been achieved *via* montmorillonite K-10 mediated multi-component reaction in a chemo- and regioselective manner. The four-component reaction of β-keto ester, arylaldehyde, malononitrile and an alcohol provided a variety of pyridine derivatives and montmorillonite K-10 was found to be a reusable catalyst. The potential of this operationally simple methodology has been demonstrated in further structure elaboration of a compound synthesized *via* C–C bond forming reactions under Suzuki, Sonogashira and Heck conditions. Some of the cyano pyridines synthesized showed PDE4B inhibitory properties *in vitro* and good interactions with PDE4B protein *in silico* suggesting cyano pyridine scaffold as a potential template for the discovery of novel PDE4 inhibitors.

1. Introduction

The prevalence of pyridines in nature (e.g., in the coenzyme vitamin B6 family and in numerous alkaloids) and their central role as versatile building blocks in the synthesis of natural products as well as biologically active compounds has led to a continued interest in the practical synthesis of pyridine derivatives [1]. The pyridine nucleus has also been found to be integral part of several potent inhibitors of phosphodiesterase 4 (PDE4) e.g. piclamilast, roflumilast etc (Fig. 1) that are known to be beneficial for the potential treatment of asthma and chronic obstructive pulmonary disease (COPD) [2a]. While roflumilast (Daxas®, Nycomed) [2b] has been launched in Europe recently, due to the adverse side effects (e.g. nausea, emesis and cardiovascular effects) shown by other inhibitors the search for new chemical class for the evaluation of selective PDE4 inhibitory properties with improved therapeutic indexes is desirable. In our effort to identify novel inhibitors of PDE4 we became interested to evaluate various 4-aryl substituted cyano pyridine derivatives (**A**, Fig. 1) for their PDE4 inhibitory potential in vitro.

The major strategies used for the synthesis of pyridine derivatives include (i) modification of the pre-formed pyridine ring or (ii) creation of the pyridine ring from suitably substituted open chain precursor. Since multi-component reactions (MCRs) have emerged as powerful tools in organic, combinatorial, and medicinal chemistry [3] particularly for easy access to diversified arrays of, e.g., valuable heterocyclic scaffolds their uses has also been reported in the straightforward synthesis of pyridines. All these approaches generally are based on carbonyl condensation chemistry and frequently involve the use of aldehydes along with other suitable reactants and reagents. Thus, a one-pot synthesis of 3,5-dicarbonitrile-pyridines has been reported by a MCR of an aldehyde, malononitrile, and a thiol in the presence of a base catalyst such as piperidine, DABCO or triethylamine [4]. Similar synthesis of pyridines was performed by using (i) a basic ionic liquid as both catalyst and reaction medium [5], (ii) a range of Lewis acids [6] (e.g. $ZnCl_2$, $AlCl_3$ and $FeCl_3$), (iii) $InCl_3$ [7], (iii) NaOH [8] and (iv) *p*-toluenesulfonic acid [9]. The use of microwave irradiation to the MCR preparation of pyridines has also been investigated [10]. While useful for the preparation of various pyridine derivatives many of these methods require the use of environmentally harmful catalysts or reagents or longer reaction time. Thus simple and greener synthetic methods for the construction of functionalized pyridine

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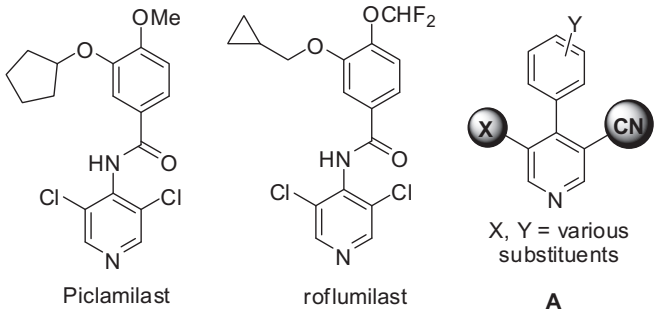


Fig. 1. Pyridine based known PDE4 inhibitors and design of new inhibitors (**A**).

ring are still desirable. In continuation of our interest in pyridine derivatives [11] of potential pharmacological interest [12] we now wish to report a one-pot synthesis of substituted cyano pyridines *via* four-component reaction of β-keto ester, arylaldehydes, malononitrile and an alcohol in the presence of montmorillonite K-10 as a green and reusable catalyst (Scheme 1).

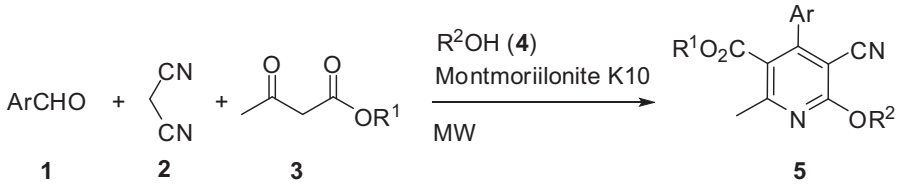
2. Results and discussion

2.1. Chemistry

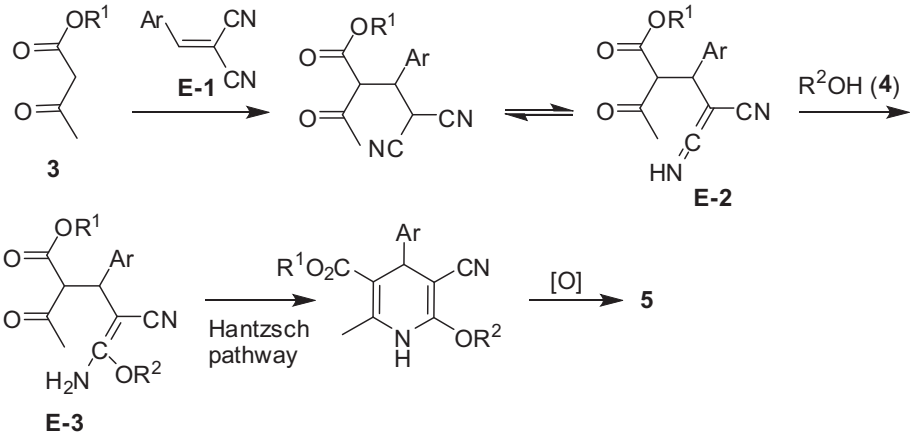
Based on earlier reports on montmorillonite K-10 catalysed Knoevenagel reaction [13] and Michael addition of 1,3-dicarbonyl compounds [14] we envisioned that a Knoevenagel adduct (**E-1**) generated *in situ* from an aldehyde and malononitrile may undergoes Michael addition with a 1,3-dicarbonyl compound **3** (Scheme 2). The tautomeric form (**E-2**) of the intermediate thus generated may reacts with an alcohol to generate an enamine (**E-3**) which *via* intramolecular cyclization (Hantzsch pathway) and subsequent aerial oxidation may provide a cyano pyridine derivative **5**.

Initially, the MCR of 4-bromo benzaldehyde (**1a**), malononitrile (**2**), ethyl acetoacetate (**3a**) and ethanol (**4a**) in the presence or absence of a solvent was examined under microwave irradiation conditions (Table 1). A number of Lewis acids such as $ZnCl_2$, $AlCl_3$ and $FeCl_3$ was examined but was found to be less effective as the expected product **5a** was isolated in 30–50% yield (entries 1–3, Table 1). All these reactions were carried out for 10 min. To develop an environmentally process we then examined the use of montmorillonite K-10 (entries 4–6, Table 1) and the reaction was carried out for 4, 8 and 10 min separately. While the reaction proceeded well in all these cases the best result however was obtained when duration was 10 min (entry 6, Table 1). Spectral analysis of the isolated product **5a** suggested that ethanol played the role of reactant as well as solvent in all these cases. However, the use of additional aprotic solvents such as dimethylformamide (DMF) and N-methyl pyrrolidone (NMP) decreased the product yield (entries 7 and 8, Table 1) indicating the use of ethanol was optimum. Notably, all these reactions required excess amount of montmorillonite K-10 (200% w/w with respect to the aldehyde used) as the decreased amount of catalyst (e.g. 75 or 150% w/w) decreased the product yield. To test the recyclability of the catalyst, montmorillonite K-10 recovered by simple filtration was reused and the MCR afforded **5a** without affecting the yield significantly (entry 6, Table 1). While the use of a similar methodology i.e. montmorillonite K-10 in combination with Pd/C under microwave irradiation towards the three component synthesis of pyridine has been reported earlier [15] to the best of our knowledge preparation of cyano pyridine **5a** or its analogues using this methodology has never been explored.

Having the optimized reaction conditions in hand we then tested the generality and scope of this green methodology. Thus reactants bearing varied substituted groups e.g. Ar, R^1 and R^2 were employed under the optimized reaction conditions established (Table 2). The reaction proceeded smoothly in all these cases to afford a variety of cyano pyridines in good to excellent yields. Aryl aldehydes **1** bearing mono (entries 1–6, 9, 14, 16, 17, Table 2), di (entries 7,8, 18, Table 2) and tri substituted aromatic ring (entries 10

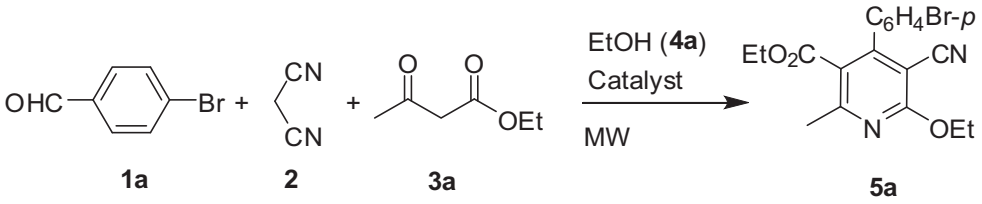


Scheme 1. Green synthesis of substituted cyano pyridines through multi-component reaction.



Scheme 2. Proposed pathway for the montmorillonite K-10 mediated synthesis of substituted cyano pyridines.

Table 1
Effect of reaction conditions on the multi-component reactions of **1a**, **2**, **3a** and **4a**.[a]



Entry	Catalyst	Amount[b]	Time (min)	% yield[c]
1.	$ZnCl_2$	2.0 equiv	10	30
2.	$AlCl_3$	2.0 equiv	10	40
3.	$FeCl_3$	2.0 equiv	10	50
4.	Montmorillonite K-10	200% w/w	4	50
5.	Montmorillonite K-10	200% w/w	8	75
6.	Montmorillonite K-10	200% w/w	10	90 (88)[d]
7.	Montmorillonite K-10	200% w/w	10	55[e]
8.	Montmorillonite K-10	200% w/w	10	50[f]

[a] All the reactions were carried out using **1a** (200 mg, 2.0 mmol), **2** (71 mg, 2.0 mmol), **3** (140 mg, 2.0 mmol), a catalyst and ethanol **4a** (5 mL) in a vessel that was sealed with a pressure cap and irradiated in a CEM Explorer microwave at 120 °C (300 W).
[b] With respect to the aldehyde **1a** used.
[c] Isolated yield.
[d] Recovered montmorillonite K-10 was used.
[e] 1.1 equiv of EtOH along with DMF (5 mL) was used.
[f] 1.1 equiv of EtOH along with NMP (5 mL) was used.

and 15, Table 2) or simple phenyl ring (entries 11–13, Table 2) were employed and found to be effective in the present MCR.

To demonstrate the potential of this cyano pyridine synthesis further structure elaboration of a compounds synthesized was carried out through transition-metal mediated C–C bond forming reactions. Thus compound **5b** was derivatized *via* Sonogashira, Suzuki and Heck reaction to afford compound **6**, **7** and **8** (Scheme 3).

Mechanistically, the present one-pot MCR seems to proceed *via* the pathway shown in Scheme 2. Thus four bonds were formed in a single pot operation in highly chemo- and regioselective manner to afford the functionalized cyano pyridines **5** under environmentally friendly conditions. Many of these cyano pyridines are amenable for further functionalization to generate diversity based library of molecules.

Table 2
Montmorillonite K-10 mediated synthesis of functionalized cyano pyridines through MCR (Scheme 1).[a]

Entry	**1**; Ar=	**3**; R^1=	**4**; R^2=	Products (**5**)	Yield[b] (%)
1.	**1a**; 4-BrC_6H_4	**3a**; Et	**4a**; Et	**5a**	90
2.	**1a**	**3b**; Me	**4b**; Me	**5b**	85
3.	**1b**; 4-$MeOC_6H_4$	**3b**	**4b**	**5c**	72
4.	**1b**	**3a**	**4a**	**5d**	75
5.	**1c**; 4-FC_6H_4	**3a**	**4a**	**5e**	81
6.	**1c**	**3b**	**4b**	**5f**	85
7.	**1d**; 2,4-di-FC_6H_3	**3b**	**4b**	**5g**	87
8.	**1d**	**3a**	**4a**	**5h**	79
9.	**1e**; 4-$CF_3C_6H_4$	**3b**	**4b**	**5i**	81
10.	**1f**; 2,6-di-F-4-BrC_6H_2	**3b**	**4b**	**5j**	71
11.	**1g**; C_6H_5	**3b**	**4b**	**5k**	82
12.	**1g**	**3a**	**4a**	**5l**	73
13.	**1g**	**3a**	**4b**	**5m**	79
14.	**1e**	**3a**	**4a**	**5n**	76
15.	**1f**	**3a**	**4a**	**5o**	75
16.	**1c**	**3a**	**4b**	**5p**	89
17.	**1a**	**3a**	**4b**	**5q**	91
18.	**1d**	**3a**	**4b**	**5r**	88

[a] All the reactions were carried out using **1** (2.0 mmol), **2** (2.0 mmol), β-keto ester **3** (2.0 mmol), montmorillonite K-10 (400 mg, 2.0 times with respect to **1**) and an alcohol **4** (5 mL) in a vessel that was sealed with a pressure cap and irradiated in a CEM Explorer microwave at 120 °C (300 W).
[b] Isolated yield.

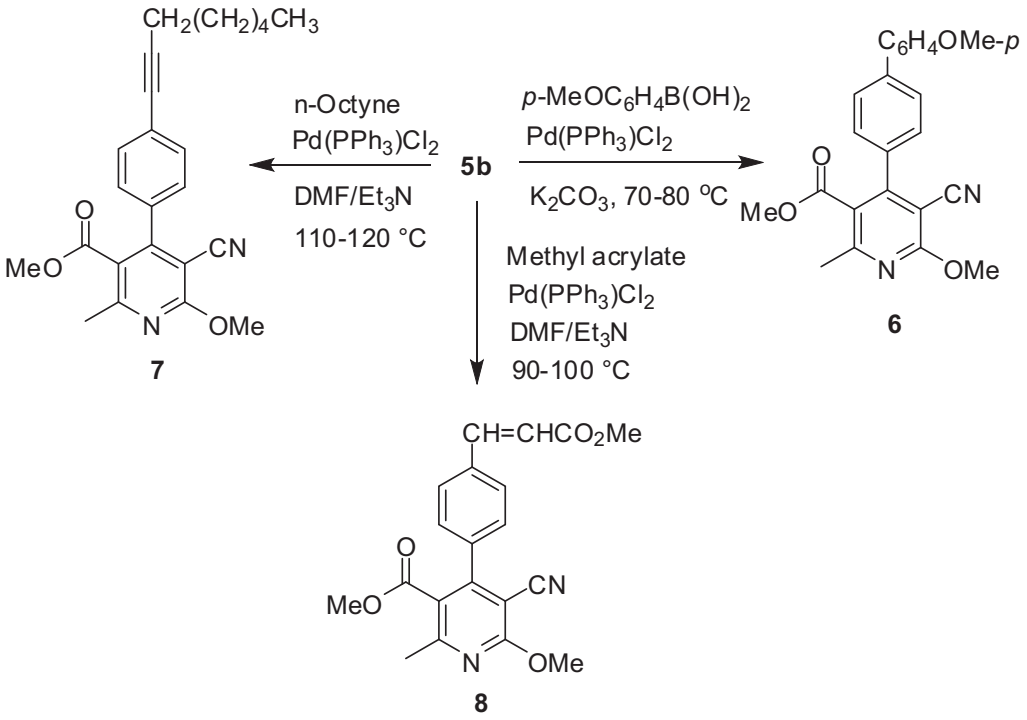
2.2. Pharmacology

The selective inhibition of PDE4A and/or PDE4B without affecting the other isoforms (thought to be responsible for undesired side effects such as nausea and emesis) is the emerging strategy to develop a safer drug [2]. On the other hand nicotinamide derivatives have been reported as inhibitors of PDE4 [16]. Due to our interest in PDE4 inhibitors [17] we therefore evaluated some of the pyridines synthesized initially for their PDE4B inhibitory potential *in vitro* at 30 μM using PDE4B enzyme assay [18] (Table 3). Rolipram [19] was used as a reference compound in this assay. The nature of substituent(s) present on the C-4 aryl moiety seemed to have significant influence on PDE4 activities of the corresponding compound. For example, a strong electron donating group e.g. OMe at the *para* position of the C-4 benzene ring (entries 3 and 4, Table 3) was found to be better than other milder donating groups e.g. F or Br (entries 1,2, 5 and 6, Table 3). The di or tri substitution on the C-4 benzene ring decreased the PDE4B inhibitory activities.

In order to understand the nature of interactions of these molecules with PDE4B docking studies were carried out using the compounds **5a** (Fig. 2) and **5d** (Fig. 3). The dock scores i.e. −17.08 and −25.47 kcal/mol obtained for **5a** and **5d** respectively suggests that these molecules bind well with PDE4B. H-bonding interaction was observed in the case of **5a** involving the –CN group and His234 residue of PDE4B in addition to arene-cation interaction with the same His234. Notably, the H-bonding interaction observed in the case of **5d** involved the –OMe group and Glu443 residue of PDE4B in addition to the arene-arene interaction with Phe446 residue.

3. Conclusions

In conclusion, a green and one-pot four-component synthesis of functionalized cyano pyridines from readily available starting materials has been developed using montmorillonite K-10 as an efficient



Scheme 3. Suzuki, Sonogashira and Heck reaction of compound **5b**.

and reusable catalyst. This operationally simple methodology provided a variety of cyano pyridines in a chemo- and regioselective manner. The potential of this methodology has been demonstrated in further structure elaboration of a compound synthesized. Some of the cyano pyridines synthesized showed PDE4B inhibitory properties in vitro and good interactions with PDE4B protein *in silico*. The cyano pyridine template presented here therefore has potential for the discovery of novel PDE4 inhibitors and the methodology therefore will find applications in the synthesis of pyridine based library of molecules of potential pharmacological interest.

4. Experimental section

4.1. Chemistry

4.1.1. General methods

Unless stated otherwise, reactions were monitored by thin layer chromatography (TLC) on silica gel plates (60 F_{254}), visualizing with ultraviolet light or iodine spray. Column chromatography was performed on silica gel (60–120 mesh) using distilled petroleum ether and ethyl acetate. ^{1}H and ^{13}C NMR spectra were determined in $CDCl_3$/DMSO-d_6 solution using 400 and 100 MHz spectrometers, respectively. Proton chemical shifts (δ) are relative to tetramethylsilane (TMS, $\delta = 0.0$) as internal standard and expressed in parts per million. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), and m (multiplet) as well as b (broad). Coupling constants (J) are given in hertz. Infrared spectra were recorded on a FTIR spectrometer. Melting points were determined by using a Buchi melting point B-540 apparatus. MS spectra were obtained on a mass spectrometer. HRMS was determined using waters LCT premier XETOFARE-047 apparatus.

4.1.2. Typical procedure for the synthesis of substituted cyano pyridine ***5a***

A mixture of ethyl acetoacetate (140 mg, 2.0 mmol), 4-bromo benzaldehyde (200 mg, 2.0 mmol), and malononitrile (71 mg, 2.0 mmol) in ethanol (5 mL) was added montmorillonite K-10 (400 mg, 200% w/w with respect to the aldehyde used). The reaction vessel was sealed with a pressure cap and irradiated in a CEM Explorer microwave for 10 min at 120 °C (300 W) (the progress of the reaction was monitored by TLC). After completion, the reaction mixture was cooled to room temperature, filtered through a celite bed and the bed was washed with dichloromethane (2 × 5.0 mL). The filtrates were collected, combined and concentrated under reduced pressure. The residue was purified by column chromatography using ethyl acetate/hexanes (0–15%) to give the desired product.

4.1.3. Ethyl 4-(4-bromophenyl)-5-cyano-6-ethoxy-2-methylnicotinate (***5a***)

Light yellow solid (379 mg, 0.90 mmol, 90%); mp 136–137 °C; IR (KBr) 2964, 2229, 1725, 1586, 1548, 1379, 1337, 1274, 1158, 1073 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.92 (t, $J = 7.2$ Hz, 3H), 1.43 (t, $J = 7.6$ Hz, 3H), 2.57 (s, 3H), 4.0 (q, $J = 7.0$ Hz, 2H), 4.53 (q, $J = 7.6$ Hz, 2H), 7.21 (d, $J = 8.4$ Hz, 2H), 7.59 (d, $J = 8.0$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.5, 14.3, 23.4, 61.6, 63.8, 93.9, 114.2, 122.6, 124.0, 129.6 (2C), 131.8 (2C), 134.0, 153.7, 159.5, 163.5, 166.8; HRMS: m/z calcd for $C_{18}H_{17}BrN_2O_3$ (M + 1) 389.0423; found 389.0507.

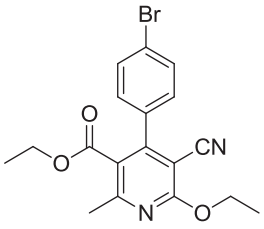
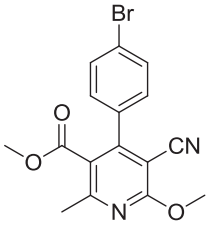
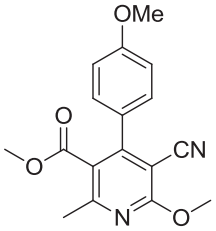
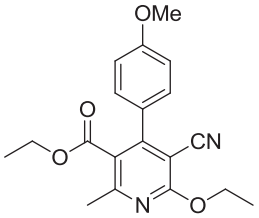
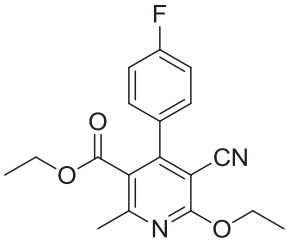
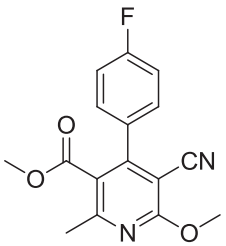
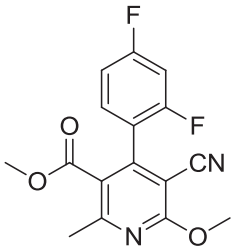
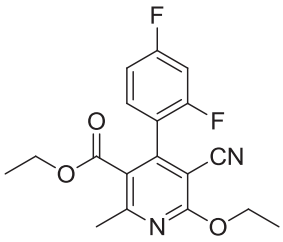
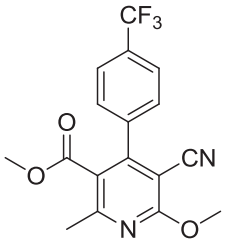
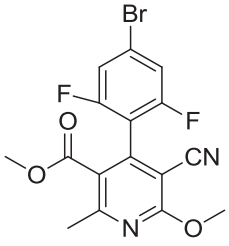
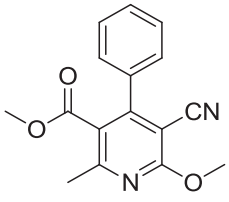
4.1.4. Methyl 4-(4-bromophenyl)-5-cyano-6-methoxy-2-methylnicotinate (***5b***)

Light yellow solid (334 mg, 0.85 mmol, 85%); mp 112–113 °C; IR (KBr) 2940, 2226, 1732, 1591, 1551, 1473, 1378, 1274, 1152, 1081 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.50 (s, 3H), 3.48 (s, 3H), 4.02 (s, 3H), 7.16 (d, $J = 8.8$ Hz, 2H), 7.52 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.5, 52.4, 54.9, 93.9, 114.1, 122.7, 124.3, 129.5, 131.9 (2C), 132.1, 133.7, 153.7, 159.5, 163.3, 167.3; HRMS: m/z calcd for $C_{16}H_{13}BrN_2O_3$ (M + 1) 361.0110; found 361.0174.

4.1.5. Methyl 5-cyano-6-methoxy-4-(4-methoxyphenyl)-2-methylnicotinate (***5c***)

Off white solid (330 mg, 0.72 mmol, 72%); mp 137–138 °C; IR (KBr) 2940, 2225, 1732, 1579, 1557, 1470, 1388, 1297, 1152, 1079 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.56 (s, 3H), 3.57 (s, 3H),

Table 3
Inhibition of PDE4B by compound **5** at 30 μM.

Entry	Compounds	Average % inhibition	SD
1.	 **5a**	43.72	3.92
2.	 **5b**	44.80	2.17
3.	 **5c**	64.10	2.15
4.	 **5d**	65.90	3.20
5.	 **5e**	42.34	1.29
6.	 **5f**	43.21	3.22
7.	 **5g**	21.46	1.34
8.	 **5h**	20.43	2.31
9.	 **5i**	23.47	1.63
10.	 **5j**	22.44	2.75
11.	 **5k**	38.42	1.56
12.	Rolipram	90.12	4.35

SD = standard deviation.

3.81 (s, 3H), 4.08 (s, 3H), 6.82 (d, J = 8.4 Hz, 2H), 7.08 (d, J = 8.0 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.3, 52.3, 54.9, 55.3, 93.9, 114.3, 115.6, 115.9, 118.4, 130.0, 130.1, 131.1, 154.6, 159.4, 159.5, 163.9, 167.6; HRMS: m/z calcd for $C_{17}H_{16}N_2O_4$ $(M + 1)^+$ 313.1110; found 313.1181.

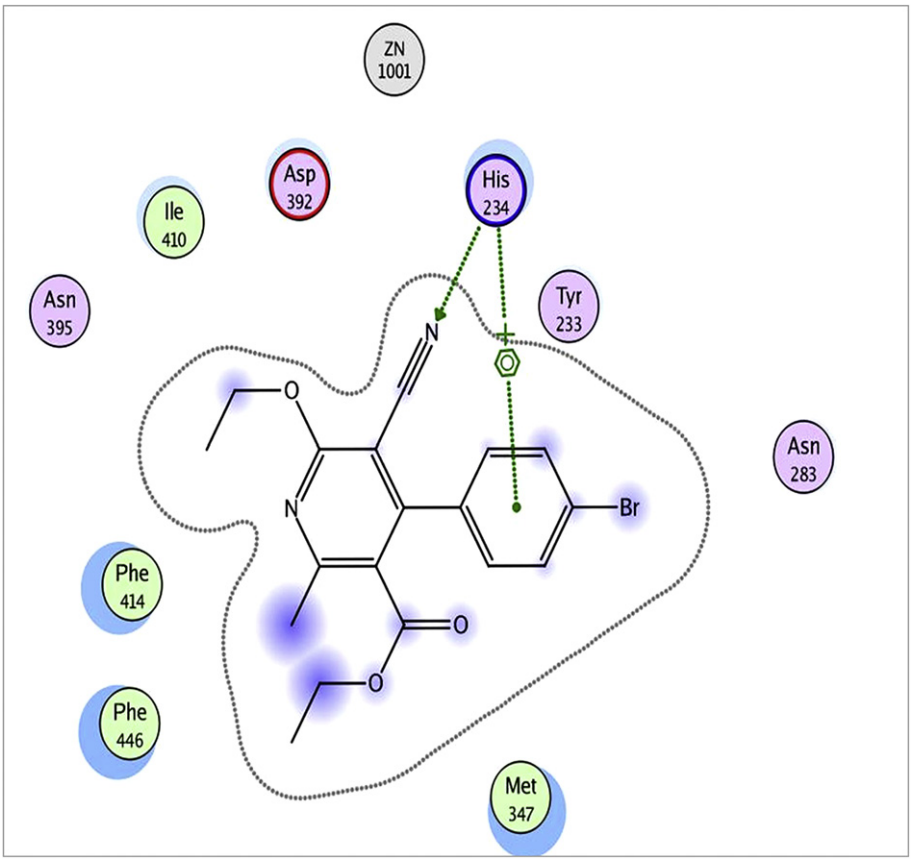


Fig. 2. Schematic representation of the interaction between **5a** and PDE4B. Hydrophobic amino acids are coloured green, polar are purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

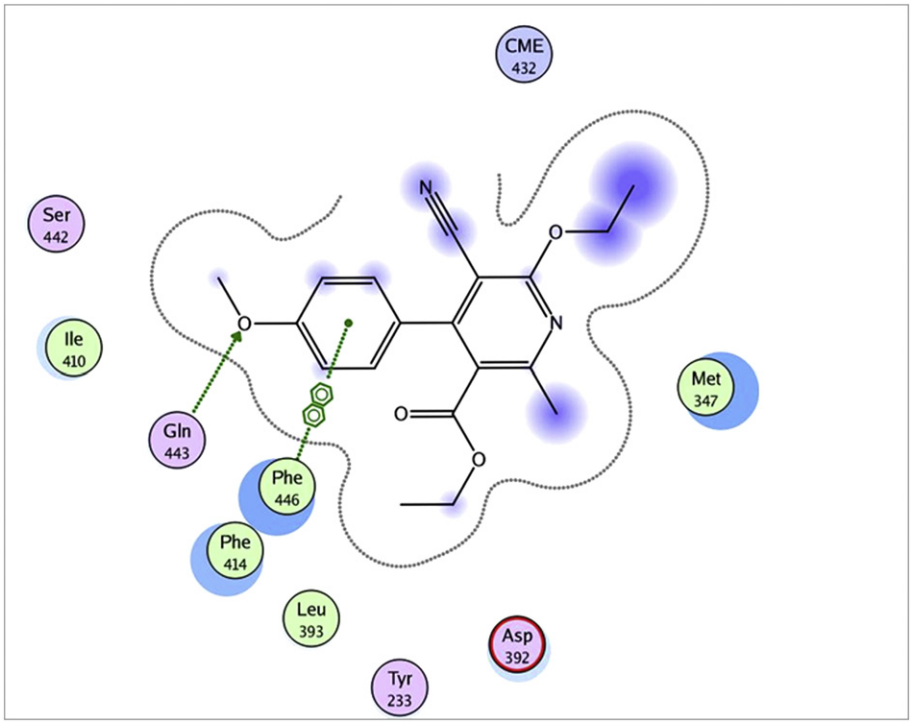


Fig. 3. Schematic representation of the interaction between **5d** and PDE4B. Hydrophobic amino acids are coloured green, polar are purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*4.1.6. Ethyl 5-cyano-6-ethoxy-4-(4-methoxyphenyl)-2-methylnicotinate (**5d**)*

Off white solid (375 mg, 0.75 mmol, 75%); mp 125–127 °C; IR (KBr) 2925, 2226, 1738, 1589, 1551, 1463, 1375, 1249, 1179, 1086 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.94 (t, $J = 7.2$ Hz, 3H), 1.42 (t, $J = 7.2$ Hz, 3H), 2.57 (s, 3H), 3.79 (s, 3H), 4.01 (q, $J = 7.2$ Hz, 2H), 4.62 (q, $J = 7.2$ Hz 2H), 6.95 (d, $J = 8.4$ Hz, 2H), 7.15 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.5, 14.2, 24.5, 55.2, 61.6, 63.8, 93.8, 114.2, 115.6, 115.9, 118.2, 130.0, 130.1, 131.0, 154.7, 159.1, 159.5, 164.0, 167.7; HRMS: *m/z* calcd for $C_{19}H_{20}N_2O_4$ (M + 1) 341.1423; found 341.1497.

*4.1.7. Ethyl 5-cyano-6-ethoxy-4-(4-fluorophenyl)-2-methylnicotinate (**5e**)*

Light yellow solid (428 mg, 0.81 mmol, 81%); mp 127–129 °C; IR (KBr) 2948, 2224, 1739, 1596, 1562, 1472, 1379, 1268, 1152, 1077 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.93 (t, $J = 7.2$ Hz, 3H), 1.43 (t, $J = 6.8$ Hz, 3H), 2.56 (s, 3H), 4.01 (q, $J = 7.0$ Hz, 2H), 4.53 (q, $J = 6.8$ Hz, 2H), 7.14 (d, $J = 7.2$ Hz, 2H), 7.52 (d, $J = 6.8$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.5, 14.3, 23.4, 61.6, 63.7, 94.0, 114.3, 115.7, 115.9, 122.8, 130.0, 130.1, 130.2, 153.8, 159.3, 162.1, 164.6, 167.0; HRMS: *m/z* calcd for $C_{18}H_{17}FN_2O_3$ (M + 1) 329.1223; found 329.1290.

*4.1.8. Methyl 5-cyano-4-(4-fluorophenyl)-6-methoxy-2-methylnicotinate (**5f**)*

Off white solid (411 mg, 0.85 mmol, 85%); mp 131–133 °C; IR (KBr) 2948, 2224, 1738, 1596, 1561, 1472, 1379, 1268, 1165, 1077 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.58 (s, 3H), 3.55 (s, 3H), 4.10 (s, 3H), 7.16 (d, $J = 8.4$ Hz, 2H), 7.35 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.4, 52.4, 54.9, 94.1, 114.2, 115.8, 116.0, 122.9, 129.9, 130.0, 130.9, 153.9, 159.3, 163.9, 164.6, 167.5; HRMS: *m/z* calcd for $C_{16}H_{13}FN_2O_3$ (M + 1) 301.0910; found 301.0972.

*4.1.9. Methyl 5-cyano-4-(2,4-difluorophenyl)-6-methoxy-2-methylnicotinate (**5g**)*

Light yellow solid (390 mg, 0.87 mmol, 87%); mp 151–152 °C; IR (KBr) 2956, 2227, 1736, 1597, 1563, 1474, 1381, 1273, 1150, 1075 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.62 (s, 3H), 3.59 (s, 3H), 4.11 (s, 3H), 6.93 (m, 2H), 7.21 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.9, 52.3, 54.9, 95.3, 104.5, 111.7, 113.7, 119.1, 122.8, 130.8, 158.0, 160.4, 162.5, 163.7, 165.0, 166.7; HRMS: *m/z* calcd for $C_{16}H_{12}F_2N_2O_3$ (M + 1) 319.0816; found 319.0885.

*4.1.10. Ethyl 5-cyano-4-(2,4-difluorophenyl)-6-ethoxy-2-methylnicotinate (**5h**)*

Light yellow solid (385 mg, 0.79 mmol, 79%); mp 145–146 °C; IR (KBr) 2979, 2226, 1731, 1620, 1559, 1477, 1343, 1270, 1177, 1100 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.98 (t, $J = 7.2$ Hz, 3H), 1.44 (t, $J = 7.6$ Hz, 3H), 2.61 (s, 3H), 4.03 (q, $J = 7.0$ Hz, 2H), 4.54 (q, $J = 7.4$ Hz, 2H), 6.93 (m, 2H), 7.22 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.5, 14.3, 23.9, 61.5, 63.8, 94.0, 104.5, 111.8, 113.8, 119.2, 122.7, 130.9, 158.0, 160.5, 162.4, 163.3, 164.9, 166.2; HRMS: *m/z* calcd for $C_{18}H_{16}F_2N_2O_3$ (M + 1) 347.1129; found 347.1207.

*4.1.11. Methyl 5-cyano-6-methoxy-2-methyl-4-(4-(trifluoromethyl)phenyl)nicotinate (**5i**)*

Light yellow solid (326 mg, 0.81 mmol, 81%); mp 112–113 °C; IR (KBr) 2954, 2230, 1731, 1620, 1556, 1472, 1379, 1276, 1154, 1067 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.60 (s, 3H), 3.53 (s, 3H), 4.12 (s, 3H), 7.46 (d, $J = 8.4$ Hz, 2H), 7.72 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.5, 52.3, 54.9, 93.9, 113.9, 122.3, 122.5, 125.0, 125.6, 128.4 (2C), 131.4, 138.5, 153.4, 159.9, 163.8, 167.0; HRMS: *m/z* calcd for $C_{17}H_{13}F_3N_2O_3$ (M + 1) 351.0878; found 351.0945.

*4.1.12. Methyl-4-(4-bromo-2,6-difluorophenyl)-5-cyano-6-methoxy-2-methylnicotinate (**5j**)*

Off white solid (256 mg, 0.71 mmol, 71%); mp 124–125 °C; IR (KBr) 2925, 2232, 1732, 1625, 1556, 1470, 1388, 1274, 1152, 1071 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.68 (s, 3H), 3.66 (s, 3H), 4.12 (s, 3H), 7.21–7.26 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 24.5, 52.4, 55.0, 95.9, 113.4, 115.6, 122.1, 124.0, 124.1, 143.7, 157.7, 160.2, 160.3, 161.6, 163.4, 165.4; HRMS: *m/z* calcd for $C_{16}H_{11}BrF_2N_2O_3$ (M + 1) 396.9921; found 396.9982.

*4.1.13. Methyl 5-cyano-6-methoxy-2-methyl-4-phenylnicotinate (**5k**)*

Light yellow solid (436 mg, 0.82 mmol, 82%); mp 122–123 °C; IR (KBr) 2949, 2226, 1732, 1558, 1496, 1391, 1276, 1152, 1078 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.58 (s, 3H), 3.51 (s, 3H), 4.10 (s, 3H), 7.31–7.39 (m, 2H), 7.41–7.46 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.4, 52.2, 54.8, 94.0, 114.3, 122.9, 127.8 (2C), 128.6 (2C), 129.6, 134.9, 155.0, 159.1, 163.9, 167.6; HRMS: *m/z* calcd for $C_{16}H_{14}N_2O_3$ (M + 1) 283.1004; found 283.1073.

*4.1.14. Ethyl 5-cyano-6-ethoxy-2-methyl-4-phenylnicotinate (**5l**)*

Light yellow solid (427 mg, 0.73 mmol, 73%); mp 137–139 °C; IR (KBr) 2980, 2227, 1723, 1557, 1446, 1383, 1340, 1272, 1154, 1077 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.98 (t, $J = 7.2$ Hz, 3H), 1.43 (t, $J = 7.2$ Hz, 3H), 2.57 (s, 3H), 3.95 (q, $J = 7.2$ Hz, 2H), 4.53 (q, $J = 7.2$ and 7.2 Hz, 2H), 7.32–7.37 (m, 2H), 7.44–7.45 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.4, 14.3, 23.3, 61.4, 63.6, 94.0, 114.3, 122.8, 128.0, 128.5, 128.7, 128.9, 129.4, 135.1, 154.9, 159.1, 163.5, 167.0; HRMS: *m/z* calcd for $C_{18}H_{18}N_2O_3$ (M + 1) 311.1317; found 311.1387.

*4.1.15. Ethyl 5-cyano-6-methoxy-2-methyl-4-phenylnicotinate (**5m**)*

White solid (441 mg, 0.79 mmol, 79%); mp 117–118 °C; IR (KBr) 2986, 2226, 1726, 1562, 1472, 1379, 1354, 1272, 1154, 1076 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, $J = 7.2$ Hz, 3H), 2.60 (s, 3H), 3.94 (q, $J = 7.2$ Hz, 2H), 4.10 (s, 3H), 7.31–7.38 (m, 2H), 7.42–7.45 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.4, 23.3, 54.8, 61.5, 93.9, 114.4, 123.1, 127.9 (2C), 128.5 (2C), 129.5, 135.0, 155.0, 159.2, 163.7, 167.0; HRMS: *m/z* calcd for $C_{17}H_{16}N_2O_3$ (M + 1) 297.1161; found 297.1234.

*4.1.16. Ethyl 5-cyano-6-ethoxy-2-methyl-4-(4-(trifluoromethyl)phenyl)nicotinate (**5n**)*

White solid (330 mg, 0.76 mmol, 76%); mp 126–128 °C; IR (KBr) 2993, 2232, 1723, 1555, 1445, 1382, 1338, 1275, 1163, 1067 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.86 (t, $J = 7.2$ Hz, 3H), 1.44 (t, $J = 7.2$ Hz, 3H), 2.60 (s, 3H), 3.96 (q, $J = 7.2$ Hz, 2H), 4.55 (q, $J = 7.2$ Hz, 2H), 7.47 (d, $J = 8.4$ Hz, 2H), 7.72 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 13.3, 14.2, 23.5, 61.6, 63.9, 93.9, 113.9, 122.4, 125.0, 125.5 (2C), 128.5 (2C), 131.4, 138.8, 153.4, 159.9, 163.5, 166.5; HRMS: *m/z* calcd for $C_{19}H_{17}F_3N_2O_3$ (M + 1) 379.1191; found 379.1256.

*4.1.17. Ethyl 4-(4-bromo-2,6-difluorophenyl)-5-cyano-6-ethoxy-2-methylnicotinate (**5o**)*

Light yellow solid (289 mg, 0.75 mmol, 75%); mp 132–133 °C; IR (KBr) 2981, 2231, 1731, 1624, 1563, 1490, 1386, 1270, 1163, 1073 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.03 (t, $J = 7.6$ Hz, 3H), 1.44 (t, $J = 7.2$ Hz, 3H), 2.67 (s, 3H), 4.09 (q, $J = 7.2$ Hz, 2H), 4.55 (q, $J = 7.4$ Hz, 2H), 7.21–7.26 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.5, 14.2, 24.5, 61.6, 64.0, 95.9, 113.3, 115.5, 122.1, 123.8, 123.9, 143.5, 157.6, 160.1, 160.2, 161.7, 163.6, 165.9; HRMS; *m/z* calcd for $C_{18}H_{15}BrF_2N_2O_3$ (M + 1) 425.0234; found 425.0303.

*4.1.18. Ethyl 5-cyano-4-(4-fluorophenyl)-6-methoxy-2-methylnicotinate (**5p**)*

White solid (450 mg, 0.89 mmol, 89%); mp 110–111 °C; IR (KBr) 2964, 2224, 1730, 1596, 1561, 1471, 1378, 1340, 1267, 1152,

1076 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.94 (t, $J = 7.2$ Hz, 3H), 2.58 (s, 3H), 4.00 (q, $J = 7.2$ Hz, 2H), 4.10 (s, 3H), 7.13 (d, $J = 8.0$ Hz, 2H), 7.32 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 13.5, 23.3, 54.8, 61.6, 94.0, 114.2, 115.6, 115.8, 123.1, 130.0, 130.1, 130.9, 153.8, 159.3, 163.7 (2C), 166.9; HRMS: m/z calcd for $C_{17}H_{15}FN_2O_3$ (M + 1) 315.1067; found 315.1134.

4.1.19. Ethyl 4-(4-bromophenyl)-5-cyano-6-methoxy-2-methylnicotinate (**5q**)

White solid (369 mg, 0.91 mmol, 91%); mp 125–127 °C; IR (KBr) 2955, 2228, 1725, 1592, 1553, 1469, 1384, 1276, 1151, 1080 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.95 (t, $J = 7.2$ Hz, 3H), 2.59 (s, 3H), 4.00 (q, $J = 7.0$ Hz, 2H), 4.10 (s, 3H), 7.22 (d, $J = 8.4$ Hz, 2H), 7.59 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.5, 23.3, 54.8, 61.6, 93.8, 114.1, 122.8, 124.1, 129.6 (2C), 131.8 (2C), 133.8, 153.7, 159.5, 163.7, 166.7; HRMS: m/z calcd for $C_{17}H_{15}BrN_2O_3$ (M + 1) 375.0266; found 375.0331.

4.1.20. Ethyl 5-cyano-4-(2,4-difluorophenyl)-6-methoxy-2-methylnicotinate (**5r**)

Light yellow solid (408 mg, 0.88 mmol, 88%); mp 147–148 °C; IR (KBr) 2956, 2228, 1736, 1596, 1563, 1473, 1380, 1269, 1168, 1075 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.98 (t, $J = 7.2$ Hz, 3H), 2.63 (s, 3H), 4.04 (q, $J = 7.2$ Hz, 2H), 4.11 (s, 3H), 6.93–6.99 (m, 2H), 7.25–7.27 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.5, 23.8, 54.9, 61.5, 95.3, 104.7, 111.8, 113.7, 119.1, 123.0, 130.8, 158.0, 160.3, 162.5, 163.5, 165.0, 166.1; HRMS: m/z calcd for $C_{17}H_{14}F_2N_2O_3$ (M + 1) 333.0972; found 333.1044.

4.1.21. Preparation of methyl 5-cyano-6-methoxy-4-(4'-methoxy-[1,1'-biphenyl]-4-yl)-2-methylnicotinate (**6**)

A mixture of bromo pyridine **5b** (200 mg, 0.55 mmol), aryl boronic acid (101 mg, 0.66 mmol), $(PPh_3)_2PdCl_2$ (19.44 mg, 0.05 mmol,) and anhydrous K_2CO_3 (230 mg, 1.65 mmol) in dioxane (5 mL) was stirred initially at room temp for 15 min and then at 70 °C for 3 h under nitrogen (progress of the reaction was monitored by TLC). After completion, the reaction mixture was cooled to room temp and filtered through celite bed. The filtrate was extracted with ethylacetate (3 × 5.0 mL). The organic layers were collected, combined, dried over anhydrous Mg_2SO_4, and concentrated under vacuum. The residue was purified by column chromatography using EtOAc/Hexane (0–15%) to give the title compound as a light yellow solid (178 mg, 0.83 mmol, 83%); mp 173–174 °C; IR (KBr) 2952, 2223, 1724, 1610, 1567, 1470, 1380, 1279, 1148, 1079 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.58 (s, 3H), 3.56 (s, 3H), 3.86 (s, 3H), 4.11 (s, 3H), 6.98 (d, $J = 2.0$ Hz, 2H), 7.40 (d, $J = 8.4$ Hz, 2H), 7.57 (d, $J = 2.0$ Hz, 2H), 7.63 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.3, 52.3, 54.8, 55.2, 93.8, 114.2, 114.5, 122.9, 126.7 (2C), 128.1 (2C), 128.3 (2C), 132.3 (2C), 132.9, 141.9, 154.7, 159.1, 159.5, 163.9, 167.7; HRMS: m/z calcd for $C_{23}H_{20}N_2O_4$ (M + 1) 389.1423; found 389.1496.

4.1.22. Preparation of methyl 5-cyano-6-methoxy-2-methyl-4-(4-(oct-1-yn-1-yl)phenyl)nicotinate (**7**)

A mixture of bromo pyridine **5b** (200 mg, 0.55 mmol), *n*-octyne (121 mg, 1.1 mmol), $(PPh_3)_2PdCl_2$ (19.44 mg, 0.05 mmol), CuI (52 mg, 0.27 mmol) and Et_3N (168 mg, 1.66 mmol) in DMF (5 mL) was stirred initially at room temp for 15 min and then at 110–120 °C for 5 h under nitrogen reaction (progress of the reaction was monitored by TLC). After completion, the reaction mixture was cooled to room temp and filtered through celite bed. The filtrate was extracted with ethylacetate (3 × 5.0 mL). The organic layers were collected, combined, dried over anhydrous Mg_2SO_4, and concentrated under vacuum. The residue was purified by column chromatography using EtOAc/Hexane (0–10%) to give the title compound as a light yellow solid (162 mg, 0.75 mmol, 75%); mp 153–154 °C; IR (KBr) 2930, 2857, 2229, 1731, 1568, 1512, 1469, 1379, 1275, 1153, 1079 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.91 (t, $J = 6.8$ Hz, 3H), 1.23–1.63 (m, 8H), 2.39 (t, $J = 6.8$ Hz, 2H), 2.61 (s, 3H), 3.54 (s, 3H), 4.05 (s, 3H), 7.17 (d, $J = 8.4$ Hz, 2H), 7.58 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.0, 22.6, 23.4, 28.5, 29.6, 31.3, 31.8, 52.3, 54.9, 79.8, 92.6, 93.8, 114.2, 122.7, 124.2, 125.7, 127.8, 129.5, 131.9, 133.7, 154.4, 159.3, 163.9, 167.5; HRMS: m/z calcd for $C_{24}H_{26}N_2O_3$ (M + 1) 391.1943; found 391.1997.

4.1.23. (E)-methyl 5-cyano-6-methoxy-4-(4-(3-methoxy-3-oxoprop-1-en-1-yl)phenyl)-2-methylnicotinate (**8**)

A mixture of bromo pyridine **5b** (200 mg, 0.55 mmol), methyl acrylate (95 mg, 1.11 mmol), $(PPh_3)_2PdCl_2$ (19.44 mg, 0.05 mmol), Et_3N (196 mg, 1.94 mmol) in DMF (5 mL) was stirred at room temp for 15 min and then at 90 °C for 6 h under nitrogen (progress of the reaction was monitored by TLC). After completion, the reaction mixture was cooled to room temp and filtered through celite bed. The filtrate was extracted with ethylacetate (3 × 5.0 mL). The organic layers were collected, combined, dried over anhydrous Mg_2SO_4, and concentrated under vacuum. The residue was purified by column chromatography using EtOAc/Hexane (0–20%) to give the title compound as a light yellow solid (158 mg, 0.78 mmol, 78%); mp 135–137 °C; IR (KBr) 2962, 2867, 2231, 1726, 1596, 1561, 1483, 1377, 1276, 1162, 1079 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.50 (s, 3H), 3.47 (s, 3H), 3.75 (s, 3H), 4.03 (s, 3H), 6.35 (d, $J = 16.0$ Hz, 1H), 7.17 (d, $J = 8.4$ Hz, 2H), 7.54 (d, $J = 8.0$ Hz, 2H), 7.61 (d, $J = 16.0$ Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.5, 52.4, 54.9, 56.6, 94.0, 114.1, 115.3, 122.7, 124.3, 129.5 (2C), 131.9 (2C), 132.1, 133.7, 153.7, 159.5, 163.9, 165.0, 167.3; HRMS: m/z calcd for $C_{20}H_{18}N_2O_5$ (M + 1) 367.1216; found 367.1255.

5. Pharmacology

5.1. Materials and methods

Cells and Reagents: HEK 293 and Sf9 cells were obtained from ATCC (Washington D.C., USA). HEK 293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen Inc., San Diego, CA, USA). Sf9 cells were routinely maintained in Grace's supplemented medium (Invitrogen) with 10% FBS. RAW 264.7 cells (murine macrophage cell line) were obtained from ATCC and routinely cultured in RPMI 1640 medium with 10% fetal bovine serum (Invitrogen Inc.). cAMP was purchased from SISCO Research Laboratories (Mumbai, India). PDElight HTS cAMP phosphodiesterase assay kit was procured from Lonza (Basel, Switzerland).

5.2. PDE4B protein production and purification

PDE4B cDNA was sub-cloned into pFAST Bac HTB vector (Invitrogen) and transformed into DH10Bac (Invitrogen) competent cells. Recombinant bacmids were tested for integration by PCR analysis. Sf9 cells were transfected with bacmid using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Subsequently, P3 viral titer was amplified, cells were infected and 48 h post infection cells were lysed in lysis buffer (50 mM Tris–HCl pH 8.5, 10 mM 2-Mercaptoethanol, 1% protease inhibitor cocktail (Roche), 1% NP40). Recombinant His-tagged PDE4B protein was purified as previously described elsewhere [18]. Briefly, lysate was centrifuged at 10,000 rpm for 10 min at 4 °C and supernatant was collected. Supernatant was mixed with Ni-NTA resin (GE Life Sciences) in a ratio of 4:1 (v/v) and equilibrated with binding buffer (20 mM Tris–HCl pH 8.0, 500 mM KCl, 5 mM imidazole, 10 mM 2-mercaptoethanol and 10% glycerol) in a ratio of 2:1 (v/v) and mixed gently on rotary shaker for 1 h at 4 °C. After incubation,

lysate-Ni-NTA mixture was centrifuged at 4500 rpm for 5 min at 4 °C and the supernatant was collected as the flow-through fraction. Resin was washed twice with wash buffer (20 mM Tris–HCl pH 8.5, 1 M KCl, 10 mM 2-Mercaptoethanol and 10% glycerol). Protein was eluted sequentially twice using elution buffers (Buffer I: 20 mM Tris–HCl pH 8.5, 100 mM KCl, 250 mM imidazole, 10 mM 2-mercaptoethanol, 10% glycerol, Buffer II: 20 mM Tris–HCl pH 8.5, 100 mM KCl, 500 mM imidazole, 10 mM 2-mercaptoethanol, 10% glycerol). Eluates were collected in four fractions and analyzed by SDS-PAGE. Eluates containing PDE4B protein were pooled and stored at −80 °C in 50% glycerol until further use.

5.3. PDE4B enzymatic assay

The inhibition of PDE4B enzyme was measured using PDElight HTS cAMP phosphodiesterase assay kit (Lonza) according to manufacturer's recommendations. Briefly, 10 ng of PDE4B enzyme was pre-incubated either with DMSO (vehicle control) or compound for 15 min before incubation with the substrate cAMP (5 μM) for 1 h. The reaction was halted with stop solution followed by incubation with detection reagent for 10 min in dark. Luminescence values (RLUs) were measured by a Multilabel plate reader (Perkin Elmer 1420 Multilabel counter). The percentage of inhibition was calculated using the following formula:

$$\%\text{inhibition} = \frac{(\text{RLU of vehicle control} - \text{RLU of inhibitor})}{\text{RLU of vehicle control}} \times 100$$

6. Docking study

The molecular docking simulation was carried out using Chemical Computing Group's Molecular Operating Environment (MOE) software 2008.10 Version, "DOCK" application Module. The molecule **5a** and **5d** was docked in the PDE4B protein and their respective docking scores and interactions were observed.

Procedure: The PDE4B receptor from the structure of PDE4B in complex with Roflumilast (PDB code 1XMU) was used for docking studies. The original 1XMU PDB file contains crystallized Zn and Mn metal ions. The PDE4B Protein was retrieved from PDB and protonated (addition of hydrogen atoms) with protonation 3D application in MOE. Connolly Molecular surface was generated around the ligand site of the protein. Gasteiger Partial charges were added to the protein and finally energy minimized to relieve bad crystallographic contacts. "Active site finder" function of the MOE software was used to denote potential docking pockets within the protein crystal structure. The molecule **5a** and **5d** was placed in the active site pocket of the protein by the "Triangle Matcher" method, which generated poses by aligning the ligand triplet of atoms with the triplet of alpha spheres in cavities of tight atomic packing. Dock scoring was performed using London d*G* method. The best 10 poses of molecules were retained and scored. The preparation of ligands for docking simulation involved energy minimization with Molecular Mechanics Force-field MMFF94x (Merck Molecular Force Field 94x). Molecules were then subjected to conformational search in MOE using the Conformations Stochastic search module to find the lowest energy conformers.

The docking results were appeared as docking score in which the docking poses are ranked by the Molecular Mechanics and Generalized Born solvation model (MM/GBVI) binding free energy. RMSD of the docking pose was compared with the docking poses to the ligand in the co-crystallized structure.

For all scoring functions, lower scores indicate more favorable poses. The unit for all scoring functions is kcal/mol. The final energy was calculated using the Generalized Born solvation model. Poses for each ligand were scored based on complementarity with the binding pocket.

The London d*G* scoring function estimates the free energy of binding of the ligand from a given pose. The functional form is a sum of terms:

$$\Delta G = c + E_{flex} \sum_{h-\text{bonds}} c_{HB} f_{HB} + \sum_{m-\text{lig}} c_M f_M \sum_{\text{atoms } i} \Delta D_i$$

where c represents the average gain/loss of rotational and translational entropy; E_{flex} is the energy due to the loss of flexibility of the ligand (calculated from ligand topology only); f_{HB} measures geometric imperfections of hydrogen bonds and takes a value in [0,1]; c_{HB} is the energy of an ideal hydrogen bond; f_M measures geometric imperfections of metal ligations and takes a value in [0,1]; c_M is the energy of an ideal metal ligation; and D_i is the desolvation energy of atom i.

To validate the Docking accuracy of the program used, the native co-crystallized Roflumilast was docked back into its binding site of PDE4B Protein.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.12.024.

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