Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 3098

www.rsc.org/obc

PAPER

A new cascade reaction: concurrent construction of six and five membered rings leading to novel fused quinazolinones†

K. Siva Kumar,[a,b] P. Mahesh Kumar,[a] V. Sreenivasa Rao,[a] Ahamed A. Jafar,[b] Chandana Lakshmi T. Meda,[c] R. Kapavarapu,[c] Kishore V. L. Parsa[c] and Manojit Pal*[c]

Received 21st December 2011, Accepted 15th February 2012
DOI: 10.1039/c2ob25296a

A one-pot cascade reaction has been developed leading to the concurrent construction of six and five membered fused *N*-heterocyclic rings of indazolo[3,2-*b*]quinazolinones. The methodology involved the reaction of isatoic anhydride, a hydrazine and *o*-iodo benzaldehyde in the presence of $Pd(PPh_3)_4$ and BINAP in MeCN. The mechanism of this cascade reaction is discussed. A variety of indazolo[3,2-*b*]-quinazolinone derivatives were prepared by using this methodology in good yields, some of which were tested for their PDE4 inhibitory properties *in vitro*. The dose response and docking study performed using a representative compound is presented.

Introduction

Multi-component reactions[1] (MCRs) have emerged as powerful tools in organic synthesis. By providing arrays of novel heterocyclic frameworks, these strategies often facilitate identification of new lead structures in the area of drug discovery. While many MCRs are well suited for the construction of heterocyclic cores their usage however for the construction of heteroaryl-based structures is rather limited.[2] Moreover, most of the marketed drugs or those in the clinical trials contain heterocyclic structures. Thus design and development of novel MCRs and their exploration as tools in combinatorial and medicinal chemistry in addition to catalysis and natural product syntheses is of high interest both in academic and industrial organizations.

Phosphodiesterase 4 (PDE4) inhibitors are known to be beneficial for the potential treatment of asthma and chronic obstructive pulmonary disease (COPD)[3] and exert anti-inflammatory and bronchodilatory effects *via* elevation of the c-AMP level. These inhibitors *e.g.* Phase III clinical candidates cilomilast, roflumilast *etc*. therefore have potential to provide relief of symptoms, prevent complications and/or progression of these diseases. Evodiamine (**A**, Fig. 1), a characteristic alkaloid extracted from Evodia fruits, has been reported to show nonselective phosphodiesterase (PDE) activity thereby preventing cyclic nucleotide degradation.[4] As part of our ongoing effort on the identification of novel inhibitors of PDE4 derived from natural products, we report the evaluation of a series of indazolo[3,2-*b*]quinazolinones (**C**, Fig. 1) designed from **A** *via* **B**. The substituents R and R′ were introduced to expand the scope of library generation. The synthesis of **C** was carried out using a conceptually new MCR in a single pot.

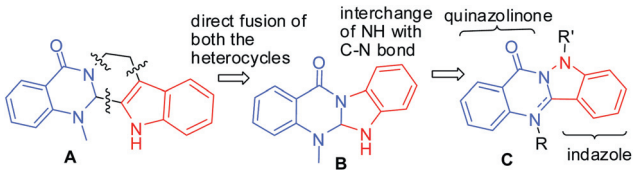


Fig. 1 Design of novel PDE4 inhibitors (**C**) derived from **A**.

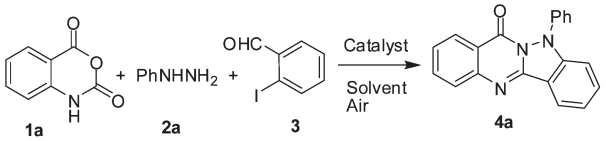
Results and discussions

While as an individual class of heterocycles, 2-aryl-2,3-dihydroquinazolin-4(1*H*)-ones[5] and indazoles[6] are well known, their combined form *i.e.* indazolo[3,2-*b*]quinazolinones (**C**; R = alkyl or aryl) are rather uncommon. Therefore, generation of a compound library based on **C** to evaluate their PDE4 inhibiting property was the major goal, whereas development of suitable methodology leading to the heterocyclic structure **C** was the major challenge. We envisaged that a combination of isatoic anhydride (**1**) and a hydrazine (**2**) could provide the required precursor of quinazolin-4-one moiety *in situ* and the use of *o*-halo benzaldehyde (**3**) could complete the construction of the fused indazole ring in the presence of a suitable catalyst. Accordingly, the reaction of **1**, phenylhydrazine (**2a**) and 2-iodobenzaldehyde

[a]*Custom Pharmaceutical Services, Dr Reddy's Laboratories Limited, Bollaram Road Miyapur, Hyderabad, 500 049, India*
[b]*PG and Research Department of Chemistry, Jamal Mohamed College, Tiruchirappalli, 620 020 Tamil Nadu, India*
[c]*Institute of Life Sciences, University of Hyderabad Campus, Gachibowli, Hyderabad, 500 046, India. E-mail: manojitpal@rediffmail.com*

†Electronic supplementary information (ESI) available: NMR spectra of all new compounds. See DOI: 10.1039/c2ob25296a

Table 1 Effect of reaction conditions on MCR using isatoic anhydride (**1**) with phenylhydrazine (**2a**) and 2-iodobenzaldehyde (**3**)[a]



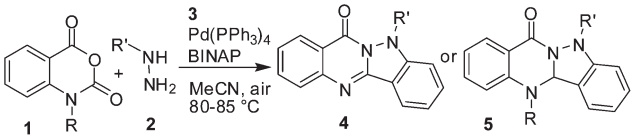
Entry	Catalyst	Solvent	*T* (°C)	Time (h)	%Yield[b]
1	$Pd(PPh_3)_2Cl_2$	DCE	80–85	48	10
2	$Pd(PPh_3)_2Cl_2$	MeCN	80–85	48	12
3	$Pd(PPh_3)_4$	MeCN	80–85	24	50
4	$Pd(PPh_3)_4$	Toluene	100–110	24	65
5	$Pd(PPh_3)_2Cl_2$/ John Phos[c]	DCE	80–85	24	20
6	$Pd(PPh_3)_4$–BINAP[c]	MeCN	80–85	4	78
7	$Pd(PPh_3)_4$–BINAP[c]	MeCN	25–30	48	35
8	$Pd(PPh_3)_4$–BINAP[c]	MeCN	80–85	24	80

[a] All the reactions were carried out using **1a** (6.13 mmol), **2a** (6.71 mmol), **3** (6.74 mmol) and a catalyst (0.60 mmol) in a solvent (10 mL). [b] Isolated yield. [c] 2.40 mmol of ligand was used. DCE = 1,2-dichloroethane.

(**3**) was examined in the presence of air under various conditions (Table 1). The initial use of catalyst $Pd(PPh_3)_2Cl_2$ at 80–85 °C (entries 1 and 2, Table 1) was not successful either in DCE or acetonitrile as the desired product **4a** was isolated in poor yield. Changing the catalyst to $Pd(PPh_3)_4$ provided **4a** in moderate yield when the reaction was performed in MeCN (entry 3, Table 1) or toluene (entry 4, Table 1) at 80–85 or 100–110 °C respectively. A combination of $Pd(PPh_3)_2Cl_2$ and the ligand John Phos provided **4a** albeit in low yield (entry 5, Table 1). Good yield of **4a** however was obtained when a combination of Pd $(PPh_3)_4$–BINAP was used in MeCN (entry 6, Table 1). The reaction was completed within 4 h. A decrease in reaction temperature to 25–30 °C was counter productive (entry 7, Table 1) whereas an increase in reaction time at 80–85 °C did not improve the product yield significantly (entry 8, Table 1). The use of a lower quantity of Pd-catalyst decreased the product yield and the MCR did not provide **4a** in the absence of Pd $(PPh_3)_4$ confirming the key role played by the catalyst. The compound **4a** was characterized by the appearance of a C═O signal at 1726 cm^{-1} in IR and 160 ppm in the ^{13}C NMR spectra.

We examined the scope of the present MCR using the optimized conditions. Depending on the nature of isatoic anhydride employed (*e.g.* **1a**; R = H) the MCR provided indazolo[3,2-*b*] quinazolinone with a variety of substitution patterns (Table 2). The reaction proceeded well with a variety of hydrazines (**2**) to give a range of 5-substituted derivatives (entries 1–7, Table 2). However, the use of *N*-substituted isatoic anhydride (*e.g.* **1b**; R = Me or **1c**; R = Bn) provided the corresponding 5,12-disubstituted-12,12*a*-dihydroindazolo[3,2-*b*]quinazolin-7(5*H*)-ones (**5**) (entries 8–12, Table 2). All the compounds synthesized were characterized by spectral and analytical data. The compound **5** was characterized by the appearance of C-12*a* proton near 6.6 δ in the ^1H NMR spectrum that was absent in the case of **4**. Moreover, the C-12*a* of **5** appeared near 78 ppm whereas that of **4** (*i.e.* C═N) appeared in the range 160–155 ppm in their corresponding ^{13}C NMR spectrum.

Table 2 Synthesis of 5,12-disubstituted 4*bH*-isoquinolino[2,1-*a*] quinazolin-6(5*H*)-ones (**4**)[a]



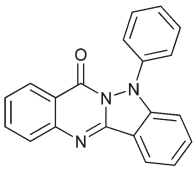
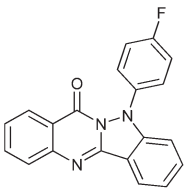
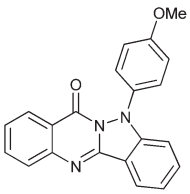
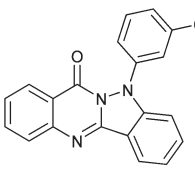
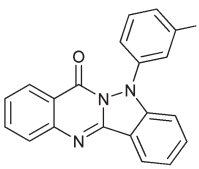
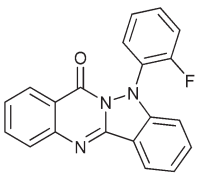
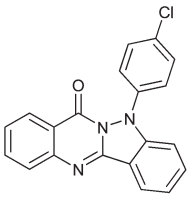
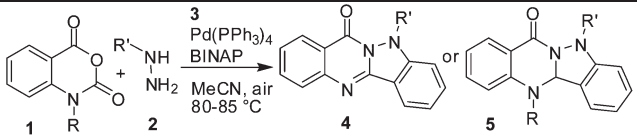
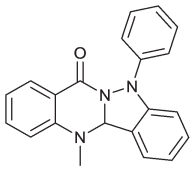
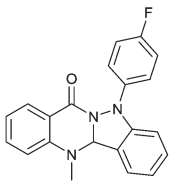
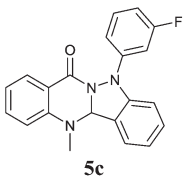
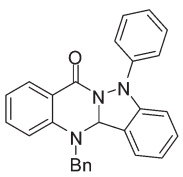
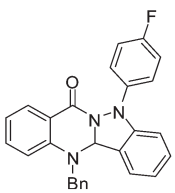
Entry	**1**; R=	**2**; R′=	Products (**4** or **5**)	Time (h); % yield[b]
1	**1a**; H	**2a**; C_6H_5	 **4a**	4; 78
2	**1a**	**2b**; C_6H_4F-*p*	 **4b**	4; 75
3	**1a**	**2c**; C_6H_4OMe-*p*	 **4c**	6; 72
4	**1a**	**2d**; C_6H_4Cl-*m*	 **4d**	5; 71
5	**1a**	**2e**; C_6H_4F-*m*	 **4e**	4; 74
6	**1a**	**2f**; C_6H_4F-*o*	 **4f**	5; 73
7	**1a**	**2g**; C_6H_4Cl-*p*	 **4g**	6; 72

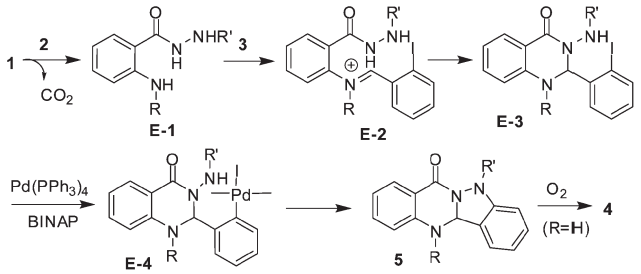
Table 2 *(Contd.)*



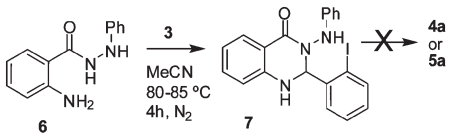
Entry	1; R=	2; R′=	Products (4 or 5)	Time (h); % yield[b]
8	**1b**; CH_3	**2a**	 **5a**	4; 72
9	**1b**	**2b**	 **5b**	5; 74
10	**1b**	**2e**	 **5c**	4; 74
11	**1c**; $C_6H_5CH_2$	**2a**	 **5d**	6; 75
12	**1c**	**2b**	 **5e**	4; 72

[a] All the reactions were carried out using **1** (6.13 mmol), **2** (6.71 mmol), **3** (6.74 mmol), $Pd(PPh_3)_4$ (0.60 mmol) and BINAP (2.40 mmol) in MeCN (10 mL) at 80–85 °C. [b] Isolated yield.

Mechanistically, the present MCR seems to proceeds (Scheme 1) *via* (i) generation of 2-aminobenzohydrazide based intermediate **E-1** from **1** and **2** *in situ* which on (ii) condensation with **3** provides the corresponding imine **E-2**. (iii) A subsequent intramolecular and regiospecific cyclization generates the corresponding 2,3-dihydroquinazolinone intermediate **E-3** containing iodoaryl moiety, which (iv) in the presence of a Pd-catalyst undergoes an intramolecular Buchwald type coupling to give **5** (*via* **E-3**). (v) *In situ* oxidation of **5** (when R = H) in the presence of air provides **4**. To gain further evidence, the intermediate **E-1** (R = H; R′ = Ph) was prepared separately and reacted with aldehyde **3** in the presence of $Pd(PPh_3)_4$–BINAP and air in MeCN at



Scheme 1 Proposed reaction mechanism.



Scheme 2 The reaction of **6** with **3** in the absence of $Pd(PPh_3)_4$–BINAP.

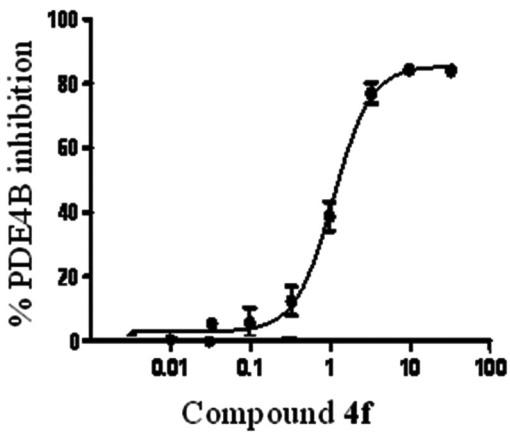


Fig. 2 Dose response study of compound **4f**.

80–85 °C for 4 h and the product **4a** was isolated in good yield. Furthermore, the same reaction was performed in the absence of $Pd(PPh_3)_4$–BINAP under nitrogen and compound **7** (or **E-3**) (R = H; R′ = Ph) (Scheme 2) was isolated, indicating the key role played by the catalyst/ligand during the formation of fused indazole ring. Since the selective inhibition of PDE4A and/or PDE4B without affecting the other isoforms (thought to be responsible for undesired side effects) is the emerging strategy to develop a safer drug,[3a] we evaluated some of the compounds synthesized initially for their PDE4B inhibitory potential *in vitro*.[7] The compounds were tested at 30 μM using PDE4B enzyme assay.[8] Rolipram[9] was used as a reference compound in this assay. Compounds **4a** (28%), **4b** (50%), **4c** (30%), **4d** (30%) and **4g** (50%) showed moderate inhibition whereas **4f** (82%) showed good inhibition of PDE4B with an IC_{50} value of ~1–2 μM in a dose response study (Fig. 2).

To understand the nature of the interactions of compound **4f** with the PDE4B protein, a docking study was performed. The results showed H-bonding between the –NH group of **4f** with the

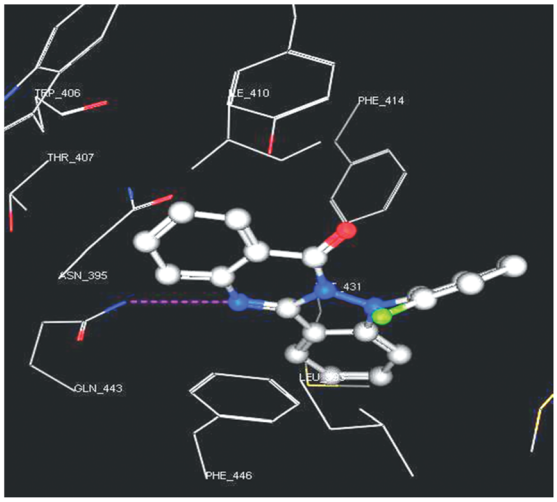


Fig. 3 Docking of **4f** at the active site of PDE4B.

Glu-443 residue of the PDE4B protein (Fig. 3). The overall binding energy (−20.11 kcal mol^{-1}) indicated a good interaction between **4f** and the PDE4B protein.

Conclusions

In conclusion, a new MCR has been developed that allowed a concurrent construction of six and five membered fused *N*-heterocyclic rings, thereby creating rapid access to a library of small molecules based on a novel structural motif. The methodology involved the reaction of isatoic anhydride, a hydrazine and *o*-iodo benzaldehyde in the presence of $Pd(PPh_3)_4$ and BINAP in MeCN. This is the first example of a one-pot and general synthesis of indazoloquinazolinones using readily available reactants, catalysts or reagents. One of these compounds showed promising inhibition of PDE4 *in vitro* and may have potential for therapeutic applications.

Experimental

Chemistry

General methods. Unless stated otherwise, reactions were performed under nitrogen atmosphere using oven dried glassware. Reactions were monitored by thin layer chromatography (TLC) on silica gel plates (60 F254), visualizing with ultraviolet light or iodine spray. Flash chromatography was performed on silica gel (230–400 mesh) using distilled hexane, ethyl acetate, dichloromethane. ^1H NMR and ^{13}C NMR spectra were determined in $CDCl_3$ or DMSO-d_6 solution by using 400 or 500 and 50 or 100 MHz spectrometers, respectively. Proton chemical shifts (δ) are relative to tetramethylsilane (TMS, $\delta = 0.00$) as internal standard and expressed in ppm. 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 an FT-IR spectrometer. Melting points were determined using melting point apparatus and are uncorrected. MS spectra were obtained on a mass spectrometer. High-resolution mass spectra (HRMS) were recorded using electron ionization (EI) mass spectrometry.

Typical method for the preparation of 4a *via* MCR. To a mixture of isatoic anhydride **1a** (6.13 mmol), phenyl hydrazine **2a** (6.71 mmol), 2-iodo benzaldehyde **3** (6.74 mmol) in acetonitrile (10 mL) was added $Pd(PPh_3)_4$ (693 mg, 0.6 mmol) and BINAP (1.53 g, 2.4 mmol) and the mixture was stirred at room temperature under air. The mixture was then stirred at 80–85 °C for 4 h under air (progress of the reaction was monitored by TLC). After completion of the reaction the mixture was concentrated under vacuum and the residue was purified by column chromatography (20% EtOAc–hexane).

5-Phenylindazolo[3,2-*b*]quinazolin-7(5*H*)-one (4a). ^1H NMR (DMSO-d_6, 400 MHz): 8.25 (d, $J = 7.9$ Hz, 1H), 8.17 (d, $J = 7.9$ Hz, 1H), 7.91–7.89 (m, 2H), 7.75 (t, $J = 7.9$Hz, 1H), 7.56–7.39 (m, 7H), 7.35 (d, $J = 7.9$ Hz, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz): 160.4, 155.2, 148.4, 148.2, 141.7, 134.0, 133.8, 129.3 (2C), 127.9, 126.9, 126.0, 125.3, 124.7, 123.7 (2C), 122.9, 119.4, 118.4, 112.4; IR (KBr): 3166, 2977, 1726, 1439, 1080 cm^{-1}; HRMS (ESI): calcd for $C_{20}H_{14}N_3O$ $(M + H)^+$ 312.1137, found 312.1125.

5-(4-Fluorophenyl)indazolo[3,2-*b*]quinazolin-7(5*H*)-one (4b). ^1H NMR ($CDCl_3$, 400 MHz): 8.33–8.28 (m, 2H), 7.92–7.80 (m, 2H), 7.64 (t, $J = 7.4$ Hz, 1H), 7.49–7.35 (m, 4H), 7.18–7.13 (m, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): 162.4, 155.3, 148.4, 148.2, 147.6, 137.9, 134.0, 133.8, 126.9, 126.5, 126.4, 125.9, 125.3, 124.7, 122.9, 119.4, 118.4, 116.2, 115.9, 112.3; IR (KBr): 3170, 2978, 1730, 1448, 1280, 1082 cm^{-1}; HRMS (ESI): calcd for $C_{20}H_{13}FN_3O$ $(M + H)^+$ 330.1043, found 330.1031.

5-(4-Methoxyphenyl)indazolo[3,2-*b*]quinazolin-7(5*H*)-one (4c). ^1H NMR (DMSO-d_6, 400 MHz): 8.23 (d, $J = 7.8$ Hz, 1H), 8.15 (d, $J = 7.8$ Hz, 1H), 7.91–7.88 (m, 2H), 7.74 (t, $J = 7.8$ Hz, 1H), 7.52–7.36 (m, 4H), 7.24 (d, $J = 7.8$ Hz, 1H), 7.00 (d, $J = 7.8$ Hz, 2H), 3.81 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): 159.6, 155.0, 148.3, 148.1, 141.5, 134.1, 133.9, 129.2 (2C), 127.9, 126.7, 126.0, 125.1, 124.5, 123.7 (2C), 122.9, 119.3, 118.4, 112.3, 55.3; IR (KBr): 3165, 2972, 1724, 1442, 1102, 1078 cm^{-1}; HRMS (ESI): calcd for $C_{21}H_{16}N_3O_2$ $(M + H)^+$ 342.1243, found 342.1236.

5-(3-Chlorophenyl)indazolo[3,2-*b*]quinazolin-7(5*H*)-one (4d). ^1H NMR (DMSO-d_6, 400 MHz): 8.23 (d, $J = 7.3$ Hz, 1H), 8.17 (d, $J = 7.3$ Hz, 1H), 7.92–7.87 (m, 2H), 7.75 (t, $J = 7.3$ Hz, 1H), 7.66 (s, 1H), 7.55–7.45 (m, 5H), 7.38 (d, $J = 7.3$ Hz, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz): 161.2, 155.5, 148.4, 148.2, 140.2, 134.2, 133.9, 132.3, 129.3 (2C), 126.9, 126.0, 125.9 (2C), 125.6, 124.9, 123.0, 119.5, 118.6, 112.5; IR (KBr): 3172, 2982, 1734, 1456, 1078 cm^{-1}; HRMS (ESI): calcd for $C_{20}H_{13}ClN_3O$ $(M + H)^+$ 346.0747, found 346.0735.

5-(3-Fluorophenyl)indazolo[3,2-*b*]quinazolin-7(5*H*)-one (4e). ^1H NMR (DMSO-d_6, 400 MHz): 8.25 (d, $J = 7.3$ Hz, 1H), 8.19 (d, $J = 7.3$ Hz, 1H), 7.91–7.89 (m, 2H), 7.77 (t, $J = 7.3$ Hz, 1H), 7.56–7.39 (m, 6H), 7.27 (t, $J = 7.3$ Hz, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz): 162.6, 160.2, 155.4, 148.4, 148.2, 147.6, 137.9, 134.1, 133.9, 130.9, 128.7, 126.9, 126.5, 124.9, 123.0, 119.4, 118.4, 116.2, 114.9, 112.4; IR (KBr): 3172, 2979, 1732, 1452,

1282, 1086 cm^{-1}; HRMS (ESI): calcd for $C_{20}H_{13}FN_3O$ $(M + H)^+$ 330.1043, found 330.1035.

5-(2-Fluorophenyl)indazolo[3,2-*b*]quinazolin-7(5*H*)-one (4f). ^1H NMR (DMSO-d_6, 400 MHz): 8.27 (d, J = 7.8 Hz, 1H), 8.17 (d, J = 7.8 Hz, 1H), 7.93–7.89 (m, 2H), 7.80–7.76 (m, 1H), 7.65–7.42 (m, 5H), 7.32 (t, J = 7.8 Hz, 1H), 7.24 (d, J = 7.8 Hz, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz): 160.8, 158.1, 155.1, 148.1, 147.6, 134.1, 134.0, 130.7, 130.6, 127.7, 126.9, 125.9, 125.5, 125.2, 124.7, 123.0, 119.2, 118.3, 116.8, 111.9; IR (KBr): 3172, 2968, 1728, 1436, 1270, 1078 cm^{-1}; HRMS (ESI): calcd for $C_{20}H_{13}FN_3O$ $(M + H)^+$ 330.1043, found 330.1041.

5-(4-Chlorophenyl)indazolo[3,2-*b*]quinazolin-7(5*H*)-one (4g). ^1H NMR (DMSO-d_6, 400 MHz): 8.25 (d, J = 7.6 Hz, 1H), 8.19 (d, J = 7.6 Hz, 1H), 7.92–7.87 (m, 2H), 7.76 (t, J = 7.6 Hz, 1H), 7.58–7.50 (m, 6H), 7.38 (d, J = 7.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz): 161.2, 155.3, 148.2, 147.7, 140.5, 134.1, 133.9, 132.2, 129.2 (2C), 126.9, 126.0, 125.9 (2C), 125.4, 124.8, 123.0, 119.4, 118.5, 112.3; IR (KBr): 3176, 2983, 1737, 1458, 1082 cm^{-1}; HRMS (ESI): calcd for $C_{20}H_{13}ClN_3O$ $(M + H)^+$ 346.0747, found 346.0739.

12-Methyl-5-phenyl-12,12*a*-dihydroindazolo[3,2-*b*]quinazolin-7(5*H*)-one (5a). ^1H NMR (DMSO-d_6, 400 MHz): 8.19 (d, J = 7.3 Hz, 1H), 7.52–7.39 (m, 6H), 7.31 (t, J = 7.3 Hz, 1H), 7.26 (d, J = 7.3 Hz, 1H), 7.20–7.13 (m, 3H), 6.95 (d, J = 7.3 Hz, 1H), 6.59 (s, 1H), 2.90 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): 160.2, 149.4, 149.1, 148.0, 133.3, 130.3, 129.5, 129.3, 128.5, 127.9, 126.6, 124.9, 123.6, 123.5, 123.4, 123.3, 122.0, 119.5, 113.7, 77.8, 35.9; IR (KBr): 3162, 2974, 1729, 1448, 1250, 1056 cm^{-1}; HRMS (ESI): calcd for $C_{21}H_{18}N_3O$ $(M + H)^+$ 328.1450, found 328.1448.

5-(4-Fluorophenyl)-12-methyl-12,12*a*-dihydroindazolo[3,2-*b*]-quinazolin-7(5*H*)-one (5b). ^1H NMR ($CDCl_3$, 400 MHz): 7.98 (d, J = 7.6 Hz, 1H), 7.54–7.45 (m, 5H), 7.33 (t, J = 7.6 Hz, 1H), 7.27 (d, J = 7.6 Hz, 1H), 7.19–7.08 (m, 3H), 6.96 (d, J = 7.6 Hz, 1H), 6.60 (s, 1H), 2.93 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): 161.4, 159.6, 149.5, 149.4, 148.3, 133.5, 130.3, 129.7, 129.5, 128.6, 127.9, 126.8, 124.9, 123.9, 123.4, 123.3, 122.2, 119.5, 113.9, 78.2, 35.9; IR (KBr): 3166, 2977, 1732, 1458, 1252, 1228, 1062 cm^{-1}; HRMS (ESI): calcd for $C_{21}H_{17}FN_3O$ $(M + H)^+$ 346.1356, found 346.1346.

5-(3-Fluorophenyl)-12-methyl-12,12*a*-dihydroindazolo[3,2-*b*]-quinazolin-7(5*H*)-one (5c). ^1H NMR (DMSO-d_6, 400 MHz): 8.24 (d, J = 7.7 Hz, 1H), 8.19 (d, J = 7.7 Hz, 1H), 7.93–7.90 (m, 2H), 7.62 (d, J = 7.7 Hz, 1H), 7.58–7.38 (m, 5H), 7.32 (t, J = 7.7 Hz, 1H), 6.96 (d, J = 7.7 Hz, 1H), 6.60 (s, 1H), 2.93 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): 161.6, 159.8, 149.6, 149.2, 148.3, 133.5, 130.5, 129.8, 129.5, 128.7, 127.9, 126.9, 124.9, 123.9, 123.5, 123.3, 122.3, 119.5, 113.9, 78.4, 35.9; IR (KBr): 3167, 2979, 1735, 1459, 1254, 1230, 1062 cm^{-1}; HRMS (ESI): calcd for $C_{21}H_{17}FN_3O$ $(M + H)^+$ 346.1356, found 346.1347.

12-Benzyl-5-phenyl-12,12*a*-dihydroindazolo[3,2-*b*]quinazolin-7(5*H*)-one (5d). ^1H NMR (DMSO-d_6, 400 MHz): 8.30 (d, J = 7.6 Hz, 1H), 8.20 (d, J = 7.6 Hz, 1H), 7.92–7.65 (m, 9H), 7.56–7.39 (m, 5H), 7.26 (t, J = 7.6 Hz, 1H), 6.92 (d, J = 7.6 Hz, 1H), 6.56 (s, 1H), 4.82 (s, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz): 161.8, 149.5, 149.4, 148.5, 133.5, 130.5, 129.8, 129.5, 128.7 (2C), 128.2, 127.9, 127.6 (2C), 126.9, 126.4, 125.5, 125.2, 124.9, 123.9, 123.5, 123.3, 122.3, 119.5, 113.9, 78.4, 52.6; IR (KBr): 3158, 2968, 1742, 1438, 1247, 1230, 1056 cm^{-1}; HRMS (ESI): calcd for $C_{27}H_{22}N_3O$ $(M + H)^+$ 404.1763, found 404.1756.

12-Benzyl-5-(4-fluorophenyl)-12,12*a*-dihydroindazolo[3,2-*b*]-quinazolin-7(5*H*)-one (5e). ^1H NMR (DMSO-d_6, 400 MHz): 8.36 (d, J = 7.8 Hz, 1H), 8.26 (d, J = 7.8 Hz, 1H), 7.90–7.82 (m, 8H), 7.58–7.39 (m, 5H), 7.28 (t, J = 7.8 Hz, 1H), 6.94 (d, J = 7.8 Hz, 1H), 6.62 (s, 1H), 4.92 (s, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz): 161.9, 155.8, 149.6, 149.4, 148.4, 133.5, 132.2, 130.6, 129.8, 129.6, 128.7 (2C), 128.6, 127.9, 127.8 (2C), 126.9, 125.5, 125.6, 124.9, 123.9, 123.5, 123.7, 119.5, 114.2, 79.2, 52.9; IR (KBr): 3156, 2975, 1742, 1450, 1256, 1232, 1054 cm^{-1}; HRMS (ESI): calcd for $C_{27}H_{21}FN_3O$ $(M + H)^+$ 422.1669, found 422.1668.

Pharmacology

Materials and methods

Cells and reagents. HEK 293 and Sf9 cells were obtained from ATCC (Washington DC, 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).

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, the 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.[9] Briefly, lysate was centrifuged at 10 000 rpm for 10 min at 4 °C and the supernatant was collected. The 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 a rotary shaker for 1 h at 4 °C. After incubation, the 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. The resin was washed twice with wash buffer (20 mM Tris–HCl pH 8.5, 1 M KCl, 10 mM 2-mercaptoethanol and 10% glycerol). The 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.

PDE4B enzymatic assay. The inhibition of the 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 the 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 the 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:

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

Docking study

The molecular docking simulation was performed using Chemical Computing Group's Molecular Operating Environment (MOE) software 2008.10 Version, "DOCK" application module. The molecule was docked in the PDE4B protein. The docking score and interactions were observed.

The purpose of the Dock application was to search for favorable binding configurations in a macromolecular target, which is usually a protein. For each ligand, a number of configurations called *poses* are generated and scored in an effort to determine favorable binding modes.

The dock workflow involves conformational analysis, placement, scoring, and force field method of refinement.

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. A 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 **4f** 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 the London dG method. The best 10 poses of the molecule were retained and scored. The preparation of ligands for docking simulation involved energy minimization with molecular mechanics force-field MMFF94× (Merck molecular force field 94×). Molecules were then subjected to conformational search in MOE using the conformations stochastic search module to find the lowest energy conformers.

The docking results appeared as a 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^{-1}. 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 dG scoring function estimates the free energy of binding of the ligand from a given pose. The functional form is a sum of terms:

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

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 the PDE4B protein.

Acknowledgements

KSK thanks Dr Vilas Dahanukar for constant support and the analytical group of DRL for spectral data. KP and MP thanks Prof. J. Iqbal for encouragements.

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