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PAPER

Novel thieno[2,3-*d*]pyrimidines: their design, synthesis, crystal structure analysis and pharmacological evaluation†

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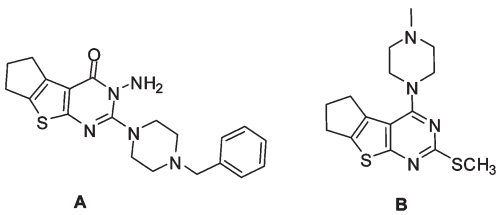
Novel thieno[2,3-*d*]pyrimidines containing a cyclohexane ring fused with a six- or five-membered heterocyclic moiety along with a benzylic nitrile were designed as potential inhibitors of PDE4. Expeditious synthesis of these compounds was carried out *via* a multi-step sequence consisting of a few key steps such as Gewald reaction, Dieckmann type cyclisation and Krapcho decarboxylation. This newly developed strategy involved construction of the thienopyrimidine ring followed by the cyclohexanone moiety and subsequently the fused heterocyclic ring. A number of thieno[2,3-*d*]pyrimidine based derivatives were synthesized using this method some of which showed promising PDE4B inhibitory properties. One of them was tested for PDE4D inhibition *in vitro* and dose dependent inhibition of TNF-α. A few selected molecules were docked into the PE4B protein the results of which showed good overall correlations to their observed PDE4B inhibitory properties *in vitro*. The crystal structure analysis of representative compounds along with hydrogen bonding patterns and molecular arrangement present within the molecule is described.

Introduction

A pyrimidine nucleus fused with another heterocycle has found wide applications in the design and discovery of novel bioactive molecules and drugs.[1] For example, thieno[2,3-*d*]pyrimidine derivatives such as **A** and **B** (Fig. 1) exhibited remarkable affinity and selectivity for the 5-HT3 receptor.[2,3] In continuation of our research under the new drug discovery program, we became interested in evaluating a library of small-molecules based on thieno[2,3-*d*]pyrimidine that were designed as potential inhibitors of PDE4 (phosphodiesterase 4). PDEs are a diverse family of enzymes that hydrolyse cyclic nucleotides and thus play a key role in regulating intracellular levels of the second messenger cAMP and cGMP, and hence cell function.[4] PDE4 is a cAMP-specific PDE and predominant isoenzyme in the majority of inflammatory cells, with the exception of platelets,



A B

Fig. 1 Examples of biologically active thieno[2,3-*d*]pyrimidine derivatives.

implicated in inflammatory airways disease. Elevated levels of cAMP play a major role in relaxation of vascular smooth muscle, which is beneficial in treating inflammatory diseases especially pulmonary diseases. Thus, inhibition of PDE4 is beneficial for the treatment of respiratory diseases including asthma and chronic obstructive pulmonary disease (COPD).[5] The use of first-generation PDE4 inhibitor rolipram[6] (**C**, Fig. 2) however was associated with dose-limiting side effects *e.g.* nausea and vomiting. While these side effects were reduced by second-generation inhibitors like cilomilast[7] (Ariflo) and roflumilast (Daxas®, Nycomed), their therapeutic index has delayed market launch so far. Recent studies have indicated that the PDE4B subtype is linked to inflammatory cell regulation[8] whereas the PDE4D subtype is implied in the emetic response.[9] However, none of the PDE4 inhibitors under development are PDE4B selective.[10a] Recently it has been demonstrated that inhibition of PDE4D by allosteric inhibitors (maximum inhibition,

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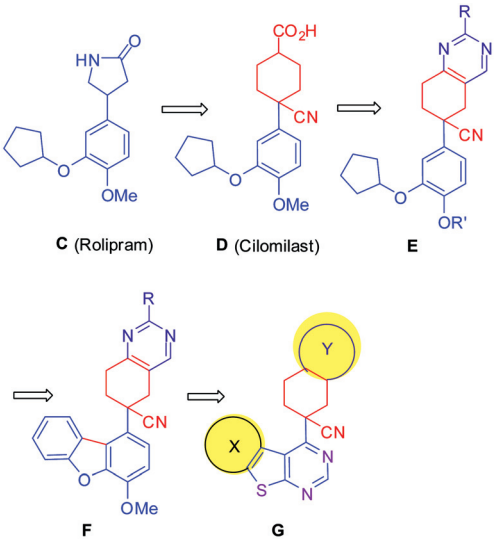


Fig. 2 Design of novel inhibitors (**G**) of PDE4 based on known inhibitors (**C**–**F**).

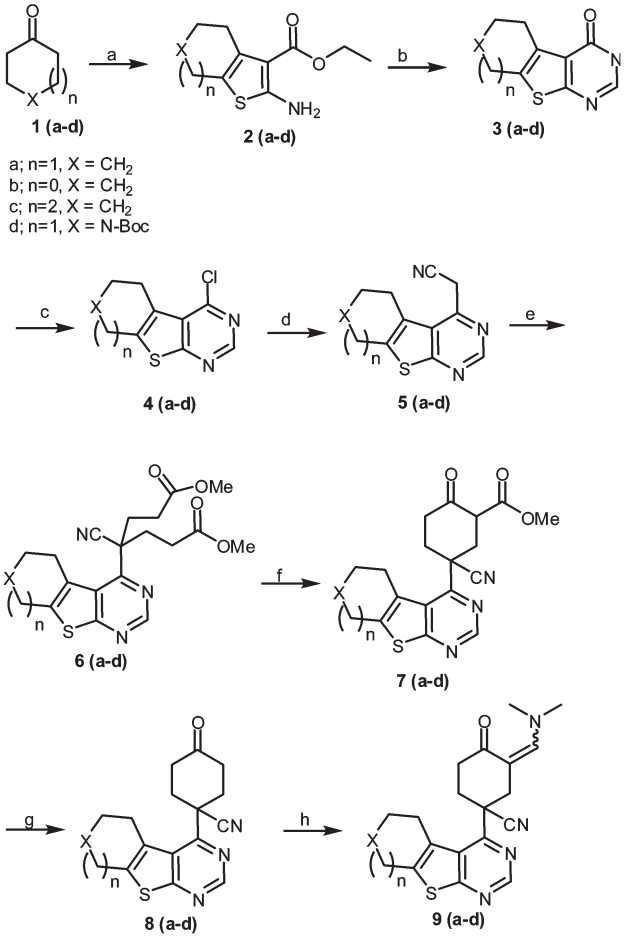
I_{max} 80–90%) did not cause emetic side effects raising a possibility that PDE4B inhibitors with partial but not complete inhibition of PDE4D (I_{max} of ~60–80%) could be developed to treat COPD and asthma without causing emetic side effects.[10b]

To identify novel and orally active PDE4 inhibitors with decreased potential for side effects the design and synthesis of 4-cyano cyclohexane-1-carboxylic acid derivatives was undertaken which resulted in significant improvement in reducing the side effects of **C**.[11] Thus, cilomilast (**D**, Fig. 2) that belongs to this class was discovered and finally entered into phase 3 clinical trials. However, to address the issue of configurational isomerism of **D** around the CO_2H group new cyclohexane derivatives (**E**, Fig. 2) were designed maintaining the benzylic nitrile as one of the pharmacophores[12] and the cyclohexane ring fused with a six- or five-membered heterocyclic moiety. Recently, cyclohexane derivatives (**F**, Fig. 2) containing a tricyclic fused aryl ring have been reported to possess PDE4 inhibitory properties.[13] Based on these observations we designed novel cyclohexane derivatives (**F**, Fig. 2) containing the thieno[2,3-*d*]pyrimidine moiety along with benzylic nitrile as potential and new inhibitors of PDE4. The cyclic rings 'X' and 'Y' were chosen to introduce diversity into the basic scaffold for the generation of library of small molecules. To the best of our knowledge template **G** has not been previously explored for the discovery of PDE4 inhibitors.

Results and discussions

Chemistry

The retro-synthetic analysis of the target compound **G** revealed that construction of a cyclohexane ring at C-4 of the thieno-[2,3-*d*]pyrimidine moiety could be a key step. Overall, we envisioned that sequential construction of (i) the thienopyrimidine ring followed by (ii) the cyclohexanone moiety and subsequently (iii) the fused heterocyclic ring could provide us with the target compounds based on **G**. While introduction of an aryl or



Scheme 1 Reagents and conditions: (a) ethyl cyanoacetate, morpholine, sulphur, ethanol, 90 °C, 3–8 h; (b) for **5a–c**: formamide, 190 °C, 2–4 h; for **5d**: formimidine acetate, DMF, 130 °C, 16 h; (c) for **5a–c**: $POCl_3$, 110 °C, 1–1.5 h; for **5d**: $POCl_3$, Et_3N, 60 °C, 2 h; (d) for **5a–b**: ethyl cyanoacetate, K_2CO_3, 130 °C, 1–2 h; for **5c–d**: (i) ethyl cyanoacetate, K_2CO_3, DMSO, 120 °C, 1–1.5 h; (ii) NaCl, H_2O, DMSO, 150 °C, 5–10 h; (e) methyl acrylate, triton-B, CH_3CN, 85 °C, 3–6 h; (f) for **5a–c**: NaH, DME, 85 °C, 2–5 h; for **5d**: NaH, THF, 60 °C, 1 h; (g) NaCl, H_2O, DMSO, 150 °C, 4–7 h; (h) for **9a–b**: DMF-DMA, Et_3N, DMF, 80 °C, 2–4 h; for **9c–d**: DMF-DMA, toluene, 95 °C, 16 h.

heteroaryl or alkyne moiety[14,15] at C-4 of the thieno[2,3-*d*]-pyrimidine moiety is known a similar method to introduce a cyclohexyl moiety at the same position was unprecedented. Nevertheless, the 4-chloro-thieno[2,3-*d*]pyrimidines (**4**) appeared to be appropriate starting materials for our synthesis and were prepared following a 3-step method (step a–c, Scheme 1) as reported earlier.[14,15] The use of **4** for the preparation of subsequent intermediates is shown in Scheme 1. Thus, the reaction[16] of ethyl cyanoacetate with chloro derivative **4** followed by *in situ* decarboxylation of the resulting ester afforded the cyano derivative **5** which on double Michael reactions with methyl acrylate furnished the diester **6**. A Dieckmann type cyclisation[17] of **6** followed by Krapcho decarboxylation[18] of the resulting β-ketoester **7** provided the cyclohexanone derivative **8** which on reaction with *N*,*N*-dimethylformamide dimethyl acetal (DMF-DMA) furnished the required 2-((dimethylamino)methylene)cyclohexanone derivative **9**. The intermediates **7** and **9** were used for the

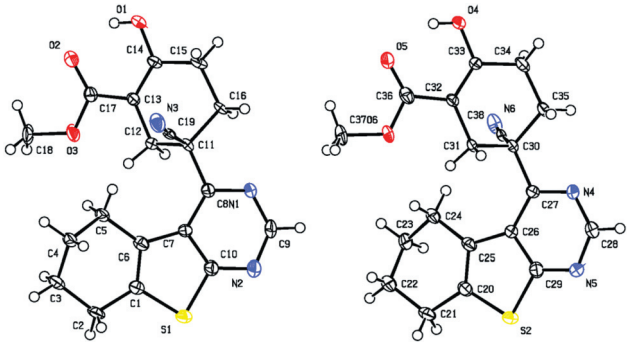


Fig. 3 ORTEP representation of the **7a** (thermal ellipsoids are drawn at 50% probability level).

preparation of the target compounds. All the intermediates synthesized were well characterized by spectral (NMR, MS and IR) data. Additionally, the molecular structure of intermediate **7a** (methyl-5-cyano-5-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]-pyrimidin-4-yl)-2-oxocyclohexanecarboxylate) was established unambiguously by single crystal X-ray diffraction (Fig. 3) the details of which are presented in the following section. The X-ray diffraction study indicated that **7a** existed in the enol tautomeric form predominantly stabilized by the 6-membered ring formed due to an intramolecular H-bond. The existence of the enol form in solution was also supported by ^{1}H NMR data as the enolic hydroxyl group appeared at ~ 12.0–12.3 δ.

Compound **7a** crystallizes in the triclinic $P\bar{1}$ space group with two symmetry molecules in the asymmetric unit ($Z = 4$, $Z' = 2$) (Fig. 3). The two molecules in the asymmetric unit contain free hydroxyl and ester functional groups. These molecules have the capability to form supramolecular synthons and are conformationally different (Fig. 4). The inversion related molecules of conformer-i, and conformer-ii are both forming the same type of intramolecular O–H···O synthon in between the substituted hydroxyl group with the *ortho* oriented methyl ester group and the C–H···O, C–H···S intermolecular hydrogen bonding. Consequently, the two groups OH and ester come closer and interact with the O–H···O synthon. These interactions propagate 3D network packing along the ac axis (see ESI†).

The reaction of **9** with guanidine or formamidine afforded the compounds **10** or **11** (Scheme 2). Similarly, the reaction of **7a** with guanidine or formamidine afforded the compounds **12** or **13** whereas treating **7a** with hydrazines afforded compounds **14** or **15** (Scheme 3). The reaction of **9a** with hydrazine provided the compound **16** (Scheme 4).[12] All the target compounds synthesized were characterized by spectral (NMR, MS and IR) data. For example, the presence of a –CN group was confirmed by an IR absorption in the region 2240–2230 cm^{-1}. The keto form of compound **12** and **13** was characterized by the IR absorption at 1650 and 1655 cm^{-1} respectively, due to the C═O moiety. The structure of compound **14** was assigned based on two broad ^{1}H NMR signals at 11.3 and 9.6 δ due to the two NH groups and an IR absorption at 1734 cm^{-1} due to the amide C═O moiety. The keto form of compound **15** was also indicated by IR absorption at 1730 cm^{-1} due to the C═O group and the absence of any NH IR absorption beyond 3000 cm^{-1}. Nevertheless, the molecular structure of a representative compound **10a**

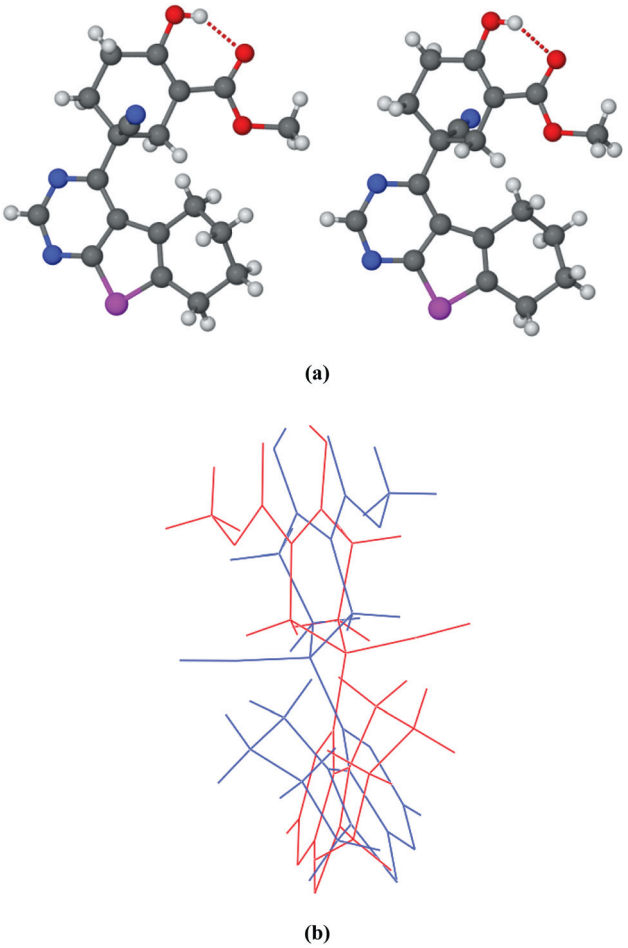
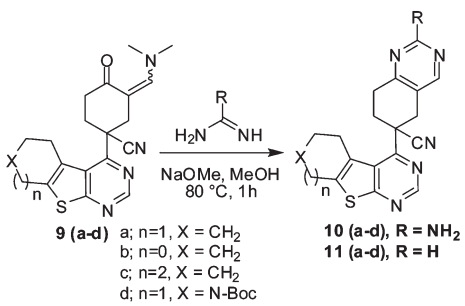


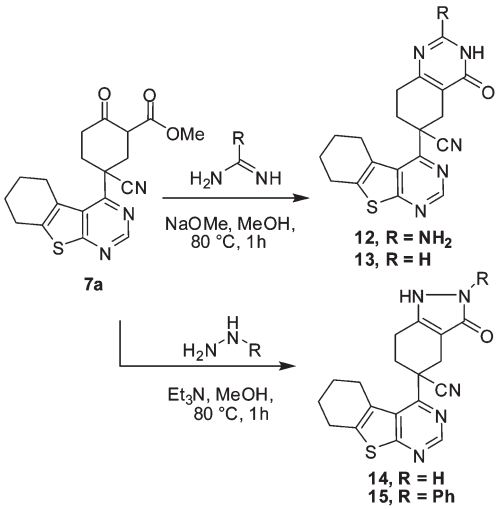
Fig. 4 (a) Showing the intramolecular hydrogen bonding present in the molecule **7a** *via* O–H···O synthon. (b) Showing the conformations present in the asymmetric unit (i) conformer-**i** in blue, (ii) conformer-**ii** in red.



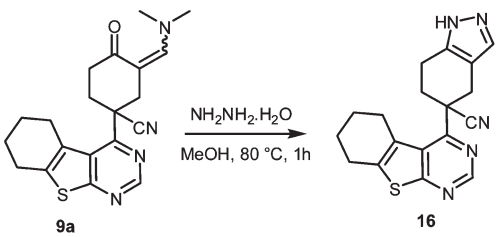
Scheme 2 Preparation of 5,6,7,8-tetrahydroquinazoline derivatives.

was established unambiguously by single crystal X-ray diffraction (Fig. 5). The X-ray diffraction study also indicated the *R*-isomer of compound **10a**.

Compound **10a** crystallizes in the triclinic $P\bar{1}$ space group with one molecule and one solvent molecule in the asymmetric unit ($Z = 6$, $Z' = 2$) (Fig. 5). The molecule in the asymmetric unit contains pyrimidine substituted with a free amine and has capability to form a supramolecular synthon *via* intermolecular



Scheme 3 Preparation of 4-oxo-3,4,5,6,7,8-hexahydroquinazoline and 3-oxo-2,3,4,5,6,7-hexahydro-1*H*-indazole derivatives.



Scheme 4 Preparation of 4,5,6,7-tetrahydro-1*H*-indazole derivative.

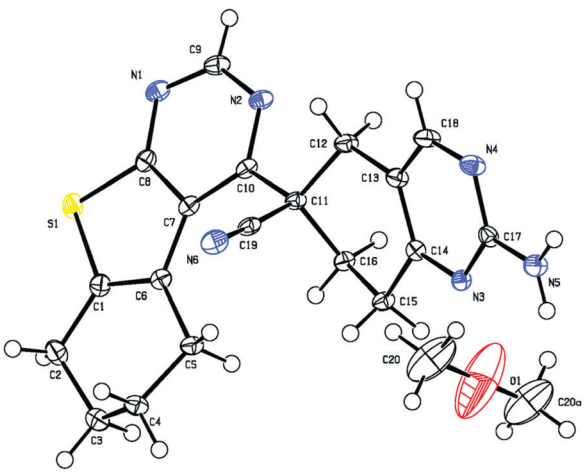


Fig. 5 ORTEP representation of the **10a** (thermal ellipsoids are drawn at 50% probability level).

hydrogen bonding. The inversion related molecule in the asymmetric unit formed the dimer synthon through pyrimidine amine like N–H···N interactions (Fig. 6) and is stabilised by C–H···C, C–H···N and C–H···S interactions with dimethoxy solvent molecules. These interactions propagate in a 3D network packing along bc axis (see ESI†).

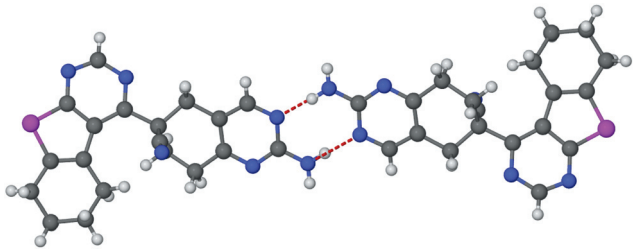


Fig. 6 Showing the intermolecular hydrogen bonding present in the molecule **10a** *via* N–H···N synthon.

While only the *R*-isomer of compound **10a** was isolated during generation of the single crystal *via* crystallization the possibility of the presence of the *S*-isomer in the solution could not be ruled out. It was therefore necessary to assess the chiral purity of **10a** and other target compounds synthesized. It is worthy to mention that the stereocentre in compounds **10–16** was generated during the conversion of intermediate **6** to **7** and **8** to **9**. This conversion was expected to afford a mixture of stereoisomers as the methodology used was not an enantiospecific one. Thus, a chiral HPLC method was used to determine the enantiomeric purity of some representative compounds *e.g.* **10a**, **13**, **14** and **16**. All these compounds were found to be a ~1 : 1 mixture of both the antipodes.

In vitro pharmacology

All the target compounds (**10–16**) synthesized were evaluated for their PDE4 inhibitory properties *in vitro*. Initially, PDE4B inhibitory potential was assessed by using PDE4B enzyme isolated from Sf9 cells.[19] Some of the compounds showed significant inhibition of PDE4B at 30 μM and the data generated for most of the compounds are summarized in Table 1. As is evident from Table 1 the change in ring size (**10a** *vs.* **10b** and **10c**) or functionalization of the saturated cycloalkyl ring (**10a** *vs.* **10d**) fused with the thiophene moiety or removal of NH_2 group from the pyrimidine ring (**10a** *vs.* **11a**) did not show significant effect on inhibitory activities. A similar trend regarding the effect of fused cycloalkyl ring was observed for compounds **11**. The functionalization of the pyrimidine ring of 5,6,7,8-tetrahydroquinazoline-6-carbonitrile moiety was tolerated (**11a** *vs.* **12** and **13**). While replacing the 5,6,7,8-tetrahydroquinazoline-6-carbonitrile moiety by 3-oxo-2,3,4,5,6,7-hexahydro-1*H*-indazole-5-carbonitrile group decreased the activity significantly (**11a** *vs.* **14** and **15**) the 4,5,6,7-tetrahydro-1*H*-indazole-5-carbonitrile moiety restored the activity (**11a** *vs.* **16**). Based on their initial PDE4B inhibitory properties compounds **10a**, **10c**, **10d**, **11a**, **11c**, **11d** and **16** were evaluated for dose dependent inhibitions (Fig. 7, see also ESI†) and the corresponding IC_{50} values are presented in Table 1. A few of these compounds were also tested in a cell based cAMP reporter assay in HEK 293 cells and their TNF-α inhibitory activity was measured in lipopolysaccharide (LPS) stimulated RAW 264.7 cells.[19] Rolipram, a well known inhibitor of PDE4 was used as a reference compound in all these assays which showed 100% inhibition of PDE4B at 30 μM. Since compound **16** appeared as the promising inhibitor among all the compounds tested its dose dependent inhibition of TNF-α was

Table 1 *In vitro* data of compounds **10–16**

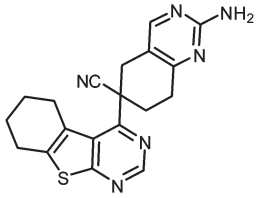
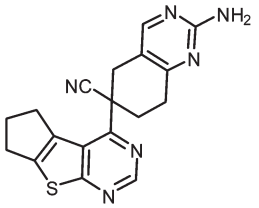
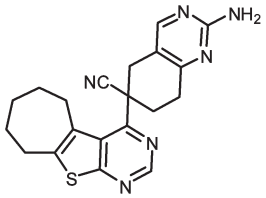
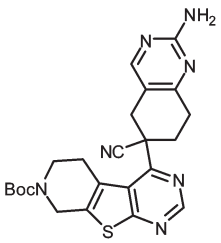
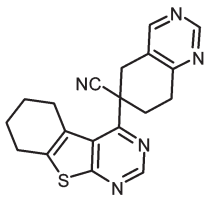
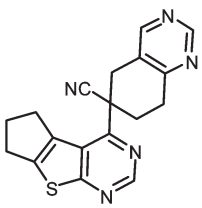
Compound	%PDE4B inhibition (30 μM)	IC_{50} (μM)	TNF-α inhibition (30 μM)	Fold elevation of cAMP[a]
 **10a**	80.0	4.48 ± 0.91	66.0	3.70
 **10b**	66.0	nd	nd	nd
 **10c**	70.4	5.64 ± 1.26	nd	3.09
 **10d**	78.9	3.24 ± 0.73	48.5	nd
 **11a**	80.0	4.51 ± 0.89	35.7	2.34
 **11b**	65.5	nd	nd	nd

Table 1 *(Contd.)*

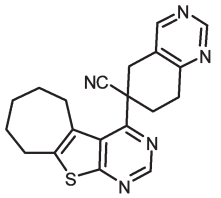
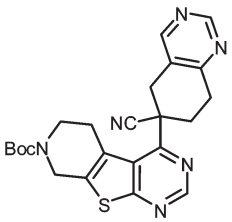
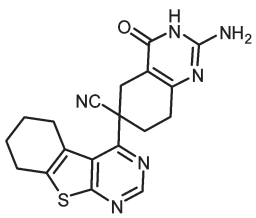
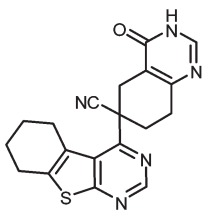
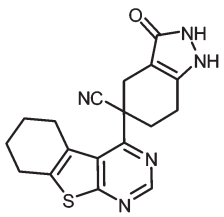
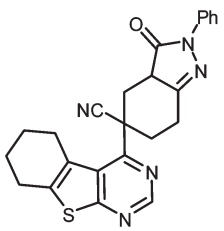
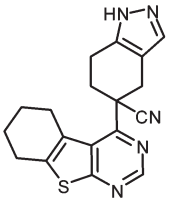
Compound	%PDE4B inhibition (30 μM)	IC_{50} (μM)	TNF-α inhibition (30 μM)	Fold elevation of cAMP[a]
 **11c**	74.8	6.19 ± 1.30	nd	nd
 **11d**	71.0	5.01 ± 0.56	32.6	nd
 **12**	68.0	nd	nd	nd
 **13**	70.7	nd	50.0	3.17
 **14**	51.4	nd	nd	nd
 **15**	41.9	nd	nd	nd

Table 1 *(Contd.)*

Compound	%PDE4B inhibition (30 μM)	IC_{50} (μM)	TNF-α inhibition (30 μM)	Fold elevation of cAMP[a]
 **16**	83.6	2.0 ± 0.41	87.4	3.90

[a] In a cell based reporter assay.

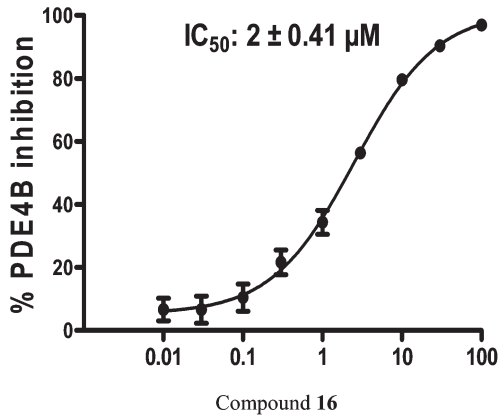


Fig. 7 Dose dependent inhibition of PDE4B by compound **16**.

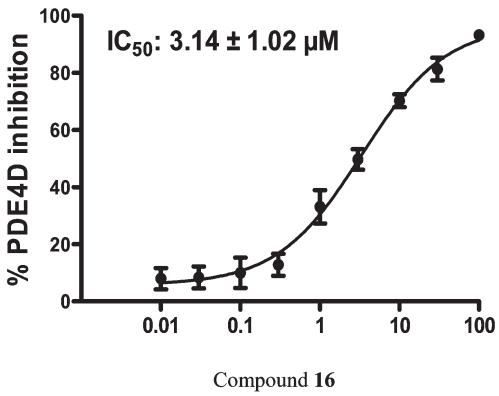


Fig. 9 Dose dependent inhibition of PDE4D by compound **16**.

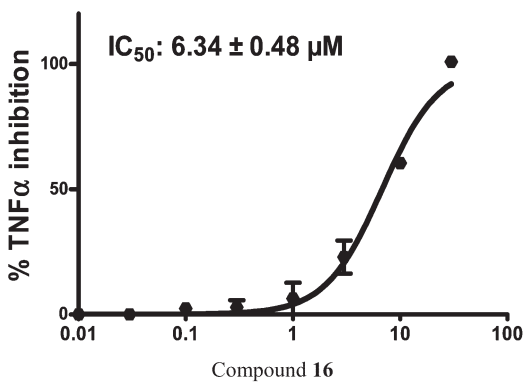


Fig. 8 Dose dependent inhibition of TNF-α by compound **16**.

also determined (Fig. 8). Further the PDE4D inhibitory potential of compound **16** was evaluated using the PDE4D enzyme assay (Fig. 9). Based on the PDE4B and D inhibitory data it is evident that compound **16** has 1.5 fold or balanced selectivity towards PDE4B. Notably potent inhibitor cilomilast (**D**, Fig. 2) showed reverse selectivity *i.e.* ~10 fold selectivity towards PDE4D over PDE4B and induced emesis at the first and/or second doses though this effect apparently disappeared with continued treatment.[19c] Overall, with respect to the *in vitro* data (PDE4 & TNF-α) compound **16** seemed to be comparable with phase 2 clinical candidate CC-1088 (PDE4 IC_{50} = 1.1 μM, TNF-α IC_{50} = 2.5 μM)[19b] of Celgene and was identified as a PDE4 inhibitor of further interest.

To understand the nature of interactions of this class of molecules with PDE4B a few selected molecules (only *R*-isomer) were docked[20] into the PDE4B protein (see ESI†). The results of this study showed good overall correlations to their observed PDE4B inhibitory properties *in vitro*. For example, due to the absence of an amine moiety on the pyrimidine ring though **11a** and **11b** showed different orientation of binding at the active site of PDE4B protein their overall interactions however were not better than **10a** or **10b** (compounds **11a** and **11d** showed better dock score but not better binding energy compared to **10a** and **10d** respectively). This is supported by the observed inhibition of PDE4B shown by compounds **10a**, **10d**, **11a** and **11d**.

Conclusions

The thieno[2,3-*d*]pyrimidine based library of small molecules containing a cyclohexane ring fused with a six- or five-membered heterocyclic moiety along with a benzylic nitrile was designed as potential inhibitors of PDE4. These molecules were prepared conveniently *via* a multi-step sequence consisting of a few key steps such as Gewald reaction, Dieckmann type cyclisation and Krapcho decarboxylation. A number of thieno[2,3-*d*]-pyrimidine based derivatives were synthesized and the molecular structure of a representative compound was established

unambiguously by single crystal X-ray diffraction. The crystal structure analysis of this compound provided an insight on the hydrogen bonding patterns and molecular arrangement present within the molecule. Many of these compounds were evaluated for their PDE4B inhibitory potential *in vitro*. Some of these compounds showed promising inhibition of PDE4B initially at a single dose and then subsequently in a dose dependent manner. One of them *i.e.* 5-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]-pyrimidin-4-yl)-4,5,6,7-tetrahydro-1*H*-indazole-5-carbonitrile was tested for PDE4D inhibition *in vitro* and dose dependent inhibition of TNF-α. The docking results of a few selected molecules showed good overall correlations to their observed PDE4B inhibitory properties *in vitro*. Overall, the strategy involving the sequential construction of the thienopyrimidine ring followed by the cyclohexanone moiety and subsequently the fused heterocyclic ring provided a new framework that appeared to be a promising template for the discovery of novel inhibitors of PDE4.

Experimental section

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. ^{1}H NMR and ^{13}C NMR spectra were determined in $CDCl_3$ or DMSO-d_6 solution by using 400 or 100 MHz spectrometers, respectively. Proton chemical shifts (δ) are relative to tetramethylsilane (TMS, δ = 0.00) as internal standard and expressed in ppm. Spin multiplicities are given as s (singlet), d (doublet), t (triplet) q (quartet) and m (multiplet) as well as bs (broad). Coupling constants (J) are given in hertz. Infrared spectra were recorded on a FT-IR spectrometer. Melting points were determined using melting point apparatus and are uncorrected. MS spectra were obtained on a mass spectrometer. Chromatographic purity by HPLC (Agilent 1200 series Chem Station software) was determined by using area normalization method and the conditions specified in each case: column, mobile phase (range used), flow rate, detection wavelength, and retention time. The enantiomeric purity of some representative compounds *e.g.* **10a**, **13**, **14** and **16** was determined by using a chiral HPLC method.

Preparation of 2-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]-pyrimidin-4-yl)acetonitrile (5a)

To a mixture of ethyl cyanoacetate (4.2 mL, 40.05 mmol) and K_2CO_3 (3.7 g, 26.70 mmol) was added compound **4a** (3 g, 13.35 mmol). The mixture was initially heated to 60 °C for 30 min and then at 140 °C for 1 h under anhydrous conditions. After completion of the reaction, the mixture was cooled to room temperature and diluted with EtOAc (60 mL). The organic layer was collected, washed with water (2 × 30 mL) followed by brine solution (30 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue isolated was purified by column chromatography using ethyl acetate–hexane (1 : 6) to give the desired product **5a** (2.4 g, 78%) as a white solid; mp 164–166 °C; R_f = 0.45 (25% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2931, 2853, 2256, 1539; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.95 (s, 1H), 4.27 (s, 2H), 2.99–2.92 (m, 4H), 1.97–1.96 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 168.8, 152.0, 151.0, 140.1, 129.0, 125.5, 115.7, 26.3, 26.0, 25.9, 22.4, 22.3; MS (ES mass): *m*/*z* 229.9 (M + 1); HPLC: 99.3%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/50, 2/50, 9/95, 12/95, 15/50, 18/50; flow rate: 1.0 mL min^{-1}; UV 240 nm, retention time 5.34 min.

Preparation of 2-(6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]-pyrimidin-4-yl)acetonitrile (5b)

Compound **5b** was synthesized in 55% yield from **4b** following a similar procedure as presented above; white solid; mp: 205–207 °C; R_f = 0.6 (30% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2911, 2861, 2261, 1552; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.95 (s, 1H), 4.18 (s, 2H), 3.18–3.09 (m, 4H), 2.64–2.56 (m, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 174.2, 152.1, 150.7, 145.6, 134.6, 125.9, 115.5, 30.0, 29.4, 27.6, 24.9; MS (ES mass): *m*/*z* 216.1 (M + 1).

Preparation of ethyl 2-cyano-2-(6,7,8,9-tetrahydro-5*H*-cyclohepta[4,5]thieno[2,3-*d*]pyrimidin-4-yl)acetate (5cc)

A mixture of **4c** (1 g, 0.42 mmol), ethyl cyanoacetate (0.04 mL, 0.42 mmol) and K_2CO_3 (86 mg, 0.63 mmol) in DMSO (10 mL) and water (1 mL) was heated at 120 °C for 1.5 h under anhydrous conditions. After completion of the reaction the mixture was cooled to room temp, diluted with water (50 mL) and extracted with ethyl acetate (3 × 30 mL). The organic layers were collected, combined, washed with brine solution (30 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue isolated was purified by column chromatography using ethyl acetate–hexane (1 : 6) to give the desired product (0.9 g, 70%) as a white solid; mp: 139–141 °C; R_f = 0.5 (25% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2927, 2856, 2200, 1656; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.83 (bs, 1H), 8.41 (s, 1H), 4.23 (q, J = 4.5 Hz, 2H), 3.05–3.02 (m, 2H), 2.95–2.92 (m, 2H), 1.88–1.83 (m, 2H), 1.68–1.54 (m, 4H), 1.28 (t, J = 4.5 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 170.5, 162.1, 152.8, 141.9, 139.1, 136.7, 121.9, 119.4, 66.7, 61.3, 32.0, 30.6, 27.9, 27.0, 14.4, 14.3; MS (ES mass): 315.5 (M + 1).

Preparation of 2-(6,7,8,9-tetrahydro-5*H*-cyclohepta[4,5]thieno-[2,3-*d*]pyrimidin-4-yl)acetonitrile (5c)

A mixture of compound **5cc** (1 g, 0.32 mmol) and NaCl (1.47 g, 2.53 mmol) in DMSO (10 mL) and water (1 mL) was heated at 150 °C for 4.5 h under anhydrous conditions. After completion of the reaction the mixture was cooled to room temp, diluted with water (50 mL) and extracted with ethyl acetate (3 × 30 mL). The organic layers were collected, combined, washed with brine solution (30 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue isolated was

purified by column chromatography using ethyl acetate–hexane (1 : 6) to give desired product (524 mg, 68%) as a white solid; mp: 133–135 °C; R_f = 0.5 (10% EtOAc–DCM); IR (KBr, cm^{-1}): 2925, 2853, 2254, 1533; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.93 (s, 1H), 4.31 (s, 2H), 3.10–3.08 (m, 2H), 3.01–2.99 (m, 2H), 2.00–1.99 (m, 2H), 1.85–1.78 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 167.6, 151.5, 150.9, 144.1, 131.0, 129.7, 115.6, 31.4, 29.9, 29.3, 26.9, 26.8, 26.5; MS (ES mass): 243.5 (M + 1).

Preparation of ethyl 2-cyano-2-(7-(*tert*-butoxycarbonyl)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-4-yl)acetate (5dd)

The compound **5dd** was synthesized in 65% yield from **4d** following a similar procedure as presented above; light brown solid; mp: 128–130 °C; R_f = 0.3 (30% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2975, 2202, 1710, 1661; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 14.75 (s, 1H), 8.08 (d, J = 3.2 Hz, 1H), 4.72 (s, 2H), 4.33 (q, J = 7.2 Hz, 2H), 3.60–3.61 (m, 2H), 3.29–3.31 (m, 2H), 1.51 (s, 9H), 1.39 (t, J = 7.2 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 179.3, 170.7, 152.5 (3C), 140.1 (2C), 134.0, 120.9, 80.6, 61.6, 43.4, 41.9, 31.0, 28.4 (3C), 25.9, 14.3. MS (ES mass): 401.1 (M + 1).

Preparation of *tert*-butyl 4-acetonitrilo-5,6,7,8-tetrahydropyrido-[4′,3′:4,5]thieno[2,3-*d*]pyrimidine-7-carboxylate (5d)

Compound **5d** was synthesized in 62% yield from **5dd** following a similar procedure as presented above; white solid; mp: 161–163 °C; R_f = 0.4 (40% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2976, 2257, 2190, 1683; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.99 (s, 1H), 4.77 (s, 2H), 4.26 (s, 2H), 3.84 (t, J = 5.6 Hz, 2H), 3.08–3.09 (m, 2H), 1.51 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 169.0, 154.2, 152.6 (3C), 128.3 (2C), 115.4, 80.9, 43.9, 40.0, 28.4 (3C), 25.9 (2C); MS (ES mass): 331.1 (M + 1).

Preparation of 4-cyano-4-(5,6,7,8-tetrahydrobenzo[*b*]thieno-[2,3-*d*]pyrimidin-4-yl)-heptanedioic acid dimethyl ester (6a)

To a solution of **5a** (2 g, 8.72 mmol) in acetonitrile (12.5 mL) was added 40% solution of triton-B (1 mL) and the mixture was heated to reflux under anhydrous conditions. To this was added methyl acrylate (7.9 mL, 87.22 mmol) in acetonitrile (12.5 mL) under refluxing conditions. The mixture was refluxed for 3 h. After completion of the reaction the mixture was cooled to room temperature and solvent as well as excess of methyl acrylate was evaporated under reduced pressure. The residue was dissolved in EtOAc (40 mL). The organic layer was washed with water (2 × 20 mL) followed by brine solution (30 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue isolated was purified by column chromatography using ethyl acetate–hexane (1 : 8) to give desired product **6a** (2.3 g, 65%) as a light yellow liquid; R_f = 0.5 (25% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2946, 2861, 2236, 1738; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.87 (s, 1H), 3.67 (s, 6H), 3.22 (bs, 2H), 2.98 (bs, 2H), 2.86–2.79 (m, 2H), 2.69–2.61 (m, 2H), 2.53–2.45 (m, 2H), 2.37–2.29 (m, 2H), 1.96 (bs, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 172.4 (2C), 170.2, 156.4, 150.2, 140.1, 129.3, 126.3, 121.7, 51.9 (2C), 32.9 (2C), 29.8 (3C), 29.6, 26.6, 23.2, 22.2; MS (ES mass): 401.9 (M + 1).

Preparation of 4-cyano-4-(6,7-dihydro-5*H*-cyclopenta[4,5]thieno-[2,3-*d*]pyrimidin-4-yl)-heptanedioic acid dimethyl ester (6b)

Compound **6b** was synthesized in 65% yield from **5b** following a similar procedure as described above; light yellow liquid; R_f = 0.7 (30% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2954, 2859, 2240, 1735; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.89 (s, 1H), 3.65 (s, 6H), 3.39–3.35 (m, 2H), 3.10 (t, J = 7.2 Hz, 2H), 2.81–2.74 (m, 2H), 2.62–2.45 (m, 6H), 2.41–2.33 (m, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 175.3, 172.4 (2C), 156.3, 150.3, 146.4, 134.6, 125.7, 121.3, 51.9 (2C), 47.3, 33.5, 32.5 (2C), 30.1, 29.8 (2C), 28.1; MS (ES mass): 387.5 (M + 1).

Preparation of 4-cyano-4-(6,7,8,9-tetrahydro-5*H*-cyclohepta[4,5]-thieno[2,3-*d*]pyrimidin-4-yl)-heptanedioic acid dimethyl ester (6c)

Compound **6c** was synthesized in 68% yield from **5c** following a similar procedure as described above; light yellow liquid; R_f = 0.55 (10% EtOAc–DCM); IR (KBr, cm^{-1}): 2929, 2853, 2233, 1738; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.88 (s, 1H), 3.68 (s, 6H), 3.32–3.29 (m, 2H), 3.03–3.00 (m, 2H), 2.88–2.80 (m, 2H), 2.73–2.64 (m, 2H), 2.60–2.48 (m, 2H), 2.39–2.32 (m, 2H), 2.03–1.95 (m, 2H), 1.78–1.72 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 172.5 (2C), 168.9, 156.3, 149.9, 144.8, 132.1, 129.1, 120.8, 51.9 (2C), 32.4 (2C), 32.3, 31.0, 30.7, 29.8 (3C), 26.8, 26.7; MS (ES mass): 415.5 (M + 1).

Preparation of 4-cyano-4-(7-(*tert*-butoxycarbonyl)-5,6,7,8-tetrahydropyrido[4′,3′: 4,5]thieno[2,3-*d*]pyrimidin-4-yl)-heptanedioic acid dimethyl ester (6d)

Compound **6d** was synthesized in 65% yield from **5d** following a similar procedure as described above; white solid; mp: 133–135 °C; R_f = 0.5 (35% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2976, 2236, 1736, 1686; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.92 (s, 1H), 4.79 (s, 2H), 3.80–3.78 (m, 2H), 3.67 (s, 6H), 3.35–3.33 (m, 2H), 2.85–2.78 (m, 2H), 2.69–2.62 (m, 2H), 2.54–2.46 (m, 2H), 2.38–2.31 (m, 2H), 1.52 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 172.3 (2C), 170.4, 152.9, 150.7 (3C), 128.4 (2C), 121.5, 80.8, 51.9 (2C), 46.4, 45.1, 32.7 (2C), 32.3, 29.7 (2C), 28.4 (3C), 28.0. MS (ES mass): 503.2 (M + 1).

Preparation of methyl 5-cyano-5-(5,6,7,8-tetrahydrobenzo[*b*]-thieno[2,3-*d*]pyrimidin-4-yl)-2-oxocyclohexanecarboxylate (7a)

A cold solution of compound **6a** (2 g, 4.98 mmol) in dry DME (15 mL) was added slowly to a mixture of 60% NaH (359 mg, 14.96 mmol) in dry DME (15 mL) at 0 °C under nitrogen atmosphere. The reaction mixture was heated at 60–70 °C for 2.5 h. After completion of the reaction, the mixture was quenched with ice cold 1 N hydrochloric acid (20 mL) and extracted with ethyl acetate (2 × 30 mL). The organic layers were collected, combined, washed with water (2 × 30 mL) followed by brine (20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column

chromatography using ethyl acetate–hexane (1 : 9) to give the desired product **7a** (1.5 g, 82%) as a white solid; mp: 171–173 °C; R_f = 0.6 (25% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 3267, 2951, 2230, 1657; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.25 (s, OH), 8.89 (s, 1H), 3.81 (s, 3H), 3.30–3.26 (m, 3H), 3.05–3.00 (m, 3H), 2.93–2.85 (m, 1H), 2.63–2.58 (m, 2H), 2.44–2.37 (m, 1H), 1.98 (bs, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 171.8, 170.6, 169.7, 157.7, 150.4, 140.0, 129.1, 126.2, 121.7, 94.5, 51.8, 42.2, 32.8, 31.3, 29.2, 26.8, 26.6, 23.2, 22.3; MS (ES mass): 369.9 (M + 1); HPLC: 98.9%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/80, 2/80, 9/98, 12/98, 15/80, 18/80; flow rate: 1.0 mL min^{-1}; UV 245 nm, retention time 4.37 min.

Preparation of methyl 5-cyano-5-(6,7-dihydro-5*H*-cyclopenta-[4,5]thieno[2,3-*d*]pyrimidin-4-yl)-2-oxocyclohexanecarboxylate (7b)

Compound **7b** was synthesized in 70% yield from **6b** following a procedure similar to that of compound **7a**; white solid; mp: 153–155 °C; R_f = 0.8 (30% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 3535, 2955, 2235, 1656; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.26 (bs, 1H), 8.90 (s, 1H), 3.81 (s, 3H), 3.41–3.36 (m, 2H), 3.25 (d, J = 16.0 Hz, 1H), 3.12 (t, J = 7.2 Hz, 2H), 2.97 (d, J = 16.5 Hz, 1H), 2.92–2.84 (m, 1H), 2.64–2.53 (m, 4H), 2.44–2.36 (m, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 174.9, 171.8, 170.6, 157.4, 150.6, 146.2, 134.8, 125.8, 121.6, 94.4, 51.8, 41.9, 33.2, 32.3, 30.4, 30.1, 28.2, 26.6; MS (ES mass): 355.4 (M + 1).

Preparation of methyl 5-cyano-5-(6,7,8,9-tetrahydro-5*H*-cyclohepta[4,5]thieno[2,3-*d*]pyrimidin-4-yl)-2-oxocyclo-hexanecarboxylate (7c)

Compound **7c** was synthesized in 72% yield from **6c** following a procedure similar to that of compound **7a**; white solid; mp: 166–168 °C; R_f = 0.7 (25% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 3482, 2931, 2232, 1656; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.26 (bs, 1H), 8.90 (s, 1H), 3.80 (s, 3H), 3.39–3.26 (m, 3H), 3.04–2.98 (m, 3H), 2.94–2.85 (m, 1H), 2.66–2.60 (m, 2H), 2.46–2.38 (m, 1H), 2.01–1.98 (m, 2H), 1.78–1.70 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 171.8, 170.7, 168.5, 157.6, 150.3, 144.7, 132.1, 128.9, 121.0, 94.5, 51.8, 42.0, 32.5, 32.4, 30.9(2C), 30.6, 26.9, 26.8, 26.7; MS (ES mass): 384.2 (M + 1).

Preparation of methyl 5-cyano-5-(7-(*tert*-butoxycarbonyl)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-4-yl)-2-oxocyclohexanecarboxylate (7d)

Compound **7d** was synthesized in 55% yield from **6d** using dry THF as a solvent following a procedure similar to that of compound **7a**; white solid; mp: 189–191 °C; R_f = 0.5 (40% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2975, 2232, 1727, 1695, 1664; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.25 (bs, 1OH), 8.94 (s, 1H), 4.81 (s, 2H), 3.81 (s, 5H), 3.42–3.35 (m, 2H), 3.26 (d, J = 16.0 Hz, 1H), 2.98 (d, J = 16.0 Hz, 1H), 2.92–2.85 (m, 1H), 2.65–2.58 (m, 2H), 2.47–2.39 (m, 1H), 1.52 (s, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 171.7, 170.6, 169.9, 158.3, 154.2, 151.0 (2C), 128.2 (2C), 121.5, 94.3, 80.7, 51.9, 42.1 (2C), 37.0, 32.7, 30.9, 29.6, 28.4 (3C), 26.7. MS (ES mass): 471.2 (M + 1). HPLC: 95.7%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 5 mM ammonium acetate in water, mobile phase B: CH_3CN, gradient (T/%B): 0/70, 2/70, 9/95, 13/95, 15/70, 18/70; flow rate: 1.0 mL min^{-1}; UV 240 nm, retention time 5.29 min.

Preparation of 1-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]-pyrimidin-4-yl)-4-oxocyclohexanecarbonitrile (8a)

A mixture of **7a** (0.5 g, 1.35 mmol) and NaCl (628 mg, 10.84 mmol) in DMSO (5 mL) and water (0.5 mL) was heated at 150 °C for 5 h under anhydrous conditions. After completion of the reaction, the mixture was cooled to room temp, diluted with water (25 mL) and extracted with ethyl acetate (3 × 20 mL). The organic layers were collected, combined, washed with brine solution (15 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The isolated residue was purified by column chromatography using ethyl acetate–hexane (1 : 6) to give desired product **8a** (240 mg, 58%) as a white solid; mp: 167–169 °C; R_f = 0.5 (30% EtOAc–hexane); IR (KBr, cm^{-1}): 2947, 2883, 2233, 1713; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.89 (s,1H), 3.28–3.26 (m, 2H), 3.01–2.99 (m, 2H), 2.96–2.87 (m, 2H), 2.87–2.76 (m, 2H), 2.67–2.56 (m, 4H), 1.99 (bs, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 206.8, 169.8, 157.2, 150.4, 140.4, 129.1, 125.9, 121.5, 43.3, 37.8 (2C), 35.5 (2C), 29.3, 26.6, 23.3, 22.3; MS (ES mass): 311.9 (M + 1); HPLC: 98.4%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/50, 2/50, 9/90, 14/90, 16/50, 20/50; flow rate: 0.8 mL min^{-1}; UV 245 nm, retention time 7.74 min.

Preparation of 1-(6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]-pyrimidin-4-yl)-4-oxocyclohexanecarbonitrile (8b)

Compound **8b** was synthesized in 62% yield from **7b** following a procedure similar to that of compound **8a**; white solid; mp: 152–154 °C; R_f = 0.5 (30% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2960, 2909, 2234, 1710; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.90 (s, 1H), 3.42 (t, J = 7.2 Hz, 2H), 3.12 (t, J = 7.2 Hz, 2H), 2.95–2.87 (m, 2H), 2.78–2.72 (m, 2H), 2.69–2.63 (m, 2H), 2.61–2.53 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 206.8, 174.9, 156.8, 150.6, 146.5, 134.6, 125.8, 121.3, 42.9, 37.8 (2C), 34.9 (2C), 33.2, 30.1, 28.2; MS (ES mass): 297.5 (M + 1).

Preparation of 1-(6,7,8,9-tetrahydro-5*H*-cyclohepta[4,5]thieno-[2,3-*d*]pyrimidin-4-yl)-4-oxocyclohexanecarbonitrile (8c)

Compound **8c** was synthesized in 65% yield from **7c** following a procedure similar to that of compound **8a**; white solid; mp: 144–146 °C; R_f = 0.5 (25% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2916, 2856, 2232, 1720; ^{1}H NMR (400 Hz, $CDCl_3$) δ: 8.89 (s, 1H), 3.37–3.34 (m, 2H), 3.05–3.02 (m, 2H), 2.96–2.87 (m, 2H), 2.80–2.76 (m, 2H), 2.67–2.55 (m, 4H), 2.04–1.98 (m, 2H), 1.81–1.74 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 206.8, 168.6, 157.0, 150.2, 144.9, 131.8, 128.9, 120.7, 43.1,

37.8 (2C), 35.1 (2C), 32.3, 30.9, 30.6, 26.9, 26.7; MS (ES mass): 326.2 (M + 1).

Preparation of 1-(7-(*tert*-butoxycarbonyl)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-4-yl)-4-oxocyclohexanecarbonitrile (8d)

Compound **8d** was synthesized in 58% yield from **7d** following a procedure similar to that of compound **8a**; white solid; mp: 259–261 °C; R_f = 0.4 (40% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 2973, 2935, 2235, 1699; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.87 (s, 1H), 4.75 (s, 2H), 3.76 (t, J = 4.8 Hz, 2H), 3.33 (bs, 2H), 2.88–2.80 (m, 2H), 2.71–2.67 (m, 2H), 2.61–2.49 (m, 4H), 1.45 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 206.6, 170.1, 150.9, 150.1, 132.1, 129.8, 128.7, 126.9, 121.2, 80.9, 43.7, 43.2, 37.7 (2C), 35.2 (2C), 29.6, 28.4 (3C), 28.3. MS (ES mass): 413.2 (M + 1). HPLC: 97.6%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 μ, mobile phase A: 5 mM ammonium acetate in water, mobile phase B: CH_3CN, gradient (T/%B): 0/50, 2/50, 9/95, 13/95, 15/50, 18/50; flow rate: 1.0 mL min^{-1}; UV 240 nm, retention time 6.72 min.

Preparation of 3-[(dimethylamino)methylene]-1-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]pyrimidin-4-yl)-4-oxocyclohexanecarbonitrile (9a)

A mixture of **8a** (0.5 g, 1.60 mmol), DMF-DMA (0.8 mL, 6.43 mmol) and Et_3N (0.7 mL, 4.82 mmol) in dry DMF (5 mL) was heated to 110 °C for 4 h under a nitrogen atmosphere. After completion of the reaction the mixture was cooled to room temp, diluted with water (25 mL) and extracted with ethyl acetate (3 × 20 mL). The organic layers were collected, combined, washed with brine solution (20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography using ethyl acetate–hexane (4 : 1) to give desired product **9a** (0.4 g, 64%) as a brown solid; mp: 214–216 °C; R_f = 0.2 (100% EtOAc); IR (KBr, cm^{-1}): 2947, 2230, 1735, 1647; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.88 (s, 1H), 7.69 (s, 1H), 3.61 (s, 2H), 3.41–3.37 (m, 1H), 3.17 (s, 6H), 3.13–3.08 (m, 1H), 2.99 (s, 2H), 2.87–2.77 (m, 1H), 2.72–2.62 (m, 1H), 2.60–2.53 (m, 1H), 2.36–2.28 (m, 1H), 1.97 (bs, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 193.7, 169.7, 158.4, 152.4, 150.4, 139.9, 129.1, 126.2, 122.2, 98.9, 43.7, 43.7, 43.2, 35.6, 34.6, 32.9, 29.1, 26.5, 23.2, 22.3; MS (ES mass): 367.0 (M + 1).

Preparation of 3-[(dimethylamino)methylene]-1-(6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidin-4-yl)-4-oxocyclohexanecarbonitrile (9b)

Compound **9b** was synthesized in 58% yield from **8b** following a procedure similar to that of compound **9a**; light brown solid; mp: 227–229 °C; R_f = 0.1 (100% EtOAc); IR (KBr, cm^{-1}): 2947, 2232, 1646, 1541; ^{1}H NMR (400 Hz, $CDCl_3$) δ: 8.89 (s, 1H), 7.70 (s, 1H), 3.57 (s, 2H), 3.51–3.43 (m, 1H), 3.34–3.25 (m, 1H), 3.17 (s, 6H), 3.11 (t, J = 7.2 Hz, 2H), 2.88–2.80 (m, 1H), 2.67–2.52 (m, 4H), 2.38–2.30 (m, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 193.6, 174.8, 158.1, 152.7, 150.6, 146.1, 134.9, 125.8, 122.0, 98.8, 43.8, 42.9, 34.8, 34.5, 33.1, 32.4, 30.1 (2C), 28.2; MS (ES mass): 353.2 (M + 1).

Preparation of 3-[(dimethylamino)methylene]-1-(6,7,8,9-tetrahydro-5*H*-cyclohepta[4,5]thieno[2,3-*d*]pyrimidin-4-yl)-4-oxocyclohexanecarbonitrile (9c)

A mixture of **8c** (0.2 g, 0.615 mmol) and DMF-DMA (0.16 mL, 1.23 mmol) in toluene (5 mL) was heated to 95 °C for 16 h under anhydrous conditions. After completion of the reaction, the mixture was cooled to room temp and the solvent was removed under reduced pressure. The residue was diluted with water (25 mL) and extracted with ethyl acetate (3 × 10 mL). The organic layers were collected, combined, washed with brine solution (10 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography using ethyl acetate–hexane (4 : 1) to give desired product **9c** (105 mg, 45%) as a white solid; mp: 185–187 °C; R_f = 0.1 (100% EtOAc); IR (KBr, cm^{-1}): 2920, 2851, 2228, 1646; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.89 (s, 1H), 7.71 (s, 1H), 3.61 (s, 2H), 3.33 (t, J = 5.6 Hz, 2H), 3.17 (s, 6H), 3.03–3.01 (m, 2H), 2.88–2.78 (m, 1H), 2.68–2.56 (m, 2H), 2.39–2.30 (m, 1H), 2.04–1.96 (m, 2H), 1.78–1.74 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 193.7, 168.4, 158.3, 152.7, 150.2, 144.5, 132.2, 128.9, 121.5, 99.0, 43.8, 43.2, 35.6, 34.6, 32.5 (2C), 32.4, 30.8, 30.6, 27.1, 26.7; MS (ES mass): 381.2 (M + 1).

Preparation of 3-[(dimethylamino)methylene]-1-(7-(*tert*-butoxycarbonyl)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno-[2,3-*d*]pyrimidin-4-yl)-4-oxocyclohexanecarbonitrile (9d)

Compound **9d** was synthesized in 45% yield from **8d** following a procedure similar to that of compound **9c**; brown solid; mp: 113–115 °C; R_f = 0.1 (100% EtOAc); IR (KBr, cm^{-1}): 2976, 2237, 1729, 1696; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.90 (s, 1H), 8.67 (s, 1H), 4.80 (s, 2H), 3.83 (bs, 2H), 3.60 (bs, 3H), 3.39–3.29 (m, 2H), 3.08 (s, 3H), 3.03 (s, 3H), 2.71–2.63 (m, 3H), 1.52 (s, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 193.5, 169.9, 159.0, 152.7 (2C), 150.9 (2C), 128.2, 122.1, 121.2, 98.7, 80.8, 43.8, 43.2, 42.3, 35.4, 34.6, 32.8, 31.0, 29.2, 28.4 (3C), 28.3. MS (ES mass): 468.2 (M + 1).

Preparation of 2-amino-6-(5,6,7,8-tetrahydrobenzo[*b*]thieno-[2,3-*d*]pyrimidin-4-yl)-5,6,7,8-tetrahydroquinazoline-6-carbonitrile (10a)

A mixture of **9a** (0.1 g, 0.27 mmol), guanidine HCl (24.1 mg, 0.41 mmol) and NaOMe (22 mg, 0.41 mmol) in methanol (8 mL) was stirred at 80 °C for 1 h under nitrogen. After completion of the reaction the excess of sodium methoxide was quenched with ice cold water and methanol was removed under reduced pressure. The residue was diluted with water (25 mL) and extracted with ethyl acetate (3 × 10 mL). The organic layers were collected, combined, washed with brine solution (10 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography

using ethyl acetate–hexane (4 : 1) to give desired product **10a** (80 mg, 78%) as a light brown solid; mp: 153–155 °C; R_f = 0.35 (100% EtOAc); IR (KBr, cm^{-1}): 3320, 3172, 2937, 2235; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.89 (s, 1H), 8.17 (s, 1H), 5.15 (s, 2H), 3.67 (d, J = 16.1 Hz, 1H), 3.43–3.36 (m, 2H), 3.26–3.16 (m, 2H), 3.01–2.91 (m, 3H), 2.79–2.75 (m, 1H), 2.45–2.37 (m, 1H), 1.99 (bs, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 169.8, 163.9, 161.9, 158.6, 157.5, 150.4, 140.3, 129.1, 126.1, 121.5, 115.7, 42.3, 35.9, 32.5, 29.3, 29.2, 26.6, 23.3, 22.3; MS (ES mass): 362.9 (M + 1); HPLC: 99.3%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN (isocratic) (A : B) 40 : 60; flow rate: 0.8 mL min^{-1}; UV 245 nm, retention time 2.9 min; chiral HPLC: column: chiral pak IC (250 × 4.6 mm) 5 µm, mobile phase: A: MeOH: B: 0.1% DEA, flow: 1.0 mL min^{-1}, wavelength: 245 nm, retention time (area %): 16.9 min (50%) and 19.4 min (50%).

Preparation of 2-amino-6-(6,7-dihydro-5*H*-cyclopenta-[4,5]thieno[2,3-*d*]pyrimidin-4-yl)-5,6,7,8-tetrahydroquinazoline-6-carbonitrile (10b)

Compound **10b** was synthesized in 70% yield from **9b** following a procedure similar to that of compound **10a**; white solid; mp: 200–202 °C; R_f = 0.3 (100% EtOAc); IR (KBr, cm^{-1}): 3318, 3161, 2950, 2242; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.89 (s, 1H), 8.16 (s, 1H), 5.10 (bs, 2H), 3.62 (d, J = 16.0 Hz, 1H), 3.52–3.45 (m, 1H), 3.40–3.36 (m, 1H), 3.34–3.17 (m, 2H), 3.15–3.11 (m, 2H), 2.96–2.89 (m, 1H), 2.78–2.72 (m, 1H), 2.62–2.54 (m, 2H), 2.46–2.38 (m, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 169.3, 163.9, 161.7, 158.6, 157.0, 150.6, 146.5, 134.7, 125.8, 121.4, 115.5, 41.9, 34.7, 33.2, 31.9, 30.1, 29.7, 28.9; MS (ES mass): 349.1 (M + 1); HPLC: 90.7%, column: X Bridge C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/30, 2/30, 9/95, 12/95, 15/30, 18/30; flow rate: 0.8 mL min^{-1}; UV 241 nm, retention time 7.0 min.

Preparation of 2-amino-6-(6,7,8,9-tetrahydro-5*H*-cyclohepta[4,5]-thieno[2,3-*d*]pyrimidin-4-yl)-5,6,7,8-tetrahydroquinazoline-6-carbonitrile (10c)

Compound **10c** was synthesized in 68% yield from **9c** following a procedure similar to that of compound **10a**; white solid; mp: 234–236 °C; R_f = 0.2 (100% EtOAc); IR (KBr, cm^{-1}): 3456, 3314, 2930, 2232; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.90 (s, 1H), 8.15 (s, 1H), 5.17 (s, 2H), 3.64 (d, J = 16.4 Hz, 1H), 3.44–3.17 (m, 4H), 3.04 (t, J = 5.4 Hz, 2H), 2.99–2.92 (m, 1H), 2.81–2.76 (m, 1H), 2.45–2.37 (m, 1H), 2.05–1.97 (m, 2H), 1.79–1.76 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 168.6, 164.1, 161.9, 158.6, 157.3, 150.3, 144.9, 131.9, 128.9, 120.8, 115.7, 42.1, 35.7, 32.4, 32.1, 30.9, 30.6, 29.1, 26.9, 26.7; MS (ES mass): 377.1 (M + 1); HPLC: 99.1%, column: X Bridge C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.1% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/50, 2/50, 9/95, 12/95, 15/50, 18/50; flow rate: 0.8 mL min^{-1}; UV 245 nm, retention time 4.5 min.

Preparation of 2-amino-6-(7-(*tert*-butoxycarbonyl)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-4-yl)-5,6,7,8-tetrahydroquinazoline-6-carbonitrile (10d)

Compound **10d** was synthesized in 55% yield from **9d** following a procedure similar to that of compound **10a**; light yellow solid; mp: 141–143 °C; R_f = 0.3 (100% EtOAc); IR (KBr, cm^{-1}): 3327, 3194, 2971, 2235, 1696; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.92 (s, 1H), 8.21 (s, 1H), 5.82 (s, 2H), 4.82 (s, 2H), 3.89–3.77 (m, 2H), 3.72 (d, J = 16.1 Hz, 1H), 3.57–3.46 (m, 1H), 3.40 (d, J = 16.3 Hz, 1H), 3.31–3.19 (m, 2H), 3.03–2.91 (m, 1H), 2.81–2.71 (m, 1H), 2.49–2.39 (m, 1H), 1.52 (s, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 175.3, 170.1, 163.8, 161.2 (2C), 158.3, 154.2, 151.0 (2C), 128.2, 121.3, 115.4, 80.9, 42.1, 35.4, 32.1, 31.5, 28.7, 28.4 (3C), 28.3, 26.9. MS (ES mass): 464.2 (M + 1). HPLC: 97.9%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 5 mM ammonium acetate in water, mobile phase B: CH_3CN, gradient (T/%B): 0/20, 2/20, 9/95, 13/95, 15/20, 18/20; flow rate: 1.0 mL min^{-1}; UV 240 nm, retention time 8.6 min.

Preparation of 6-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]-pyrimidin-4-yl)-5,6,7,8-tetrahydroquinazoline-6-carbonitrile (11a)

Compound **11a** was synthesized in 45% yield from **9a** and formimidine acetate (1.5 mmol) following a procedure similar to that of compound **10a**; brown solid; mp: 182–184 °C; R_f = 0.45 (100% EtOAc); IR (KBr, cm^{-1}): 2941, 2868, 2234, 1557; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.05 (s, 1H), 8.86 (s, 1H), 8.61 (s, 1H), 3.99 (d, J = 16.9 Hz, 1H), 3.54 (d, J = 16.9 Hz, 1H), 3.46–3.34 (m, 2H), 3.15–3.02 (m, 4H), 2.86–2.82 (m, 1H), 2.51–2.43 (m, 1H), 2.05–1.99 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 169.9, 162.9, 157.1, 157.0, 156.7, 150.4, 140.6, 129.0, 126.7, 125.9, 121.4, 41.7, 36.3, 32.3, 29.3, 29.1, 26.6, 23.2, 22.2; MS (ES mass): 347.9 (M + 1); HPLC: 98.5%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN (Isocratic) (A:B) 40 : 60; flow rate: 0.8 mL min^{-1}; UV 245 nm, retention time 4.2 min.

Preparation of 6-(6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]-pyrimidin-4-yl)-5,6,7,8-tetrahydroquinazoline-6-carbonitrile (11b)

Compound **11b** was synthesized in 78% yield from **9b** and formimidine acetate (1.5 mmol) following a procedure similar to that of compound **10a**; white solid; mp: 248–250 °C; R_f = 0.5 (100% EtOAc); IR (KBr, cm^{-1}): 2954, 2863, 2243, 1535; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.05 (s, 1H), 8.86 (s, 1H), 8.60 (s, 1H), 3.91 (d, J = 16.8 Hz, 1H), 3.57–3.49 (m, 2H), 3.41–3.25 (m, 2H), 3.13 (t, J = 7.2 Hz, 2H), 3.08–3.01 (m, 1H), 2.84–2.77 (m, 1H), 2.65–2.54 (m, 2H), 2.51–2.44 (m, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 175.0, 162.9, 157.2, 157.1, 156.3, 150.5, 146.8, 134.6, 126.5, 125.7, 121.2, 41.3, 34.9, 33.2, 31.9, 30.2, 28.9, 28.2; MS (ES mass): 334.1 (M + 1); HPLC: 97.9%. column: X Bridge C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN, gradient

(T/%B): 0/30, 2/30, 9/95, 12/95, 15/30, 18/30; flow rate: 0.8 mL min^{-1}; UV 241 nm, retention time 7.9 min.

Preparation of 6-(6,7,8,9-tetrahydro-5*H*-cyclohepta[4,5]thieno-[2,3-*d*]pyrimidin-4-yl)-5,6,7,8-tetrahydroquinazoline-6-carbonitrile (11c)

Compound **11c** was synthesized in 67% yield from **9c** and formimidine acetate (1.5 mmol) following a procedure similar to that of compound **10a**; white solid; mp: 157–159 °C; R_f = 0.4 (100% EtOAc); IR (KBr, cm^{-1}): 2934, 2853, 2230, 1551; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.06 (s, 1H), 8.88 (s, 1H), 8.61 (s, 1H), 3.97 (d, J = 16.8 Hz, 1H), 3.55 (d, J = 16.8 Hz, 1H), 3.43–3.28 (m, 3H), 3.13–3.00 (m, 3H), 2.87–2.84 (m, 1H), 2.50–2.42 (m, 1H), 2.10–1.95 (m, 2H), 1.80–1.77 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 168.7, 163.0, 157.2, 157.1, 156.6, 150.2, 145.2, 131.8, 128.9, 126.7, 120.6, 41.6, 36.1, 32.4, 31.9, 30.9, 30.6, 29.1, 26.9, 26.7; MS (ES mass): 362.1 (M + 1); HPLC: 97.3%. column: X Bridge C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.1% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/50, 2/50, 9/95, 12/95, 15/50, 18/50; flow rate: 0.8 mL min^{-1}; UV 245 nm, retention time 6.4 min.

Preparation of 6-(7-(*tert*-butoxycarbonyl)-5,6,7,8-tetrahydro-pyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-4-yl)-5,6,7,8-tetrahydro-quinazoline-6-carbonitrile (11d)

Compound **11d** was synthesized in 50% yield from **9d** and formimidine acetate (1.5 mmol) following a procedure similar to that of compound **10a**: brown solid; mp: 191–193 °C; R_f = 0.5 (100% EtOAc); IR (KBr, cm^{-1}): 2975, 2930, 2228, 1698; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.11 (s, 1H), 8.89 (s, 1H), 8.72 (m, 1H), 4.84 (s, 2H), 4.08 (d, J = 16.0 Hz, 1H), 3.83 (s, 2H), 3.66–3.51 (m, 2H), 3.51–3.37 (m, 1H), 3.35–3.03 (m, 2H), 2.89–2.77 (m, 1H), 2.60–2.48 (m, 1H), 1.52 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 170.3 (2C), 163.3, 156.8, 155.9, 154.2, 150.9 (3C), 128.1, 121.1, 109.9, 80.9, 41.5, 35.9, 32.0 (2C), 28.8 (2C), 28.4 (3C), 28.3; MS (ES mass): 449.1 (M + 1). HPLC: 98.2%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 5 mM ammonium acetate in water, mobile phase B: CH_3CN, gradient (T/%B): 0/50, 2/50, 9/95, 13/95, 15/50, 18/50; flow rate: 0.8 mL min^{-1}; UV 240 nm, retention time 6.6 min.

Preparation of 2-amino-6-(5,6,7,8-tetrahydrobenzo[*b*]thieno-[2,3-*d*]pyrimidin-4-yl)-4-oxo-3,4,5,6,7,8-hexahydroquinazoline-6-carbonitrile (12)

A mixture of **7a** (0.1 g, 0.27 mmol), guanidine HCl (48 mg, 0.81 mmol) and NaOMe (73 mg, 1.35 mmol) in methanol (8 mL) was stirred at 80 °C for 1 h under nitrogen. After completion of the reaction the excess sodium methoxide was quenched with ice cold water and methanol was removed under reduced pressure. The residue was diluted with water (25 mL) and extracted with ethyl acetate (3 × 10 mL). The organic layers were collected, combined, washed with brine solution (10 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography using methanol–DCM (1 : 19) to give desired product **12** (73 mg, 72%) as a white solid; mp: 279–281 °C; R_f = 0.5 (10% MeOH–DCM); IR (KBr, cm^{-1}): 3448, 3314, 3125, 2940, 2229, 1650; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.8 (bs, 1H), 9.04 (s, 1H), 6.46 (bs, 2H), 3.28–3.26 (m, 3H), 3.23–3.21 (m, 3H), 2.81–2.63 (m, 3H), 2.39–2.36 (m, 1H), 1.94 (bs, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 168.8, 162.9, 158.3, 154.1, 150.6, 145.1, 139.7, 128.4, 126.1, 122.1, 104.4, 42.2, 32.0, 31.6, 28.9, 28.5, 25.9, 22.7, 21.8; MS (ES mass): 378.9 (M + 1); HPLC: 97.4%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/20, 2/20, 9/95, 12/95, 15/20, 18/20; flow rate: 1.0 mL min^{-1}; UV 246 nm, retention time 6.2 min.

Preparation of 6-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]-pyrimidin-4-yl)-4-oxo-3,4,5,6,7,8-hexahydroquinazoline-6-carbonitrile (13)

Compound **13** was synthesized in 68% yield from **7a** and formimidine acetate (3 mmol) following a procedure manner similar to that of compound **12**; white solid; mp: 202–204 °C; R_f = 0.6 (10% MeOH–DCM); IR (KBr, cm^{-1}): 3153, 2943, 2233, 1655; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.89 (s, 1H), 8.12 (s, 1H), 3.58 (d, J = 17.6 Hz, 1H), 3.46–3.34 (m, 2H), 3.26–3.18 (m, 2H), 3.01 (s, 2H), 2.95–2.89 (m, 1H), 2.78–2.74 (m, 1H), 2.55–2.47 (m, 1H), 1.99–1.98 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 169.8, 163.5, 160.6, 157.2, 150.4, 145.9, 140.2, 129.1, 126.1, 121.7, 119.1, 41.5, 32.4, 31.8, 29.2, 29.1, 26.6, 23.2, 22.2; MS (ES mass): 363.9 (M + 1); HPLC: 98.6%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/20, 2/20, 9/95, 12/95, 15/20, 18/20; flow rate: 0.8 mL min^{-1}; UV 244 nm, retention time 8.1 min; chiral HPLC: column: chiral pak AD (250 × 4.6 mm) 3 µm, mobile phase: A: *n*-hexane: B: 0.1% IPA, flow: 0.8 mL min^{-1}, wave length: 245 nm, retention time (area %): 12.6 min (49.5%) and 15.8 min (50.5%).

Preparation of 5-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]-pyrimidin-4-yl)-3-oxo-2,3,4,5,6,7-hexahydro-1*H*-indazole-5-carbonitrile (14)

A mixture of **7a** (0.1 g, 0.27 mmol), hydrazine (0.03 mL, 0.54 mmol) and Et_3N (0.09 mL, 0.81 mmol) in methanol (8 mL) was stirred at 80 °C for 1 h under nitrogen. After completion of the reaction methanol was removed under reduced pressure. The residue was diluted with water (25 mL) and extracted with ethyl acetate (3 × 10 mL). The organic layers were collected, combined, washed with brine solution (10 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The isolated residue was purified by column chromatography using methanol–DCM (1 : 49) to give desired product **14** (75 mg, 76%) as a white solid; mp: 271–273 °C; R_f = 0.5 (5% MeOH–DCM); IR (KBr, cm^{-1}): 3231, 2944, 2234, 1734; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.31 (bs, 1H), 9.61 (bs, 1H), 9.00 (s, 1H), 3.22–3.15 (m, 4H), 3.02 (s, 2H), 2.87 (s, 2H), 2.66–2.62 (m, 1H), 2.38–2.28 (m, 1H), 1.91 (s, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 168.7, 158.5, 150.5, 150.3, 139.6, 137.9, 128.3, 126.1, 122.1, 109.5, 43.6, 32.3, 30.5, 28.5, 25.9,

22.7, 21.8, 19.4; MS (ES mass): 351.9 (M + 1); HPLC: 97.7%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/20, 2/20, 9/95, 12/95, 15/20, 18/20; flow rate: 0.8 mL min^{-1}; UV 245 nm, retention time 7.9 min; chiral HPLC: column: Lux Cellulose-2 (250 × 4.6 mm) 3 µm, mobile phase: A: *n*-hexane: D: 0.1% TFA in EtOH, flow: 0.8 mL min^{-1}, wavelength: 245 nm, retention time (area %): 16.9 min (45.7%) and 20.9 min (49.8%).

Preparation of 5-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]-pyrimidin-4-yl)-3-oxo-2-phenyl-3,3a,4,5,6,7-hexahydro-2*H*-indazole-5-carbonitrile (15)

Compound **15** was prepared in 65% yield from **7a** and phenyl hydrazine (2 mmol) following a procedure similar to compound **14**; white solid; mp: 218–220 °C; R_f = 0.3 (70% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 3062, 2861, 2237, 1730; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.87 (s, 1H), 7.87 (d, J = 8.0 Hz, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.41 (t, J = 7.6 Hz, 1H), 7.35 (t, J = 7.6 Hz, 1H), 7.22–7.13 (m, 1H), 3.41–3.33 (m, 2H), 3.27–3.17 (m, 2H), 3.09–2.84 (m, 5H), 2.76–2.65 (m, 1H), 2.40–2.33 (m, 1H), 2.01–1.94 (m, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 169.8, 159.2, 157.9, 150.4, 148.1, 140.1, 129.1, 128.9, 128.9 (2C), 126.3, 125.7, 121.7, 120.3, 118.9, 42.8, 37.3, 32.1, 30.5, 29.2, 26.6, 23.2, 22.2, 20.4; MS (ES mass): 427.9 (M + 1); HPLC: 97.9%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.05% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/50, 2/50, 9/95, 12/95, 15/50, 18/50; flow rate: 0.8 mL min^{-1}; UV 245 nm, retention time 5.9 min.

Preparation of 5-(5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]-pyrimidin-4-yl)-4,5,6,7-tetrahydro-1*H*-indazole-5-carbonitrile (16)

A mixture of **9a** (0.1 g, 0.27 mmol) and hydrazine (0.02 mL, 0.41 mmol) in methanol (5 mL) was stirred at 80 °C for 1 h under nitrogen. Then, methanol was removed under reduced pressure. The residue was diluted with water (25 mL) and extracted with ethyl acetate (3 × 10 mL). The organic layers were collected, combined, washed with brine solution (10 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography using ethyl acetate–*n*-hexane (3 : 2) to give desired product **16** (65 mg, 72%) as a light brown solid; mp: 109–111 °C; R_f = 0.3 (70% EtOAc–*n*-hexane); IR (KBr, cm^{-1}): 3647, 3248, 2230, 1513; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.89 (s, 1H), 7.54 (s, 1H), 6.45 (bs, 1H), 3.61 (d, J = 16.0 Hz, 1H), 3.48 (d, J = 16.0 Hz, 1H), 3.38–3.09 (m, 4H), 3.00 (bs, 2H), 2.83–2.78 (m, 1H), 2.48–2.43 (m, 1H), 1.98 (bs, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 169.5, 158.2, 150.2 (2C), 139.8 (2C), 129.0, 126.1 (2C), 121.8, 43.5, 33.4, 31.9, 29.1, 26.4, 23.1, 22.1, 20.1. MS (ES mass): 336.2 (M + 1); HPLC: 99.1%, column: ZORBAX XDB C-18 150 × 4.6 mm 5 µ, mobile phase A: 0.1% formic acid in water, mobile phase B: CH_3CN, gradient (T/%B): 0/20, 2/20, 9/95, 13/95, 15/20, 18/20; flow rate: 1.0 mL min^{-1}; UV 245 nm, retention time 8.5 min. Chiral HPLC: column: chiral pak IC (250 × 4.6 mm) 5 µm, mobile phase: A: MeOH: B: 0.1% DEA, flow : 1.0 mL min^{-1}, wave length: 295 nm, retention time (area %): 8.8 min (50%) and 10.9 min (50%).

Single crystal X-ray data for compound 7a and 10a

Single crystals suitable for X-ray diffraction of **7a** and **10a** were grown from methanol. The crystals were carefully chosen using a stereo zoom microscope supported by a rotatable polarizing stage. The data was collected at room temperature on Bruker's KAPPA APEX II CCD Duo with graphite monochromated Mo-Kα radiation (0.71073 Å). The crystals were glued to a thin glass fibre using FOMBLIN immersion oil and mounted on the diffractometer. The intensity data were processed using Bruker's suite of data processing programs (SAINT), and absorption corrections were applied using SADABS.[21] The crystal structure was solved by direct methods using SHELXS-97 and the data was refined by full matrix least-squares refinement on F^2 with anisotropic displacement parameters for non-H atoms, using SHELXL-97.[22]

Crystal data of **7a**: Molecular formula = $C_{19}H_{19}N_3O_3S$, formula weight = 369.44, crystal system = triclinic, space group = $P\bar{1}$, a = 11.092 (5) Å, b = 11.448 (5) Å, c = 15.672 (7) Å, V = 1761.7 (13) $Å^3$, T = 296 K, Z = 4, D_c = 1.401 Mg m^{-3}, μ(Mo-Kα) = 0.21 mm^{-1}, 20 533 reflections measured, 7395 independent reflections, 5076 observed reflections [$I > 2.0\sigma(I)$], R_1_obs = 0.081, goodness of fit = 1.003. CCDC 864130.

Crystal data of **10a**: Molecular formula = $C_{19}H_{18}N_6S$, formula weight = 362.13, crystal system = triclinic, space group = $P\bar{1}$, a = 7.625 (4) Å, b = 10.1757 (5) Å, c = 12.243 (6) Å, V = 903.66 (8)$Å^3$, T = 296 K, Z = 6, D_c = 1.387 Mg m^{-3}, μ(Mo-Kα) = 0.21 mm^{-1}, 15 612 reflections measured, 3949 independent reflections, 3342 observed reflections [$I > 2.0\sigma(I)$], R_1_obs = 0.029, goodness of fit = 0.876. CCDC 864129.

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). PDE4B1 clone was from OriGene Technologies (Rockville, MD, USA). PDE4D2 enzyme was purchased from BPS Bioscience (San Diego, CA, USA). Lipopolysaccharide (LPS) was from *Escherichia coli* strain 0127:B8 obtained from Sigma (St. Louis, MO, USA). Mouse TNF-α ELISA kit was procured from R&D Systems (Minneapolis, MN, USA).

Evaluation of PDE4 inhibitory potential by cell based cAMP reporter assay. One day prior to transfection, HEK 293 cells were seeded in p60 cell culture dish (Tarsons Inc.). These were

transfected using Lipofectamine 2000 (as per the manufacturer's instructions) with 2.4 μg of PDE4B1 expression plasmid and 4.0 μg of pCRELuc plasmid. After 5 h of transfection, medium was aspirated, cells were trypsinized and seeded in 96 well plates at a density of 60 000 cells per well. Plates were incubated overnight in a CO_2 incubator set to 37 °C and 5% CO_2. Twenty four hours post transfection, cells were pre-treated with various concentrations (0.001 to 30 μM) of compounds for 30 min, followed by stimulation with 5 μM forskolin for 4 h. Subsequently medium was removed and cells were lysed in reporter lysis buffer (Promega Inc) for 15 min with gentle rocking at RT. Luciferase activity in the lysates was measured by a Multilabel Plate Reader (Perkin Elmer 1420 Multilabel Counter). Fold elevation of cAMP is calculated using the following formula.

$$\text{Fold activation} = \frac{(\text{RLU of compound} - \text{Rlu of vehicle control})}{(\text{Rlu of forskolin} - \text{RLU of vehicle control})}$$

PDE4B protein production and purification. PDE4B1 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.[19a] 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 hour 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.

PDE4 enzymatic assay. The inhibition of PDE4 enzyme was measured using PDElight HTS cAMP phosphodiesterase assay kit (Lonza) according to manufacturer's recommendations. Briefly, 10 ng of in house purified PDE4B1 or 0.5 ng commercially procured PDE4D2 enzyme was pre-incubated either with DMSO (vehicle control) or compound for 15 min before incubation with the substrate cAMP (5 μM) for 1 hour. The reaction was halted with stop solution and reaction mix was incubated with detection reagent for 10 min in dark. Dose response studies were performed at 13 different concentrations ranging from 200 μM to 0.001 μM. Luminescence values (RLUs) were measured by a Multilabel Plate Reader (PerklinElmer 1420 Multilabel Counter). The percentage of inhibition was calculated using the following formula and the IC_{50} values were determined by a nonlinear regression analysis from dose response curve using Graphpad Prism software (San Diego, USA). IC_{50} values are presented as mean ± SD.

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

TNF-α production assay. RAW 264.7 cells were pre-incubated either with DMSO (vehicle control) or compound for 30 min and then stimulated with 1 μg mL^{-1} of LPS overnight. Dose response studies were carried out at eight different concentrations (30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01 μM). Post-stimulation, cell supernatants were harvested, centrifuged to clear cell debris and the amount of TNF-α in the supernatants was measured using mouse TNF-α DuoSet ELISA kit from R&D Systems according to manufacturer's recommendations. The percentage of inhibition was calculated using the following formula:

$$\%\,\text{inhibition} = 100 - \left[\frac{(\text{LPS stimulated}_{\text{compound}} - \text{unstimulated})}{(\text{LPS stimulated}_{\text{DMSO}} - \text{unstimulated})}\right] \times 100$$

The IC_{50} values were determined by a nonlinear regression analysis from dose response curve using Graphpad Prism software (San Diego, USA). IC_{50} values are expressed as mean ± SD.

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