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Triazole-Pyridine Dicarbonitrile Targets Phosphodiesterase 4 to Induce Anticancer Activity in Lung Carcinoma Cells

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Abstract

Phosphodiesterase 4 (PDE4) is a key enzyme involved in the hydrolysis of cyclic adenosine monophosphate (cAMP) and widely expressed in several types of cancers. The inhibition of PDE4 results in an increased concentration of intracellular cAMP levels that imparts the anti-inflammatory response in the target cells. In the present report, two series of triazolo-pyridine

dicarbonitriles and substituted dihydropyridine dicarbonitriles were synthesized using green protocol (TBAB in refluxed water). We next evaluated the new compounds for their cytotoxicity towards lung cancer (A549) cells and identified 7-(4-(methylsulfonyl)phenyl)-5-oxo-3,5-dihydro-1*H*-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (**5h**) and 7-(1-methyl-1*H*-imidazol-2-yl)-5-oxo-3,5-dihydro-1*H*-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (**5j**) as lead analogs with the IC_{50} value of 15.2 and 24.1 μM respectively. Furthermore, all the new compounds were tested for PDE4 inhibitory activity and **5j** showed relatively good inhibitory activity towards PDE4 with inhibition of 50.9% at 10 μM. In silico analysis demonstrated the favorable interaction of the title compounds with the target enzyme. Taken together, the present study introduces a new scaffold for the development of novel PDE4 inhibitors to fight against inflammatory diseases.

Keywords: Phosphodiesterase 4, Anticancer, Synthetic small molecule, Chemotherapy.

Introduction

PDE4 catalyzes the hydrolysis of cyclic adenosine monophosphate (cAMP), an important second messenger involved in the regulation of various biological functions such as apoptosis, inflammation and lipid metabolism.[1] The relay of signals from the activated membrane spanned G-protein coupled receptors lead to the stimulation of adenylyl cyclase which generates intracellular cAMP from adenosine triphosphate. The cAMP, in turn, activates protein kinase A that phosphorylates multiple transcription factors which are involved in the modulation of expression of the cAMP target genes.[2] PDE4 hydrolyzes and reduces the cAMP level and counterbalance its cellular effects.[3] PDE4 inhibitors increase the levels of intracellular cAMP which possess a wider spectrum of anti-inflammatory effects on the effector cells associated with inflammatory bowel disease, chronic obstructive pulmonary diseases, psoriasis and asthma.[4-6] Studies have demonstrated that PDE4 is widely expressed in cancers of brain, lung, colorectal, prostate and promote their growth. Moreover, PDE4

inhibitors suppressed cell proliferation and induced tumor regression in the tested preclinical cancer models.[7-11] Taken together, these evidences suggested that PDE4 as a potential molecular therapeutic target for cancer therapy.

The antitumor efficacy of triazoles and pyridines have been well-documented in several preclinical cancer studies.[12-17] Furthermore, the antitumor potential of triazole conjugated pyridine has also been established in various malignancies.[18-21] Some of the also reports indicated that triazolo-pyridine conjugates target PDE4 to induce their biological effect.[22, 23] Specifically, synthesis of a PDE4 inhibitor, 5,6-Dihydro-(9H)-pyrazolo[3,4-c]-1,2,4-triazolo[4,3-a]pyridine was described.[23] In another study, the substitution at the 3-position of 5,6-Dihydro-(9*H*)-pyrazolo[3,4-c]-1,2,4-triazolo[4,3-a]pyridine tricycle led to a 2-thienyl analog (Tofimilast) which is a potent PDE4 inhibitor with low oral bioavailability.[24] Apart, there are limited number of reports on the preparation of triazolo pyridine dicarbonitriles and many of them employ the use of strong base, hazardous organic solvents, expensive catalysts and tedious laboratory procedures.[25] Considering these drawbacks, we used environmentally benign conditions such as water as solvent and tetrabutyl ammonium bromide (TBAB) as a mild water-soluble catalyst for the synthesis of triazolo-pyridine dicarbonitriles and dihydropyridine carbonitriles. In our effort to explore the pharmacological properties of synthetic small molecules,[26-30] new spiro[1,2,4]triazolo[1,5-a]pyridine dicarbonitriles were synthesized and evaluated for their possible anticancer effect towards lung cancer cells and PDE4 inhibitory activity.

Results and Discussion

Chemistry

We reported the synthetic methodologies for the preparation of various bioactive heterocyclic moieties.[31-34] In the present study, the green procedure for the synthesis of triazolo-pyridine dicarbonitriles and dihydropyridine dicarbonitriles catalyzed by TBAB in water has been

reported. In a similar eco-friendly approach, the synthesis of highly functionalized pyridines, 1,2,4-triazolo[1,5-a]pyridines, and 1,2,4-triazolo[1,5-a]isoquinolines were reported earlier [35, 36]. The reaction between 4-bromo benzaldehyde (**1**), malononitrile (**2**), cyanoacetohydrazide (**3**) and cyclohexanone (**4**) was considered to optimize the reaction conditions using various solvents and catalysts (Table 1).

Initially, we attempted the reaction in refluxed water with organic bases such as piperidine (2 eq) and triethylamine (2 eq) which resulted in no product formation even after refluxing for 8h. The usage of inorganic catalysts such as sodium silicate, p-TSA in refluxed water resulted in relatively low yield of 7-(4-bromophenyl)-5-oxo-3,5-dihydro-1*H*-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (**5a**). The reaction with TBAB in organic solvents including ethanol, ethyl acetate, and toluene resulted in a low yield. TBAB in an equal ratio of refluxed ethanol and water gave **5a** with a yield of 72%. Finally, TBAB (1.5 eq) in refluxed water resulted in the formation of **5a** in relatively higher yield (92%). Thus, the TBAB (2 eq) in refluxed water was considered for further reactions with another aryl/heteroaryl aldehydes containing electron-withdrawing and electron donation groups to prepare new series of spiro[1,2,4]triazolo[1,5-a]pyridine dicarbonitriles (**5a-l**) (Table 2).

We next employed this protocol to synthesize substituted dihydropyridine dicarbonitriles (**6a-i**) (Table 3). The formation of (**6a-i**) from a variety of aryl and heteroaryl aldehydes bearing electron-withdrawing and electron donation groups resulted in high yields of products within 15-20 min. Hence, the present protocol can be applied to a broad choice of differently substituted aldehydes. Though we have not performed the mechanistic studies, we propose the theoretical possible mechanism in the formation of spiro[1,2,4]triazolo[1,5-a]pyridine dicarbonitriles and substituted dihydropyridine dicarbonitriles. The compound **1** and malononitrile condense to form arylidinemalononitrile which combines with hydrazone of

cyanoacetohydrazide and cyclohexanone resulting in the formation of **5a-i**. Similarly, arylidinemalononitrile reacts with cyanoacetohydrazide to yield **6a-i**.

Spiro[1,2,4]triazolo[1,5-a]pyridine dicarbonitriles elicit cytotoxicity towards lung carcinoma cells

The newly synthesized triazolo-pyrimidines were investigated for their cytotoxic potential towards lung carcinoma (A549) cells using *in vitro* cytotoxicity assay [37, 38]. Among the new compounds, 7-(4-(methylsulfonyl)phenyl)-5-oxo-3,5-dihydro-1*H*-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (compound **5h**) and 7-(1-methyl-1*H*-imidazol-2-yl)-5-oxo-3,5-dihydro-1*H*-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (compound **5j**) displayed good anticancer activity with the IC_{50} value of 15.2 and 24.1 μM respectively. The other derivatives presented the moderate growth inhibitory activity with the IC_{50} values ranging between 26 to 36 μM (Table 3). These inhibitory values of title compounds indicate that the electron withdrawing polar functional groups are effective when compared to electron releasing groups.

Pharmacology

Compound 5j inhibits PDE4 enzyme activity in vitro

All the newly synthesized compounds were tested for PDE4 inhibitory activity at the concentration of 10 μM (Table 3). Compounds **5j** showed relatively good inhibitory activity towards PDE4 compared to the other structural analogs with inhibition of 50.9% at 10 μM. Moreover, most of the other compounds displayed moderate to marginal inhibition ranging between 10 to 44% at 10 μM. The compounds with <10% inhibition were considered as inactive. Rolipram was used as the positive control which showed inhibition of 87.3%. This result supports the compound **5j** that showed anti-cancer activity by targeting PDE4 enzyme of human lung cancer cells.

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In silico analysis

For nine out of the twelve compounds docking identified a common binding mode. The predicted binding mode of the best-scored compound is shown in Figure 1A. Docking predicts hydrogen bond interactions with His-234 and a water molecule anchored to Asp-392 and Asn-395. The compound also forms a face to face stacking interaction with Phe-446 and an edge to face interaction with Phe-414. Comparing the binding mode to that of the co-crystallized ligand it is similar with a large volume overlap. Both the hydrogen bond to water and the *pi*-stacking interactions are formed by the co-crystallized ligand as well (Figure 1B).

Experimental Section

All chemicals used were of reagent grade and used without further purification. Thin layer chromatography (TLC) was performed on 0.25 mm silica gel plates ($60F_{254}$, Merck). Column chromatography separations were obtained on silica gel (200-400 mesh). ^{1}H NMR spectra were recorded on Bruker NMR 400 MHz instrument in DMSO-d_6 solvent [39]. ^{13}C NMR spectra were obtained on Agilent NMR instrument at 100 MHz in $CDCl_3$/DMSO-d_6 solvent. Chemical shifts are expressed in ppm downfield relative to TMS. LC-MS analysis was performed on Agilent LC-MS with electron ionization (ESI) +ve and –ve mode. Elemental analyses were recorded using PerkinElmer CHNS analyzer.

General procedure for the synthesis of spiro[[1,2,4]triazolo[1,5-a]pyridine dicarbonitriles

In a 50 mL round bottom flask, 2-cyano acetohydrazide (1.0 eq) and cyclohexanone (1.0 eq) were stirred at 90 ºC in tetrabutyl ammonium bromide solution (8 mL, 10 mol%) for 20 min. To this reaction mixture, an aromatic/heterocyclic carbaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 1-2.5 h at 100 ºC (scheme 1). The reaction was monitored by TLC using n-hexane and ethyl acetate eluent. After completion of the reaction, solid was filtered through Whatman filter paper, air dried and purified by column chromatography.

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Synthesis of (7-(4-bromophenyl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile) (5a)

The product **5a** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 4-bromobenzaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 45 min at 100 ºC. This compound was obtained as light brown color solid with 92% yield. IR (KBR ν in cm^{-1}): 3415, 3360, 2220, 2225, 1660; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 7.75-7.71 (d, 2H, Ar-H), 7.52 (s, 1H, C-NH), 7.35-7.31 (d, 2H, Ar-H), 2.19 (s, 1H, N-NH), 1.82-1.71 (m, 4H, Ali-H), 1.42-1.31 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 170.12, 161.54, 160.25, 130.76, 128.41, 121.53, 110.20, 114.61, 85.15, 76.62, 33.11, 23.53, 22.11; **Anal. Calcd.** for $C_{19}H_{16}BrN_5O$: C, 55.62; H, 3.93; N, 17.07; found C, 55.58; H, 3.90; N, 17.10 % , **LCMS** (MM:ES+APCI) $(M+H)^+$ 410.

Synthesis of 7-(3-bromo-4-fluorophenyl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5b)

The product **5b** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 3-bromo-4-fluoro benzaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 1 hour at 100 ºC. This compound was obtained as yellow solid with 90% yield. IR (KBR ν in cm^{-1}): 3420, 3350, 2221, 2219, 1635; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 7.69 (s, 1H, Ar-H), 7.41 (s, 1H, C-NH), 7.14-7.04 (m, 2H, Ar-H), 2.14 (s, 1H, N-NH), 1.72-1.60 (m, 4H, Ali-H), 1.39-1.23 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 170.31, 165.56, 161.45, 129.59, 128.38, 126.11, 118.19, 115.81, 110.80, 87.12, 78.23, 34.95, 26.16, 24.21; **Anal. Calcd.** for $C_{19}H_{15}BrFN_5O$: C, 53.29; H, 3.53; N, 16.35; found C, 53.32; H, 3.49; N, 16.31 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 428.

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Synthesis of 7-(3-bromo-4-methoxyphenyl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5c)

The product **5c** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 3-bromo-4-methoxy benzaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 45 min at 100 ºC. This compound was obtained as yellow solid with 94% yield. IR (KBR ν in cm^{-1}): 3420, 3380, 2219, 2220, 1645; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 7.35-7.25 (m, 3H, Ar-H), 7.04 (s, 1H, C-NH), 3.52 (s, 3H, -OCH_3), 2.57 (s, 1H, N-NH), 1.92-1.70 (m, 4H, Ali-H), 1.44-1.21 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 167.28, 158.04, 157.13, 154.02, 131.19, 130.26, 128.07, 114.97, 113.32, 110.81, 87.90, 74.08, 56.19, 34.01, 26.21, 23.05; **Anal. Calcd.** for $C_{20}H_{18}BrN_5O_2$: C, 54.56; H, 4.12; N, 15.91; found C, 54.50; H, 4.15; N, 15.88 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 440.

Synthesis of 7-(3,4-dimethoxyphenyl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5d)

The product **5d** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 3, 4-dimethoxy benzaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 45 min at 100 ºC. This compound was obtained as white solid with 93% yield. IR (KBR ν in cm^{-1}): 3390, 3356, 2220, 2224, 1650; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 7.42-7.46 (m, 3H, C-NH), 7.15 (s, 1H, Ar-H), 6.92-6.90 (m, 2H, Ar-H), 3.71 (s, 6H, -CH_3), 2.12 (s, 1H, N-NH), 1.55-1.49 (m, 4H, Ali-H), 1.36-1.29 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 19.51, 160.54, 157.26, 134.88, 130.90, 128.41, 115.21, 114.93, 113.21, 85.90, 79.32, 56.24, 32.65, 26.81, 21.93; **Anal. Calcd.** for $C_{21}H_{21}N_5O_3$: C, 64.44; H, 5.41; N, 17.89; found C, 64.40; H, 5.38; N, 17.92 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 392.

Synthesis of 5-oxo-7-(4-propoxyphenyl)-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8- dicarbonitrile (5e)

The product **5e** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 4-propoxy benzaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 45 min at 100 ºC. This compound was obtained as white solid with 92% yield. IR (KBR ν in cm^{-1}): 3410, 3375, 2220, 2215, 1650; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 7.84-7.79 (d, 2H, Ar-H), 7.52 (s, 1H, C-NH), 7.16-7.11 (d, 2H, Ar-H), 6.42-6.35 (q, 2H, -CH_2-), 2.17 (s, 1H, N-NH), 1.76-1.70 (m, 2H, -CH_2-), 1.60-1.52 (m, 4H, Ali-H), 1.33-1.21 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 168.39, 158.18, 156.27, 153.69, 128.81, 123.42, 116.30, 115.27, 113.18, 84.01, 75.35, 70.42, 32.01, 24.00, 22.46, 21.84, 10.52; **Anal. Calcd.** for $C_{22}H_{23}N_5O_2$: C, 67.85; H, 5.95; N, 17.98; found C, 67.81; H, 5.98; N, 18.00 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 390.

Synthesis of 7-(4-nitrophenyl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5f)

The product **5f** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 4-nitro benzaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 1 hour at 100 ºC. This compound was obtained as yellow solid with 89% yield. IR (KBR ν in cm^{-1}): 3395, 3382, 2221, 2220, 1648; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 8.39-8.28 (m, 2H, Ar-H), 7.64-7.56 (m, 2H, Ar-H), 7.41 (s, 1H, C-NH), 2.27 (s, 1H, N-NH), 1.70-1.66 (m, 4H, Ali-H), 1.28-1.18 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 167.03, 159.26, 157.64, 148.47, 140.21, 130.35, 122.18, 115.8 114.13, 88.50, 74.25, 34.42, 27.18, 25.29; **Anal. Calcd.** for $C_{19}H_{16}N_6O_3$: C, 60.63; H, 4.28; N, 22.33; found C, 60.59; H, 4.30; N, 22.30 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 377.

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Synthesis of 5-oxo-7-(4-(trifluoromethyl)phenyl)-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5g)

The product **5f** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 4-trifluoromethyl benzaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 1 hour at 100 ºC. This compound was obtained as yellow solid with 88% yield. IR (KBR ν in cm^{-1}): 3390, 3350, 2220, 2222, 1650; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 8.39-8.28 (m, 2H, Ar-H), 7.64-7.56 (m, 2H, Ar-H), 7.41 (s, 1H, C-NH), 2.27 (s, 1H, N-NH), 1.70-1.66 (m, 4H, Ali-H), 1.28-1.18 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 167.03, 159.26, 157.64, 148.47, 140.21, 130.35, 122.18, 115.8 114.13, 88.50, 74.25, 34.42, 27.18, 25.29; **Anal. Calcd.** for $C_{20}H_{16}F_3N_5O$: C, 60.15; H, 4.04; N, 14.27; found C, 60.11; H, 4.07; N, 14.23 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 400.

Synthesis of 7-(4-(methylsulfonyl)phenyl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5h)

The product **5h** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 4-(methylsulfonyl) benzaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 1.5 hours at 100 ºC. This compound was obtained as white solid with 90% yield. IR (KBR ν in cm^{-1}): 3418, 3345, 2222, 2218, 1640; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 8.05-7.90 (m, 4H, Ar-H), 7.32 (s, 1H, C-NH), 3.84 (s, 3H, -CH_3), 2.30 (s, 1H, N-NH), 1.69-1.57 (m, 4H, Ali-H), 1.31-1.19 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 170.20, 161.93, 160.24, 139.85, 136.27, 129.29, 127.51, 116.22, 114.34, 87.56, 78.11, 48.50, 34.83, 25.16, 22.17; **Anal. Calcd.** for $C_{18}H_{15}BrN_6O$: C, 52.57; H, 3.68; N, 20.44; found C, 52.52; H, 3.70; N, 20.25 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 411.

Synthesis of 7-(6-bromopyridin-3-yl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5i)

The product **5i** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 4-bromopyrine-3-carbaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 1.5 hours at 100 ºC. This compound was obtained as white solid with 87% yield. IR (KBR ν in cm^{-1}): 3395, 3348, 2220, 2219, 1650; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 8.35 (s, 1H, Py-CH), 7.83-7.80 (d, 1H, Ar-H), 7.56-7.53 (d, 1H, Ar-H), 7.40 (s, 1H, C-NH), 2.49 (s, 1H, N-NH), 1.75-1.65 (m, 4H, Ali-H), 1.30-1.18 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 168.32, 159.61, 158.09, 150.04, 140.19, 136.67, 130.79, 122.81, 116.02, 88.08, 74.21, 35.76, 26.85, 22.11; **Anal. Calcd.** for $C_{20}H_{19}N_5O_3S$: C, 58.67; H, 4.68; N, 17.10; found C, 58.60; H, 4.70; N, 17.12 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 410.

Synthesis of 7-(1-methyl-1H-imidazol-2-yl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5j)

The product **5j** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 1-methyl-1H-imidazole-2-carbaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 2 hours at 100 ºC. This compound was obtained as off-white solid with 90% yield. IR (KBR ν in cm^{-1}): 3390, 3345, 2222, 2221, 1638; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 7.74 (s, 1H, C-NH), 7.25-7.22 (m, 2H, Imi-H), 3.87 (s, 3H, -CH_3), 2.05 (s, 1H, N-NH), 1.64-1.60 (m, 4H, Ali-H), 1.37-1.31 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 170.75, 160.22, 159.93, 136.01, 127.99, 120.48, 119.98, 102.04, 88.07, 74.01, 36.06, 33.12, 25.21, 22.52; **Anal. Calcd.** for $C_{17}H_{17}N_7O$: C, 60.88; H, 5.11 N, 29.24; found C, 60.85; H, 5.07; N, 29.35 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 336.

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Synthesis of 7-(1H-indol-3-yl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5k)

The product **5k** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 1H-indole-3-carbaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 2 hours at 100 ºC. This compound was obtained as brown solid with 91% yield. IR (KBR ν in cm^{-1}): 3410, 3385, 2218, 2220, 1645; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 10.50 (s, 1H, Ind-NH), 7.88 (s, 1H, Ind-CH), 7.67 (s, 1H, C-NH), 7.44-7.40 (m, 4H, Ar-H), 2.16 (s, 1H, N-NH), 1.63-1.54 (m, 4H, Ali-H), 1.48-1.39 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 170.12, 159.51, 158.18, 138.27, 130.11, 125.61, 121.78, 120.91, 115.63, 110.84, 106.45, 86.21, 74.17, 35.21, 26.42, 23.41; **Anal. Calcd.** for $C_{21}H_{18}N_6O$: C, 68.09; H, 4.90; N, 22.69; found C, 68.11; H, 4.85; N, 22.72 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 371.

Synthesis of 7-(2-methyl-1H-indol-3-yl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile (5l)

The product **5l** was obtained by stirring 2-cyano acetohydrazide (1.0 eq) and cycloxexanone (1.0 eq) at 90 ºC in 8 mL of 10 mol% of tetrabutyl ammonium bromide solution for 20 min. To this reaction mixture, 2-methyl-1H-indole-3-carbaldehyde (1.0 eq) and malononitrile (1.2 eq) were added and stirred further for 2.5 hours at 100 ºC. This compound was obtained as brown solid with 85% yield. IR (KBR ν in cm^{-1}): 3390, 3360, 2221, 2222, 1646; **^{1}H NMR** (DMSO-d_6, 400 MHz δ in ppm): 8.35 (s, 1H, Ind-NH), 7.42-7.38 (m, 2H, Ar-H), 7.26 (s, 1H, C-NH), 7.08-7.00 (m, 2H, Ar-H), 2.22 (s, 3H, -CH_3), 2.01 (s, 1H, N-NH), 1.43-1.39 (m, 4H, Ali-H), 1.24-1.21 (m, 6H, Ali-H); **^{13}C NMR** ($CDCl_3$, 100 MHz δ in ppm) 169.41, 157.23, 156.45, 133.11, 128.23, 124.25, 117.86, 114.86, 110.11, 86.93, 79.90, 34.20, 24.51, 21.91;

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Anal. Calcd. for $C_{22}H_{20}N_6O$: C, 68.73; H, 5.24; N, 21.86; found C, 68.77; H, 5.22; N, 21.80 %; **LCMS** (MM:ES+APCI) $(M+H)^+$ 385.

General procedure for the synthesis of 1,6-diamino-2-oxo-4-phenyl substituted-1,2-dihydropyridine-3,5-dicarbonitriles

In a 50 mL round bottom flask, aldehyde (1.0 eq), malononitrile (1.0 eq), tetra butyl ammonium bromide solution was taken and stirred for 10 min at 50ºC, followed by addition of 2-cyanoacetohydrazide (1.0 eq) and stirring at 70ºC for 10 min (Scheme 2). The progress of the reaction was monitored using TLC and after completion of the reaction, solid was filtered, dried and purified by column chromatography.

Synthesis of 1,6-diamino-2-oxo-4-phenyl-1,2-dihydropyridine-3,5-dicarbonitrile (6a)

Melting point 238-240ºC.[40]

Synthesis of 1,6-diamino-4-(4-bromophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (6b)

The product **6b** was obtained from 4-bromobenzaldehyde (1.0 eq), malononitrile (1.0 eq) and 2-cyanoaceto hydrazide (1.0 eq). This compound was obtained as light brown color solid with 95% yield. Melting point: 232-234 ºC IR (KBR ν in cm^{-1}): 3406, 2244, 1674; **^{1}H NMR (400 MHz, DMSO-d_6, δ in ppm):** 5.63(s, 2H, N-NH_2), 7.48-7.51 (m, 2H, Ar-H), 7.57-7.62 (m, 2H, Ar-H), 8.47 (s, 2H, C-NH_2); **^{13}C NMR (100 MHz, DMSO-d_6, δ in ppm):** 159.98, 159.89, 159.75, 156.98, 130.33, 130.29, 124.98, 116.89, 116.33, 115.76, 86.20, 74.40; **Anal. Calcd.for $C_{13}H_8BrN_5O$**: C, 47.29; H, 2.44; N, 21.21; found C, 47.19; H, 2.58; N, 21.28%; **LCMS** (MM:ES+APCI) $[M-1]^-$ 330.

Synthesis of 1,6-diamino-2-oxo-4-(4-propoxyphenyl)-1,2-dihydropyridine-3,5-dicarbonitrile (6c)

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The product **6c** was obtained from 4-propoxybenzaldehyde (1.0 eq), Malononitrile (1.0 eq) and 2-cyanoaceto hydrazide (1.0 eq). This compound was obtained as light brown color solid with 95% yield. Melting point: 226-228 ºC, IR (KBR ν in cm^{-1}): 3395, 2250, 1678; **^{1}H NMR (400 MHz, DMSO-*d*$_6$, δ inppm):** 0.99 (t, 3H, CH_3), 1.72 (m, 2H, **CH_2**-CH_3), 3.99 (t, 2H, **CH_2**-CH_2), 5.60 (s, 2H, N-NH_2), 7.03 (d, 2H, J = 8.8, Ar-H), 7.38 (d, 2H, J = 9.2, Ar-H), 8.35 (s, 2H, C-NH_2); **^{13}C NMR (100 MHz, DMSO-*d*$_6$, δ in ppm):** 159.77, 158.23, 157.29, 157.07, 132.99, 129.93, 128.29, 116.94, 115.92, 113.04, 112.79, 110.72, 86.94, 74.84, 69.92, 22.74, 11.84; **Anal. Calcd.for $C_{16}H_{15}N_5O_2$**: C, 62.13; H, 4.89; N, 22.64 ; found C, 61.99; H, 4.68; N, 22.78% ; **LCMS** (MM:ES+APCI) $[M-1]^-$ 308.

Synthesis of 1,6-diamino-4-(3,4-dimethoxyphenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (6d)

Melting point 291-292 ºC.[36]

Synthesis of 1,6-diamino-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (6e)

Melting point 3359- 361 ºC.[36]

Synthesis of 1,6-diamino-4-(4-(methylsulfonyl)phenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (6f)

The product **6f** was obtained from 4-(methylsulfonyl)benzaldehyde (1.0 eq), Malononitrile (1.0 eq) and 2-cyanoaceto hydrazide (1.0 eq). This compound was obtained as light brown color solid with 97% yield., IR (KBR ν in cm^{-1}): 3390, 2245, 1676; **^{1}H NMR (400 MHz, DMSO-*d*$_6$, δ in ppm):** 3.29 (s, 3H, -CH_3), 5.66 (s, 2H, N-NH_2), 7.74-7.77 (m, 2H, Ar-H), 8.07-8.10 (m, 2H, Ar-H), 8.53(s, 2H, C-NH_2); **^{13}C NMR (100 MHz, DMSO-*d*$_6$, δ in ppm):** 159.77, 159.68, 157.09, 150.75, 148.69, 126.90, 126.11, 117.08, 116.22, 86.08, 74.59, 45.16; **Anal.**

Calcd.for $C_{14}H_{11}N_5O_3S$: C, 51.06; H, 3.37; N, 21.27 ; found C, 50.99; H, 3.48; N, 21.38%; **LCMS** (MM:ES+APCI) $[M-1]^-$ 328.

Synthesis of 1,6-diamino-4-(3-bromo-4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (6g)

The product **6g** was obtained from 3-bromo-4-methoxy benzaldehyde (1.0 eq), malononitrile (1.0 eq) and 2-cyanoaceto hydrazide (1.0 eq). This compound was obtained as brown solid with 93% yield. IR (KBR ν in cm^{-1}): 3394, 2242, 1669; **^{1}H NMR (400 MHz, DMSO-d_6, δ in ppm):** 3.91 (s, 3H, $-OCH_3$), 5.63 (s, 2H, $N-NH_2$), 7.47-7.50 (m, 2H, Ar-H), 7.71 (s, 1H, Ar-H), 8.40 (s, 2H, $C-NH_2$); **^{13}C NMR (100 MHz, DMSO-d_6, δ in ppm): :** 159.62, 158.14, 157.19, 157.02, 132.89, 129.83, 128.29, 116.84, 115.97, 113.03, 112.89, 110.87, 86.84, 74.84, 56.37; **Anal. Calcd.for $C_{14}H_{10}BrN_5O_2$**: C, 46.69; H, 2.80; N, 19.44 ; found C,46.50; H,2.71; N, 19.29%; **LCMS** (MM:ES+APCI) $[M-2]^-$ 358.

Synthesis of 1,6-diamino-4-(4-hydroxyphenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (6h)

The product **6h** was obtained from 4-hydroxy benzaldehyde (1.0 eq), Malononitrile (1.0 eq) and 2-cyanoaceto hydrazide (1.0 eq). This compound was obtained as light brown solid with 94% yield. IR (KBR ν in cm^{-1}): 3401, 2235, 1668; **^{1}H NMR (400 MHz, DMSO-d_6, δ in ppm):** 5.62 (s, 2H, $N-NH_2$), 5.81 (s, 1H, O-H), 7.02-7.04 (m, 2H, Ar-H), 7.07-7.09 (m, 2H, Ar-H), 8.36 (s, 2H, $C-NH_2$); **^{13}C NMR (100 MHz, DMSO-d_6, δ in ppm):** 160.01, 159.82, 159.75, 157.10, 130.39, 130.25, 125.24, 117.14, 116.26, 115.67, 86.16, 74.56; **Anal. Calcd.for $C_{13}H_9N_5O_2$**: C, 58.43; H, 3.39; N, 26.21 ; found C,58.50; H,3.25; N, 26.29%; **LCMS** (MM:ES+APCI) $[M-1]^-$ 266.

Synthesis of 1,6-diamino-4-(1H-indol-3-yl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (6i)

The product **6i** was obtained from indole-3-carbaldehyde (1 eq), Malononitrile (1.0eq) and 2-cyanoaceto hydrazide (1eq). This compound was obtained as brown solid with 94% yield. IR (KBR ν in cm^{-1}): 3402, 2240, 1670; **^{1}H NMR (400 MHz, DMSO-*d*$_6$, δ in ppm):** 5.60 (s, 2H, N-NH_2), 7.08-7.20 (m, 2H, Ar-H), 7.44-7.48 (m, 2H, Ar-H), 7.78 (s, 1H, Ar-H), 8.25 (s, 2H, C-NH_2), 11.81(s, 1H, Ind-NH); **^{13}C NMR (100 MHz, DMSO-*d*$_6$, δ in ppm):** 160.12, 157.94, 157.34, 157.17, 133.04, 132.84, 130.03, 129.77, 128.29, 116.97, 115.84, 113.03, 112.72, 111.03, 87.3, 74.96; **Anal. Calcd.for $C_{15}H_{10}N_6O$**: C, 62.06; H, 3.47; N, 28.95 ; found C,62.15; H,3.35; N, 28.79%, **LCMS** (MM:ES+APCI) $[M-1]^-$ 289.

Pharmacology

MTT assay

The antiproliferative effect of triazolo-pyridines towards lung cancer cells were investigated by the MTT dye uptake method as described earlier.[41, 42] Briefly, A549 cells ($2.5X10^4$/ml) were incubated in triplicate in a 96-well plate in the presence or absence of the indicated concentrations of compounds in a final volume of 0.2 ml for different time intervals at 37°C. Thereafter, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well. After 2h of incubation at 37°C, 0.1 ml of lysis buffer (20% SDS, 50% dimethylformamide) was added and incubated 1h at 37 °C, and subsequently, the optical density was measured at 570 nm.

PDE4 assay

The PDE4 inhibition assay was performed at Prof. Manojith Pal's laboratory, Institute of Life Sciences, University of Hyderabad, Hyderabad, India. The PDE4 inhibitory activity of newly synthesized compounds as described previously.[43]

In silico analysis

A crystal structure of PDE4 (PDB ID 3D3P) was retrieved from the Protein Data Bank [44] and prepared for docking using the Protein Preparation Wizard [45] in Maestro [46] of default

settings and keeping only water molecule with at least three hydrogen bond interactions. The docking was done using Glide SP at default settings.[47-49]

Conclusions

The library of new triazole-pyridine conjugates was synthesized as anticancer agents targeting PDE4. All the new compounds were investigated for anticancer activity against lung cancer cells. Compound 5h (7-(4-(methylsulfonyl)phenyl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile) and compound **5j** (7-(1-methyl-1H-imidazol-2-yl)-5-oxo-3,5-dihydro-1H-spiro[[1,2,4]triazolo[1,5-a]pyridine-2,1'-cyclohexane]-6,8-dicarbonitrile) showed good cytotoxicity. Further investigation revealed that **5j** displayed good inhibition *in vitro* towards PDE4 at a single dose (10 μM). In silico analysis conclusively supported the *in vitro* results and docking predicted hydrogen bond interactions with His-234 and a water molecule anchored to Asp-392 and Asn-395. Overall, this is a preliminary report that presents compound **5j** as an inhibitor of PDE4 and further chemical modifications of the lead structure may yield a potent inhibitor.

Author Contributions

HKK, SM, HB, SR, and FS performed the experiments. AB, CDM, B, KSR, and RB designed the experiments and provided the resources. CDM, B, and KSR wrote the paper.

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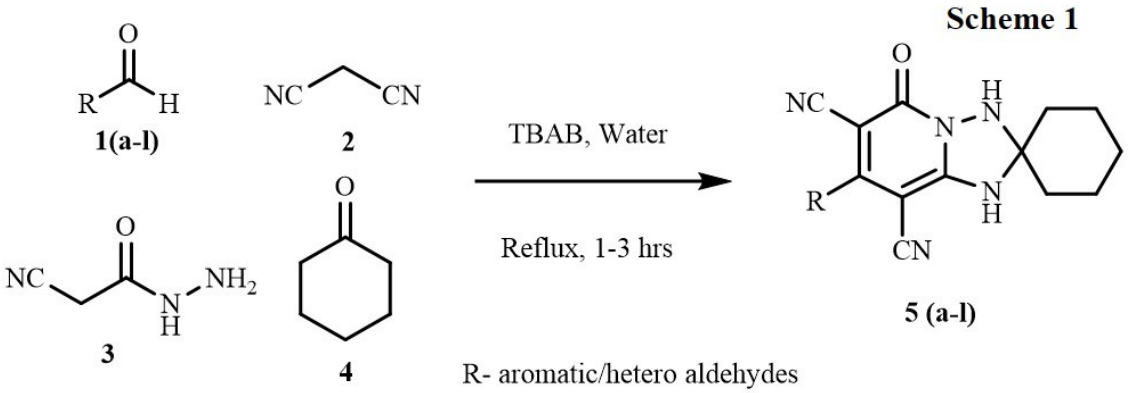
Figure legends

Scheme 1 and 2: Synthetic procedure for the preparation of substituted pyridines.

Figure 1: A. The predicted binding mode of the best-scored compound. B. Docking predicts hydrogen bond interactions with His-234 and a water molecule anchored to Asp-392 and Asn-395. The compound also forms a face to face stacking interaction with Phe-446 and an edge to face interaction with Phe-414.



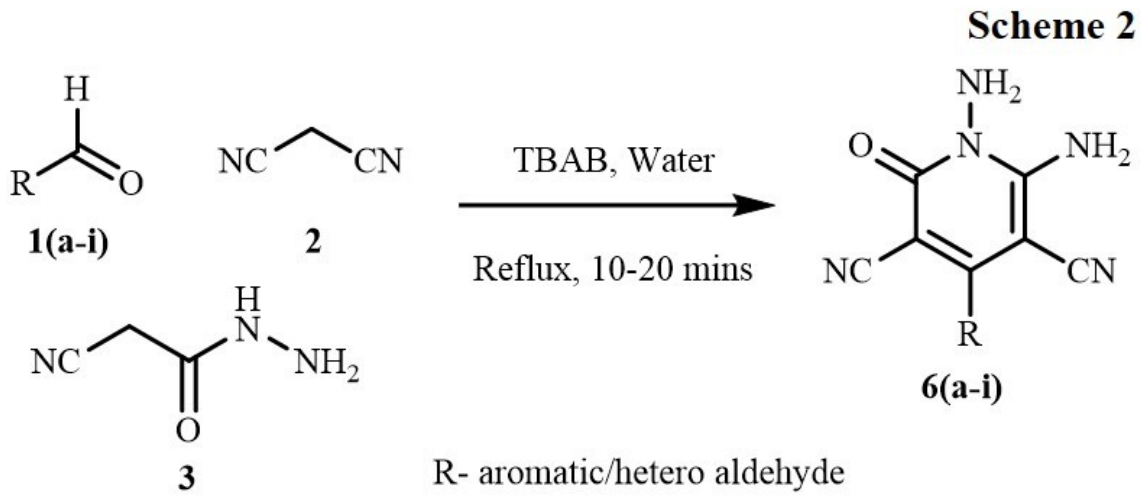


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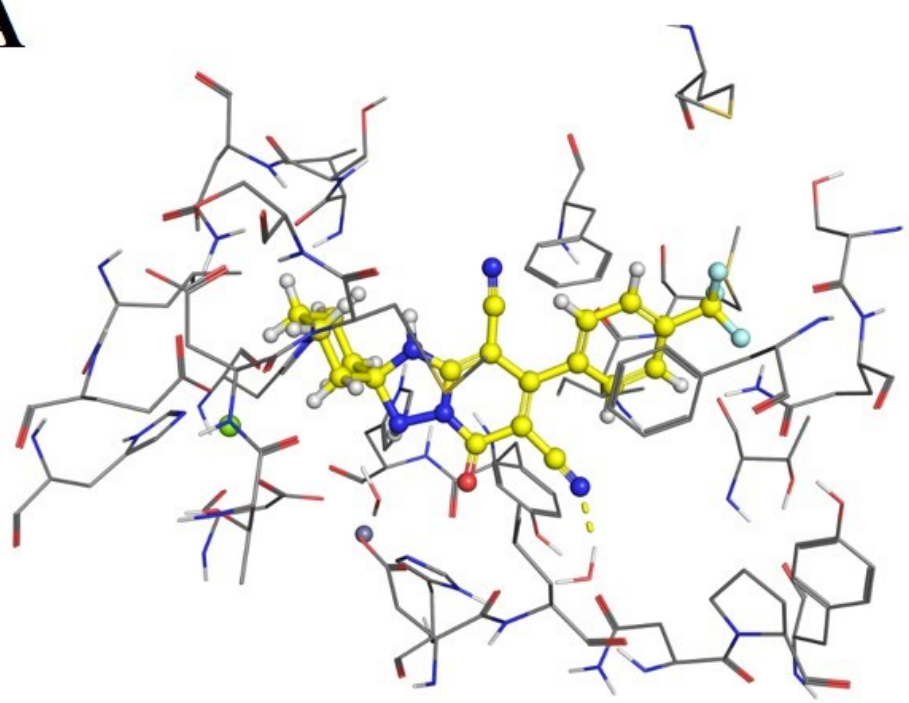
Figure 1

A



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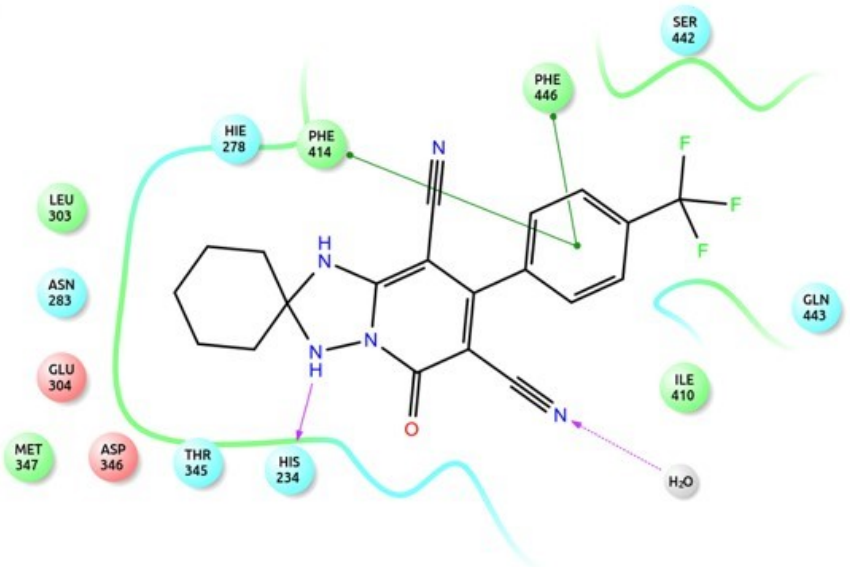

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Graphical abstract

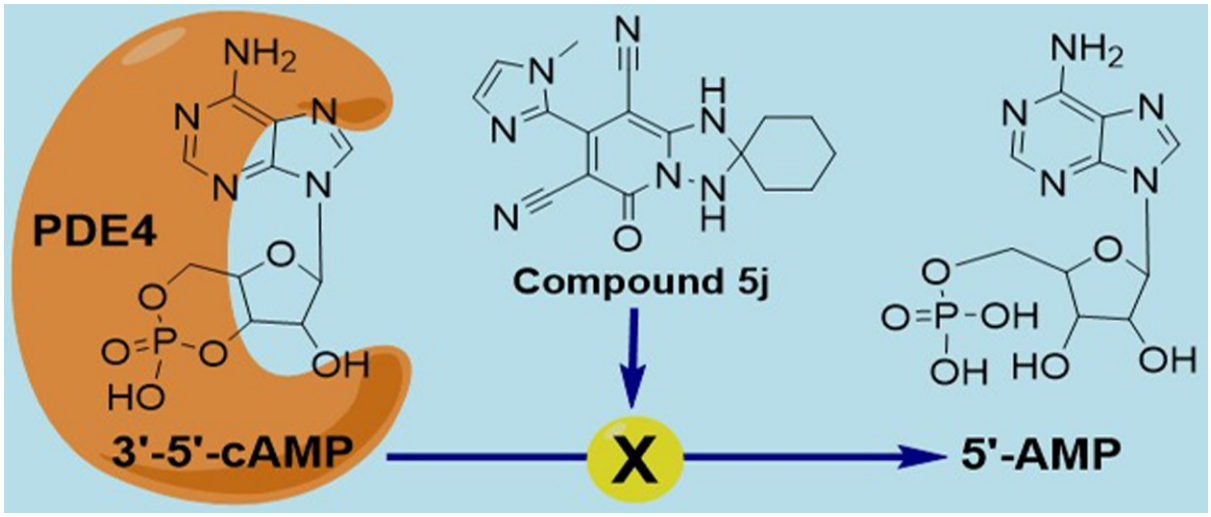

Table 1: Optimization of a solvent-catalyst system for the preparation of **5a**

Entry	Solvent	Catalyst	Time (h)	Yield [a] (%)
1	Water	Piperidine	8	-
2	Water	Triethylamine	8	-
3	Water	TBAB	1	92
4	Water	Sodium Silicate	2.5	63
5	Water	p-TSA	3	Traces
6	Ethanol	Piperidine	3	58
7	Ethyl acetate	TBAB	10	Traces
8	Toulene	TBAB	10	Traces
9	Ethanol:Water (5:5)	TBAB	6	72

Table 2: Reaction conditions employed for the synthesis of substituted pyridines

Entry	R	5(a-l)	Time (H)	6(a-i)	Time (min)

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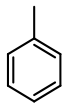
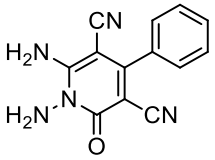
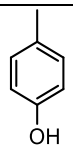
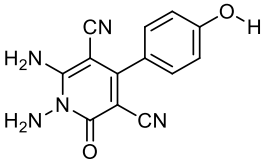
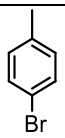
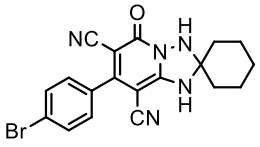
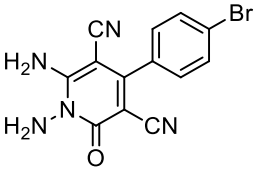
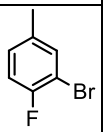
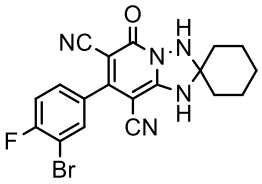
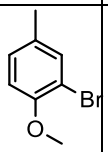
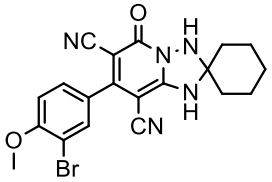
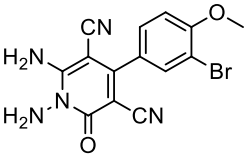
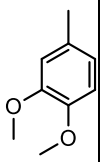
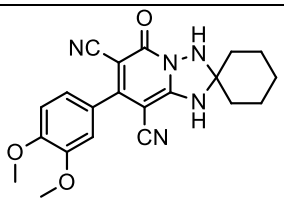
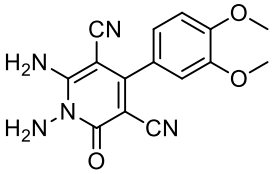
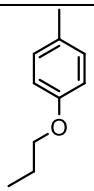
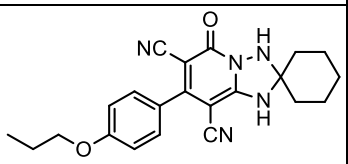
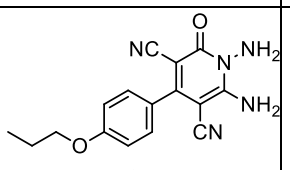
1		-	-	 6a	10[a]
2		-	-	 6h	15
3		 5a	1	 6b	12
4		 5b	1.5	-	-
5		 5c	1	 6g	15
6		 5d	1	 6d	10[a]
7		 5e	1	 6c	10

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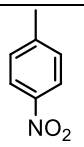
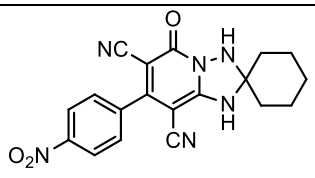
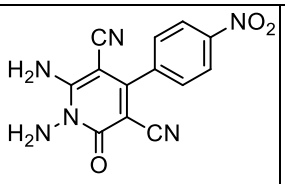
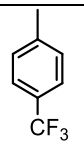
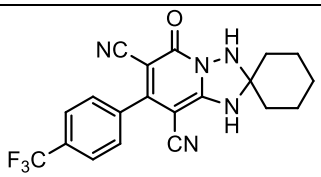
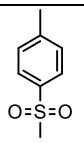
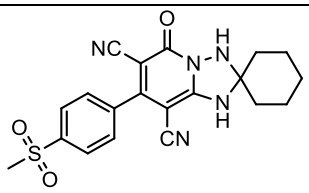
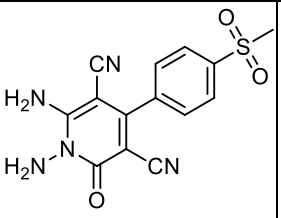
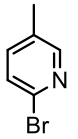
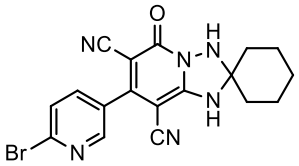
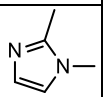
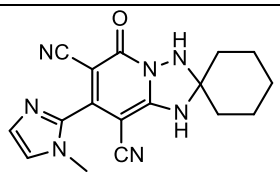
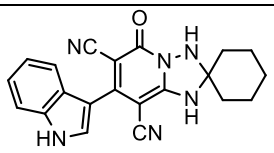
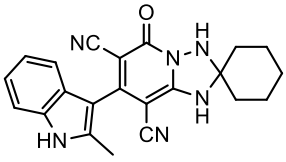
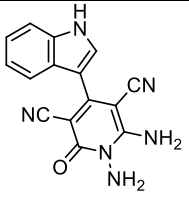
8		 5d	1.5	 6e	18[a]
9		 5f	1.5	-	-
10		 5h	2	 6f	10
11		 5i	2	-	-
12		 5j	2.5	-	-
13		 5k	2.5	-	-
14		 5l	3	 6i	20

10.1002/cbdv.201900234

[a] Literature reported compounds

Table 3: Substituted pyridines showing IC_{50} values and % inhibition studies of human lung cancer cells and PDE4 enzymes.

Compound	A549 Cells IC_{50} (µM)	Average PDE4 inhibition (%)
5a	28.1±0.11	22.09
5b	35.5±0.12	-0.39
5c	26.1±0.15	33.86
5d	NA	34.90
5e	30.2±0.32	-
5f	25±0.6	-
5g	NA	-
5h	15.2±0.12	27.40
5i	30.2±0.05	-
5j	**24.1±0.03**	**50.90**
5k	35.6±0.23	1.81
5l	31.2±0.25	44.80
6a	NT	-
6b	NT	6.61
6c	NT	10.75
6d	NT	1.67
6e	NT	26.25
6f	NT	-1.36
6g	NT	12.07

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6h	NT	9.77
Rolipram	-	87.30
Paclitaxel	0.0046	-
Paclitaxel represents the positive control and the IC_{50} value of paclitaxel is taken from our previous report.[41]		