Modulating the Cyclic Guanosine Monophosphate Substrate Selectivity of the Phosphodiesterase 3 Inhibitors by Pyridine, Pyrido[2,3-*d*]-pyrimidine Derivatives and Their Effects upon the Growth of HT-29 Cancer Cell Line

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Analogues with the scaffolds of 3-cyano-4-alkoxyphenyl-6-bromoaryl-2-pyridone and 2-amino-3-cyano-4-alkoxyphenyl-6-bromoarylpyridine were synthesized. Cyclization of the 2-amino derivatives with formic acid and formamide gave the corresponding pyrido[2,3-*d*]pyrimidin-4(3*H*)-one and the pyrido[2,3-*d*]-pyrimidin-4-amine derivatives, respectively. Active phosphodiesterase 3 (PDE3) inhibitors were identified from each of the four aforementioned scaffolds. This is the first report that pyrido[2,3-*d*]pyrimidin-4(3*H*)-one and pyrido[2,3-*d*]pyrimidin-4-amine derivatives can inhibit PDE3. The analogues with the pyridone and pyrido[2,3-*d*]pyrimidin-4(3*H*)-one scaffolds inhibited both cAMP and cyclic guanosine monophosphate (cGMP) hydrolysis by PDE3, while the amine containing scaffolds were more selective for cGMP hydrolysis. This observation may set the base for substrate-selective pharmacological modulation of this important class of drug targets and with less side effects, particularly tachcardia. The dual inhibitors of PDE3 were more potent inhibitor towards the growth of HT-29 cancer cell lines.

Key words phosphodiesterase 3; substrate-modulation; pyridine derivative

Phosphodiestrases (PDEs) are large family of enzymes that initiate the hydrolysis of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) into adenosine monophosphate (AMP) and guanine monophosphate (GMP), respectively; thus regulating a wide range of biological responses stimulated by various signaling molecules such as hormones, neurotransmitters, and cytokines.[1] The superfamily of PDEs comprises 11 different isozyme families; each having different isoforms of each isozyme. It is known that PDE1, 2, 3, 10, 11 isozymes can hydrolyze both cAMP and cGMP, while PDE4, 7, 8 hydrolyze cAMP, and PDE5, 6, 9 hydrolyze cGMP. The specificity of a PDE isozyme to bind cAMP and/or cGMP depends on the orientation of the terminal carboxamide of the conserved glutamine residue in the catalytic site of the respective PDE, where the neighboring residues serve to anchor the glutamine into different orientations that define the specificity for either or both cAMP and cGMP.[2–4]

PDE3 isozymes are also unique by their sensitivity to a number of specific inhibitors, including milrinone, cilostamide, enoximone, and lixazinone (Fig. 1), which are chemically related as being 2-pyridone, cyclic amides or 3-cyano-2-pyridone derivatives. PDE3 inhibitors are mainly used as cardiotonics or for the treatment of peripheral artery disease. Additional experimental uses are treatment of cystic fibrosis and cancer, although these indications may be limited by toxicity relating to the activation of protein kinase A (PKA) as a consequence of elevating intracellular cAMP levels.[5–7]

Previously, our laboratory reported a multi-component reaction that leads to the formation of cyanopyridones and their isosteric cyanoiminopyridines with anticancer activity towards leukemia and colorectal cancer cell line HT-29.[8,9] Based on the above findings, it deemed of interest to combine the chemicals and biological concepts and design novel molecules and chemical scaffolds derived from the above mentioned derivatives, namely: 3-cyano-4,6-disubstituted-2-pyridones, 2-amino-3-cyano-4,6-disubstituted pyridines, 5,7-diaryl-pyrido[2,3-*d*]pyrimidin-4(3*H*)-one and 5,7-diaryl-pyrido[2,3-*d*]pyrimidin-4-amine derivatives and their evaluation as PDE3B inhibitors and tumor cell growth inhibition.

Chemistry The general synthesis of the 3-cyano-2-pyridone and the 3-cyano-2-aminopyridine derivatives is illustrated in Chart 1, utilizing an in-solution, one-step synthesis. Whereby, the respective aromatic ketone or aldehyde, ammonium acetate and ethyl cyanoacetate or malononitrile,

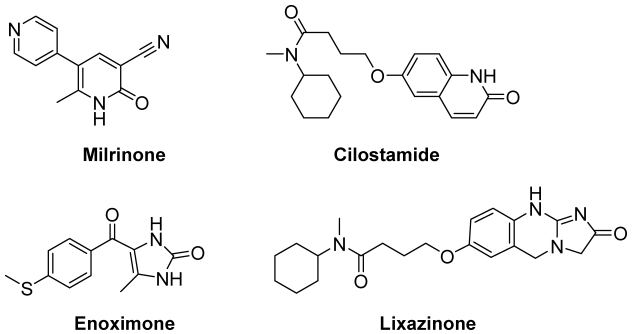

Fig. 1. Chemical Structures of 2-Pyridone or 3-Cyano-2-pyridone Containing PDE3 Inhibitors, Namely: Milrinone (Upper Left), Cilostamide (Upper Right), Enoximone (Lower Left), and Lixazinone (Lower Right)

The authors declare no conflict of interest.

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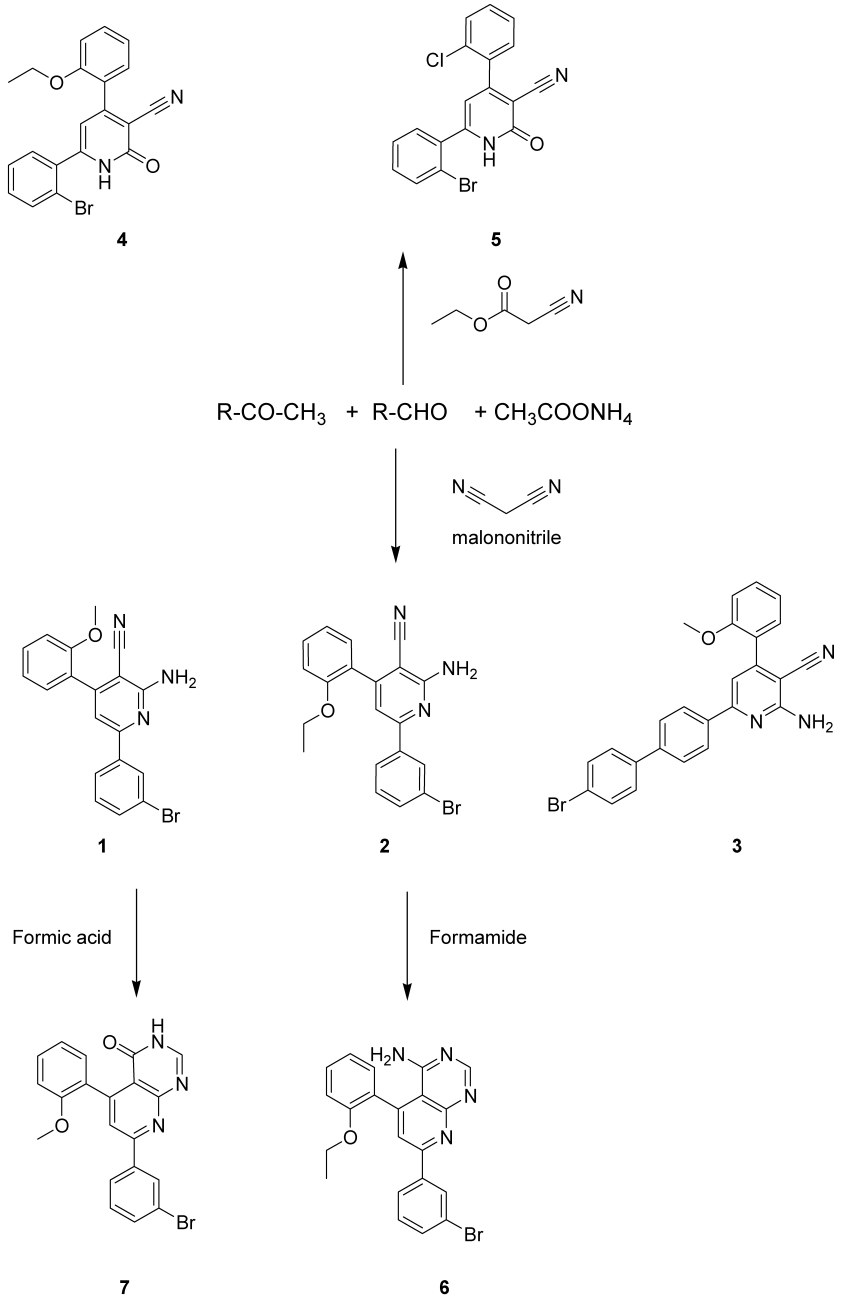
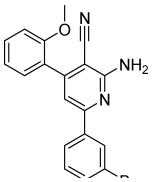
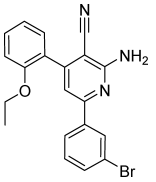
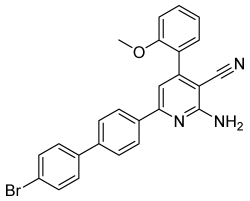
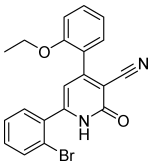
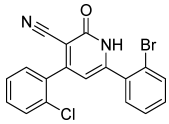
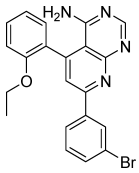
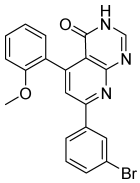


Chart 1

were refluxed in ethanol. The desired compounds were purified by re-crystallization from a mixture of *N*,*N*-dimethylformamide (DMF)–ethanol in different ratios or by column chromatography. Cyclization to the corresponding 5,7-diaryl-pyrido[2,3-*d*]pyrimidin-4(3*H*)-one and 5,7-diaryl-pyrido[2,3-*d*]pyrimidin-4-amine derivatives **6**, **7** was accomplished by refluxing in formic acid or formamide, respectively which act both as solvents and reactants. In the ^{1}H-NMR spectra compounds **1**–**5** showed aromatic protons as multiplet peaks at δ 6.95–8.50 ppm. The aromatic proton at position 5 of the pyridine ring appeared as singlet peak at about 6.90 ppm. Mass spectrometry of the synthesized compounds showed molecular ion peak [M^+] corresponding to either the exact mass or the molecular weight of the target compounds. The molecular ion peaks were also the base peaks indicating the stable nature of these compounds. All compounds are bromine containing derivatives and showed molecular ion peak at [M^+] and [M^++2] in a ratio of almost 1 : 1 due to the isotopic nature of the bromine atom. Infrared spectra of all compounds **1**–**5** showed bands at about 3100–3480 cm^{-1} (NH stretching) stretching and a band at 2200 cm^{-1} due to –CN stretching, the latter disappears in compounds **6** and **7**. The 3-cyano-2-pyridone derivatives showed an extra band at 1660–1700 cm^{-1} for the pyridone carbonyl amide stretching.

Table 1. Inhibitory Effect of Compounds **1**–**7** upon the Growth of the Colorectal HT-29 Cancer Cell Line and Phosphodiesterase 3B

Compound No.	Chemical structure	HT-29 growth inhibition		PDE3 inhibition			
		% Growth inhibition at 50 μM	IC_{50} μM	% PDE3 inhibition at 50 μM		IC_{50} μM	
				cAMP	cGMP	cAMP	cGMP
1		31.14	ND	42.75	104.6	ND	4
2		78.73	43	22.90	38.50	ND	ND
3		75	32	55	85	35.6	13.4
4		68	15.5	70	72	14.7	11.8
5		81	10.4	77	75	6.2	10.5
6		86.10	18.27	48	85	ND	10.91
7		97.98	1.34	62	78.6	24.21	10.75
8	Milrinone	10	>50	77	95	11.4	3.6

Results and Discussion

All compounds were tested for their ability to inhibit PDE3B using the recombinant enzyme and tumor cell growth using the human HT-29 colon tumor cell line, which is known to express multiple PDE isozymes, including PDE3. Compounds were evaluated in 2 steps; first, the percentage inhibition at a concentration of 50 μM was performed in triplicate, the compounds displaying >50% were evaluated by testing a range of 10 concentrations with at least two replicates per concentration to calculate an IC_{50} value. The results are summarized in Table 1.

For the aminopyridine derivatives **1**–**3**, compound **2** did not inhibit PDE3 by >50% at 50 μM, regardless of whether cAMP or cGMP were used as substrates. Compound **1** selectively

inhibited PDE3 with an IC_{50} of 4 μM when cGMP was used as the substrate, but did not cause >50% inhibition at the 50 μM screening concentration in the case of cAMP. In addition, the bulkier compound **3** inhibited PDE3B when either cAMP or cGMP were used as substrates, but similar to compound **1** was selective inhibition of cGMP hydrolysis compared with cAMP hydrolysis with IC_{50} values of 13.4 and 35.6 μM, respectively.

Only compounds **2** and **3** showed modest tumor cell growth inhibitory potency with IC_{50} values of 43.0 and 32 μM, respectively. Compound **1** did not cause >50% inhibition at the 50 μM screening concentration. Relative to compounds **4**–**7**, compounds **1**–**3** displayed the lowest tumor cell growth activity and PDE3B (using cAMP as a substrate) inhibitory activity. Cyclization of compound **2** by formamide resulted into compound **6** that did not inhibit PDE3 when cAMP was used as the substrate and with the third least active to inhibit tumor cell growth. However, compound **6** inhibited PDE3 with an IC_{50} of 10.9 μM using cGMP as the substrate. The development of substrate selective inhibitors to dual acting enzymes as PDE3 is a new development and opens the horizons towards modulating the level of one substrate rather than the other, which may have safety and/or efficacy consequences for drug discovery.

The 2-pyridone derivatives **4** and **5** and the cyclized pyridopyrimidone derivative **7** inhibited tumor cell growth with IC_{50} as low as 1.34 μM for compound **7**. These three compounds effectively inhibited PDE3 with cAMP and cGMP as substrates. This suggests that inhibition of both cAMP and cGMP hydrolysis to increase intracellular levels of both signaling molecules may be optimal for anticancer activity. Since the PDE3 and the growth inhibitory activities are not in parallel, thus other phosphodiesterases and/or off targets may be contributing to the anticancer activity, this is confirmed by the inactivity of the positive control milrinone to induce apoptosis to the HT-29 cell line although of its dual inhibition to PDE3.

Docking of compound **4** and compound **7** to the catalytic domain of PDE3B (PDB 1SOJ) using MOE software[10] showed that the bromophenyl is involved in π–π stacking with the phenyl of Phe991, while the –C=O and –CN functions of **4** are involved indirectly through H_2O molecules with multiple interactions including Mg ions, Tyr736, His737, His741, Asp822, Asp937. With regard to compound **7**, its bromophenyl group is also involved in π–π stacking with the phenyl of Phe991, while the carbonyl and amino of the newly formed ring are involved in interaction indirectly through H_2O molecules with Mg ions, His821, Asp822, Glu851, His854, Thr893 and Asp937, Fig. 2. It is also worthy to mention that the ligand (IBMX) in 1SOJ showed 4 interactions with Gln988 through H bonding, with Phe991 through π–π stacking and with Tyr736 and Asp937 through H_2O molecules,[11] Fig. 2. In our compounds, the higher electronegativity of the oxo substituents at position 2 on the pyridine rather than the amino, may explain the stronger ability of cyanopyridones rather than the aminocyanopyridines to establish indirect hydrogen bonding and the more efficient inhibition of PDE3.

From a conformational point of view, it seems that an alkoxy substituent at the *ortho* position of the phenyl at position 4 and a bromo substituent at the *ortho* position of the phenyl at position 6 will lead to non-coplanarity between the 2 aryls and the pyridone, as in Fig. 3. The enforcement of non-coplanarity of the bromophenyl seems essential to allow π–π stacking with Phe991.

In conclusion, selective inhibition of cGMP but not of the cAMP substrate hydrolysis of PDE3 by replacement of the oxygen atom of cyano-2-pyridones by an amino may set the base for substrate-selective pharmacological modulation of this important class of drug targets.

Experimental

Chemistry All reactions were performed with commercially available reagents and were used without further purification. Solvents were dried by standard methods and stored over molecular sieves. All reactions were monitored by thin-layer chromatography (TLC) carried on fluorescent precoated plates and detection of the components was made by short UV light. Melting points were determined in open capillaries using MEL-TEMP II and Buchi B-540 melting point apparatus and are uncorrected. FT-IR spectra were recorded on Nicolet Avatar 380 spectrometer. ^{1}H-NMR spectra were recorded on Varian Mercury VX-300 MHz spectrometer. Mass spectra were obtained with Hewlett Packard GC-MS, model 5890, series II at an ionization potential of 70 eV. Elemental analyses were performed by the Microanalytical Unit, Faculty of Science, Cairo University. All values were within ±0.4% of the theoretical ones, unless otherwise indicated. Yields were not optimized

General Procedure for the Preparation of 4,6-Diaryl-2-imino-1,2-dihydropyridine-3-carbonitrile (1–3) The respective aromatic ketone (1 mmol), together with the respective aromatic aldehyde (1 mmol), malononitrile (1 mmol), and ammonium acetate (8 mmol) were dissolved in ethanoll (30 mL) and put under reflux for 10–12 h. The precipitate obtained was filtered, washed with ethyl alcohol and dried. For the purification purpose, the precipitate was subjected either to re-crystallization from a mixture of DMF–ethanol (1 : 10), or to column chromatography on silica gel, eluting with chloroform and/or methylene chloride.

2-Amino-6-(3-bromophenyl)-4-(2-methoxyphenyl)-nicotinonitrile (1) Synthesized Using 3-Bromoacetophenone and 2-Methoxybenzaldehyde: Yield 80%, mp 276–274°C; IR (cm^{-1}) 2205 (–C≡N), 3482 (NH); ^{1}H-NMR (DMSO-d_6) δ: 3.89 (s, 3H, $-OCH_3$), 7.08–7.13 (t, 1H, aromatic), 7.20–7.54 (m, 5H, aromatic), 7.63 (d, 1H, aromatic), 7.72–7.75 (d, 1H, aromatic), 7.81 (s, 1H, aromatic); MS (electron ionization (EI)) *m/z* 379 (M^+; 100%), *m/z* 381 (M^++2); *Anal.* ($C_{19}H_{14}BrN_3O$) C, H, N.

2-Amino-6-(3-bromophenyl)-4-(2-ethoxyphenyl)nicotinonitrile (2) Synthesized Using 3-Bromoacetophenone and 2-Ethoxybenzaldehyde: Yield 60%; mp 235–237°C; IR (cm^{-1}) 2222 (CN), 2284 (NH); ^{1}H-NMR (DMSO-d_6) δ: 1.36–1.40 (t, 3H, CH_3), 4.12–4.19 (q, 2H, $-OCH_2$), 7.05–7.10 (t, 1H, aromatic), 7.14–7.17 (d, 1H, aromatic), 7.25 (s, 1H, aromatic), 7.43–7.52 (m, 2H, aromatic), 7.60–7.62 (d, 1H, aromatic), 7.70–7.78 (t, 2H, aromatic), 7.89–7.93 (d, 1H, aromatic); MS (EI) *m/z* 393 (M^+; 100%), *m/z* 395 (M^++2); *Anal.* ($C_{20}H_{16}BrN_3O$) C, H, N.

2-Amino-6-(4'-bromobiphenyl-4-yl)-4-(2-methoxyphenyl)nicotinonitrile (3) Synthesized from 1-(4′-Bromobiphenyl-4-yl)ethanone and -2-Methoxybenzaldehyde: Yield 85%; mp 210–212°C; IR (cm^{-1}) 2213 (–CN–), 3338 (–NH–); ^{1}H-NMR (DMSO-d_6) 3.91 (s, 3H, $-OCH_3$), 6.57 (s, 1H, C-5 pyridine), 7.27–7.59 (m, 4H, aromatic), 7.60–7.73 (m, 5H,

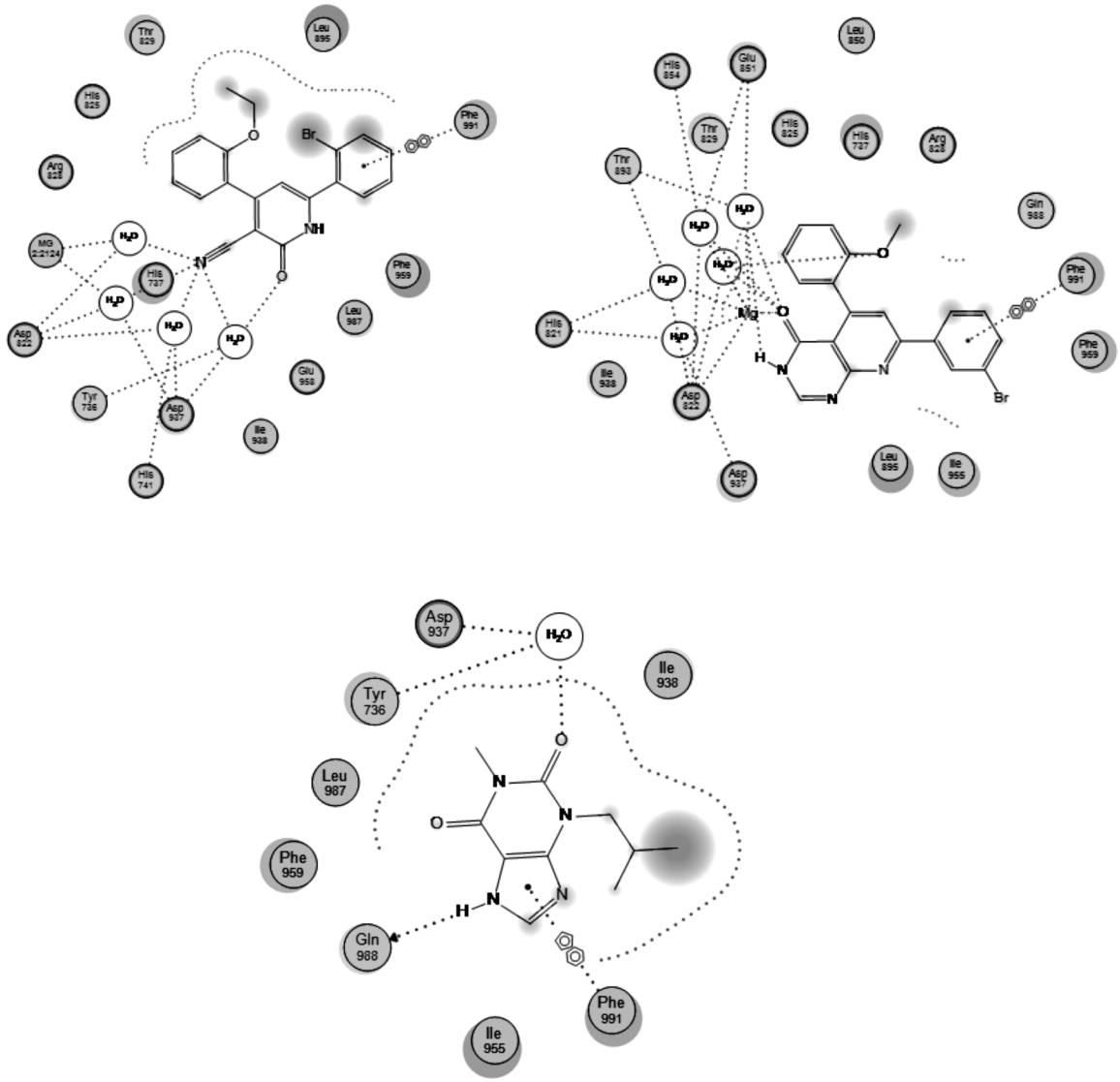


Fig. 2. Detailed 2D Interaction of Compound **4** (Upper Left), **7** (Upper Right) and the Ligand IBMX (Lower) with the Catalytic Site of Human PDE3B

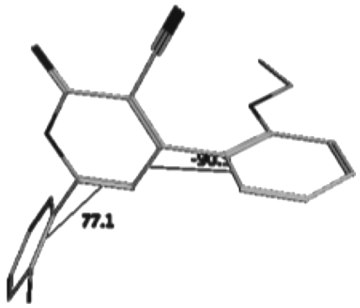


Fig. 3. The Energy Minimized Form of Compound **4** Showing Non-coplanarity of the Substituents at Position 4 and Position 6 Relative to the Pyridine

The non-coplanarity of the bromophenyl may be responsible for the potential π–π stacking with Phe991.

aromatic), 7.75–8.29 (m, 5H, aromatic+NH_2) ; MS (EI) *m/z* 455 (M^+; 100%), *m/z* 457 (M^++2); *Anal.* ($C_{25}H_{18}BrN_3O$) C, H, N.

General Procedure for the Preparation of 4,6-Diaryl-2-oxo-1,2-dihydropyridine-3-carbonitriles (4, 5) The respective aromatic ketone (1 mmol), together with the respective aromatic aldehyde (1 mmol), ethyl cyanoacetate (1 mmol), and ammonium acetate (8 mmol) were dissolved in ethyl alcohol (30 mL) and put under reflux for 10 to 12 h. The precipitate obtained was filtered, washed with ethyl alcohol and dried. For the purification purpose, the precipitate was subjected either to re-crystallization from a mixture of DMF–ethanol (1 : 10), or to column chromatography on silica gel, eluting with chloroform.

6-(2-Bromophenyl)-4-(2-ethoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (4) Synthesized from 2-Bromoacetophenone and 2-Ethoxybenzaldehyde: Yield 55%; mp 237–239°C; IR (cm^{-1}) 1699 (–CO–), 2225 (–CN–), 3616 (–NH–); ^{1}H-NMR ($CDCl_3$) 1.32–1.45 (t, 3H, $-CH_3$), 4.10–4.53 (q, 2H, $-O-CH_2-CH_3$), 6.55 (s, 1H, C-5 pyridone), 6.90–7.10 (m, 2H, aromatic), 7.07–7.31 (m, 2H, aromatic), 7.32–7.52 (d, 2H, aromatic), 7.72–7.75 (d, 2H, aromatic); MS (EI) *m/z* 394 (M^+; 100%), *m/z* 396 (M^++2); *Anal.* ($C_{20}H_{15}BrN_2O_2$) C, H, N.

6-(2-Bromophenyl)-4-(2-chlorophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (5) Synthesized from 2-Bromoacetophenone and 2-Chlorobenzaldehyde: Yield 86%;

mp 269–271°C; IR (cm^{-1}) 1657 (–CO–), 2220 (–CN–), 3300 (–NH–); ^{1}H-NMR ($CDCl_3$) 6.52 (s, 1H, C-5 pyridone), 7.26–7.54 (d, 5H, aromatic), 7.55–7.76 (m, 3H, aromatic); MS (EI) *m/z* 384 (M^+; 100%), *m/z* 386 (M^++2); *Anal.* ($C_{18}H_{10}BrClN_2O$) C, H, N.

7-(3-Bromophenyl)-5-(2-ethoxyphenyl)pyrido[2,3-*d*]-pyrimidin-4-amine (6) A mixture of compound **2** (0.5 g) with an excess amount of formamide (20 mL) was heated under reflux with stirring for 3 d. The reaction mixture was poured into an ice bath; the formed precipitate was filtered, washed with water, dried and crystallized from DMF–ethanol 3 : 1. Yield 50%; mp 225–227°C; IR (cm^{-1}) 3320, 3280 (NH_2); ^{1}H-NMR: (DMSO-d_6) δ: 1.30–1.35 (t, 3H, $-CH_3$), 3.6 (br s, 2H, NH_2), 4.10–4.23 (q, 2H, $-O-CH_2-CH_3$), 6.89 (s, 1H, aromatic), 7.12–7.53 (m, 4H, aromatic), 7.81–7.92 (m, 4H, aromatic), 9.24 (s, 1H, aromatic). MS (EI) *m/z* 420 (M^+; 100%), *m/z* 422(M^++2); *Anal.* ($C_{21}H_{17}BrN_4O$) C, H, N.

7-(3-Bromophenyl)-5-(2-methoxyphenyl)pyrido[2,3-*d*]-pyrimidin-4(3*H*)-one (7) A mixture of compound **1** (0.5 g) with an excess amount of formic acid (20 mL) was heated under reflux with stirring for 7 d. The reaction mixture was poured into an ice bath; the formed precipitate was filtered, washed with water, dried and crystallized from DMF–ethanol 3 : 1. Yield 42%, mp 201–203°C; IR (cm^{-1}) 1680 (CO); ^{1}H-NMR (DMSO-d_6) δ: 3.93 (s, 3H, $-OCH_3$), 6.92 (s, 1H, aromatic), 7.07–7.24 (t, 2H, aromatic), 7.29–7.74 (m, 4H, aromatic), 7.82–7.85 (d, 1H, aromatic), 8.13 (s, 1H, aromatic), 9.40 (s, 1H, aromatic), 10.82 (s, 1H, –NH). MS (EI) *m/z* 408 (M^+; 100%), *m/z* 410 (M^++2); *Anal.* ($C_{20}H_{14}BrN_3O_2$) C, H, N.

Biology. Phosphodiesterase Inhibitory Activity PDE activity was measured using an adaptation of the IMAP fluorescence polarization phosphodiesterase assay (Molecular Devices). PDE hydrolysis of the fluorescent-labeled substrate allows it to bind the IMAP_ reagent, which increases fluorescence polarization (FP). The assay used fluorescein (Fl)-cAMP and tetramethylrhodamine (TAMRA)-cGMP as substrates. The different excitation and emission spectra of the substrates (485–530 nm for Fl and 530–590 nm for TAMRA) allowed for simultaneous measurement of cAMP and cGMP hydrolysis in the same well. The assays were performed in 96-well microtiter plates using a reaction buffer containing 10 mM Tris–HCl (pH 7.2) 10 mM $MgCl_2$, 0.05% NaN_3 and 0.1% phosphate-free BSA as the carrier. Each well contained 20 μL of recombinant enzyme (BPS Biosciences, San Diego, CA, U.S.A.) and 10 μL inhibitor. The reaction was initiated by the addition of 10 μL of a substrate solution containing 50 nM Fl-cAMP and/or TAMRA-cGMP. After incubating at room temperature for 60 min, the reaction was terminated by adding 120 μL of binding solution. FP was measured with a BioTek Synergy 4.

Molecular Modeling The crystal structure of the catalytic domain of the human PDE3 in complex with IBMX (PDB 1SOJ) was downloaded from the Protein Data Bank and opened with MOE software. The pocket was identified and the ligand was removed. Compounds **4** and **7** were docked into the binding pocket. The placement method was the Triangle Matcher and Scoring function was London dG. The best scored 30 poses were retained and the best scoring one was considered as the right pose by default.

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