Bioorganic Chemistry 89 (2019) 103022

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Design, synthesis and biological evaluation of pyrazolopyrimidinone based potent and selective PDE5 inhibitors for treatment of erectile dysfunction

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ARTICLE INFO

Keywords:
Pyrazolopyrimidinone derivatives
PDE5 inhibitors
PDE6 enzyme
Sildenafil
Erectile dysfunction

ABSTRACT

Our previous discovery of series of pyrazolopyrimidinone based PDE5 inhibitors led to find potent leads but with low aqueous solubility and poor bioavailability, and low selectivity. Now, a new series of same pyrazolopyrimidinone scaffold is designed, synthesized and evaluated for its PDE5 inhibitory potential. In this study, some of the molecules are found more potent and selective PDE5 inhibitors *in vitro* than sildenafil. The studies revealed that compound **5** is 20 fold selective to PDE5 against PDE6. As PDE6 enzyme is involved in the phototransduction pathway in the retina and creates distortion problem, the selectivity for PDE5 specifically against PDE6 enzyme is preferred for any development candidate and in present study, compound **5** has been found to be devoid of this liability of selectivity issue. Moreover, compound **5** has shown excellent *in vivo* efficacy in conscious rabbit model, it's almost comparable to sildenafil. The preclinical pharmacology including pharmacokinetic and physicochemical parameter studies were also performed for compound **5**, it was found to have good PK properties and other physicochemical parameters. The development of these selective PDE5 inhibitors can further lead to draw strategies for the novel preclinical and/or clinical candidates based on pyrazolopyrimidinone scaffold.

1. Introduction

PDE5 is the major cGMP-hydrolyzing enzyme in human corpus carvernosal tissue. Elevation of cGMP is directly correlated with vascular smooth muscle relaxation. This enzyme is distributed in lung, kidney, spleen, endothelial cells, and smooth muscle cells, etc. and plays a key role in the regulation of the cellular level of cGMP [1].

As reported in literature pyrazolopyrimidinones are very well exploited for their PDE inhibitory activities [2–9]. The first successful drug 'Sildenafil' was structurally based on pyrazolopyrimidinone scaffold [10]. Effectiveness of sildenafil in treating erectile dysfunction (ED) was discovered serendipitously in phase II angina trials and this discovery has led to put several molecules from pyrazolopyrimidinone class in different preclinical and clinical developmental stages [11]. Still, this molecule is being used as effective therapy for ED besides its several notable side effects such as headache, nausea, cutaneous flushing, and the major one is visual disturbance caused due to this molecule, and these side effects are because of its non selectivity and off-target activity. This limited selectivity of sildenafil mostly against PDE1 and PDE6 needs improvement [12]. Now, the drugs like vardenafil or tadalafil have come up with reduced side effects because of their higher selectivity towards PDE5 isozyme [13]. Looking into this aspect, there is a need to develop more selective new candidates as PDE5 inhibitors. In connection with our continuous efforts in search of newer molecules as PDE5 inhibitors, we have discovered a series of molecules based on pyrazolopyrimidinone scaffold as potent and

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https://doi.org/10.1016/j.bioorg.2019.103022
Received 11 February 2019; Received in revised form 14 March 2019; Accepted 29 May 2019
Available online 31 May 2019
0045-2068/

selective PDE5 inhibitors.

Previously, we reported the discovery of a series of pyrazolopyrimidinones as potent inhibitors of PDE5, which was shown to be as efficacious as sildenafil [14]. In an attempt to improve the potency and selectivity of present series, modification or replacement towords the piperazine ring of the structure were undertaken with the inputs of informatics in designing. A series of designer molecules derived from pyrazolopyrimidinone scaffold that are further augmented with better PDE5 activity and selectivity conducted in wet lab experiments and supported with significant *in vivo* efficacy than sildenafil is presented here. Our studies commenced with the design of molecules *in silico* by studying their interactions with PDE5 protein followed by their chemical synthesis and biological studies, the details are described below.

1.1. In silico studies

It is reported that the hydrophobic favored regions around the alkoxy group of the phenyl ring and methyl piperazine group of sildenafil are favorable for substitution [15]. The 'Q' pocket accommodates the pyrazolopyrimidinone group of sildenafil, strongly suggests that the chemically similar guanidine group of cGMP also binds at this region. The amide moiety of the pyrazolopyrimidinone group forms a bidentate hydrogen bond with the γ-amide group of Gln817. The ethoxyphenyl group of sildenafil fits into the hydrophobic H-pocket formed by Phe786, Ala783, Leu804 and Val782. The 'L' region composed of residues Tyr664, Met816, Ala823 and Gly819 surrounds the methylpiperazine group of sildenafil.

Using the PDB code 2H42 [16], the binding site analysis of PDE5 was carried out with respect to the standard molecule sildenafil. It was observed that in lid region there is a scope of modification at *N*-methyl piperazine ring position by the substitution of hydrophobic moiety followed by the hydrogen bond donor or accepter (Fig. 1A). Studies have shown that piperazine moiety is not crucial for PDE inhibitory activity and compounds with replacement of piperazine substituents charged or larger than a methyl group is of benefit for better inhibitory activity [17]. Opening the piperazine ring represents a crucial alteration that gives more flexibility to the molecules allowing different conformations of the supported functional groups [17]. Based on the *in silico* findings and above available literature reports, sildenafil derivatives were designed and synthesized. The sildenafil and its two most active derivatives i.e. compound **5** and **6** (Fig. 1B–D) showed similar π-π and H-bonding interaction with Tyr612, and the bidentate H-bond formation with the Gln817. In case of compound **5**, Phe786 forms π-π interaction (Fig. 1C) whereas in compound **6**, Met816 forms H-bonds, and stabilize the PDE5-sildenafil derivatives complex. Moreover, the active compound **5** and **6** showed comparable docking scores of −10.298 and −10.757 w.r.t. sildenafil (-10.844). The best docked conformations of sildenafil (**23**) and its most active derivatives are represented in Fig. 1E. It was observed that the selected derivatives retain the same orientation as that of sildenafil in the active pocket of PDE5, therefore most of the interacting residues are similar in all the three studied systems.

To address the selectivity of sildenafil and its derivatives among PDE5 and PDE6, further molecular interaction studies were carried out with PDE6 using PDB ID 5ML3 (Fig. 2). It was observed that sildenafil derivatives did not show any interaction with Arg61, Gln78, Tyr149 (Fig. 2A and 2B), which are reported to be pivotal residues for PDE6 inhibition [18]. The standard, sildenafil showed H-bonding with Arg61 and Gln78, and core moiety i.e. pyrazolo-pyrimidione form π-π interaction with Trp90 (Fig. 2C). The sildenafil derivative compound **6** formed H-bond with the Glu88 (Fig. 2B). The docking score of these derivatives with PDE6 (Sildenafil (23): −8.806, compound **5**: −8.211, and compound **6**: −8.564) is also less in comparison to PDE5. The orientation difference of sildenafil derivatives w.r.t. sildenafil and absence of any interactions with the important residues Arg61, Gln78, Tyr149 (for PDE6) may be the reason for their selectivity towards PDE5.

1.2. Chemistry and Biological activity

The synthesis of inhibitors of PDE5 based on pyrazolopyrimidinone is described here. The synthesis was started from reaction of 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide with 2-ethoxybenzaldehyde in the presence of $CuCl_2$ in EtOH solvent at 70 °C for 3 h gave cyclized compound **3** i.e. 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one. Chlorosulfonation of **3**, in the presence of chlorosulfonic acid at 0 °C for 5 h afforded 75% of compound **4** i.e. 4-ethoxy-3-(1-methyl-7-oxo-3-propyl-6,7-dihydro-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl)benzene-1-sulfonyl chloride. Compound **4** was then treated with different secondary amines in the presence of DIPEA as base in dry DCM, at room temperature for 4–6 h, which furnished final compounds (**5**-**22**), Scheme 1. All modifications were carried out towards piperazine ring of the structure and the final step was modified accordingly.

The newly synthesized all sildenafil analogs (compounds **5**-**22**) were screened for PDE5 and PDE6 (for selectivity) inhibition by *in vitro* PDE5 and PDE6 enzyme based assay and the IC_{50} values were calculated. Interestingly, all the analogs were found to be very potent (in nanomolar range) inhibitors of the PDE5 and some of the molecules found better than sildenafil (Table 1). All compounds were screened for PDE5 selectivity against PDE6 enzyme. Some compounds **5**, **17**, **21** and **22** have shown good PDE5 fold selectivity like 20, 33, 18.5 and 18 respectively against PDE6, which is better than sildenafil (4.5 fold selectivity for PDE5 against PDE6).

The effect of substitutions at R position in structure A (Table 1) were found to be crucial. Some of the cyclic piperidine ring based modified analogs have shown excellent IC_{50} value and good PDE5 selectivity compared to sildenafil (**23**).

Based on these results, the compound with lower PDE5 IC_{50} and good selectivity against PDE6, compound **5** was taken for further biological investigational studies (i.e. isozymes selectivity, physicochemical properties, *in vivo* efficacy and pharmacokinetic profiling).

The substitutions on piperidine or piperazine ring affected the activity of compounds. Piperidone, the C4-carbonyl piperidine based analog (compound **5**) was found to be highly active among all synthesized compounds and its reduced analog **6** (C4-OH) was comparatively found little less active and less selective but improved water solubility was noted for this compound, could be because of hydrogen bonding. However, there was not much difference found in activity when this reduced analog was protected with methyl group (compound **7**), but it increased the selectivity for PDE5. The terminal hydroxylated analogs (compound **8** and **9**) with varying alkyl chain lengths on C4 of piperidine ring were active against PDE5 but were having less selectivity. The benzyl (compound **10**) and methyl (compound **11**) substituted C4 piperidine analog was less active and selective. C4 amino substituted Boc protected analog (compound **12**) was active but free amino group bearing (compound **13**) and hetero cyclic ring (compound **14**) were found comparatively less active on C4 position of piperidine was less active, both compounds were found with equal selectivity. The fluorinated compounds **15** to **17**, did not show much alteration in activity, but one of the -CF_3 bearing analog (compound **17**) has shown better selectivity (33 fold, the best in series) towards PDE5 against PDE6 but this has not given good results when tested for *in vivo* efficacy, and this could be because of less solubility. The 2-piperazone analog (compound **18**) was little less active and lacks selectivity as well. The extended bulkier group substitutions like aryl and heteroaryl (compounds **19** to **22**) on C4 of piperazine were less active. The increased selectivity was observed for compounds **21** and **22**, which suggest that pyrimidyl kind of substitutions towards *N*-piperazine ring could be a good choice to work further for medicinal chemistry to generate better PDE5 selective candidates. Eventhough, the overall observation on pattern of substitutions could not be concluded with a proper structure activity relationship on the substitutions towards piperidine or piperazine ring of the sildenafil core, but all designed and synthesized

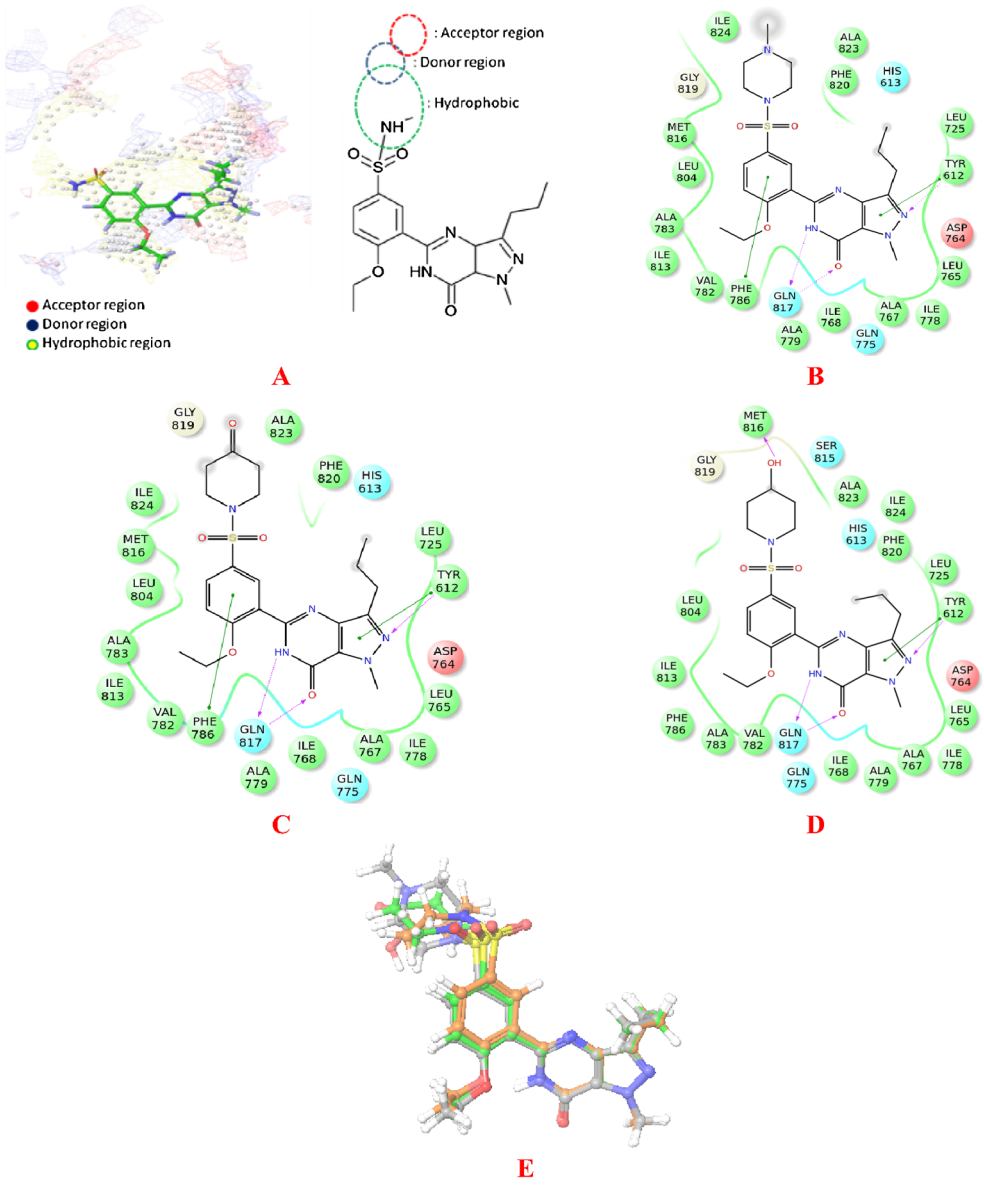


Fig. 1. Sildenafil and its derivatives (A) Scope of modification around the sildenafil (standard compound), Interaction of PDE5 in complex with (B) Sildenafil **23**, (C) compound **5**, (D) compound **6** (with the enzyme residues shown within 4 Å from the centroid of the active site). (E) Docked conformation of Sildenafil (grey), compound **5** (green), and **6** (orange) within the active site of PDE5.

analogs were found to have good activity. Based on these inputs, from *in silico* and *in vitro* activity profile, compound **5** was chosen for further detailed studies.

1.3. Isozyme selectivity profiling of compound 5

From the *in vitro* PDE5 inhibition screening results compound **5** (low IC_{50} 0.8 nM and 20 fold selectivity against PDE6) was taken for further studies. We tested compound **5** for isozymes selectivity against all PDEs. The screening results are shown in Table 2. Even though, the selectivity of compound **5** towards PDE5 against PDE6 was better, however, it has shown poor selectivity towards PDE1 isozyme, this issue could be further addressed as a future activity with the help of in silico approach.

1.4. Physicochemical properties of compound 5

Significant results of *in vitro* study prompted us to carry out detailed preclinical characterization of compound **5**. In physicochemical properties we checked the plasma protein binding affinity of compound **5**. Human serum protein binding affinity, affects the drug distribution in body and this also have impact on the drug-drug interaction. In plasma; drug exist in bound ↔ unbound form. Compound **5** was found to have fraction unbound = 0.039 and % bound = 96.13. In the context of compounds partitioning and dissociation, pKa (Dissociation constant) and Log P (Partition coefficient) are useful parameters to anticipate behavior of drug molecules in body. At different pH, a molecule exist in different ionic species which impact on the ADME (pharmacokinetic). Thus partition coefficient (Log P) and dissociation constant (pKa) are useful parameter to predict the distribution of a drug compound in a biological system. Compound **5** has optimum pKa = 8.7 and LogP = 2.5 respectively. Caco-2 permeability is also an important parameter to predict human intestinal permeability and bioavailability of orally administered drugs. An efflux ratio of 1 is indicative of passive diffusion, value less than 0.5 and > 2 regarded as active influx and active efflux, respectively. For compound **5**, Caco-2 permeability (10^{-6} cm/*sec*) showed; A to B as 0.211; B to A as 0.30, and efflux ratio of compound **5** in Caco-2 monolayer permeability model was (B/A/A/B = 1.4; Table 3). Solubility is one of the important parameters to

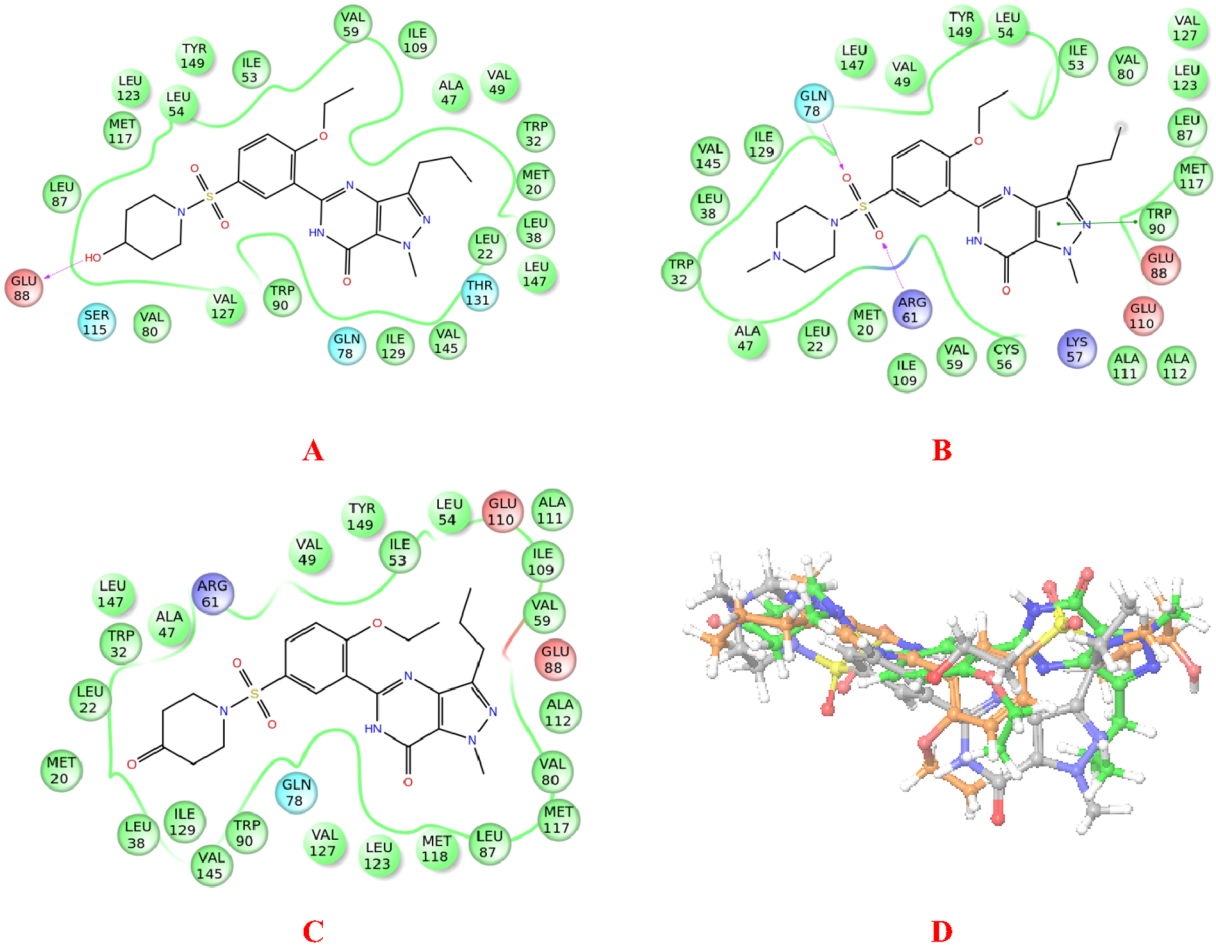
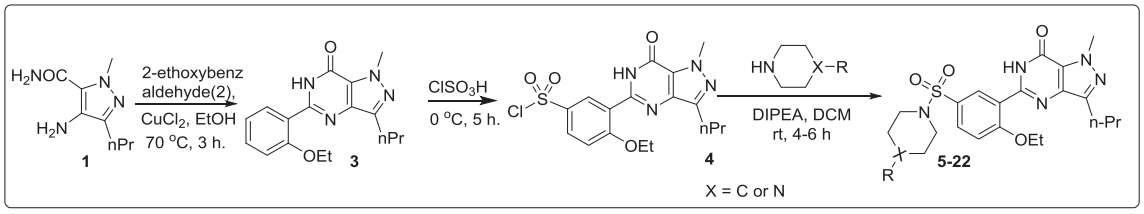


Fig. 2. 2D Interaction diagram of sildenafil and its derivatives in complex with PDE6. (A) compound 6, (B) Sildenafil **23**, (C) Compound **5** (D) 3D docked conformation of compound **5** (green), and **6** (orange) and sildenafil **23** (grey) within the active site of PDE6.



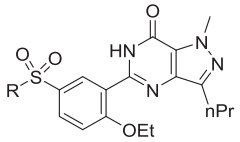
Scheme 1. Synthesis of sildenafil based analogs.

achieve desired concentration of drug in systemic circulation for desired pharmacological response and it is also an important parameter to be considered during dosing, formulation and administration of drug. Compound **5** was having moderate solubility in water = 10, PBS = 90, SGF = 91 and SIF = 169 μM respectively. Compound **5** has little low solubility in water but has good solubility in PBS, SGF and SIF. However, sildenafil has low solubility in water and PBS, moderate to good solubility in SIF and has high solubility in SGF. Further to know the metabolism and involvement of CYPs, we carried CYP inhibition of compound **5** and 23. Cytochrome P450 is a drug metabolizing enzyme, its induction or inhibition affects the blood level of a drug, and hence the toxicity and efficacy also. In further study, compound **5** showed no significant liability against CYP3A4 (66% inhibition) and CYP2C9 (21% inhibition) at 10 μM concentration.

1.5. Pharmacokinetic studies of compound 5

Pharmacokinetics study was carried out following an oral administration of compound **5** and **23** (sildenafil) individually at a single dose of 30 mg/kg in BALB/c mice. Mean plasma concentration versus time profiles and pharmacokinetic parameters of **5** and **23** are represented in Fig. 3 and Table 4, respectively. Both **5** and **23** was absorbed rapidly as maximum plasma concentration (1024 and 1452 ng/mL) was achieved within 0.5 h of dose administration that can satisfy the proposed therapeutic application. Areas under the curve ($AUC_{0\text{-}t}$ & $AUC_{0\text{-}\infty}$) were 803 and 1462 ng.h/mL, respectively for **5** and 1111 & 1650 ng.h/mL, respectively for **23**. Difference in oral exposure of **5** with **23** is possibly related to the lower solubility in SGF and higher value of pKa. However, compound **5** is expected to be better *in vivo* efficacy based on the comprehensive results of oral exposure and *in vitro* efficacy. Half life of **5** (1.3 h) is lower in comparison to **23** (1.5 h). Moreover, half life of both the compounds is correlated to its corresponding clearance rate from the body. The clearance of this molecule with an effective time frame of around 2 hr will be extremely suitable for the patients because too high lead to therapeutic failure and too low clearance may be unnecessary for patients compliance or increase in

Table 1
PDE5 potency (IC_{50}), PDE6 selectivity (Fold) and solubility of synthesized compounds.



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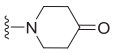
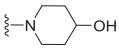
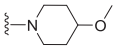
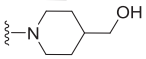
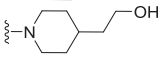
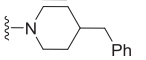
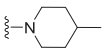
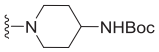
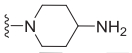
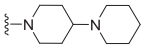
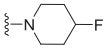
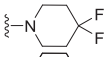
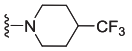
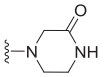
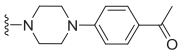
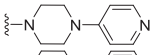
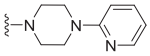
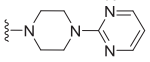
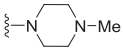
Sr No.	Compound	R	IC_{50}(nM) PDE5A1	Fold selectivity against PDE6	Solubility (μM)			
					Water	PBS	SGF	SIF
	5		0.8	20	10	90	91	169
	6		4.1	2.6	20	20	10	5
	7		4.27	9.78	5	5	1	1
	8		1.3	6.23	5	5	5	1
	9		7.5	1.5	5	5	1	1
	10		10	7.5	ND	ND	ND	ND
	11		18.5	10.6	5	5	1	1
	12		1.9	7.89	ND	ND	ND	ND
	13		26	7.3	ND	ND	ND	ND
	14		107	2.0	40	20	800	1
	15		1.1	6.09	1	1	1	1
	16		2.1	6.19	1	1	1	5
	17		6.0	33	1	1	1	1
	18		16	1.6	10	1	1	1
	19		6.4	7.9	1	1	1	5
	20		5.7	4.9	200	120	200	20
	21		9.3	18.5	10	1	200	1
	22		5.8	18.0	10	10	1	1
	23 (Sildenafil)		5.6	4.2	40	10	800	20

ND: not determined

liability if associated to it. Overall, compound **5** has satisfactory pharmacokinetic profile that insinuates further development.

1.6. In vivo efficacy study of compound 5 in conscious rabbit model

The animal studies for compound **5** and sildenafil were carried out in rabbits after approval from the IIIM-Institutional biosafety committee. Nitric oxide is the main neurotransmitter which mediates smooth muscle relaxation, necessary for penile erection, by activating the guanylyl cyclase and raising the cGMP levels. In penile tissue, the cGMP is predominantly metabolized by PDE5, hence an inhibitor of PDE5 increases cGMP levels, enhances relaxation of smooth muscle in the corpus cavernosum, and induces penile erection [20]. In order to check and compare the activity of the compound **5** with sildenafil in animal model of erectile dysfunction, we followed a well accepted model for penile erection called conscious rabbit model [21]. Briefly, compound **5** and sildenafil were injected into the ear vein of a group of five healthy adult male rabbits each. Another injection of sodium nitroprusside (SNP), which acts as a donor of nitric oxide, was given five minutes later. The SNP injection induced the penile erection in all the animals and length of the uncovered penile mucosa was measured at different time points, by using vernier calliper. The effect of SNP in all the experiments was removed from the readings obtained from the control experiments performed with the SNP alone injections. A graph was plotted between the length of uncovered penile mucosa verses the time and the area under the curve was calculated for comparison. The results (Fig. 4) show that compound **5** is indeed able to sustain the penile erection in the rabbit model, with almost the same, rather slightly higher efficiency as that of sildenafil. The area under curve of compound **5** (640.2) is almost same to that of sildenafil (601.9).

The overall observation on the studies conducted on compound **5** have produced relevant data as per design of the analogs based on

Table 2
Isozymes selectivity profile of compound **5** and sildenafil against all 11 PDEs.

Enzyme	Sildenafil (23)[a]		Compound 5	
	Mean IC_{50}, nM	PDE/PDE5 selectivity, fold difference	Mean IC_{50}, nM	PDE/PDE5 selectivity, fold difference
PDE1A1	600	107	41	51
PDE2A1	63,000	11,250	7200	9000
PDE3A	26,000	4642	> 10,000	> 12500
PDE4A1	5,000	892	> 10,000	> 12500
PDE5A1	**5.6**	**1**	**0.8**	**1**
PDE6C	**24**	**4.2**	**16**	**20**
PDE7A1	22,000	3928	> 10,000	> 12500
PDE8A1	> 100,000	> 17,857	> 10,000	> 12500
PDE9A2	3,600	642	4,300	5375
PDE10A1	5,400	964	8,000	10,000
PDE11A4	7,800	1392	2200	2750

[a] IC_{50} values of sildenafil against all other PDEs, except PDE5 and PDE6 have been taken from published data [19]

pyrazolopyrimidinone scaffold. To summarize this work with achieved end results, we have identified a series of potent and selective PDE5 inhibitors based on a pyrazolopyrimidinone scaffold. Compound **5**, has shown comparatively excellent *in vitro* activity with greater selectivity for PDE5 against PDE6 along with comparable *in vivo* efficacy to sildenafil. Compound **5** has also shown good PK profile and found good or rather better, in other physico-chemical parameter studies also, like solubility, CYP liabilities, permeability metabolic stability (MLM) and plasma protein binding (PPB). This series of molecules are useful for extending further efforts in medicinal chemistry for identifying better lead candidate for ED.

2. Experimental section

2.1. Chemistry

2.1.1. General procedure for synthesis of compound-5: Synthesis of 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (3)

4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide (5.0 g, 2.74 mmol) and 2-ethoxybenzaldehyde (4.32 g, 2.88 mmol) were suspended in ethanol and the mixture was heated at 70 °C for 1.5 h after confirmation of forming an imine by TLC. Added $CuCl_2$ (10.87 g, 8.2 mmol) and the reaction mixture again heated at 70 °C under O_2 for 1.5 h. After completion of reaction, ethanol was removed under vacuum and to this crude residue was added ethyl acetate (50 mL) and water (50 mL). The organic layer was separated and water layer was re-extracted with (2 × 50 mL) ethyl acetate. The combined organic layers were washed with brine solution, concentrated under vacuum and purified by using Column chromatography afforded the title compound as a white solid. Yield 85%.

2.1.2. Synthesis of 4-ethoxy-3-(6, 7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo [4, 3-d] pyrimidin-5-yl) benzene-1-sulfonyl chloride (4)

To the chlorosulphonic acid (3.0 mL, 36.58 mmol) was added 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one (1 g, 2.4 mmol) while maintaining the temperature 0 °C, then

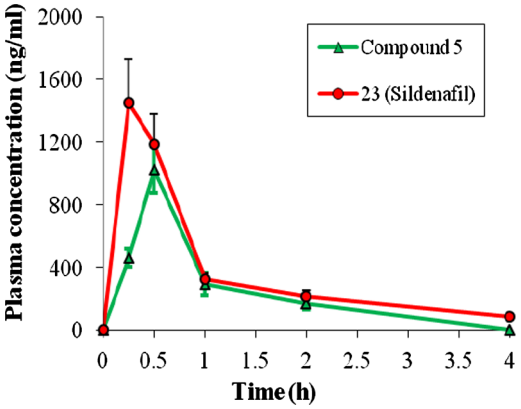


Fig. 3. Comparative plasma concentration vs. time profiles after oral administration of **5** and **23** (Sildenafil) individually at 30 mg/kg in BALB/c mice.

Table 4
Pharmacokinetic parameters of compound **5** and **23** (sildenafil) after oral administration at 30 mg/kg in BALB/c mice.

Pharmacokinetic parameter	5	23
C_{max} (ng/mL)	1024	1452
T_{max} (h)	0.50	0.25
$T_{1/2}$ (h)	1.3	1.5
AUC_{0-t} (ng.h/mL)	803	1462
$AUC_{0-\infty}$ (ng.h/mL)	1111	1650
V_d /F (l/kg)	49.2	39.6
Cl/F (l/h/kg)	27.0	18.2
MRT (h)	1.61	1.64

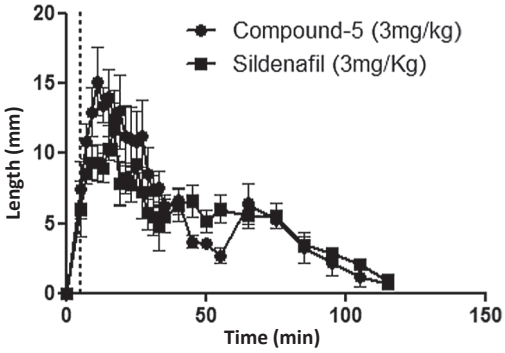


Fig. 4. Compound **5** is able to sustain SNP induced penile erection in male rabbits with comparable AUC to that of sildenafil. A single dose (3 mg/kg) of sildenafil and compound **5** were given intravenously to a group of 5 male rabbits each, which was followed, 5 min later, by an injection of sodium nitroprusside (0.2 mg/kg) in all animals. Another group of 5 male rabbits received only SNP and served as a control group. Length of uncovered penile mucosa in all the rabbit groups was measured at different time points. A graph of the length verses time was plotted to calculate the area under curve (AUC) for all experiments.

Table 3
Study of physicochemical properties and pre-formulation data for compound **5.**

Compound Code	Solubility (μM)				Plasma protein binding		Caco-2 permeability (10^{-6} cm/*sec*)			% Metabolized in MLM	LogP	pKa
	Water	PBS	SGF	SIF	Fraction unbound (fu)	% Bound	A to B	B to A	Efflux ratio			
5	10	90	91	169	0.039	96.13	0.211	0.30	1.4	> 95	2.5	8.7
23 (Sildenafil)	40	10	800	20	0.066	96.0	37.9	45.5	1.2	90	2.69	4.86

reaction was allowed to proceed at 0–5 °C until TLC analysis indicated the absence of starting material. After completion, to the reaction mixture, cold $CHCl_3$ was added and to it ice was added in portions. Organic layer was separated and re-extracted the water layer with 2 × 100 mL cold $CHCl_3$ and the combined organic layer are washed with brine solution, concentrated under vacuum; Yield 75%.

2.1.3. Synthesis of 5-(2-ethoxy-5-((4-oxopiperidin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one) (5)

Piperidin-4-one (18.0 mg, 0.18 mmol) was dissolved in dry DCM and added DIPEA (95.0 μL, 0.54 mmol) stirred the reaction mixture 10 min at 15 °C added 4-ethoxy-3-(1- methyl-7-oxo-3-propyl-6,7-dihydro-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl)benzene-1-sulfonyl chloride (75 mg, 0.18 mmol) and stirred the reaction for 6 h at 25 °C. After completion of reaction, added 30 mL DCM, 30 mL water and organic layer was separated. Water layer re-extracted with 20 mL DCM and the combined organic layer washed with brine solution, concentrated under vacuum and purified by using Column chromatography afforded the title compound as a white solid. Yield 90%.

2.1.4. 5-(2-Ethoxy-5-((4-hydroxypiperidin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-7(6H)-one (6)

White solid; yield 85%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.3; m.p. 180–181 °C; ^{1}H NMR (500 MHz $CDCl_3$): δ 10.84 (s, 1H), 8.83 (d, J = 2.4 Hz, 1H), 7.84 (dd, J = 8.8, 2.4 Hz, 1H), 7.17 (d, J = 8.8 Hz, 1H), 4.34 (q, J = 6.8 Hz, 2H), 4.27 (s, 3H), 3.85–3.76 (m, 1H), 3.61 (t, J = 7.6 Hz, 2H), 2.97–2.90 (m, 4H), 1.96–1.87 (m, 4H), 1.69–1.63 (m, 5H) 1.02 (t, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.2, 153.6, 146.9, 146.5, 138.3, 131.5, 130.9, 129.7, 124.4, 121.0, 113.0, 66.0, 65.6, 43.1, 38.2, 33.2, 27.7, 22.2, 14.5, 14.0; HRMS (ESI) *m/z* calcd. for $C_{22}H_{30}N_5O_5S$ $[M+H]^+$: 476.1968, found 476.1967.

2.1.5. 5-(2-Ethoxy-5-((4-methoxypiperidin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-7(6H)-one (7)

White solid; yield 80%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.5; m.p. 172–173 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.84 (s, 1H), 8.83 (d, J = 2.4 Hz, 1H), 7.84 (dd, J = 8.7, 2.4 Hz, 1H), 7.15 (d, J = 8.7 Hz, 1H), 4.38 (q, J = 7.0 Hz, 2H), 4.28 (s, 3H), 3.34–3.28 (m, 1H), 3.26 (s, 3H), 3.22–3.16 (m, 2H), 3.12–3.04 (m, 2H), 2.93 (t, J = 7.5 Hz, 2H), 1.95–1.71 (m, 6H), 1.65 (t, J = 7.0 Hz, 3H), 1.03 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 157.5, 152.0, 145.3, 144.9, 136.8, 129.9, 129.3, 128.3, 122.9, 119.4, 111.5, 72.2, 64.4, 54.2, 41.3, 36.6, 28.1, 26.1, 20.7, 13.0, 12.4; HRMS (ESI) *m/z* calcd. for $C_{23}H_{32}N_5O_5S$ $[M+H]^+$: 490.2124, found 490.2128.

2.1.6. 5-(2-Ethoxy-5-((4-(hydroxymethyl)piperidin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (8)

White solid; yield 70%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.3; m.p. 205–206 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.82 (s, 1H), 8.82 (d, J = 2.3 Hz, 1H), 7.85 (dd, J = 8.7, 2.3 Hz, 1H), 7.15 (d, J = 8.8 Hz, 1H), 4.38 (q, J = 6.9 Hz, 2H), 4.27 (s, 3H), 3.89 (d, J = 11.6 Hz, 2H), 3.51–3.45 (m, 2H), 2.93 (t, J = 7.5 Hz, 2H), 2.40–2.32 (m, 2H), 1.91–1.80 (m, 2H), 1.67 (t, J = 6.9 Hz, 3H), 1.51–1.12 (m, 5H), 1.03 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.1, 153.6, 146.9, 146.5, 138.3, 131.5, 130.8, 129.9, 124.4, 121.0, 113.0, 67.0, 66.0, 46.1, 38.2, 37.8, 28.0, 27.7, 22.2, 14.5, 14.0; HRMS (ESI) *m/z* calcd. for $C_{23}H_{32}N_5O_5S$ $[M+H]^+$: 490.2124, found 490.2124.

2.1.7. 5-(2-Ethoxy-5-((4-(2-hydroxyethyl)piperidin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (9)

White solid; yield 65%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.25; m.p. 195–197 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.83 (s, 1H), 8.82 (d, J = 2.3 Hz, 1H), 7.84 (dd, J = 8.7, 2.4 Hz, 1H), 7.15 (d, J = 8.8 Hz, 1H), 4.38 (q, J = 7.0 Hz, 2H), 4.27 (s, 3H), 3.85–3.82 (m, 2H), 3.66 (t, J = 6.3 Hz, 2H), 2.93 (t, J = 7.5 Hz, 2H), 2.35 (t, J = 11.5 Hz, 2H), 1.90–1.81 (m, 2H), 1.77 (d, J = 11.5 Hz, 2H), 1.65 (t, J = 7.0 Hz, 3H), 1.53–1.40 (m, 2H), 1.14–1.25 (m, 3H), 1.03 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.1, 153.6, 146.9, 146.5, 138.4, 131.6, 130.9, 129.6, 124.4, 120.9, 112.9, 66.0, 60.0, 46.4, 38.6, 38.2, 31.7, 31.4, 27.7, 22.2, 14.5, 14.0; HRMS (ESI) *m/z* calcd. for $C_{24}H_{34}N_5O_5S$ $[M+H]^+$: 504.2281, found 504.2287.

2.1.8. 5-(5-((4-Benzylpiperidin-1-yl)sulfonyl)-2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-7(6H)-one (10)

White solid; yield 70%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.6; m.p. 186–188 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.84 (s, 1H), 8.82 (d, J = 2.3 Hz, 1H), 7.84 (dd, J = 8.7, 2.3 Hz, 1H), 7.28–7.24 (m, 2H) 7.20–7.15 (m, 2H), 7.09 (d, J = 7.1 Hz, 2H), 4.39 (q, J = 6.9 Hz, 2H), 4.29 (s, 3H), 3.86 (t, J = 11.5 Hz, 2H), 2.93 (t, J = 7.5 Hz, 2H), 2.54 (d, J = 6.6 Hz, 2H), 2.29 (t, J = 11.5 Hz, 2H), 1.91–1.80 (m, 2H), 1.76–1.61 (m, 6H), 1.46–1.36 (m, 2H), 1.02 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.0, 153.6, 146.9, 146.5, 139.7, 138.4, 131.5, 130.9, 129.8, 128.9, 128.2, 126.0, 124.4, 120.9, 112.9, 66.0, 46.5, 42.6, 38.2, 37.3, 31.2, 27.7, 22.2, 14.5, 14.0; HRMS (ESI) *m/z* calcd. for $C_{29}H_{36}N_5O_4S$ $[M+H]^+$: 550.2488, found 550.2495.

2.1.9. 5-(2-Ethoxy-5-((4-methylpiperidin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-7(6H)-one (11)

White solid; yield 80%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.5; m.p. 206–208 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.83 (s, 1H), 8.82 (d, J = 2.4 Hz, 1H), 7.85 (dd, J = 8.8, 2.4 Hz, 1H), 7.15 (d, J = 8.8 Hz, 1H), 4.38 (q, J = 7.0 Hz, 2H), 4.28 (s, 3H), 3.80 (d, J = 11.0 Hz, 2H), 2.93 (t, J = 7.6 Hz, 2H), 2.34 (t, J = 11.0 Hz, 2H), 1.91–1.80 (m, 2H), 1.71–1.60 (m, 6H), 1.36–1.28 (m, 2H), 1.03 (t, J = 7.4 Hz, 3H), 0.92 (d, J = 5.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.1, 153.6, 147.0, 146.6, 138.4, 131.6, 130.9, 130.2, 124.5, 121.0, 113.0, 66.0, 46.4, 38.2, 33.4, 30.1, 27.7, 22.2, 21.4, 14.5, 14.0; HRMS (ESI) *m/z* calcd. for $C_{23}H_{32}N_5O_4S$ $[M+H]^+$: 474.2175, found 474.2173.

2.1.10. Tert-Butyl(1-((4-ethoxy-3-(1-methyl-7-oxo-3-propyl-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimi din-5-yl)phenyl)sulfonyl)piperidin-4-yl)carbamate (12)

White solid; yield 75%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.4; m.p. 227–228 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.85 (s, 1H), 8.82 (d, J = 1.9 Hz, 1H), 7.84 (dd, J = 8.7, 1.8 Hz, 1H), 7.16 (d, J = 8.8 Hz, 1H), 4.39 (q, J = 6.9 Hz, 2H), 4.28 (s, 3H), 3.79–3.70 (m, 2H), 3.49–3.38 (m, 1H), 2.94 (t, J = 7.5 Hz, 2H), 2.57–2.46 (m, 2H), 2.02–1.96 (m, 2H), 1.91–1.80 (m, 2H), 1.66 (t, J = 6.9 Hz, 3H), 1.56–1.46 (m, 2H), 1.41 (s, 9H), 1.03 (t, J = 7.3 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.8, 152.5, 151.2, 144.5, 144.0, 135.9, 129.0, 128.5, 127.2, 122.0, 118.7, 110.7, 77.2, 63.6, 44.5, 42.8, 35.8, 29.3, 25.9, 25.3, 19.8, 12.1, 11.6; HRMS (ESI) *m/z* calcd. for $C_{27}H_{39}N_6O_6S$ $[M+H]^+$: 575.2652, found 575.2629.

2.1.11. 5-(5-((4-Aminopiperidin-1-yl)sulfonyl)-2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-7(6H)-one (13)

To a solution of **12** (1 mmol) in CH_2Cl_2 was added 30% TFA in CH_2Cl_2 solution and stirred for 2 h at rt. The reaction mixture poured onto crushed ice and adjust the pH = 12 with NaOH solution and extracted with CH_2Cl_2. The combined organic layer was dried on Na_2SO_4 and concentrated *in vacuo*, affording white solid with 80% yield. TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.2; m.p. 206–207 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (d, J = 2.3 Hz, 1H), 7.85 (dd, J = 8.7, 2.4 Hz, 1H), 7.15 (d, J = 8.8 Hz, 1H), 4.38 (q, J = 6.9 Hz, 2H), 4.27 (s, 3H), 3.75 –3.72 (m, , 2H), 2.93 (t, J = 7.5 Hz, 2H), 2.69 (m, 1H), 2.56–2.50 (m, 2H), 1.92–1.81 (m, 4H), 1.64 (t, J = 7.0 Hz, 3H), 1.50–1.46 (m, 2H), 1.02 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.2, 153.6, 146.9, 146.5, 138.3, 131.5, 130.8, 129.8, 124.4, 121.0, 113.0, 66.0, 47.6, 44.9, 38.2, 34.7, 27.7, 22.2, 14.5, 14.0; HRMS (ESI) *m/z* calcd. for $C_{22}H_{31}N_6O_4S$ $[M+H]^+$: 475.2127, found 475.2127.

2.1.12. 5-(5-([1,4′-Bipiperidin]-1′-ylsulfonyl)-2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo [4,3-d] pyrimidin-7(6H)-one (14)

White solid; yield 65%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.3; m.p. 217–218 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.83 (s, 1H), 8.84 (d, J = 2.2 Hz, 1H), 7.87 (dd, J = 8.8, 2.2 Hz, 1H), 7.17 (d, J = 8.8 Hz, 1H), 4.40 (q, J = 7.0 Hz, 2H), 4.30 (s, 3H), 3.94 (d, J = 11.5 Hz, 2H), 2.95 (t, J = 7.5 Hz, 2H), 2.51–2.49 (m, 4H), 2.35 (s, 2H), 1.93–1.82 (m, 4H), 1.75–1.27 (m, 12H), 1.05 (t, J = 7.3 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.9, 151.4, 144.7, 144.2, 136.1, 129.4, 128.7, 127.3, 122.2, 118.8, 110.8, 63.8, 59.5, 47.7, 44.0, 36.0, 25.5, 24.8, 23.8, 22.3, 20.0, 12.3, 11.8; HRMS (ESI) m/z calcd for $C_{27}H_{41}N_6O_4S$ $[M + 3H]^{+2}$: 272.6455, found 272.6431.

2.1.13. 5-(2-Ethoxy-5-((4-fluoropiperidin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-7(6H)-one (15)

White solid; yield 75%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.55; m.p. 199–200 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.83 (s, 1H), 8.84 (d, J = 2.1 Hz, 1H), 7.85 (dd, J = 8.8, 2.1 Hz, 1H), 7.16 (d, J = 8.8 Hz, 1H), 4.85–4.71 (m, 1H), 4.39 (q, J = 6.9 Hz, 2H), 4.28 (s, 3H), 3.50–3.40 (m, 2H), 3.0–2.90 (m, 4H), 2.04–1.91 (m, 4H), 1.92–1.80 (m, 2H), 1.65 (t, J = 6.9 Hz, 3H), 1.02 (t, J = 7.3 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 158.4, 152.8, 146.1, 145.6, 137.5, 130.6, 130.1, 128.7, 123.6, 120.2, 112.3, 85.2 (d, J = 177.66 Hz), 65.2, 40.9 (d, J = 3.78 Hz), 37.4, 29.6 (d, J = 22.68 Hz), 26.9, 21.4, 13.7, 13.2; ^{19}F NMR (376 MHz, $CDCl_3$) δ −185.8; HRMS (ESI) m/z calcd. for $C_{22}H_{29}FN_5O_4S$ $[M+H]^+$: 478.1924, found 478.1928.

2.1.14. 5-(5-((4,4-Difluoropiperidin-1-yl)sulfonyl)-2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-7(6H)-one (16)

White solid; yield 75%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.55; m.p. 205–207 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.81 (s, 1H), 8.84 (d, J = 2.4 Hz, 1H), 7.85 (dd, J = 8.8, 2.4 Hz, 1H), 7.17 (d, J = 8.8 Hz, 1H), 4.39 (q, J = 7.0 Hz, 2H), 4.28 (s, 3H), 3.32–3.24 (m, 4H), 2.93 (t, J = 7.5 Hz, 2H), 2.15–2.05 (m, 4H), 1.92–1.80 (m, 2H), 1.66 (t, J = 7.0 Hz, 3H), 1.03 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.4, 153.6, 146.9, 146.3, 138.3, 131.3, 130.8, 129.6, 124.4, 121.2, 120.7 (t, J = 241.92 Hz), 113.2, 66.1, 43.3 (t, J = 5.04 Hz), 38.2, 33.5 (t, J = 23.94 Hz), 27.7, 22.2, 14.5, 14.0; ^{19}F NMR (376 MHz, $CDCl_3$) δ −99.2; HRMS (ESI) m/z calcd. For $C_{22}H_{28}F_2N_5O_4S$ $[M+H]^+$: 496.1830, found 496.1819.

2.1.15. 5-(2-Ethoxy-5-((4-(trifluoromethyl)piperidin-1-yl)sulfonyl) phenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (17)

White solid; yield 70%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.55; m.p. 203–204 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.75 (s, 1H), 8.74 (d, J = 2.2 Hz, 1H), 7.77 (dd, J = 8.8, 2.3 Hz, 1H), 7.09 (d, J = 8.8 Hz, 1H), 4.31 (q, J = 6.9 Hz, 2H), 4.20 (s, 3H), 3.88 (d, J = 11.3 Hz, 2H), 2.86 (t, J = 7.5 Hz, 2H), 2.31 (t, J = 11.3 Hz, 2H), 1.97–1.83 (m, 3H), 1.83–1.74 (m, 2H), 1.70–1.62 (m, 2H), 1.58 (t, J = 6.9 Hz, 3H), 0.95 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.3, 153.6, 146.9, 146.4, 138.3, 131.5, 130.9, 129.5, 124.4, 121.1, 120.6, 113.1, 66.1, 45.0, 39.6, 38.2, 27.7, 24.1, 22.2, 14.5, 14.0; ^{19}F NMR (376 MHz, $CDCl_3$) δ −73.7; HRMS (ESI) m/z calcd. for $C_{23}H_{29}F_3N_5O_4S$ $[M+H]^+$: 528.1892, found 528.1894.

2.1.16. 5-(2-Ethoxy-5-((3-oxopiperazin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d] pyrimidin-7(6H)-one (18)

White solid; yield 70%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.3; m.p. 262–263 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.88 (s, 1H), 8.82 (d, J = 2.4 Hz, 1H), 7.88 (dd, J = 8.8, 2.4 Hz, 1H), 7.19 (d, J = 8.8 Hz, 1H), 6.43 (s, 1H), 4.39 (q, J = 6.9 Hz, 2H), 4.28 (s, 3H), 3.74 (s, 2H), 3.54–3.44 (m, 2H), 3.43–3.34 (m, 2H), 2.94 (t, J = 7.5 Hz, 2H), 1.91–1.81 (m, 2H), 1.65 (t, J = 7.0 Hz, 3H), 1.03 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.8, 159.7, 153.6, 147.0, 146.2, 138.3, 131.7, 131.1, 128.6, 124.5, 121.6, 113.4, 66.2, 48.6, 42.6, 41.2, 38.2, 27.7, 22.2, 14.5, 14.0; HRMS (ESI) m/z calcd. for $C_{21}H_{27}N_6O_5S$ $[M+H]^+$: 475.1764 found 475.1767.

2.1.17. 5-(5-((4-(4-Acetylphenyl)piperazin-1-yl)sulfonyl)-2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (19)

White solid; yield 65%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.4; m.p. 228–229 °C; ^{1}H NMR (400 MHz $CDCl_3$): δ 10.80 (s, 1H), 8.87(d, J = 2.4 Hz, 1H), 7.88–7.81 (m, 3H), 7.17 (d, J = 8.8 Hz, 1H), 6.81 (d, J = 8.8 Hz, 2H), 4.38 (q, J = 7.0 Hz, 2H), 4.27 (s, 3H), 3.45–3.43 (m, 4H), 3.25–3.23 (m, 4H), 2.94 (t, J = 7.6 Hz, 2H), 2.50 (s, 3H), 1.92–1.81 (m, 2H), 1.64 (t, J = 7.0 Hz, 3H), 1.03 (t, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 196.5, 159.5, 153.5, 153.3, 146.9, 146.3, 138.3, 131.6, 131.1, 130.3, 128.6, 124.4, 121.2, 114.2, 113.2, 66.1, 47.3, 45.7, 38.2, 27.7, 26.2, 22.3, 14.5, 14.1; HRMS (ESI) m/z calcd. For $C_{29}H_{35}N_6O_5S$ $[M+H]^+$: 579.2390, found 579.2397.

2.1.18. 5-(2-Ethoxy-5-((4-(pyridin-4-yl)piperazin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (20)

White solid; yield 70%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.3; m.p. 213–215 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.82 (s, 1H), 8.84 (s, 1H), 8.26 (d, J = 4.8 Hz, 2H), 7.85 (d, J = 8.8 Hz, 1H), 7.17 (d, J = 8.8 Hz, 1H), 6.60 (d, J = 5.0 Hz, 2H), 4.38 (q, J = 6.9 Hz, 2H), 4.27 (s, 3H), 3.45 (t, J = 4.8 Hz, 4H), 3.20 (t, J = 4.8 Hz, 4H), 2.94 (t, J = 7.5 Hz, 2H), 1.91–1.81 (m, 2H), 1.64 (t, J = 6.9 Hz, 3H), 1.04 (t, J = 7.3 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.5, 154.2, 153.5, 150.3, 146.9, 146.3, 138.3, 131.5, 131.1, 128.7, 124.5, 121.4, 113.2, 108.7, 66.1, 45.7, 45.4, 38.2, 27.7, 22.2, 14.5, 14.0; HRMS (ESI) m/z calcd. for $C_{26}H_{32}N_7O_4S$ $[M+H]^+$: 538.2236, found 538.2239.

2.1.19. 5-(2-Ethoxy-5-((4-(pyridin-2-yl)piperazin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (21)

White solid; yield 70%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.35; m.p. 186–187 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.80 (s, 1H), 8.85 (d, J = 2.3 Hz, 1H), 8.14 (d, J = 3.6 Hz, 1H), 7.86 (dd, J = 8.7, 2.3 Hz, 1H), 7.48–7.40 (m, 1H), 7.15 (d, J = 8.7 Hz, 1H), 6.64–6.58 (m, 2H), 4.37 (q, J = 7.0 Hz, 2H), 4.27 (s, 3H), 3.67 (t, J = 4.8 Hz, 4H), 3.18 (t, J = 4.8 Hz, 4H), 2.94 (t, J = 7.5 Hz, 2H), 1.91–1.81 (m, 2H), 1.64 (t, J = 7.0 Hz, 3H), 1.04 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.4, 158.6, 153.6, 148.0, 147.0, 146.3, 138.3, 137.7, 131.6, 131.1, 128.7, 124.4, 121.1, 114.0, 113.1, 107.2, 66.1, 45.8, 44.7, 38.2, 27.7, 22.3, 14.5, 14.1; HRMS (ESI) m/z calcd. for $C_{26}H_{32}N_7O_4S$ $[M+H]^+$: 538.2236, found 538.2244.

2.1.20. 5-(2-Ethoxy-5-((4-(pyrimidin-2-yl)piperazin-1-yl)sulfonyl) phenyl)-1-methyl-3-propyl-1H -pyrazolo[4,3-d]pyrimidin-7(6H)-one (22)

White solid; yield 65%; TLC (MeOH:$CHCl_3$; 1:9): R_f = 0.35; m.p. 209–210 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.81 (s, 1H), 8.86 (d, J = 2.3 Hz, 1H), 8.28 (d, J = 4.7 Hz, 2H), 7.87 (dd, J = 8.7, 2.3 Hz, 1H), 7.17 (d, J = 8.7 Hz, 1H), 6.51 (t, J = 4.7 Hz, 1H), 4.38 (q, J = 7.0 Hz, 2H), 4.29 (s, 3H), 3.98 (t, J = 4.8 Hz, 4H), 3.15 (t, J = 4.8 Hz, 4H), 2.95 (t, J = 7.5 Hz, 2H), 1.95–1.82 (m, 2H), 1.65 (t, J = 7.0 Hz, 3H), 1.06 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.1, 159.4, 157.7, 153.6, 147.0, 146.3, 138.3, 131.6, 131.0, 128.7, 124.4, 121.1, 113.1, 110.5, 66.0, 45.9, 42.9, 38.2, 27.7, 22.2, 14.5, 14.0; HRMS (ESI) m/z calcd. for $C_{25}H_{31}N_8O_4S$ $[M+H]^+$: 539.2189, found 539.2196.

2.2. In silico[22−23] methodology

The computational studies against PDE5 and PDE6 were carried out using the Schrodinger suite 2015-1 molecular modeling software. The coordinates of PDE5 in complex with sildenafil inhibitor (PDB code: 2H42, having 2.3 Å resolution) and PDE6 in complex with Deltasonamide1 (PDB code: 5ML3, having 1.4 Å resolution) were obtained from protein data bank [22–23]. The selected protein-inhibitor complexes were prepared for molecular docking studies using the

protein preparation wizard, where hydrogen were added to the protein with assigning bond order and only that water residues were kept which were interacting with protein as well as heteroatom [23]. Then the heteroatom was extracted and protein was refined by assigning H-bonds and minimization at OPLS 2005 force field. A grid was generated at active site, identified on the bases of already co-crystallized ligand to the receptor using receptor grid generation module [24]. In order to standardize the docking protocol, the co-crystallized ligand was re-docked on to the prepared protein structure and RMSD was calculated between its docked and bound conformation. Herein at XP scoring function of Glide an RMSD of 1.02 Å (for PDE5) and 0.973 (for PDE6) was observed which was better than SP and HTVS. Thus all the molecular docking studies were performed using XP scoring function of Glide.

2.3. Pharmacological studies:

(a) *In vitro* PDE screening: The PDE5 inhibitory activity (IC_{50}) of different compounds was checked by using commercially available purified human PDE5A active (Signalchem, Canada: Cat No. P93-31G), expressed by baculovirus in sf9 insect cells and PDE Glo Phosphodiesterase Assay kit from Promega (Cat No.V1361). The assays were performed by following the manufacturer recommended protocol. In order to determine the IC_{50} of the active molecules a nine point titration in duplicate against the PDE5A was performed. Data analysis was performed using GraphPad Prism®, version 5.00, for Windows using a sigmoidal dose-response (variable slope) equation. Each point represents an average of two replicates per concentration. IC_{50} values of Compound **5** against all other PDE isoforms was carried out at BPS Biosciences, USA.

(b) *In vivo* activity: The *in vivo* study for compound **5** was carried out at Reliance Life Sciences, Mumbai. In order to check the *in vivo* activity of the most effective molecule, we followed a well accepted model for penile erection called conscious rabbit model [19,20]. Briefly, compound **5** as well as sildenafil **23** were dissolved in Transcutol and diluted with 20% cremophor-EL in distilled water at a ratio of 3:7. This solution was injected into the ear vein of a group of five healthy male rabbit (3–3.5 kg weight) in a volume of 0.5 mL/kg. The event was followed 5 min later by another equal volume saline injection containing sodium nitroprusside (0.2 mg/kg), which acts as a donor of nitric oxide (NO). Length of uncovered penile mucosa was measured using a sliding caliper at different time points for 70 min after administration of the test compounds. The area under the curve (AUC) was calculated by Graph Pad Software. Controls experiments were performed with the SNP alone injections.

2.4. Pharmacokinetic study

[24] Compounds were administered by oral route to Swiss mice as mentioned (5 mice in each group) at a dose of 1, 2.5 and 10 mg/kg respectively. For p.o. compound **5** was triturated in 2% gum acacia (w/v) and 10 mL distilled water added to form 10 mg/10 mL as suspension. Samples derived from plasma at different time points were then analyzed by LC-MS/MS to generate the required pharmacokinetic parameters.

Acknowledgements

GLR, MID thanks CSIR/UGC for the award of senior research fellowship. The funding support from CSIR grant HCP0008 and SERB-DST grant ECR/2016/000625 is gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103022.

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