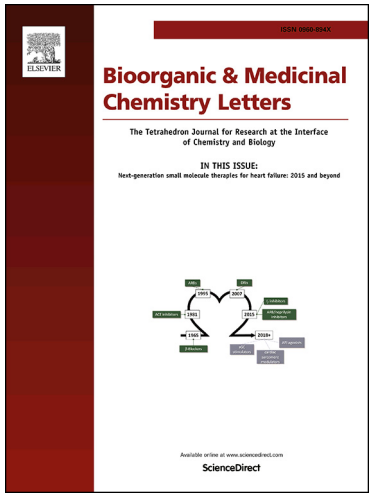

Design, synthesis of novel purin-6-one derivatives as phosphodiesterase 2 (PDE2) inhibitors: the neuroprotective and anxiolytic-like effects

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Design, synthesis of novel purin-6-one derivatives as phosphodiesterase 2 (PDE2) inhibitors: the neuroprotective and anxiolytic-like effects

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ABSTRACT: Phosphodiesterase 2 (PDE2) has received much attention for the potential treatment of the central nervous system (CNS) disorders. Herein, based on the existing PDE2 inhibitors and their binding modes, a series of purin-6-one derivatives were designed, synthesized and evaluated for PDE2 inhibitory activities, which led to the discovery of the best compounds **6p** and **6s** with significant inhibitory potency (IC_{50}: 72 and 81 nM, respectively). Docking simulation was performed to insert compound **6s** into the crystal structure of PDE2 at the active site to determine the binding mode. Furthermore, compound **6s** significantly protected HT-22 cells against corticosterone-induced cytotoxicity and rescued corticosterone-induced decreases in cAMP and cGMP levels. It also produced anxiolytic-like effect in the elevated plus-maze test and exhibited favorable pharmacokinetic properties *in vivo*. These results might bring significant instruction for further development of potent PDE2 inhibitors.

Keywords: PDE2 inhibitors; purin-6-one; anxiolytic-like effects

Phosphodiesterases (PDEs) are a class of enzymes degrading second messengers including cAMP and cGMP, which play a key role in mediating cellular responses to various hormones and neurotransmitters. Currently, eleven PDE families with distinct tissue distribution and selectivity for cAMP and cGMP have been identified. PDEs 1, 2, 3, 10, 11 are considered dual specificity enzymes, hydrolyzing both cAMP and cGMP. PDEs 4, 7, and 8 specifically hydrolyze cAMP, whereas PDEs 5, 6, and 9 prefer to hydrolyze cGMP.[1] PDEs are highly expressed in the human brain and their inhibitors regulate neurodegenerative processes by increasing the concentration of cAMP and cGMP in brain tissue and thereby modulate a variety of neuronal processes.[2,3] Noticeably, inhibitors of PDEs 2, 3, 4, 5 and 9 have been shown to significantly enhance memory *in vitro* and *in vivo*. [4-8] Modulation of cyclic nucleotide (cNT) levels by PDE inhibitors is becoming a particularly attractive therapeutic approach to treating disorders of mood and emotion.

PDE2A is a dual-specificity phosphodiesterase controlling the cellular levels of both cAMP and cGMP in the brain areas where mood signaling is regulated. Inhibition of PDE2A increases cNT levels in brain to evoke antianxiety, antidepressant, and pro-cognitive effects in normal and stressed rodents.[6,9-12] In addition, PDE2 inhibition can promote procognitive activity, and restore hippocampal function. For a growing body of preclinical research, PDE2A has recently received intensive attention for its therapeutic potential to treat central nervous system (CNS) disorders, including schizophrenia and Alzheimer's disease. Although several PDE2 inhibitors have been shown to be promising for treatment of mood and cognition disorders in the past two decades, none of them have reached the market yet. EHNA [Figure 1] is a first generation of PDE2 inhibitors with IC_{50} of 800 nM but low-selectivity over other PDEs. [13] Another PDE2 inhibitor, BAY60-7550,

selectively inhibited PDE2 with IC_{50} of 4.7 nM, increased cGMP in neuronal culture and hippocampal slice. Besides, this compound also could improved acquisition and consolidation in the tests of object recognition and social recognition of young adult and aged rats. [14] However, the clinical application of BAY60-7550 was limited due to moderate pharmacokinetic properties. A pyrazolopyrimidine PDE2 inhibitor, PF-05180999 reported by Pfizer in 2012, was in phase I clinical trial for the treatment of schizophrenia and migraine, but there was no further report. [9,15,16] Overall, development of novel PDE2 inhibitors for treatment of neurodiseases is still urgently needed.

In the previous studies, we had reported a selective PDE2 inhibitor, Hcyb1, with purin-6-one structure, which was a moderate PDE2 inhibitor with an IC_{50} value of 0.57 μM and produced neuroprotective and antidepressant-like effects most likely mediated by cAMP/cGMP-CREB-BDNF signaling *in vitro* and *in vivo*. [17] As the continuing work for searching more potent PDE2 inhibitors to treat CNS disorders, herein, further optimization of Hcyb1 was carried out and 20 derivatives were designed, synthesized and tested with biological assays.

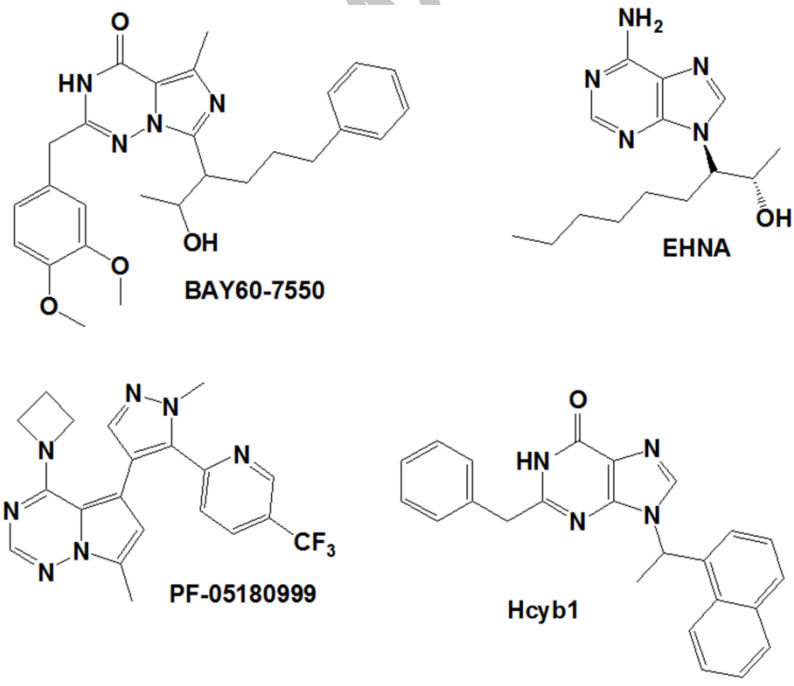
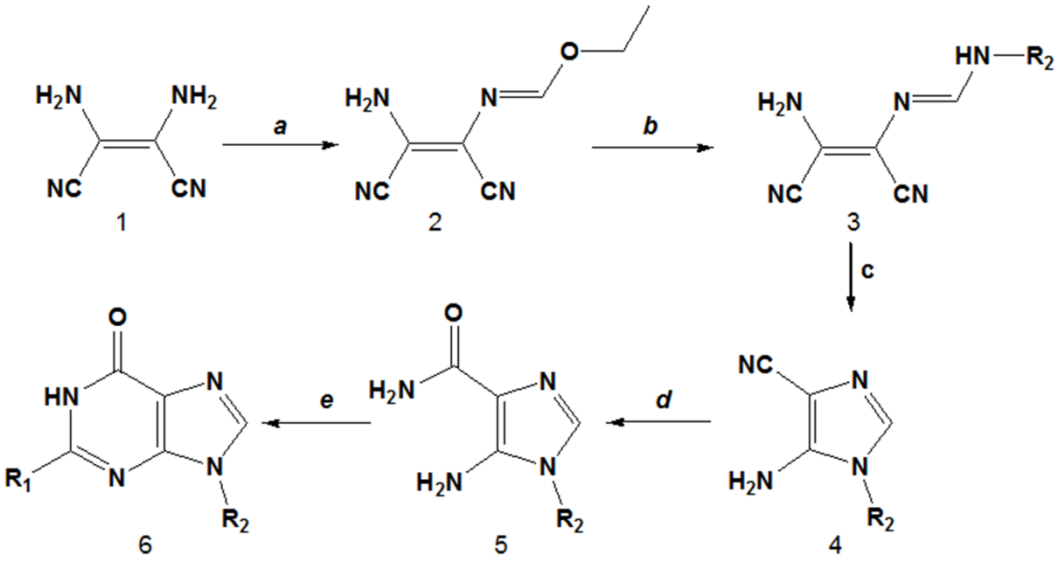


Figure 1. Structures of known PDE2 inhibitors

The X-ray crystal structure of PDE2 in complex with BAY60-7550 revealed that its heterocyclic core of is anchored via π-stacking against Phe862 and Phe830 [9,18,19] and the pyrimidone forms two hydrogen bonds respectively with Gln859 and Gln812. The latter of hydrogen bond is unique to PDE2, while the hydrogen bond with Gln859 is common for all PDEs. Interestingly, the propylphenyl group of BAY60-7550 interacts with a hydrophobic pocket that was

thought to play an important role in the enhancement of binding affinity and selectivity for PDE2.[18] BAY60-7550 also interacts with the surface hydrophobic patch through the dimethoxybenzyl group. Other PDE2 inhibitors reported recently bear various heterocyclic cores such as purin-6-one of Hcyb1 may have a similar binding pattern of stacking and hydrogen-bonding to that of BAY60-7550 or ENHA. We therefore reasoned that replacement of the imidazo[1,5-f][1,2,4]triazin-4(3*H*)-one core in BAY60-7550 with purin-6-one core will lead to a novel type of PDE2 inhibitors. Molecular docking suggest that the 5,6-fused heteroaromatic rings of Hcyb1 and BAY60-7550 have similar interactions with PDE2 and the phenyl and other substitution groups (we denote as R1 and R2) can be further modified to lead more potent PDE2 inhibitors. Therefore, we designed and synthesized 20 derivatives with purin-6-one scaffold.

The general routes for synthesizing derivatives **6a**–**6t** are shown in Scheme 1. Diaminomaleonitrile **1** was refluxed with triethylorthoformate in dioxane, to afford compound **2**. Compound **2** was reacted with the amines containing various desired substitutions to generate **3**, which was further treated with an aqueous solution of potassium hydroxide to obtain molecules **4**. The remaining cyano group was oxidized with 30% peroxide in aqueous ammonium hydroxide to yield **5**. Cyclization by reacting with esters that contain the desired substitutions in a solution of dioxane/sodium hydride and heating generated the targeted derivatives **6a-6t**.



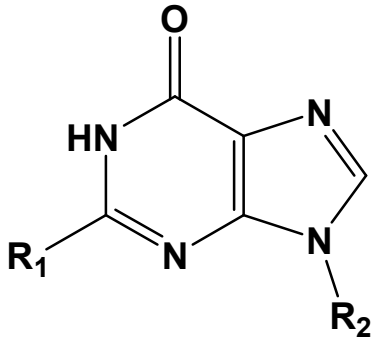
Scheme 1. Synthesis of compounds **6**. Reagents and conditions: (a) $HC(OC_2H_5)_3$, 1, 4-dioxane, reflux, 2 h; (b) R_2NH_2,EtOH, 30 °C overnight; (c) 1 mol/L KOH, rt, 0.5 h (65–75%); (d) NH_4OH, 50% H_2O_2, EtOH, 30 °C 6 h; (e) $R_1COOC_2H_5$, NaH, 1,4-dioxane.

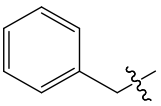
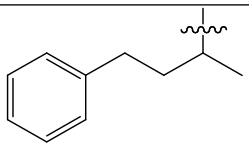
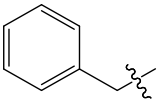
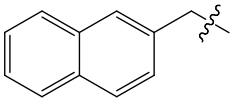
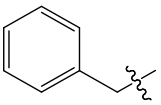
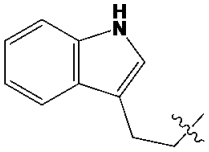
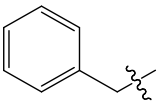
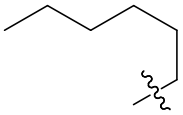
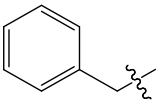
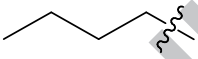
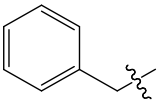
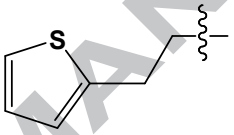
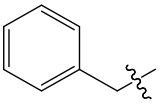
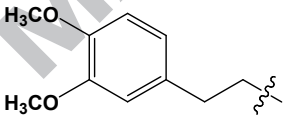
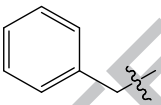
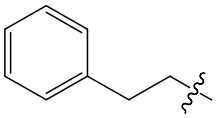
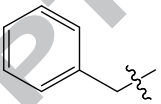
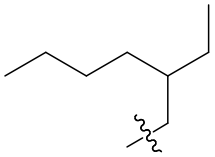
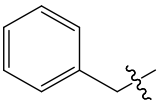
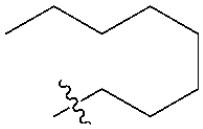
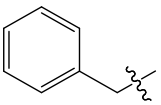
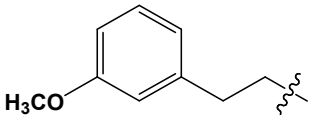
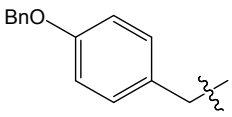
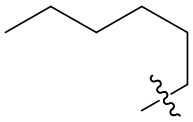
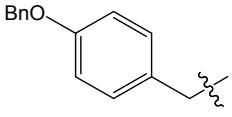
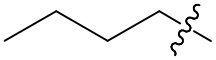
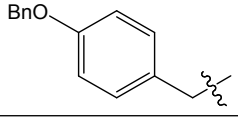
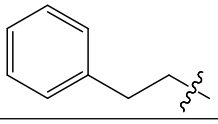
The *in vitro* inhibitory activity of compounds **6a-6t** against PDE2 is listed in Table 1. Our initial exploration was focused on introducing different substituents in R2 of the purin-6-one

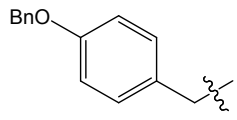
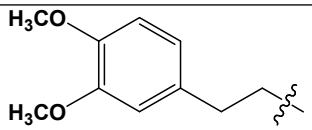
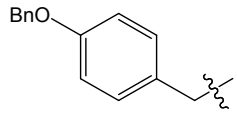
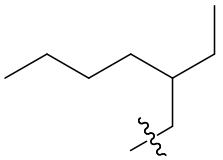
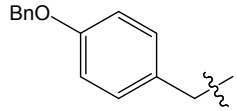
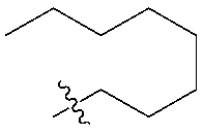
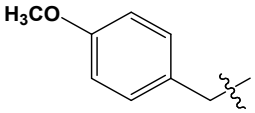
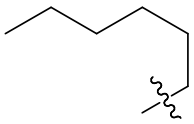
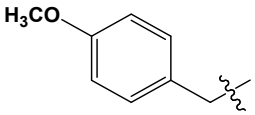
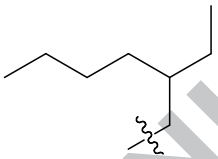
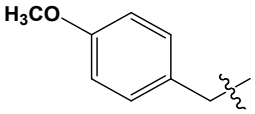
scaffold, while keeping the benzyl group (R1) fixed. As mentioned above, The propylphenyl group of classic PDE2 inhibitor BAY60-7550 is accommodated very well through a hydrophobic induced binding pocket located under L770 of the protein. Thus, for purin-6-one derivatives in this report, only by bearing certain length and lipophilicity can the R2 group interact properly with the pocket. Based on this rule, various R2 groups in Table 1 were selected to evaluated their effects on PDE2 inhibitory activities. Most of the derivatives have appropriate values of CLogP (2.0-5.0), indicating good blood-brain barrier penetration. Compounds **6a-6k** were tested for *in vitro* PDE2 inhibitory activities and showed that R2 group remarkably affected the PDE2 inhibitory activities. The substitutions with hydrophilic groups significantly sacrified the inhibitory potency, as seen in the examples of compounds **6f** and **6g** that have the IC_{50} values of 17 μM and 40 μM, respectively. Compound **6c** did not inhibit PDE2 with IC_{50}> 50μM. Then we introduced 4-benzyloxy (**6l-6q**) group to the 4-position of the benzene of R1 and varies R2. Overall, most of these compounds displayed good PDE2 inhibitory activities. Especially, compound **6p** showed an IC_{50} of 72nM. And substitution of R2 with 3,4-Dimethoxyphenethyl group (compound **6o**) almost abolish the inhibitory activity on PDE2, as shown by IC50 > 50 μM [Table 1]. The data showed that both of R1 and R2 groups can significantly affect the PDE2 inhibitory activities of the purin-6-one scaffold. Replacement with the methoxybenzyl led to compounds **6r-6t**, of which **6s** also showed high inhibitory potency against PDE 2 (IC_{50} = 81 nM).

The *in vitro* inhibitory selectivity of compounds **6p** and **6s** over other PDEs (PDE1, 4, 5, 9 and 10) was evaluated [Table 2]. The two compounds displayed > 5000-fold selectivity with IC_{50} > 500 μM against PDE 1, 4, 5, 10, and inhibited moderately PDE9 with IC_{50} values of 13.9 and 32.3 μM, respectively. Although inhibition of **6p** and **6s** on PDE2 is less potent than BAY60-7550, they achieved significant selectivity for closely-related PDE enzymes. For example, > 5000-fold selectivity of **6p** and **6s** over PDE1, in comparison with 50-fold selectivity of BAY-7550 over PDE1 [12] represents a significant progress, and the poor selectivity over PDE1 for PDE2 inhibitors could often lead to side effect of headache.

Table 1. In vitro PDE2 inhibitory activity of compounds **6a-6t**



Compounds	R1	R2	PDE2 IC_{50} (μM)[a]	CLogP
6a			0.31±0.023	2.85
6b			3.99±0.29	3.41
6c			> 50	2.81
6d			5.94±0.47	3.36
6e			4.32±0.32	2.31
6f			16.98±1.23	2.46
6g			40.35±3.89	2.47
6h			7.27±0.58	2.81
6i			1.24±0.08	4.29
6j			4.28±0.51	4.42
6k			11.02±1.33	2.73
6l			3.91±0.36	5.05
6m			5.46±0.60	3.99
6n			5.8±0.57	4.50

6o			>50	4.16
6p			0.072±0.006	5.98
6q			2.63±0.11	6.10
6r			3.79±0.35	3.28
6s			0.081±0.009	4.12
6t			1.94±0.20	2.22
Bay60-7550			4.8±0.20nM	2.64

[a] Results are expressed as the mean of at least three experiments.

Table 2. PDE selectivity of compounds **6p** and **6s**

Compounds	PDE IC_{50} (μM)[a]					
	PDE2	PDE1	PDE4	PDE5	PDE9	PDE10
6p	0.072±0.006	> 500	> 500	> 500	13.9±1.5	> 500
6s	0.081±0.009	> 500	> 500	> 500	32.3±3.9	> 500

[a] Results are expressed as the mean of at least three experiments.

Compound **6s** was docked into the binding pocket of PDE2 by using Discovery Studio 2017. The simulation indicated that the compound has a lower CDOCKER interaction energy when its R2 group bears the (R)-stereochemistry. As predicted, this compound binds to the catalytic domain in a cGMP-like binding mode shown by the orientation of Gln859 [Figure 2]. Two hydrogen bonds were observed between the carbonyl and lactam groups of compound **6s** and Gln859. As shown in Figure 2, the aromatic ring of the purin-6-one is anchored in the active binding site through a face-face p-stacking interaction with Phe862 and Phe830, and these interactions are commonly referred to in PDE nomenclature as the hydrophobic clamp region. The isooctyl group is situated in a

binding-induced hydrophobic pocket between Ile866 and Leu770 which is unique to PDE2 and has been described in earlier publications.[18] Also, the methoxybenzyl moiety of compound **6s** interacts with the surface hydrophobic patch of the binding pocket of PDE2, which can enhance the PDE2 inhibitors' potency and selectivity. [18]

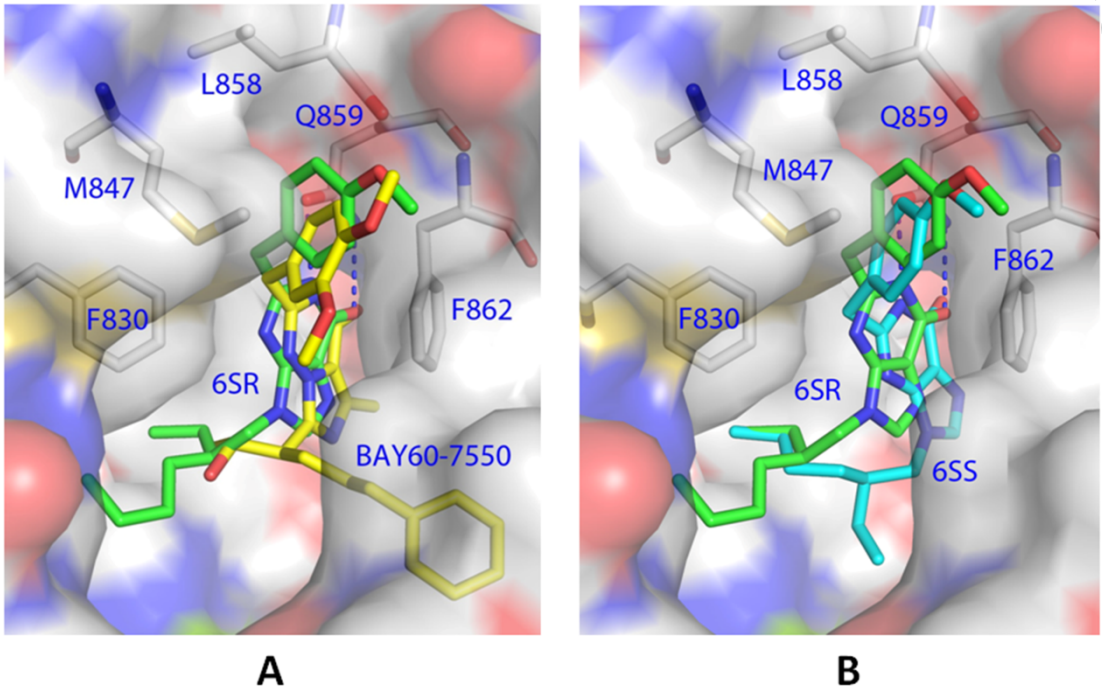


Figure2. Binding mode of compound 6s (R: green, S:) and BAY60-7550 (yellow) within the active pocket of PDE2 (PDB ID: 4HTX). Hydrogen bonds are represented by dashed lines (blue).

HT-22 cells, which are immortalized mouse hippocampal neuronal precursor cells, were used to understand cellular potency of **6p** and **6s** relevant to the hippocampus activity. [20] To determine the neuroprotective effect against corticosterone-induced toxicity, HT-22 cell viability in the presence of 100 μM CORT (positive control) or absence of CORT (vehicle) and presence of various concentrations of **6p** and **6s** was assayed. As shown in Figure 3, the cell viability was significantly decreased (about 60% survived) when HT-22 cell was treated with 100 μM CORT ($p < 0.001$). Compound **6s** protected HT-22 cells from corticosterone-induced death at all the tested concentrations between 0.01 and 1 μM, and the best concentration was 0.1 μM ($p < 0.01$). In contrast, compound **6p** did not show the similar effects. We speculated that the lack of activity of **6p** *in vitro* may be attributed to its low cell membrane penetration resulting from higher molecular weight compared with compound **6s**, but it need more detailed study.

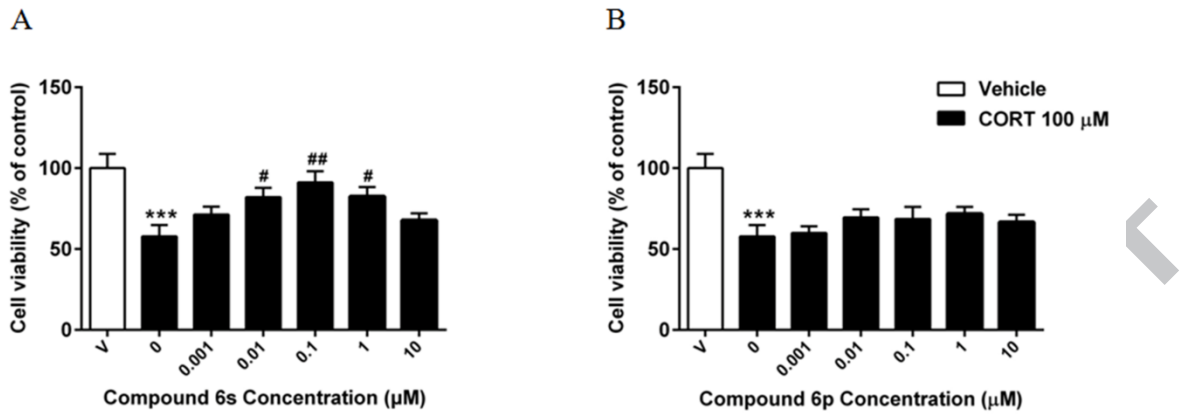


Figure 3. Compounds **6s** and **6p** protected cells against corticosterone (CORT)-induced cytotoxicity in a concentration-dependent manner. (A) HT-22 cells were treated with 100 M CORT for 30 min and Compound **6s** was added for 24 hr. (B) HT-22 cells were treated with 100 M CORT for 30 min and Compound **6p** was added for 24 hr. Cell viability was measured by MTS assay. Results are expressed as the mean ± standard error of the mean (SEM) of six independent experiments performed in triplicates. $^{***}p < 0.001$, compared to control group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared to vehicle-treated CORT group.

To explore whether its cell protection activity is achieved *via* inhibition on PDEs, the effects of compound **6s** on accumulation of cGMP and cAMP in HT-22 cells were further evaluated. Cyclic nucleotide (cAMP and cGMP) signaling in brain is fundamentally involved in mechanisms that require for the neuronal activity and energy production, metabolic processes and synaptic physiology. [21,22] A decrease in cGMP-dependent signal transduction has been demonstrated in hippocampus during aging and Alzheimer's disease. [23-25] Our results revealed a significant decrease in cGMP expression when HT-22 cells were exposed to 100 μM corticosterone ($p < 0.01$). The decrease of cGMP level was rescued by treatment with compound **6s** at concentrations of 0.01, 0.1 and 1.0 μM, when compared to corticosterone-treated group ($p < 0.05$; $p < 0.01$; $p < 0.05$). However, cAMP levels did not show significant changes even though there was slight increase. These effects were similar to those of the positive control drug at concentration of 1.0 μM. The present results may indicate that compound **6s** probably protected HT-22 cells from corticosterone-induced death by activation of cAMP and cGMP-dependent signaling.

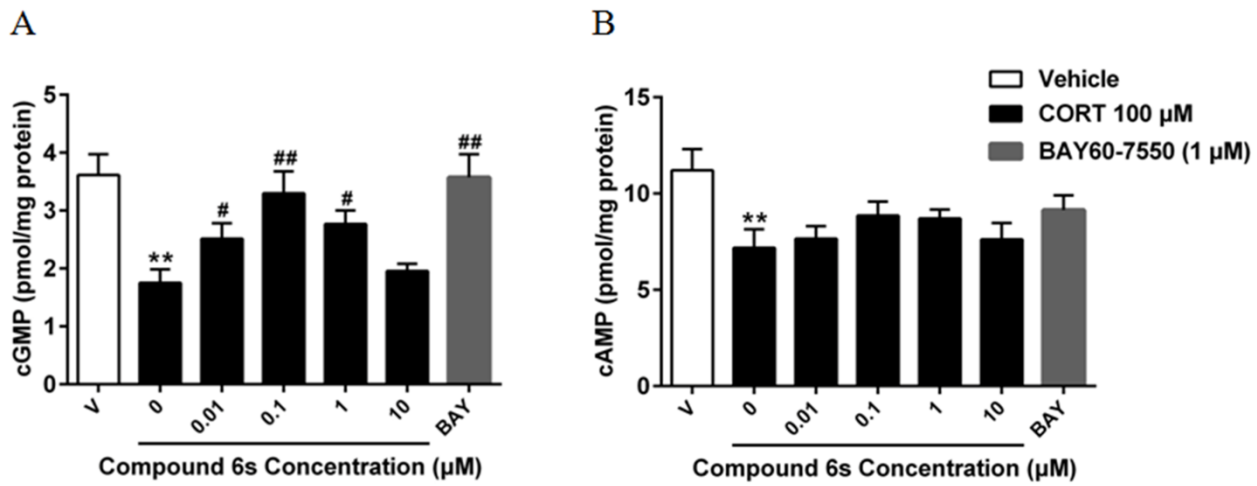


Figure 4. The effects of Compound 6s on CORT-induced cGMP and cAMP reduced levels in concentration-dependent manners in the HT-22 cells. HT-22 cells were treated with 100 M CORT for 30 min and added compound **6s** for 24 hr. The results represent the mean ± SEM, n = 6. $^{**}p < 0.01$, compared to control group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared to vehicle-treated CORT group.

To detect if compound **6s** has physiological effects *in vivo*, we examined the effects of compound **6s** *in vivo* and evaluated behavioral changes of chronic stress-induced mice using the elevated plus-maze test. As shown in Figure 5, chronic stress induced a significant decrease in the open-arm entry time ($p < 0.001$), and compound **6s** reversed the effects in dose-dependent manner in comparison to vehicle group. The maximum increases were observed at a dose of 2.0 mg/kg (p.o.) [$F(4, 32) = 16.23$, $p<0.01$; $F(4, 32) = 10.87$, $p<0.01$]. The classical drug diazepam showed similar anxiolytic-like effects, but Bay 60-7550 did not exhibited the effects at doses of 0.5 mg/kg *via* i.p. Their locomotion counts were measured 10 minutes after a single treatment to rule out whether compound **6s** had any CNS stimulating or inhibiting effects on mice. The results showed that none of doses of compound **6s** affected locomotor activity, indicating the anxiolytic-like effects of compound **6s** were not due to central stimulation or inhibition. Due to the promising activity *in vivo*, compound **6s** was evaluated for pharmacokinetic properties [Table 3]. A preliminary pharmacokinetic analysis revealed that compound **6s** has C_{max} of 511 ng/mL, T_{max} of 0.6 h and $T_{1/2}$ of 1.7 h in the oral administration mode. Especially, the compound achieved good brain exposure (AUC of 646 h·ng/ml) and acceptable B/P ratio (B/P of 0.56). From these studies it could be concluded that compound **6s**, although less potent, had better pharmacokinetic profile than BAY60-7550.

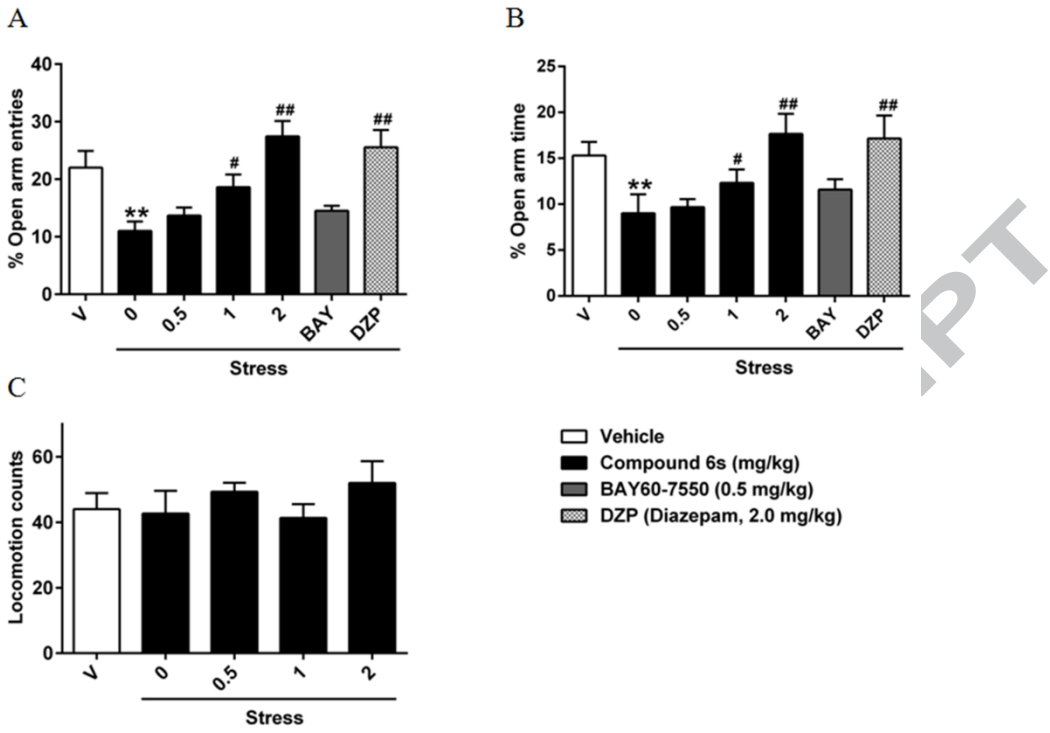
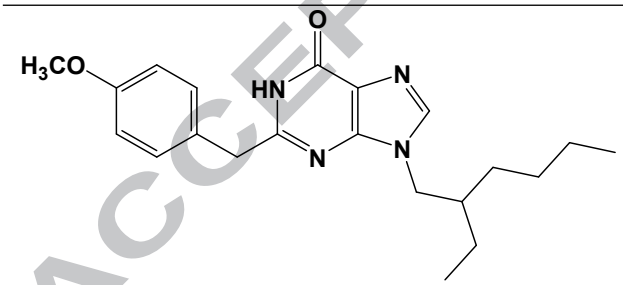


Figure 5. The effects of compound **6s** on chronic stress-induced anxiety-like behaviors and cognitive impairment in EPM (A and B) and LA (C) tests. The results represent the mean ± SEM, n = 6. $^{**}p < 0.05$, compared to control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to vehicle-treated stressed group. DZP (diazepam, 0.5 mg/kg, i.p.).

Table 3. Pharmacokinetic profile of compound **6s** in mice[a]

Compound	Parameters	
	$T_{1/2}$ (h)	1.4 ± 0.16
	T_{max} (h)	0.53± 0.02
	C_{max} (ng/ml)	511 ± 83
	$AUC_{0-\infty}$ (h·n g /ml)	646 ± 83
	B/P ratio[b]	0.56 ± 0.03

[a] Dose: 2 mg/kg, p.o. (oral administration). [b] B/P ratio: Brain to plasma ratio at 0.5 h after oral administration.

In summary, a series of purin-6-one derivatives with a new scaffold were designed and synthesized, of which compounds **6p** and **6s** showed high inhibitory activities with the IC_{50} values of 72 and 81 nM, respectively, and > 5000 fold selectivity over PDEs 1, 4, 5 and 10. Compound **6s** protected the HT-22 cells from the corticosterone-induced death and rescued corticosterone-induced decrease of cAMP and cGMP. The *in vivo* study suggested that compound **6s** dose-dependently ameliorated acute stress-induced anxiety impairment and possessed acceptable brain uptake. Thus,

our study provided a basis for the rational design of novel PDE2 inhibitors with high affinity, and subsequent efforts on further optimization of this structural class would lead to more potent and selective PDE2 inhibitors with favorable pharmacokinetic properties.

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Design, synthesis of novel purin-6-one derivatives as phosphodiesterase 2 (PDE2) inhibitors: the neuroprotective and anxiolytic-like effects

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