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# Synthesis of hybrid molecules of caffeine and eudistomin D and its effects on adenosine receptors

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**Abstract**—Four hybrid molecules (**1** and **12**–**14**) of caffeine and eudistomin D, a β-carboline alkaloid from a marine tunicate, were synthesized, and their affinity and selectivity for adenosine receptors $A_1$, $A_{2A}$, and $A_3$ were examined. It was found that all the compounds showed better potency as adenosine receptor ligands as compared with caffeine. Among them, a compound (**13**) possessing a nitrogen at the δ–position of the pyridine ring (δ-*N* type) showed the most potent affinity for adenosine receptor $A_3$ subtype, while *N*-methylation (**14**) of a pyrrole ring in **13** significantly lowered the potency as adenosine receptor ligands. Compounds (**1** and **12**) having a nitrogen at the β-position of the pyridine ring (β-*N* type) showed lower affinity than the corresponding δ-*N* type compounds (**13** and **14**), while compounds (**10**, **11**, and **17**) lacking a pyrrole ring between the pyridine and pyrimidine rings exhibited almost no affinity to the adenosine receptor subtypes examined.
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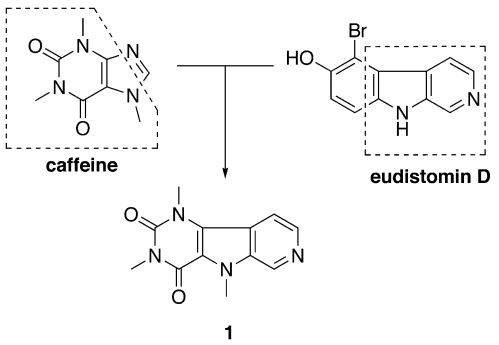
## 1. Introduction

Caffeine exhibits a variety of physiological activities (or action) including regulation of the blood pressure, respiratory functioning, gastric and colonic activity, urine volume, and exercise performance.[1] The mechanism of action of caffeine is reported to be competitive antagonism to $A_1$ and $A_{2A}$ adenosine receptors,[2] induction of $Ca^{2+}$-release from sarcoplasmic reticulum (SR), inhibition of phosphodiesterase, and so on. During our continuing search for bioactive compounds from marine organisms, we have found that eudistomin D, a β-carboline alkaloid isolated from a marine tunicate *Eudistoma olivaceum*,[3] and its analogs are potent inducers of $Ca^{2+}$-release from SR[4] as well as inhibitors of phosphodiesterase.[5] In the present study, to obtain a useful bioprobe to examine the mechanism of action of caffeine in details, we synthesized hybrid molecules of caffeine and eudistomin D such as compound **1** (Scheme 1) by two routes, **A** and **B**, as shown in Scheme 2.

## 2. Results and discussion

### 2.1. Chemistry

A key at route **A** is photoreaction of **2**, derived from **3** and **4** employing Pd-catalyzed coupling, while that at route **B** is Pd-catalyzed intramolecular cyclization of **5**, obtained from **6** and **7** employing Stille coupling reaction. The synthesis of **1** via route **A** is summarized in Scheme 3. Coupling of 5-amino-1,3-dimethyluracil (**8**)[6] and 3-chloropyridine (**9**) in DMF with $Pd_2(dba)_3$,




**Scheme 1.** A hybrid compound (**1**) of caffeine and eudistomin D.

*Keywords*: Eudistomin D; Cafffeine; Adenosine receptor; SAR.
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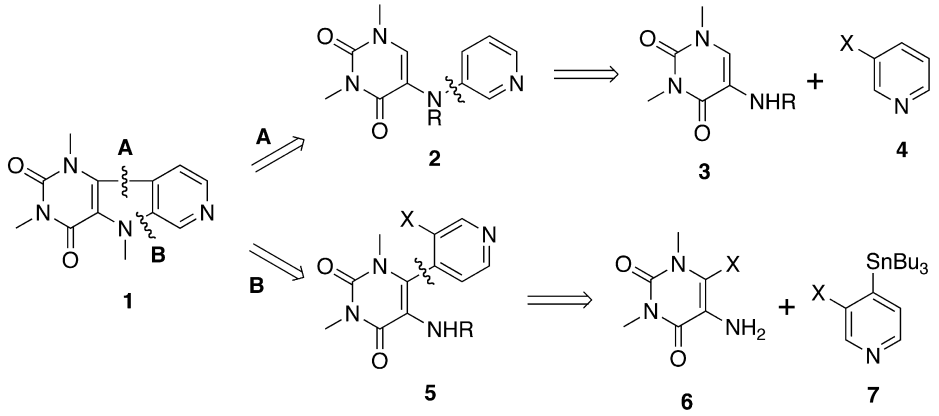
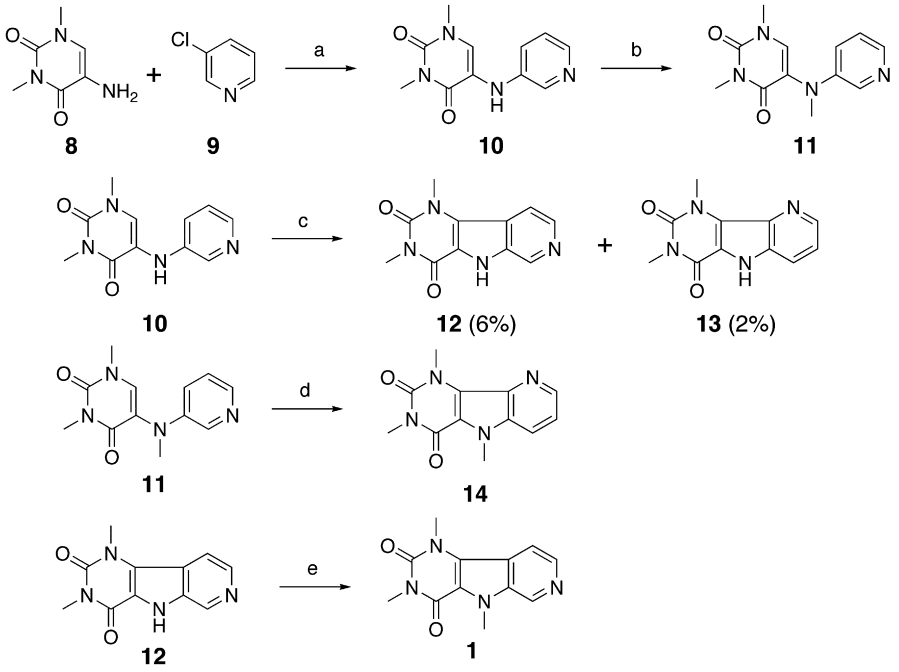


**Scheme 2.** Retrosynthetic analysis of compound **1** through two routes **A** and **B**.



**Scheme 3.** Synthesis of compound **1** and its congeners (**12**–**14**) via route **A**. Reagents: (a) $Pd_2(dba)_3$, XPhos, $Cs_2CO_3$/DMF (87%); (b) MeI, NaH/DMF (82%); (c) *hν*/*i*-PrOH; (d) *hν*/acetone (6%); (e) MeI, NaH/DMF (86%).

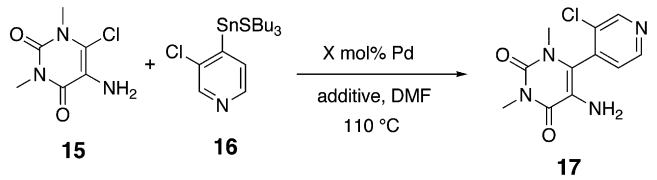
Xphos,[7] and $Cs_2CO_3$ afforded **10** in 87% yield. Methylation of **10** in DMF with MeI and NaH provided **11**. Photocyclization of **10** in *i*-PrOH afforded **12** and **13**, while that of **11** in acetone gave **14**. Methylation of **12** in DMF with MeI and NaH furnished **1** in 86% yield.

Alternative synthesis of **1** via route **B** is summarized in Table 1 and Scheme 4. A survey of additives for Stille coupling reaction of 5-amino-6-chloro-1,3-dimethyluracil (**15**)[8] and 4-tributylstannyl-3-chloropyridine (**16**)[9] in DMF revealed that the coupling reaction proceeded with CuI (entries 3 and 5–9). Switching ligand from $AsPh_3$ to $PPh_3$ improved yields of **17** (entries 7 vs 8). Increasing catalyst up to 100 mol% gave **17** in 55% yield (entries 7 vs 9). Intramolecular cyclization of **17** with $Pd_2(dba)_3$, Xphos, and $Cs_3CO_3$ provided **12**. Methylation of **12** in DMF with MeI and NaH furnished **1** in 86% yield.

## 2.2. Biological evaluation

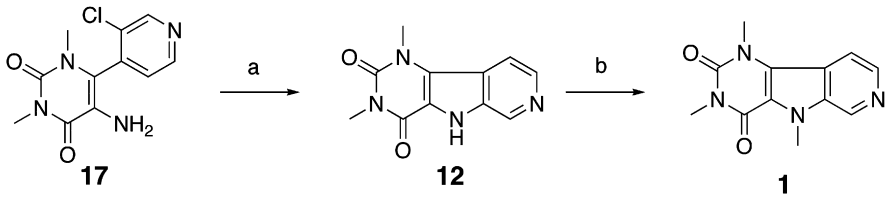
Previously, our group has found that a β-carboline compound, 7-bromo-eudistomin D (BED), is a good $Ca^{2+}$-releaser from SR and its *N*-methyl derivative of the pyrrole ring (9-methyl-7-bromoeudistomin D) is 10 times more potent than BED.[10] The presence of a nitrogen atom in the pyridine ring is essential for the $Ca^{2+}$-releasing activity, while the position of a nitrogen in the pyridine ring does not affect the potency of the $Ca^{2+}$-releasing activity. Actually, compounds possessing a nitrogen at the α, γ, or δ position of the pyridine ring have almost the same activity as the corresponding β-carboline, such as BED. Therefore, we first had a plan to synthesize a hybrid molecule (**1**) of caffeine and eudistomin D, expecting as a caffeine analog that could show a high potency as adenosine receptor ligands. On the way to the synthesis of des-*N*-methyl derivative

**Table 1.** Preparation of compound **17** by Stille coupling of **15** with **16**




| Compound | Palladium | Ligand | mol% | Additives | Yield % |
|---|---|---|---|---|---|
| **1**[a] | $Pd(PPh_3)_4$ | — | 10 | — | — |
| **2** | $Pd(PPh_3)_4$ | — | 30 | CuO (1.4 equiv.) | — |
| **3** | $Pd(PPh_3)_4$ | — | 10 | CuI, LiCl (1.4 equiv.) | 4 |
| **4** | $Pd_2(dba)_3$ | $AsPh_3$ | 10 | — | 0 |
| **5** | $Pd(PPh_3)_4$ | — | 20 | CuI, LiCl (1.4 equiv.) | 4[b] |
| **6** | $Pd(PPh_3)_4$ | — | 30 | CuI, LiCl (1.4 equiv.) | 12 |
| **7** | $Pd_2(dba)_3$ | $PPh_3$ | 30 | CuI, LiCl (1.4 equiv.) | 16 |
| **8** | $Pd_2(dba)_3$ | $AsPh_3$ | 30 | CuI, LiCl (1.4 equiv.) | 12 |
| **9** | $Pd_2(dba)_3$ | $PPh_3$ | 100 | CuI, LiCl (1.4 equiv.) | 55 |

[a] 65 °C.
[b] Determined by NMR.




**Scheme 4.** Synthesis of compound **1** via route **B**. Reagents: (a) $Pd_2(dba)_3$, XPhos, $Cs_2CO_3$/DMF (71%); (b) MeI, NaH/DMF (86%).

(**12**) of **1** having a nitrogen at the β-position of the pyridine ring (β-*N* type), a compound (**13**) possessing a nitrogen at the δ-position of the pyridine ring (δ-*N* type) was produced. The *N*-methyl derivatives (**1** and **14**, respectively) of **12** and **13** were also prepared to examine the effect of the *N*-methyl group on the activity of adenosine receptors.

The potency of these newly synthesized compounds as adenosine receptor ligands was investigated in radioligand binding assays at human recombinant adenosine $A_1$, $A_{2A}$, and $A_3$ receptors expressed in membranes of HEK293T cells as described under Section 4. The results expressed as $K_i$ values are presented in Table 2. When the affinity of the test compounds was very low, percentage of inhibition at 100 μM was shown. Affinities of reference ligands, that is, the non-selective caffeine and 5′-(*N*-ethylcarboxamido)adenosine (NECA), the $A_1$-selective xanthine amine congener (XAC), and the $A_{2A}$-selective CGS21680, were also shown for comparison. These compounds showed reasonable affinity for each receptor confirming the validity of our assay.

Among all the compounds examined (Table 2), a δ-*N* type compound **13** exhibited most potent affinity to all the three adenosine receptors, $A_1$ $A_{2A}$, and $A_3$, especially very high affinity for $A_3$. The $K_i$ value (0.0139 μM) of **13** to adenosine $A_3$ receptor was comparable to that ($K_i$ = 0.020 μM) of the potent $A_3$ agonist, NECA. For affinity to the adenosine $A_1$ receptors, the δ-*N* type compound (**13**) showed a moderate potency ($K_i$ = 0.379 μM) but much better than the corresponding β-*N* type compound (**12**), while *N*-methylation of the pyrrole ring (**14** and **1**, respectively) in **13** and **12** resulted in significant loss of the affinity to the receptors. Similarly, the δ-*N* type compound (**13**) showed higher affinity for adenosine $A_{2A}$ receptors than the corresponding β-*N* type compound (**12**). *N*-Methylation (**1**) of the pyrrole ring in **12** showed a better affinity to $A_{2A}$ receptor than the parent molecule (**12**). However, *N*-methylation (**14**) of the pyrrole ring in **13** showed a lower affinity to $A_{2A}$ receptor than the parent molecule (**13**). Compounds (**10**, **11**, and **17**) lacking a pyrrole ring between the pyridine and pyrimidine rings exhibited almost no affinity to any adenosine receptors examined.

## 3. Conclusion

Overall, it was found that (1) the hybrid molecules (**1** and **12**–**14**) of caffeine and eudistomin D synthesized here showed better potency as adenosine receptor ligands than caffeine, (2) the δ-*N* type compound (**13**) showed the most potent activity for adenosine receptors tested in this study, especially for $A_3$ subtype, and (3) *N*-methylation (**14**) of the pyrrole ring in **13** significantly lowered the potency as adenosine receptor ligands.

## 4. Experimental

### 4.1. Chemistry

**4.1.1. Instruments and analyses.** The IR spectrum was recorded on a JASCO FT/IR-5300 spectrometer. Proton and carbon NMR spectra were recorded on a Bruker

**Table 2.** Affinities of caffeine and eudistomin D hybrid molecules (**1** and **12**–**14**) and its related compounds at human adenosine $A_1$, $A_{2A}$ and $A_3$ receptors

| Compound | $K_i$[a] (μM) or % inhibition[b] | | |
|---|---|---|---|
| | $A_1$ | $A_{2A}$ | $A_3$ |
| **13** | 0.379 ± 0.043 | 0.893 ± 0.086 | 0.0139 ± 0.0032 |
| **12** | 11.7 ± 6.2 | 10.1 ± 0.01 | 0.526 ± 0.156 |
| **14** | 13.5 ± 3.2 | 6.94 ± 2.04 | 0.332 ± 0.074 |
| BED (7-bromo-eudistomin D) | 7.37 ± 2.13 | 4.92 ± 1.17 | 2.05 ± 0.69 |
| **1** | 27% | 4.19 ± 1.87 | 3.19 ± 0.08 |
| **10** | 22% | 38% | 25% |
| **11** | 9% | 29% | 0.2% |
| **17** | 8% | 15% | 3% |
| Caffeine | 49.0 ± 19.6 | 18.1 ± 5.9 | 9% |
| XAC | 0.009 ± 0.001 | nd | nd |
| CGS21680 | nd | 0.0462 ± 0.0084 | nd |
| NECA | nd | nd | 0.020 ± 0.009 |

[a] The $K_i$ values are means ± SEM of two or three separate assays, each performed in duplicate.

[b] Percentage of inhibition (%) of specific [$^3$H]DPCPX (for $A_1$), [$^3$H]CGS21680 (for $A_{2A}$) or [$^3$H]NECA (for $A_3$) binding by test compounds at 100 μM concentration. The binding of each radioactive ligand to membranes prepared from HEK293T cells expressing human adenosine $A_1$, $A_{2A}$, or $A_3$ receptors was best-fitted to a one-site model of binding with estimated $K_d$ (dissociation constant) values of 5, 52, and 6.5 nM, respectively, and $B_{max}$ values of 8600, 7000, and 310 fmol/mg protein, respectively. nd; not determined.

500 and/or 600 MHz and JEOL 400 MHz spectrometer. ESI mass spectra were obtained on a JEOL JMS-SX102A spectrometer.

**4.1.2. 1,3-Dimethyl-5-(pyridin-3-ylimino)-imidazolidine-2,4-dione (10).** DMF (0.25 mL) was added to an oven-dried Schlenk tube charged with 5-amino-1,3-dimethyluracil (**8**) (78.0 mg, 0.5 mmol), $Pd_2(dba)_3$ (23.0 mg, 25 μmol), 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (24.2 mg, 50 μmol), and $Cs_2CO_3$ (230.3 mg, 0.71 mmol). The mixture was stirred for 10 min at ambient temperature. 3-Chloropyridine (**9**; 48 μL, 0.5 mmol) was added and then stirred for 18 h at 65 °C. The reaction mixture was filtered through Celite and the residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH, 60:1 → 20:1) to give **10** (101.1 mg, 87%) as a brown oil. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.48 (1H, d, $J$ = 8.4 Hz), 8.27 (1H, dt, $J$ = 4.5, 1.6 Hz), 7.38–7.29 (2H, m), 6.15 (1H, s), 3.55 (3H, s), 3.54 (3H, s), 2.52 (1H, s); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 161.0, 149.9, 141.6, 139.5, 138.8, 125.9, 123.6, 117.4, 37.3, 28.6; IR (KBr) $\nu_{max}$ 3428, 3318, 1698, 1640 cm$^{-1}$; HRESIMS calcd for $C_{11}H_{12}N_4O_2$ ($M^+$) $m/z$ 232.0960, found $m/z$ 232.0971.

**4.1.3. 1,3-Dimethyl-5-(methylpyridin-3-yl-amino)-1*H*-pyrimidine-2,4-dione (11).** To a solution of **10** (51.2 mg, 0.22 mmol) in DMF (1 mL) was added NaH (8.1 mg, 0.33 mmol). After 10 min at room temperature, to the reaction mixture was added MeI (14 μL, 0.22 mmol) and then stirred for 3 h. The reaction mixture was filtered through Celite and the residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH, 5:1) to give **11** (44.4 mg, 82%) as a brown oil. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.98 (1H, d, $J$ = 3.0 Hz), 7.92 (1H, d, $J$ = 4.5 Hz), 7.34 (1H, s), 7.00 (1H, dd, $J$ = 8.4, 4.6 Hz), 6.88 (1H, dd, $J$ = 8.5, 3.0 Hz), 3.31 (3H, s), 3.24 (3H, s), 3.06 (3H, s); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 161.0, 151.0, 144.8, 142.6, 139.4, 135.7, 123.3, 120.3, 119.7, 39.5, 37.1, 28.2, ; IR (KBr) $\nu_{max}$ 1706, 1652, 1488 cm$^{-1}$; HRESIMS calcd for $C_{12}H_{14}N_4O_2$ ($M^+$) $m/z$ 246.1116, found $m/z$ 246.1125.

**4.1.4. 2,4-Dimethyl-4,9-dihydro-2,4,5,9-tetraaza-fluorene-1,3-dione (12) and 2,4-dimethyl-4,9-dihydro-2,4,7,9-tetraaza-fluorene-1,3-dione (13).** A solution of **10** (100.2 mg, 0.43 mmol) in *i*-PrOH (40 mL) was irradiated with tungsten lamp for 24 h at ambient temperature. The mixture was concentrated in vacuo and purified by flash column chromatography on silica gel ($CHCl_3$/MeOH, 5:1, hexane/EtOAc/acetone, 2:1:1) to give **12** (6.1 mg, 6%) and **13** (2.2 mg, 2%) as brown oil, respectively.

Compound **12**: $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.2 (1H, s), 8.51 (1H, dd, $J$ = 1.4, 4.2 Hz), 7.87 (1H, dd, $J$ = 1.4, 8.6 Hz), 7.42 (1H, dd, $J$ = 4.4, 8.4 Hz), 3.99 (3H, s), 3.34 (3H, s); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 156.2, 151.0, 143.5, 132.9, 125.1, 121.2, 120.8, 115.8, 101.5, 32.1, 27.9; IR (KBr) $\nu_{max}$ 1698, 1650 cm$^{-1}$; HRESIMS calcd for $C_{11}H_{10}N_4O_2$ ($M^+$) $m/z$ 230.0804, found $m/z$ 230.0809.

Compound **13**: $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.6 (1H, s), 8.86 (1H, s), 8.23 (1H, d, $J$ = 4.7 Hz), 8.00 (1H, d, $J$ = 5.6 Hz), 3.80 (3H, s), 3.34 (3H, s); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 156.2, 150.8, 137.8, 137.1, 133.7, 125.6, 119.0, 116.3, 115.2, 32.3, 28.0.; IR (KBr) $\nu_{max}$ 1698, 1638 cm$^{-1}$; HRESIMS calcd for $C_{11}H_{10}N_4O_2$ ($M^+$) $m/z$ 230.0804, found $m/z$ 230.0796.

**4.1.5. 2,5,9-Trimethyl-4,9-dihydro-2,4,5,9-tetraaza-fluorene-1,3-dione (14).** A solution of **11** (46 mg, 0.19 mmol) in acetone (160 mL) was irradiated with tungsten lamp for 20 h at ambient temperature. The mixture was concentrated in vacuo and purified by $C_{18}$ column chromatography (MeOH/$H_2O$, 20:80 → 80:20) to give **14** (2.9 mg, 6%) as a brown solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.54 (1H, dd, $J$ = 4.4, 1.2 Hz), 8.15 (1H,

dd, $J = 8.7$, 1.1 Hz), 7.49 (1H, dd, $J = 8.6$, 4.4 Hz), 4.09 (3H, s), 4.00 (3H, s), 3.37 (3H, s); HRESIMS calcd for $C_{12}H_{12}N_4O_2$ ($M^+$) *m/z* 244.0960, found *m/z* 244.0962.

**4.1.6. 2,4,9-Trimethyl-4,9-dihydro-2,4,7,9-tetraaza-fluorene-1,3-dione (1).** To a solution of **12** (7.0 mg, 0.03 mmol) in DMF (2 mL) was added NaH (16.2 mg, 0.7 mmol). After 30 min at room temperature, to the reaction mixture was added a solution of MeI (2 μL, 0.032 mmol) in DMF (0.2 mL) and then stirred for 2 h. The reaction mixture was filtered through Celite and the residue was purified by chromatography on a $C_{18}$ column ($MeOH/H_2O$, 50:50 → 100:0) to give **1** (6.4 mg, 86%) as a yellow solid. $^1H$ NMR (600 MHz, DMSO-$d_6$) $\delta$ 9.11 (1H, br s), 8.29 (1H, br s), 8.01 (1H, br s), 4.42 (3H, s), 3.90 (3H, s), 3.51 (3H, s); HRESIMS calcd for $C_{12}H_{12}N_{12}O_2$ ($M^+$) *m/z* 244.0960, found *m/z* 244.0963.

**4.1.7. 5-Amino-6-(3-chloropyridin-4-yl)-1,3-dimethyl-1*H*-pyrimidine-2,4-dione (17).** DMF (1.5 mL) was added to an oven-dried Schlenk tube charged with 5-amino-6-chloro-1,3-dimethyluracil (**15**) (50.0 mg, 0.262 mmol), $Pd_2(dba)_3$ (120.2 mg, 0.131 mmol), $PPh_3$ (69.3 mg, 0.262 mmol), CuI (70.7 mg, 0.367 mmol), and LiCl (16.0 mg, 0.367 mmol). The mixture was stirred for 30 min at 110 °C. 3-Chloro-4-tributyltinstannylpyridine (**16**; 80 μL, 0.262 mmol) was added and then stirred for 19 h at 110 °C. The reaction mixture was filtered through Celite and the residue was purified by flash column chromatography on silica gel (hexane/$CHCl_3$, 60:1 → 20:1, and then hexane/EtOAc 6:1 → 2:3) to give **17** (38.2 mg, 55%) as a brown oil. $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.83 (1H, s), 8.70 (1H, d, $J = 4.9$ Hz), 7.32 (1H, d, $J = 4.9$ Hz), 3.47 (3H, s), 3.08 (3H, s), 2.83 (2H, br s); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 159.8, 150.9, 150.1, 149.1, 137.8, 131.4, 125.0, 124.1, 119.9, 33.3, 28.7.; HRESIMS calcd for $C_{11}H_{11}N_4O_2Cl$ ($M^+$) *m/z* 266.0570, found *m/z* 266.0569.

### 4.2. Biological assays

**4.2.1. Radioligand materials.** [$^3$H]-8-Cyclopentyl-1,3-dipropylxanthine ([$^3$H]DPCPX), [$^3$H]-2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine ([$^3$H]CGS21680), and [$^3$H]-5′-*N*-ethylcarboxamidoadenosine ([$^3$H]NECA) were purchased from Perkin-Elmer (Boston, MA, USA). Unless otherwise stated, all other materials used for ligand binding assay were purchased from Sigma–Aldrich (St. Louis, MO, USA).

**4.2.2. Membrane preparations.** HEK293T cell lines transiently expressing human adenosine $A_1$, $A_{2A}$, and $A_3$ receptors were used as the receptor source in this study. Plasmids encoding human adenosine $A_1$, $A_{2A}$ or $A_3$ receptor construct obtained from UMR cDNA Resource Center (Rolla, MO, USA) were transiently transfected into HEK293T cells using Effectene (Quiagen). Cells were maintained at 37 °C in humidified air containing 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum, 100 μg/mL kanamycin for 48 h. The cells were harvested and homogenized in lysis buffer containing 50 mM Tris–HCl buffer (pH 7.4) with a protease-inhibitor mixture (Roche Diagnostics) and subjected to low-speed centrifugation to remove organelles and nuclei. The resulting supernatant was subjected to centrifugation at 30,000*g* for 20 min, and precipitated cell membranes were collected, washed twice, resuspended in the lysis buffer, and stored at −80 °C until use.

**4.2.3. Adenosine receptor binding assays.** Radioligand binding experiments to adenosine $A_1$, $A_{2A}$, and $A_3$ receptors were carried out by using [$^3$H]DPCPX, [$^3$H]CGS21680, and [$^3$H]NECA, respectively. Cell membranes expressing adenosine $A_1$, $A_{2A}$, and $A_3$ receptors were incubated with 4 nM [$^3$H]DPCPX, 15 nM [$^3$H]CGS21680, or 32 nM [$^3$H]NECA, respectively, in the presence of 9 to 10 different concentrations of test compounds in 250 μL of assay buffer containing 50 mM Tris–acetate buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, and 1 U/mL adenosine deaminase for 60 min at 25 °C. The incubated mixture was harvested on Whatman GF/B filters pre-soaked in 0.1% polyethyleneimine by a cell harvester and washed three times with 50 mM Tris–HCl buffer (pH 7.4). The radioactivity on the filter was measured by a scintillation counter. All experiments were carried out two or three times in duplicate. The nonspecific binding for adenosine $A_1$, $A_{2A}$ and $A_3$ receptors was defined as the binding activity in the presence of XAC, CGS21680, and NECA, respectively, at 10 μM each. $K_d$ and $B_{max}$ values in saturation and inhibition studies were determined using one-site binding model by non-linear regression analysis (GraphPad Prism 4; GraphPad, San Diego, CA, USA).

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