Original Article

Adenosine A₁ receptor agonist N⁶-cyclohexyladenosine induced phosphorylation of delta opioid receptor and desensitization of its signaling

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Aim: To define the effect of adenosine A₁ receptor (A₁R) on delta opioid receptor (DOR)-mediated signal transduction.

Methods: CHO cells stably expressing HA-tagged A₁R and DOR-CFP fusion protein were used. The localization of receptors was observed using confocal microscope. DOR-mediated inhibition of adenylyl cyclase was measured using cyclic AMP assay. Western blots were employed to detect the phosphorylation of Akt and the DOR. The effect of A₁R agonist N⁶-cyclohexyladenosine (CHA) on DOR down-regulation was assessed using radioligand binding assay.

Results: CHA 1 μmol/L time-dependently attenuated DOR agonist [D-Pen^{2,5}]enkephalin (DPDPE)-induced inhibition of intracellular cAMP accumulation with a $t_{1/2}$ =2.56 (2.09–3.31) h. Pretreatment with 1 μmol/L CHA for 24 h caused a right shift of the dose-response curve of DPDPE-mediated inhibition of cAMP accumulation, with a significant increase in EC₅₀ but no change in E_{max}. Pretreatment with 1 μmol/L CHA for 1 h also induced a significant attenuation of DPDPE-stimulated phosphorylation of Akt. Moreover, CHA time-dependently phosphorylated DOR (Ser363), and this effect was inhibited by A₁R antagonist 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX) but not by DOR antagonist naloxone. However, CHA failed to produce the down-regulation of DOR, as neither receptor affinity (K_d) nor receptor density (B_{max}) of DOR showed significant change after chronic CHA exposure.

Conclusion: Activation of A₁R by its agonist caused heterologous desensitization of DOR-mediated inhibition of intracellular cAMP accumulation and phosphorylation of Akt. Activation of A₁R by its agonist also induced heterologous phosphorylation but not down-regulation of DOR.

Keywords: adenosine A₁ receptor; N⁶-cyclohexyladenosine; delta opioid receptor; cAMP; Akt

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Introduction

Accumulating evidence supports an interaction between adenosine and opioids in the central nervous system and the myocardium^[1]. For example, previous studies indicate an interaction between adenosine A₁ receptor (A₁R) and delta opioid receptor (DOR) in analgesia^[2] and cardioprotection^[3]. Moreover, A₁R has also been shown to be involved in the development of opioid dependence^[4]. A₁R and DOR both belong to the G protein-coupled receptor (GPCR) superfamily. Acute activation of A₁R^[5] and DOR^[6] inhibit adenylyl cyclase activity *via* inhibitory G protein (G_i), and lead to a decrease in intracellular cAMP levels. However, upon prolonged exposure to their agonists, these receptors undergo desensitization which is indicated by the decreased ability to mediate agonist-

induced inhibition of adenylyl cyclase^[7–11]. Receptor desensitization was also measured by the activities of other downstream effectors stimulated by selective receptor agonists in various signaling pathway such as ERK1/2 and Akt/PKB^[12], which depends on not only the functional status of the receptor but also the signal transduction amplification between the receptor and the effectors^[13].

In cells expressing multiple GPCRs, prolonged stimulation of these GPCRs have been shown to result in not only homologous desensitization, but sometimes also heterologous desensitization^[14–16], which refer to a process whereby the activation of one type of receptor results in the desensitization of other types of receptor. It has been reported that prolonged A₁R agonist (–)N⁶-phenylisopropyl adenosine (PIA) treatment causes heterologous desensitization of PGE₁ receptor to inhibit lipolysis^[17], and cross desensitization has also been shown between DOR and CB₁-cannabinoid receptors^[18], D₂-dopaminergic receptor, and α₂-adrenergic receptor^[19]. However, it is

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unknown whether prolonged exposure to A₁R agonist could lead to a heterologous desensitization of DOR.

It is generally thought that receptor desensitization is relevant to receptor phosphorylation and subsequent internalization and down-regulation. [D-Pen^{2,5}]enkephalin (DPDPE)-induced homologous desensitization of the DOR was significantly attenuated by mutation of the primary phosphorylation site Ser363 to alanine^[9, 13], and heterologous desensitization was suggested to correlate with heterologous receptor phosphorylation^[20]. DOR undergoes down-regulation after chronic selective agonist exposure, for different agonists the magnitude and time-course of receptor down-regulation varied^[21–23], nevertheless, all recent studies favor the idea that reduction of active opioid receptors from the cell surface would potentiate their desensitization.

The present study was undertaken to investigate the effect of prolonged A₁R agonist N⁶-Cyclohexyladenosine (CHA) exposure on DOR-mediated regulation of intracellular cAMP levels and Akt phosphorylation in the CHO cell line stably co-expressing A₁R and DOR, and to determine whether prolonged CHA exposure could cause the phosphorylation and down-regulation of DOR.

Materials and methods

Materials

Plasmid encoding N-terminal 3xHA (Hemagglutinin)-tagged human Adenosine A₁ receptor was purchased from UMR cDNA Resource Center (Rolla, MO, USA). CHA, DPDPE, DPCPX, Naloxone, and monoclonal Anti-HA-TRITC antibody were purchased from Sigma (St Louis, MO, USA). Anti-phospho-Akt1/2/3 (Ser 473) and anti-Akt1/2/3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-DOR (Ser363) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). [8-³H]adenine and ECL plus Western Blotting Detection Reagents were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Cell culture

CHO cells were maintained in F12 medium (Gibco) with 10% fetal calf serum, and incubated in a humidified atmosphere consisting of 5% CO₂ at 37 °C. For the CHO-A₁R-DOR cells, 0.5 mg/mL G418 was added to maintain selection.

Transfection

The plasmids containing cDNA of N-terminal 3xHA-tagged human Adenosine A₁ receptor (HA-A₁R) were transfected into CHO cells by using Lipofectamine 2000 (Invitrogen). Cells stably expressing HA-A₁R were selected by culture with 1.0 mg/mL G418 for two weeks, then labeled with monoclonal anti-HA-TRITC antibody, and screened by fluorescence microscope (Olympus Optical Co Ltd). Subsequently, the CHO-A₁R cells were transfected with the plasmids containing cDNA of mouse delta opioid receptor-enhanced cyan fluorescent fusion protein (DOR-CFP). Cells stably co-expressing DOR-CFP and HA-A₁R were screened by fluorescence microscope.

Confocal microscopy

Confocal microscopy test was preformed as described previously^[24]. Briefly, cells were seeded onto poly-D-lysine coated coverslips placed in a 24-well plate at 37 °C. Cells were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were incubated with 2% BSA as blocking solution and with anti-HA-TRITC antibody at room temperature for 1 h, washed, and incubated with Hoechst for 15 min. Fluorescence was observed with a Leica TCS NT laser scanning confocal microscope.

Cyclic AMP assay

The levels of intracellular cAMP were measured as described^[25, 26]. In brief, cells were cultured in 24-well plates, serum starved, and incubated at 37 °C for 2 h in 0.5 mL/well of fresh growth medium containing 5 µCi/mL [8-³H]-adenine, 0.5 mmol/L 1-methyl-3-isobutylxanthine (IBMX), and with or without 1 µmol/L CHA of indicated duration. The incubation with [8-³H]-adenine and different time course pretreatment of CHA always ended at the same time. After incubation and extensive washing to remove residual, cells were incubated in an Assay Mixture (10 µmol/L Forskolin, 0.5 mmol/L IBMX, dissolved in Krebs-Ringer HEPES buffer) with or without the indicated concentrations of DPDPE at 37 °C for 15 min. 50 µL of 2.2 mol/L HCl was added to terminate the reaction. The cAMP was separated by Alumina column chromatography, and radioactivity was determined by liquid scintillation counting.

Western blot analysis

Cells were seeded in 24-well plates, incubated at 37 °C for 24 h, and starved in serum free media overnight. After treated with indicated chemicals, cells were lysed immediately by RIPA extraction buffer, and boiled for 10 min. Cell extracts were subjected to 10%-SDS polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membrane (Amersham Biosciences). The immunoblots were detected as described previously^[24]. Membranes were blocked with 5% non-fat dried milk dissolved in PBS/0.1% Tween 20 (PBS/T) for 1 h, and incubated overnight at 4 °C with primary antibodies diluted in PBS/T containing 5% non-fat dried milk. Membranes were subjected to 4 washes with PBS/T before incubating for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (Calbiochem). Chemiluminescence detection was performed with ECL Plus Western Blotting Detection Reagent (GE Healthcare). Immunoblots were quantified by densitometry with Quantity One (Bio-Rad). For repeated immunoblotting, membranes were stripped in ReBlot Plus Mild Antibody Stripping Solution for 15 min (Millipore Chemicon).

Plasma membrane preparation

Cell were seeded in 100-mm diameter dishes, incubated for 24 h to 90% confluence, and treated with or without CHA (1 µmol/L) for 72 h, the reaction is terminated on the ice. Cells were detached, and collected by centrifugation. Plasma

membrane fractions were prepared with a Dounce homogenizer as described^[27]. Cells were detached by incubation with phosphate-buffered saline containing 1 mmol/L EDTA and centrifuged at 1000×g for 10 min. The cell pellet was suspended in ice-cold homogenization buffer composed of 50 mmol/L HEPES, pH 7.4, 1 mmol/L MgCl₂, and 1 mmol/L EGTA. Cells were homogenized with 20 strokes using a glass Dounce homogenizer. After centrifugation at 40000×g for 10 min (4 °C), pellets were resuspended in homogenization buffer, homogenized, and centrifuged again as described. This procedure was repeated twice more. The final pellets were resuspended in a 50 mmol/L Tris-HCl buffer, pH 7.4. Protein concentration was determined and aliquots were stored at -80 °C.

Radioligand binding assay

Radioligand binding assay was performed as described previously^[27]. The DOR number were detected by incubating [³H]-diprenorphine (0.1–1.2 nmol/L) with membranes (10–15 µg of protein) in a final volume of 0.1 mL of binding buffer (Tris-HCl 50 mmol/L, pH 7.4) at 37 °C for 30 min. Naloxone (10 µmol/L) was used to define nonspecific binding. Bound and free [³H]-diprenorphine were separated by filtration under reduced pressure with GF/B filters (Whatman). Radioactivity on filters was determined by liquid scintillation counting method (Beckman LS6500).

Statistical analysis

All statistical and curve-fitting analysis was performed by the GraphPad Prism 4.0 software (GraphPad software, San Diego, CA, USA). Data are presented as mean±SEM, except the EC₅₀ values which are presented as mean (95% confidence interval), from at least three separate experiments. Statistical significance was determined by unpaired *t*-test.

Results

Coexpression of HA-A₁R and DOR-CFP in CHO cells

In order to study the possible cross-talk in signaling transduc-

tion between the A₁R and DOR, HA-tagged A₁R and CFP-tagged DOR were stably transfected into CHO cells. Co-expression of A₁R and DOR was visualized by confocal microscope, and functional activity was examined by assessing the effect of selective A₁R and DOR agonists on intracellular cAMP accumulation, because both A₁R and DOR inhibit adenylyl cyclase activity via G_i when acutely activated by their agonists, leading to reduction of intracellular cAMP levels^[5, 6, 28]. As shown in Figure 1, functional A₁R and DOR coexpressed in the CHO cells. Confocal images showed that A₁R and DOR co-localized on the plasma membrane of the CHO cells (Figure 1A). A₁R agonist CHA (1 µmol/L) and DOR agonist DPDPE (1 µmol/L) both significantly inhibited forskolin-stimulated cAMP accumulation (Figure 1B).

CHA pretreatment decreased the ability of DOR agonist DPDPE to inhibit adenylyl cyclase and phosphorate (activate) Akt in the CHO-A₁R/DOR cells

To determine whether there is an interaction between the A₁R and DOR in signal transduction, we first examined the effect of A₁R agonist CHA pretreatment on DOR-mediated inhibition of adenylyl cyclase. Like many other GPCRs, the opioid receptors undergo phosphorylation, desensitization, internalization, and down-regulation after prolonged exposure to agonists and these receptor regulatory mechanisms may play an important role in regulation of opioid receptor function. DPDPE dose dependently inhibited forskolin stimulated cAMP accumulation with EC₅₀ value of 0.095 nmol/L and *E*_{max} value of 83.64% (Figure 2A, Table 1). Pretreatment of CHO-A₁R/DOR cells with 1 µmol/L CHA for 24 h caused a right shift of the dose-response curve (Figure 2A), with a 4.38 fold increase in EC₅₀ value, while the *E*_{max} showed no statistically significant differences between pretreatment with and without CHA (Table 1). CHA time dependently attenuated DOR-mediated inhibition of intracellular cAMP, with a *t*_{1/2}=2.56 (2.09–3.31) h (Figure 2B). It indicated that pretreatment of CHO-A₁R /DOR cells with A₁R agonist led to heterologous desensitization of DOR-mediated inhibition of intracellular cAMP accumulation.

Figure 1. HA-A₁R and DOR-CFP co-localized on the plasma membrane. (A) Cells stably co-expressing HA-A₁R and DOR-CFP were stained with anti-HA-TRITC antibody and Hoechst, and detected with confocal microscopy as described in Methods. Images of HA-A₁R (red), CFP (cyan), and nucleus (blue) merged in the right image, which showed the overlap of images of HA-A₁R and DOR-CFP on the plasma membrane. Scale bar, 10 µm. (B) Acute CHA and DPDPE mediated inhibition of intracellular cAMP. Cells incubated with [8-³H]-adenine as described, washed, and then incubated in an Assay Mixture with or without 1 µmol/L CHA or 1 µmol/L DPDPE for 15 min. Data represent means±SEM from three independent experiments performed in duplicate.

Figure 2. Effect of CHA pretreatment on acute DPDPE inhibition of intracellular cAMP levels. (A) Chronic CHA exposure caused the shift of the dose-response curve of DOR-mediated inhibiting cAMP accumulation. Cells were pretreated with or without 1 μ mol/L CHA for 24 h, washed with PBS three times to remove residual, and then incubated in Assay Mixture for 15 min with or without increasing concentrations of DPDPE to inhibit forskolin stimulated cAMP accumulation. Both basal level of cAMP with (CPM: 12999 $\pm$ 860) and without (CPM: 3958 $\pm$ 262) CHA pretreatment were defined as 100%. (B) Time course of the heterologous desensitization of DOR-mediated inhibition of cAMP accumulation. Cells were pretreated with or without 1 μ mol/L CHA for the indicated time, washed, then incubated in Assay Mixture for 15 min with or without 1 nmol/L DPDPE. One-phase exponential decay equation was used to fit the curve. Data represent means $\pm$ SEM from three independent experiments performed in duplicate.

Table 1. The EC₅₀ and E_{max} value of inhibiting cAMP accumulation by DPDPE after pretreated with or without CHA. ^cP<0.01 vs control.

	EC ₅₀ (nmol/L)	E _{max} (%)
Control	0.095 (0.064–0.141)	83.64 $\pm$ 2.33
CHA pretreated	0.416 (0.228–0.760) ^c	92.92 $\pm$ 4.43

The EC₅₀ and E_{max} value were obtained from the dose-response curve of DOR mediated inhibition of cAMP accumulation described in Figure 2A. After CHA pretreatment, the EC₅₀ of inhibition cAMP accumulation by DPDPE increased significantly, while the E_{max} showed no statistically significant differences between pretreatment with and without CHA. EC₅₀ values are presented as mean (95% confidence interval), while E_{max} values are represented as means $\pm$ SEM, from three independent experiments performed in duplicate.

The serine–threonine kinases Akt is one of the key downstream targets of PI3K signaling, which regulates cell growth, differentiation, survival and functions. Activation of DOR by its agonist leads to activation of PI3K/Akt signaling pathway^[29]. To further confirm the desensitization of DOR-

mediated signaling by A₁R agonist CHA treatment, we examined the effect of CHA pretreatment on DPDPE-stimulated phosphorylation (activation) of Akt. Both CHA and DPDPE induced a robust but transient phosphorylation of Akt1/2/3 (Ser473, Figure 3A). However, pAkt induced by CHA appears to be more persistent than that induced by DPDPE. As anticipated, pretreatment of cells with 1 μ mol/L CHA for 1 h abolished the phosphorylation of Akt by DPDPE (Figure 3B, 3C). Taken together, these results clearly indicate that CHA pretreatment led to the heterologous desensitization of DOR-mediated signaling.

Figure 3. Effect of CHA pretreatment on DPDPE-stimulated Akt phosphorylation. (A) The time course of Phospho-Akt stimulated by 1 μ mol/L CHA or 10 nmol/L DPDPE. (B) Cells were pretreated with or without 1 μ mol/L CHA for 1 h, washed, and followed by incubation with 10 nmol/L DPDPE for an additional 5 min period. Cells were immediately washed and lysed. Cell lysates were subjected to SDS-PAGE as described in Methods. Membranes were immunoblotted sequentially with anti-phospho-Akt1/2/3 (Ser473) and anti-Akt1/2/3 antibodies. Representative immunoblots from three to four independent experiments are shown. (C) Data represent means $\pm$ SEM from four independent experiments. ^bP<0.05.

CHA heterologously phosphorylated DOR and this effect was inhibited by A₁R antagonist DPCPX but not by opioid receptor antagonist naloxone

It is generally considered that the molecular mechanisms underlying G protein coupled receptors (GPCRs) desensitization included receptor phosphorylation and subsequent down-regulation. Phosphorylation of Ser363 residue in the C-terminal tail of DOR has been shown to be important for agonist-mediated homologous desensitization and internalization^[9, 30]. Our recent study demonstrated that DPDPE treatment induced rapid phosphorylation of Ser363 residue in the C-terminal tail of DOR and led to desensitization of the DOR^[24]. To determine the mechanisms by which CHA pretreatment resulted in heterologous desensitization of DOR-mediated signaling, we examined the effect of CHA pretreatment on the phosphorylation of DOR. By Western blot analysis with a specific phospho-DOR (Ser363) primary antibody, we found

that both 1 $\mu\text{mol/L}$ CHA and 10 nmol/L DPDPE time-dependently phosphorylated DOR (Ser363) (Figure 4A). We also found that the heterologous phosphorylation of DOR by 1 $\mu\text{mol/L}$ CHA was completely blocked by concomitant treatment of the cells with a selective A_1R antagonist DPCPX (100 $\mu\text{mol/L}$) but not opioid receptor antagonist naloxone (100 $\mu\text{mol/L}$) (Figure 4B), suggesting that the DOR and A_1R may undergo cross-talk at receptor levels.

Figure 4. CHA stimulated heterologous phosphorylation of DOR. (A) Time-curves of phospho-DOR-Ser363 stimulated by 1 $\mu\text{mol/L}$ CHA and 10 nmol/L DPDPE. (B) Cells were treated with or without 100 $\mu\text{mol/L}$ DPCPX or 100 $\mu\text{mol/L}$ naloxone for 15 min, and followed by adding 1 $\mu\text{mol/L}$ CHA or not for an additional 5 min period. Cells were immediately washed, and lysed. Cell lysates were subjected to SDS-PAGE as described in Methods. Membranes were immunoblotted with anti-phospho-DOR-Ser363 antibody. Representative immunoblots from three to four independent experiments are shown.

Chronic CHA pretreatment caused no significant down-regulation of the DOR

Changes in receptor number or affinity provide an attractive mechanism to explain the loss of receptor responsiveness that is characteristic of desensitization. Previous studies showed that A_1R agonist (R)-PIA induced a time-dependent reduction in cell surface adenosine A_1 receptor radioligand binding sites, which reached a maximum at 48–72 h^[31, 32]. To examine whether heterologous desensitization of the DOR by CHA was attributed to receptor down-regulation, saturation binding was used to assess receptor affinity (K_d) and receptor density (B_{max}) of DOR in plasma membranes prepared from cells pretreated with or without 1 $\mu\text{mol/L}$ CHA for 72 h. Saturation curves of DOR (Figure 5A) and the Scatchard analysis of the saturation binding (Figure 5B) were present in Figure 5, which showed no significant change of receptor numbers and affinity after chronic CHA exposure. There were no statistically significant differences in B_{max} (Control, 2.587 ± 0.359 pmol/mg protein; CHA pretreated, 2.454 ± 0.390 pmol/mg protein) or K_d (Control, 0.7269 ± 0.0543 nmol/L; CHA pretreated, 0.7372 ± 0.0916 nmol/L), indicating that chronic CHA treatment failed to induce DOR down-regulation. This result suggests that heterologous desensitization of DOR-mediated signaling by CHA may not be due to the down-regulation of the DOR.

Figure 5. Saturation curves of [^3H]-Diprenorphine binding to plasma membranes pretreated with (■) or without (□) 1 $\mu\text{mol/L}$ CHA for 72 h. (A) Saturation curve. Membranes bound with increasing concentrations of [^3H]-Diprenorphine as described in Materials and methods. (B) Scatchard analysis of the saturation binding. Data shows a representative result of five independent experiments performed in duplicate.

Discussion

Previous studies revealed a tight cross-talk between A_1R and DOR. The present study demonstrated that prolonged exposure to A_1R agonist CHA resulted in heterologous desensitization of DOR-mediated inhibition of intracellular cAMP accumulation and Akt phosphorylation in CHO- A_1R /DOR cells. The heterologous desensitization of DOR-mediated inhibition of intracellular cAMP accumulation was partly, with a significant increase in EC_{50} but no change in B_{max} , whereas heterologous desensitization of DOR-mediated Akt signaling was almost completely, and preceded the desensitization of cAMP signaling, suggesting that the heterologous desensitization of DOR was specific in different signaling pathway. These results support that there is a tight cross-talk between A_1R and DOR in the regulation their functions. Desensitization of opioid receptors and its link with opiate tolerance and dependence have been extensively investigated^[33, 34]. Furthermore, cross tolerance between A_1R and μ opioid receptor has been observed previously^[35]. Thus, the heterologous desensitization may be a possible mechanism underlying opiate tolerance *in vivo*.

Previous study showed that DOR underwent phosphory-

lation and down-regulation after prolonged agonist exposure, which contributed to the homologous desensitization of DOR^[24]. In this study, prolonged CHA exposure caused heterologous phosphorylation of DOR, which was blocked by DPCPX but not naloxone, indicating that DOR was phosphorylated by CHA via A₁R activation. Although receptor phosphorylation independent of homologous^[11] and heterologous^[36] desensitization was demonstrated, there is strong evidence showing the causal relationship between desensitization and receptor phosphorylation^[9, 13]. Thus, we speculate that CHA-induced phosphorylation of the DOR may be a potential mechanism underlying the heterologous desensitization of DOR-mediated signaling by CHA treatment. However, further work is needed to confirm this speculation.

Receptor down-regulation is known to be subsequent to receptor phosphorylation and internalization. After internalization, receptor could either recycle from endosomes to the plasma membrane and reduce desensitization, or degraded in lysosomes to decrease functional receptors on cell membrane and enhance desensitization^[23]. In this study, prolonged CHA treatment did not induce DOR down-regulation, which suggests that receptor down-regulation is not a necessary consequence of phosphorylation. As prolonged CHA exposure caused no down-regulation of DOR, it seems that receptor down-regulation may not be the mechanism of heterologous desensitization of DOR by A₁R agonist CHA.

In conclusion, we found that prolonged A₁R stimulation resulted in heterologous desensitization of DOR-mediated inhibition of intracellular cAMP levels and Akt phosphorylation. We also found that activation of A₁R by its agonist induced heterologous phosphorylation but not down-regulation of the DOR. The findings of the present study suggest that receptor phosphorylation, but not down-regulation, may contribute to the heterologous desensitization of DOR-mediated signaling by CHA. These results may shed some light on the molecular mechanism of interaction between A₁R and DOR. Since desensitization of opioid receptor signaling can also result from changes at post-receptor components such as G-proteins, effectors, or their regulators, further work is needed to elucidate the mechanisms by which CHA induces heterologous desensitization of the DOR signaling.

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Author contribution

Yun CHENG and Jing-gen LIU designed research. Yun CHENG performed research, analyzed data, and wrote the paper. Jing-gen LIU revised the paper. Yi-min TAO helped with Radioligand binding assay. Jian-feng SUN, Yu-hua

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