EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 39, No. 3, 335-342, June 2007

An inhibitory compound against the interaction between $G\alpha_s$ and the third intracellular loop region of serotonin receptor subtype 6 ($5\text{-}HT_6$) disrupts the signaling pathway of $5\text{-}HT_6$

Yun Hee Choi[1], Hatan Kang[1], Won Kyu Lee[1], Taehyun Kim[2], Hyewhon Rhim[2] and Yeon Gyu Yu[3,4]

[1]Life Sciences Division
Korea Institute of Science and Technology
Seoul 130-650, Korea
[2]Biomedical Research Center
Korea Institute of Science and Technology
Seoul 136-791, Korea
[3]Department of Chemistry, Kookmin University
Seoul 136-702, Korea
[4]Corresponding author: Tel, 82-2-910-4619;
Fax, 82-2-910-4415; E-mail, ygyu@kookmin.ac.kr

Accepted 23 March 2007

Abbreviations: 5-HT, serotonin; $5\text{-}HT_6$, serotonin receptor subtype 6; FITC, fluorescein isothiocyanate; GPCR, G-protein coupled receptor; GST, glutathione S-transferase; iL3, intracellular loop 3; IPTG, isopropyl-β-D-1-thiogalactopyranoside

Abstract

Serotonin receptor subtype 6 ($5\text{-}HT_6$) is a neurotransmitter receptor, which is involved in various brain functions such as memory and mood. It mediates signaling via the interaction with a stimulatory G-protein. Especially, the third intracellular loop (iL3) of $5\text{-}HT_6$ and the α subunit of stimulatory G protein ($G\alpha_s$) are responsible for the signaling process of $5\text{-}HT_6$. Chemical compounds that could inhibit the interaction between the iL3 region of $5\text{-}HT_6$ and $G\alpha_s$ were screened from a chemical library consisted of 5,600 synthetic compounds. One of the identified compounds bound to $G\alpha_s$ and effectively blocked the interaction between $G\alpha_s$ and the iL3 region of $5\text{-}HT_6$. The identified compound was further shown to reduce the serotonin-induced accumulation of cAMP in 293T cells transformed with $5\text{-}HT_6$ cDNA. It also lowered the Ca^{2+} efflux induced by serotonin in cells expressing $5\text{-}HT_6$ and chimeric $G\alpha_{s5/q}$. These results indicate that the interaction between the iL3 of $5\text{-}HT_6$ and $G\alpha_s$ can be exploited for screening of regulatory compounds against the signaling pathway of $5\text{-}HT_6$.

Keywords: GTP-binding protein α subunits, Gs; serotonin; serotonin 6 receptor

Introduction

The neurotransmitter 5-hydroxytryptamine (5-HT), known as serotonin, is involved in various physiological functions such as learning, mood and food uptake. Improper levels of serotonin in brain are related to neurological disorders such as anxiety, depression or schizophrenia. The receptors for serotonin ($5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_3$ and $5\text{-}HT_4$) have been identified from pharmacological investigation (Brandley *et al*., 1986; Bockaert *et al*., 1990; Baxter *et al*., 1995; Kilpatrick *et al*., 1991). Moreover, three more families of serotonin receptors ($5\text{-}HT_5$, $5\text{-}HT_6$ and $5\text{-}HT_7$) were discovered from molecular biological studies (Ree *et al*., 1994; Kohen *et al*., 1996; Jasper *et al*., 1997). These serotonin receptors, except $5\text{-}HT_3$, belong to G-protein coupled receptors (GPCRs), and they transduce the signal via the interaction with different types of G-proteins. The stimulatory G-protein (G_s) is coupled to $5\text{-}HT_4$, $5\text{-}HT_6$ and $5\text{-}HT_7$ (Hoyer and Martin, 1997), whereas inhibitory G-protein (G_i) to $5\text{-}HT_1$, $5\text{-}HT_5$ (Hoyer and Schoeffter, 1991; Francken *et al*., 1998).

The serotonin receptor subtype 6 ($5\text{-}HT_6$) has been identified from rat, mouse and human (Monsma *et al*., 1993; Unsworth and Molinoff, 1994; Kohen *et al*., 1996). Human $5\text{-}HT_6$ is highly expressed in basal ganglia and limbic structure of brain (Kohen *et al*., 1996). The localization of $5\text{-}HT_6$ to these brain tissues suggested that it might participate in the serotonergic control of motor function, mood-dependent behavior, depression, and cognition (Meneses, 2001; Rogers and Hagan, 2001; Woolley *et al*., 2001). Furthermore, high binding affinities of $5\text{-}HT_6$ to antipsychotic agents such as chlorpromazine, amoxapine, clozapine and olanzapine, (Monsma *et al*., 1993; Roth *et al*., 1994; Kohen *et al*., 1996) indicated that $5\text{-}HT_6$ was related to the pathogenesis of psychiatric disorders. The involvement of $5\text{-}HT_6$ in mental function and high affinities against antipsychotic agents nominate $5\text{-}HT_6$ as a potential target for the development of antidepressant and antipsychotic drugs. Few antagonists that could selectively bind to

5-HT_6 had been developed and characterized (Boess *et al*., 1998; Hirst *et al*., 2000).

However, selective pharmacological tools for the characterization of 5-HT_6 *in vivo* are limited, except the use of antisense oligonucleotides that reduce the expression of the receptor (Bourson *et al*., 1995; Sleight *et al*., 1996). Hence, discovery of new class of regulator of 5-HT_6 is demanding for the investigation of *in vivo* function of 5-HT_6 as well as for the development of novel antipsychotic drugs.

The signaling of GPCR is mediated by specific interaction with G-proteins. In case of 5-HT_6, it interacts with stimulatory G protein, which results in the increase of cAMP by stimulation of adenylate cyclase (Hoyer and Martin, 1997). Among the three subunits of G protein, the alpha subunit ($G\alpha$) is responsible for the interaction with the receptor. Previously, we investigated the signaling mechanism of 5-HT_6 by dissecting the interactions between $G\alpha_s$ and the intracellular regions of 5-HT_6, and showed that the third intracellular loop region (iL3) of 5-HT_6 specifically bound to $G\alpha_s$ using surface plasmon resonance analysis (Kang *et al*., 2005).

In this study, we have tested whether the interaction between $G\alpha_s$ and the iL3 region of 5-HT_6 could be exploited as target site for screening of chemical compounds that could disrupt the signaling pathway of 5-HT_6. An assay system that could measure the interaction between $G\alpha_s$ and the iL3 of 5-HT_6 using 96-well plate was established, and it was utilized to screen chemical library. One of the identified compounds was shown to interrupt the serotonin-induced signaling pathway in cells expressing 5-HT_6.

Materials and Methods

Materials

The human cDNA library was purchased from Novagen (Madison, WI), and the 96-well polystyrene plate was obtained from Corning costar (NY). Reagents for cell culture were purchased from Gibco Ltd (Paisley, UK). All of the other chemicals were reagent grade.

Preparation of proteins

The expression vector pHGα_s (Kang *et al*., 2005) was used for the expression of H_6-$G\alpha_s$, the alpha subunit of stimulatory G protein containing N-terminus His_6 tag sequence. The expression of the H_6-$G\alpha_s$ was induced in *E. coli* BL21 (DE3) RP codon plus cells (Stratagene, La Jolla, CA) harboring pHGα_s by addition of 30 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 14 h at 18°C, and the expressed protein was purified using Ni^{2+}-charged HiTrap chelating HP column (GE Healthcare, Piscataway, NJ) and Mono Q HR column as described previously (Kang *et al*., 2005). The fluorescence probe labeled H_6-$G\alpha_s$ was prepared by labeling the purified protein with flourescein isothiocyanate (FITC) according to the manufacturer's manual (Sigma). The expression vector pGST-iL3 was used for the preparation of GST-iL3, the glutathione S-transferase fusion protein in which the third intracellular loop of 5-HT_6 (iL3; residue 209-265) was fused at the C-terminus of GST. The expression of GST-iL3 in *E. coli* DH5α cells was induced by adding 0.5 mM IPTG when the optical density of the culture at 600 nm (OD_{600}) reached to 0.6. After incubation of the cell culture for 16 h at 18°C, cells were harvested, and GST-iL3 was purified using a glutathione affinity column, GSTPrep FF 16/10 (GE Healthcare), as described previously (Kang *et al*., 2005).

Measurement of the interaction between H6-Gαs and GST-iL3

The interaction between H_6-$G\alpha_s$ and GST-iL3 was measured using a modified ELISA method. Briefly, H_6-$G\alpha_s$ (5 μg/ml) in 100 μl of 50 mM phosphate buffer (pH 8.0) was incubated in a 96-well medium binding polystyrene plate (Corning costar) for overnight at 4°C. After blocking the H_6-$G\alpha_s$ coated plate with 5% of (w/v) skim milk for 1 h, the plate was washed with PBS-T (50 mM sodium phosphate, pH 7.4, 150 mM NaCl and 0.5% Tween 20) for 6 times. Then, 1 μM of GST-iL3 in PBS (50 mM Sodium phosphate, pH 7.4 and 150 mM NaCl) was incubated in the plate (100 μl/well) for 2 h at room temperature. The unbound GST-iL3 was washed 6 times (300 μl/well) with PBS-T, and the plate was incubated for 1 h in the presence of 100 μl of 2,000 fold-diluted HRP conjugated anti-GST goat antibody (Sigma, St. Louis, MO). After washing the plate 6-times with PBS-T, 100 μl of 1 mg/ml of OPD in the stable peroxide substrate buffer (Pierce, Rockford, IL) was added to the plate and incubated for 10 min. The color developing reaction was terminated by adding 100 μl of 2.5 M sulfuric acid, and the absorbance of the reaction mixture at 490 nm was measured using a Spectra Max 340 spectrophotometer (Molecular Device Corp., Sunnyvale, CA). Alternatively, the interaction between GST-iL3 and the FITC-labeled $G\alpha_s$ was analyzed by measuring the amount of bound $G\alpha$ protein on immobilized GST-iL3. GST-iL3 (0.5 μg) in 100 μl PBS-T were attached to a glutathione plate (BD Biosciences) by incubation at room temperature for 1 h. The well was washed six times with 200 μl of PBS-T, and blocked with 200 μl of 5% (w/v) skim milk in PBS-T for 1 h at room temperature. After washing six times with 200 μl of PBS-T, the well was

incubated with FITC-labeled Gα_s (100 μl/well) in buffer B for 1 h at room temperature. After washing six times with 200 μl of PBST, the bound FITC-labeled Gα_s was eluted into solution by incubation with 100 μl of 8 M urea solution for 15 min. The intensity of fluorescence in the solution was measured at the excitation wavelength of 495 nm and at the emission wavelength of 525 nm using a TRIAD microplate reader (Dynex Technologies).

Screening of chemical library

The compounds of the in-house chemical library of Korea Institute of Science and Technology were tested for their inhibitory activities against the interaction between H$_6$-Gαs and GST-iL3. For the screening of the library using the modified ELISA assay, 1 μM of GST-iL3 fusion proteins in PBS (50 mM sodium phosphate, pH 7.4, and 150 mM NaCl) was mixed with 50 μM of compound and incubated in the plate (100 μl/well). Binding of HRP conjugated anti-GST goat antibody and the color development reaction were performed as described above, and the percentage of inhibition was calculated from $(A_o\text{-}A)/A_o \times 100$ (%), where A and A_o were the absorbance values at 490 nm of the reaction mixtures with or without chemical compounds, respectively.

Measurement of the serotonin-induced stimulation of cyclic AMP in HEK 293 cells transfected with 5-HT$_6$ cDNA

Human embryonic kidney (HEK) 293T cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a 5% CO_2 incubator at 37°C as previously described (Byun *et al*., 2006). The cells were plated onto 100-mm dishes, and they were transiently transfected with pCMV3b- 5-HT$_6$ using lipofectamine when they had reached 60-70% confluence as described in the manufacturer's instruction. After 48 h of transfection, cells were stimulated with 100 μM 5-HT in the presence or absence of various concentrations of selected chemicals for 20 min. The intracellular level of cyclic AMP was determined by a [^{3}H]-cyclic AMP competition method using a cAMP binding protein as described previously (Brown *et al*., 1971; Suh *et al*., 2001).

Measurement of intracellular Ca^{2+} level in CHO-K1 cells transfected with 5-HT$_6$

CHO-K1 cells were maintained in Nutrient F-12 Ham's medium supplied with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humid atmosphere of 5% CO_2 and 95% air. The human 5-HT$_6$ (Guthrie Resource Center, Sayre, PA) and the chimeric G-protein α-subunit G$\alpha_{s5/q}$ (from Dr. Conklin, University of California, San Francisco) were expressed in CHO-K1 cells using PolyFect (Qiagen, Germany) according to the manufacturer's protocol. The chimera protein (G$\alpha_{s5/q}$) has the same sequence as Gα_q except its C-terminal 5 amino acids were replaced by Gα_s residues (EYNLV to QYELL). This construct allows G_s-coupled receptors to stimulate phospholipase C and subsequent intracellular Ca^{2+} release (Conklin *et al*., 1996). The level of Ca^{2+} in cells transfected with 5-HT$_6$ and G$\alpha_{s5/q}$ was measured using acetoxymethyl-ester form of fura-2 (fura-2/AM; Molecular probes, Eugene, OR), a fluorescent Ca^{2+} indicator, and fluorometric imaging plate reader (Coward *et al*., 1999). The effects of the compounds on the stimulated activity of 5-HT$_6$ in transfected cells after the treatment of 100 μM of serotonin were measured indirectly using chimeric G-protein. The transfected cells were treated with 100 μM of serotonin, and then incubated with 5 μM fura-2/AM and 0.001% Pluronic F-127 in reaction buffer (115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM HEPES, 13.8 mM glucose, 2.5 mM probenecid and 0.1% BSA, pH 7.4) at room temperature for 1 h. After they were washed, cells were illuminated with a xenon arc lamp. The required excitation wavelengths (340 and 380 nm) were selected by a computer-controlled filter wheel (Sutter Instruments). Data were acquired every 2 s and a shutter between exposures in the light path was interposed in order to protect the cells from photo-toxicity. Emitter fluorescence was reflected through a 515 nm long- pass filter to a frame transfer cooled CCD camera. The ratios of emitted fluorescence were then calculated using a digital fluorescence analyzer and subsequently converted to an intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). All imaging data were collected and analyzed using Universal Imaging software (West Chester, PA). Tested compounds or SB258585, a potent and selective antagonist of 5-HT$_6$, were incubated along with serotonin to examine their effect on the signaling pathway of 5-HT$_6$.

Results

Measurement of the interaction between Gα_s and iL3 loop region of 5-HT$_6$

The iL3 of 5-HT$_6$ representing amino acid 209-265 between the 5th and 6th transmembrane helixes of 5-HT$_6$ was attached at the C-terminus of GST to produce GST-iL3 (Figure 1A). GST-iL3 and the His-tag labeled Gα_s (H$_6$-Gα_s) protein (Figure 1B) were expressed in *E. coli* and purified as previously de-

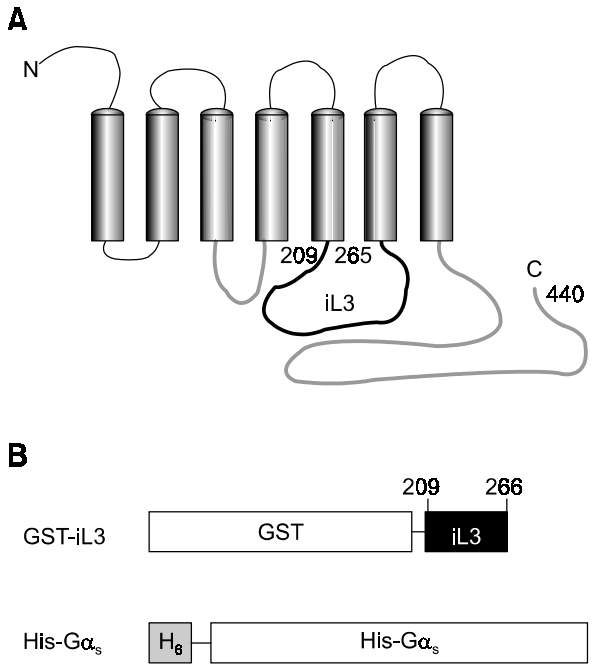

Figure 1. Schematic representation of H_6-Gα_S and GST-iL3. (A) The transmembrane topology of 5-HT_6. The third intracellular loop region (iL3) represents amino acid 209-265 of 5-HT_6 and is indicated as thick line. (B) GST-iL3 contains the iL3 region of 5-HT_6 at C-terminus of glutathion S-transferase (GST). H_6-Gα_s has N-terminus his tag sequence.

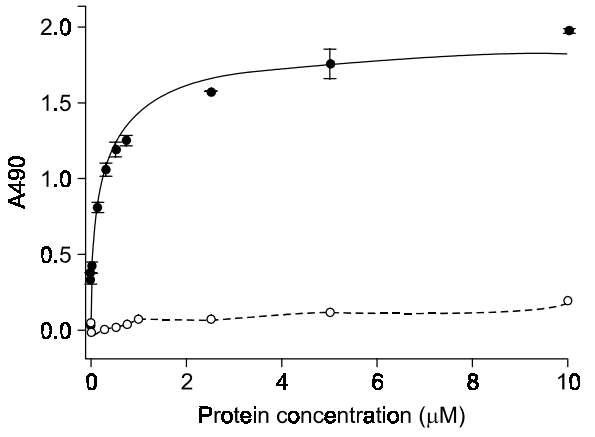

Figure 2. Measurement of the interaction between GST-iL3 and H_6-Gα_s by a modified ELISA method. The amount of bound GST (○) or GST-iL3 (●) on H_6-Gα_s coated plate was measured using a modified ELISA method as described in "Materials and Methods".

scribed (Kang *et al*., 2005). The specific interaction between Gα_s and iL3 of 5-HT_6 was examined by a modified ELISA method using GST-iL3 and H_6-Gα_s. The amount of bound GST-iL3 increased as the concentration of GST-iL3 in the plate coated with H_6-Gα_s

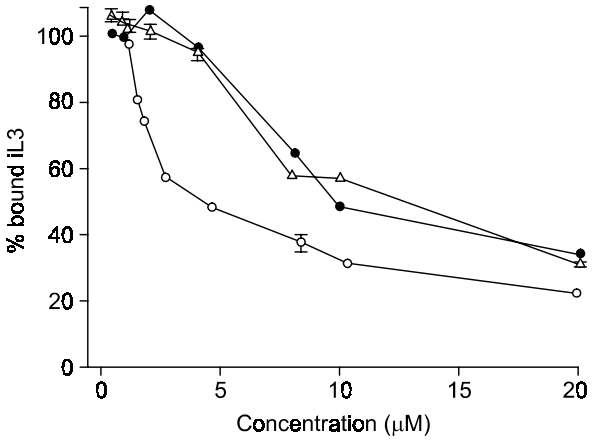

Figure 3. Inhibitory activity of identified compounds on the interaction between GST-iL3 and H_6-Gα_s. GST-iL3 was incubated with various concentration of KKJ00016 (●), KKJ00583 (△) and KYS00423 (○) in H_6-Gα_s coated plate. The relative amount of GST-iL3 bound on the plate was measured using the modified ELISA method.

increased. The amount of bound GST-iL3 became saturated when the concentration of GST-iL3 was 2 μM or higher (Figure 2). When GST was applied to the plate coated with H_6-Gα_s, negligible amount of GST was detected (Figure 2, open circle). The dissociation constant (K_D) was calculated as 0.5×10^{-6} M from the binding curve of GST-iL3. These results indicated that GST-iL3 specifically bound to the immobilized H_6-Gα_S via the interaction between the iL3 region of the fusion protein and Gα_s. The modified ELISA method was further applied for the screening of chemical library for potential inhibitors against the interaction between 5-HT_6 and Gα_s.

Screening of chemical compounds that block the interaction between Gα_s and iL3 of 5-HT_6 receptor

A chemical library consisted of 5,600 compounds was screened to identify inhibitory compounds against the interaction between Gα_s and the iL3 region of 5-HT_6 using the modified ELISA method. About 40 compounds that could inhibit more than 50% of the binding of GST-iL3 to immobilized H_6-Gα_s were identified from primary screening. The inhibitory activities of these compounds were further examined by the modified ELISA method, and 3 compounds that could effectively inhibit the interaction between with GST-iL3 and H_6-Gα_s were selected. The concentration dependent inhibitory activities of KKJ00016, KKJ00583 and KYS00423 were measured (Figure 3), and the IC_{50} values of KKJ00016, KKJ00583 and KYS00423 were calculated as 8.0, 8.0 and 1.2 μM,

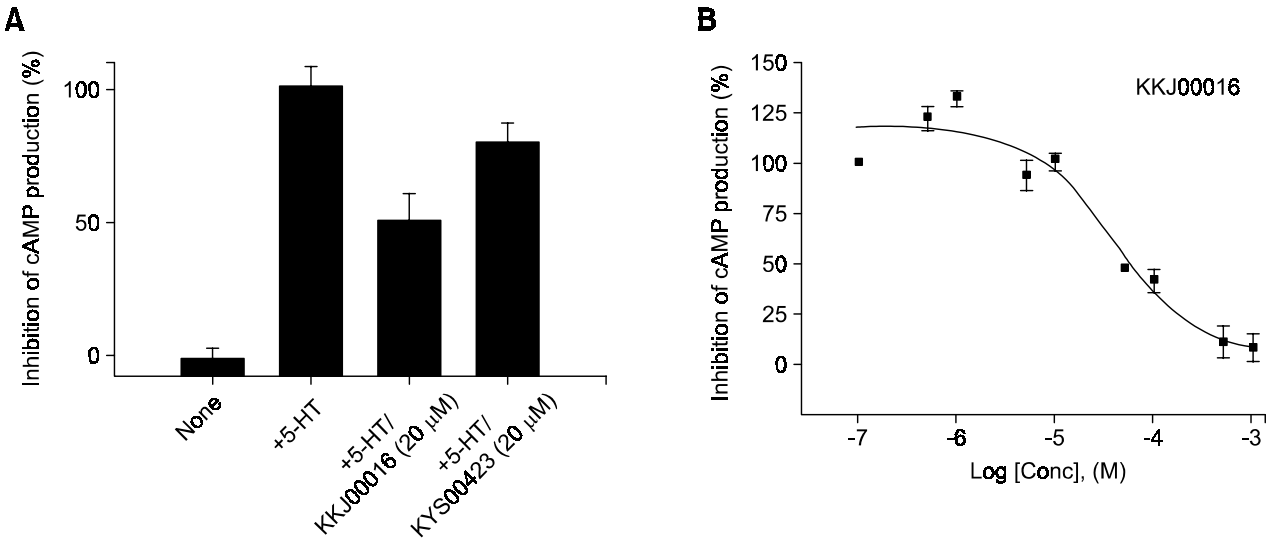

Figure 4. Inhibition of cellular level of accumulated cAMP of cells transfected with 5-HT$_6$ by selected compounds. (A) The level of cAMP in HEK 293 cells expressing wild-type 5-HT$_6$, were measured after the treatment of 100 μM of 5-HT. The relative inhibitions of cAMP production in the cells expressing 5-HT$_6$ treated with 20 μM of the KYS00423 and KKJ00016 were compared with that of 0.5% DMSO. (B) The concentration dependent inhibition of cAMP production in HEK 293 cells expressing 5-HT$_6$ receptor with KKJ00016. Standard deviation from three measurements was obtained and represented.

respectively.

Inhibition of serotonin induced cAMP production by KKJ00016 and KYS00423

The three compounds selected from the primary screening of the chemical library were further tested whether they could disrupt the signaling pathway of 5-HT$_6$. To examine the effect of the isolated compounds on the serotonin-induced production of cellular cAMP, HEK293T cells transformed with 5-HT$_6$ were treated with the compounds, and then the amount of cAMP produced by the treatment of 5-HT was measured. The treatment of serotonin activated the 5-HT$_6$ in the transformed HEK293T cells and stimulated the production of cAMP (Figure 4A). When the transfected cells were treated with 20 μM of KKJ00016, KKJ00583 or KYS00423, only KKJ-00016 significantly reduced the levels of cAMP. The induction of cAMP in KKJ00016-treated cells was 50% compared to un-treated cells, and KYS00423 only marginally reduced the level of cAMP (Figure 4A). In contrast, KKJ00583 rather enhanced the level of cAMP (data not shown). The inhibitory activity of KKJ00016 on the serotonin-induced production of cAMP was further examined at various concentrations. As the concentration of KKJ00016 increased, the level of cAMP induced by serotonin decreased, and the IC_{50} value of KKJ00016 in this assay system was calculated about 15 μM (Figure 4B). These results indicated that KKJ0016 that could block the interaction between the iL3 of 5-HT$_6$ and Gα_s, and it also prevented the serotonin-induced production of cAMP in the cell transfected with 5-HT$_6$.

KKJ00016 and KYS00423 bind to Gα_s rather than the iL3 region of 5-HT$_6$

To investigate the inhibitory mechanism of the identified compounds, the proteins bound to the compounds were examined. Immobilized GST-iL3 on glutathione plate was pretreated with 20 μM of KKJ-00016 or KYS00423 and incubated with FITC-labeled Gα_s after washing the unbound compounds. Then, the amount of bound Gα_s was measured and compared to the bound Gα_s from the incubation mixture in the presence or absence of the compounds. As shown in Figure 4, the binding of H_6-Gα_s to GST-iL3 is effectively prevented by KKJ00016 or KYS00423 (Figure 5, black boxes). However, pretreatment of these compounds to GST-iL3 failed to prevent the interaction between GST-iL3 and H_6-Gα_s (Figure 5, grey boxes). These results indicate that KKJ00016 or KYS00423 binds to Gα_s rather than GST-iL3 and prevents the interaction between Gα_s and GST-iL3.

Inhibition of serotonin induced Ca^{2+} signaling by KKJ00016

The inhibitory activity of KKJ00016 on the signaling pathway of 5-HT$_6$ was also examined using cells ex-

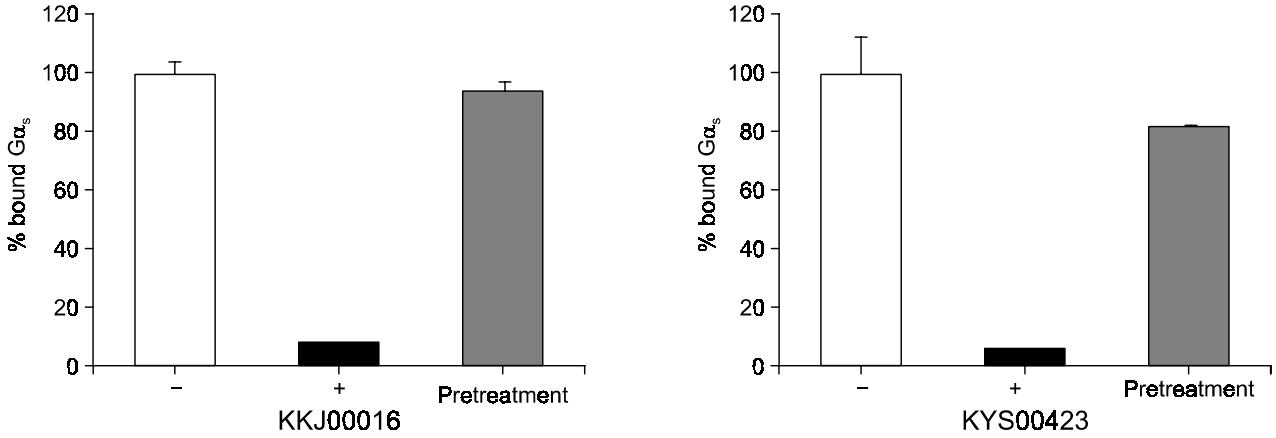

Figure 5. Inhibitory activities of KKJ00016 and KYS00423 on the interaction between GST-iL3 and H_6-Gα_s. The amount of bound FITC-labeled Gα_s to the immobilized GST-iL3 in the presence (black box) or absence (white box) of 20 μM of KKJ00016 (left panel) or KYS00423 (right panel) was measured. Also the bound Gα_s to the GST-iL3 pre-treated with 20 μM of KKJ00016 or KYS00423 was measured (black boxes) using a modified ELISA method as described in "Materials and Methods".

Table 1. Inhibition effect profile of target compounds for 5-HT_6 receptor.

Compound	Concentration (μM)	% Inhibition*
SB258585	10	100
KKJ00016	1	10.4 ± 2.2
	10	37.5 ± 7.2

*Inhibition effects of the compounds were compared to the inhibitory activity of SB258585, a potent antagonist. The inhibitory activities were determined using the maximum change in fluorescence over baseline. All data represent the mean of duplicate determinations from a typical experiment which was repeated at least more than 10 times.

pressing a chimeric G protein, G$\alpha_{s5/q}$. The chimera protein interacted with GPCRs coupled with G_s protein and increased the cellular level of calcium ion. The cells transfected both with the gene of G$\alpha_{s5/q}$ and 5-HT_6 showed increased Ca^{2+} level when they were activated by serotonin (Table 1). When the cells were treated with SB258585, the antagonist of 5-HT_6 (Hirst *et al*., 2000), the Ca^{2+} efflux induced by serotonin was effectively inhibited indicating that the signaling pathway of 5-HT_6 was coupled with the chimera G$\alpha_{s5/q}$ in the transfected cells. When the cells were treated with KKJ00016, the serotonin induced Ca^{2+} efflux was substantially inhibited (Table 1). About 37% of Ca^{2+} level was reduced at 10 μM of KKJ00106. These results indicated that KKJ00016 could interfere the signaling pathway of 5-HT_6, by inhibiting the activation of G_s protein.

Discussion

The interaction between the intracellular region of receptor and G-protein has been exploited for the screening of small molecule regulators of 5-HT_6 signaling pathway. Among the intracellular regions of 5-HT_6, the iL3 was identified as a critical region for the interaction with Gα_s, whereas the second intracellular region between the transmembrane helix 3 and 4 or the C-terminus region failed to bind to the Gα_s (Kang *et al*., 2005). The dissociation constant of iL3 on the Gα_s ($K_D = 0.5 \times 10^{-6}$ M) obtained by a modified ELISA method in this study was comparable to the previously obtained value ($K_D = 0.9 \times 10^{-6}$ M) by surface plasmon resonance analysis (Kang *et al*., 2005). Glutathione sepharose bead and GST-fusion protein had been used to measure the protein interaction *in vitro* (Kim *et al*., 2006). However, this method is inadequate for screening large number of chemical compounds. In contrast, the modified ELISA method in this study could be adapted in 96-well plate system and applied for the screening of chemical library for potential inhibitors against the interaction between iL3 of 5-HT_6 and Gα_s. The identified compounds in this study were shown to interact with Gα_s since the compounds pretreated with GST-iL3 were easily washed out and failed to prevent the interaction with Gα_s (Figure 4). The direct binding of these compounds to Gα_s was further confirmed by co-elution of these compounds along with H_6-Gα_s in size exclusion chromatography. When the mixture of H_6-Gα_s and KKJ00016 or KYS00423 was separated using desalting column, KKJ00016 or KYS00423 was detected in the protein fractions by thin layer chromatography. However, they were not detected in the protein fraction when they were mixed with GST-iL3 (data not shown).

One of the identified compounds from the primary

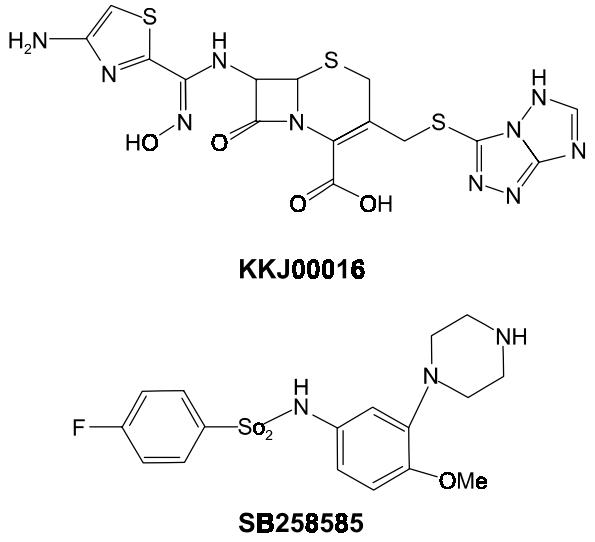

Figure 6. Chemical structure of KYS00423, KKJ00016 and SB258585.

screening was shown to interrupt the signaling of 5-HT$_6$. KKJ00016 prevented the accumulation of cAMP induced by serotonin in 5-HT$_6$ transfected cells with IC$_{50}$ value of 15 μM, which was comparable to the IC$_{50}$ value (8 μM) measured from the modified ELISA method. Also, 10 μM of KKJ00016 inhibited about 37% of Ca^{2+} efflux in cells transfected with 5-HT$_6$ and chimera G protein, which triggers Ca^{2+}-efflux after stimulation of serotonin. The similar ranges of the inhibitory concentrations of KKJ00016 in different assay systems imply that the disruption of the interaction between 5-HT$_6$ and G$_s$ protein is directly related to the interference of the signaling pathway of 5-HT$_6$. The GPCRs, activated by ligands, induce the exchanges of GDP for GTP on Gα subunit. The activated Gα-GTP dissociates from the Gβγ subunits and activates its downstream effectors. Chemicals that interfere with the interaction between GPCR and G-protein may perturb this signaling pathway and abolish the ligand-dependent activation of G-protein.

It should be noted that KKJ00016 is a derivative of cephalosporin containing aminothiazole and triazole groups (Figure 6), which has different structure of SB258585, an antagonist of 5-HT$_6$ (Hirst *et al*., 2000). Since KKJ00016 binds Gα$_s$ rather than the serotonin-binding site of 5-HT$_6$, KKJ00016 was expected to have different structures than the general antagonist or agonists of 5-HT receptors. It should be noted that Gα$_s$ mediates signaling of 5-HT$_6$ as well as 5-HT$_4$, 5-HT$_7$ or other G-protein coupled receptors. Hence, direct application of KKJ00016 as inhibitor of 5-HT$_6$ will be limited due to the possible non-specific disruption of Gα$_s$ mediated signaling pathway, although KKJ00016 could inhibit serotonin-induced signaling pathway of 5-HT$_6$ (Figure 4). However, the assay system targeted the interaction between the iL3 of 5-HT$_6$ and Gα$_s$ could applied to screen chemical compounds that show specificity to the iL3 region of 5-HT$_6$.

In this study, we have identified chemical compounds that could block the specific interaction between 5-HT$_6$ and Gα$_s$ using a convenient assay system that measuring the interaction between iL3 of 5-HT$_6$ and Gα$_s$. The inhibitory activities of the identified compound on the serotonin-induced production of cAMP suggested that the specific interaction between 5-HT$_6$ and Gα$_s$ could serve as a novel target site for developing 5-HT$_6$ regulators. Interference of the interaction between GPCR and G-protein would have advantages compared to the classical antagonist or agonist of GPCR targeted to the ligand binding site. This approach may generate subtype-specific drugs which could be difficult to obtain. Also high resolution structure of the G-protein and intracellular loop complex would be used to design high affinity inhibitors that block the signaling process of GPCRs.

Acknowledgement

This work was supported by grants from the Functional Proteomics Research (FPR05B2040), Ministry of Science and Technology, Korea. Hyewhon Rhim thanks to the grants from KIST Core-Competence and Brain Research (M103KV010007-07K2201-00710).

References

Baxter G, Kennett G, Bladey F, Blackburn T. 5-HT$_2$ receptor subtypes: a family re-united? Trends Pharmacol Sci 1995;16: 105-10

Bockaert J, Sebben M, Dumuis A. Pharmacological characterization of 5-hydroxytryptamine$_4$ (5-HT$_4$) receptors positively coupled to adenylate cyclase in adult guinea pig hippocampal membrane; effect of substituted bezamide derivatives. Mol Pharmacol 1990;37:408-11

Boess FG, Monsma FJJ, Carolo C, Meyer V, Rudler A, Zwingelstein C, Sleight AJ. Functional and radioligand binding characterization of rat 5-HT$_6$ receptors stably expressed in HEK293 cells. Neuropharmacology 1997;36:713-20

Boess FG, Riemer C, Bos M, Bentley J, Bourson A, Sleight AJ. The 5-hydroxytryptamine 6 receptor-selective radioligand [^{3}H]Ro 63-0563 labels 5-hydroxytryptamine receptor binding sites in rat and porcine striatum. Mol Pharmacol 1998;54: 577-83

Bourson A, Borroni E, Austin RH, Monsma F, Sleight AJ. Determination of the role of the 5-HT$_6$ receptor in the rat brain: a study using antisense oligonucleotides. J Pharmacol Exp Ther 1995;274:173-80

Bradley PB, Engel G, Feniuk W, Fozard JR, Humphrey PP, Middlemiss DN, Mylecharane EJ, Richardson BP, Saxena PR. Proposals for classification and nomenclature of functional receptors for 5-hydroxytryptamine. Neuropharmacology 1986;25:563-76

Brown BL, Albano JDM, Ekins RRP, Sgherzi AM, Tampion W. A simple and sensitive saturation assay method for the measurement of adenosine 3', 5'-cyclic monophosphate. Biochem J 1971;121:561-2

Byun MS, Choi J, Jue DM. Cysteine-179 of IκB kinase β plays a critical role in enzyme activation by promoting phosphorylation of activiation loop serines. Exp Mol Med 2006;38: 545-52

Conklin BR, Herzmark P, Ishida S, Voyno-Yasenetskaya TA, Sun Y, Farfel Z, Bourne HR. Carboxyl-terminal mutations of Gq alpha and Gs alpha that alter the fidelity of receptor activation. Mol Pharmacol 1996;50:885-90

Coward P, Chan SD, Wada HG, Humphries GM, Conklin BR. Chimeric G proteins allow a high-throughput signaling assay of Gi-coupled receptors. Anal Biochem 1999;270:242-8

Francken BJ, Jurzak M, Vanhauwe JF, Luyten WH, Leysen JE. The human 5-HT_{5A} receptor couple to G_i/G_o proteins and inhibits adenylate cyclase in HEK 293 cells. Eur J Pharmacol 1998;361:299-309

Hirst WD, Minton JA, Bromidge SM, Moss SF, Latter AJ, Riley G, Routletge C, Middledmiss DN, Price GW. Characterization of [^{125}I]-SB-258585 binding to human recombinant and native 5-HT(6) receptors in rat, pig and human brain tissue. Br J Pharmacol 2000;130:1597-605

Hoyer D, Schoeffter P. 5-HT receptors: subtypes and second messengers. J Recept Res 1991;11:197-214

Hoyer D, Martin G. 5-HT receptor classification and nomenclature: Towards a harmonization with human genome. Neuropharmacology 1997;36:419-28

Jasper JR, Kosaka A, To ZP, Chang DJ, Eglen RM. Cloning, expression and pharmacology of a truncated splice variant of the human 5-HT_7 receptor (h5-HT_{7b}). Br J Pharmacol 1997; 122:126-32

Kang H, Lee WK, Choi YH, Vukoti KM, Bang WG, Yu YG. Molecular analysis of the interaction between the intracellular loops of the human serotonin receptor type 6 (5-HT_6) and the alpha subunit of G_s protein. Biochem Biophys Res Commun 2005;329:684-92

Kilpatrick GJ, Barnes NM, Cheng CHK, Costal B, Naylor RJ, Tyers MB. The pharmacological characterization of 5-HT_3 receptor binding sites in rabbit ileum; comparison with those in rat ileum and rat brain. Neurochem Int 1991;19:389-96

Kim SK, Choi JH, Suh PG, Chang JS. Pleckstrin homology domain of phospholipase C-γ1 directly binds to 68-kDa neurofilament light chain. Exp Mol Med 2006;38:265-72

Kohen R, Metcalf MA, Khan N, Druck T, Huebner K, Lachowicz JE, Meltzer HY, Sibley DR, Roth BR, Hamblin MW. Cloning, characterization and chromosomal localization of a human 5-HT_6 serotonin receptor. J Neurochem 1996;66:47-56

Meneses A. Effects of the 5-HT6 receptor antagonist Ro 04-6790 on learning consolidation, Behav Brain Res 2001;118: 107-10

Monsma FJ, Shen Y, Ward RP, Hamblin MW, Sibley DR. Cloning and expression of a novel serotonin with high affinity for tricyclic psychotropic drugs. Mol Pharmacol 1993;43: 320-7

Ree S, Dendaas I, Foord S, Goodson S, Bull D, Kilpatrick G, Lee M. Cloning and characterization of the human 5-HT_{5A} serotonin receptor. FEBS Lett 1994;355:242-6

Rogers DC, Hagan JJ. 5-HT_6 receptor antagonists enhance retention of a water maze task in the rat. Psychopharmacology 2001;158:114-9

Roth BL, Craigo SC, Choudhary MS, Uluer A, Monsma FJ, Shen Y, Meltzer HY, Sibley DR. Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors. J Pharmacol Exp Ther 1994;268: 1403-10

Sleight AJ, Monsama F, Borroni E, Austin RH, Bourson A. Effects of altered 5-ht6 expression in the rat: functional studies using antisense oligonucleotides. Behav Brain Res 1996;73: 245-8

Suh BC, Kim TD, Lee JU, Seong JK, Kim KT. Pharmacological characterization of adenosine receptors in PGT-β mouse pineal gland tumor cells. Br J Pharmacol 2001;134:132-42

Unsworth CD, Molinoff PB. Characterization of a 5-hydroxytryptamine receptor in mouse neuroblastoma N18TG2 cells. J Pharmacol Exp Ther 1994;269:246-55

Woolley ML, Bentley JC, Sleight AJ, Marsden CA, Fone KC. 5-HT_6 receptors, Neuropharmacology 2001;41:210-9