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ST1936 stimulates cAMP, Ca2+, ERK1/2 and Fyn kinase through a full activation of cloned human 5-HT$_6$ receptors

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ABSTRACT

5-HT$_6$ receptor is one of the most recently cloned serotonin receptors, and it might play important roles in Alzheimer's disease, depression, and learning and memory disorders. Availability of only very few 5-HT$_6$ receptor agonists, however, does not allow examining their contribution in psychopharmacological processes. Therefore, a new 5-HT$_6$ receptor agonist, ST1936, was synthesized. ST1936 binds to human 5-HT$_6$ receptors with good affinity (K_i = 28.8 nM). ST1936 also exhibited some moderate binding affinity for 5HT$_{2B}$, 5HT$_{1A}$, 5HT$_7$ receptors and adrenergic α receptors. ST1936 behaved as a full 5-HT$_6$ agonist on cloned cells and was able to increase Ca^{2+} concentration, phosphorylation of Fyn kinase, and regulate the activation of ERK1/2 that is a downstream target of Fyn kinase. These effects were completely antagonized by two 5-HT$_6$ receptor antagonists, SB271046 and SB258585. The other 5-HT$_6$ receptor agonist, WAY181187 also increased Fyn kinase activity. These results suggest that both ST1936 and WAY181187 mediate 5-HT$_6$ receptor-dependent signal pathways, such as cAMP, Fyn and ERK1/2 kinase, as specific agonists.

1. Introduction

5-HT$_6$ receptors are linked to G-protein, which stimulates adenylate cyclase via Gαs-coupling (Kohen et al., 2001; Plassat et al., 1993; Ruat et al., 1993). They are predominantly expressed in the rat and human central nervous systems, particularly in the cerebral cortex, striatum, hippocampus, nucleus accumbens, and olfactory tubercles (Gerard et al., 1997; Monsma et al., 1993; Ruat et al., 1993; Ward et al., 1995). Because of their distribution in limbic areas and the cerebral cortex, 5-HT$_6$ receptor are proposed to be involved in cognitive processes, novelty-seeking behavior as well as mood regulation (Ballaz et al., 2007; Svenningsson et al., 2007; Wesolowska and Nikiforuk, 2007). However, while 5-HT$_6$ receptor antagonists available at this time are numerous and some are even in clinical development for cognitive disorders or obesity (Heal et al., 2008; Ruiz and Olsina, 2010) the availability of only very few 5-HT$_6$ receptor agonists does not allow examining their contribution in psychopharmacological processes. For instance, the role of 5-HT$_6$ receptor is not clearly defined in the context of depression due to contradictory results, as both antagonists and agonists were shown to exert antidepressant-like effects (Carr et al., 2010; Svenningsson et al., 2007; Wesolowska and Nikiforuk, 2007) or procognitive effects (Fone, 2008). To shed some light to this controversial issue, we synthesized and investigated the new 5-HT$_6$ receptor agonist ST1936 in selected in vitro studies. We also compared ST1936 with WAY181187, one of the very few 5-HT$_6$ receptor agonists available (Schechter et al., 2008). SB271046 and SB258585 were used as 5-HT$_6$ receptor antagonists (Routledge et al., 2000; Wesolowska et al., 2007). Fyn is a member of the Src family of non-receptor proteintyrosine kinases (Semba et al., 1986) that is expressed to a high degree in neurons, glia, and oligodendrocyte and its physiological importance has been suggested in the central nervous system. A recent study demonstrated that activation of 5-HT$_6$ receptor activated ERK 1/2 via an Fyn-dependent pathway, suggesting that Fyn may play an important role in 5-HT$_6$ receptor-mediated signaling pathways (Yun et al., 2007). In the present study we showed that both 5-HT$_6$ receptor agonists ST1936 and WAY181187 mediated Fyn and ERK1/2 kinase pathways. Preliminary results on ST1936 on cAMP and binding sites were presented at a conference (Borsini et al., 2008).

2. Materials and methods

2.1. Chemical synthesis

The synthesis of compound N,N-Dimethyl-2-(5-chloro-2-methyl-1H-indol-3-yl)ethylamine (ST1936), is described in the *patent* number EP1404317.

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2.2. 5-HT_6 receptor binding assay

Human astrocytoma 1321N1 cells, stably expressing the human serotonin 5-HT_6 receptor gene (cat. ES-316-CV, clone C1, PerkinElmer, Boston, MA, USA), were grown in DMEM (Cambrex, Verviers, Belgium) supplemented with 10% FBS (Cambrex), 1 mM sodium pyruvate and 400 μg/mL G418 (Sigma-Aldrich, St Louis, MO) for receptor expression selection, at 37 °C in a 5% CO_2 atmosphere. For radioligand binding experiments, the cells were collected in PBS/EDTA (Lonza, Basel, Switzerland, cat.# BE02-017F) and centrifuged at 1000×*g* for 10 min. The pellet obtained was resuspended in the same buffer used in binding experiments and homogenized with Ultra Turrax. The homogenate was centrifuged at 48,000×*g* for 90 min. The final pellet was stored at −80 °C until the day of the experiment. The protein concentration of membrane suspension was determined using the Bradford method (Pierce, Rockford, IL, USA) with bovine albumin as standard. Competition binding experiments were performed in 96-well filter plates (MultiScreen system, cat # MAFBN0B10, Millipore, Billerica, MA, USA), incubating cell membranes (10–20 μg of protein/sample) with a single concentration of [N-methyl-^{3}H]LSD (Lysergic acid Diethylamide) (Perkin Elmer, cat NET-638), corresponding to its K_d, in the presence of seven concentrations (ranging from 10^{-5} to 10^{-11} M) of test compounds for 1 h at 37 °C in a total volume of 200 μl/well of appropriate buffer (Tris–HCl 50 mmol/l, pH 7.4, containing 10 μmol/l pargyline, 5 mmol/l $MgCl_2$, 0.5 mmol/l EDTA). Nonspecific binding was obtained in presence of 100 μmol/l 5-HT (Sigma Aldrich). At the end of incubation, bound and free radioligands were separated by filtering the 96-well filter plates using a Millipore filtration apparatus (MultiscreenHTS vacuum manifold). Filter plates were then washed several times with ice-cold buffer (50 mmol/l Tris–HCl, pH 7.4) and filter-bound radioactivity measured using a MicroBeta counter (PerkinElmer) after addition of 30 μl/well of OptiPhase SuperMix scintillation cocktail (PerkinElmer).

Data were analyzed using nonlinear regression with GraphPad PRISM commercial software and expressed as IC_{50}, defined as the concentration of compounds that inhibits 50% of specific radioligand binding. Inhibitory binding constant (K_i) values were calculated from IC_{50} values according to the Cheng and Prusoff equation $K_i = IC_{50}/(1 + [C]/K_d)$, where [C] is the concentration of the radioligand and K_d its dissociation constant.

2.3. cAMP accumulation

cAMP quantitative determination was performed by a commercial kit (cAMP-Glo™ Assay, Promega), which provides a homogeneous, bioluminescent and high throughput assay to measure cAMP levels in cells. Briefly, 1321N1 cells stably transfected with the human 5-HT_6 receptor were plated in complete medium on 96-well dishes at a concentration of 5×10^3 cells/well 24 h before test compound exposure. After a brief washing with "induction buffer" [PBS containing the phosphodiesterase inhibitors IBMX (0.5 mM) and Ro 20–1724 (0.1 mM) (both from Sigma-Aldrich)], cells were stimulated with scalar concentrations (0.01 nM–10 μM) of the receptor agonists 5-HT, ST1936 and WAY181187 resuspended in induction buffer. Reversal of the agonist-mediated response was evaluated with a fixed concentration of the antagonist (1 μM) in the presence of three different concentrations of the agonist (10, 1 and 0.1 μM). After 20 min treatment with the compounds, the reaction was terminated by the addition of 20 μl of the "cAMP-Glo™ Lysis Buffer". According to manufacturer's instructions, after further 15 min at room temperature, the following components were added in sequence to each well: 40 μl of "cAMP Detection Solution" (containing Protein Kinase A) and after another 20 min at room temperature, 80 μl of "Kinase-Glo® Reagent". The plates were then incubated for 10 min at room temperature and the luminescence measured by a plate-reading luminometer (GLOMAX; Promega). Data from agonist response curve were analyzed using nonlinear regression with GraphPad PRISM commercial software and expresses as EC_{50} (concentration causing a half maximal stimulation of control values).

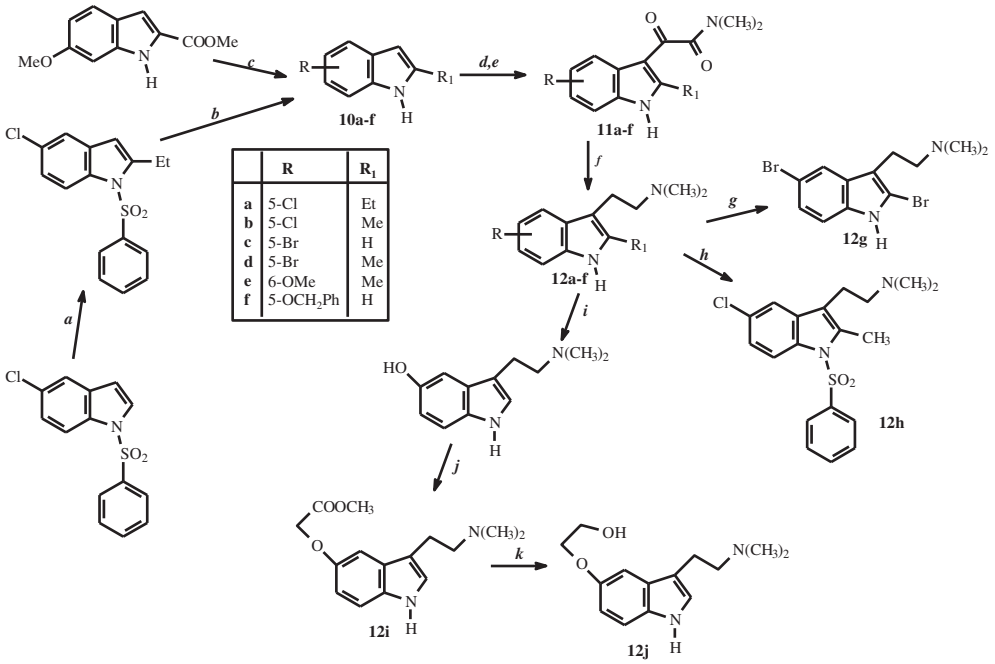

Fig. 1. Chemical synthesis of ST1936 (compound 12b) and analogues. Reagents: (a) *t*-BuLi, THF, −20°; EtI, −78° to room temperature, 2 h; (b) 2 N NaOH, MeOH, reflux, 20 h; (c) $LiAlH_4$, dioxane, 80 °C, 16 h; (d) $(COCl)_2$, THF, r.t., 1 h; (e) $NH(CH_3)_2$, THF, r.t., 6 h; (f) $LiAlH_4$, THF, reflux, 1 h; (g) Br_2, AcOH, r.t., 4 h; (h) NaH, benzenesulfonyl chloride, DMF, r.t., 16 h; (i) H_2, 10% Pd-C, 4 atm., r.t. 5 h; (j) NaH, $ClCH_2COOCH_3$, DMF −10° to r.t.; (k) $LiAlH_4$, THF, r.t.

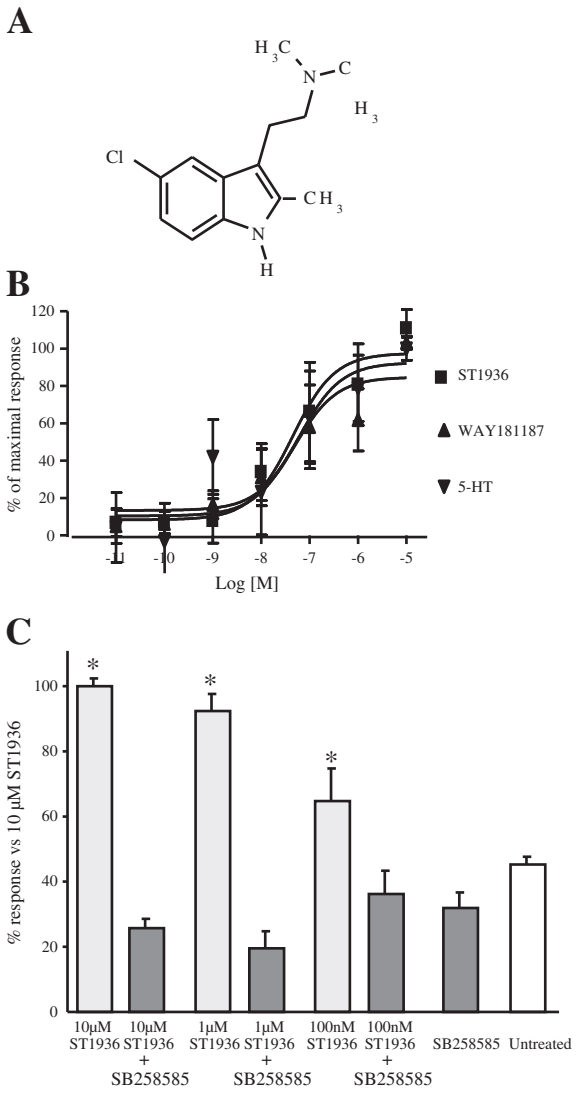

Fig. 2. Structure of ST1936 and stimulation of cAMP accumulation in human astrocytoma 1321N1 cells stably expressing the 5-HT_6 receptor. (A) Structural formula of ST1936. (B) Stimulation of cAMP accumulation. Cells were incubated for 20 min with scalar concentration of either 5-HT, ST1936 or WAY181187. cAMP determination was performed by a commercial kit (cAMP-GloTM Assay, Promega). Data (mean values ± S.D. obtained in three independent experiments preformed in duplicate) were analyzed using nonlinear regression with GraphPad PRISM commercial software. (C) Inhibition of ST1936-mediated cAMP accumulation by the antagonist SB258585. 1321N1 cells stably expressing the 5-HT_6 receptor were incubated for 20 min with a fixed concentration of SB258585 (1 μM) in the presence of three different concentrations of the agonist (10, 1, or 0.1 μM). Data (mean values ± S.E.D., obtained in four independent experiments performed in duplicate) are expressed as % vs maximal response elicited by 10 μM ST1936. One-Way ANOVA: $F_{[11, 82]} = 29.16$ and $P < 0.001$; Tukey Test:* $P < 0.05$ vs untreated cells.

Data from experiments of inhibition of agonist response in the presence of the antagonist were expressed as % vs maximal values.

2.4. Binding or functional analysis to other receptors

ST1936 was tested for binding in a broad cross-reactivity panel comprising G protein-coupled receptors, ion channel binding sites, enzymes, and transporters (CEREP, Celle l'Evescault, France; Caliper Discovery Alliances & Services, Hanover, MD, USA). In case of significant receptor occupancy, or in order to better characterize the affinity of ST1936 for particular receptors, concentration–response curves were performed.

2.5. Assay of 5-HT_6 receptor activity using a FDSS6000 system

5-HT_6 receptor activity was measured using an FDSS6000 96-well fluorescence plate reader (Hamamatsu Photonics, Japan) as previously described [18, 19]. Briefly, human embryonic kidney (HEK293) cells stably expressing HA-5-HT_6 receptor (HEK/HA-5-HT_6 receptor) were transiently transfected with $G\alpha_{15}$ protein for 24 h. HEK/HA-5-HT_6 receptor were grown in DMEM supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and G-418 (400 μg/ml) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. 5-HT-induced Ca^{2+} increases via activation of 5-HT_6 receptor were measured using the Fluo-4-AM fluorescence dye with the FDSS6000 system. After determination of a short baseline, 100 nM or one of various indicated doses of ST1936 or 5-HT was added to the cells, and the Ca^{2+} response was measured at 480 nM. For antagonist experiments, cells were pre-incubated with compounds for 15 min before the addition of an agonist. All data were collected and analyzed using the FDSS6000 system and related software (Hamamatsu Photonics, Japan).

2.6. Phospho-ERK1/2 activity assay

After serum starvation, HEK/HA-5-HT_6 receptor cells were treated with ST1936, WAY181187 or 5-HT in the absence or presence of the indicated drugs and gently lysed in lysis buffer A containing 1 mM DTT, 5 mM Na_3VO_4 and 10 mM $Na_4P_2O_7$, and protease inhibitor cocktail. Equal amounts of lysates were prepared and immunoblotted with anti-ERK1/2 (1:2000, Cell Signaling) and anti-*p*-ERK1/2 (1:2000, Cell Signaling) antibodies. For western blot analysis, the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) after 10% SDS-PAGE, and the membrane was blocked with 1 × Tris-buffered saline (TBS) containing 0.1% Tween 20, and 5% BSA or 5% skim milk for 1 h at room temperature. After blocking, the membranes were incubated overnight at 4 °C with the respective primary antibodies. The membranes were washed thrice, and incubated with diluted horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000, Jackson ImmunoReserch, West Grove, PA) for 1 h at room temperature. After three washes, the membranes were visualized using the enhanced chemiluminescence (ECL) kit (Millipore, Bedford, MA).

2.7. Fyn kinase activity assay

After HEK/HA-5-HT_6 receptor cells were kept in serum-free DMEM for 16 h, the cells were treated with ST1936 or WAY181187 in the absence or presence of SB258585, and then the cells lysed in lysis buffer A (50 mM Tris–HCl, pH 7.4, 130 mM NaCl, 1% Triton X-100, 10% glycerol, 20 mM NaF, 2 mM EDTA, 2 mM EGTA) containing 1 mM DTT,

Table 1
Binding affinities of ST1936 for 5-HT receptors and for other receptors which showed K_i of less than 1 μM or displacement of more than 50% at 1 μM concentration.

Receptor	Displacement at 1 μM (%)	K_i (nM)	Origin
5-HT_{1A}	41		Rat cerebral cortex
5-HT_{1B}	0		Rat cerebral cortex
5-HT_{1D}	26		Bovine caudate
5-HT_{2A}	0		Rat cerebral cortex
5-HT_{2B}		245	H recombinant (CHO cells)
5-HT_{2C}	36		H recombinant (CHO cells)
5-HT_3	17		NIE-115 cells
5-HT_4	0		Guinea pig striatum
5-HT_{5a}	0		H recombinant (HEK cells)
5-HT_6		28.8	H recombinant (1321N1 cells)
5-HT_7		290	H recombinant (1321N1 cells)
5-HTT	20		Rat cerebral cortex
α_1		390	Rat cerebral cortex
α_2		300	H recombinant (CHO cells)
α_2		300	Rat cerebral cortex

10 mM Na_3VO_4 and 10 mM $Na_4P_2O_7$, and protease inhibitor cocktail. Equal amounts of lysates were immunoprecipitated with 4 μg of anti-Fyn (Upstate Biotechnology Inc., Lake Placid, NY) and 50 μl of ImmunoPure Immobilized Protein G Plus, followed by immunoblotting with anti-Fyn (1:2000) and anti-*p*Y-416 Src (1:2000, Upstate) antibodies that cross-react with *p*Y-420 Fyn.

2.8. Drugs

ST1936, other 5-HT_6 derivatives and 2-(1-{6-chloroimidazo[2,1-b][1,3]thiazole-5-sulfonyl}-1H-indol-3-yl)ethan-1-amine (WAY181187), were synthesized in Sigma-Tau's Chemistry Department or in Istituto di Chimica Farmaceutica (University of Urbino). 5-HT and selective 5-HT_6 receptor antagonists, 4-Iodo-N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl] benzenesulfonamide (SB258585) and 5-chloro-*N*-(4-methoxy-3-piperazin-1-ylphenyl)-3-methyl-1-benzothiophene-2-sulfonamide, (SB271046) were purchased from Sigma-Aldrich and Tocris (Bristol, UK), respectively.

2.9. Statistics

According to the experimental scheme, ANOVA followed by Dunnett or Tukey's test, was used. Sigma-Stat was used as statistical program.

3. Results

3.1. Binding and cAMP response

Chemical synthesis of ST1936 is shown in Fig. 1. Structural formula is depicted in Fig. 2A. ST1936 displaced [^{3}H]LSD binding with K_i (95% confidence intervals) of 28.8 (16.5–50.0) nM. The affinity value for 5-HT was 75.7 nM, with 95% confidence values between 40.3 and 142 nM. ST1936 appeared to be rather selective for 5-HT_6 receptor, but it showed additional affinity for α1 and α2 adrenoceptors, as well as for and 5-HT_{2B}R and 5-HT_7R (Table 1). Although selectivity is only about 10 fold towards 5-HT_{2B} and 5-HT_7 receptors, ST1936 exhibited in vitro some agonistic activity to 5-HT_{2B} receptors (EC_{50}<100 nM), only a weak agonistic effects to human 5-HT_{1A} receptor (EC_{50} = 8.9 μM), neither antagonistic nor agonistic effects to 5-HT_7R or adrenergic α1 and α2 receptors at 1 μM.

ST1936 has no affinity or very low affinity for all the other receptors (adenosine, beta-adrenergic, benzodiazapine, dopamine, GABA, histamine, melatonin, NMDA, nicotinic, opiate, and vasopressin) that were investigated, including serotonin, dopamine and noradrenaline transporter. ST1936 was also inactive in reducing MAO-A and MAO-B activities (IC_{50}>10 μM). ST1936 showed the same efficacy and potency as the other 5-HT6 agonist WAY181187 in increasing cAMP levels (EC_{50} values = 44.8 and 48.5 nM for ST1936 and WAY181187, respectively). The unselective 5-HT stimulated cAMP accumulation with EC_{50} = 61.4 nM (Fig. 2B). The effect of ST1936 was antagonized by the selective 5-HT_6 receptor antagonist SB258585 which completely abolished cAMP accumulation obtained at all concentrations of ST1936 (Fig. 2C).

3.2. Fluorescence derived calcium assay of 5-HT_6 receptor activity

We previously demonstrated that the activity of 5-HT_6 receptor can be measured as 5-HT-induced Ca^{2+} increases using a promiscuous $G\alpha_{15}$ protein or a chimeric $G\alpha_{qG66Ds5}$ protein (Kim et al., 2008; Yun et al., 2007), which allows coupling of $G\alpha_S$-coupled receptors to phospholipase C and consequent intracellular Ca^{2+} release. Therefore, effects of

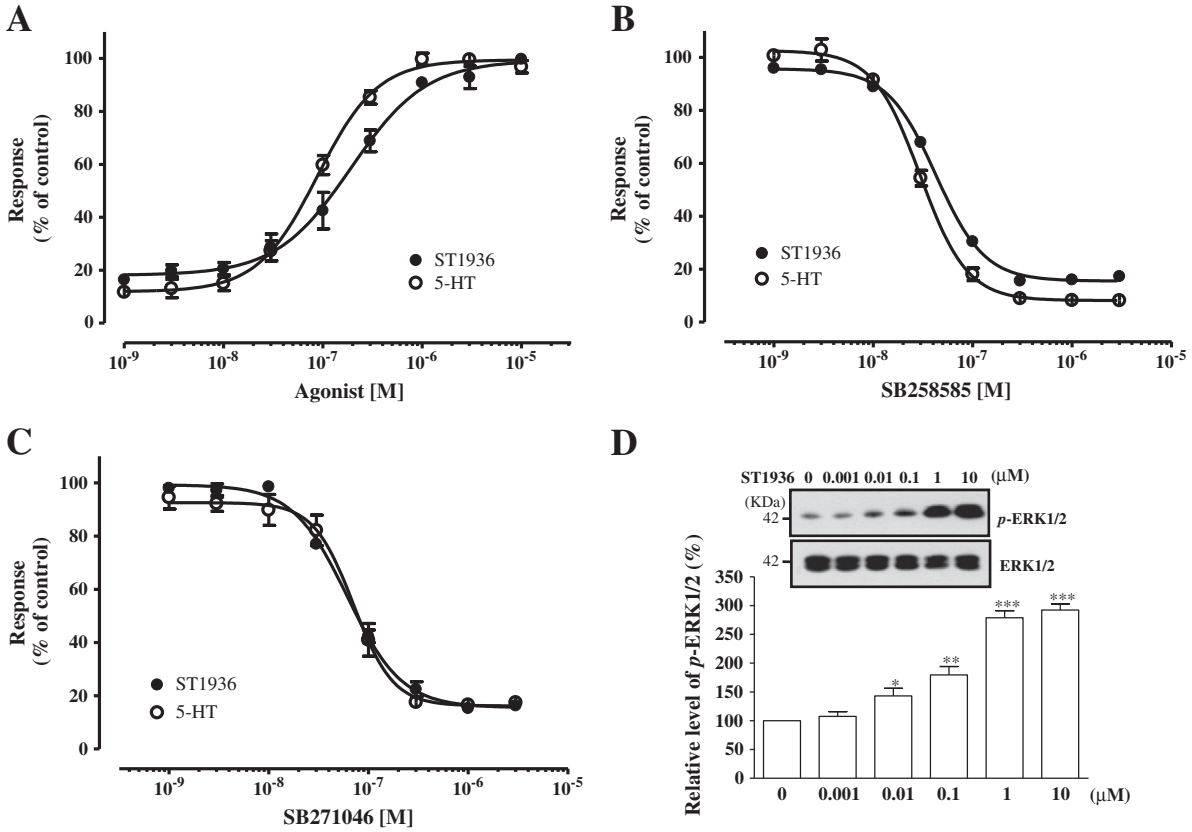

Fig. 3. ST1936 on 5-HT_6 receptor-mediated Ca^{2+} increases and ERK1/2 activities. (A) Dose-dependent curves of ST1936 and 5-HT on 5-HT_6 receptor-mediated Ca^{2+} increases in HEK/HA-5-HT_6 receptor cells transfected with $G\alpha_{15}$. (B) & (C) Antagonist effects of SB258585 (B) or SB271046 (C) on ST1936- or 5-HT-mediated Ca^{2+} increases. (D) Dose-dependent responses of ST1936 on ERK1/2 activities in HEK/HA-5-HT_6 receptor cells. *P<0.05, **P<0.01, ***P<0.001.

ST1936 on 5-HT_6 receptor were also tested using an FDSS6000 96-well fluorescence plate reader. As shown in Fig. 3A, ST1936 and 5-HT produced dose-dependent Ca^{2+} increases in HEK/HA-5-HT_6 receptor cells transfected with $G\alpha_{15}$. EC_{50} values of ST1936 and 5-HT were 192.6 and 88.6 nM, respectively. ST1936- or 5-HT-induced Ca^{2+} increases were antagonized by the selective 5-HT_6 receptor antagonists, SB258585 (Fig. 3B) and SB271046 (Fig. 3C). IC_{50} values for SB258585 were 42.6 nM and 29.7 nM when ST1936 and 5-HT were used at the concentration of 100 nM, respectively. For SB271046, IC_{50} values were 61.1 nM and 70.5 nM when ST1936 and 5-HT were used, respectively. These results suggested the role of ST1936 as a selective 5-HT_6 receptor agonist in the HEK/HA-5-HT_6 receptor/FDSS6000 assay system.

3.3. Activation of ERK1/2 kinase activity

The 5-HT_6 receptor is a $G\alpha_S$-coupled receptor that couples with adenylate cyclase and consequently triggers a cAMP-dependent signaling pathway. A key signal transduction mediator in this pathway is the extracellular signal-regulated kinase 1/2 (ERK1/2). Therefore, we next examined whether ST1936 and WAY181187 regulate the activation of ERK1/2 that is a downstream target of Fyn kinase in a 5-HT_6 receptor-dependent pathway. The treatment with ST1936 for 5 min produced a dose-dependent ERK1/2 phosphorylation in HEK/HA-5-HT_6 receptor cells. The activation of ERK1/2 was significantly observed from 10 nM and saturated at 1 μM ST1936 (Fig. 3D). Furthermore, we examined whether ST1936-induced activation of ERK1/2 was blocked by SB258585. As shown in Fig. 4A, the pretreatment with 10 μM SB258585 significantly decreased 1 μM or 10 μM ST1936-induced activation of ERK1/2. However, the treatment with SB258585 alone did not increase activation of ERK1/2. WAY181187, both at 1 and 10 μM concentrations, also increased activation of ERK1/2, which was significantly blocked by SB258585 (Fig. 4B). As a further detailed mechanism of ST1936-mediated activation of ERK1/2, we examined an involvement of Fyn kinase or c-AMP-dependent protein kinase (PKA). HEK/HA-5-HT_6 receptor cells were pretreated with PP2, a Fyn inhibitor, or H89, a PKA inhibitor (Yun et al., 2007). The pretreatment with 1 μM PP2 led to a significant decrease in ST1936-mediated activation of

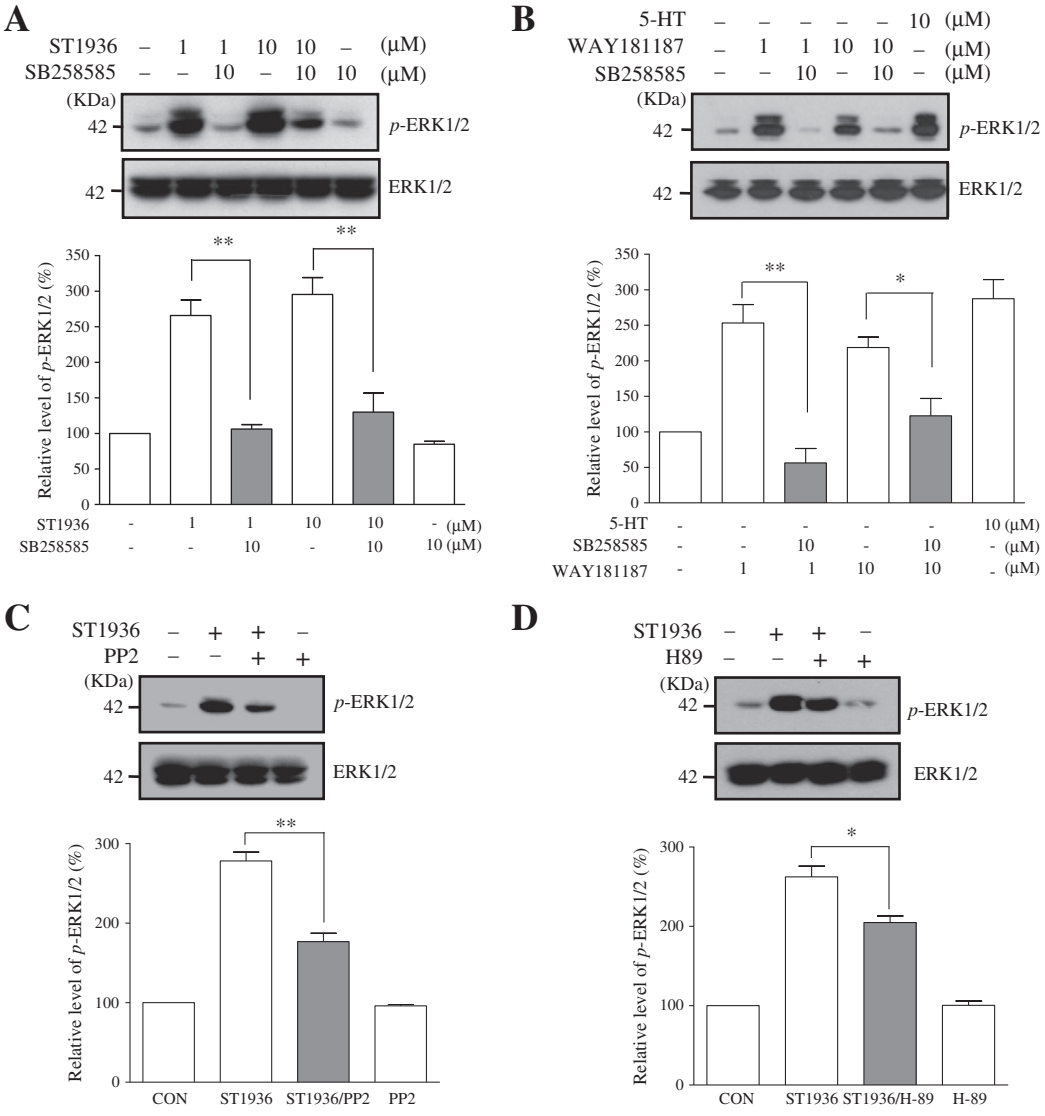

Fig. 4. ST1936 and WAY181187 increase ERK1/2 activities via 5-HT_6 receptors. (A) & (B) HEK/HA-5-HT_6 receptor cells were treated with each agonist (ST1936, WAY181187, or 5-HT) for 5 min in the absence or presence of 10 μM SB258585 for 15 min. (C) & (D) After the cells were treated with 10 μM ST1936 for 5 min, 1 μM PP2, Fyn inhibitor or 10 μM H89, PKA inhibitor, was further treated for 15 min. The representative immunoblotting shown here was detected using anti-ERK1/2 and anti-*p*-ERK antibodies. $^*P<0.05$, $^{**}P<0.01$ compared to ST1936 or WAY181187.

ERK1/2 (Fig. 4C) and 10 μM H89 partially but significantly reduced ST1936-mediated activation of ERK1/2 (Fig. 4D).

3.4. Effects on phosphorylation of Fyn kinase

In a previous study, 5-HT increased phosphorylation of Fyn kinase, which has been shown to interact with 5-HT_6 receptors (Yun et al., 2007). Therefore, we investigated whether ST1936 modulates Fyn kinase activity as a 5-HT_6 receptor agonist in HEK/HA-5-HT_6 receptor cells. As shown in Fig. 5A, the treatment with ST1936 at the concentration of 10 μM induced a significant increase in phosphorylation of Fyn (148.7 ± 5.7% of control, $n = 4$). This ST1936-mediated phosphorylation of Fyn returned to control level by pretreatment with 10 μM SB258585, a specific 5-HT_6 receptor antagonist (107.0 ± 8.8% of control, $n = 4$). When the other 5-HT_6 receptor agonist WAY181187 was examined, it also increased Fyn kinase activity, which was significantly blocked by SB258585 (Fig. 5B). Taken together, our data suggest that ST1936 and WAY181187 can mediate a 5-HT_6 receptor-dependent signal pathway, such as Fyn and ERK1/2 kinase, as specific agonists.

4. Discussion

Our results showed that ST1936 possesses affinity for the 5-HT_6 receptor, upon which it behaves as a full agonist, as demonstrated by its intrinsic activity on cAMP accumulation or a promiscuous $G\alpha_{15}$ protein-mediated Ca^{2+} increases, and by the antagonistic effect exhibited by the specific 5-HT_6 receptor antagonists SB252585 and SB271046. ST1936 seems to be rather selective within 5-HT receptors, although it shows some affinity also for 5-HT_{2B}, 5-HT_{1A} and 5-HT_7 receptors. It also binds α-adrenergic receptors. ST1936 does not bind to any other receptors, enzymes, and transporters tested.

ST1936's dose-dependent Ca^{2+} increases in HEK/HA-5-HT_6 receptor cells transfected with $G\alpha_{15}$ demonstrated that it is a highly efficacious full agonist because activates both cAMP and Ca^{2+} responses, in contrast with the 5-HT_6 receptor agonist WAY181187 that is full agonist for cAMP response but partial for the Ca^{2+} response (Codony et al., 2010) or in a GαS-based assay (Dupuis et al., 2008). The other 5-HT_6 receptor agonist WAY208466 (Schechter et al., 2008) also behaved as partial agonist at 5-HT6 receptors coupled to Gαs (Dupuis et al., 2008).

The cellular mechanisms of 5-HT_6 receptor-mediated signal pathways are not well explored except the common GαS-protein mediated PKA. In a previous paper, it was demonstrated that activation of 5-HT_6 receptor by 5-HT modulates Fyn kinase (Yun et al., 2007), a member of the Src family of tyrosine kinases (Thomas and Brugge, 1997). Here we showed for the first time that two specific 5HT_6 agonists, ST1936 and WAY181187, also modulate Fyn kinase showing similar activities. The two agonists also interact with PKA pathways that mediate ERK1/2 phosphorylation. It would be interesting in future experiments to check whether different 5-HT_6 receptor agonists or antagonists interact with Fyn kinase.

The importance of Fyn kinase has been suggested in a number of brain diseases including Alzheimer Disease (AD) where the distribution and levels of Fyn are altered in AD brains (Shirazi and Wood, 1993) and Bipolar Disorder where the FYN gene has been associated particularly with type I illness and early age of onset of the disease (Szczepankiewicz et al., 2009).

Differences in interaction with Fyn kinase might also explain some contradictory results obtained with 5-HT_6 receptor agonists and antagonists in behavioral experiments. Fyn signal pathways could be differentially affected by agonists and antagonists (Fone, 2008). Therefore, it is very important to elucidate the mechanisms responsible for 5-HT_6 receptor-mediated cognition and mood changes in the brain.

Scarcity of 5-HT_6 receptor agonists available had limited the research in this field. ST1936 has recently been shown to increase dopamine (DA) and noradrenaline (NA) in the nucleus accumbens and in the prefrontal cortex after systemic administration in rats and these effects were prevented by systemic administration of the two 5-HT_6 receptor antagonists, SB271046 and SB399885 (Valentini et al., 2011). ST1936 has an excellent brain penetration with a mean elimination half-life of approximately 1 h in both plasma and brain (Mancinelli, 2010).

We hope that ST1936 may represent a new pharmacological tool to study 5-HT_6 receptor activation and psychopharmacological process.

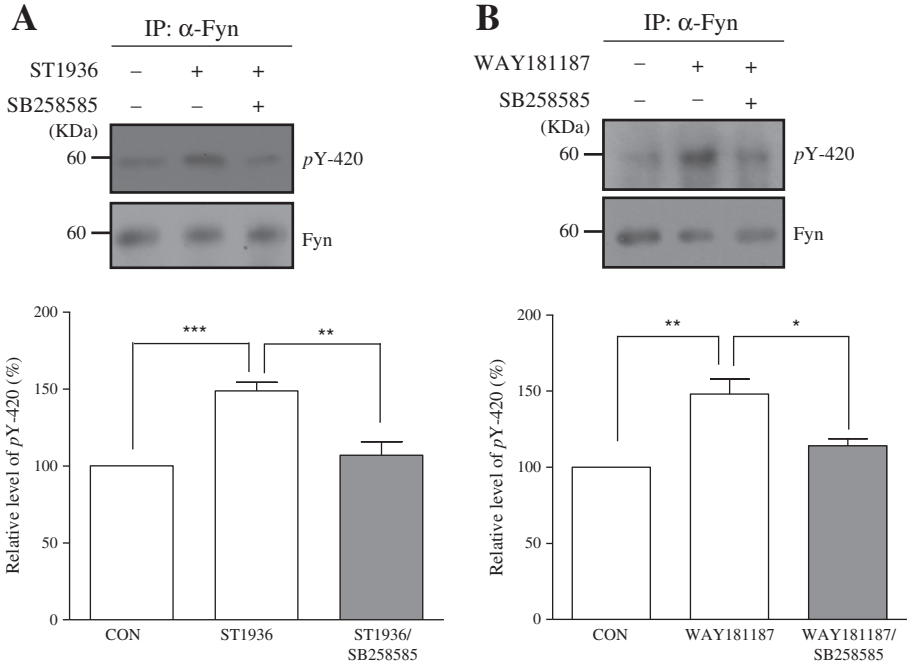

Fig. 5. ST1936 and WAY181187 increase phosphorylation of Fyn via 5-HT_6 receptors. After HEK/HA-5-HT_6 receptor cells were treated with 10 μM ST1936 (A) or 10 μM WAY181187 (B) for 5 min, the cells were lysed and immunoprecipitated using anti-Fyn antibodies. The representative immunoblotting shown in A & B was detected with anti-pY-420 or anti-Fyn antibodies. For blockage experiments, the cells were further treated with 10 μM SB258585 for 15 min. The bar graphs represented the pooled data from multiple independent experiments ($n > 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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