SKF83959 is a potent allosteric modulator of sigma-1 receptor

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Abbreviations: BD1047, N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide; cAMP, cyclic adenosine monophosphate; D1 receptor, dopamine receptor-1; DTG, 1,3-di(2-tolyl)-guanidine; G protein, guanine nucleotide-binding protein; HEK, human embryonic kindey; IP3, inositol-1,4,5-triphosphate; SCH23390, (R)-(+) -7-chloro -8- hydroxy-3-methyl-1-phenyl -2, 3,4,5- tetrahydro-1H-3-benzazepine hydrochloride; SKF38393, (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8- diol hydrobromide; SKF83959, 3-methyl-6-chloro-7,8-hydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-1H-3-benzazepine.
Abstract

SKF83959, an atypical dopamine receptor-1 (D₁ receptor) agonist, has showed many D₁ receptor-independent effects, such as neuroprotection, blockade of Na⁺ channel, and promotion of spontaneous glutamate release, which somehow resemble the effects of the sigma-1 receptor activation. In the present work, we explored the potential modulation of SKF83959 on the sigma-1 receptor. The results indicated that SKF835959 dramatically promoted the binding of ³H(+)-pentazocine (a selective sigma-1 receptor agonist) to the sigma-1 receptor in brain and liver tissues, but produced no effect on ³H-progesterone binding (a sigma-1 receptor antagonist). The saturation assay and the dissociation kinetics assay confirmed the allosteric effect. We further demonstrated that the SKF83959 analogs, such as SCH22390 and SKF38393, also showed the similar allosteric effect on the sigma-1 receptor in the liver tissue but not in the brain tissue. Moreover, all three tested chemicals elicited no significant effect on ³H-1,3-di(2-tolyl)-guanidine (³H-DTG) binding to the sigma-2 receptor. The present data uncovered a new role of SKF83959 and its analogs on the sigma-1 receptor, which, in turn, may reveal the underlying mechanism for the D₁ receptor-independent effect of the drug.
Introduction

The atypical dopamine receptor-1 (D₁ receptor) agonist, 3-methyl-6-chloro-7,8-hydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF83959), has shown a various biological functions in vitro or in intact animals. Unlike the typical D₁ receptor agonists, SKF83959 doesn’t stimulate cAMP production via D₁-like receptor mediated activation of Gₛ protein (Andringa et al., 1999a; Fujita et al., 2010; Makihara et al., 2007; Rashid et al., 2007); instead, it selectively activates Gₛ protein via D₁-like receptor and results in the IP₃ production (Cools et al., 2002; Jin et al., 2003; Ming et al., 2006; O’Sullivan et al., 2004; Panchalingam and Undie, 2001; Sahu et al., 2009; Undieh, 2010). In animals, the drug was found to increase eye blinking in monkeys and rats, and to elicit excellent anti-Parkinsonian’s effects in the primate model as well as in the unilateral lesioned rodent model (Andringa et al., 1999b; Gnanalingham et al., 1995; Zhang et al., 2007). The anti-Parkinsonian’s effect was shown to be independent of D₁ dopamine receptor-stimulated cAMP and may associate with the drug-activated Gₛ/phospholipase C pathway (Zhang et al., 2007; Zhen et al., 2005).

In addition to receptor-mediated events, recent information indicates that the D₁ receptor-independent pharmacological effects may play important roles in SKF83959-mediated biological responses. For instance, we have found that the potent neuronal protection of the drug was only partially dependent on D₁ receptor (Yu et al., 2008), and that SKF83959 blocked Na⁺ channel (Chu et al., 2011), modulated the delayed rectifier K⁺ channel (Chen et al., 2009) and promoted the spontaneous release of glutamate in the rat somatosensory cortical neurons (Chu et al., 2010).
In review of the chemical structure of SKF83959 (Fig. 1), it shares the similar pharmacophore with some of sigma-1 receptor ligands (N,N-diakyl or N-alkyl-N-aralkyl pharmacophore, Fig. 1) (Prezzavento et al., 2007). Moreover, the activation of sigma-1 receptor inhibits the voltage-gated Na⁺ channel, Ca²⁺ channel and K⁺ channel (Aydar et al., 2002; Cheng et al., 2008; Zhang and Cuevas, 2002). Over-expression of sigma-1 receptor enhances the spontaneous release of glutamate (Dong et al., 2007; Meyer et al., 2002). All of these effects resemble the D₁ receptor-independent effect of SKF83959. We wonder if SKF83959 and its analogs are sigma-1 receptor modulators. In the present study, by employing radio-labeled ligands, such as ³H(+)-pentazocine (sigma-1 receptor agonist) and ³H-progesterone(sigma-1 receptor antagonist), we found that SKF83959 potentiates the binding of sigma-1 receptors with ³H(+)-pentazocine in the allosteric manner. This result may provide a new mechanism for the D₁ receptor-independent effects of SKF83959 and its analogs.
Materials and Methods

Animals

Male SD rats, weighting 180-200 g, were housed in SPF conditions (temperature: 21±1°C) with air exchange every 20 min and an automatic 12 h light/dark cycle (light on from 7:00 h-19:00 h). Animals were fed on a standard laboratory diet and water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committees of Shanghai Institute of Materia Medica, Chinese Academy of Sciences (SIMM-2011-06-ZXC-07) and were in compliance with the Guidance for the Care and Use of Laboratory Animals (National Research Council, People’s Republic of China,1996).

Drugs and chemicals

3-methyl-6-chloro-7,8-hydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF83959, Fig. 1) was synthesized in Synthetic Organic & Medicinal Chemistry Laboratory, Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

[Ring-1,3-3H]-(+)-2-dimethylallyl-5,9-dimethyl-2'-hydroxybenzomorphan (3H(+)-pentazocine), [1,2,6,7-3H(N)]- pregn-4-ene-3,20-dione (3H-progesterone) and 3H-1,3-di(2-tolyl)- guanidine (3H-DTG) were purchased from PerkinElmer Inc.(Waltham, MA, USA). (R)-(+) -7-chloro-8- hydroxy-3-methyl-1-phenyl -2, 3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390, Fig. 1), (±)-1-phenyl-2,3,4,5-tetrahydro- (1H)-3-benzazepine-7,8-diol hydrobromide (SKF38393, Fig. 1), N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2- (dimethylamino) ethylamine dihydrobromide (BD1047), 4-[4-(4-chlorophenyl)-4
-hydroxy-1-piperidyl]-1-(4-fluorophenyl) -butan-1-one (haloperidol), water-soluble
pregn-4-ene-3,20-dione (progesterone) and 5,5-diphenylimidazolidine-2,4-dione (phenytoin) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). SKF83959, SCH23390, SKF38393, haloperidol, BD1047 and phenytoin were dissolved in dimethyl sulfoxide (DMSO) at the concentration of 100 mM as the stock solutions. Before the experiments, the stock solutions were diluted with Kreb’s solution (NaCl 118 mM, KCl 4.7 mM, CaCl₂ 2.5 mM, MgSO₄ 1.2 mM, KH₂PO₄ 1.2 mM, NaHCO₃ 25 mM, glucose 11.1 mM, pH 7.2-7.4) to the designed concentrations (1 nM-1 mM). Water-soluble progesterone was dissolved directly in Kreb’s solution prior to use.

The construction of sigma-1 receptor stable-expressed HEK293 cell line

The human sigma-1 receptor plasmid was kindly denoted by Dr. Ebru Aydar (Division of Cellular and molecular Biology, Faculty of Natural Sciences Imperial College, London, United Kingdom). The stably-expressed HEK293 cell line was constructed as previously described (Palmer et al., 2007) with some modification. Briefly, the plasmid was transfected into HEK293 cells under the guidance of PolyJet tranfection kit. After 36 h, G418 was added to the culture media and maintained at the concentration 600 μg/ml. After 10 days, the monoclonal cell lines resisting G418 was selected and proliferated. The expression of sigma-1 receptor was confirmed by RT-PCR and western blotting. The stable cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal serum and 100 μg/ml G418). The culture dishes were maintained in the 37°C incubator with a humidified atmosphere of 5% CO₂.
Membrane Preparation

Synaptosomes were obtained with the method described previously (Gonzalez et al., 2001). Briefly, rats were killed by decapitation and the brains were dissected. The tissues were homogenized in 10 volumes (ml/g) of 10 mM Tris HCl containing 0.32 M sucrose (pH 7.4) with a glass homogenizer. The homogenates were centrifuged at 1000g for 10 min at 4°C. The resulting pellets were discarded and the supernatants were centrifuged again at 1000g for 10 min. The supernatants were then centrifuged at 35,000g for 30 min. The pellets were re-suspended in 10 mM Tris HCl containing 0.32 M sucrose, and carefully layered onto 1.2 M sucrose and centrifuged at 100,000g for 150 min. The synaptosomes were collected which lay at the interface between the 1.2 M and 0.32 M sucrose layers. The synaptosomes were diluted with Kreb’s solution and used for the following experiments.

The liver plasma membranes were isolated and purified with the sucrose density gradient ultracentrifugation (Zhang et al., 2010). Briefly, the liver tissues were homogenized and centrifuged at 1000g for 20 min. The pellets were re-suspended in the Kreb’s solution containing 50% of sucrose and ultracentrifuged using sucrose density gradient of 44.0% and 42.3% at 100,000g for 2.5 h. The crude plasma membranes at the top of 42.3% sucrose were collected for further ultracentrifuge at the gradients of 44%, 42.8%, 42.3%, 41.8%, 41.0%, 39.0%, and 37.0% at 100,000g for 6 h. At last, the purified plasma membranes at the top of 37.0% sucrose were collected and used for the binding tests.

HEK293 membrane P2 fractions were prepared using the method described previously (Sun et al., 2009). Briefly, cells were collected and lysed in hypotonic buffer (5 mM
Tris-HCl and 2 mM EDTA, pH 7.4), containing a protease inhibitor mixture (Sigma) and sonicated three times for 18 s. After the lysates were centrifuged (1000g, 10 min, 25°C), the pellets were discarded and the supernatants were re-centrifuged at 35,000g for 30 min at 4°C. The following steps were the same as those in the synaptosomes preparation.

Protein concentrations were measured by the BCA protein assay kit (Pierce Biotechnology, Thermo Scientific; Rockford, IL USA), according to the guidance of the supplier.

**Receptor binding assays**

$^3$H(+)-pentazocine is a highly specific ligand for sigma-1 receptor and the binding with $^3$H(+)-pentazocine is a prerequisite for sigma-1 receptor (Cagnotto et al., 1994; Cobos et al., 2008; Hellewell et al., 1994). So, the binding activities of sigma-1 receptors with $^3$H(+)-pentazocine was firstly examined. For $^3$H(+)-pentazocine binding assays, membrane fraction aliquots were diluted with Kreb’s solution in a final protein concentration of 2 mg/ml. $^3$H(+)-pentazocine was diluted with Kreb’s solution (the final concentration: 0.3 nM in the concentration-response assays and 0.1-60 nM in the saturation experiments). The binding assay buffer consisted of 100 µl membrane aliquots, 20 µl $^3$H(+)-pentazocine, 20 µl tested drugs and 60 µl Kreb’s solution. BD1047 was used for non-specific binding at the concentration of 10 µM. In the control tubes, the tested drugs were replaced with the equal volume of Kreb’s solution. After incubation of 2.5 h at 30°C, the bound and free radioligand were separated by rapid filtration under a vacuum with a Brandel harvester over Whatmann GF/B glass fiber filters. Radioactivity was measured with a liquid scintillation spectrometer (*PekinElmer*).
In the $^{3}$H-progesterone binding assay, $^{3}$H(+)-pentazocine was replaced with equal amount of $^{3}$H-progesterone (the final concentration: 10 nM). The other procedure was the same with that of $^{3}$H(+)-pentazocine.

For the sigma-2 receptor binding assay, $^{3}$H-1,3-di(2-tolyl)-guanidine ($^{3}$H-DTG), a common used probe for sigma-2 receptor, was adapted for monitoring the sigma-2 binding activity (the final concentration: 10 nM). 10 μM BD1047 was used to block the sigma-1 binding and the non-specific binding activity was determined by 10 μM haloperidol.

**Sigma-1 receptor dissociation kinetics studies**

The membrane aliquots were firstly incubated with $^{3}$H(+)-pentazocine for 2.5 h to reach the binding equilibrium. 10 μl tested compound (the final concentration: 100 μM for SKF83959, SCH2390 and SKF38393, 1000 μM for phenytoin) or its vehicle (Kreb’s solution) were then added into the reaction buffer. Followed was the addition of BD1047 (10 μM) to initiate the dissociation process. Then, samples were filtered and the radioactivities were measured at the indicated time points.

**Data analysis**

Data in the saturation binding assays were analyzed with the one-site model (Eq.1), one site with cooperative binding (Hill equation, Eq.2) or the two-sites binding model (Eq.3). Comparison between models was performed using an extra-sum-of-squares test ($F$ test).

\[
B = \frac{B_{\text{max}}\times[A]^{n}}{[A]^{n}+K_{d}}
\]  

(1)
Where $B$ denotes the bound receptor number per $mg$ protein; $K_d$ denotes the equilibrium dissociation constant of $^3$H(+)-pentazocine; $[A^+]$ denotes the concentration of $^3$H(+)-pentazocine(M).

$$B = \frac{B_{max} \times [A^+]^{nH}}{[K_d]^{nH} + [A^+]^{nH}}$$

(2)

Where $nH$ denotes the Hill coefficient, other symbols are the same to those in Eq.1.

$$B = \frac{B_{max-HI} \times [A^+]^{nH}}{[A^+] + K_{d-HI}} + \frac{B_{max-LOW} \times [A^+]^{nH}}{[A^+] + K_{d-LOW}}$$

(3)

Where $B_{max-HI}$ denotes the maximal number of the high-affinity population, $n$, $B_{max-LOW}$ denotes the maximal number of the low-affinity population; $K_{d-HI}$ denotes the equilibrium dissociation constant for the high-affinity subpopulation, $K_{d-LOW}$ denostes the dissociation constant for the subpopulation low-affinity subpopulation; other symbols are the same to those in Eq.1.

The displacing binding data were fitted to the one-site (Eq.4) inhibition curve.

$$B = B_{basal} + \frac{B_{max-Basal}}{1 + 10^{[L]-log IC_{50}}}$$

(4)

Where $B$ means the binding activity, $B_{max}$ means the binding of the radioligand in the absence of any competing ligand, $B_{basal}$ is the nonspecific binding of the radioligand, $[L]$ denotes the concentration of progesterone, $IC_{50}$ denotes the concentration at which the binding strength is equal to $(B_{max-Basal})/2$.

The $K_i$ value of progesterone (which indicates the affinity of progesterone for the sigma-1
receptor) was calculated with the Cheng-Prusoff equation (Eq. 5):

$$\frac{I_{C_50}}{K_i} = \frac{[A^*]}{K_d} + 1$$  \hspace{1cm} (5)

where $[A^*]$ denotes the concentration of radioligand used, and $K_d$ denotes the equilibrium dissociation constants of $^{3}$H(+)-pentazocine, which was obtained with the nonlinear regression analysis from the saturation experiment.

The data obtained from the concentration-response assays were fitted to the ternary complex model (Eq. 6) (Christopoulos, 2000).

$$\frac{B}{B_{basal}} = \frac{\frac{[A^*]}{K_d}(1+\alpha\frac{[X]}{K_X})}{\frac{[A^*]}{K_d}(1+\alpha\frac{[X]}{K_X}) + \frac{[X]}{K_X} + 1}$$  \hspace{1cm} (6)

Where $B/B_{basal}$ denotes the fractional receptor occupancy; $K_d$ and $K_X$ denote the equilibrium dissociation constants of $^{3}$H(+)-pentazocine and the modulator (SKF83959, etc), respectively, at the free receptor; $\alpha$ denotes the cooperativity factor for allosteric interaction between radioligand $^{3}$H(+)-pentazocine and the modulator; $[A^*]$ denotes the concentration of radioligand $^{3}$H(+)-pentazocine (M); $[X]$ denotes the concentration of modulator (M).

The data from the saturation studies in the presence of tested compounds were fitted by the one-site hyperbola model (Eq.7).

$$B = \frac{B_{max} \times [A^*]}{K_{app} + [A^*]}$$  \hspace{1cm} (7)

Where $B$ denotes the bound receptor number per mg protein, $B_{max}$ denotes the maximal
receptor number per mg protein, \([A^*]\) denotes the concentration of \(^3\text{H}(+)-\text{pentazocine}\) (nM). \(K_{\text{app}}\) denotes the dissociation constant in the presence of the modulator (SKF83959, etc). When the modulator is absent, \(K_{\text{app}}\) is the \(K_d\) value of \(^3\text{H}(+)-\text{pentazocine}\).

In the ternary complex model,

\[
K_{\text{app}} = \frac{K_d(1+\frac{|X|}{K_X})}{1+\alpha\frac{|X|}{K_X}} \tag{8}
\]

Where \(K_d\) and \(K_X\) denote the equilibrium dissociation constants of \(^3\text{H}(+)-\text{pentazocine}\) and the modulator (SKF83959, etc), respectively, at the free receptor; \(\alpha\) denotes the cooperativity factor for the allosteric interaction between radioligand \(^3\text{H}(+)-\text{pentazocine}\) and the modulator; \(|X|\) denotes the concentration of the modulator (M).

Theoretically, when \(|X|\) is infinite, \(K_{\text{app}} = \frac{K_d}{\alpha}\), that is,

\[
\alpha = \frac{K_d}{K_{\text{app}}} \tag{9}
\]

The dissociation kinetic data were fitted in the mono-exponential decay equation (Eq.10).

\[
B_t = B_0 \times e^{-K_{\text{off-obs}}t} \tag{10}
\]

Where \(B_t\) denotes the radioactivity at the \(t\) time, \(B_0\) denotes that radioactivity before the addition of SKF83959 (time=0), and \(K_{\text{off-obs}}\) denotes the observed radioligand dissociation rate constant in presence of the modulator. When the modulator is absent, \(K_{\text{off-obs}}\) is \(K_{\text{off}}\) of \(^3\text{H}(+)-\text{pentazocine}\).
The parameters mentioned above were calculated by means of non-linear regression methods. Except some parameters specially noted, data were expressed as mean±S.E.M. For multiple comparisons of parameters, one-way ANOVA was used and Dunnett’s post-hoc test was followed. Student’s t-test was used to compare the difference between two groups. Difference was considered significant when P<0.05. Data were analyzed with the Graphpad Prism software (Version 5.0).
Results

The sigma-1 receptors from different tissues or stable transfected cells displayed the similar affinities with $^3$H(+)-pentazocine.

We firstly examined the affinities of sigma-1 receptors from different tissues or transfected cells with $^3$H(+)-pentazocine. Data were fitted to the one-site binding model (Eq. 1), Hill equation (Eq. 2) or the two-site model, separately (Eq. 3). The nHs in Hill equation were 1.01±0.10 in brain tissues, 0.97±0.09 in liver and 0.89±0.10 in constructed HEK293 cells, which were not significantly different (one-way ANOVA, P>0.05). Moreover, the extra-sum-of-squares test also showed that the one-site model was preferred. This suggested that a single population existed in each tissue and no cooperative effect existed in the binding process of $^3$H(+)-pentazocine with the sigma-1 receptors. The $K_d$ values of $^3$H(+)-pentazocine were 6.78±0.24 nM for brain, 6.74±0.62 nM for liver, 7.10±0.85 nM for stably-expressed HEK293 cells (Fig. 2). There were no significant differences (one-way ANOVA, P>0.05).

Pentazocine and progesterone bound to the sigma-1 receptor in the competition manner.

Next, we examined the manner in which $^3$H(+)-pentazocine and progesterone interacted with the sigma-1 receptors. In the displacement binding test, the IC$_{50}$ values of progesterone (fitted to Eq. 4) increased with the concentrations of $^3$H(+)-pentazocine in the brain (Fig. 3A, 3E), liver (Fig. 3B, 3E) and the stably-expressed HEK293 cells (Fig. 3C,
which was in accordance with competitive antagonism. This was tested by comparison of the data to the Cheng-Prusoff equation (Eq. 5). The regression was linear and the slopes (brain: 0.96±0.05, liver: 0.94±0.04 and HEK293: 1.08±0.06) were not significantly from unity (t-test, P>0.05, Fig. 3D), indicating that \(^{3}\text{H}(+)\)-pentazocine and progesterone bound with the sigma-1 receptor in the competition manner.

The affinities of progesterone with sigma-1 receptors were also determined (Eq. 5). The \(K_i\) values for progesterone were 1.54±0.18 µM in brain, 1.49±0.28 µM in liver, 1.80±0.35 µM in HEK293 cells, respectively. Again, there was no significant difference among them (one-way ANOVA, P>0.05), suggesting that \(^{3}\text{H}(+)\)-pentazocine and progesterone bound to the sigma-1 receptor at the same sites.

**SKF83959 enhanced the binding activity of \(^{3}\text{H}(+)\)-pentazocine with the sigma-1 receptor.**

As shown in Fig. 4A (brain) & 4B (liver), SKF83959 enhanced the binding of \(^{3}\text{H}(+)\)-pentazocine with sigma-1 receptors in the dose-dependent manner. The concentration-response curves were fitted into Eq. 6, based on the ternary complex model. The cooperativity factor (\(\alpha\)) were 2.277 (95% Credit Interval (CI): 2.009-2.581) in the brain and 2.604 (95% CI: 1.959-3.462) in the liver. The values of \(K_X\), which indicated the affinity of modulator with the sigma-1 receptor, were 311 nM (95% CI: 162-598 nM) in brain and 629 nM (95% CI: 186-2126 nM) in liver.

We also tested the effects of structural analogs of SKF83959 on the binding of \(^{3}\text{H}(+)\)-pentazocine to sigma-1 receptors. Unlike SKF83959, SCH23390 and SKF38393-modulated bindings of \(^{3}\text{H}(+)\)-pentazocine on sigma-1 receptor were only observed in
liver tissues (Fig. 4B), no detectable effects were seen in brain tissues. The cooperativity factor ($\alpha$) values were 2.507 (95% CI: 2.212-2.840) for SCH23390 and 1.882 (95% CI: 1.567-2.259) for SKF38393 in the liver. The $K_X$ values were 1802 nM (95% CI: 987-3290 nM) for SCH23390 and 212 nM (95% CI: 60-758 nM) for SKF38393.

Phenytoin is the first recognized sigma-1 allosteric modulator (DeHaven-Hudkins et al., 1993). We also re-examined the effect of phenytoin. The modulation of phenytoin was only observed in the brain tissue (Fig. 4A) but not in the liver tissue. The cooperativity factor ($\alpha$) of phenytoin was 1.562 (95% CI: 1.490-1.638), less than that of SKF83959 ($t$-test, $P<0.05$). And $K_{X\text{-phenytoin}}$ was 72.98 $\mu$M (95% CI: 46.68-114 $\mu$M), higher than that of SKF83959 ($t$-test, $P<0.05$).

**SKF83959 shifted the saturation curve toward the left.**

The one-site binding model was preferred to fit the saturation binding (Eq. 7) regardless of the presence or absence SKF83959 (Fig. 5A (brain) & 5B (liver)). The $K_{app}$ in the absence of SKF83959 (i.e. $K_d$) was 6.80±0.24 nM for the brain tissue (Fig. 5A) and 6.74±0.62 nM for the liver tissue (Fig. 5B). In the presence of 100 $\mu$M SKF83959, $K_{app}$ was 3.42±0.52 nM in the brain tissue, and 2.66±0.28 nM in the liver tissue, significantly lower than the values of $K_d$ ($t$-test, and $P<0.05$). The $K_{app}$ for 1000 $\mu$M phenytoin was 4.54±0.21 nM.

Similarly, the $K_{app}$ in the presence of 100 $\mu$M SCH3390 (3.16±0.48nM) or 100 $\mu$M SKF38393 (3.56±0.36 nM) in liver tissue was significantly decreased ($P<0.05$), compared to that of the vehicle group (6.74±0.62 nM).
As indicated in Eq. 8, when the concentration of modulator is infinite, the value of $\alpha$ is equal to the ratio of $K_d$ to $K_{app}$ (Eq.9). As shown in Fig. 4A (brain) and 4B (liver), the maximal modulation effect reached at the concentration of 100 $\mu$M (for SKF83959, SCH23390, and SKF38393) or 1000 $\mu$M (for phenytoin). The estimated values of $\alpha$ were not different from those obtained from the concentration-response assays (Fig. 5C), which confirmed the modulatory effects of SKF83959 and its analogs on the sigma-1 receptors.

**SKF83959 delayed the dissociation process of $^3$H(+) -pentazocine from the bound sigma-1 receptor.**

So far, we had shown that SKF83959 and its analogs modulated the binding of sigma-1 receptor in the allosteric manner. To further confirm this observation, we performed the dissociation kinetics studies. As showed in Fig 6A (brain) & 6B (liver), the dissociation processes of $^3$H(+) -penzacozine from the bound sigma-1 receptor fitted the best to the monoexponential model (Eq.10). SKF83959 (100 $\mu$M) significantly inhibited the dissociation rate in brain tissues ($K_{off-obs}$: 0.0063±0.0004 min$^{-1}$ v.s 0.0135±0.0007 min$^{-1}$ for the vehicle control, P<0.05, Fig 6A) and in the liver tissues ($K_{off-obs}$: 0.0043±0.0002 min$^{-1}$ v.s. 0.0111±0.0006 min$^{-1}$ for the vehicle control, P<0.05; Fig. 6B). The $K_{off-obs}$ of phenytoin in the brain was 0.009011±0.0004 min$^{-1}$, higher than that of SKF83959 (t-test, P<0.05).

Similar inhibitions were found for SCH23390 and SKF38393 in the liver tissues. The values of $K_{off-obs}$ were 0.0055±0.0002 min$^{-1}$ for SCH23390 and 0.0069±0.0003 min$^{-1}$ for SKF38393, which were significantly lower than that of the vehicle control (0.0111±0.0006 min$^{-1}$, P<0.05).
The effects of SKF83959 on sigma-1 receptor ligand binding were of ligand and receptor selectivity.

In order to test the ligand selectivity for SKF83959 and its analogs in allosteric modulation on sigma-1 receptor, we studied the effect of SKF83959 on the binding of sigma-1 receptor antagonist ³H-progesterone. As shown in Fig. 7A (brain) and 7B (liver), SKF835959 failed to alter the binding activity of ³H-progesterone on sigma-1 receptor. This result implies that the allosteric modulation on sigma-1 receptor of SKF83959 is of ligand selectivity.

To test whether the modulation was selective for sigma-1 receptor, we investigated the effect of SKF83959 and its analogs on the binding of ³H-DTG, a relatively selective sigma-2 receptor ligand, on the sigma-2 receptor. All tested drugs at the concentration of 100 μM and 10 μM did not elicit any significant modulation on the binding affinity of ³H-DTG to sigma-2 receptor either in brain (Fig. 8A) or liver (Fig. 8B) tissues.

SKF83959, SCH23390, SKF38393 and phenytoin failed to modulate the binding activity of ³H(+)-pentazocine and sigma-1 receptor in constructed HEK293 cells.

The stable sigma-1 receptor–expressed HEK293 cells were prepared as described in Methods. We characterized the effects of each tested chemical on the binding activity of ³H(+)-pentazocine in the cells. The density of sigma-1 receptors was 0.46±0.09 pmol/mg protein in the wild type of HEK293 cells. In the constructed HEK293 cells, the amount of sigma-1 receptors was 9.88±0.32 pmol/mg protein. Although the sigma-1 receptors from transfected HEK293 cells displayed the similar affinity with that of brain (Fig. 2 & 3), SKF83959 and its analogs failed to change the binding activity of ³H(+)-pentazocine on
the sigma-1 receptors in the receptor stably-expressed HEK293 cells (Fig. 9A). The failure on binding modulation was also observed while phenytoin (10 and 100 μM), a well-known sigma-1 receptor allosteric modulator, was applied. Moreover, all tested compounds didn’t change the binding activity of 3H-progesterone with the sigma-1 receptor in the constructed HEK293 cells (Fig. 9B).
Discussion

The present studies demonstrated that SKF83959, an atypical D₁ receptor agonist, and its analogs, such as SCH23390 and SKF38393, produce potent allosteric modulations on the binding of $^3$H(+)-pentazocine to sigma-1 receptor. We further demonstrated this modulation on sigma-1 receptor binding was ligand-dependent, and was selective for sigma-1 receptor. To our knowledge, this is the first report depicting the role of SKF83959 and its analogs in modulating sigma-1 receptor binding.

It is known that the positive allosteric modulators on ligand binding share some common features: promoting the binding of specific ligands for the receptor to the orthodox site, reducing the dissociation rate and being of ligand selectivity (Cobos et al., 2006). Ideally, the choice of orthosteric probes would be dictated by the endogenous ligands. However, it is difficult for the sigma-1 receptor due to the obscurity of endogenous ligands. We thus adapted two surrogate ligands to examine the allosteric effect of SKF83959. We found that SKF83959 increased the binding of $^3$H(+)-pentazocine to sigma-1 receptor in the brain and liver tissues, shifted the saturation curve toward the left and decreased the dissociating rate in the binding kinetic analysis. In addition, the allosteric modulation was of ligand and receptor selectivity. It is comfortable to conclude that SKF83959 and its analogs are the positive allosteric modulators for the binding activities of $^3$H(+)-pentazocine with sigma-1 receptors.

The simple allosteric ternary complex model is the most common model for quantification of allosteric interactions (Christopoulos and Kenakin, 2002). In this model, the orthosteric ligand (A) and the allosteric modulator (X), bind to the receptor (R) and form
a ternary complex (ARX). The binding of X with R changes the affinity of A with R. The capability of X affecting the affinity A is named as the cooperativity factor and denoted as $\alpha$. When $\alpha > 1$, the interaction between X and A is positive cooperative. As showed in Fig.4, the value of $\alpha > 1$, it is conceivable that the interaction between SKF83959 and $^3$H(+) -pentazocine is positively cooperative, and SKF83959 potentiates the binding of $^3$H(+) -pentazocine with sigma-1 receptor.

There are no other allosteric modulators found since the first report describing phenytoin as an allosteric modulator for the sigma-1 receptor (Cobos et al., 2005; DeHaven-Hudkins et al., 1993). We also compared the allosteric capability of SKF83959 with that of phenytoin. It was noted that SKF83959 seems to be more potent than phenytoin, as the $\alpha$ value of SKF83959 is greater than that of phenytoin. Moreover, the affinity of SKF83959 with the sigma-1 receptor is also higher than that of phenytoin ($K_{X-SKF83959} < K_{X-phenytoin}$).

Based on the principles of the two-state theory of receptor activation and the ternary complex model of allosteric modulation, the extended two-state model of receptor activation was proposed to explain the behavior of allosteric modulators both in terms of binding and of functional activation of the receptor (Bruns and Fergus, 1990; Hall, 2000). In this model, the receptor consists of two states, which are kept in dynamic balance between the inactive (R) state and active (R$^*$) state. The allosteric agonist makes the shift from the R state to active R$^*$ state. The sigma-1 receptors displayed the constitutive activity when excessively expressed in the MCF-7 tumor cells (Hayashi and Su, 2005). Therefore, we attempted to explain some biological behavior of SKF83959 using this model. SKF83959 has shown a number of D$_1$ receptor independent effects in brain tissue.
For instance, the neuroprotective effect of the drug is found only partially to depend on D₁ receptor activation (Yu et al., 2008); SKF83959 also inhibited delayed rectifier K⁺ channel in primary culture neurons and suppresses excitatory synaptic transmission and voltage-activated Na⁺ current in rat hippocampus (Chen et al., 2009; Chu et al., 2011; Noriyama et al., 2006). Many of those D₁ receptor-independent biological responses are unable to reproduce in D₁ receptor-expressed systems (HEK293 or CHO cells). Given the wide spectrum effects of sigma-1 receptor, our findings may imply that so-called D₁ receptor independent effects in brain tissue elicited by SKF83959 could result from the positive allosteric modulation of the drug on the sigma-1 receptors: SKF83959 promotes the accumulation of R⁺ and enhances the function of sigma-1 receptor. The details are currently under investigation.

It is interesting to note that the tissue difference exists in the modulating effect of tested compounds on sigma-1 receptor binding. Although SKF83959, SCH23390 and SKF38393 all elicited allosteric modulation on sigma-1 receptor binding in liver tissue, only SKF83959 displayed the allosteric effect on sigma-1 receptor in brain tissue. In contrast, phenytoin modulated the sigma-1 receptor binding only in the brain tissues. In addition, we failed to detect any significant allosteric effects of SKF83959, its analogs and phenytoin on the sigma-1 receptor binding in the receptor stable-expressed HEK293 cells. Given the fact that ³H(+)-pentazocine binds to the sigma-1 receptors with the same affinities in brain, liver and HEK293 cells (Fig.2 & 3), we proposed that the failure in allosteric modulation of sigma-1 receptor binding in constructed HEK293 cells may result from the complication of the receptor structure in different tissues or the interacting partners or cell contents, rather than from the distinct binding sites. Different structures of sigma-1 re-
ceptor have been proposed. For example, a single transmembrane domain structure was firstly reported in the liver microsome (Hanner et al., 1996), however, it was showed later that the receptor in the plasma membrane has two transmembrane segments with the NH and COOH termini on the cytoplasmic side of the membrane when the receptor was expressed in Xenopus laevis oocytes (Aydar et al., 2002). More recently, two additional hydrophobic segments were proposed except the two putative transmembrane domains in the liver microsome (Pal et al., 2007). Moreover, it is widely accepted consensus that the allosteric site is distinct from the orthosteric site, the former is less well-conserved than the orthosteric site (Christopoulos and Kenakin, 2002). Therefore, we believed that the failure for the allosteric modulation on sigma-1 receptor in the constructed system in vitro could be attributed to differences in sigma-1 receptor structure, cellular contents or auxiliary proteins. However, we can’t completely rule out the the potential contribution of sequence differences between species in the sigma-1 receptor, although the sigma-1 receptor gene is highly conserved cross different species and there are over 85% similarities of sigma-1 receptor mRNA between guinea pig, human, mouse and rat (Hanner et al., 1996; Kekuda et al., 1996; Mei and Pasternak, 2001; Seth et al., 1998).

In summary, the present data demonstrated that SKF83959 and its analogs are the potent allosteric modulators of sigma-1 receptor for the binding activities of $^3$H(+)-pentazocine with sigma-1 receptors. Given facts that SKF83959 elicits a wide range of biological responses that are independent of D$_1$ receptor, the present finding may shed light on the underlying mechanism for SKF83959-elicted D$_1$ receptor independent response. Meanwhile, allosteric modulation on various receptors is an important target for pharmaceutical designing, the finding of novel sigma-1 receptor modulators may provide new strate-
gy for drug discovery for sigma-1 receptor–associated diseases.
Authorship contributions

Participated in research design: Guo, Zhen, B. Zhao, Wang

Conducted experiments: Guo, J. Zhao

Contributed news reagents: Zhang

Performed data analysis: Guo, Jin

Wrote or contributed to the writing of the manuscript: Guo, Zhen
References


Footnotes

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Figure Legends

Figure 1. The chemical structures of sigma-1 receptor pharmaophore, SKF83959, SCH23390 and SKF38393.

Figure 2. The saturation binding assays of \(^3\)H(+)-pentazocine with the sigma-1 receptors in various tissues or cells. Inset: the Scatchard plot from Fig. 2. Synaptosomes, purified liver plasma membranes or membrane P2 fractions of sigma-1 receptor expressing HEK293 cells were incubated for 2.5 h at 30°C with \(^3\)H(+)-pentazocine (0.1-60 nM). BD1047 10 \(\mu\)M was used to define nonspecific binding. Each experiment was replicated at least three times. Data were expressed as Mean±S.EM.

Figure 3. Displacement of \(^3\)H(+)-pentazocine bound with the sigma-1 receptor by progesterone. (A) The brain tissues. (B) The liver tissues. (C) The sigma-1 receptor stably-expressed HEK293 cells. (D) Multiple values of the \(K_i\) for progesterone as a function of the concentration of \(^3\)H(+)-pentazocine. Linear relationship showed the increase in observed IC\(_{50}\) of progesterone with the increasing concentrations of \(^3\)H(+)-pentazocine. Synaptosomes, purified liver plasma membranes, or constructed HEK293 cell membrane P2 fractions were incubated with \(^3\)H(+)-pentazocine (0.2-20 nM) at 30°C for 2.5 h. BD1047 10 \(\mu\)M was used to define the nonspecific binding. Each experiment was replicated at least three times. Data were expressed as Mean±S.EM.

Figure 4. The effects of respective SKF83959, SCH23390, SKF38393 and phenytoin
on the binding of $^3$H(+)pentazocine to sigma-1 receptor. (A) The concentration-response curves of SKF83959 and phenytoin in the brain tissues. (B) The concentration-response curves of SKF83959, SCH23390 and SKF38393 in the liver tissues. The basal binding activity was the specific binding value (dpm) in the absence of tested drugs, and was defined as 100%. Synaptosomes or purified liver plasma membranes were incubated with $^3$H(+)pentazocine (0.3 nM) at 30°C for 2.5 h. BD1047 (10 μM) was used to define the nonspecific binding. Each experiment was replicated at least three times. Data were expressed as Mean±S.EM.

Figure 5. The effects of respective SKF83959, SCH23390, SKF38393 and phenytoin on the saturation binding. (A) Saturation binding curves in the brain synaptosomes; Inset(A) The Scatchard plot redrawn from (A). (B) Saturation binding curves in the purified liver plasma membranes; Inset(B):The Scatchard plot redrawn from (B). (C) The parameters of $K_{app}$, $K_X$, and $α$. Synaptosomes or purified liver plasma membranes were incubated for 2.5 h at 30°C with $^3$H(+)-pentazocine (0.1-60 nM) in the presence of SKF83959, SCH23390, SKF38393, phenytoin or vehicle, respectively. BD1047 10 μM was used to define the nonspecific binding. Each experiment was replicated at least three times. Data were expressed as Mean±S.EM. *P<0.05, compared with the vehicle group, #P<0.05, compared with the phenytoin group.

Figure 6. The effects of respective SKF83959, SCH23390, SKF38393 and phenytoin on the dissociation kinetics. (A) Dissociation curves in the brain synaptosomes. (B) Dissociation curves in the purified liver plasma membranes. Dissociation was initiated by addition of 10 μM BD1047 after co-incubation of synaptosomes or purified liver plasma
membranes with 10 nM $^3$H-pentazocine at 30°C for 2.5 hours. BD1047 (10 μM) was used to define nonspecific the binding. Each experiment was replicated at least three times. Data were expressed as Mean±S.EM.

Figure 7. The effects of respective SKF83959, SCH23390, SKF38393, phenytoin on the binding activity of $^3$H-progesterone at sigma-1 receptor in brain (A) and liver tissues (B). The binding assays were conducted as described in Methods. The final concentration of $^3$H-progesterone was 10 nM. BD1047 (10 μM) was used to define the nonspecific binding. The basal binding activity was the specific binding value (dpm) in the absence of tested drugs, and was defined as 100%. Each experiment was replicated at least three times. Data were expressed as Mean±S.EM.

Figure 8. The effects of respective SKF83959, SCH23390, SKF38393 and phenytoin on the binding activity of $^3$H-DTG to sigma-2 receptor in brain (A) and liver (B) tissues. The binding assays were conducted as described in Methods. The final concentration of $^3$H-DTG was 10 nM. The basal binding activity was the specific binding value (dpm) in the absence of tested drugs, and was defined as 100%. Haloperidol (10 μM) was used to define the nonspecific binding. Each experiment was replicated at least three times. Data were expressed as Mean±S.EM.

Figure 9. The effects of respective SKF83959, SCH23390, SKF38393 and phenytoin on the binding activity of $^3$H(+)-pentazocine (A) or $^3$H(+)-progesterone (B) at sigma-1 receptor from the stably-expressed HEK293 cells. The binding assays were conducted as described in Methods. The final concentration of $^3$H(+)-pentazocine (or
$^3$H(+)-progesterone) was 10 nM. BD1047 (10 μM) was used to define the nonspecific binding. The basal binding activity was the specific binding value (dpm) in the absence of tested drugs, and defined as 100%. Each experiment was replicated at least three times. Data were expressed as Mean±S.EM.
Figure 1

Sigma-1 receptor pharmacophore

SKF83959

SCH23390

SKF38393
Figure 3

A. Brain

- $[^3H](+)$-pentazocine, $K_d = 0.3$
- $[^3H](+)$-pentazocine, $K_d = 1$
- $[^3H](+)$-pentazocine, $K_d = 3$
- $[^3H](+)$-pentazocine, $K_d = 10$

Bound (fmol/mg protein) vs. log [progesterone (M)]

B. Liver

- $[^3H](+)$-pentazocine, $K_d = 0.3$
- $[^3H](+)$-pentazocine, $K_d = 1$
- $[^3H](+)$-pentazocine, $K_d = 3$
- $[^3H](+)$-pentazocine, $K_d = 10$

Bound (fmol/mg protein) vs. log [progesterone (M)]

C. HEK293

- $[^3H](+)$-pentazocine, $K_d = 0.3$
- $[^3H](+)$-pentazocine, $K_d = 1$
- $[^3H](+)$-pentazocine, $K_d = 3$
- $[^3H](+)$-pentazocine, $K_d = 10$

Bound (fmol/mg protein) vs. log [progesterone (M)]

D. Cheng-Prusoff equation

IC$_{50}$/Ki vs. $[^3H](+)$-pentazocine, $K_d$

- Brain
- Liver
- HEK293

E. Table

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<td>$K_d$ (nM)</td>
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Figure 4

A. Brain

% basal binding activity vs. log[compound(M)]

- SKF83959
- Phenytoin

B. Liver

% basal binding activity vs. log[compound(M)]

- SKF83959
- SCH23390
- SKF38393
Figure 6

A. Brain

B. Liver