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Pharmacological Characterization of (10b*S*)-1,2,3,5,6,10b-hexahydropyrrolo[2,1-*a*]isoquinoline Oxalate (YSL-3S) as a New α_2-Adrenoceptor Antagonist

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α_2-Adrenoceptor antagonists, which can enhance synaptic norepinephrine levels by blocking feedback inhibition processes, are potentially useful in the treatment of disease states such as depression, memory impairment, impotence and sexual dysfunction. (10b*S*)-1,2,3,5,6,10b-Hexahydropyrrolo[2,1-*a*]isoquinoline oxalate (YSL-3S) was evaluated in several *in vitro* biological tests to establish its pharmacological profile of activities as an α_2-adrenoceptor antagonist. Saturation binding assay revealed that [^{3}H]rauwolscine bound to the α_2-adrenoceptors with a Kd value of 6.3 ± 0.5 nM and a Bmax value of 251 ± 39 fmol/mg protein in rat cortical synaptic membranes. Competitive binding assay showed that YSL-3S inhibited the binding of [^{3}H]rauwolscine (1 nM) in a concentration-dependent manner with a Ki value of 98.2 ± 12.1 nM while it did not inhibit the binding of [^{3}H]cytisine (1.25 nM) to neuronal nicotinic cholinergic receptors. The Ki values of yohimbine, clonidine and norepinephrine for [^{3}H]rauwolscine binding were 15.8 ± 1.0, 40.1 ± 5.9 and 40.0 ± 11.5 nM, respectively. In addition, the binding affinity of YSL-3S for a_2-adrenoceptors was higher than that of its antipode and the racemic mixture. The functional activity of YSL-3S at the presynaptic α_2-adrenoceptors was assessed using the prostatic portion of the rat vas deferens. Clonidine inhibited field-stimulated contractions of the vas deference in a dose-dependent manner. The presence of YSL-3S or yohimbine caused a parallel, rightward the dose-response curve of clonidine in a dose-dependent manner, indicating an antagonistic action at the presynaptic α_2-adrenoceptors. The pA_2 values of yohimbine and YSL-3S were 7.66 ± 0.13 and 6.64 ± 0.18, respectively. The results indicate that YSL-3S acts as a competitive antagonist at presynaptic α_2-adrenoceptors with a potency approximately ten times lower than yohimbine, but is devoid of binding affinity for neuronal nicotinic cholinergic receptors.

Key words: α_2-Adrenoceptors, Depression, YSL-3S, Yohimbine, Clonidine, [^{3}H]Rauwolscine, [^{3}H]Cytisine, Cerebral cortex, Vas deferens

INTRODUCTION

Presynaptically located a_2-adrenoceptors play an important role in the regulation of neurotransmitter release from sympathetic nerve endings. Presynaptic α_2-adrenoceptors may also mediate the inhibition of a release of neurotransmitters other than norepinephrine (NE) in the central and peripheral nervous systems. α_2-Adrenoceptors are also located at postsynaptic sites, including many types of neurons in the brain (Lefkowitz *et al.*, 1996). Activating the α_2-adrenoceptors in the pontomedullary region of the central nervous system (CNS) inhibits sympathetic nervous system activity and leads to a fall in blood pressure; these receptors are activity sites for certain centrally acting antihypertensives, such as clonidine, guanabenz and guanfacine. A blockade of α_2-adrenoceptors with selective antagonists (e. g. yohimbine) block feedback inhibition processes, there by enhancing the release of NE from sympathetic nerve endings, which leads to the activation of α_1 and β_1 receptors in the peripheral vasculature and heart with a consequent rise in blood pressure (Goldberg and Robertson, 1983). It has been suggested recently that α_2-adrenoceptor

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antagonists are potentially useful in the treatment of disease states such as depression, age-dependent memory impairment, impotence and sexual dysfunction, and a variety of vascular disorders. One interesting approach based on the work of Weich and Ursillo (1980) is the potential use of α_2-adrenoceptor antagonists as antide-pressants. Presynaptic a_2-adrenoceptor antagonists can enhance the synaptic NE and/or serotonin levels by blocking feedback inhibition processes. Clinically effective antide-pressants such as the classical tricyclic antide-pressants and the selective serotonin reuptake inhibitors, are thought to exert their therapeutic effects by increasing the concentrations of NE and/or serotonin in the synaptic cleft (Richelson, 1987; Pinder and Wieringa, 1993).

The α_2-adrenoceptor antagonist mirtazapine (Organon) was introduced in the Netherlands in 1994 and is now in use in the United States for the treatment of depression (Prous, 1995). Scientists at the University of Tasmania (Australia) have synthesized ADT-16, a peripherally and centrally active heterocyclic antagonist of 5-HT_2 and α_2-adrenoceptor-mediated responses (Leitch *et al.*, 1994). Moreover, ABT-200 was synthesized for the purpose of combining the dual mechanisms of reuptake inhibition and α_2-adrenoceptor antagonism. It exhibited moderate inhibition of NE reuptake with blockage of the α_2-adrenoceptors and was devoid of antihistaminic or anticholinergic activity. This compound may have the potential to act more rapidly and with greater efficacy than existing agents (Hancock, 1995).

Recently, we reported the syntheses of five pyrrolidinoisoquinoline (Lee *et al.*, 1995) and four imidazoloquinolizidine (Lee *et al.*, 1994) derivatives. Due to the structural similarities of these compounds to ADT-16, their binding affinities for α_2-adrenoceptors were initially evaluated. Since (10bS)-1,2,3,5,6,10b-hexahydropyrrolo [2,1-a] isoquinoline oxalate (YSL-3S) showed the highest affinity among them, its pharmacological profile for α_2-adrenoceptors was characterized through a series of *in vitro* assays in the present study. In addition, since YSL-3S was regarded as a conformationally constrained nicotine, the binding affinity for neuronal nicotinic cholinergic receptors was also examined. The chemical structure of YSL-3S is shown in Fig. 1.

MATERIALS AND METHODS

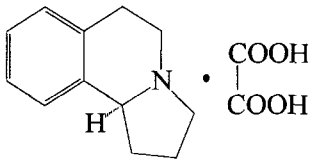

Fig. 1. Chemical structure of (10bS)-1,2,3,5,6,10b-hexahydropyrrolo [2,1-a]isoquinoline oxalate, YSL-3S

Materials

Male Sprague-Dawley (SD) rats weighing 250-350 g were purchased from Daehan Laboratory Animal Research Center Co., Ltd. (Daejeon, Korea). They were maintained at 23 ± 2°C and 55 ± 1% relative humidity with food and water available *ad libitum,* and were acclimated for a week prior to the experiments. The light/dark cycle was 12 h of light and 12 h of dark, with the onset of darkness at 21:00 h. [^{3}H]Rauwolscine (83.0 Ci/mmol) and [^{3}H] cytisine (39.7 Ci/mmol) were purchased from Dupont NEN (Boston, MA, USA). Yohimbine HCl, clonidine HCl, (-)-norepine-phrine bitartrate, (-)-nicotine bitartrate, bovine serum albumin and polyethylenimine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents were of analytical grade. YSL-3S, its antipode (YSL-3R) and the racemic mixture were obtained from the Korea Institute of Science and Technology (KIST).

Crude synaptic membrane preparation

The crude synaptic membranes were prepared according to the method of Zukin *et al.* (1974) with minor modifications. The male SD rats were decapitated with a guillotine and their brains were rapidly removed. The cerebral cortex was dissected out on ice according to the method of Glowinski and Iversen (1996), and the cortical tissues were homogenized in 15 volumes of 0.32 M sucrose with a Janke-Kunkel Ultra-Turrax T25 tissue homogenizer at low speed. The homogenate was centrifuged at 1000 × g for 10 min.

The supernatant was centrifuged at 20,000 × g for 20 min to obtain a crude mitochondrial pellet. The pellet was resuspended in ice-cold deionized water and dispersed with the homogenizer. The suspension was centrifuged at 8000 × g for 20 min.

The supernatant was collected and the pellet, a bilayer with a soft, buffy uppe-rcoat, was rinsed carefully with the supernatant fluid to collect the upper layer. The combined supernatant fraction was then centrifuged at 48,000 × g for 20 min. The crude synaptic membrane pellet was resuspended in 50 mM Tris-citrate buffer (pH 7.1 at 4°C) and centrifuged at 48,000 × g for 20 min. The resulting pellet was suspended in the same buffer and stored at -80°C until used for the receptor binding assay. The frozen membranes were thawed and centrifuged twice at 48,000 × g for 20 min and suspended in the assay buffer to yield a protein concentration of approximately 1.5 mg/ml. The protein concentrations in the final preparation were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. All procedures were performed at 4°C.

α_2-Adrenoceptor binding assay

The binding assay was performed according to the

method of Perry and U'Prichard (1981) with minor modifications. The binding of [^{3}H]rauwolscine to the synaptic memranes was measured by a filtration assay. The membranes (approximately 0.3 mg of protein) were incubated at 4°C for 120 min in a 50 mM Tris-HCl buffer (pH 7.1 at 4°C) containing [^{3}H]rauwolscine alone or in the presence of 100 mM (-)-NE or seven graded concentrations of competitors (10^{-9}-10^{-5} M), where the total incubation volume was 0.6 ml. In the saturation binding experiments, eight concentrations of [^{3}H] rauwolscine between 0.02 and 20 nM were used.

The competition experiments were performed in the presence of 1 nM [^{3}H]rauwolscine. After incubation, the reactions were terminated by rapid filtration through Whatman GF/B glass fiber filters presoaked with 0.05% polyethylenimine using a Brandel M-24R cell harvester. The filters were washed twice with 5 ml of ice-cold buffer, dried and then placed in scintillation vials containing 7 ml of Packard Ultima Gold scintillation cocktail. After shaking the vials and allowing them to reach equilibrium overnight, the radioactivity trapped on each filter was measured with a Packard 2000CA liquid scintillation counter. The nonspecific [^{3}H]rauwolscine binding was defined as that determined in the presence of 100 μM (-)-NE. All assays were performed in duplicate.

Neuronal nicotinic cholinergic receptor binding assay

The binding of [^{3}H]cytisine to the neuronal nicotinic cholinergic receptors was determined according to the method of Pabreza *et al.* (1991) with minor modifications. The competition experiments were performed with four graded concentrations of drugs (10^{-11}-10^{-5} M) in the presence of 1.25 nM [^{3}H]cytisine. The assay buffer used was 50 mM Tris-HCl buffer (pH 7.4 at 4°C) containing 120 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$, and 2 mM $MgCl_2$. The mixture was incubated at 4°C for 75 min. The nonspecific [^{3}H]cytisine binding was defined as that determined in the presence of 10 μM (-)-nicotine.

Functional *in vitro* studies for presynaptic α_2-adrenoceptors

The presynaptic α_2-adrenoceptor interactions of YSL-3S and yohimbine were assayed using a vas deferens preparation (Doxey *et al.*, 1977; Giardina *et al.*, 1993). Male SD rats weighing 245-265 g were sacrificed by decapitation with a guillotine. The prostatic end (15 mm) of the vas deferens was removed and placed in Krebs solution (NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 0.6 mM, $NaHCO_3$ 25 mM and dextrose 11.1 mM, pH 7.4 at 37°C when saturated with 95% O_2 and 5% CO_2). The tissue was gently cleaned of extraneous connective tissue, mounted between two platinum stimulating electrodes and suspended in a 10 ml organ bath.

The tissue was stimulated transmurally by rectangular electrical pulses of 30 volts each lasting 3 ms duration at a frequency of 0.01 hertz until the preparation was stabilized, usually 60-90 min. The stimulus was controlled by a Grass model S48 stimulator and constant current/voltage device. The isometric tension was recorded on a Model 7D Grass polygraph using FT03 transducers. After the twitch became cons-tant, the same stimulus was applied at a frequency of 0.1 hertz for the duration of the dose-response.

Clonidine was used as the control agonist for inhibiting the twitch. Ten minutes of contact was used to obtain a stable recording. Subsequent doses were added in a cumulative fashion until the stimulated twitch was attenuated maximally to zero. The stimulus was then reduced to 0.01 hertz, and the tissue was washed until the twitch regained most of its control value, usually for 60-70 min. After this stabilization period, the 0.1 hertz protocol was reapplied beginning 15 min before adding of the antagonist; the clonidine concentration-response challenge was repeated beginning 30 min after adding the antagonist. Three different concentrations of each antagonist were used. All responses to the control agonist were expressed as a percentage of the maximum response.

Data analysis

The data were expressed as mean ± S.E. The binding constants (Kd, Bmax and Ki values) were calculated using the EBDA/LIGAND computer program (Elsevier-Biosoft, UK). The Ki values were calculated from the IC_{50} values using the equation, $Ki=IC_{50}/(1+[ligand]/Kd)$ (Cheng and Prusoff, 1973). The functional potency of each antagonist was expressed as a pA_2 value (Schild, 1947; Tallarida *et al.*, 1979) calculated using the PHARM/PCS computer program (MicroComputer Specialists, PA).

RESULTS

Radioligand binding assays

The specific binding of [^{3}H]rauwolscine in synaptic membranes prepared from the rat cerebral cortex was saturable with increasing concentrations of the ligand and represents 60-90% of the total binding at all concentrations (Fig. 2). A scatchard analysis of the saturation binding isotherms for [^{3}H]rauwolscine indicated that a one-site binding model fist best with a Kd value of 6.3 ± 0.5 nM and a Bmax value of 251 ± 39 fmol/mg protein (n=3, inset of Fig. 2). In addition, the one-site binding model was also indicated by the Hill coefficient (n_H), which was very close to 1.

To assess the binding affinities of pyrrolidinoisoquinoline and imidazoloquinolizidine derivatives synthesized at the KIST for α_2-adrenoceptors, a competitive receptor binding assay was performed in the presence of 1 nM of [^{3}H]rauwolscine. α_2-Adrenoceptor agonists (NE and cloni-

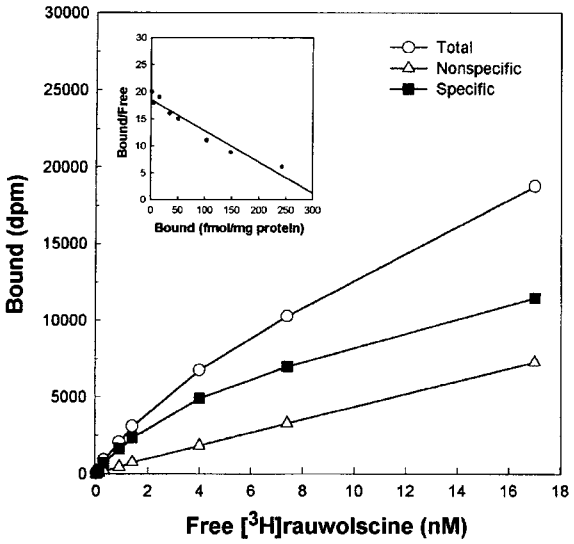

Fig. 2. [^{3}H]Rauwolscine binding in rat cerebral cortical membranes as a function of increasing radioligand concentration. Nonspecific binding was determined in the presence of 100 μM (-)-norepinephrine. Specific binding was defined as the difference between total and nonspecific binding. *Inset*, Scatchard plot of the specific binding data. It shows representative one of three separate experiments each performed in duplicate

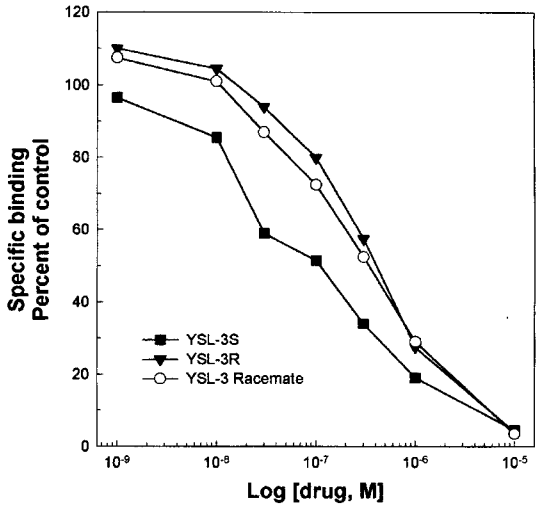

Fig. 4. Inhibitory effects of YSL-3S, YSL-3R (R enantiomer) and YSL-3 racemate on [^{3}H]rauwolscine binding in rat cerebral cortical membranes. The concentratin of the radioligand used was 1 nM. The results are shown as percent of control (the specific [^{3}H]rauwolscine binding without any competitors). Each point indicates the mean of three separate experiments each performed in duplicate

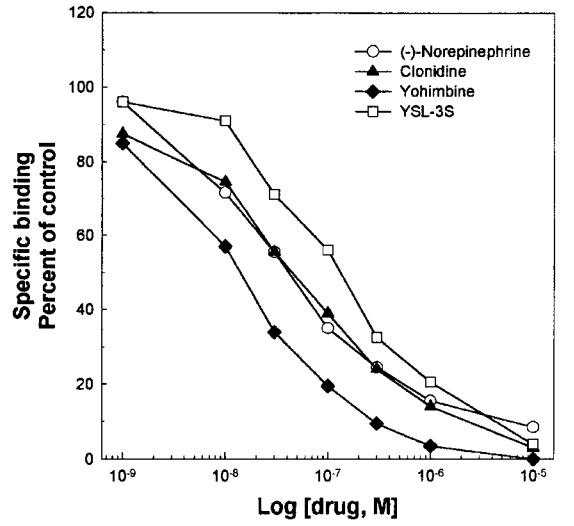

Fig. 3. Inhibitory effects of (-)-norepinephrine, clonidine, yohimbine and YSL-3S on [^{3}H]rauwolscine binding in rat cerebral cortical membranes. The concentration of the radioligand used was 1 nM. The results are shown as percent of control (the specific [^{3}H]rauwolscine binding without any competitors). These drugs inhibited α_2-adrenoceptor binding in a concentration-dependent manner. Each point indicates the mean of three separate experiments each performed in duplicate

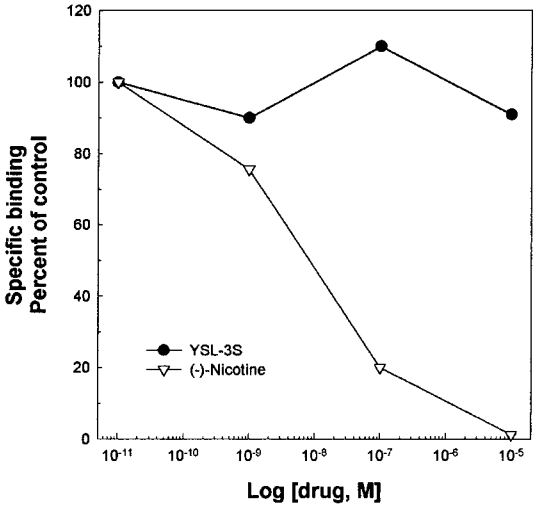

Fig. 5. Effect of YSL-3S on [^{3}H]cytisine binding to nicotinic cholinergic receptors in rat cerebral cortical membranes. YSL-3S did not show any affinity for the receptors. The concentration of the radioligand used was 1.25 nM. Nonspecific binding was determined in the presence of 10 μM (-)-nicotine

dine) and an antagonist (yohimbine) were included as reference compounds. Among the derivatives, (10bS)-1,2,3,5,6,10b-hexahydropyrrolo[2,1-a]isoquinoline oxalate (YSL-3S) showed the highest affinity for α_2-adrenoceptors (data not shown). It inhibited the binding of [^{3}H]rauwolscine to α_2-adrenoceptors in a concentration-dependent manner with an affinity approximately six times lower than yohimbine and two and one-half times lower affinity than clonidine and NE (Fig. 3).

The measured Ki values of yohimbine, clonidine, NE and YSL-3S were 15.8 ± 1.0, 40.1 ± 5.9, 40.0 ± 11.5 and 98.2 ± 12.1 nM, respectively. Therefore, the affinities for α_2-adrenoceptors were in the order of yohimbine>

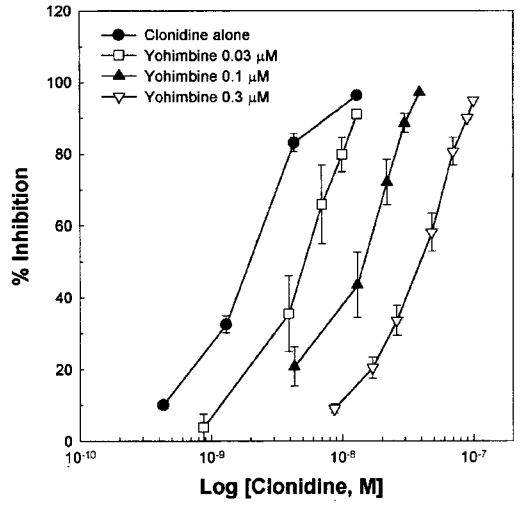

Fig. 6. Concentration-dependent inhibitory effects of clonidine alone or in the presence of 0.03, 0.1 and 0.3 μM yohimbine on electrically induced contraction of the rat vas deferens. Each point indicates the mean ± S.E. of 3 to 10 experiments

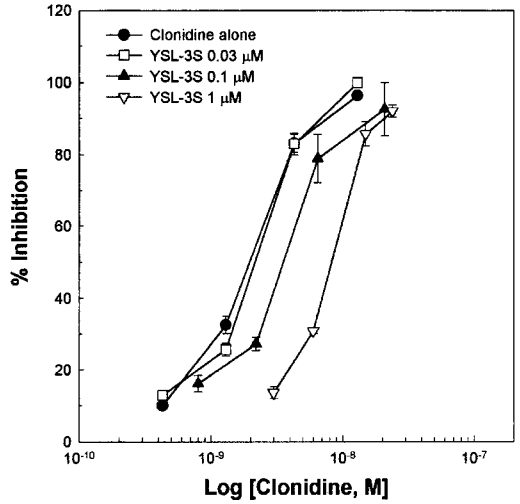

Fig. 7. Concentration-dependent inhibitory effects of clonidine alone or in the presence of 0.03, 0.1 and 1 μM YSL-3S on electrically induced contraction of the rat vas deferens. Each point indicates the mean ± S.E. of 3 to 10 experiments.

clonidine≒NE>YSL-3S. When compared with its antipode (YSL-3R) and the racemic mixture to examine stereospecificity, YSL-3S showed an affinity approximately three times higher, suggesting its enantiomeric specificity (Fig. 4). The measured Ki values of YSL-3R and the racemic mixture were 302 and 276 nM, respectively. However, YSL-3S did not inhibit the binding of [^{3}H] cytisine to neuronal nicotinic cholinergic receptors in the rat cerebral cortex (Fig. 5).

Functional *in vitro* bioassays

Table I. Potencies of α_2-adrenoceptor antagonists (pA_2) against clonidine at the presynaptic α_2-adrenoceptors in the isolated rat vas deferens and slopes of the Schild regression lines

Compound	pA_2	Slope
Yohimbine	7.66 ± 0.13	1.04 ± 0.17
YSL-3S	6.64 ± 0.18	0.96 ± 0.27

Each value represents the mean ± S.E. of 3-10 separate experiments.

Stimulating the intramural nerves of the rat vas deferens at a low frequency (0.01 hertz), at 30 volts for 3 ms, produced regular contractions of the tissue. There was little variation in the sensitivity of different preparations to clonidine, and the concentration-response curves of clonidine could be constructed repeatedly, again with little change in sensitivity in individual experiments. Initially, the antagonistic property of YSL-3S was revealed by reversing the clonidine-induced inhibition of the vas deferens contrations by post-treatment with 1 mM of YSL-3S.

To determine the antagonistic potency of YSL-3S in comparison with yohimbine, cumulative concentration-response curves of clonidine were constructed in the presence of different concentrations of the antagonists. The concentration-response curves of clonidine showed a parallel shift to the right in the presence of yohimbine (0.03, 0.1 and 0.3 μM, Fig. 6) or YSL-3S (0.1 and 1 μM, Fig. 7) in a concentration-dependent manner, indicating competitive antagonism. However, 0.03 μM of YSL-3S produced little effect on the concentration-response curves of clonidine (Fig. 7). Table I shows the functional α_2 antagonistic activity of YSL-3S and yohimbine expressed as pA_2 values, and the slopes of the Schild plot. YSL-3S (pA_2= 6.64) was approximately ten times less potent for the presynsptic α_2-adrenoceptors than for the yohimbine (pA_2=7.66).

DISCUSSION

The attempt to develop α_2-adrenoceptor antagonists was initially made at KIST by synthesizing pyrrolidinoisoquinoline and imidazoloquinolizidine derivatives due to their structural similarities to ADT-16. Since YSL-3S showed the highest binding affinity for α_2-adrenoceptors among them, its pharmacological profile of activity on α_2-adrenoceptors was further examined using *in vitro* functional bioassays in comparison with yohimbine. The results indicate that YSL-3S is a competitive α_2-adrenoceptor antagonist. α_2-Adrenoceptor antagonism is a theoretical approach to enhancing the synaptic concentrations of NE and/or serotonin (Pinder and Wieringa, 1993). Mirtazapine (Organon) and ADT-16 were synthesized as α_2-adrenoceptor antagonists for the treatment of depression (Leitch *et al.*, 1994; Prous, 1995). Moreover, ABT-200, an agent with the dual mechanisms of NE reuptake inhibition and

α_2-adrenoceptor antagonism, was recently synthesized for having the potential to act more rapidly and with greater efficacy than the existing agents (Hancock *et al.*, 1995).

Perry and U'Prichard (1981) reported that [^{3}H]rauwolscine specifically labeled α_2-adrenoceptors in bovine brain membranes. Due to the greater pharmacological specificity of rauwolscine, it is a more useful radioligand than [^{3}H]dihydroergocryptine or [^{3}H]yohimbine in brain and other tissues containing, mixed α_1 and α_2-adrenoceptor population. The radioligand binding assays using [^{3}H]rauwolscine showed that YSL-3S possessed a binding affinity of 10^{-7} molar range for α_2-adrenoceptors in the rat cerebral cortex, although its affinity was 2.5- and 6.2-times lower than those of clonidine and yohimbine, respectively. In addition, its affinity was several times higher than those of its antipode (YSL-3R) and the racemic mixture, indicating the stereospecificity of the binding. However, YSL-3S did not show any binding affinity for the neuronal nicotinic cholinergic receptors in the rat cerebral cortex.

The rat vas deferens subjected to a low frequency field stimulation provides an alternative method for studying the effects of agonists and antagonists at presynaptic α_2-adrenoceptors (Doxey *et al.*, 1977). The functional *in vitro* bioassays using that preparation were performed to determine the antagonistic activity of YSL-3S at the presynaptic α_2-adrenoceptors in comparison with the reference α_2-adrenoceptor antagonist, yohimbine. The pA_2 value for yohimbine (7.66 ± 0.13) was similar to those previously reported for rauwolscine (α-yohimbine; Hancock *et al.*, 1995) and yohimbine (Doxey *et al.*, 1977). The two diastereoisomers were reported to be approximately equipotent α_2-adrenoceptor antagonists in functional studies (Hedler *et al.*, 1981). YSL-3S acted as a competitive α_2-adrenoceptor antagonist with a pA_2 value of 6.64 ± 0.18, indicating that it was approximately ten-times less potent than yohimbine. However, the difference in potency determined by the pA_2 values measured in the functional bioassays correlated well with the difference in potency determined by the Ki values measured in the radioligand binding assays between these two test compounds, although the former was somewhat greater than the latter.

In conclusion, YSL-3S is a novel competitive antagonist of presynaptic α_2-adrenoceptors without binding affinity for neuronal nicotinic cholinergic receptors, and can serve as a lead compound. Further studies to search for more potent α_2-adrenoceptor antagonists are in progress.

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