Assessment of Affinity and Dissociation Ability of a Newly Synthesized 5-HT₂ Antagonist, AT-1015: Comparison With Other 5-HT₂ Antagonists

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ABSTRACT—This study investigated the binding affinities of a newly synthesized 5-HT₂ antagonist, AT-1015 (N-[2-[4-(5H-dibenzo[a,d]cyclohepten-5-ylidene)-piperidino]ethyl]-1-formyl-4-piperidinecarboxamide monohydrochloride monohydrate) for [³H]ketanserin bindings to 5-HT₂ receptors in the rabbit cerebral cortex membranes using the radioligand binding assay method. The affinity of this compound was also compared with other 5-HT₂-selective antagonists such as ketanserin, sarpogrelate, cyproheptadine and ritanserin, and the results showed that AT-1015 has a high pKᵢ value for the 5-HT₂ receptor. The rank order of these antagonists are: ritanserin > ketanserin = AT-1015 > cyproheptadine = sarpogrelate. We also evaluated the dissociation ability (slow or rapid) of AT-1015 in the rabbit cerebral cortex membrane and compared it with other 5-HT₂ antagonists using the radioligand binding assay method. The blockade of [³H]ketanserin binding sites in the rabbit cerebral cortex induced by ketanserin and sarpogrelate was readily reversed by washing, whereas the inhibition by AT-1015, cyproheptadine and ritanserin was not readily reversed by washing. The % of control after washing are 76.10% and 49.55% for AT-1015 at 10⁻⁷.5 and 10⁻⁷.0 M, 67.32% and 50.17% for cyproheptadine at 10⁻⁷.5 and 10⁻⁷.0 M, and 72.38% and 39.80% for ritanserin at 10⁻⁹.5 and 10⁻⁹.0 M concentrations, respectively. Thus, these findings suggest that AT-1015 has antagonistic properties towards the 5-HT₂ receptor and also shows that AT-1015 slowly dissociates from the 5-HT₂ receptor, whereas, ketanserin and sarpogrelate dissociate rapidly from the 5-HT₂ receptor, which do not correlate with their affinity.

Keywords: AT-1015, 5-HT₂-receptor antagonist, Cerebral cortex (rabbit)

Through its interactions with different receptor subtypes in the central and peripheral nervous system, 5-hydroxytryptamine (5-HT), a neurotransmitter, has diverse physiological functions (1). These diverse responses are elicited through the activation of a large family of 5-HT-receptor subtypes. So far, more than 14 kinds of 5-HT-receptor subtypes have been confirmed (2). Ketanserin, the first selective [³H]-ligand for labelling the 5-HT₂ receptors, contributed to further investigations to determine the localization of 5-HT₂ receptors in the central nervous system and peripheral tissues (3). 5-HT₂ receptors are demonstrated on both platelet membranes and brains by radioligand binding assay methods (4 – 6).

Previously we (7) reported that AT-1015 (N-[2-[4-(5H-dibenzo[a,d]cyclohepten-5-ylidene)-piperidino]ethyl]-1-formyl-4-piperidinecarboxamide monohydrochloride monohydrate) was a strong noncompetitive 5-HT₂ antagonist, comparing it with ketanserin, and it inhibited the contraction response of porcine coronary arteries induced by 5-HT and (α-methylserotonin). Kihara et al. (8) also reported that AT-1015, which is newly synthesized as a 5-HT₂ antagonist, blocked vascular and platelet 5-HT₂ receptors and prevented laurate-induced peripheral vascular lesion in rats. The activity of AT-1015 on selective inhibition of 5-HT₂A-mediated platelet aggregation was almost equivalent to ketanserin, but 100 times more potent than sarpogrelate. In this study, we demonstrated the binding affinity of AT-1015 on 5-HT₂ receptors in rabbit cerebral cortex membrane using the radioligand binding assay method. Moreover, we evaluated its dissociation ability from 5-HT₂ receptors in the cerebral cortex and compared these find-
ings with those of other 5-HT₂ antagonists such as ketanserin, sarpogrelate, cyproheptadine and ritanserin.

MATERIALS AND METHODS

Materials

[³H]Ketanserin (63.3 Ci/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA, USA). AT-1015 used in the study was donated by Ajinomoto Co., Ltd. (Tokyo). Ketanserin and ritanserin were obtained from RBI (Research Biochemicals Incorporated, Natick, MA, USA) and cyproheptadine was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sarpogrelate was obtained from Mitsubishi Chemical Corporation (Tokyo). Their chemical structures are shown in Fig. 1.

Preparation of membrane-enriched fraction

The membrane-enriched fraction of rabbit cerebral cortex was prepared by the method described previously (9). In brief, the brain was removed from a male rabbit (Japanese white, 3–4 kg), after the rabbits were anesthetized with sodium pentobarbital (25 mg/kg, i.v.) and exsanguinated. The cerebral cortex was then separated from the whole brain and minced in 10 vol sucrose buffer (10 mM

Fig. 1. Chemical structures of serotonin (5-HT) and 5-HT₂ antagonists.
Tris-HCl, 0.25 M sucrose, pH 7.4) and was homogenized by using a glass-homogenizer. The homogenate was then centrifuged at 40,000 × g for 30 min at 4°C. The supernatant was discarded and the resultant pellet was suspended in the incubation medium containing 120 mM Tris-HCl and 40 mM MgCl₂ (pH 7.4). The membrane-enriched fraction was then immediately frozen in liquid nitrogen and stored at −80°C until used. Membrane protein concentrations were determined by the method of Bradford (10) with bovine serum albumin used as the standard.

Radioligand binding assay

The radioligand binding assays for the determination of affinity for 5-HT₁₂-receptor subtype were performed according to the method described previously (11). Experiments were performed at a final volume of 200 μl of assay buffer (60 mM Tris-HCl, 20 mM MgCl₂, 0.1% ascorbic acid and 1.0 μM pargyline, pH 7.4) with 50 μl of [³²P]ketanserin, 100 μl of membrane preparation (0.1 mg of protein) and 50 μl of vehicle or test solution containing various concentrations of the test compounds. The concentration of [³²P]ketanserin ranged from 0.12 nM to 8.0 nM for the saturation binding experiments, and a concentration of 1.25 nM was used for the competition binding experiments. After 30 min incubation at 23°C, the reaction mixture was rapidly filtered under vacuum through a Whatman GF/C glass fiber filter and was followed by five 2.0-ml washes with ice-cold buffer of 50 mM Tris-HCl (pH 7.4). The filters were pre-soaked in 0.1% polyethyleneimine (PEI) to reduce filter binding of free radioligand. The retained radioactivity in the vial which contained the filter with 2 ml of toluene-trinitron based scintillation fluid was determined using a liquid scintillation counter (Packard 2200 Tri-Carb Scintillation Analyzer; Packard Instrument Co., Inc., Meriden, CT, USA). The specific binding was determined by subtracting the remaining nonspecific binding in the presence of 1.0 μM ritanserin from the total binding.

The dissociation ability of 5-HT₁₂ antagonists, that is, the inhibitory effects of [³²P]ketanserin binding by 5-HT₁₂ antagonists after washout of the membranes pretreated with drugs were studied according to the method of Hosohata et al. (9) with slight modifications. Estimation of the dissociation abilities were carried out at a final volume of 500 μl of assay buffer (60 mM Tris-HCl, 20 mM MgCl₂, 0.1% ascorbic acid and 1.0 μM pargyline, pH 7.4) with 50 μl of [³²P]ketanserin, 250 μl of membrane preparation (0.2 mg of protein) and 50 μl of vehicle or test compounds. The membranes were preincubated with or without antagonists added for 30 min at 23°C. In these experiments, two concentrations of each antagonist were used for investigating the dissociation ability. The concentrations of the drugs used were AT-1015 at 10⁻⁷.⁵ and 10⁻⁷.⁰ M, ketanserin at 10⁻⁹ and 10⁻⁸.⁰ M, sarpogrelate at 10⁻⁶.⁵ and 10⁻⁶.⁰ M, cyproheptadine at 10⁻⁷.⁵ and 10⁻⁷.⁰ M, and ritanserin at 10⁻⁹.⁵ and 10⁻⁹.⁰ M, respectively. The respective first and second concentrations of these 5-HT₁₂ antagonists used here inhibited 40 – 50% and 20 – 30% of [³²P]ketanserin bindings to 5-HT₁₂ receptors in the rabbit cerebral cortex membranes. The preincubated membranes were washed twice with the incubation buffer by centrifugation at 33,000 × g for 20 min at 4°C. The washed membranes were then assayed for the [³²P]ketanserin binding using the conditions described above. The concentration of the [³²P]ketanserin used was 1.0 nM. For the determination of nonspecific binding, nonlabeled ritanserin (10⁻⁶ M) was added throughout the experiment. The membranes preincubated without antagonists were also assayed using the conditions mentioned above.

Kinetic analyses

Data were given as the means ± S.E.M. The dissociation constants (Kᵦ) and the density of 5-HT₁₂-receptor binding sites (Bₘₐₓ) in the membrane preparation of the rabbit cerebral cortex were determined by linear regression analysis of the saturation binding curves transformed to Scatchard plots. IC₅₀ (inhibitory concentration 50%), and Kᵦ (inhibition constant) values were determined with Prism, a nonlinear curve-fitting program (Graph Pad Software, Inc., San Diego, CA, USA). Most of the Kᵦ values are expressed as pKᵦ (−log Kᵦ) in this report. Significance of the data was evaluated by Student’s t-test.

RESULTS

Binding affinity

The binding of [³²P]ketanserin to rabbit cerebral cortex membranes occurred at a saturable single site with a Bₘₐₓ value of 175.46 ± 81.43 fmol/mg protein and a Kᵦ value of 6.85 ± 1.94 nM. Binding studies for Scatchard analysis were repeated 4 times with duplicate samples for each concentration in each experiment. The average specific binding represented 60 – 65% of the total radioactivity.

Table 1. pKᵦ values of several 5-HT₁₂ antagonists to 5-HT₁₂ receptor in rabbit cerebral cortex membrane

| Drugs        | pKᵦ    |
|--------------|--------|
| Ritanserin   | 8.98 ± 0.31 (4) |
| Ketanserin  | 8.23 ± 0.16 (7) |
| AT-1015      | 7.94 ± 0.26 (8) |
| Cyproheptadine | 7.54 ± 0.32 (4) |
| Sarpogrelate | 7.22 ± 0.07 (5) |

Values are the mean ± S.E.M. Numbers in parentheses indicate the number of experiments. The concentration of [³²P]ketanserin used was 1.25 nM.
bound to the cerebral cortex membranes.

Table 1 summarizes the affinities (pKᵢ values) of AT-1015, ketanserin, sarpogrelate, cyproheptadine and ritanserin on [³H]ketanserin bindings to 5-HT₂ receptors in rabbit cerebral cortex membrane. The result showed that ritanserin had the highest affinity binding to 5-HT₂ receptors (pKᵢ value 8.98); and in contrast, the affinity of AT-1015 (pKᵢ value 7.94) was almost equivalent to that of ketanserin (pKᵢ value 8.23) and also higher than that of sarpogrelate (pKᵢ value 7.22) for [³H]ketanserin binding. Moreover, the affinity of AT-1015 was almost similar to that of cyproheptadine (pKᵢ value 7.54).

Dissociation ability (slow or rapid)

The inhibition of [³H]ketanserin binding by AT-1015 and other 5-HT₂ antagonists to rabbit cerebral cortex membrane are shown in Fig. 2. The % of control in the specific [³H]ketanserin bindings for the first concentrations of each 5-HT₂ antagonist (without washout, i.e., preincubation without drugs) were between 12.5% and 28.7%; also for the second concentrations, they were between 35.9% and 58.1% (open columns in Fig. 2). When membranes previously preincubated with AT-1015 and other 5-HT₂ antagonists were washed extensively and subsequently assayed for [³H]ketanserin binding, there was little recovery of specific [³H]ketanserin binding in membranes treated with the second concentration of AT-1015, cyproheptadine and ritanserin (hatched columns in Fig. 2), and this recovery was the lowest with the treatment of ritanserin; the % of control with washout for the first concentrations of these three antagonists were almost identical (76.1%, 67.3% and 72.4%, respectively). In contrast, binding was significantly restored following treatment with either concentration of ketanserin, and the % of specific binding in the membranes treated with the first and second concentrations of sarpogrelate were 96.4% and 77.27% of the control, respectively.

DISCUSSION

AT-1015 showed a strong inhibitory effect on contraction of porcine coronary arteries mediated by 5-HT₂ sub-

![Graph showing dissociation ability of 5-HT₂ antagonists](image-url)
types, and it was a strong non-competitive 5-HT\textsubscript{2} antagonist compared to ketanserin (7). AT-1015 selectively inhibited in vitro 5-HT\textsubscript{2A}-receptor-mediated platelet aggregation, and this activity is almost equivalent to that of ketanserin and 100 times more potent than sarpogrelate. AT-1015 also slightly reduced maximal contraction induced by 5-HT in rat blood vessel, which is also unlike competitive inhibitors such as ketanserin and sarpogrelate. AT-1015 was also very effective and more potent in preventing the progression of peripheral vascular lesions compared with ketanserin, sarpogrelate and cilostazol (8). Because AT-1015 is a very potent 5-HT\textsubscript{2} antagonist, we wanted to investigate its affinity and also its dissociation ability from 5-HT\textsubscript{2} receptors in rabbit cerebral cortex by the radioligand binding assay method. We also evaluated its dissociation ability from 5-HT\textsubscript{2} receptors in rabbit cerebral cortex by comparing it with ketanserin and sarpogrelate as well as other potent 5-HT\textsubscript{2} antagonists such as cyproheptadine and ritanserin. Ritanserin is a very potent and long acting 5-HT\textsubscript{2} antagonist compared with ketanserin (12).

In the present study, AT-1015 showed a high affinity to 5-HT\textsubscript{2} receptors using \textsuperscript{3}H\textsuperscript{19}ketanserin binding in rabbit cerebral cortex. The affinities of all other antagonists (ketanserin, sarpogrelate, cyproheptadine and ritanserin) to the 5-HT\textsubscript{2} receptor in this study were almost the identical as those reported in rat frontal lobe (13–15); rabbit, cat and human platelet membranes (16–18); vascular smooth muscle cell (19, 20); and cloned 5-HT\textsubscript{2A} receptor (21).

The present study showed that ritanserin had the highest binding affinity to the 5-HT\textsubscript{2} receptor among all of these antagonists used in these investigations. The basic chemical structure of 5-HT consists of a benzene ring, heterocyclic ring, ethylene chain and amine. Ritanserin possesses all of these basic groups in its structure. These investigations showed almost the same affinities between ketanserin and AT-1015 and these two antagonists demonstrate similar basic groups (N-ethyl piperidine) in their structures and they also possess the same basic chemical structure of serotonin. Our previous report (7) showed the major difference in the pharmacological profiles between AT-1015 and ketanserin may be due to different chemical substituents in their chemical structures. Cyproheptadine has a different type of chemical structure from those of AT-1015, ketanserin and ritanserin, but it also has the almost similar affinity to that of AT-1015. It has no basic chemical structure (N-ethyl piperidine) like ketanserin and AT-1015, and it also does not contain the basic chemical structure of serotonin. However, it contains the dibenzo-cycloproten group attached to piperidine rings, similar hydrophobic residues on 4-position of the piperidine ring of the basic chemical structure of AT-1015 (Fig. 1). On the other hand, the chemical structure of sarpogrelate is also totally different from that of other antagonists used in this study reported by Maruyama et al. (14), and it has slightly lower affinity than those of ketanserin and AT-1015. Sarpogrelate does not contain N-ethyl piperidine, the basic chemical structure of ketanserin and AT-1015. The chemical structure of sarpogrelate contains two methyl groups in amine position and a large groups on \(\beta\)-carbon of ethyl-group (Fig. 1).

Moreover, we also evaluated the dissociation ability of AT-1015 and compared it with other 5-HT\textsubscript{2} antagonists (ketanserin, sarpogrelate, cyproheptadine and ritanserin) to rabbit cerebral cortex membrane using \textsuperscript{3}H\textsuperscript{19}ketanserin by the radioligand binding assay reported by Hosohata (9). We observed here that AT-1015, cyproheptadine and ritanserin dissociated slowly from the 5-HT\textsubscript{2} receptor of the rabbit cerebral cortex membrane. They were not readily removed by washing. Among these three antagonists, ritanserin has the slowest dissociation from the 5-HT\textsubscript{2} receptors, and it was bound to the receptor very strongly. Leysen et al. (12) also reported that ritanserin dissociated very slowly from 5-HT\textsubscript{2} \((t_{1/2} = 160\text{ min})\); and in contrast to ritanserin, ketanserin dissociated rapidly from the 5-HT\textsubscript{2} receptors \((t_{1/2} = 5\text{ min})\). The result showed that the dissociation ability of AT-1015 and cyproheptadine from the 5-HT\textsubscript{2} receptor were almost similar.

On the other hand, we observed that ketanserin and sarpogrelate rapidly dissociated from the 5-HT\textsubscript{2} receptor after washout. Thus, pretreatment of ketanserin and sarpogrelate prevented the inhibitory effects of \textsuperscript{3}H\textsuperscript{19}ketanserin in the rabbit cerebral cortex membrane after washing and specific \textsuperscript{3}H\textsuperscript{19}ketanserin binding recovered to the control levels, but pretreatment with AT-1015, cyproheptadine or ritanserin could not recovered specific \textsuperscript{3}H\textsuperscript{19}ketanserin binding to the control levels (Fig. 2). Thus the result obtained here imply that AT-1015 could tightly bind to 5-HT\textsubscript{2} receptors in membranes, suggesting that the dissociation of AT-1015 from bound 5-HT\textsubscript{2} receptors was definitely slower when compared to other 5-HT\textsubscript{2} antagonists.

In conclusion, a) AT-1015 showed a high affinity for 5-HT\textsubscript{2} receptors in rabbit cerebral cortex membranes using \textsuperscript{3}H\textsuperscript{19}ketanserin as determined by the radioligand binding assay method. This affinity was almost similar to that of ketanserin, but higher than that of sarpogrelate. The affinity of the 5-HT\textsubscript{2} antagonists used in these experiments were in the following rank order: ritanserin > ketanserin \(\cong\) AT-1015 > cyproheptadine \(\cong\) sarpogrelate. b) AT-1015 was a slowly dissociating 5-HT\textsubscript{2} receptor antagonist compared to other 5-HT\textsubscript{2} antagonists, whereas ketanserin and sarpogrelate dissociate rapidly from the 5-HT\textsubscript{2} receptors. Thus we may conclude that the affinities of these antagonists do not correlate with the dissociation ability from the receptor site, as ketanserin has high affinity, but it dissociates rapidly from the 5-HT\textsubscript{2} receptor. This variation may be due to their hydrophobicity or hydrophilicity and the mode of inter-
action of their functional groups with amino acids of the receptor site. Now, we are doing molecular modelling to find out the interaction sites and the binding mode of these antagonists to 5-HT₂-receptor subtypes.

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