Binding Affinity of a Newly Synthesized 5-HT₂ Antagonist, AT-1015 (*N*-[2-[4-(5*H*-Dibenzo[*a,d*]cyclohepten-5-ylidene)-piperidino]ethyl]-1-formyl-4-piperidinecarboxamide Monohydrochloride Monohydrate), in the Rabbit Platelet Membrane

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The object of this study was to investigate the binding affinity of a newly synthesized 5-HT₂ antagonist, (*N*-[2-[4-(5*H*-dibenzo[*a,d*]cyclohepten-5-ylidene)-piperidino]ethyl]-1-formyl-4-piperidinecarboxamide monohydrochloride monohydrate) (AT-1015), in the rabbit platelet membrane using [³H]-ketanserin by radioligand binding assay method and to compare the results with other selective 5-HT₂ antagonists. The results showed that AT-1015 displayed high affinity to 5-HT₂ receptors in rabbit platelet membranes. The p*K*_i value of AT-1015 was 7.40, which is slightly lower than that of ketanserin, but higher than that of cyproheptadine. On the other hand, the displacement potency of AT-1015 for 5-HT₂ receptors in rabbit platelets was similar to those of sarpogrelate and ritanserin. This is the first report of the high affinity of AT-1015 in rabbit platelets.

Key words AT-1015; 5-HT₂ receptor; rabbit platelet

Serotonin (5-HT) induces shape change and aggregation of platelets, vasoconstriction and increase of vascular permeability and cell proliferation,^{1–5)} after release from the site of vascular injury. The 5-HT_{2A} receptor is thought to be involved in all of these 5-HT mediated pathophysiological effects.^{6–8)}

Kihara *et al.*⁹⁾ reported that AT-1015 (*N*-[2-[4-(5*H*-dibenzo[*a,d*]cyclohepten-5-ylidene)-piperidino]ethyl]-1-formyl-4-piperidinecarboxamide monohydrochloride monohydrate), a newly synthesized 5-HT₂ antagonist, blocked vascular and platelet 5-HT_{2A} receptors and prevented laurate-induced peripheral vascular lesion in rats. The inhibitory activity and selectivity of AT-1015 towards 5-HT_{2A} receptor-mediated platelet aggregation was almost equivalent to ketanserin. Previously, we reported that AT-1015 was a strong noncompetitive 5-HT₂ antagonist compared to ketanserin and that it inhibited the contraction response of porcine coronary arteries induced by 5-HT and α -methylserotonin.¹⁰⁾ In this study, we demonstrated the binding affinity of AT-1015 to 5-HT₂ receptor in rabbit platelet membranes using [³H]-ketanserin in a radioligand binding assay method and make a comparison of these findings 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, U.S.A.). AT-1015 used in the study was donated by Ajinomoto Co., Ltd., Tokyo, Japan. Ketanserin and ritanserin were obtained from RBI (Research Biochemicals Incorporated, Natick, MA, U.S.A.) and cyproheptadine was obtained from SIGMA (Sigma Chemical Co., St. Louis, MO, U.S.A.). Sarpogrelate was obtained from Mitsubishi Chemical Corporation, Tokyo, Japan. The chemical structure of AT-1015 is shown in Fig. 1.

Preparation of Membrane-Enriched Fraction Male rabbits (Japanese, white, 3–4 kg) were anesthetized by i.v.

injection of sodium pentobarbital (25 mg/kg). A polyethylene extension tube was introduced in the carotid artery *via* an incision in the neck. The first 2–3 ml blood was discarded, and the blood was at first collected by a 50 ml plastic syringe containing 0.38% trisodium citrate and then it was gently decanted to a 50 ml plastic centrifuge tube. Membrane preparation of platelet was performed according to the method of Tsuchihashi *et al.*¹¹⁾ with slight modification. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 180×*g* for 15 min at 10 °C. Residual erythrocytes and leukocytes were removed by another centrifugation at 200×*g* for 15 min at 10 °C. The PRP was then centrifuged at 30000×*g* for 10 min at 4 °C and the resultant platelet-rich pellet was suspended in hypotonic Tris–EDTA (5 mM Tris–HCl, 0.1% EDTA, pH 7.5) using Polytron homogenization at a setting of 7 for 10 s. The suspension was centrifuged at 40000×*g* for 15 min at 4 °C and the membranes were re-suspended in the same buffer using Polytron homogenization as described above. After the same centrifugation procedure, the pellet was finally re-suspended in the assay buffer, which consisted of 60 mM Tris–HCl, 20 mM MgCl₂, pH 7.4. The platelet 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 Bradford¹²⁾ with bovine serum albumin used as standard.

Binding Assay The radioligand binding assays for the

AT-1015

Fig. 1. The Chemical Structure of AT-1015

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determination of affinity for 5-HT₂ receptor subtype were performed according to the method described previously.¹³⁾ Experiments were performed at a final volume of 500 μ l of assay buffer (60 mM Tris-HCl, 20 mM MgCl₂, pH 7.4) with 50 μ l of [³H]-ketanserin, 100 μ l of membrane preparation (0.1 mg of protein) and 50 μ l of vehicle or test solution containing various concentrations of the test compounds. The concentration of [³H]-ketanserin ranged from 0.12 nM to 10.0 nM for the saturation binding experiments, and a concentration of 1.0 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 50 mM Tris-HCl buffer (pH 7.4). The filter mats were pre-soaked in 0.1% polyethyleneimine (PEI) to reduce filter binding. 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 Analyser). The specific binding was determined by subtracting the remaining nonspecific binding in the presence of 0.1 mM 5-HT from the total binding.

Kinetic Analysis Data are given as the means $\pm$ S.E. The dissociation constants (K_d) and the density of 5-HT₂ receptor binding sites (B_{max}) in the membrane preparation of the rabbit platelet membrane were determined by linear regression analysis of the saturation binding curves transformed to Scatchard plots. IC₅₀ (inhibitory concentration 50%) and K_i (inhibition constant) were analyzed using Prism, a nonlinear curve-fitting program (Graph Pad Software, Inc. CA, U.S.A.). Most of the K_i values are expressed as pK_i (-log K_i) in this report. Significance of the data was evaluated using Student's *t*-test.

RESULTS

The specific binding of [³H]-ketanserin to rabbit platelet membranes occurred at a saturable single site in the range from 0.12 to 10.0 nM. Specific binding represented 40–60% of the total radioactivity bound to the platelet membranes. The B_{max} value and K_d value were 806.72 ± 82.41 fmol/mg protein and 4.64 ± 1.18 nM, respectively (Table 1).

The pK_i values of AT-1015 and other 5-HT₂ antagonists to 5-HT₂ receptors in rabbit platelet are shown in the Table 2. The pK_i value of ketanserin was higher than those of other 5-HT₂ antagonists. The pK_i value of the newly synthesized 5-HT₂ antagonist, AT-1015 also showed a high value (7.40). The pK_i values of ritanserin, sarpogrelate and cyproheptadine were 7.25, 7.20 and 6.20 respectively.

DISCUSSION

Our investigation showed that the specific binding of [³H]-ketanserin to rabbit platelet membranes was saturable in the range from 0.12 to 10.0 nM and the specific binding was 40–60% of the total radioactivity bound to the platelet membrane. These results were similar to those reported by Tsuchihashi *et al.*¹¹⁾ in rabbit platelet and Leysen *et al.*¹⁴⁾ in cat platelet. These authors reported that only 40% binding of the total [³H]-ketanserin binding represented true 5-HT₂ receptor binding and the other 60% of the binding was at an

Table 1. K_d and B_{max} Values of 5-HT₂ Receptors in Rabbit Platelet Membrane as Assayed by Scatchard Analysis

	K_d (nM)	B_{max} (fmol/mg protein)
5-HT ₂ receptors	$4.64 \pm 1.18(3)$	$806.72 \pm 82.41(3)$

Values are mean $\pm$ S.E. Numbers in parenthesis indicate the number of experiments.

Table 2. pK_i Values of Various 5-HT₂ Antagonists in Rabbit Platelets

5-HT ₂ antagonists	pK_i values
Ketanserin	$8.63 \pm 0.18(5)**$
AT-1015	$7.40 \pm 0.40(5)$
Ritanserin	$7.25 \pm 0.24(4)$
Sarpogrelate	$7.20 \pm 0.65(5)$
Cyproheptadine	$6.20 \pm 0.22(3)*$

Values are mean $\pm$ S.E. Numbers in parenthesis indicate the number of experiments. The concentration of [³H]-ketanserin used was 1.0 nM. Significant difference between AT-1015 and other antagonists: * $p < 0.05$, ** $p < 0.01$.

other binding site when the displacement experiments were carried out using 5-HT₂ antagonists, and nonspecific binding was determined in the presence of ketanserin. They also reported that this 60% of [³H]-ketanserin binding was inhibited by high concentrations of ketanserin between 10 and 100 nM. The concentration of serotonin (5-HT) for determination of non-specific binding was therefore set at 0.1 mM and the specific binding was determined by subtracting the non-specific binding from the total binding.

The present study examined the displacement potencies of 5-HT₂ antagonists for [³H]-ketanserin binding to 5-HT₂ receptor in rabbit platelet membrane. Ketanserin had the highest affinity for the 5-HT₂ receptor site in the rabbit platelet membrane among the 5-HT₂ antagonists used in the present study. AT-1015, a newly synthesized 5-HT₂ antagonist, also had high affinity for this binding site although its affinity was slightly lower than ketanserin. Sarpogrelate and ritanserin also had high affinity, but lower than ketanserin. In contrast, the displacement potency of cyproheptadine was lower for this binding site. Previously, Tsuchihashi *et al.*¹¹⁾ and Maruyama *et al.*¹⁵⁾ also reported that sarpogrelate had high affinity for the 5-HT₂ receptor site in both rabbit platelet and rat frontal lobe, while the value for cyproheptadine was significantly lower than those in the rat frontal lobe.

Kihara *et al.*⁹⁾ reported that AT-1015, a novel 5-HT₂ receptor antagonist, selectively inhibited *in vitro* 5-HT_{2A} receptor-mediated platelet aggregation and effectively prevented progression of peripheral vascular lesions in rats. Our previous paper¹⁰⁾ showed that AT-1015 was a strong noncompetitive 5-HT₂ antagonist compared with ketanserin and inhibited the contraction response of porcine coronary arteries mediated by 5-HT and α -methylserotonin. The present study also confirmed the high affinity of AT-1015 in the platelet membrane. Thus, we predict that the high affinity of AT-1015 in rabbit platelet may play an important role in its biological effects *i.e.* its inhibitory effect on platelet aggregation⁹⁾ and inhibition of contraction response in porcine coronary artery¹⁰⁾ and this agent may be expected to be useful for the therapeutic treatment of peripheral, cerebral or coronary ischemia caused by the aggregation of platelets mediated by 5-HT₂ receptor activation.

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