Antagonistic Activity of Y-25130 on 5-HT₃ Receptors

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ABSTRACT—This paper describes the 5-hydroxytryptamine₃ (5-HT₃) receptor antagonism of Y-25130 ((±)-N-(1-azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-8-carboxamide monohydrochloride) in the rat cerebral cortex, isolated rabbit heart and isolated guinea pig ileum. In an in vitro binding assay, Y-25130 inhibited the specific binding of [³H]quipazine to 5-HT₃ receptors at the synaptic membranes of the rat cerebral cortex with a K_i value of 2.9 nM, the same as that of ondansetron. Metoclopramide, 5-HT and 2-methyl-5-HT also showed an inhibitory effect, but their affinities for 5-HT₃ receptors were lower than that of Y-25130. Y-25130 showed low affinity for histamine H₁ receptors (IC₅₀ = 4.4 μM) but it could not reveal any affinities for the other receptors (5-HT_{1A}, 5-HT₂, dopamine D₁, dopamine D₂, α₁-adrenoceptor, α₂-adrenoceptor, muscarine and benzo-diazepine) even at a 10 μM concentration. In the isolated rabbit heart, Y-25130 antagonized the indirect sympathomimetic responses to 5-HT (pA₂ value = 10.06) and this effect was more potent than that of metoclopramide. In the isolated longitudinal smooth muscle of the guinea pig ileum, concentration-contraction effect curves for 5-HT were biphasic in the presence of ketanserin. Y-25130 shifted to the right only in the second phase of concentration-effect curves for 5-HT (pA₂ value = 7.04) and its activity was more potent than that of metoclopramide. These results indicate that Y-25130 is a potent and selective 5-HT₃ receptor antagonist.

Keywords: Y-25130, 5-HT₃ receptor, Heart (isolated), Ileum (isolated)

Gaddum and Picarelli (1) reported that 5-hydroxytryptamine (5-HT) induces contractions in the guinea pig ileum in vitro by activating two distinct 5-HT receptor subtypes: the neuronal 'M' receptor and the 'D' receptor located on the longitudinal smooth muscle, subsequently referred to as 5-HT₃ and 5-HT₂ receptors, respectively (2).

Selective 5-HT₃ receptor antagonists such as ondansetron (3) and granisetron (4) have been developed and are now used clinically for the prevention of chemotherapy-induced nausea and vomiting.

Y-25130 ((±)-N-(1-azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-8-carboxamide monohydrochloride) is now being developed as an antiemetic drug for the prevention of emesis induced by anticancer therapy. According to Fukuda et al. (5), Y-25130 was effective against emesis induced in dogs and ferrets by cytotoxic drugs or by total body irradiation. It has been subsequently reported that Y-25130 is a potent inhibitor of the von Bezold-Jarisch effect induced by 5-HT or 2-methyl-5-HT in rats (6). The von Bezold-Jarisch effect is thought to be induced by activation of

5-HT₃ receptors on sensory vagal afferents (2). Recently, Richardson and Engel (7) have reported 5-HT₃ receptor antagonism in different bioassay systems.

The purpose of this study is to examine the antagonistic activities of Y-25130 on 5-HT₃ receptors in vitro. The interaction of Y-25130 with 5-HT₃ receptors has been investigated in rat cerebral cortex, which showed high affinity and saturation for 5-HT₃ receptors; in isolated rabbit heart, where stimulation of 5-HT₃ receptors results in noradrenaline release from the terminal sympathetic nerve fibers; and in isolated guinea pig ileum, where excitatory 5-HT₃ receptors mediate acetylcholine release from the intramural cholinergic nerves.

MATERIALS AND METHODS

Drugs used

Y-25130, ondansetron, 2-methyl-5-HT hydrobromide, ketanserin hydrochloride and ICS 205-930 were synthesized at the Yoshitomi Research Laboratories; and metoclopramide hydrochloride, 5-HT creatinine sulfate and atropine sulfate were obtained from Sigma (USA).

[³H]Quipazine (2520 GBq/mmol), [³H]ketanserin (2276 GBq/mmol), [³H]prazosin (3034 GBq/mmol) and [³H]QNB (33.1 GBq/mmol) were obtained from New England Nuclear (USA). [³H]8-OH-DPAT (7437 GBq/mmol), [³H]SCH23390 (2220 GBq/mmol), [³H]clonidine (24.0 GBq/mmol), [³H]mepyramine (1040 GBq/mmol) and [³H]diazepam (3070 GBq/mmol) were obtained from Amersham (England). Ketanserin was dissolved in 0.005% dimethyl sulfoxide. All other drugs were dissolved in distilled water.

Radioligand binding to 5-HT₃ receptors

Radioligand binding assays were performed according to the methods described by Peroutka and Hamik (8). Briefly, rat cerebral cortex was homogenized in 20 volumes of 0.32 M sucrose and centrifuged at 35000 × g for 15 min. The supernatant was discarded, and the pellet was resuspended in the same volume of Krebs-HEPES buffer solution (25 mM HEPES, 180 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂ and 1.2 mM MgCl₂, pH 7.4). After a 10 min incubation at 37°C, the tissue was recentrifuged to obtain a crude synaptosomal fraction. The final pellet was resuspended in 80 volumes of Krebs-HEPES buffer. Each test tube contained 50 µl of [³H]quipazine, 50 µl of a known concentration of test compounds and a 900-µl aliquot of crude synaptosomal membranes. Following a 30 min incubation at 30°C, the mixtures were rapidly filtered under vacuum through Whatman GF/B glass filters which had been pre-soaked in 0.1% polyethyleneimine. The filters were washed 3 times with 3 ml of ice cold 50 mM Tris-HCl buffer (pH 7.7). The radioactivity on the filters was measured by a liquid scintillation counter (Beckman LS 3801). Specific binding was defined as the difference between the total and nonspecific binding. Nonspecific binding was determined in the presence of 0.1 µM ICS 205-930. All experiments were performed in triplicate.

Radioligand binding to several neurotransmitter receptors

The binding studies of several neurotransmitter receptors (5-HT_{1A}, 5-HT₂, dopamine D₁, dopamine D₂, α₁-adrenoceptor, α₂-adrenoceptor, muscarine, histamine H₁ and benzodiazepine) were carried out according to the previously published methods (9–17).

Isolated rabbit heart

Male white rabbits (2.6–4.4 kg, Seiwa Experimental Animals, Ltd., Fukuoka, Japan) were injected with heparin (500 U/kg) into a marginal ear vein. From 2 min to 5 min later, they were sacrificed by a blow on the head. The hearts were rapidly removed and perfused with Tyrode solution at 35.5°C by the Langen-

dorff technique. The Tyrode solution (137.0 mM NaCl, 1.8 mM CaCl₂, 2.7 mM KCl, 1.05 mM MgCl₂, 11.9 mM NaHCO₃, 0.4 mM NaH₂PO₄, and 5.6 mM glucose) was gassed with a mixture of 95% O₂ and 5% CO₂. Perfusion pressure was maintained at approximately 60 cm water. Atropine (1.4 µM) was added routinely to the perfusate to prevent interference from indirect muscarinic activity. Right atrio-ventricular tension as well as ventricular rates were recorded as previously described by Fozard and Mwaluko (18).

Dose-response curves for 5-HT on ventricular rate were established using bolus injections in 1 sec by a microliter syringe. 5-HT was injected immediately before the perfusate entered into the heart. The volume of injected 5-HT was maintained at 100 µl.

Usually the interval between the doses was 5 min, but if the effects of a previous dose were still in evidence, this interval was extended. In the control experiments, three successive dose-response curves were established with a 15-min interval among them.

The effects of Y-25130 and metoclopramide were assessed by adding each compound to the perfusate in increasing concentrations prior to the subsequent curves and comparing the changes observed in the control group where no modifying drug was present.

To study the antagonism, the method described by Arunlakshana and Schild (19) for the determination of pA₂ and pD'₂ values was adopted using cardiac rate as the quantified response.

Isolated longitudinal guinea pig ileum

Male Hartley guinea pigs (470–760 g; Seiwa Experimental Animals, Ltd., Fukuoka, Japan) were sacrificed by a blow on the head. A length of the small intestine was taken about 2 cm from the ileo-caecal valve. The mesentery was carefully removed, and then the ileum was stretched over a glass rod. The longitudinal muscle layer was separated from the underlying circular muscle.

Longitudinal muscle strips, 3–4 cm in length, were mounted in a 5-ml organ bath containing Tyrode solution at 37°C and bubbled with a mixture of 95% O₂ and 5% CO₂. Ketanserin (1 µM) was added to the Tyrode solution to avoid the interference from 5-HT₂ receptors. The strips were placed under a resting tension of 500 mg. Contractions were recorded isometrically using an F-D pick up.

After equilibration for 30 min, non-cumulative concentration-effect curves for 5-HT were established by adding increasing concentrations of 5-HT to the organ bath at 15-min intervals. Preceding experiments showed that the intervals were long enough to avoid tachyphylaxis. Each concentration was kept in contact

with the tissue for 1 min. Each strip was used to record two concentration-effect curves: the first one for 5-HT alone and the second one for 5-HT in the presence of a set concentration of antagonists, each strip thus serving as its own control. Antagonists were allowed to pre-equilibrate for at least 10 min prior to the addition of 5-HT. The contractions were expressed as the percentage of the maximal response to 5-HT obtained from several preparations which were plotted as the mean values to obtain log-concentration-effect curves. Inhibition constants were expressed in the form of pA₂ values which were graphically determined according to conventional methods (19, 20).

RESULTS

Radioligand binding to 5-HT₃ receptors

Increasing concentrations of [³H]quipazine (0.2–5 nM) were incubated with the rat cortical membranes. Scatchard analysis showed that the radioligand displayed a high affinity ($K_d = 6.4$ nM) for the binding sites, and the density of these sites in the rat cortex (B_{max}) was 43.1 fmol/mg protein. Drug competitive studies demonstrated that Y-25130 displayed a high affinity for [³H]quipazine-labeled sites (K_i value = 2.9 nM). K_i values of ondansetron, metoclopramide, 5-HT and 2-methyl-5-HT were 3.5, 660, 200 and 800 nM, respectively (Fig. 1). None of the following compounds: 8-OH-DPAT (5-HT_{1A} receptor agonist), ketanserin (5-HT₂ receptor antagonist), spiperone (dopamine D₂ and 5-HT₂ receptor antagonist) and prazosin (α_1 -adrenoceptor antagonist) showed any affinity for [³H]quipazine-labeled sites at a concentration of 1 μ M.

Radioligand binding to several neurotransmitter receptors

The potency of Y-25130, ondansetron and metoclopramide to bind other neurotransmitter receptors was assayed in radioligand-binding models for 5-HT_{1A}, 5-HT₂, dopamine D₁, dopamine D₂, α_1 -adrenoceptor, α_2 -adrenoceptor, muscarinic, histamine H₁ and benzodiazepine receptor sites. Y-25130 showed low affinity for histamine H₁ receptors ($IC_{50} = 4.4$ μ M), but it revealed no affinities for the other receptors at a 10- μ M concentration. Ondansetron showed low affinities for α_1 -adrenoceptors (2.5 μ M) and muscarinic receptors (3.8 μ M). Metoclopramide showed the highest affinity for dopamine D₂ receptors (0.038 μ M). It showed relatively lower affinities for 5-HT_{1A} (3.0 μ M), 5-HT₂ (1.8 μ M) receptors and α_2 -adrenoceptors (2.0 μ M) (Table 1).

Fig. 1. Inhibition by a test compound of [³H]quipazine binding to rat cortical membranes. ○: Y-25130, ▲: ondansetron, ●: metoclopramide, □: 5-HT, ■: 2-methyl-5-HT. Results are expressed as means $\pm$ S.E. (bar) of 2–4 experiments, each performed in triplicate.

Table 1. Effect of Y-25130, ondansetron and metoclopramide on the binding of various radioligands to rat synaptosomal membranes

Receptor	Ligand	IC ₅₀ (μ M)		
		Y-25130	Ondansetron	Metoclopramide
5-HT _{1A}	[³ H]8-OH-DPAT	> 10	> 10	3.0
5-HT ₂	[³ H]Ketanserin	> 10	> 10	1.8
Dopamine D ₁	[³ H]SCH23390	> 10	> 10	> 10
Dopamine D ₂	[³ H]Spiperone	> 10	> 10	0.038
α_1 -Adrenoceptor	[³ H]Prazosin	> 10	2.5	> 10
α_2 -Adrenoceptor	[³ H]Clonidine	> 10	> 10	2.0
Muscarine	[³ H]QNB	> 10	3.8	> 10
Histamine H ₁	[³ H]Mepyramine	4.4	> 10	> 10
Benzodiazepine	[³ H]Diazepam	> 10	> 10	> 10

IC₅₀ values were derived from 1–2 determination, each involving 3 to 8 concentrations of each agent evaluated in triplicate.

Isolated rabbit heart

Bolus injections of 5-HT (5–320 nmol) produced a dose-related increase both in the force of contraction and the heart rate. The same procedure was repeated 3 times at an interval of 15 min until the same response was observed (Fig. 2).

The results of adding Y-25130 and metoclopramide to the perfusion fluid 15 min prior to establishing the dose-response curves for 5-HT are shown in Fig. 3A.

Y-25130 (30–300 pM) produced parallel shifts to the right of the dose-response curves in the case of 5-HT and it suppressed the maximum attainable response to 5-HT. The pA_2 value (with 95% confidence limits) was 10.06 (9.76–10.35). Schild plots gave linear regression; the slope of the regression was not significantly different from unity and was 0.69 (0.19–1.19). The pD'_2 value was 9.45 (9.08–9.82).

Metoclopramide (0.1–1 μ M) also produced parallel shifts to the right of the dose-response curves for 5-HT, but it could not depress the maximum attainable response to 5-HT (Fig. 3B). Its pA_2 value was 7.20 (7.03–7.37). Schild plots gave linear regression; the slope of the regression was not significantly different from unity and was 1.01 (0.79–1.22).

Isolated longitudinal guinea pig ileum

5-HT (0.001–30 μ M) caused concentration-related contractions in the presence of ketanserin in the isolated longitudinal muscle strip from guinea pig ileum (Fig. 4). The concentration-effect curves for 5-HT were biphasic, consisting of a first phase occurring between concentrations of 0.001 μ M and 0.3 μ M and a second phase between 1 μ M and 30 μ M.

In the presence of Y-25130 (0.1–10 μ M), the responses to 5-HT in the first phase of the curves were not affected. However, the second phase of the concentration-effect curves was shifted to the right in a concentration-dependent manner (Fig. 5A). The pA_2 value was 7.04 (6.76–7.32). Schild plots gave linear regression; the slope of the regression was not significantly different from unity and was 0.81 (0.64–0.97).

Metoclopramide (3–30 μ M) slightly inhibited the responses to 5-HT in the first phase of the curves and it shifted to the right in the second phase in a concentration-dependent manner (Fig. 5B). The pA_2 value in the second phase was 5.69 (5.31–6.07). Schild plots gave linear regression; the slope of the regression was not significantly different from unity and was 1.33 (0.69–1.98).

Fig. 2. Effect of bolus injections of 5-HT in isolated rabbit heart. The upper trace is the right ventricular tension (g = gram); the middle one is the right atrial tension; and the lower one is the ventricular rate (b/min = beats per minute).

Fig. 3. Effect of Y-25130 and metoclopramide on the changes in ventricular rate of isolated rabbit hearts evoked by bolus injections of 5-HT. (A) Dose-response curves for 5-HT in the absence of antagonist ($\circ$, $n = 12$) and in the presence of Y-25130: 30 pM ($\bullet$, $n = 4$), 100 pM ($\square$, $n = 4$) and 300 pM ($\blacksquare$, $n = 4$). (B) Dose-response curves for 5-HT in the absence of antagonist ($\circ$, $n = 12$) and in the presence of metoclopramide: 0.1 μ M ($\bullet$, $n = 4$), 0.3 μ M ($\square$, $n = 4$) and 1 μ M ($\blacksquare$, $n = 4$). Only one concentration of antagonists was tested per heart; and since the control curves did not differ significantly from each other, they were pooled. Results are expressed as means $\pm$ S.E. (bar).

Fig. 4. Original recording of the concentration-related contractile responses (displacement) to 5-HT in the isolated longitudinal guinea pig ileum.

Fig. 5. Effect of Y-25130 and metoclopramide on the contractile responses to 5-HT in the presence of ketanserin in isolated longitudinal guinea pig ileum. (A) Concentration-effect curves for 5-HT in the absence of antagonist ($\circ$, $n = 30$) and in the presence of Y-25130: 0.1 μ M ($\bullet$, $n = 10$), 1 μ M ($\square$, $n = 10$) and 10 μ M ($\blacksquare$, $n = 10$). (B) Concentration-effect curves for 5-HT in the absence of antagonist ($\circ$, $n = 30$) and in the presence of metoclopramide: 3 μ M ($\bullet$, $n = 10$), 10 μ M ($\square$, $n = 10$) and 30 μ M ($\blacksquare$, $n = 10$). Only one concentration of antagonists was tested in each preparation; and since the control curves did not differ significantly from each other, they were pooled. Results are expressed as means $\pm$ S.E. (bar).

DISCUSSION

Peroutka and Hamik (8) have reported that [³H]quipazine can label 5-HT₃ recognition sites in the rat cortical membranes. In our experiments, it was shown that 5-HT and 2-methyl-5-HT, which is a specific 5-HT₃ receptor agonist, were bound to the [³H]-quipazine-labeled sites; and none of the following ligands bound to those sites: 5-HT_{1A}, 5-HT₂, dopamine D₂ receptor and α_1 -adrenoceptor ligands. Y-25130 showed a high affinity for the [³H]quipazine-labeled sites, with a K_i value of 2.9 nM. The affinity of Y-25130 towards histamine H₁ receptors was about 1500 times less than that to 5-HT₃ receptors. Y-25130 failed to show specific affinities in vitro for the other receptors (5-HT_{1A}, 5-HT₂, dopamine D₁, dopamine D₂, α_1 -adrenoceptor, α_2 -adrenoceptor, muscarine and benzodiazepine) even at a concentration of 10 μ M. Ondansetron showed an affinity for 5-HT₃ receptors (K_i value = 3.5 nM), which was almost the same as that of Y-25130, and some affinities for α_1 -adrenoceptors and muscarinic receptors. Metoclopramide also showed an affinity for 5-HT₃ receptors (K_i value = 660 nM), but it was about 230 times lower than that of Y-25130. Similarly, metoclopramide revealed affinities for several other neurotransmitters, especially dopamine D₂ receptors in which case the affinity was more potent than that for 5-HT₃ receptors. These results indicate that Y-25130 has a potent and selective affinity for 5-HT₃ receptors, like ondansetron, and a superior potency and selectivity to metoclopramide.

Richardson and Engel (7) have reported that the 5-HT₃ receptors were present at the cardiac parasympathetic and sympathetic nerve endings. In the isolated rabbit heart, 5-HT stimulates transmitter release from the terminals of the sympathetic nerve fibers via activation of specific receptor sites (21). Y-25130 inhibited the indirect sympathomimetic responses to 5-HT in the rabbit heart. Its pA_2 and pD'_2 values were 10.06 and 9.45, respectively, while the pA_2 value for metoclopramide was 7.20. These results indicate that the antagonistic activity of Y-25130 is about 720 times more potent than that of metoclopramide in the isolated rabbit heart.

In the isolated guinea pig ileum, concentration-effect curves for 5-HT are biphasic even in the presence of methysergide (22) and according to Butler et al. (23), it is the second phase (higher concentrations) that is sensitive to the effects of 5-HT₃ receptor antagonists. Only the second phase of the concentration-effect curves for 5-HT was shifted to the right by Y-25130. The pA_2 value was 7.04, while that of metoclopramide was 5.69. The antagonistic activity of Y-25130 was

about 20 times more potent than that of metoclopramide in the isolated guinea pig ileum. The first phase which was suppressed by metoclopramide might be induced by other 5-HT subtype agonists. However, detailed studies are necessary to clarify this point.

Comparative antagonistic effects of Y-25130 obtained in the isolated rabbit heart ($pA_2 = 10.06$) and in the isolated guinea pig ileum ($pA_2 = 7.04$) revealed marked differences. There was some indication that there may be species variants of the 5-HT₃ receptors (24). Also, the relative potency of Y-25130 to metoclopramide obtained in the isolated heart was markedly different from that of the isolated ileum. This may be related to the evidence that metoclopramide is not a pure 5-HT₃ receptor antagonist. However, these observations need to be fully investigated before reaching firm conclusions.

It has been previously reported that Y-25130 is effective against emesis induced by cytotoxic drugs or by total body irradiation (5) and is a potent inhibitor of the von Bezold-Jarisch effect induced by 5-HT (6). Moreover, Y-25130 inhibited the response of 5-HT to frog sensory neuron (T. Yakushiji et al., personal communication). In addition to the above findings, this report has demonstrated that Y-25130 is a potent and selective 5-HT₃ receptor antagonist.

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