High Affinity Binding of Azasetron Hydrochloride to 5-Hydroxytryptamine_3 Receptors in the Small Intestine of Rats

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ABSTRACT—The binding affinity of azasetron hydrochloride (azasetron) for the 5-hydroxytryptamine_3 (5-HT_3) receptor in a tissue preparation of rat small intestine was investigated by using [3H]granisetron as a radioligand. Scatchard analysis of specific [3H]granisetron binding revealed a single population of saturable binding sites in the tissue preparation. At this site, azasetron was concentration-dependently competitive with [3H]granisetron, and it inhibited the specific [3H]granisetron binding with a K_i value of 0.33 nM. Azasetron has a high affinity for 5-HT_3 receptor in the gastrointestinal organ, the very site of its antiemetic action against chemotherapy-induced emesis.

Keywords: Azasetron, 5-HT_3 receptor, Small intestine

Nausea and vomiting are severe side effects of cancer chemotherapeutic agents such as cisplatin and cyclophosphamide. The antagonists against 5-HT_3 receptors effectively inhibit the chemotherapy-induced emesis in humans (1) and ferrets (2). Cisplatin-induced emesis can also be abolished by a combination of vagotomy and greater splanchnicectomy in dogs (3), and the treatment with cisplatin increases the level of 5-HT in dog ileum (4). These observations suggest that 5-HT released from enterochromaffin cells binds to 5-HT_3 receptors located on the end of the afferent vagus and the greater splanchnic nerve, and the 5-HT initiates sensory signals to finally stimulate the vomiting center (1, 5). Therefore, 5-HT_3 receptors existing in the gastrointestinal organ is widely considered as the site of action of 5-HT_3-receptor antagonists in preventing the chemotherapy-induced emesis.

Azasetron, a potent and selective 5-HT_3-receptor antagonist (6–8), inhibits the chemotherapy-induced nausea and vomiting in animals (9, 10) and humans (11). The affinity of azasetron for the 5-HT_3 receptor examined in the cerebral cortex of rats indicated that the agent binds to the receptor competitively with [3H]granisetron with a K_i value of 0.54 nM (12). However, the gastrointestinal organ would be the very site of action of 5-HT_3-receptor antagonists, and the affinity should be evaluated in such an organ. Herein we establish an assay system that uses the gastrointestinal organ, and we describe the affinity of azasetron and other antiemetics for the 5-HT_3 receptor.

Male Wistar rats (222–357 g; Charles River Japan, Yokohama) were sacrificed by a blow on the head and bled. From each rat, the small intestine was separated from the end of the duodenum to the ileocaecal junction and cut in segments of approximately 10 cm in length. Each segment was placed over a glass rod, and the longitudinal muscle coat with attached myenteric plexus (LMMP) was separated by blunt dissection from the serosal surface. The LMMP was stored at −80°C until used. For the binding study, the LMMP was finely chopped with scalpel blades and then placed in 25 vol. of 50 mM ice-cold HEPES buffer (pH 7.4). The minced LMMP was homogenized in a Polytron PT-10 (dial 7, 15 sec ×2), and the suspension was filtered through three layers of nylon mesh (pore size of 250 µm). After the filtration, the suspension was finally diluted to the appropriate protein concentration for assay (1.2 mg protein per assay tube) with HEPES buffer and used as the tissue preparation. The protein concentration was measured with the Bio-Rad protein assay system (Nippon Bio-Rad Labs. KK, Tokyo) (13) with bovine serum albumin as a standard.

Azasetron [(±)-N-(1-azabicyclo[2.2.2.]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-8-carboxamide mono-hydrochloride], granisetron hydrochloride (granisetron), ondansetron hydrochloride (ondansetron), tropisetron and zacopride hydrochloride (zacopride) were synthesized at the Research Laboratories of Yoshitomi Pharmaceutical Industries, Ltd. Cisapride...
was obtained from Janssen (Beerse, Belgium). 5-Hydroxytryptamine creatinine sulphate (5-HT) was purchased from E. Merck (Darmstadt, Germany). Metoclopramide hydrochloride (metoclopramide), 5-methoxytryptamine hydrochloride (5-methoxytryptamine) and ketanserin were purchased from Sigma Chemicals (St. Louis, MO, USA). 2-Methyl-5-HT maleate (2-methyl-5-HT) was purchased from Research Biochemicals, Inc. (Natic, MA, USA). Methysergide was a gift from Sandoz (Basle, Switzerland). Cisapride was dissolved in 4% lactic acid solution. Tropisetron and ketanserin were initially dissolved in dimethyl sulfoxide and diluted with distilled water. The other drugs were dissolved in distilled water.

For the saturation analysis, the tissue preparation was incubated with \[^{3}H\]granisetron (83–85 Ci/mmol, New England Nuclear, Boston, MA, USA) ranging from 0.1 to 6 nM. \[^{3}H\]Granisetron at the concentration of 2 nM was used for the competition study. \[^{3}H\]Granisetron at concentrations of 0.05 to 3 nM was used to evaluate the manner of interaction between azasetron and the 5-HT\(_3\) receptor. Assay tube contained 50 \(\mu\)l of HEPES buffer or a solution of the test agents, 50 \(\mu\)l of \[^{3}H\]granisetron, and 900 \(\mu\)l of tissue preparation (1.2 mg protein). The total volume in each assay tube was 1 ml. Each tube was incubated for 60 min at 0°C, and the reaction was terminated by rapid filtration through a Whatman GF/B filter (pre-soaked in 0.01% v/v polyethyleneimine). The filter was washed 4 times with 3 ml of ice-cold HEPES buffer. Then the filter was placed in 3 ml of scintillator, and the radioactivity was determined by scintillation counting in a Beckman model LS3801 scintillation counter (Fullerton, CA, USA). Specific binding was defined in the presence of tropisetron (2 \(\mu\)M). To evaluate 5-HT as a competing agent, pargyline (1 mM) was added to the assay system to prevent the degradation of 5-HT by monoamine oxidase.

Unless otherwise stated, each result is expressed as the mean±S.E.M. The IC\(_{50}\) value and Hill coefficient were determined by non-linear regression of the displacement curve, and the K\(_d\) value was calculated according to the formula (K\(_d = IC_{50} / (1 + L / K_d)\)), where L is the concentration of radioligand and K\(_d\) is the dissociation constant of the radioligand (14). Significant difference of Hill coefficient against unity was assessed using a program employing the partial F-test.

Endogeneous 5-HT was not detected in the tissue preparation (measured by using HPLC), and no divalent cation that has influence upon the assay system was contained in the tissue preparation because EDTA (up to 2 mM) did not affect the radioligand binding. The specific radioligand binding reached the maximum level when the pH of the assay buffer was 7.4, and the binding was linear over 0.4–1.4 mg protein of the tissue preparation per assay tube (data not shown). The level of the specific binding was up to 1,000 dpm, and this was about 60% of the total binding at the used protein concentration. In this assay system, methysergide, ketanserin and 5-methoxytryptamine hardly inhibited specific \[^{3}H\]granisetron binding at concentrations up to 10 \(\mu\)M.

Scatchard analysis revealed that \[^{3}H\]granisetron binds to a single and saturable site in the tissue preparation of the small intestine of rats with the K\(_d\) value of 0.37±0.04 nM and B\(_{max}\) value of 7.57±0.43 fmol/mg protein (Fig. 1: A and B). Here the Hill coefficient was 0.95±0.07.

The affinity of azasetron was compared with that of other antiemetics including a 5-HT\(_3\)-receptor antagonist and that of some drugs structurally related to 5-HT

\[ \text{Fig. 1.} \quad \text{[^{3}H]Granisetron binding as a function of increasing concentrations of [^{3}H]granisetron.} \quad \text{A: Saturation analysis of [^{3}H]granisetron (0.1–6 nM) binding, where \(\circ\) is the total binding, \(\bullet\) is the specific binding and \(\bigtriangleup\) is the non-specific binding determined by the addition of 2 \(\mu\)M tropisetron.} \quad \text{B: Scatchard plot of specific [^{3}H]granisetron (0.1–6 nM) binding.} \quad \text{The data were analyzed by linear regression (\(r\) means correlation coefficient). Results are from a typical experiment representing four experiments.} \]
Azasetron inhibited the specific $[^3H]$granisetron binding with the $K_i$ value of 0.33 nM, which was 230, 670, 480 and 3300 times that of metoclopramide, cisapride, 5-HT and 2-methyl-5-HT, respectively. Zacopride, tropisetron, granisetron and ondansetron also showed high affinity to the 5-HT$_3$ receptor, and the $K_i$ values were similar with that of azasetron. The rank order of affinities of agonists and antagonists are similar to that found in the cerebral cortex of rats (12, 15).

In conclusion, azasetron was confirmed to show a high affinity to the 5-HT$_3$ receptor at the very site of its antiemetic action. The current study should offer a probable explanation for the potent antiemetic effect of azasetron.

### Table 1. Binding affinities of 5-HT$_3$-receptor ligands in small intestine of rats

| Drugs        | $K_i$ value (nM) | (95% confidence limits) | Hill coefficient |
|--------------|------------------|-------------------------|------------------|
| Azasetron    | 0.33             | (0.27–0.40)             | 0.80±0.06**      |
| Zacopride    | 0.20             | (0.16–0.26)             | 0.97±0.10        |
| Tropisetron  | 0.75             | (0.62–0.90)             | 0.97±0.07        |
| Granisetron  | 0.84             | (0.67–1.1)              | 1.06±0.10        |
| Ondansetron  | 1.3              | (0.92–1.7)              | 0.85±0.10        |
| Metoclopramide| 76               | (55–110)                | 1.14±0.18        |
| 5-HT         | 160              | (130–180)               | 1.02±0.06        |
| Cisapride    | 220              | (160–300)               | 1.36±0.18**      |
| 2-Methyl-5-HT| 1100             | (860–1400)              | 0.83±0.07*       |

$K_i$ values are the means of three experiments, and 95% confidence limits are represented in parenthesis. Hill coefficients are the means±S.E. of three experiments. **P<0.01, *P<0.05, significantly different from unity (partial F-test).

### Table 2. Effects of azasetron on kinetic parameters of $[^3H]$granisetron

| Treatments | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) | Hill coefficient |
|------------|------------|-----------------------------|------------------|
| Control    | 0.35±0.04  | 5.64±0.19                   | 0.98±0.05        |
| Azasetron  | 0.78±0.05  | 7.45±0.44                   | 1.01±0.01        |
| 0.3 nM     | 1.62±0.30**| 7.75±0.79                   | 0.99±0.01        |
| 1 nM       |            |                             |                  |

Values are the means±S.E. of three experiments. **P<0.01, significantly different from the control (Dunnett).

(Tables I). Azasetron binding with the $K_i$ value of 0.33 nM, which was 230, 670, 480 and 3300 times that of metoclopramide, cisapride, 5-HT and 2-methyl-5-HT, respectively. Zacopride, tropisetron, granisetron and ondansetron also showed high affinity to the 5-HT$_3$ receptor, and the $K_i$ values were similar with that of azasetron. The rank order of affinities of agonists and antagonists are similar to that found in the cerebral cortex of rats (12, 15).

Haga et al. reported that antiemetic potency of azasetron against cisplatin- and doxorubicin/cyclophosphamide-induced emesis was similar to those of granisetron and ondansetron, but superior to that of metoclopramide (10). The antiemetic potency of these compounds well-correlated with the affinity to the 5-HT$_3$ receptor determined here.

The Hill coefficient of azasetron (0.80) was significantly different from unity. In order to determine the manner of interaction between azasetron and the 5-HT$_3$ receptor, we performed Scatchard analysis of the specific $[^3H]$granisetron binding in the presence of azasetron. Azasetron concentration-dependently increased the $K_d$ value of $[^3H]$granisetron without any influence upon the $B_{max}$ value and the Hill coefficient of the radioligand, and the Hill coefficient approximated unity (Table 2). This result indicates that azasetron is competitive with $[^3H]$granisetron in the binding to the receptor, and the binding behavior indicates neither negative cooperation nor receptor heterogeneity. Further investigation will be necessary to clarify the reason why azasetron possesses a low Hill coefficient.

REFERENCES

1. Cubeddu LX, Hoffmann IS, Fuenmayor NT and Finn AL: Efficacy of ondansetron (GR38032F) and the role of serotonin in cisplatin induced nausea and vomiting. N Engl J Med 322, 810–816 (1990)
2. Costall B, Domeney AM, Naylor RJ and Tattersall FD: Emesis induced by cisplatin in the ferret as a model for the detection of anti-emetic drugs. Neuropharmacology 26, 1321–1326 (1987)
3. Fukui H, Yamamoto M and Sato S: Vagal afferent fibers and peripheral 5-HT$_3$ receptors mediate cisplatin-induced emesis in dogs. Jpn J Pharmacol 59, 221–226 (1992)
4. Fukui H, Yamamoto M, Ando T, Sakaki S and Sato S: Increase in serotonin levels in the dog ileum and blood by cisplatin as measured by microdialysis. Neuropharmacology 32, 959–968 (1993)
5. Andrews PLR, Rapeport WG and Sanger GJ: Neuropharmacology of emesis induced by anti-cancer therapy. Trends Pharmacol Sci 9, 334–341 (1988)
6. Inaba K, Morimoto Y, Fukuda T and Setoguchi M: Inhibition by Y-25130 of the von Bezold-Jarisch effect evoked by 5-HT- or 2-methyl-5-HT in anesthetized rats. Folia Pharmacol Jpn 98, 293–299 (1991) (Abstr in English)
7. Sato N, Sakamori M, Haga K, Takehara S and Setoguchi M: Antagonistic activity of Y-25130 on 5-HT$_3$ receptors. Jpn J Pharmacol 59, 443–448 (1992)
8. Yakushiji T and Akaike N: Blockade of 5-HT$_3$ receptor-mediated currents in dissociated frog sensory neurones by benzoxazine derivative, Y-25130. Br J Pharmacol 107, 853–857 (1992)
9. Fukuda T, Setoguchi M, Inaba K, Shoji H and Tahara T: The antiemetic profile of Y-25130, a new selective 5-HT$_3$ receptor antagonist. Jpn J Pharmacol 106, 309–315 (1991)
10. Haga K, Inaba K, Shoji H, Morimoto Y, Fukuda T and Setoguchi M: The effects of orally administered Y-25130, a selective serotonin$_3$-receptor antagonist, on chemotherapeutic agent-induced emesis. Jpn J Pharmacol 63, 377–383 (1993)
11. Niitani H, Ota K, Taguchi T, Takeuchi S, Tsukagoshi S, Furue H, Furuse K, Machida T, Waku A, Sakuma A, Sakurai Y and Tomina T: Clinical evaluation of Y-25130 against nausea and vomiting induced by anticancer drugs: Multi-centered, placebo-controlled, double-blind comparative study. Jpn Pharmacol Ther 20, 2525–2542 (1992)
12. Sakamori M, Takehara S and Setoguchi M: High affinity bind-
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ing of Y-25130 for serotonin 3 receptor. Folia Pharmacol Jpn 100, 137–142 (1992) (Abstr in English)

13 Bradford M: A rapid and sensitive method for quantitation of microgram quantities of proteins utilizing the principles of protein-dye binding. Anal Biochem 72, 248–254 (1976)

14 Cheng Y and Prusoff WH: Relationship between the inhibition constant (Kᵢ) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. Biochem Pharmacol 22, 3099–3108 (1973)

15 Nelson DR and Thomas DR: [³H]-BRL 43694 (Granisetron), a specific ligand for 5-HT₃ binding sites in rat brain cortical membranes. Biochem Pharmacol 38, 1693–1695 (1989)