Comparative Study of [^{3}H]Ramosetron and [^{3}H]Granisetron Binding in the Cloned Human 5-Hydroxytryptamine$_3$ Receptors

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ABSTRACT—Characteristics of the binding of [^{3}H]ramosetron to cloned human 5-hydroxytryptamine$_3$ (5-HT$_3$) receptors were investigated and directly compared to those of [^{3}H]granisetron binding. Saturation studies revealed that [^{3}H]ramosetron labeled more sites with high affinity (K_d=0.15±0.01 nM, B_{max} =653±30 fmol/mg protein) than [^{3}H]granisetron (K_d=1.17±0.25 nM, B_{max}=427±43 fmol/mg protein). Kinetic studies revealed that dissociation of [^{3}H]ramosetron was slower than that of [^{3}H]granisetron. These results suggest that ramosetron is a highly potent 5-HT$_3$-receptor antagonist.

Keywords: Ramosetron, Granisetron, 5-HT$_3$ receptor

The 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor is a ligand-gated ion channel that mediates fast synaptic transmission in the peripheral and central nervous systems (1). The receptor is thought to exist as a pentamer of identical or homologous subunits (2). A single subunit of the 5-HT$_3$ receptor was initially cloned from the NCB20 neuroblastoma cell line (3). Later a cDNA encoding the human 5-HT$_3$ receptor subunit from hippocampus was isolated by homology screening and characterized by expression in COS-1 cells and Xenopus oocytes (4). Subsequently, many 5-HT$_3$-receptor-selective antagonists, some of which are now used clinically, were used to study this receptor (5). In addition, several radiolabeled ligands such as [^{3}H]GR65630, [^{3}H]granisetron and [^{3}H]zacopride have been used to characterize the 5-HT$_3$ receptor (6). Recently, structurally different radioligands such as [^{3}H]-quipazine, [^{3}H]granisetron and [^{3}H]GR65630 have been shown to recognize different sites on the 5-HT$_3$ receptor in rats or in rabbits (7). Moreover, [^{3}H]BRL46470 labeled twice the number of sites compared with [^{3}H]granisetron. However, these differences have not been apparent in the human 5-HT$_3$ receptor.

Ramosetron is a potent and selective 5-HT$_3$-receptor antagonist (8). Ramosetron prevents the chemotherapy-induced nausea and vomiting in ferrets (9) and humans (10). In ferrets, the duration of antiemesis due to ramosetron was longer than that of granisetron (9). However, there is no direct evidence that explains the action of ramosetron's increased duration. [^{3}H]Ramosetron (11, 12) and [^{3}H]granisetron (13) both recognize 5-HT$_3$ receptors with high affinity. Therefore, in the present study, the binding characteristics of [^{3}H]ramosetron were investigated and directly compared to those of [^{3}H]-granisetron using cloned human 5-HT$_3$ receptors.

The isolation of the human 5-HT$_3$ receptor cDNA and its nucleotide sequence were previously reported (5). The cDNA fragment was subcloned into the mammalian expression vector pEF-BOS. COS-1 cells were transfected with plasmid using the DEAE-dextran/chloroquine method. COS-1 cells ($1-2\times10^6$ cells) were incubated overnight, exposed to the plasmid DNA (15 μg) with DEAE-dextran (0.25 mg/ml) for 14 hr, and further exposed to 0.1 mM chloroquine for 2.5 hr. After 3 days culture, the transfected cells were homogenized in 50 mM HEPES, pH 7.4, and centrifuged at 48,000×g for 10 min. The membrane homogenates were stored at −80°C until required for the radioligand binding assay.

[^{3}H]Ramosetron (78 Ci/mmol) was specially synthesized by Amersham International (Buckinghamshire, England). [^{3}H]Granisetron (85 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Ondansetron hydrochloride (ondansetron) was synthesized at Yamanouchi Pharmaceutical Co., Ltd. (Tsukuba).

Saturation studies were performed using six concentrations of [^{3}H]ramosetron (0.02-2 nM) or [^{3}H]granisetron (0.2-6 nM). A mixture consisting of 0.05 ml of radioligands, 0.35 ml of buffer or ondansetron (1 μM, to define nonspecific binding), and 0.1 ml of membrane

homogenates was used. All assays were carried out in incubation buffer consisting of 50 mM HEPES buffer (pH 7.4). The mixture was incubated at 25°C for 60 min. For association studies, the binding reaction was initiated by addition of radioligands. For dissociation studies, membranes were incubated with radioligand for 60 min, and then dissociation was initiated by addition of ondansetron (1 μM). The incubation was terminated by rapid filtration through Whatman GF/B filters using a Brandel cell harvester (Brandel, Gaithersburg, MD, USA), followed by washing the filter three times with 3 ml of ice-cold HEPES buffer. Radioactivity retained on the filters was counted with a liquid scintillation counter (2500CA; Packard, Tokyo). The protein content of each membrane suspension was measured with protein assay dye reagent (Bio-Rad, Tokyo).

Each result is expressed as the mean±S.E.M. Statistical significance was determined by Dunnett's two-tailed test. The maximal binding sites (B_{max}) of radioligand and the equilibrium constant (K_D) were derived directly from saturation curves fitted with a one site ligand binding model. The observed association rate constant (K_{obs}) and dissociation rate constant (K_{-1}) were determined using nonlinear curve-fitting programs in the Prism software package (GraphPad Software, San Diego, CA, USA). The association rate constant was calculated by: $K_{+1} = (K_{obs} - K_{-1}) / [L]$. The kinetic dissociation constant K_D was calculated by: $K_D = K_{-1} / K_{+1}$.

On membranes prepared from COS-1 cells transiently expressing human 5-HT_3 receptors, [^{3}H]ramosetron and [^{3}H]granisetron exhibited specific, high-affinity binding to an apparently homogeneous and saturable population of binding sites. The affinity of [^{3}H]ramosetron for the receptor was 8-fold higher than that of [^{3}H]granisetron, and the density of sites labeled with [^{3}H]ramosetron was 1.5-fold higher than those labeled with [^{3}H]granisetron (Table 1). The difference in the densities of binding sites of the 5-HT_3 receptor had previously been demonstrated using [^{3}H]quipazine, [^{3}H]granisetron and [^{3}H]GR65630 in rat cerebral cortex and rabbit ileal homogenates (7). In addition, differences in binding densities had also been reported between [^{3}H]BRL46470 and [^{3}H]granisetron in rat cerebral cortex and hippocampus, rat ileum, NG-108-15 cells and HEK-5-HT_3As cells (14). The human 5-HT_3 receptor was transiently expressed on COS-1 cells and no specific [^{3}H]ramosetron or [^{3}H]granisetron binding was observed in cells not transfected with the 5-HT_3 receptor (data not shown). This excludes the possibility of the presence of another binding site of [^{3}H]ramosetron on COS-1 cells. Furthermore, cooperative interaction is unlikely to contribute to the difference in binding densities, because the Hill coefficients of [^{3}H]ramosetron and [^{3}H]-granisetron were each near to unity (Table 1). Further investigations are therefore needed to characterize the difference in binding densities.

In order to investigate kinetic differences between ramosetron and granisetron at the 5-HT_3 receptor, association and dissociation studies were performed (Figs. 1 and 2). Results show that [^{3}H]ramosetron and [^{3}H]-granisetron associated rapidly with the cloned human 5-HT_3 receptor and reached equilibrium within 60 min. The observed association rate for [^{3}H]ramosetron and [^{3}H]-granisetron was $0.054\pm0.006\ min^{-1}$ and $0.114\pm0.020\ min^{-1}$, respectively. Dissociation of [^{3}H]ramosetron had a rate constant (K_{-1}) of $0.009\pm0.002\ min^{-1}$. K_{+1} was calculated to be $0.023\times10^{10}\ min^{-1}M^{-1}$, and the kinetically determined K_D was estimated to be 0.04 nM. In contrast, the dissociation of [^{3}H]granisetron had a rate constant (K_{-1}) of $0.029\pm0.001\ min^{-1}$. K_{+1} was calculated to be $0.043\times10^{9}\ min^{-1}M^{-1}$, and the kinetically determined K_D was estimated to be 0.67 nM. The kinetically determined K_D for [^{3}H]ramosetron and [^{3}H]granisetron were in agreement with the K_D determined by saturation binding studies. Although association of both radioligands in cloned human 5-HT_3 receptor was almost the same, dissociation of [^{3}H]ramosetron ($t_{1/2}=108$ min) was much slower than that of [^{3}H]granisetron ($t_{1/2}=24$ min). The difference of binding densities between [^{3}H]-ramosetron and [^{3}H]granisetron may be related to this difference in dissociation, because similar features have been observed in experiments using [^{3}H]BRL46470 and [^{3}H]granisetron (14).

The effect of ramosetron on reducing emesis induced by anticancer agents in ferrets had a longer duration compared with that of granisetron (9). Similar features were observed in inhibition experiments of 5-HT-induced

Table 1. Saturation studies of [^{3}H]ramosetron and granisetron

	K_D (nM)	B_{max} (fmol/mg protein)	Hill coefficient
[^{3}H]Ramosetron	0.15±0.01*	653.7±30.1*	0.96±0.03
[^{3}H]Granisetron	1.17±0.25	427.2±42.7	0.99±0.01

Values are means±S.E.M. of three experiments. *$P<0.05$, significantly different from the data of [^{3}H]granisetron.

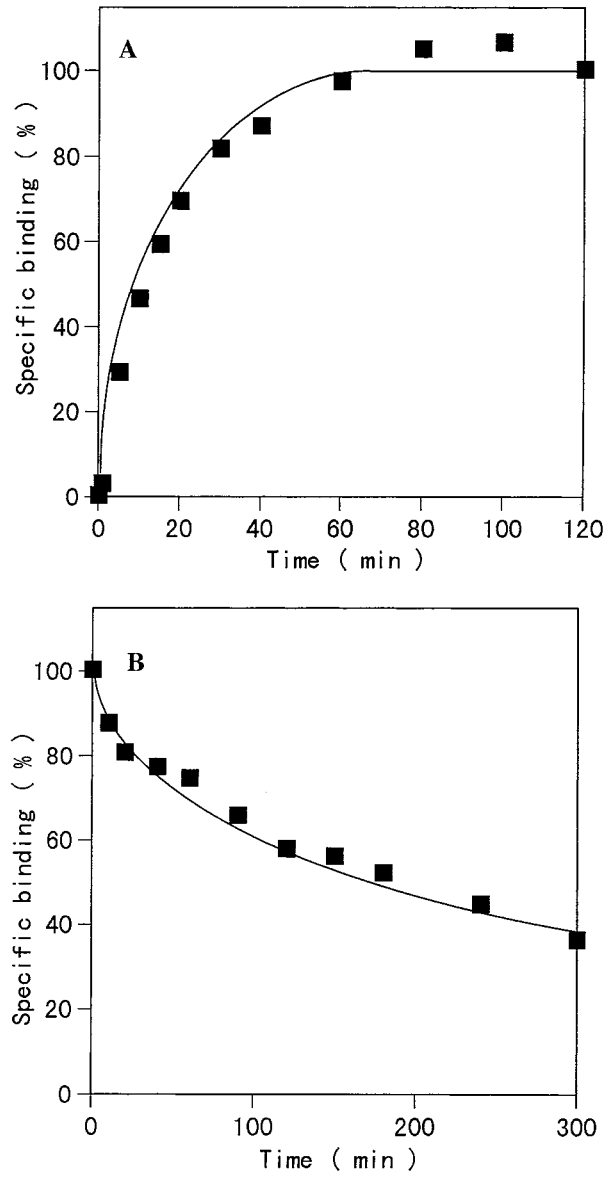

Fig. 1. Representative kinetic curves for [^{3}H]ramosetron in the cloned human 5-HT$_3$ receptors. A: Association curves for [^{3}H]-ramosetron in the cloned human 5-HT$_3$ receptors. B: Dissociation curves for [^{3}H]ramosetron in the cloned human 5-HT$_3$ receptors. Results are from a typical experiment representing three experiments.

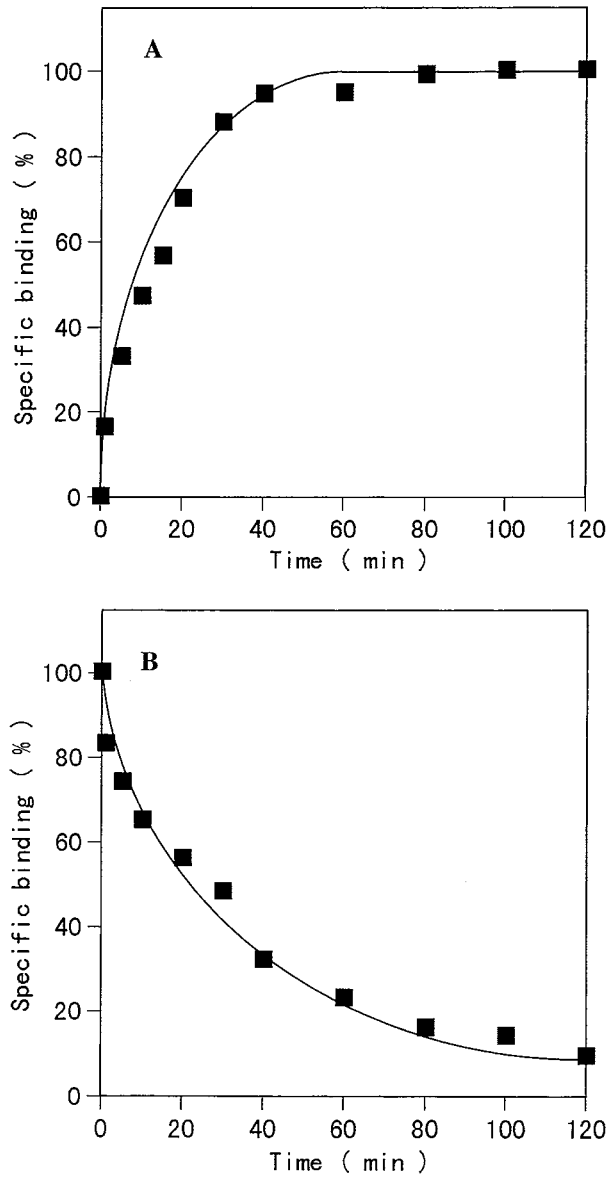

Fig. 2. Representative kinetic curves for [^{3}H]granisetron in the cloned human 5-HT$_3$ receptors. A: Association curves for [^{3}H]-granisetron in the cloned human 5-HT$_3$ receptors. B: Dissociation curves for [^{3}H]granisetron in the cloned human 5-HT$_3$ receptors. Results are from a typical experiment representing three experiments.

bradycardia in anesthetized rats (15). Slow dissociation may be one explanation for the long duration of ramosetron compared with that of granisetron. Moreover, the antiemetic effect of ramosetron may have a longer lasting duration in humans, because slow dissociation of [^{3}H]-ramosetron was observed in the cloned human 5-HT$_3$ receptors. However, clinical evaluation is necessary to determine whether ramosetron has a longer duration of action in vivo.

In summary, [^{3}H]ramosetron recognized cloned human 5-HT$_3$ receptors with high affinity at more sites than [^{3}H]-granisetron. Furthermore, dissociation of [^{3}H]ramosetron from the receptor was markedly slower than that of [^{3}H]granisetron. Taken together, ramosetron is a highly potent 5-HT$_3$-receptor antagonist.

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