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Characterization of [^{3}H]YM060, a potent and selective 5-HT$_3$ receptor radioligand, in the cerebral cortex of rats

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Abstract

The binding properties of a new radioligand, [methyl-^{3}H]-(−)-(*R*)-5-[(1-methyl-1*H*-indol-3-yl)carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride ([^{3}H]YM060), were studied in membranes of the rat cerebral cortex. [^{3}H]YM060 rapidly associated with its binding sites in membranes and reversibly dissociated. Saturation analysis revealed that the specific binding of [^{3}H]YM060 was saturable and non-specific binding was low. Scatchard analysis yielded a linear plot, suggesting a single population of binding sites with a dissociation constant (K_d) of 8.4 ± 0.2 pM ($n = 3$) and the kinetic K_d determined from the association constant (K_{+1}) and the dissociation rate constant (K_{-1}) was similar. The maximum number of binding sites (B_{max}) was 37.0 ± 0.8 fmol/mg protein ($n = 3$). [^{3}H]YM060 binding was potently and stereospecifically inhibited by serotonin (5-HT)$_3$ receptor agonists and antagonists. Other 5-HT receptor ligands such as 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin), methysergide and ketanserin were inactive to inhibit specific binding at 10^{-4} M. These results suggest that [^{3}H]YM060 is a highly potent and selective 5-HT$_3$ receptor radioligand and will be useful in the further analysis of 5-HT$_3$ receptors.

Keywords: [^{3}H]YM060 ([methyl-^{3}H]-(−)-(*R*)-5-[(1-methyl-1*H*-indol-3-yl)carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride); 5-HT$_3$ receptor; Cerebral cortex, rat

1. Introduction

Serotonin (5-HT) is an important neurotransmitter which exerts its effects through several pharmacologically distinct receptors. 5-HT receptors can be divided into at least four (5-HT$_1$, 5-HT$_2$, 5-HT$_3$ and 5-HT$_4$ receptor), possibly up to seven (5-HT$_5$, 5-HT$_6$ and 5-HT$_7$ receptor) types (Hoyer et al., 1994). Of these, the 5-HT$_3$ receptor is unique in that it is a ligand-gated ion channel (Derkach et al., 1989). The 5-HT$_3$ receptor is widely distributed throughout the peripheral (Fozard, 1984) and central nervous systems (Kilpatrick et al., 1987), and has been proposed as the mediator of many physiological actions such as emesis (Fozard, 1987), anxiety (Jones et al., 1988), cognitive function (Barnes et al., 1990) and stress-induced defecation (Miyata et al., 1992). Recently, cDNA encoding a single subunit of the 5-HT$_3$ receptor has been isolated from NCB-20 cells. The predicted protein is 487 amino acids long and contains four hydrophobic transmembrane spanning domains (Maricq et al., 1991). Species differences of the 5-HT$_3$ receptor have been reported for radioligand binding affinity and electrophysiological characteristics (Ito et al., 1992; Wong et al., 1993). No strong evidence is reported for the existence of different 5-HT$_3$ receptors within the same species except for different affinity of ligands in two mouse tissues (Bonhaus et al., 1993).

A number of radioligands have been used in the identification of 5-HT$_3$ receptors, including [^{3}H]-GR65630 (Kilpatrick et al., 1987), [^{3}H]ICS205-930 (Hoyer and Neijt, 1988), [^{3}H]BRL43694 (Nelson and Thomas, 1989), [^{3}H]zacopride (Barnes et al., 1988b), [^{125}I]zacopride (Laporte et al., 1992), [^{3}H]GR67330 (Kilpatrick et al., 1990b) and [^{3}H]RS-42358-197 (Wong

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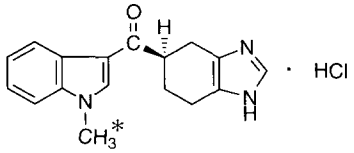

Fig. 1. Chemical structure of [methyl-^{3}H]-(−)-(*R*)-5-[(1-methyl-1*H*-indol-3-yl)carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride ([^{3}H]YM060). * Indicates the site at which the compound was radiolabeled with tritium.

et al., 1993). Most of these are useful for cells or tissues which have a high concentration of 5-HT_3 receptors. However, the detailed characterization of 5-HT_3 receptor binding sites is hampered by their generally low concentration in many species, including the rabbit, guinea pig, ferret and human (Barnes et al., 1988a; Kilpatrick et al., 1989).

YM060 is a tetrahydrobenzimidazole derivative which possesses potent 5-HT_3 receptor blocking properties both in vitro and in vivo (Miyata et al., 1991a,b). It inhibits [^{3}H]GR65630 binding in N1E-115 neuroblastoma cells with a K_i value of 0.091 nM (Ito et al., 1992). As it is likely that a ligand with high affinity for a receptor possesses high specific binding as a radioligand, we synthesized [^{3}H]YM060 (Fig. 1) and characterized it as a radioligand for 5-HT_3 receptors in cerebral cortex of rats.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats weighing 250–300 g were used. The rats were decapitated, their brains were rapidly removed and the cerebral cortex was dissected. The tissues were finely minced with scissors and homogenized in 30 volumes of ice-cold 50 mM Hepes buffer (pH 7.4 at 4° C) with a Polytron (Kinematica, Lucerne, Switzerland). The homogenate was centrifuged at $48\,000 \times g$ for 10 min. The pellet was resuspended in Hepes buffer and recentrifuged as above. The final pellet was suspended in Hepes buffer at a concentration of 0.8–1 mg protein ml^{-1}.

2.2. Radioligand binding assay

For saturation studies, membranes (0.1 mg protein) were incubated with increasing concentrations of [^{3}H]YM060 (0.01–0.2 nM) in a final volume of 0.5 ml for 30 min at 25° C. For competition studies, a single concentration (0.03 nM) of [^{3}H]YM060 and four to six concentrations of agonists and antagonists were used. Incubation was terminated by rapid filtration through Whatman GF/B filters using a Brandel cell harvester (Brandel, Gaithersburg, MD), followed by washing of the filter 3 times with 3 ml of ice-cold Hepes buffer. Radioactivity retained on the filters was counted with a liquid scintillation counter (Packard 2000CA). Non-specific binding was determined in the presence of 1 μM of tropisetron. The protein content of each membrane suspension was measured by the method of Bradford (1976).

2.3. Analysis of data

Values were expressed as the means ± S.E.M. Comparisons between values from different groups were evaluated by analysis of variance. Probabilities of $< 5\%$ ($P < 0.05$) were considered significant. IC_{50} values, the concentration required to inhibit specific binding by 50%, were calculated by logit-log analysis from the following equation (DeLean et al., 1978): $\log[(B_o - B_i)/(B_i - B_n)] = n[\log(\text{antagonist concentration} - \log(IC_{50})]$ where B_o and B_i are binding in the absence and presence of the antagonist to be tested, respectively; B_n is non-specific binding and n is the slope factor identical to the Hill coefficient. The inhibition constants (K_i values) were calculated from IC_{50} values using the following equation (Cheng and Prusoff, 1973): $K_i = IC_{50}/(1 + [L]/K_d)$ where [L] is the radioligand concentration and K_d is the dissociation constant of the radioligand.

Kinetic data were analyzed according to the procedure of Weiland and Molinoff (1981). The observed association rate constant (K_{obs}) was calculated as the slope of the plot $\ln[B_e/(B_e - B_t)]$ versus time, according to the equation $B_t = B_e[1 - e^{-(K_{obs} \cdot t)}]$, where B_t is the amount of radioligand specifically bound at equilibrium. The dissociation rate constants were determined according to the equation $B_t = B_0^{-(K-1 \cdot t)}$, by plotting $\ln(B_t/B_0)$ versus time; the slope of this plot is equal to K_{-1}. The association constant (K_{+1}) was calculated using the equation: $K_{+1} = (K_{obs} - K_{-1})/L$ where L is the radioligand concentration. Kinetic K_d was calculated using the equation: $K_d = K_{-1}/K_{+1}$.

2.4. Drugs

[^{3}H]YM060 (78 Ci/mmol) was specially synthesized by Amersham International (Buckinghamshire, England) for Yamanouchi Pharmaceutical Co. (Tsukuba city, Japan). YM060, its enantiomer (*S*-form), YM114 (KAE-393, (*R*)-5-[(1-indolinyl)carbonyl]-4,5,6,7-tetrahydro-1*H*-benzimidazole monohydrochloride), ondansetron (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazole-1-yl)methyl]-4*H*-carbazole-4-one monohydrochloride), granisetron (BRL43694, *endo*-1-methyl-*N*-(9-methyl-azabicyclo[3.3.1]non-3-yl)-1*H*-indazole-3-carboxamide), tropisetron (ICS205-930, *endo*-8-methyl-8-azabicyclo[3.2.1]oct-3-yl-1*H*-indole-3-car-

boxylate), *m*-chlorophenylbiguanide and 2-methyl-5-HT were prepared by Yamanouchi Pharmaceutical Co. 5-HT creatinine sulfate was purchased from E. Merck (Darmstadt, Germany). 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin) and ketanserin (1-(2,5-dimethoxy-4-indophenyl)-2-(di-*N*-propylamino)tetralin hydrobromide) were purchased from Research Biochemicals (Natick, MA). Methysergide (9,10-didehydro-*N*-[1-(hydroxymethyl)propyl]1,6-dimethylergoline-8-carboxamide) was kindly donated by Sandoz (Basle, Switzerland). For the competition analysis, YM060, YM114, granisetron and the enantiomer of YM060 were dissolved in 100% dimethyl sulfoxide (final 1%) and diluted with Hepes buffer.

3. Results

3.1. Association and dissociation analysis

The time course of association and dissociation of [^{3}H]YM060 binding was examined in rat cerebral cortex membranes. [^{3}H]YM060 rapidly associated with its binding sites in membranes, and reached steady state by 30 min at 25° C (Fig. 2A). Binding remained at steady state for at least 70 min. The observed association rate constant (K_{obs}) was 0.18 ± 0.03 min^{-1} (n = 3). Dissociation could be monitored by the addition of 1 μM tropisetron after 30 min of association (Fig. 2B). The association constant (K_{+1}) was 4.46 ± 0.99 × 10^9 M^{-1} s^{-1} (n = 3). The dissociation rate constant (K_{-1}) was 0.05 ± 0.01 min^{-1} (n = 3). The kinetically determined K_d was 12.2 ± 0.2 pM (n = 3).

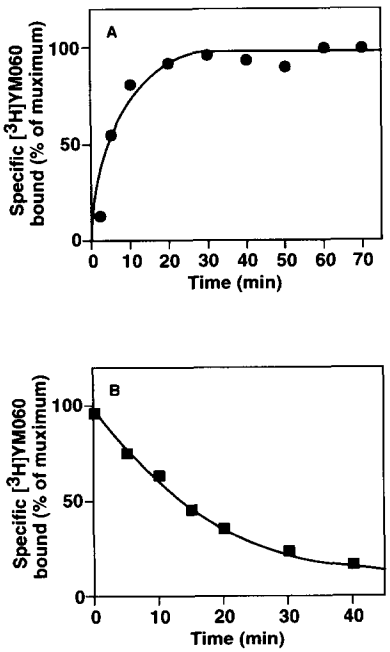

Fig. 2. (A) Time course of association and (B) dissociation of specific [^{3}H]YM060 binding to rat cerebral cortex. [^{3}H]YM060 (0.03 nM) binding was quantified as a function of time from the addition of ligand. The dissociation of the [^{3}H]YM060-receptor complex was monitored by the addition of 1 μM tropisetron. Each point represents the average of three experiments in triplicate.

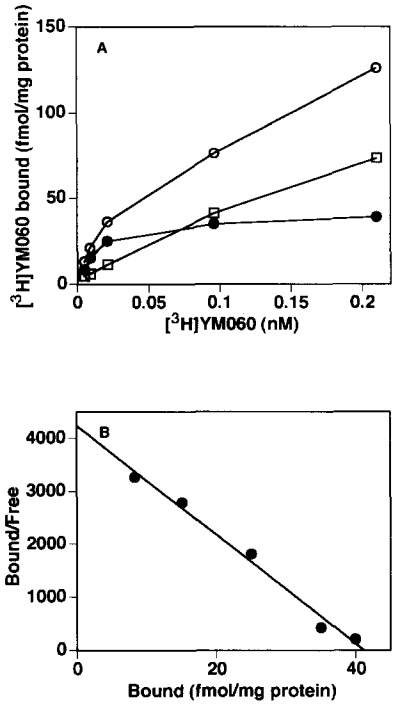

Fig. 3. (A) Total (○), specific (●) and non-specific (□) binding of [^{3}H]YM060 to rat cerebral cortex and (B) Scatchard plot. Rat cerebral cortex membranes were incubated with increasing concentrations of [^{3}H]YM060 (0.01–0.20 nM) at 25° C for 30 min. Non-specific binding was determined in the presence of 1 μM tropisetron. These plots are representative of three separate determinations in triplicate.

3.2. Saturation analysis

Identification of [^{3}H]YM060 binding sites was carried out in membranes of rat cerebral cortex. Specific binding of [^{3}H]YM060 (0.01–0.2 nM, defined using 1 μM tropisetron) in rat cortex was saturable, whilst non-specific binding increased linearly with ligand concentration (Fig. 3A). Scatchard analysis revealed that binding was apparently to a single site with high affinity (Fig. 3B) (K_d = 8.4 ± 0.2 pM, B_{max} = 37.0 ± 0.3 fmol/mg protein, n = 3).

3.3. Competition analysis

A variety of 5-HT$_3$ receptor agonists and antagonists, and ligands for other 5-HT receptors were tested for their ability to inhibit specific binding of [^{3}H]YM060 in rat cerebral cortex membranes. The 5-HT$_3$ receptor agonists and antagonists dose dependently competed

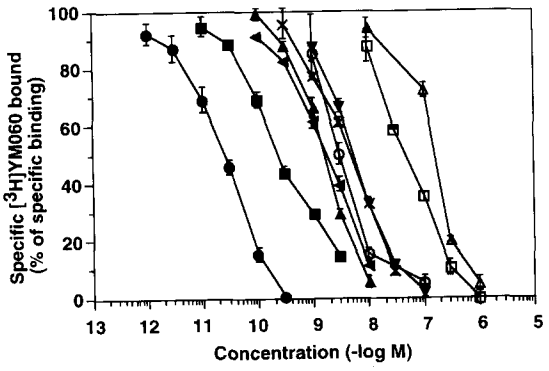

Fig. 4. Inhibition of specific [^{3}H]YM060 binding to rat cerebral cortex by YM060 (●), YM114 (■), granisetron (▲), tropisetron (◀), ondansetron (▼), the enantiomer of YM060 (*S*-form) (×), *m*-chlorophenylbiguanide (○), 5-HT (□) and 2-methyl-5-HT (△). Competitive binding experiments with [^{3}H]YM060 were performed in the absence and presence of four to eight concentrations of various 5-HT$_3$ receptor agonists and antagonists. Each point represents the mean ± S.E.M. of three experiments in triplicate.

with [^{3}H]YM060 binding (Fig. 4). pK_i values estimated for a series of compounds are shown in Table 1. The rank order of affinity was as follows: YM060 > YM114 > tropisetron > granisetron > *m*-chrolophenylbiguanide > the enantiomer of YM060 (*S*-form) > ondansetron > 2-methyl-5-HT > 5-HT. Ligands for other 5-HT receptors such as 8-OH-DPAT, methysergide and ketanserin at 10^{-4} M were inactive to inhibit [^{3}H]YM060 binding.

Table 1
Inhibition of [^{3}H]YM060 binding by various compounds in rat cerebral cortex

Compounds	p*K*i (− log M)	n_H
Antagonists		
YM060	11.47 ± 0.16	1.13 ± 0.07
YM114	10.76 ± 0.19	0.98 ± 0.19
Tropisetron	9.74 ± 0.18	0.94 ± 0.22
Granisetron	9.55 ± 0.09	0.98 ± 0.09
Ondansetron	8.89 ± 0.09	1.12 ± 0.08
S-form of YM060	9.12 ± 0.02	1.23 ± 0.07
Agonists		
m-Chlorophenylbiguanide	9.22 ± 0.07	1.36 ± 0.21
5-HT	7.94 ± 0.18	1.41 ± 0.15
2-Methyl-5-HT	7.68 ± 0.11	1.35 ± 0.29
Others		
8-OH-DPAT	< 4	
Methysergide	< 4	
Ketanserin	< 4	

Membranes were incubated with [^{3}H]YM060 (0.03 nM) and four to six concentrations of each compound. p*K*i values represent the means ± S.E.M. of three separate experiments, except for the inactive compounds. The Hill coefficient represents the mean ± S.E.M. of three separate experiments.

4. Discussion

The present study demonstrated that [^{3}H]YM060 selectively binds to 5-HT$_3$ receptors in the rat cerebral cortex in a reversible and saturable manner with high affinity.

Competition analysis revealed that [^{3}H]YM060 selectively recognized 5-HT$_3$ receptors. YM060, YM114, tropisetron, granisetron and ondansetron were used as selective 5-HT$_3$ receptor antagonists. These have been pharmacologically characterized with functional methods such as 5-HT- and 2-methyl-5-HT-induced contraction of the isolated colon of guinea pigs (Miyata et al., 1991a), the Bezold-Jarisch reflex in anesthetized rats (Miyata et al., 1991b, Miyata et al., 1993) and binding assays in brain and ileum of mice, nodose ganglion of rabbits and N1E-115 neuroblastoma cells (Bonhaus et al., 1993; Ito et al., 1992). The rank order of inhibitory activity in the present study (YM060 > YM114 > tropisetron > granisetron > ondansetron) was similar to that in these reports. *m*-Chlorophenylbiguanide and 2-methyl-5-HT were used as selective 5-HT$_3$ receptor agonists (Kilpatrick et al., 1990a). Both agonists also inhibited specific binding of [^{3}H]YM060. However, other 5-HT receptor ligands such as 8-OH-DPAT (5-HT$_{1A}$ receptor agonist), methysergide (5-HT$_{1,2}$ receptor antagonist) and ketanserin (5-HT$_2$ receptor antagonist) were inactive to inhibit specific binding. These findings therefore confirm that [^{3}H]YM060 is a 5-HT$_3$ receptor-specific radioligand.

The affinity of [^{3}H]YM060 in the rat cerebral cortex (K_d = 8.4 ± 0.2 pM) was significantly higher than that of the other 5-HT$_3$ receptor radioligands. The K_d value was similar to the K_i value obtained for unlabeled YM060 in competition analysis (pK_i = 11.47 ± 0.16) and kinetically determined K_d value (12.0 ± 0.2 pM). Specific binding of [^{3}H]YM060 ranged from 65% to 70% when the ligand concentration was 0.03 nM. In an experiment with the same conditions, the specific binding of [^{3}H]GR65630 and [^{3}H]BRL43694 in the rat cerebral cortex was so small (20% and 30%) that it was difficult to analyze the subsequent data (data not shown). There still remain many problems regarding the 5-HT$_3$ receptor that are not clarified, such as structural characteristics, transductional characteristics and inter- or intra-species heterogeneity (Martin and Humphrey, 1994). For the further characterization of 5-HT$_3$ receptor, radioligand binding experiments using native tissue will be needed. However, the concentration of 5-HT$_3$ receptors in native tissue such as rat, guinea pig, rabbit and human is low (Barnes et al., 1988a; Kilpatrick et al., 1989). [^{3}H]YM060 can be used as a 5-HT$_3$ receptor radioligand in tissues with low receptor concentration. Although the specific activity of [^{3}H]YM060 is high (78 Ci/mmol) among the tritiated radioligands, there is an iodinated radioligand,

[^{125}I]zacopride, which has higher specific activity (1100 Ci/mmol) than tritiated radioligands (Laporte et al., 1992). [^{125}I]Zacopride is a useful radioligand for autoradiographic assays. In contrast, [^{3}H]YM060 has higher affinity than [^{125}I]zacopride ($K_d = 4.3$ nM). Furthermore, low non-specific binding of [^{3}H]YM060 is more important in binding assays. In general, [^{3}H]YM060 has characteristics that make it advantageous as radioligand for 5-HT$_3$ receptor study.

[^{3}H]YM060 may have several features that will be useful for 5-HT$_3$ receptor analysis. First, Bonhaus et al. (1993) reported that the affinity of YM060 in CD-1 mouse cortex using [^{3}H]RS-42358-197 ($pK_i = 9.5 \pm 0.18$, $n_H = 0.70 \pm 0.09$) was different from that in CD-1 mouse ileum ($pK_i = 10.0 \pm 0.12$, $n_H = 0.94 \pm 0.2$). Compared with RS-42358-197, YM060 had a higher affinity in ileum than in cortex. Second, the K_d value of YM060 (8.4 pM) obtained in the present study was about 10-fold higher than that (0.091 nM) obtained from [^{3}H]GR65630 binding in N1E-115 cells (mouse neuroblastoma) (Ito et al., 1992). This result may suggest that the affinity of [^{3}H]YM060 is different in different species. However, further studies will be needed to establish these features. High affinity and low non-specific binding of [^{3}H]YM060 will be helpful.

The Hill coefficient of 5-HT$_3$ receptor agonists and antagonists in competition analysis showed the same trend as that for other 5-HT$_3$ receptor radioligands: the Hill coefficient of antagonists was close to unity, but that of agonists was greater than unity (not statistically significant) in our studies. The same results have been reported for [^{3}H]GR65630 (Kilpatrick et al., 1987), [^{3}H]GR67330 (Kilpatrick et al., 1990b) and [^{3}H]RS-42358-197 (Wong et al., 1993) in rat brain membrane. These results have not been well explained, but may reflect positive cooperativity.

In conclusion, [^{3}H]YM060 is a highly potent and selective antagonist radioligand for the 5-HT$_3$ receptor. The specific binding of [^{3}H]YM060 is high enough to study the 5-HT$_3$ receptor. Further study is now underway in tissues with low receptor concentration, not only from rats but also other species. Thus, [^{3}H]YM060 is likely to be a very useful radioligand for further characterizing the 5-HT$_3$ receptor.

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