2002

Functional group interactions of a 5-HT3R antagonist

Padmavati Venkataraman

Prasad Joshi

Srinivasan P. Venkatachalan

Mani Mathalagi

Harish S. Parihar
Philadelphia College of Osteopathic Medicine, harishpa@pcom.edu

See next page for additional authors

Authors

Padmavati Venkataraman, Prasad Joshi, Srinivasan P. Venkatachalan, Mani Mathalagi, Harish S. Parihar, Karen S. Kirschbaum, and Marvin K. Schulte

BMC Biochemistry

Functional group interactions of a 5-HT$_3$R antagonist

Padmavati Venkataraman[2], Prasad Joshi[1], Srinivasan P Venkatachalan[1], Mani Muthalagi[1], Harish S Parihar[1], Karen S Kirschbaum[1] and Marvin K Schulte*[1]

Address: [1]Department of Basic Pharmaceutical Sciences, College of Pharmacy, The University of Louisiana at Monroe, Monroe, LA 71209, USA and [2]Department of Neurobiology and Physiology Northwestern University Evanston, IL 60208-3520, USA

E-mail: Padmavati Venkataraman - padma964@hotmail.com; Prasad Joshi - joship@tribe.ulm.edu; Srinivasan P Venkatachalan - pv_srinivasan@yahoo.com; Mani Muthalagi - muthalagiss@yahoo.com; Harish S Parihar - harishparihar@hotmail.com; Karen S Kirschbaum - pyKirschbaum@ulm.edu; Marvin K Schulte* - pyschulte@ulm.edu

*Corresponding author

Abstract

Background: Lerisetron, a competitive serotonin type 3 receptor (5-HT$_3$R) antagonist, contains five functional groups capable of interacting with amino acids in the 5-HT$_3$R binding site. Site directed mutagenesis studies of the 5-HT$_{3A}$R have revealed several amino acids that are thought to form part of the binding domain of this receptor. The specific functional groups on the ligand that interact with these amino acids are, however, unknown. Using synthetic analogs of lerisetron as molecular probes in combination with site directed mutagenesis, we have identified some of these interactions and have proposed a model of the lerisetron binding site.

Results: Two analogs of lerisetron were synthesized to probe 5-HT$_3$R functional group interactions with this compound. Analog 1 lacks the N1 benzyl group of lerisetron and analog 2 contains oxygen in place of the distal piperazine nitrogen. Both analogs show significantly decreased binding affinity to wildtype 5-HT$_{3AS}$Rs. Mutations at W89, R91, Y142 and Y152 produced significant decreases in binding compared to wildtype receptors. Binding affinities of analogs 1 and 2 were altered only by mutations at W89, and Y152.

Conclusions: Based on the data obtained for lerisetron and analogs 1 and 2, we have proposed a tentative model of the lerisetron binding pocket of the 5-HT$_{3AS}$R. According to this model, The N-benzyl group interacts in a weak interaction with R91 while the benzimidazole group interacts with W89. Our data support an interaction of the distal amino nitrogen with Y142 and Y152.

Background

The cysteine-loop family of ligand gated ion channels (LGIC) is comprised of receptors with pentameric quaternary structure and at least two ligand binding sites present at the subunit interfaces [1,2]. This receptor family is characterized by the presence of a critical disulfide loop structure within the binding site and an integral ion selective channel. LGIC receptors are found in both the peripheral and central nervous systems. Members of this family include the acetylcholine receptors [3,4], the γ-amino butyric acid type A receptor (GABA$_A$R) [5], and the glycine receptor (GlyR) [6].

The first subunit of the 5-HT$_3$R was cloned in 1991 [7]. The sequence of this subunit was shown to be highly homologous to LGIC receptors and thus identified the 5-HT$_3$R receptor as another member of this superfamily [7–9]. Similar to other LGIC receptors, more than one subtype has been identified. Two splice variants of an A subunit (long and short forms), and a single B subunit have been cloned [10–13]. Both the long and short forms of the A subunit are capable of forming functional homomeric receptors [5-HT$_{3AL}$R and 5-HT$_{3AS}$R] although some differences between an agonist and partial agonist activity have been observed [14]. A third subtype is formed by a combination of the A and B subunits to produce a heteromeric receptor of unknown stoichiometry [10]. Heteromeric receptors are pharmacologically and functionally distinct from the homomeric 5-HT$_{3AL}$ and 5-HT$_{3AS}$ receptors [11].

5-HT$_3$Rs are distributed throughout the central and peripheral nervous system, playing a significant role in phenomenon such as anxiety, emesis and alcoholism. Antagonists to 5-HT$_3$Rs are clinically efficacious in the treatment of chemotherapy-induced emesis [15] and recent studies on human subjects have suggested their potential application in the treatment of early onset alcoholism [16,17].

Hibert *et al* proposed an early model for the antagonist pharmacophore of the 5-HT$_3$R [18]. According to this model, all 5-HT$_3$R antagonists contain an aromatic ring, a carbonyl oxygen or bioisosteric equivalent, and a basic nitrogen. According to Hibert's model, the basic nitrogen is located 5.2A° from the centre of the aromatic ring and approximately 1.7A° above plane of the ring. The carbonyl oxygen and the aromatic ring are coplanar and separated by a distance of 3.3A°. Recent studies have expanded on this model to include another lipophilic region and a second hydrogen bonding interaction two atoms away from the first [19–21]. A compound that contains all five pharmacophoric regions was synthesized by Orjales *et al*[22]. This compound (1-(phenylmethyl)-2-piperizinyl benzimidazole or lerisetron) is shown in figure 1 and is a potent 5-HT$_3$R antagonist. Functional groups on this compound capable of forming interactions with the receptor are the distal amino group, a benzimidazole and a benzyl group in the N1 position of the benzimidazole. While Lerisetron contains no carbonyl group, the second nitrogen contained in the benzimidazole heterocycle could act as bioisostere of this functional group [22]. Orjales demonstrated the importance of the N-benzyl group by synthesizing several N1 substituted analogs of Lerisetron. Removal of the N-benzyl group produced a 80-fold decrease in affinity, indicating a role for this group in interacting with the 5-HT$_3$R. Other studies have supported this observation and suggest a more specific electrostatic interaction [23].

While structure-activity relationship studies and molecular modeling have led to the development of a detailed pharmacophore model, determining specific point interactions between 5-HT$_3$ antagonists and binding site amino acids has proven difficult. Mutagenesis studies have identified the interaction of amino acids W89 and R91 in the binding of 5-HT$_3$R ligands [24,23]. Studies conducted in our laboratory have identified three additional putative binding site residues (Y140, Y142, and Y152) [25]. W89 and R91 are present in a conserved region of LGIC receptors often referred to as loop D [24,26]. Similarly, Y140, Y142 and Y152 are located in the region homologus to E loop region of nicotinic AchR.

In this study, we have endeavoured to identify the amino acids interacting with the different functional groups present on the lerisetron molecule in order to develop a model for interaction of this compound with the 5-HT$_3$R. Using site directed mutagenesis in combination with analogs of lerisetron, we have identified amino acids that appear to interact selectively with the terminal amino group, the N-benzyl group and the aromatic benzimidazole.

Results
Functional activity of lerisetron
Whole cell patch-clamp experiments were performed to test the functional activity of lerisetron. No response was observed when lerisetron was applied alone (data not shown). When co-applied with 5-HT, lerisetron inhibited the absolute magnitude of the response with no apparent alteration of the response profile (Figure 2). The combination of several identical inhibition experiments produced a K_i value of 0.2 ± 0.03 nM for lerisetron inhibition of the 5-HT induced response. These data correspond well with previously reported data for this compound [22] and verify the competitive antagonist action of lerisetron. Analogs of lerisetron have been shown to inhibit 5-HT$_3$Rs in a similar manner [23].

Importance of the N-benzyl and distal piperazine nitrogen to binding of lerisetron
The K_i value for lerisetron inhibition of [^{3}H]-granisetron binding to wildtype receptors was determined to be 0.8 ± 0.19 nM (Figure 3 and Table 1). This value agrees with previously published data for this compound. The K_i values for analogs 1 and 2 under identical conditions are 25 ± 3.2 nM and 320 ± 82 nM respectively (Figure 4A, Table 1). The observed change in K_i represents the decreases in binding energy resulting from removal of the N-benzyl group (analog 1) and the distal piperazine nitrogen (analog 2).
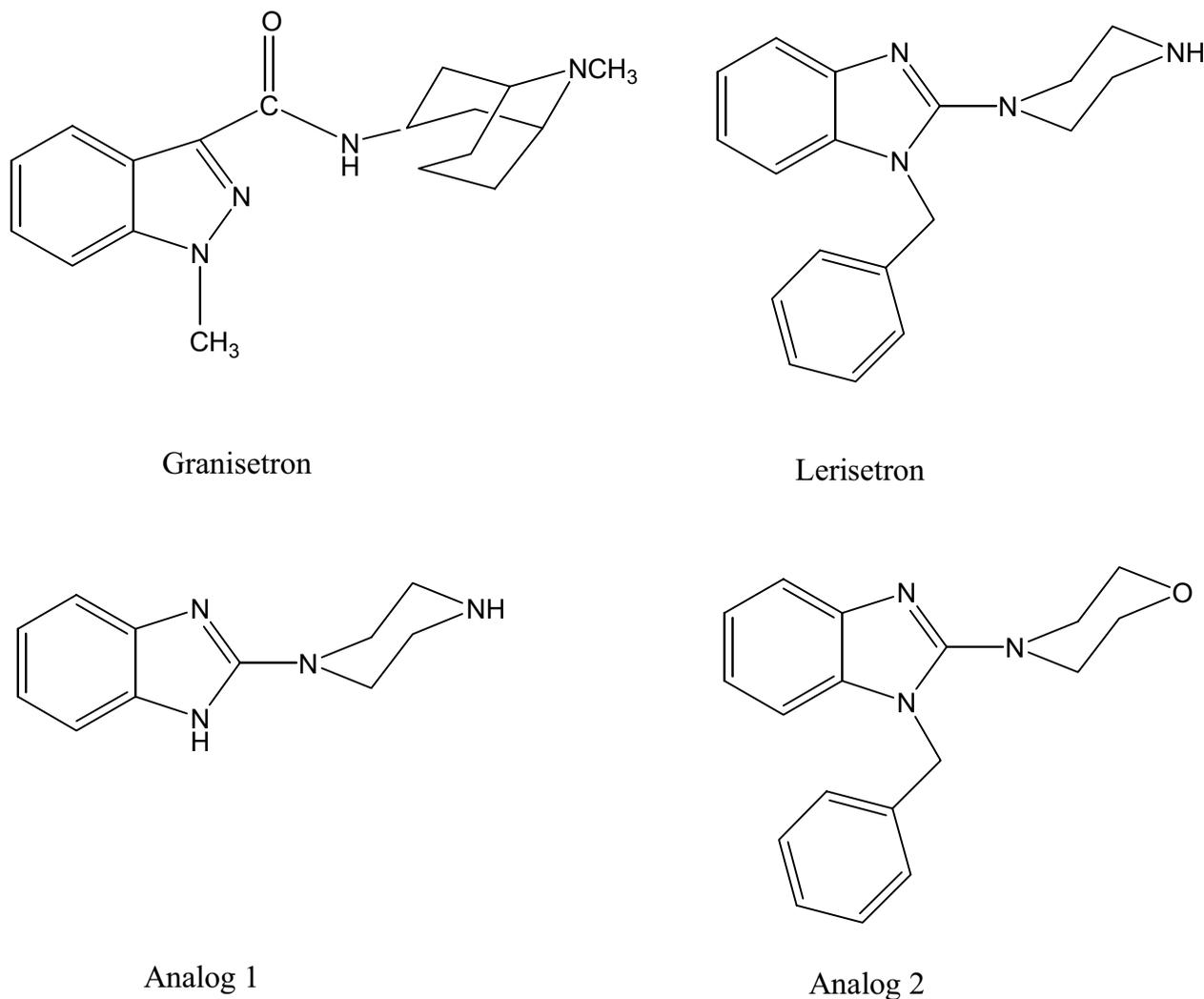
Figure 1
5-HT$_3$R antagonists. Granisetron and Lerisetron are shown along with the two analogs utilized to probe the functional group interactions of binding site amino acids. Analog 1 lacks the N-benzyl functional group. Analog 2 contains oxygen in place of the distal piperazine nitrogen.

Identification of amino acids interacting with Lerisetron
In order to determine the nature of the amino acids interacting with the distal amino and N-benzyl groups of lerisetron, we constructed 5-HT$_{3AS}$Rs containing mutations at W89, R91, Y140, Y142 and Y152. Figure 3 shows inhibition of [³H]-granisetron binding by lerisetron on wildtype and mutant receptors. For most amino acids, an alanine substitution was constructed in order to effectively remove any amino acid interaction with the ligand. For W89, an alanine substitution has been shown to prevent binding of [³H]granisetron; therefore a less severe mutation was constructed.

The W89F mutation produces a 18-fold change in K$_d$ for [³H]-granisetron binding (18 ± 2 nM) and the W89Y mutation produces a 5.8-fold change in K$_d$ (5.7 ± 0.7 nM). Mutation of amino acid R91 to alanine produced a 5-fold change in K$_d$ for [³H]granisetron binding (4.9 ± 0.7 nM) (Table 1). These data agree well with previously reported values [24]. Alanine mutations at the tyrosine positions Y140, Y142 and Y152 also produced minor increases in K$_d$ for [³H]granisetron binding (2.7 ± 0.19 nM, 4.5 ± 0.5 nM and 7.8 ± 1.1 nM respectively) [25] (Table 1).
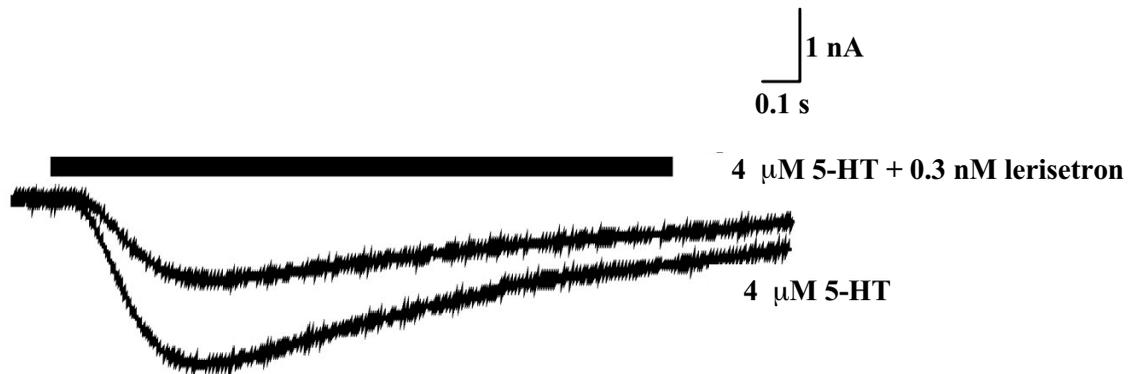
Figure 2
Inhibition of 5-HT induced responses by Lerisetron. tsA 201 cells transfected with 5-HT$_{3A_S}$R cDNA were clamped at a holding potential of -60 mV and perfused with 4 μM 5-HT (lower trace) or 4 μM 5-HT and 0.3 nM lerisetron (upper trace). Lerisetron reduced the peak 5-HT induced currents from 2.0 nA to 1.0 nA with no change in the response characteristics. An IC$_{50}$ value was determined by a combination of similar experiments and determined to be 0.4 ± 0.03 nM.

Only small changes in K$_i$ for lerisetron were observed for the Y140A mutation while the Y142A and Y152A mutations produced large increases in the K$_i$ (Table 1 and Figure 3). For W89F and W89Y, the changes in K$_i$ observed for lerisetron were much smaller than for the alanine mutations at Y142A and Y152A, as would be expected for the less severe nature of these mutations. The changes were, however significant (p < 0.001 in both cases) and are similar to the changes in K$_d$ reported for [3H]granisetron. The increase in K$_i$ on the W89F mutant receptor was 4.8 ± 0.56 fold and the increase in K$_i$ on the W89Y receptor was 3.6 ± 0.4 fold. The R91A mutant produced an increase in K$_i$ of 7.6 ± 1.5 fold as compared to the wildtype receptor. These data indicated potential interactions of lerisetron with W89, R91, Y142 and Y152.

Mutation of W89
As mentioned above, the lack of [3H]-granisetron binding to W89A mutant receptors necessitated the use of W89F and W89Y mutations to analyze functional group interactions. The effects of these mutations on the K$_i$ for analogs 1 and 2 are shown in figure 4B, 4C and Table 1. Analog 1 inhibited [3H]-granisetron binding to W89F receptors with a K$_i$ of 170 ± 54 nM (7 ± 3.2 fold increase, p < 0.001) and W89Y receptors with a K$_i$ of 81 ± 14 nM (3.2 ± 0.6 fold increase, p < 0.001). This reflects a significant increase in K$_i$ and reflects a potential interaction of analog 1 with W89. The strength of this interaction is apparently similar to the strength of the interaction with [3H]-granisetron and lerisetron since the magnitude of the change is

similar in both cases. Analog 2 also showed a significant increase in K$_i$ as a result of the W89F and W89Y mutations. The magnitude of the change for W89F (5.1 ± 1.3 fold, p < 0.05) was similar to that observed for lerisetron and analog 1. The W89Y mutation produced a 6.8 ± 1.6 fold change in K$_i$ (p < 0.05). Thus, all three compounds appear to form similar interactions with W89.

Mutation of R91
Mutation of R91 to alanine (R91A) resulted in a significant, but small increase in K$_i$ for lerisetron of 7.6 ± 1.5 fold (p < 0.01). Figure 4D shows the inhibition of [3H]-granisetron binding by analogs 1 and 2 at R91A mutant receptors. No significant change in K$_i$ was observed on these receptors for either analog 1 (0.9 ± 0.28 fold) or analog 2 (0.56 ± 0.14 fold) (Table 1) as compared to the wildtype receptor.

Mutation of Y142
Mutation of Y142 to alanine produced one of the largest observed changes in K$_i$ for lerisetron (Figure 3 and Table 1). The K$_i$ obtained for lerisetron was 130 ± 28 nM, reflecting a change of 160 ± 37 fold compared to wildtype receptors. The K$_i$ value for analog 1, in contrast, increased only 6.8 ± 2.3 fold (p < 0.01) as a result of this mutation (Figure 4E and Table 1). The K$_i$ for analog 2 showed a similar change of 17 ± 0.77 fold (p < 0.01). While these K$_i$ values are significantly different from wildtype values for each analog, the lack of larger effects suggests that neither analog 1 nor analog 2 bind as strongly as lerisetron to Y142.
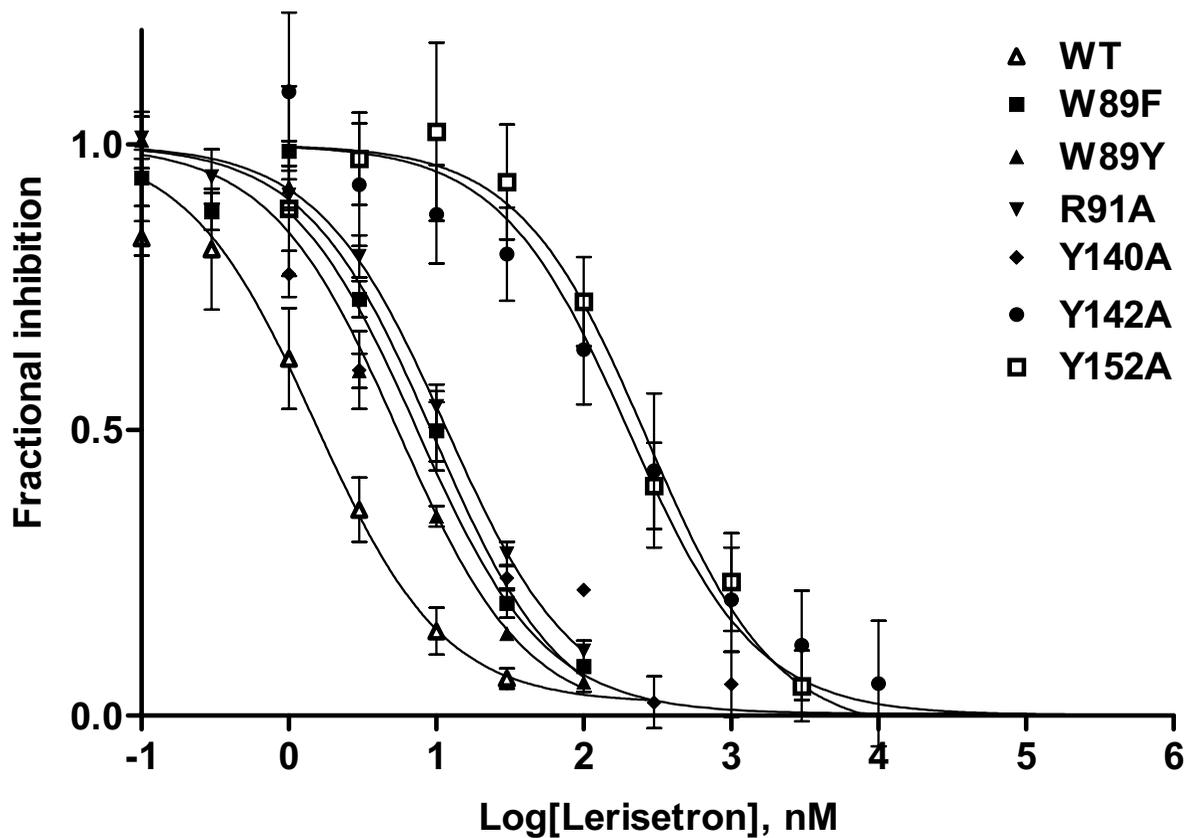
Figure 3
Inhibition of [3H]-granisetron binding to mutant and wildtype receptors by lerisetron. Competition binding assays were performed as described in Materials and Methods. Each point represents a mean $\pm$ SE of n>4 experiments. K_i values calculated from this data and the relative magnitude of the shift in Ki compared to wildtype receptors are shown in Table 2. Y140A produced no significant change in K_i values. W89F, W89Y and R91A mutations produced moderate increases in K_i. Y142A and Y152A mutations resulted in increases in K_i of 160 and 190 fold respectively.

Mutation of Y152

The Y152A mutation showed the most variability in its effects on K_i values for lerisetron, analog 1 and analog 2 (Figure 3, Figure 4F and Table 1). Lerisetron inhibited [3H]-granisetron binding with a K_i value of 150 $\pm$ 36; an increase of 190 $\pm$ 43 fold compared to wildtype values. The K_i value for analog 1 increased from 25 $\pm$ 3.2 nM (wildtype) to 2.5 $\pm$ 0.40 μM. This change of 100 $\pm$ 16 fold is slightly smaller, but not significantly different from the relative change observed for lerisetron. The K_i for analog 2 increased from 0.32 $\pm$ 0.08 μM on wildtype to 13 $\pm$ 4.2 μM on Y152A mutant receptors (40 $\pm$ 12 fold increase). The increase observed for analog 2 was significantly less than that observed for both lerisetron and analog 1. The smaller change in K_i for analog 2 suggests that analog 2 binds weakly to Y152 while lerisetron and analog 1 bind more tightly.

Discussion

The purpose of this study was to ascertain the functional group interactions of lerisetron with specific amino acids present in the 5-HT$_3$R binding site. To this end, we examined the effects of specific amino acid mutations on the binding of lerisetron and two analogs. These analogs, shown in Figure 1, are identical to lerisetron in structure; however, each analog lacks a single functional group found in lerisetron. By comparing the effects of a specific amino acid mutation on the K_i for inhibition of [3H]-granisetron binding, we can begin to identify the functional groups likely to participate in that interaction. Five different amino acids were tested: W89, R91, Y140, Y142 and Y152. Each of these amino acids has been shown in previous studies to alter the binding of 5-HT$_3$R ligands [24,25]. A summary of the changes in K_i is presented as a bar chart in Figure 5A and 5B.
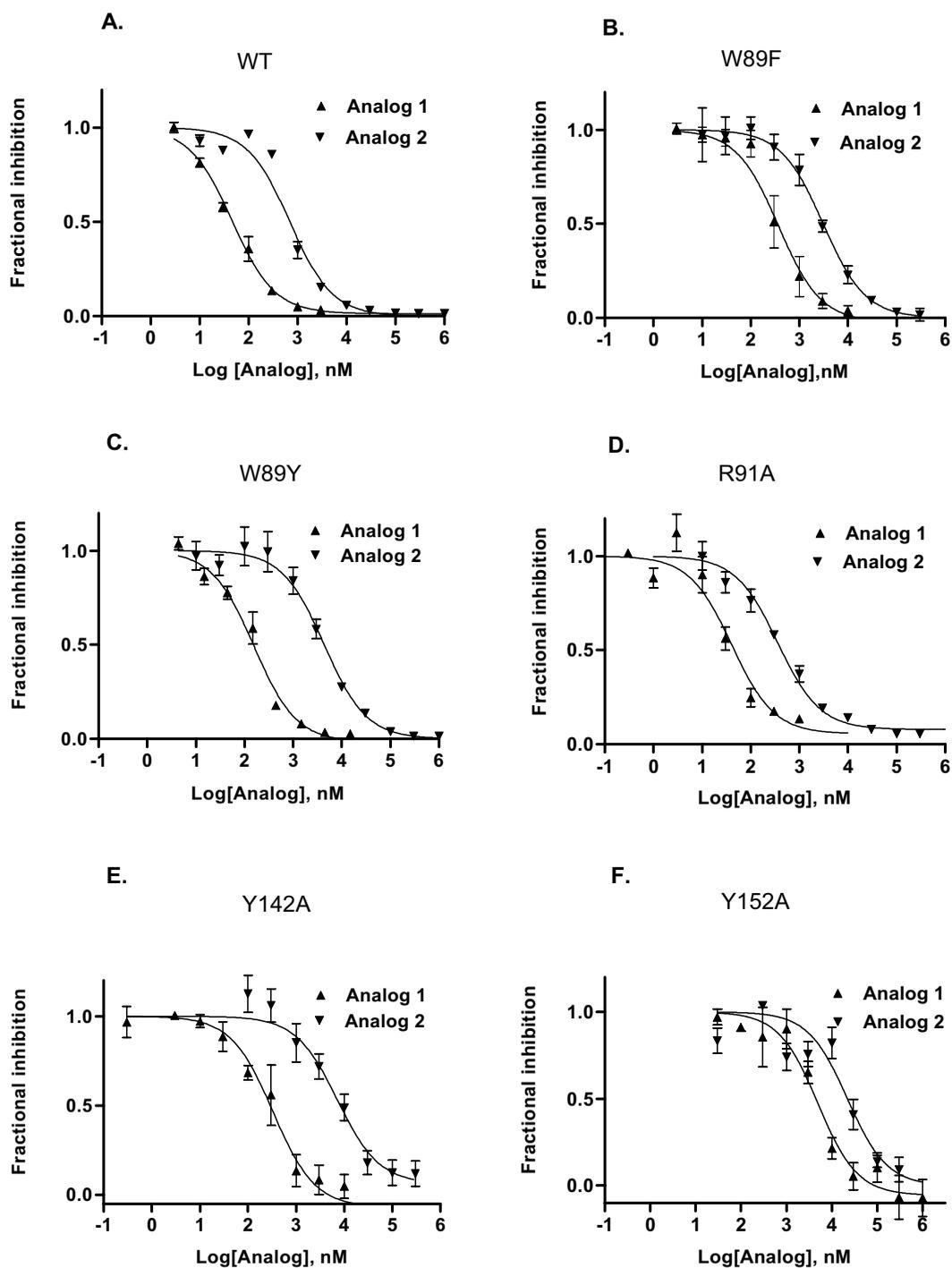
Figure 4
Inhibition of [^{3}H]-granisetron binding to wildtype and mutant receptors by analog 1 and analog 2. Competition binding assays were performed as described in Materials and Methods. Each point represents a mean ± SE of n> 4 experiments. IC_{50} values were determined from the data and a K_i value calculated using the Cheng-Prusoff equation. K_i values for inhibition on each mutant are shown in Table 1 for all three compounds. **A.** Wildtype **B.** W89F **C.** W89Y **D.** R91A **E.** Y142A **F.** Y152A

Table 1: Summary of inhibition data obtained for lerisetron, analog 1 and analog 2 on mutant and wildtype receptors. K_i values were calculated from IC_{50} values as previously described. All data is the result of n > 4 experiments and is expressed as the mean $\pm$ SE. The relative change in K_i was calculated as the K_i (mutant) / K_i (wildtype) and indicates the increase in K_i relative to wildtype $\pm$ SE. * indicates significant difference in fold change value. K_d values for [3H]granisetron binding to wt and mutant receptors were reproduced from reference 25.

Ligand	[3H] Granisetron		Lerisetron		Analog 1		Analog 2	
Amino acid	K_d (nM)	Fold change	K_i (nM)	Fold change	K_i (nM)	Fold change	K_i (nM)	Fold change
WT	0.98 ± 0.12	1	0.8 ± 0.19	1	25 ± 3.2	1	320 ± 82	1
W89F	5.7 ± 0.7	5.8 ± 0.7	3.8 ± 0.46	4.8 ± 0.56*	170 ± 54	7.0 ± 3.2*	1600 ± 400	5.1 ± 1.3*
W89Y	18 ± 2.0	18 ± 2.1	2.8 ± 0.14	3.5 ± 0.38*	81 ± 14	3.2 ± 0.6*	2200 ± 520	6.8 ± 1.6*
R91A	4.9 ± 0.7	5 ± 0.8	6.1 ± 1.16	7.6 ± 1.45*	22.7 ± 8	0.9 ± 0.28	180 ± 27	0.56 ± 0.14
Y140A	2.7 ± 0.2	2.8 ± 0.3	2.1 ± 0.33	2.6 ± 0.37	88 ± 45	3.5 ± 1.7	470 ± 210	1.4 ± 0.65
Y142A	4.5 ± 0.5	4.6 ± 0.5	130 ± 28	160 ± 37	170 ± 58	6.8 ± 2.3*	5500 ± 240	17 ± 0.77
Y152A	7.8 ± 1.1	8 ± 1.1	150 ± 36	190 ± 43	2500 ± 400	98 ± 16	13000 ± 4200	40 ± 12

While lerisetron binds to wildtype receptors with high affinity, analog 1 binds with lower affinity (31 fold increase in K_i compared to lerisetron). Analog 2, which lacks the terminal nitrogen of lerisetron, binds with even lower affinity (a 400-fold fold increase in K_i compared to lerisetron – Table 1). These data demonstrate the relative importance of the N-benzyl and the terminal nitrogen moieties for lerisetron binding. Cation-pi interactions involving aromatic residues and a quaternary ammonium have been demonstrated to play a major role in binding of nicotinic ligands to nAchRs [29,30]. Our data support a similar role for Y142 and Y152 in binding of lerisetron to the 5-HT$_{3A}$ receptor.

Functional group interactions of W89
The W89F mutation produced a significant increase in K_i for all three compounds. The magnitude of the change was similar in all cases. In addition, the increase in K_i was identical to the increase in K_d that has been observed for [3H]granisetron binding on this mutant [24]. Alterations in K_i resulting from the W89Y mutation were slightly less, however the change was again the same for lerisetron, analog 1 and analog 2. These data suggest that all three compounds form binding site interactions with W89. The interaction between lerisetron and W89 is unlikely to be via the N-benzyl functional group since the K_i for analog 1 was also altered by this mutation. The same argument can be made for the distal piperazine nitrogen since the K_i for analog 2 also increased. The portion of the molecule common to all three compounds, the aromatic benzimidazole, is thus the most likely point of interaction for W89.

***Functional group interactions of R91***
The R91A mutation increased the K_i value for lerisetron inhibition of [3H]granisetron binding by 7.6 fold. This is

a moderately small change for an alanine mutation, particularly considering that the smallest change in K_i for removal of a functional group on lerisetron (the N-benzyl group) was 31 fold. It is therefore likely that this interaction is either extremely weak or the change in K_i is the result of a structural change in the binding site. Previous studies concluded that R91 was an important interaction for the 5-HT$_3$R agonist 5-hydroxytryptamine (5-HT), since the K_i for 5-HT inhibition increased over 3000 fold as a result of the R91A mutation [24]. A change in K_d for [3H]granisetron binding to R91A was also observed. In order to determine whether the N-benzyl or distal piperazine nitrogen of lerisetron was involved in an interaction with R91, we tested both analog 1 and 2 on R91A mutant receptors. No change in K_i was observed for either compound. This result makes it much more difficult to assign the correct functional group to this amino acid since it suggests that one or both of the compounds is no longer binding the receptor in precisely the same manner as lerisetron. Considering the small change observed for lerisetron binding as a result of this mutation, even a slight reorientation of the molecule in the binding site could result in the loss of this interaction.

Functional group interactions of Y142
The K_i values for inhibition of [3H]granisetron binding by analogs 1 and 2 were altered only slightly by the Y142A mutation. The magnitude of the increase in K_i for lerisetron, however, was considerably larger (160 fold) and is indicative of an important interaction of the compound with Y142. The lack of a large change in K_i for both analogs makes it difficult to interpret this data since one or both of the compounds appears to be interacting differently with the binding site than lerisetron.

A.
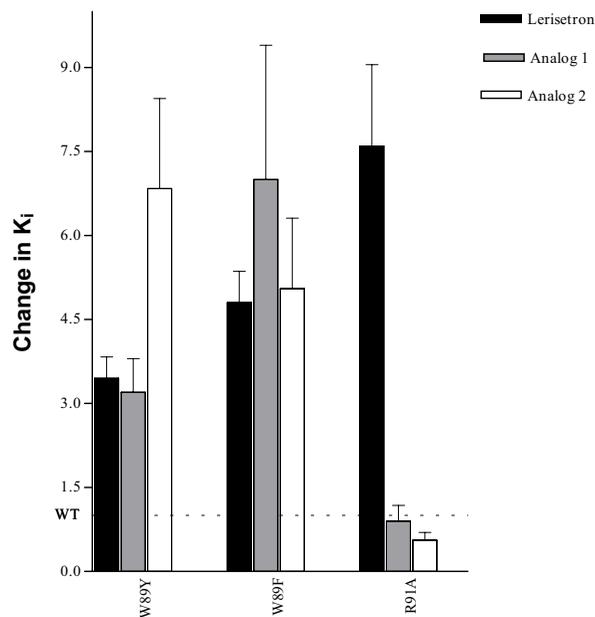
B.

Figure 5
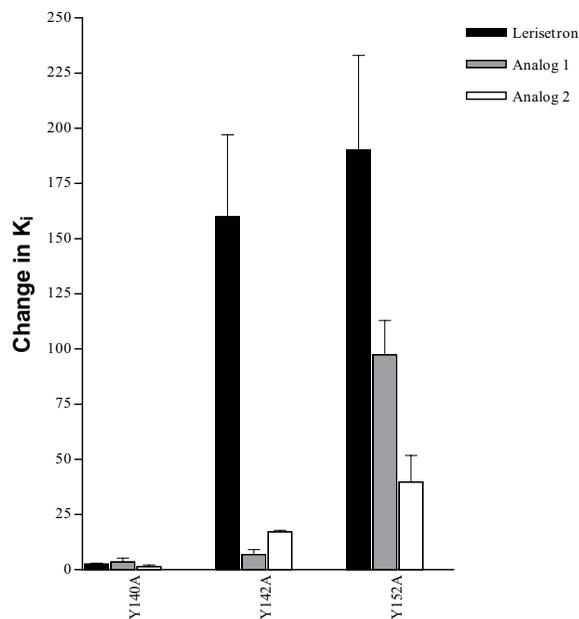**Relative increases in K_i for lerisetron, analog 1 and analog 2 on mutant and wildtype receptors.** The relative change in K_i was determined as K_i (mutant)/ K_i (wildtype) for each compound. **A.** W89Y, W89F, R91A **B.** Y140A, Y142A, Y152A. Lerisetron showed a significant increase in K_i for all mutants except Y140A. Increases in K_i were observed for analog 1 and 2 on W89 and Y152A.

Analogs 1 and 2 differ from each other both in the functional groups contained in the molecule and their structural similarity to lerisetron. Analog 2 is most similar in overall structure. The substitution of oxygen for the distal amino nitrogen alters the potential interactions formed at this position, but is likely to have a small effect on the overall size and shape of the molecule. Analog 1 is far less similar to lerisetron and more similar to the 5-HT$_3$R antagonist granisetron. Previous studies have shown that the binding of granisetron is not affected by the Y142A mutation [25]. Analog 1 may bind more similar to granisetron than lerisetron and thus would be unaffected by mutations at Y142. This is less likely to be the case with analog 2.

The strength of the putative interaction at Y142 can be identified by examining the change in binding of lerisetron as a result of the Y142A mutation. The Y142A mutation produced a 160 fold change in K$_i$. This change reflects the binding energy lost as a result of the alanine substitution. The observed change in Ki on wt receptors is much larger than that observed for removal of the N-benzyl group (31 fold), but is similar to that observed for substitution of the distal amino nitrogen in analog 2 (400 fold). Taken together with the close structural similarity of analog 2 to lerisetron, it can be concluded that comparison of analog 2 and lerisetron should provide the best means of identifying the interaction at Y142. No change in K$_i$ was observed for analog 2 as a result of the Y142A mutation indicating a lack of any significant interaction of this compound with Y142. These data support our hypothesis that Y142 interacts with the distal piperazine nitrogen of lerisetron. A second amino acid may also be involved since the change in K$_i$ for lerisetron binding as a result of the Y142A mutation was smaller than the change produced by substitution of the piperazine nitrogen. As described below, one candidate for this second amino acid is Y152.

Functional group interactions of Y152
The Y152A mutation produced increases in K$_i$ for all three compounds although the magnitude of the change differed. The increases in the K$_i$ values were 190 fold for lerisetron, 98 fold for analog 1 and only 40 fold for analog 2. Thus, analog 1 retains much of its ability to interact with Y152 despite the absence of the N-benzyl group, while analog 2 interacts more weakly with this amino acid. Since the K$_i$ for analog 1 is increased by the Y152A mutation, it is unlikely that the N-benzyl group interacts with Y152. The small change in K$_i$ for analog 2 supports a partial interaction of Y152 with the distal piperazine nitrogen although some interaction with another group is also apparent. This other group would be expected to be in close proximity to the distal nitrogen. The most likely candidate is the other nitrogen of the piperazine ring. Thus Y152A

may form a partial interaction with both piperazine nitrogens.

Conclusions
Figure 6 shows a hypothetical model of the lerisetron-binding site supported by our observations. The model illustrates the secondary structure of the region of the receptor from Y140 – Y152 in a loop configuration. This structure is supported by site-directed mutagenesis data [25] as well as structural predictions obtained from other LGIC receptors [31]. The recent determination of the structure of a nicotinic acetylcholine binding protein [27] that shares significant homology with the LGIC family also supports a loop structure in this part of the protein. The region from W89 through Y93 is shown as a β-sheet as has been hypothesized based on site-directed mutagenesis studies of this strand of the 5-HT$_3$R [24]. Our data indicate the functional groups of lerisetron that may interact with W89, R91, Y142 and Y152.
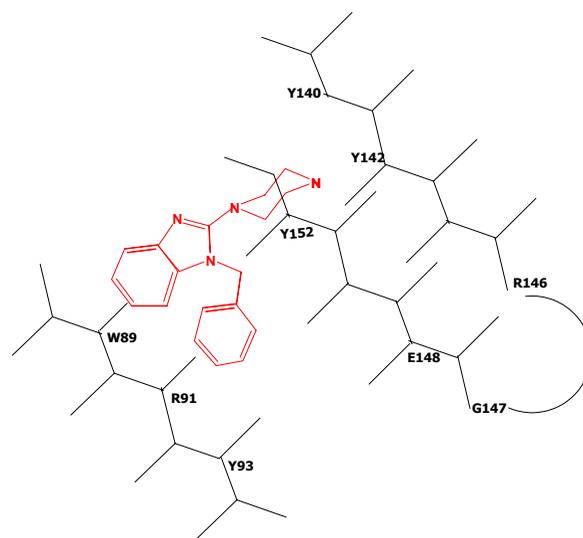

Figure 6
Model of the 5-HT$_3$R binding site. The model illustrates the major points discussed in the text. Lerisetron is shown spanning the region between the D and E loops of the receptor. Both loops are illustrated as β-sheets as described. The side chains are presumed to point in or out of the page depending on their position. W89, R91, Y140, Y142 and Y152 all presumably extend into the binding site. The terminal piperazine nitrogen is shown interacting primarily with Y142, but is also capable of forming an interaction with Y152. Y152 may also form an interaction with the second piperazine nitrogen. W89 is illustrated as forming an aromatic interaction with the benzimidazole. While R91 is shown in close contact to the N-benzyl group, the data support only a weak interaction at this point.

W89 is shown interacting with the aromatic benzimidazole group of lerisetron although the precise position of W89 relative to this group is not known. The W89 interaction with this group is supported by the observed increase in K_i for lerisetron, analog 1 and analog 2. Since the benzimidazole group is common to all three compounds it is the most likely point of interaction. W89 also represents a common interaction in the binding site for both lerisetron and [3H]granisetron.

Y142 is shown interacting with the distal piperazine nitrogen possibly through a cation-π interaction. This orientation of an amino group interacting with an aromatic amino acid in a cation-π interaction has been shown for the nicotinic acetylcholine receptor and has been hypothesized for many LGIC receptors [28–30,26]. This conclusion is based on both the magnitude of the change observed on the wild type receptor for removal of the amino group (400 fold) compared to the effect of the Y142A mutation on lerisetron binding (160 fold) and the lack of any major change in K_i for analog 2 as a result of this mutation. Our data does not support an interaction of this amino acid with either the N-benzyl or benzimidazole portions of lerisetron.

Y152 is shown positioned between the two piperazine nitrogens. This conclusion is supported by the smaller increase in K_i for analog 2 (40 fold) compared to that observed for lerisetron (190 fold). These results suggest a partial interaction of Y152 with the distal piperazine nitrogen. Since some change was observed, a second interaction is also likely. The functional group in closest proximity to the distal piperazine nitrogen is the other nitrogen on the piperazine ring. Another possibility would be the N-benzyl interaction, however, since the Y152A mutation also produced a large increase in K_i for analog 2, this conclusion is not supported by our data.

R91 is shown as interacting with the N-benzyl group. This is a difficult conclusion to make considering the small effect of the R91A mutation on lerisetron binding. The interaction is included in the model based on structural information obtained from the crystal structure of AChBP [27]. The region of this protein homologous to loop E and loop D of the 5-HT$_{3A}$ receptor suggests a loop structure from Y140 to Y152 and a 3-residue turn containing a glycine at position 147 and the β-strand from W89 through Y93 oriented as shown in Figure 6. The orientation of lerisetron between W89 and Y142A as shown would enable the N-benzyl group to be positioned in close proximity to R91. If this is the case, then a small alteration in position of analog 1 or 2 in the binding site could result in the loss of this presumably weak interaction. The apparent alterations in the binding site location of analog 1 would be consistent with this hypothesis. An alternate hypothesis

would place the N-benzyl group in a different position, interacting with another amino acid; either solely or in concert with R91.

Our data support a binding site for lerisetron on the 5-HT$_3$R that spans the D and E loop regions. Table 2 shows the sequence alignment for the 5-HT$_3$R, the α7 receptor and the AChBP for these loops. Sequence alignment of mouse 5-HT$_3$ AR, α7 nAchR and AChBP result in alignment of the proposed D and E loop of the 5-HT$_3$ AR with corresponding regions of the α7 nAchR and AChBP. The amino acids W89, R91 Y140, Y142 and Y152 of the 5HT$_3$ A R can be aligned with W53, Q55, L102, R104, and M114 of the AChBP (Figure 6). These amino acids form a cluster in the proposed acetylcholine binding domain of AChBP similar to that proposed in our model. Both loops have been identified on the complementary face of the binding site of the nAChR. It is unknown if lerisetron utilizes amino acids on the principal face although none have been identified. The model for lerisetron binding will be further refined as its interactions with other binding site amino acids are investigated [26,32,33]. Of particular interest would be potential interactions of the N-benzyl group that would account for the decrease in binding affinity of analog 2. Additional information gained from comparison of our model with the recent crystal structure of the AChBP demonstrates that lerisetron can be roughly 'fit' into the binding site such that all the residues line up as shown in our model. While this is not direct evidence that the model is correct, subsequent molecular modeling of the data presented in this paper may provide further support for our hypothesis. Our current model provides an initial working hypothesis that can form the basis of further investigation. Also, while it is unclear whether the information obtained in this study can be extended to other 5-HT$_3$R ligands, a similar approach would be useful in identifying functional group interactions for mCPBG, 5-HT, dtC and granisetron.

Materials and Methods
Mutagenesis
Wild type 5-HT$_{3AS}$ mouse receptor cDNA was derived from N1E-115 neuroblastoma cells as previously described [24]. Mutant receptors were constructed using polymerase chain reaction (Quick change mutagenesis kit, Promega). All mutations were confirmed by DNA sequencing.

Cell culture methods and transfections
tsA201 cells (a derivative of the HEK293 cell line) were grown in Dulbecco's modified Eagles medium (D-MEM) containing 10% FBS and 100-units/ml penicillin/streptomycin. Cultures were maintained in humidified atmosphere of 5% CO_2 at 37°C. For binding studies, tsA201 cells were plated at a density of 5×10^6 cells/75 cm^2 and

Table 2: Sequence alignment of the mouse 5-HT$_3$R with the mouse nicotinic α7 receptor and the AChBP. Two sequences are shown. The top sequence is the putative D loop containing W89 and R91 (homologous to W53 and Q55 of the AChBP). The lower sequence shows the putative E loop and contains Y140, Y142 and Y152 (homologous to L102, R104 and M114 of the AChBP).

													53		55				
AChBP	E	V	N	E	I	T	N	E	V	D	V	V	F	**W**	Q	**Q**	T	T	W	S
5-HT$_{3A}$	N	V	D	E	K	N	Q	V	L	T	T	Y	I	**W**	Y	**R**	Q	Y	W	T
α$_7$ nAChR	D	V	D	E	K	N	Q	V	L	T	T	N	I	**W**	L	**Q**	M	S	W	T

					102		104									114				
AChBP	V	L	T	P	Q	**L**	A	**R**	V	V	S	D	G	E	V	L	Y	**M**	P	S
5-HT$_{3A}$	P	N	I	P	-	**Y**	V	**Y**	V	H	H	R	G	E	V	Q	N	**Y**	K	P
α$_7$ AChR	A	T	F	H	T	**N**	V	**L**	V	N	S	S	G	H	C	Q	Y	**L**	P	P

grown for 9 hours prior to transfection. Cells were transfected with 10 μg murine 5-HT$_{3AS}$R cDNA using calcium phosphate co-precipitation (New Life Technologies, NY), then incubated 36 hours prior to harvesting. For whole cell patch clamp experiments, tsA201 cells were plated at a density of 0.25×10^6 cells/27 cm^2 dish and grown 12 hours prior to transfection. Cells were washed with fresh culture medium then transfected with 10 μg 5-HT$_{3AS}$R cDNA using Qiagen Superfect transfection reagent (Qiagen, CA). Transfected cells were incubated with this mixture for 2.5 hours, then divided into 35 mm culture dishes at a density of approximately 5×10^4 cells/dish and incubated for 24 hours at 37°C before recording.

Radioligand Binding Assay

Transfected cells were scraped from the dishes, washed twice with Dulbecco's PBS (New Life Technologies, NY), then resuspended in 1.0 ml PBS/100 mm dish. Cells were either used fresh or frozen at this step until needed. Immediately prior to use, cells were homogenized in PBS using a glass tissue homogenizer then centrifuged at $35\,000 \times g$ for 30 minutes in a Beckman JA20 rotor. Membranes were washed once more with PBS then resuspended in 1 ml PBS/100 mm dish. Protein content was determined using a Lowry assay (Sigma. Diagnostics, St. Louis, MO). Binding assays were performed in PBS. For K$_d$ determinations, 100 μl of homogenate was incubated at 37°C for 1 hour with varying concentrations of [^{3}H] granisetron (NEN, MA). Specific binding of [^{3}H] granisetron was determined as the bound [^{3}H] granisetron not displaced by a saturating concentration of a competing ligand (100 μM mCPBG

or 10 μM MDL-72222). K$_d$ values were determined by fitting the binding data to the following equation using GraphPad PRISM (San Diego CA): B = Bmax [L] n / ([L] n + Kn), where θ is bound ligand, Bmax is the maximum binding at equilibrium L is the free ligand concentration and n is the Hill coefficient. For K$_i$ determinations, 100 μl of homogenate was incubated at 37°C for 2 hours with varying concentrations of inhibitor and [^{3}H] granisetron (NEN, MA). Binding was terminated by rapid filtration onto a GF/B filters. The IC$_{50}$ values were calculated by fitting the data to the following equation using GraphPad PRISM (San Diego CA): θ = 1/ (1+(L/IC$_{50}$)), where θ is the fractional amount of [^{3}H] granisetron bound in the presence of inhibitor at concentration L as compared to the amount of [^{3}H] granisetron bound in the absence of inhibitor. IC$_{50}$ is the concentration at which θ = 0.5. The K$_i$ is calculated from the IC$_{50}$ value using the Cheng-Prusoff equation.

Electrophysiological Methods

Transfected tsA201 cells were transferred to a recording chamber and submerged in extracellular recording buffer containing 25 mM HEPES pH 7.4, 140 mM NaCl, 1.7 mM MgCl$_2$, 5 mM KCl, 1.8 mM CaCl$_2$. Patch electrodes (2–2.5 MΩ) were filled with intracellular recording buffer containing 25 mM HEPES pH 7.4, 145 mM KCL, 2 mM MgCl$_2$ and 1 mM EGTA. Cells were clamped in whole cell configuration at a holding potential of -60 mV. Currents elicited by agonist application were measured using an Axopatch 200B amplifier (Foster City, CA) under computer control (DataPac 2000, RUN Technologies). Agonists and antago-

nists were dissolved in extracellular solution and delivered to cells using a rapid perfusion system (Warner Instruments, Hamden, CT). For EC_{50} determinations, responses were normalized to the maximum response obtained from the full agonist 5-HT and fitted to the equation $\Psi = 1/1+(EC50/[C]^n)$, where Ψ is the normalized current at 5-HT concentration [C], EC50 is the concentration of 5-HT needed to obtain half maximal activation and n is the apparent Hill coefficient. For inhibition experiments, cells were exposed to inhibitor alone for 30 s prior to co-exposure with 5-HT. Inhibited responses were calculated as a fraction of the response to 5-HT alone. Data were plotted as the fractional response versus the concentration of inhibitor and analysed using GraphPad software. The IC_{50} value was calculated as the concentration of antagonist inhibiting the 5-HT evoked response by 50%. A K_i value was calculated from the IC_{50} using the Cheng-Prusoff equation.

Synthesis of Lerisetron and its analogs
All target molecules were prepared according to a general 2-step synthesis reported previously by Orjales *et al*. with only slight modification [22,23]. Commercially available 2-chlorobenzimidazole, in dry DMF was treated with a slight excess of NaH, (1.1eq). After stirring for 1 hour at room temperature, one equivalent of the appropriate alkyl bromide was added slowly and the reaction mixture heated under reflux for > 5 hours (the reaction was monitored by TLC). Reaction product was partitioned between water and methylene chloride; organic layer was dried (Na_2SO_4) and concentrated in vacuum. The solid residue was purified by Flash chromatography, which afforded the corresponding N-substituted 2-Chlorobenzimidazole intermediates in good yield. The final step involved a nucleophilic substitution of the 2-chloro group by piperazine at high temperatures. The reaction was performed neat using 4–10 fold excess piperazine and typically heated for a short period only, (30–45 min). Similar work-up afforded a residue that was purified by either crystallization or chromatography. The yields ranged from 40–95%. All compounds were characterized by NMR, MS, HRMS, and /or elemental analysis or were identical to literature reports.

Materials
D-MEM, Penicillin-Streptomycin, fetal bovine serum, and Trypsin were obtained from New Life Technologies. 5-HT and MDL-72222 were obtained from RBI. [^{3}H]-granisetron (84 Ci/mmol) was purchased from New England Nuclear.

Acknowledgements

References
1. Arias HR: **Localization of agonist and competitive antagonist binding sites on nicotinic acetylcholine receptors.** *Neurochem Int* 2000, **36**:595-645
2. Miyazawa A, Fujiyoshi Y, Stowell M, Unwin N: **Nicotinic acetylcholine receptor at 4.6 A resolution: transverse tunnels in the channel wall.** *J Mol Biol* 1999, **288**:765-786
3. Devillers-Thiery A, Galzi JL, Eisele JL, Bertrand S, Bertrand D, Changeux JP: **Functional architecture of the nicotinic acetylcholine receptor: a prototype of ligand-gated ion channels.** *J Membr. Biol,* 1993, **136**:97-112
4. Karlin A, Akabas MH: **Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins.** *Neuron* 1995, **15**:1231-1244
5. Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA: **Sequence and functional expression of the GABA A receptor shows a ligand-gated receptor superfamily.** *Nature* 1987, **328**:221-227
6. Grenningloh G, Rienitz A, Schmitt B, Methfessel C, Zensen M, Beyreuther K, Gundelfinger ED, Betz H: **The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors.** *Nature* 1987, **328(6127)**:215-220
7. Maricq AV, Peterson AS, Brake AJ, Myers RM, Julius D: **Primary structure and functional expression of the 5HT3 receptor, a serotonin-gated ion channel.** *Science* 1991, **254**:432-437
8. Jackson MB, Yakel JL: **The 5-HT3 receptor channel.** *Annu Rev Physiol.* 1995, **57**:447-468
9. Gill CH, Peters JA, Lambert JJ: **An electrophysiological investigation of the properties of a murine recombinant 5-HT3 receptor stably expressed in HEK 293 cells.** *Br J Pharmacol* 1995, **114**:1211-1221
10. Davies PA, Pistis M, Hanna MC, Peters JA, Lambert JJ, Hales TG, Kirkness EF: **The 5-HT3B subunit is a major determinant of serotonin-receptor function.** *Nature* 1999, **397**:359-363
11. Dubin AE, Huvar R, D'Andrea MR, Pyati J, Zhu JY, Joy KC, Wilson SJ, Galindo JE, Glass CA, Luo L, Jackson MR, Lovenberg TW, Erlander MG: **The Pharmacological and Functional Characteristics of the Serotonin 5-HT3A Receptor Are Specifically Modified by a 5-HT3B Receptor Subunit.** *J. Biol. Chem.* 1999, **274**:30799-30810
12. Downie DL, Hope AG, Lambert JJ, Peters JA, Blackburn TP, Jones BJ: **Pharmacological characterization of the apparent splice variants of the murine 5-HT3 R-A subunit expressed in Xenopus laevis oocytes.** *Neuropharmacology.* 1994, **33**:473-482
13. Hope AG, Downie DL, Sutherland L, Lambert JJ, Peters JA, Burchell B: **Cloning and functional expression of an apparent splice variant of the murine 5-HT3 receptor A subunit.** *Eur J Pharmacol.* 1993, **245**:187-192
14. Niemeyer MI, Lummis SC: **Different efficacy of specific agonists at 5-HT3 receptor splice variants: the role of the extra six amino acid segment.** *Br J Pharmacol.* 1998, **123**:661-666
15. Gandara DR, Roila F, Warr D, Edelman MJ, Perez EA, Gralla RJ: **Consensus proposal for 5HT3 antagonists in the prevention of acute emesis related to highly emetogenic chemotherapy. Dose, schedule, and route of administration.** *Support Care Cancer* 1998, **6(3)**:237-343
16. Johnson BA, Roache JD, Javors MA, DiClemente CC, Cloninger CR, Prihoda TJ, PS Bordnick, Ait-Daoud N, Hensler J: **Ondansetron for reduction of drinking among biologically predisposed alcoholic patients: A randomized controlled trial.** *JAMA* 2000, **284(8)**:963-971
17. Ait-Daoud N, Johnson BA, Prihoda TJ, Hargita ID: **Combining ondansetron and naltrexone reduces craving among biologically predisposed alcoholics: preliminary clinical evidence.** *Psychopharmacology (Berl).* 2001, **154**:23-27
18. Hibert MF, Hoffmann R, Miller RC, Carr AA: **Conformation-activity relationship study of 5-HT3 receptor antagonists and a definition of a model for this receptor site.** *J Med Chem* 1990, **33**:1594-1600
19. Clark RD, Miller AB, Berger J, Repke DB, Weinhardt KK, Kowalczyk BA, Eglen RM, DW Bonhaus, Lee CH, Michel AD: **2-(Quinuclidin-3-yl) pyrido [4,3-b] indol-1-ones and isoquinolin-1-ones. Potent conformationally restricted 5-HT3 receptor antagonists.** *J Med Chem.* 1993, **36(18)**:2645-2657

20. Daveu C, Bureau R, Baglin I, Prunier H, Lancelot JC, Rault S: **Definition of a pharmacophore for partial agonists of serotonin 5-HT3 receptors.** *J Chem Inf Comput Sci.* 1999, **39:**362-369

21. Swain CJ, Baker R, Kneen C, Herbert R, Moseley J, Saunders J, Seward EM, Stevenson GI, Beer M, Stanton J: **Novel 5-HT3 antagonists: indol-3-ylspiro (azabicycloalkane-3, 5'(4'H)-oxazoles).** *J Med Chem* 1992, **35:**1019-1031

22. Orjales A, Mosquera R, Labeaga L, Rodes R: **New 2-piperazinyl-benzimidazole derivatives as 5-HT3 antagonists. Synthesis and pharmacological evaluation** *. J. Med. Chem* 1997, **40:**586-593

23. Parihar HS, Suryanarayanan A, Ma C, Joshi P, Venkataraman P, Schulte MK, Kirschbaum KS: **5-HT (3) R binding of lerisetron: an interdisciplinary approach to drug-Receptor interactions.** *Bioorg Med Chem Lett* 2001, **11:**2133-2136

24. Yan D, Schulte MK, Bloom KE, White MM: **Structural features of the ligand-binding domain of the serotonin 5HT3 receptor.** *J Biol Chem* 1999, **274:**5537-5541

25. Venkataraman P, Schulte MK: **Site directed mutagenesis of Y140 – K153 of the serotonin type 3 receptor binding site supports a** β**-configuration.** *Soc. Neurosci. Abstracts* 2000, **26:**613.13 (abstract).

26. Spier AD, Lummis SCR: **The role of tryptophan residues in the 5-Hydroxytryptamine (3) receptor ligand binding domain.** *J Biol Chem* 2000, **275:**5620-5625

27. Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB, Sixma TK: **Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors.** *Nature* 2001, **411:**269-276

28. Dougherty DA: **Cation-pi interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp.** *Science* 1996, **271:**163-168

29. Zhong W, Gallivan JP, Zhang Y, Li L, Lester HA, Dougherty DA: **From ab initio quantum mechanics to molecular neurobiology: a cation-pi binding site in the nicotinic receptor.** *Proc Natl Acad Sci U S A* 1998, **95:**12088-12093

30. Li L, Zhong W, Zacharias N, Gibbs C, Lester HA, Dougherty DA: **The tethered agonist approach to mapping ion channel proteins–toward a structural model for the agonist binding site of the nicotinic acetylcholine receptor.** *Chem Biol.* 2001, **1:**47-58

31. Kwasigroch J, Chomileir J, Mornon J: **A global taxonomy of loops in globular proteins.** *J. Mol. Biol.* 1996, **259:**855-872

32. Steward LJ, Boess FG, Steele JA, Liu D, Wong N, Martin IL: **Importance of phenylalanine 107 in agonist recognition by the 5-hydroxytryptamine (3A) receptor.** *Mol Pharmacol* 2000, **57:**1249-55

33. Boess FG, Steward LJ, Steele JA, Liu D, Reid J, Glencorse TA, Martin IL: **Analysis of the ligand binding site of the 5-HT3 receptor using site directed mutagenesis: importance of glutamate 106.** *Neuropharmacology.* 1997, **36:**637-47