Identification of Amino Acid Residues Important for Sarpogrelate Binding to the Human 5-Hydroxytryptamine2A Serotonin Receptor

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Abstract. The purpose of the present study was to examine 5-hydroxytryptamine (5-HT)2A-receptor sarpogrelate interactions by site-directed mutagenesis. Based on molecular modeling studies, aspartic acid (Asp)155[3.32] and tryptophan (Trp)151[3.28] in transmembrane helix (TMH) III and Trp336[6.48] in TMH VI of the 5-HT2A receptor were found to interact with sarpogrelate. All of these residues were mutated to alanine (Ala). The Asp3.32Ala mutant did not exhibit any affinity for [3H]ketanserin, whereas the Trp3.28Ala mutant showed a markedly decrease in the binding affinity for [3H]ketanserin (Kd >10,000 nM). Therefore, it was not possible to find any sarpogrelate affinity to the mutants using [3H]ketanserin. The mutation also abolished agonist-stimulated formation of [3H]inositol phosphates (IP) in both cases. On the other hand, the Trp6.48Ala mutant showed reduced binding affinity for [3H]ketanserin (Kd 2.0 nM vs 0.8 nM for the wild-type receptor) and had reduced affinity for sarpogrelate (pKi value of 5.71 vs 9.06 for the wild-type receptor). The Trp6.48Ala mutant also showed the greatest decrease in sensitivity to sarpogrelate (pKb value 8.81 for wild-type and 5.11 for mutant) in inhibiting agonist-stimulated IP formation. These results provide direct evidence that Asp3.32, Trp3.28, and less importantly, Trp6.48 are responsible for the interaction between the 5-HT2A receptor and sarpogrelate. In addition, these results support the findings obtained from molecular modeling studies.

Keywords: 5-hydroxytryptamine (5-HT), 5-HT2A receptor, sarpogrelate, site-directed mutagenesis, molecular modeling

Introduction

Serotonin (5-hydroxytryptamine, 5-HT), an indoleamine neurotransmitter, plays an important role in a wide variety of activities in the central nervous, gastrointestinal, and cardiovascular systems (1). The biological actions of 5-HT in these systems are mediated by seven major families of 5-HT receptors: 5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT5, 5-HT6, and 5-HT7. To date, fifteen distinct subtypes of these receptors have been identified by molecular techniques on the basis of their binding profile, functional activity of ligands, sequence homology, and common secondary messenger coupling (2). Among them, Gq-coupled 5-HT2A receptors in platelets and vascular smooth muscle cells are mainly associated with the regulation of cardiac and vascular events in the cardiovascular system (3). The 5-HT2A receptors mediate 5-HT-induced platelet aggregation, thrombus formation, coronary artery spasm, and vascular smooth muscle contraction (4). All of these processes play an important role in the pathogenesis of a wide variety of ischemic heart diseases. Sarpogrelate, a 5-HT2 antagonist, has been found to have therapeutic potentials on...
these pathophysiological effects (5).

Efforts to improve clinical therapies by developing more receptor-specific, novel therapeutic agents have been hampered in part by a lack of knowledge concerning the precise three-dimensional structure of ligand-receptor interactions. In the absence of a high-resolution crystal structure for 5-HT$_{2A}$ (or any other G-protein-coupled receptor: GPCR), site-directed mutagenesis accompanied by molecular modeling has been used to uncover the details of ligand binding. Based on radioligand binding and functional studies, it was shown that sarpogrelate exhibits specificity toward 5-HT$_2$ receptors since it has lack of significant 5-HT$_3$, 5-HT$_4$, $\alpha_1$-, $\alpha_2$-, and $\beta$-adrenergic receptor, histamine H$_1$, H$_3$, and muscarinic M$_3$ antagonistic activity (6 – 8). It was also shown that sarpogrelate has high affinity to 5-HT$_{2A}$ receptors (9). However, the molecular mechanisms by which sarpogrelate binds to 5-HT$_{2A}$ receptors remain unknown. Molecular modeling of sarpogrelate to 5-HT$_{2A}$ receptors predicted that sarpogrelate makes strong electrostatic interactions towards aspartic acid (Asp)3.32 and tryptophan (Trp)3.28 in transmembrane helix (TMH) 3 and Trp6.48 in TMH 6 of 5-HT$_{2A}$ receptors (9). In the present study, we have used site-directed mutagenesis techniques to identify these amino acid residues that are important for sarpogrelate binding and agonist-stimulated inositol phosphates (IP) accumulation. The effects of two mutations in TMH 3 (Asp3.32 alanine (Ala) and Trp3.28Ala) and one mutation in TMH 6 (Trp6.48Ala) of the human 5-HT$_{2A}$ receptor on sarpogrelate binding and agonist-stimulated IP accumulation were examined. Our findings are consistent with the molecular modeling data that these mutations are the most important for sarpogrelate binding as demonstrated by the marked decrease in affinity of sarpogrelate for the mutant receptors.

Materials and Methods

DNA constructs

A cDNA clone encoding human 5-HT$_{2A}$ receptor was generously provided by Dr. Stuart C. Sealfon (10). The insert was subcloned from pAlter (Promega, Madison, WI, USA) by SmaI-XbaI digestion. For expression, the insert was subcloned into the EcoRV and XbaI sites of pcDNA3 (Invitrogen Life Technologies, Carlsbad, CA, USA). 5-HT$_{2A}$-receptor mutants were constructed with the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mutations were confirmed by sequencing the mutation site in the expression vector.

Cell culture and transfection

Both COS-7 and HEK293 cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. COS-7 cells were transiently transfected in 10-cm dishes at about 80% confluence with Polyfect Transfection Reagent using the manufacturer’s protocol (Qiagen, Valencia, CA, USA). Stably expressing cell lines were constructed in HEK293 cells by transfecting with Lipofectamine$^{\text{TM}}$ 2000 reagent and selecting with 1.0 mg/ml G418-containing growth medium as detailed by the manufacturer’s protocol (Invitrogen Life Technologies, Rockville, MD, USA).

IP accumulation assay

Accumulation of total $[^3]$H]IPs was assayed as described previously (11). Stably transfected HEK293 cells at about 90% confluent in 10-cm dishes were seeded into 24-well plates. Twenty-four hours after seeding, cells were washed with serum-free DMEM and labeled with 1 $\mu$Ci/ml $[^3]$H]myo-inositol in serum-free DMEM for 18 h. After labeling, the medium was replaced with the assay medium (Hank’s buffered salt solution containing 20 mM HEPES and 20 mM LiCl, pH 7.4, 37°C). The cells were incubated for 15 min at 37°C by floating the plates in a temperature-controlled water bath. Both agonists and antagonists in assay medium were added to each well and incubation continued for an additional 18 min. Assay medium was removed and the reaction was stopped by adding 1 ml of 10 mM formic acid (previously stored at 4°C) to each well. The plates were stored at 4°C for 2 h and cells were neutralized by adding 1 ml 500 mM KOH and 9 mM sodium tetraborate per well. The contents of each well were extracted and centrifuged for 5 min at 1400 × g and the upper layer loaded onto a 1-ml AG1-X8 resin (100 – 200 mesh; Assist Co., Tokyo) column. Columns were washed 2 times with 5 ml 60 mM sodium formate and 5 mM borax. Total IPs were eluted with 5 ml 1 M ammonium formate and 0.1 M formic acid. Radioactivity was measured by liquid scintillation spectrophotometry.

Ligand binding assay

Transiently transfected COS-7 cells were grown in 100-mm plates. The plates were washed three times with ice-cold phosphate-buffered saline (PBS), and cells were harvested by scraping with a rubber policeman. Cells were centrifuged at 14,000 × g for 2 min. After removing the supernatant, cell pellets were stored at −80°C until use. All membrane preparation procedures were carried out at 4°C. Cell pellets were thawed and
homogenized in 1 ml of 50 mM Tris-HCl (pH 7.4 at room temperature) (buffer A) with a polytron homogenizer (Kinematica AG, Lucerne, Switzerland) (setting 7 for 8 s). The homogenates were centrifuged at 35,000 × g for 15 min. The membrane pellets were resuspended in buffer A with a Teflon glass homogenizer (10 strokes by hand). Protein content was measured by the method of Lowry et al. (12). Each binding incubation tube contained approximately 50 µg of membrane protein, [³H]ketanserin (NEN Life Sciences, Boston, MA, USA), unlabeled drug as required, and buffer A in a final volume of 1.0 ml. Both saturation and competition binding assays were carried out as described previously (11). Briefly, for saturation binding studies, seven to eight concentrations (0.1 – 10 nM for both wild type and mutants) of [³H]ketanserin (specific activity of 76.5 Ci/mmol) were tested in duplicate. For competition binding studies, the concentration of [³H]ketanserin with wild-type receptors was 1.0 nM and 10 concentrations of competing ligands were used in duplicate. Because mutation of the 5-HT₂A receptor resulted in a reduced affinity for [³H]ketanserin, competition studies for the Trp6.48Ala mutant were carried out with 2.0 nM [³H]ketanserin. Nonspecific binding was defined with the use of 10 µM mianserin. For receptor binding using radioligand, incubations were carried out for 30 min at 37°C and were terminated by rapid filtration through Whatman GF/C filters that had been presoaked in 0.3% polyethyleneimine followed by washing with 10 ml ice-cold buffer A. The radioactivity retained on the filters was quantitated by liquid scintillation spectrophotometry.

Western blotting

Transiently transfected COS-7 cells and stably transfected HEK293 cells expressing 5-HT₂A wild type and mutant receptors were constructed according to the methods described above. Confluent layers of both cells were washed three times with ice-cold PBS, lysed in the sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol), and then incubated for 5 min at 95°C. Protein concentration was determined by the method of Lowry et al. (12). Equal amounts (20 µg/lane) of various protein samples were resolved by SDS-polyacrylamide gel electrophoresis (12.5% gradient gels) and transferred onto ProBlott membranes (Applied Biosystems, Foster city, CA, USA) using a semi dry system in immuno-transfer buffer. The membranes were blocked in blocking buffer (PBS with 5% nonfat dried milk) overnight at 4°C and then incubated for 2 h at room temperature with mouse anti-5-HT₂A R monoclonal antibody (clone G186-1117; BD PharminGen, Oxford, UK) at 1:1000 dilutions in blocking buffer. The membranes were washed three times with PBS containing 0.05% Tween-20 and incubated with alkaline phosphatase-conjugated anti-mouse goat IgG for 1 h at room temperature at 1:2000 dilutions in blocking buffer. The membranes were washed and then the proteins on the membranes were visualized by adding Western blue (Promega) as a substrate.

Residue numbering scheme

Receptor residues are numbered according to a consensus numbering scheme described previously (13). On the basis of this scheme, the most conserved helix 3 residue, R173, is designated with the index number 3.50 and is thus referred to as R3.50. The ‘3’ designates helix 3 and ‘.50’, the position of the most conserved locus in the helix. Residues C-terminal to this conserved locus are numbered in increasing order and N-terminal residues are numbered in decreasing order. Thus the Asp155 and the Trp151 are referred to as Asp3.32 and Trp3.28, respectively. Similarly, the Trp336 in the helix 6 is referred to as Trp6.48.

Molecular modeling

The approaches utilized to construct a three-dimensional model of the helix bundle of the 5-HT₂A receptor with sarpogrelate based on the structure of rhodopsin have been described previously (9).

Data analysis

Nonlinear regression analysis of saturation and competition binding assay were performed using GraphPad Prism software (San Diego, CA, USA). pKᵢ = negative logarithm of the equilibrium dissociation constant in nM. The pKᵢ values were estimated from the inhibitory effects of the antagonists on the concentration-dependent total inositol phosphate accumulation curve for serotonin according to the literature by Furchgott (14). pKₛ = negative logarithm of Kₛ value, where Kₛ value was determined using the following equation:

\[
\text{EC}_{50} \text{ antagonist} / \text{EC}_{50} \text{ agonist} - 1 = [B] / K_b,
\]

where, EC₅₀ antagonist = Concentration of agonist in presence of a particular concentration of antagonist ([B]) at which 50% of total IP is produced, EC₅₀ agonist = Concentration of agonist at which 50% of total IP is produced, [B] = Concentration of antagonist that is in 85 – 100 times higher than Kᵢ value, Kₛ = Dissociation constant of antagonist. The agonist and antagonist EC₅₀ values were calculated by nonlinear analysis using GraphPad Prism software. Statistical analyses were performed by Student’s t-test.
Ketanserin, mianserin, and ritanserin were obtained from RBI (Research Biochemical Incorporated, Natick, MA, USA) and sarpogrelate was from Mitsubishi Chemical Corporation, Tokyo. Serotonin was obtained from E. Merck, Darmstadt, Germany.

Results

Radioligand binding of wild-type and mutant receptors

Saturation binding analysis: [3H]Ketanserin was used to determine the dissociation constant ($K_d$) and maximum bound ($B_{\text{max}}$) values for each of the mutant and the wild-type receptors. Representative saturation isotherms for two of the constructs are shown in Fig. 1, A and B. The wild-type receptor bound the ligand with high affinity ($K_d = 0.8 \pm 0.01 \text{nM}$) (Table 1), whereas no detectable affinity with ligand was found with the Asp3.32Ala mutant and markedly reduced affinity (>10,000 nM) found with the Trp3.28Ala mutant (Table 1). The Trp6.48Ala mutant exhibited 2.5-fold lower affinity than the wild-type receptor ($K_d = 2.0 \pm 0.02 \text{nM}$). On the other hand, $B_{\text{max}}$ values, calculated from the maximal specific binding of [3H]ketanserin, were dependent on the particular construct. No $B_{\text{max}}$ values were obtained with the Asp3.32Ala mutant and the Trp3.28Ala mutant receptors, whereas the levels of expression for the Trp6.48Ala mutant ($B_{\text{max}} = 434.3 \pm 46.0$) were 3-fold lower than those for the wild-type receptor ($B_{\text{max}} = 1464.0 \pm 46.7$). The expression level for a given construct was found to be highly consistent in separate preparations.

Agonist competition: Displacement of [3H]ketanserin by the 5-HT$_{2A}$-receptor agonist, 5-HT, was analyzed and the wild-type values were compared with the properties of the mutant receptors. As the Asp3.32Ala and the Trp3.28Ala mutants eliminated detectable [3H]ketanserin binding, it was not possible to find 5-HT affinity to the mutants using [3H]ketanserin. However, the Trp6.48Ala mutant showed a significant decrease in binding of 5-HT ($pK_i$ value $5.2 \pm 0.02$ vs $7.1 \pm 0.04$ for the wild-type receptor) (Fig. 2, Table 2).

Competition by antagonists: The affinities ($pK_i$ values) of several 5-HT$_2$ antagonists, sarpogrelate, ketanserin, and ritanserin for the wild-type receptors were determined in competition binding experiments for sites labeled with [3H]ketanserin. Figure 3A shows representative competition curves, and all data are summarized in Table 3. The results showed that ketanserin had the highest binding affinity ($pK_i$ value $9.23 \pm 0.04$) among all the antagonists. Sarpogrelate also showed very high binding affinity ($pK_i$ value $9.06 \pm 0.06$), but its affinity is lower than that of ketanserin. Ritanserin ($pK_i$, value $8.34 \pm 0.07$) also had high binding affinity. Sarpogrelate was examined to determine whether it would exhibit a change in binding affinity for the mutant receptors. In the case of both the Asp3.32Ala mutant and the Trp3.28Ala mutant receptors, it was not possible to find any sarpogrelate
binding affinity to the mutants as the mutants were not able to bind \[^{3}H\]ketanserin (Table 4). Sarpogrelate showed a decreased binding affinity (pK\(_i\) value 5.71 ± 0.03) in the Trp6.48Ala mutant receptors (Fig. 3B, Table 4).

Agonist-stimulated IP accumulation

The wild-type and mutant receptors were expressed in stably transfected HEK293 cells, and the concentration–response curves for 5-HT-stimulated IP accumulation were determined. The EC\(_{50}\) value measured for 5-HT on the wild-type receptor was 16.83 ± 0.4 nM (Fig. 4, Table 2). The data of inhibition of 5-HT-stimulated IP formation by several 5-HT\(_2\) receptor antagonists are summarized in Table 3, and these results were qualitatively similar to the binding assay results. Ketanserin (5 × 10\(^{-7}\) M) showed highest potency in inhibiting 5-HT-stimulated IP formation with a pK\(_b\) value of 9.31 ± 0.10. Sarpogrelate (8 × 10\(^{-8}\) M) inhibited 5-HT-stimulated IP formation with a pK\(_b\) value of 8.81 ± 0.11. Ritanserin (4 × 10\(^{-7}\) M) also showed high potency in inhibiting 5-HT-stimulated IP formation and its pK\(_b\) value was 8.57 ± 0.16.

Fig. 2. 5-HT competition for \[^{3}H\]ketanserin binding to wild-type and Trp6.48Ala mutant 5-HT\(_{2A}\) receptors. The data represent the mean ± S.E.M. of three separate experiments performed in duplicate.

Table 2. 5-HT competition for \[^{3}H\]ketanserin binding (pK\(_i\)) and 5-HT efficacy (EC\(_{50}\)) to 5-HT\(_{2A}\) wild-type and mutant receptors

<table>
<thead>
<tr>
<th>Receptors</th>
<th>5-HT</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT(_{2A})-WT</td>
<td>7.1 ± 0.04 (3)</td>
<td>16.83 ± 0.4 (4)</td>
<td></td>
</tr>
<tr>
<td>Asp3.32Ala</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Trp3.28Ala</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Trp6.48Ala</td>
<td>5.2 ± 0.02 (3)*</td>
<td>3856.0 ± 67 (4)*</td>
<td></td>
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</tbody>
</table>

The data shown indicate the mean ± S.E.M. and numbers in parentheses indicate number of experiments. *P < 0.0001 vs wild-type.

Fig. 3. Competition studies of several 5-HT\(_2\) antagonists for \[^{3}H\]ketanserin binding to wild-type 5-HT\(_{2A}\) receptor (A) and sarpogrelate for \[^{3}H\]ketanserin binding to Trp6.48Ala mutant 5-HT\(_{2A}\) receptor (B). The data represent the mean ± S.E.M. of four to five separate experiments performed in duplicate.

Table 3. Binding affinities (pK\(_i\)) and functional affinities (pK\(_b\)) of several 5-HT\(_2\) antagonists for the wild-type 5-HT\(_{2A}\) receptor

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Wild-type 5-HT(_{2A})</th>
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</thead>
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<tr>
<td></td>
<td>pK(_i)</td>
</tr>
<tr>
<td>Ketanserin</td>
<td>9.23 ± 0.04 (5)</td>
</tr>
<tr>
<td>Sarpogrelate</td>
<td>9.06 ± 0.06 (5)</td>
</tr>
<tr>
<td>Ritanserin</td>
<td>8.34 ± 0.07 (4)</td>
</tr>
</tbody>
</table>

The data shown indicate the mean ± S.E.M. and numbers in parentheses indicate number of experiments.

Mutation of the Asp3.32 to Ala eliminated detectable IP accumulation. However, introducing the mutation in helix 7 (Trp6.48Ala) reduced 5-HT-induced IP formation (229-fold) as indicated by an increased EC\(_{50}\) value for 5-HT (3856.0 ± 67 nM) (Fig. 4, Table 2). In the case of the Trp6.48Ala mutant receptor, sarpogrelate (2 × 10\(^{-4}\) M) inhibited 5-HT
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stimulated IP formation with a pKᵦ value of $5.11 \pm 0.13$ (Table 4).

**Western blot analysis**

As the Asp3.32Ala and Trp3.28Ala mutants did not exhibit detectable affinity for the $[^3H]$ketanserin and eliminated detectable 5-HT-stimulated IP accumulation, Western blotting was performed in lysates prepared from COS-7 and HEK293 cells that were transfected with the 5-HT$_{2A}$ wild-type and mutant receptors. Western blots probed with the anti-5-HT$_{2A}$R antibody detected an immunoreactive band of about 55 kDa in both the wild-type and mutant receptor-expressing cells (Fig. 5: A and B), as reported earlier for this antibody in NIH-3T3 cells (15), suggesting that the mutations in TMH 3 did not cause any abnormality in the receptor proteins. It is also suggested that the mutant proteins are inactive but are still expressed and detectable by antibody.

**Discussion**

The present study was undertaken to examine the binding sites of sarpogrelate to 5-HT$_{2A}$ receptor first by mutating the amino acids in the third and sixth transmembrane helices of the receptor and then by comparing the radioligand-binding properties and agonist-stimulated IP production of the wild-type receptors with those of the mutant receptors. Based on the molecular modeling studies of sarpogrelate to 5-HT$_{2A}$ receptor (9), Asp3.32 and Trp3.28 in helix 3 and Trp6.48 in helix 6 may be the important binding sites of sarpogrelate. In addition to the interaction with the cationic head (hydrogen atom of the trimethyl ammonium) of sarpogrelate, Asp3.32 (acidic function oxygen) interacts with hydrogen atom of carboxylate

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**Table 4.** Analysis of binding affinity (pKᵢ) and functional affinity (pKᵦ) of sarpogrelate for mutant 5-HT$_{2A}$ receptors

<table>
<thead>
<tr>
<th>Receptors</th>
<th>pKᵢ</th>
<th>pKᵦ</th>
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</thead>
<tbody>
<tr>
<td>5-HT$_{2A}$-WT</td>
<td>9.06 ± 0.06 (5)</td>
<td>8.81 ± 0.11 (4)</td>
</tr>
<tr>
<td>Asp3.32Ala</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Trp3.28Ala</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Trp6.48Ala</td>
<td>5.71 ± 0.03 (5)**</td>
<td>5.11 ± 0.13 (4)***</td>
</tr>
</tbody>
</table>

WT, wild type. N.D., not determined. The data shown indicate the mean ± S.E.M. and numbers in parentheses indicate number of experiments. ***$P<0.0001$ vs wild-type.

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**Fig. 4.** 5-HT-stimulation of IP production in stably transfected HEK293 cells expressing wild-type and the Trp6.48Ala mutant 5-HT$_{2A}$ receptor. Cells were labeled with myo-$[^3H]$inositol and challenged with increasing concentrations of 5-HT. The data are expressed as a percentage of maximal IP production. Data are the mean ± S.E.M. of four separate experiments performed in duplicate.

**Fig. 5.** Western blot analysis of Asp3.32Ala and Trp3.28Ala mutant receptors and comparison with the wild-type 5-HT$_{2A}$ receptor in transiently transfected COS-7 cells (A) and stably transfected HEK 293 cells (B). Lane 1, human 5-HT$_{2A}$ wild-type; lane 2, Asp3.32Ala mutant; lane 3, Trp3.28Ala mutant.
group of sarpogrelate. The peptide bond oxygen of Trp3.28 interacts with the hydrogen atom of the trimethyl ammonium group of sarpogrelate and finally, the indole NH group hydrogen of Trp6.48 interacts with the carboxylate group oxygen of sarpogrelate (Fig. 6). According to the molecular modeling, these amino acids were mutated to alanine as alanine mutations introduce changes in both charge and H-bonding capabilities and in size, potentially leaving a cavity in comparison to other side chains. Since the evaluation of the mutant receptors using both ligand binding and functional assays of coupling to signal transduction provides an insight into the basis of the various functional effects observed following introduction of a mutation, we, therefore, evaluated the mutant receptors by both ligand binding and signal transduction assays. The effects of the mutations studied on the affinity of sarpogrelate correlated fully with the expectations from the computational modeling of sarpogrelate with the 5-HT$_{2A}$ receptor.

We mentioned earlier that COS-7 cells were transiently transfected to examine the radioligand binding properties of both wild type and mutant 5-HT$_{2A}$ receptor. However, these cells were not suitable for the production of stable cell lines expressing 5-HT$_{2A}$ receptors as this line developed resistance to the antibiotics used commonly as selection agents. Therefore, we constructed the stably expressing cell lines expressing 5-HT$_{2A}$ receptors and examined the agonist-stimulated IP production of both wild type and mutant receptors using HEK293 cells. Previous studies have shown that both COS-1 and COS-7 cells were not suitable for stable cell lines expressing 5-HT$_{2A}$ receptors (11, 16).

**Binding affinity and inhibitory effects of sarpogrelate for IP turnover: comparison with other 5-HT$_2$ antagonists**

The present study showed that sarpogrelate had higher binding affinity to 5-HT$_{2A}$ receptor in comparison to other 5-HT$_2$ antagonists, but its affinity is slightly lower than that of ketanserin (Table 3). It has been reported that sarpogrelate, a selective 5-HT$_{2A}$ antagonist, and ketanserin, a 5-HT$_2$ antagonist, showed high affinities to the 5-HT$_{2A}$ receptor (9, 17). It was also reported that ritanserin, a 5-HT$_{2A}$/2C antagonist, had high binding affinity to the 5-HT$_{2A}$ receptor (18, 19). Our data are also consistent with the previous reports. The difference in the binding affinities of several 5-HT$_2$ antagonists

![Fig. 6. Molecular model of interaction between the 5-HT$_{2A}$ receptor and sarpogrelate. Dotted lines symbolize hydrogen bonds. Residues are numbered according to a consensus numbering scheme (13). Green colored residues represent hydrogen bonding and blue colored residues represent hydrophobic bonding.](image)
may be due to the nonspecific binding sites of the antagonists, the chemical structures of the antagonists, and the amino acid interaction sites of the 5-HT₂A receptor. Our data on inhibition of 5-HT-stimulated IP formation by the 5-HT₂ antagonists are almost similar to the binding assay results.

**Effects of sarpogrelate on Asp3.32Ala and Trp3.28Ala mutants**

The affinity of the ligands for the mutant receptors is consistent with the inference from the studies of many neurotransmitter receptors, including the 5-HT₂A receptor (20), that the interaction with the highly conserved residue Asp3.32 provides a major component of the binding affinity. A prior study on Asp3.32Ala mutant 5-HT₂A receptor revealed that the Asp3.32Ala receptor was unable to bind radioligands and activate second messenger production (16). Our study indicated that mutating the Asp3.32 to Ala eliminated [³H]5-HT and [³H]ketanserin binding as well as sarpogrelate and 5-HT-stimulated IP formation. Mutating the Trp3.28 to Ala also eliminated radioligand binding and 5-HT-stimulated IP formation (Table 4). Therefore, it is evident that sarpogrelate could not interact with the side chain of the mutant receptor and other receptor loci. Although the precise mechanisms underlying this remain unknown, we can assume that this may be due to a change in the specific physicochemical properties of the side chain of the receptor such as charge, hydrogen bonding potential, hydrophobicity, volume, and shape. However, the functional effects of a mutation depend not merely on the common role of the side chain but, more importantly, on the local microenvironment that is unique to the particular receptor studied (21). Therefore, our results indicate that Asp3.32 and Trp3.28 in the third transmembrane domain of the 5-HT₂A receptor play a very important and crucial role in the interaction between sarpogrelate and 5-HT₂A receptor.

**Effects of sarpogrelate on Trp6.48Ala mutant**

The interaction between sarpogrelate and the Trp6.48Ala mutant did not eliminate the affinity of [³H]ketanserin and 5-HT-stimulated IP accumulation to the receptor, but a large decrease (2200-fold decrease) in affinity of sarpogrelate and great loss of potency (2240-fold) in inhibiting 5-HT-stimulated IP formation were found with this mutation. This result indicates that the Trp6.48Ala mutant did not induce direct actions (i.e., interaction of the side chain with sarpogrelate), but might induce a variety of indirect effects due to interaction with other receptor loci. The disruption of function of the receptor could result indirectly from this mutation inducing a structural perturbation of the binding pocket or global receptor conformation that leads indirectly to decreased affinity (11). Thus, Trp6.48 in the sixth transmembrane helix of the 5-HT₂A receptor plays a vital, but is less important than Asp3.32 and Trp3.28, role in the interaction between sarpogrelate and 5-HT₂A receptor.

Our data from mutagenesis and molecular modeling provide insight into the molecular mechanisms underlying sarpogrelate–5-HT₂A-receptor interactions and explain how such subtle alterations in the structure of the receptor can lead to altered sarpogrelate binding. We propose that the interactions of sarpogrelate with Asp3.32Ala, Trp3.28Ala, and Trp6.48Ala mutants of the 5-HT₂A receptor markedly reduce the affinity of sarpogrelate and 5-HT-stimulated IP accumulation. These results thus implicate these amino acid residues as the major determinants of sarpogrelate affinity.

In conclusion, our results show in a dramatic and novel fashion that Asp3.32 and Trp3.28 in helix 3 and Trp6.48 in helix 6 of the 5-HT₂A receptor are the most important sites for sarpogrelate binding.

**References**


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