We modelled the N-terminal ligand-binding domain of the \( \rho1 \) GABA\(_c\) receptor based on the *Lymnaea stagnalis* acetylcholine-binding protein (L-AChBP) crystal structure using comparative modelling and validated using flexible docking guided by known mutagenesis studies. A range of known \( \rho1 \) GABA\(_c\) receptor ligands comprising seven full agonists, 10 partial agonists, 43 antagonists and 12 inactive molecules were used to evaluate and validate the models. Of the 50 models identified, six models that allowed flexible ligand docking in accordance with the experimental data were selected and used to study detailed receptor-ligand interactions. The most refined model to accommodate all known active ligands featured a cavity comprising of a volume of 488 Å\(^3\). A detailed analysis of the interaction between the \( \rho1 \) GABA\(_c\) receptor model and the docked ligands revealed possible H-bonds and cation-\( \pi \) interactions between the different ligands and binding site residues. Based on quantum mechanical/molecular mechanical (QM/MM) calculations, the model showed distinctive conformations of loop C that provided a molecular basis for agonist and antagonist actions. Agonists elicit loop C closure, while a more open loop C was observed upon antagonist binding. The model differentiates the role for key residues known to be involved in either binding and/or gating.

**Key words:** channel gating, \( \rho1 \) GABA\(_c\) receptors, homology modelling, ligand docking, ligand-gated ion channels

**Abbreviations:** GABA, \( \gamma \)-aminobutyric acid; LGICs, ligand-gated ion channels; AChBP, acetylcholine-binding protein; QM/MM, quantum mechanical/molecular mechanical calculations

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GABA\(_c\) receptors are members of the cysteine loop (Cys-loop) family or ligand-gated ion channel superfamily (LGICs) that includes the nicotinic acetylcholine receptors (nAChR), serotonin type 3 receptors (5-HT\(_3\)), GABA\(_A\) and glycine receptors. LGICs are formed of homo- or heteropentamers with their respective subunits. Strong evidence suggests a shared 3D structure by all subunits within the Cys-loop family (1,2), with the main features displaying an extracellular N-terminal domain which incorporates the highly conserved Cys-loop and is believed to incorporate the orthosteric binding site, followed by the four transmembrane (M1–M4) domains and ending with an extracellular C-terminal region. The second transmembrane domain (M2) forms the lining of the channel pore and is implicated in selecting the type of ions that pass through the channel (3). The Cys-loop is located in a position close to the transmembrane region suggesting a potential role in activating the channel via an interaction with the transmembrane region of the receptor (1,2).

The agonist and/or competitive antagonist binding site of the Cys-loop family is expected to be on the N-terminal region at the interface of two adjacent subunits (4). It is believed that the binding site is formed by a series of loops, termed loops A–F. The first subunit accounts for loops A, B and C forming the plus (+) side of the binding site, whereas the minus (−) side is formed by loops D, E and F of the facing complementary subunit. The (+) side is expected to be entirely formed of loop regions, whereas the (−) side is formed from \( \beta \) strands (4). The exact amino acid residues that constitute the binding site varies between the receptor classes (5–8). High surface complementarity between the adjacent subunits is necessary to maintain the functionality of the receptor (4,9). Despite the identification of the binding site residues for the different Cys-loop family members, the exact orientation of the residues and loops are yet to be confirmed by a high-resolution crystal structure of the receptor (4,9). The lack of information about the residues involved in ligand binding and their specific conformations has hindered the efforts for the design of selective active ligands (4,9,10).

GABA\(_c\) receptors are homomeric chloride channels formed from a single subunit subtype, comprising of \( \rho \) subunits. The importance of the GABA\(_c\) receptor has evolved in recent years and they have been implicated in sleep-waking processes (11), memory enhancement (12) and modulation of eye growth and refractive development (13), making drugs acting on GABA\(_c\) receptors, potential treatments for sleep disturbances, cognitive disorders and myopia. Three subtypes are known and termed as \( \rho1 \), \( \rho2 \) and \( \rho3 \). Unlike other members of the Cys-loop family, the binding site of GABA\(_c\) receptor is composed of only five loops, termed loops A–E. In the case of \( \rho1 \), loops A–C of one \( \rho1 \) subunit forming the \( \rho1 (+) \) side of the...
binding site, and loops D and E from the facing p1 subunit account-
ing for the p1 (−) side of the binding site, lacking loop F (14). Despite
the identification of the different residues forming the binding
site, their exact role in either binding or gating is yet to be con-
firmed with a crystal structure of the p1 GABA<sub>C</sub> receptors (14–17).

However, site-directed mutagenesis and electrophysiological studies
have established a suggested role that each of these residues play
in ligand binding and/or channel gating (14–18).

GABA<sub>C</sub> receptor-ligands range from agonists (including partial agon-
ists), antagonists and allosteric modulators. The pharmacological
profile for this receptor differs from both GABA<sub>A</sub> and GABA<sub>0</sub> recep-
tors. GABA<sub>C</sub> receptors are not blocked by the alkaloid bicuculline,
which inhibits GABA<sub>A</sub> receptors nor activated by baclofen, the
GABA<sub>C</sub> selective agonist. Instead, GABA<sub>C</sub> receptors are selectively
activated by cis-aminocrotonic acid (CACA, cis-4-aminobut-2-enoic
acid), (±)-cis-2-aminoethylcyclopropane ([±]-CAMP) carboxylic acid
specifically (+)-(1R,2S)-CAMP, and the 5-Me, 5-Ph, 5-p-Me-Ph, and
5-p-F-Ph imidazole-4-acetic acid analogues (1,19). In general, p1
GABA<sub>C</sub> receptor agonists and partial agonists are carboxylic acid
GABA analogues, but the acidic moiety can vary to incorporate an
isoxazole or sulphonic acid group (Table 1). Trans-4-amino-2-enoic
acid (TACA) and trans-4-amino-2-fluorobutanoic acid (2-FTACA)
show pure agonist activity on GABA<sub>C</sub> receptors, while CACA, homo-
hypotaurine (4-aminosulphonic acid), (±)-cis-2-aminoethylcyclo-
propane ([±]-CACP) carboxylic acid, 1-imidazole-4-acetic acid (I4AA),
isoquavine [4-aminocyclohex-1-ene-1-carboxylic acid], muscimol (5-
aminomethyl-3-hydroxy-isoxazole) and (±)-trans-2-aminoethylcyclo-
propane-1-carboxylic acid ([±]-TAMP) display partial agonist activity.

Structure-activity relationship studies (SAR) indicate that a partially
folded conformation of GABA is the conformation that binds to the
p1 GABA<sub>C</sub> receptor (20). Such a conformation can also be attained
by TACA, CACA and (±)-CAMP (21).

A number of compounds have been shown to selectively inhibit p1
GABA<sub>C</sub> receptor and mainly consist of phosphonic and alkyl-phosphi-
nic acid derivatives (1,22–29). The most selective antagonists
include: (1,2,5,6-tetrahydrodipyriridine-4-yl) methylphosphonic acid
[TPMPA], (1,2,5,6-tetrahydrodipyriridine-4-yl) ethylphosphonic acid
[TEPA], (±)-cis- and (±)-trans-[3-aminocyclopropyl]methylphosphonic
acid [ACMPA] and (±)-cis- and (±)-trans-[3-aminocyclopropyl]butyl-
phosphonic acid) [ACPBuPA], whose structures are, in general, larger
than the agonists (Table 1). Despite these examples, there are not
many ligands that are potent and purely selective for this class of
receptor (26,30–32). In order to understand the role of GABA<sub>C</sub>
receptors in the brain, a potent selective and lipophilic ligand is
required. The lack of a crystal structure of the GABA<sub>C</sub> receptor and
the small number of selective ligands available have hindered com-
puter-aided drug design for designing selective ligands.

The acetylcholine-binding protein (AChBP) is a soluble protein iso-
lated from Lymnaea stagnalis (PDB code: 1I9B). From its crystal struc-
ture, L-AChBP was expected to be a structural and functional
homologue of the N-terminal region of the α7 nAChRs (33) and
was found to bind the known nAChR ligands such as acetylcholine, nico-
tine, d-tubocurarine and α-bungarotoxin (4,34,35). Therefore, despite
the low sequence identity between the amino acids of the L-AChBP
and the extracellular domain of the different members of the Cys-loop
family (15–30%) (4), many researchers have used the protein to con-
struct homology models for the different Cys-loop family (8,9,36–42).

Moreover, the newly revealed crystal structures of Aplysia Californi-
aca AChBP (PDB code: 2BYQ) (35) crystallized with a num-
ber of nicotinic agonists and antagonists has established the key
role played by loop C in channel activation and inhibition (35). This
study showed that the apo form of the A-ChBP had a more open
loop C when compared with when the agonist was bound. Further-
more, loop C appeared to wrap around the agonists, tightening the
binding pocket resulting in conformational changes in the binding
site leading to the channel opening (35). However, when antago-
nists were bound, loop C acquired a more open conformation to
accommodate the ligand (35). It is thus hypothesized that all
Cys-loop members bind agonists and antagonists in this manner
(43–46).

Different models for the GABA<sub>C</sub> receptor based on L-AChBP have
previously been reported (14,36,40). These models showed different
binding modes for GABA. In these models, GABA was docked into
the binding site and the interactions between the different GABA
agonists and the binding residues were reported (14,36,40). Although
different models for ligand binding were proposed, little information
was provided about the role of the residues involved in binding;
and apart from GABA, no other ligand interactions in the
binding site were reported (14,36,40).

In this paper, we present a model for the p1 GABA<sub>C</sub> receptor
ligand-binding domain that was achieved using a combination of
different computational techniques. Initially, models were generated
using comparative modelling and subsequently the best of the gener-
ated models were selected using flexible docking guided by
known mutagenesis studies. Further optimization of the model was
performed using a combination of quantum mechanics/molecular
mechanics (QM/MM) simulations with further runs of flexible dock-
ing. The different binding modes of the docked poses were exten-
sively analysed and found to provide a theoretical understanding
of the pharmacological activities observed among different ligands.
The conformational changes in loop C observed after the QM/MM
simulations suggest mechanisms by which the channel may be acti-
vated or inhibited.

Computational Methods

Comparative modelling

The amino acid sequences human p1 chain precursor (gi106050,
A38627) and L-AChBP (gi14277797, PDB: 1I9B, chain E) were
obtained from Entrez PubMed database at the US National Library
of Medicine.

The N-terminal binding domain of the human p1 subunit (gi106050,
A38627) was identified by generating a hydrophobicity plot, the
N-terminal domain was considered to be formed from the first 260
amino acids.

Multiple alignments between the N-terminal domain and the L-AChBP
sequence were generated using various alignment matrices and gap
Table 1: The \( \rho_1 \) GABA\(_C\) receptor ligands and their spectrum of activities

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agonist</strong></td>
<td></td>
</tr>
<tr>
<td>GABA</td>
<td>4-Aminobutanoic acid</td>
</tr>
<tr>
<td>(+)-ACPECA</td>
<td>(1R,4S)-4-Aminocyclopent-2-ene-1-carboxylic acid</td>
</tr>
<tr>
<td>(+)-CAMP</td>
<td>(+)-Cis-2-Aminomethylcyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>2-FTACA</td>
<td>Trans-4-Amino-2-fluorobutanoic acid</td>
</tr>
<tr>
<td>(S)-2-MeGABA</td>
<td>4-Amino-(S)-2-methylbutanoic acid</td>
</tr>
<tr>
<td>TACA</td>
<td>Trans-4-Aminobut-2-enico acid</td>
</tr>
<tr>
<td>(−)-TACP</td>
<td>(−)-Trans-(1R,3R)-3-aminocyclopentane-1-carboxylic acid</td>
</tr>
<tr>
<td><strong>Partial agonist</strong></td>
<td></td>
</tr>
<tr>
<td>CACA</td>
<td>Cis-4-Aminobut-2-enico acid</td>
</tr>
<tr>
<td>(−)-CADP</td>
<td>(−)-Cis-1R, 4S-2-aminomethylcyclopropane carboxylic acid</td>
</tr>
<tr>
<td>(+)-CADP</td>
<td>(+)-1S, 4R-Cis-3-aminocyclopentane carboxylic acid</td>
</tr>
<tr>
<td>Homohypotaurine</td>
<td>4-Aminosulphonic acid</td>
</tr>
<tr>
<td>I4AA</td>
<td>1-Imidazole-4-acetic acid</td>
</tr>
<tr>
<td>Isoguavacine</td>
<td>4-Aminocyclohex-1-ene-1-carboxylic acid</td>
</tr>
<tr>
<td>Muscimol</td>
<td>5-Aminomethyl-3-hydroxy-isaxazole</td>
</tr>
<tr>
<td>Chemical name</td>
<td>Structure</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>(+)-TAMP</td>
<td><img src="image1.png" alt="Structure" /></td>
</tr>
<tr>
<td>(-)-TAMP</td>
<td><img src="image2.png" alt="Structure" /></td>
</tr>
<tr>
<td>(+)-TACP</td>
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</tr>
<tr>
<td>Antagonist</td>
<td></td>
</tr>
<tr>
<td>(+)-4-ACPCA</td>
<td><img src="image4.png" alt="Structure" /></td>
</tr>
<tr>
<td>(-)-ACPECA</td>
<td><img src="image5.png" alt="Structure" /></td>
</tr>
<tr>
<td>(-)-CAMP</td>
<td><img src="image6.png" alt="Structure" /></td>
</tr>
<tr>
<td>(±)-Trans-ACPMPA</td>
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</tr>
<tr>
<td>(R)-2-MeGABA</td>
<td><img src="image8.png" alt="Structure" /></td>
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<tr>
<td>2-MeTACA</td>
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<td>3-Cis-ACBMPA</td>
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<tr>
<td>3-Cis-ACPMPA</td>
<td><img src="image11.png" alt="Structure" /></td>
</tr>
<tr>
<td>3-Trans-ACBMPA</td>
<td><img src="image12.png" alt="Structure" /></td>
</tr>
<tr>
<td>3-APMPA</td>
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</tr>
<tr>
<td>Chemical name</td>
<td>Structure</td>
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<td>---------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>4-(S)-ACP-1-BPA</td>
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<tr>
<td>4-(S)-ACP-1-MPA</td>
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<tr>
<td>4-(R)-ACP-1-BPA</td>
<td><img src="structure.png" alt="4-(R)-ACP-1-BPA" /></td>
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<td>4-(R)-ACP-1-MPA</td>
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<tr>
<td>AEMI</td>
<td><img src="structure.png" alt="AEMI" /></td>
</tr>
<tr>
<td>AMPA</td>
<td><img src="structure.png" alt="AMPA" /></td>
</tr>
<tr>
<td>APPiA</td>
<td><img src="structure.png" alt="APPiA" /></td>
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<tr>
<td>APPoA</td>
<td><img src="structure.png" alt="APPoA" /></td>
</tr>
<tr>
<td>APSA</td>
<td><img src="structure.png" alt="APSA" /></td>
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<tr>
<td>Aza-THIP</td>
<td><img src="structure.png" alt="Aza-THIP" /></td>
</tr>
<tr>
<td>CGP38593</td>
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<tr>
<td>CGP44530</td>
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</tr>
<tr>
<td>Chemical name</td>
<td>Structure</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>CGP70522</td>
<td>$Z$-3-(Aminopropen-1-yl)phosphinic acid</td>
</tr>
<tr>
<td>CGP70523</td>
<td>$Z$-3-(Aminopropen-1-yl)methylphosphinic acid</td>
</tr>
<tr>
<td>DAVA</td>
<td>$\delta$-Aminovaleric acid</td>
</tr>
<tr>
<td>4-OH-P4PiA</td>
<td>4-Hydroxy piperidin-4-yl-phosphinic acid</td>
</tr>
<tr>
<td>Isonipecotic acid</td>
<td>Piperidin-4-ylcarboxylic acid</td>
</tr>
<tr>
<td>Iso-THIP</td>
<td>4,5,6,7-Tetrahydroisothiazolo[5,4-c]pyridin-3-ol</td>
</tr>
<tr>
<td>P4MPA</td>
<td>Piperidin-4-yl methylphosphinic acid</td>
</tr>
<tr>
<td>P4S</td>
<td>Piperidine-4-sulphonic acid</td>
</tr>
<tr>
<td>P4PiA</td>
<td>Piperidin-4-yl-phosphinic acid</td>
</tr>
<tr>
<td>P4PoA</td>
<td>Piperidin-4-yl-phosphonic acid</td>
</tr>
<tr>
<td>SEPI</td>
<td>Piperidin-4-yl-seleininic acid</td>
</tr>
</tbody>
</table>
Table 1: Continued

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-95331 2-(3-Carboxypropyl)-3-amino-6-(p-methoxyphenyl) pyridazinium bromide</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>SGS742 3-Aminopropyl-n-butyl-phosphinic acid</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>THAZ 5,6,7,8-Tetrahydro-4H-isoxazolo[4,5-d] azepin-3-ol</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>Thio-THIP 4,5,6,7-Tetrahydroisoxazolo[3,4-c] pyridin-3-ol</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>THIP 4,5,6,7-Tetrahydroisoxazolo[5,4-c] pyridin-3-ol</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
<tr>
<td>TPMPA (1,2,5,6-Tetrahydropyridine-4-yl)methylphosphinic acid</td>
<td><img src="image6" alt="Structure" /></td>
</tr>
<tr>
<td>ZAPA Z-3-[Aminiminomethyl] thio]prop-2-enoic acid</td>
<td><img src="image7" alt="Structure" /></td>
</tr>
</tbody>
</table>

**Inactive compounds**

| (-)-4-ACPCA  (-)-4R-Aminocyclopent-1-ene-1-carboxylic acid | ![Structure](image8) |
| 3-Ca-AHCBMPA  C6c-3-amino, 3-hydroxy cyclobutyl-methylphosphinic acid | ![Structure](image9) |
| 1-Me-aza-THIP  1-Methyl-4,5,6,7-tetrahydropyrazolo[5,4-c]pyridin-3-ol | ![Structure](image10) |
The alignment with the least gaps particularly in the binding site loops was used in generating the model (Figure 1).

Modelling was performed with DS Modelling 1.1\textsuperscript{a} from Accelrys using the alignment shown in Figure 1. Models were developed by multi-subunit modelling in which all five subunits were modelled in a single run based on the 3D structure of \textit{L}-AChBP. Initially, 30 different models were generated. An additional 20 models were generated from a ‘Refine Loops’ run. This run was carried out on loops A–E of one of the generated models from the first modelling run. The loops were defined as consecutive amino acid sequences of the binding site forming loops.

### Model validation
The strategies adapted for the initial validation of the generated models included identification of the presence of known binding site residues at the interface between the two adjacent subunits and generation of Ramachandran plots for the different models.

After this refinement, 30 models were selected to be used in the subsequent docking studies.

### Ligand docking and model selection
All ligands shown in Table 1 were used in the docking studies. These comprised of 7 full agonists, 10 partial agonists, 43 antagonists and 12 inactive molecules. Ligands were built using the maestro software from Schrödinger\textsuperscript{b} in their zwitterionic form (20,21,30). All ligands were minimized using the Impact energy minimization module in \textsc{maestro}\textsuperscript{b}. Docking was carried out using the Glide module from Schrödinger\textsuperscript{c}.

Models were prepared for docking by selecting two adjacent subunits in each of the 30 pentameric models. This represented one of the potential binding sites of the \textit{GABA}_C receptor. The two subunits were then imported into the \textsc{maestro} interface and hydrogens were added. As Glide requires the refinement of the protein binding site around a specified ligand\textsuperscript{d}, a minimized GABA molecule was manually placed into the putative binding site of the model before

### Table 1: Continued

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Me-aza-THIP</td>
<td>2-Methyl-4,5,6,7-tetrahydropyrazolo[5,4-c]pyridin-3-ol</td>
</tr>
<tr>
<td>THIA</td>
<td>5,6,7,8-Tetrahydro-4H-isoxazolo[5,4-c]isoxazin-3-ol</td>
</tr>
<tr>
<td>3-Trans-AChBMPA</td>
<td>Trans-3-amino, 3-hydroxy-cyclobutyl-methylphosphinic acid</td>
</tr>
<tr>
<td>2-Me-TPMPA</td>
<td>2-Methyl (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid</td>
</tr>
<tr>
<td>2-Et-TPMPA</td>
<td>2-Ethyl (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid</td>
</tr>
<tr>
<td>3-Me-TPMPA</td>
<td>3-Methyl (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid</td>
</tr>
<tr>
<td>5-Me-TPMPA</td>
<td>5-Methyl (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid</td>
</tr>
</tbody>
</table>
proceeding with protein preparation steps. Subsequently, each model was prepared and refined. The protein preparation process corrects H-bonding conflicts between the different amino acids, while the refinement component runs a series of restrained, partial minimizations using Impact on the protein structure. This protein preparation allows the relaxation of the receptor structure so that it will accommodate the native ligand. No neutralization zone around the ligand was selected and the minimization was stopped when root mean square deviation (RMSD) reached 0.30 Å. The RMSD between each refined model and its parent counterpart was determined. No deviation was observed between the input model and its refined and prepared counterpart.

Thirty grids to be used for docking were generated from each model, which represent the shape and properties of the receptor’s binding site. The grid was determined as a centroid formed of the major binding residues: Y102, R104, W133, P136, F138, F139, V140, S142, F146, S168 and R170, Y198, Y200, Y241, S243, T244 and Y247 (14–18). To take account of the differences observed among the different conformations of the binding site residues, the van der Waals radii of non-polar receptor atoms were not scaled down for grid generation.

As the Glide docking assumes the rigidity of the receptor, scaling down of van der Waals radii allows the docking of ligands with close contacts with the receptor, minimizing the steric clashes of the docked ligand with the scaled van der Waals radii of the different residues.

Initially, the standard precision module was used for ligand docking, which allows the ligands to dock with fewer penalties, consequently exploring the conformational space for the ligands in the binding site. This was required to generate the highest number of possible docked poses per ligand that provided a unique position and orientation relative to the receptor as well as core and rotamer-group conformations. No binding constraints were imposed on ligand docking in order to explore all possible residues that may be contributing to ligand binding. A total of six models that showed agonists and antagonists to have comparable binding modes and maximal interaction with known binding residues were selected.

Further docking was performed to further establish the validity of the selected models using the extensive extra-precision module in Glide. The extra-precision module rejects false positives and provides a better correlation between good poses and good scores, thus providing more reliable poses. Thus, the docked poses are expected to represent favourable hydrophobic contacts between the receptor and the ligand and provide appropriate H-bond data.

Quantum mechanics/molecular mechanics calculations
Quantum mechanics/molecular mechanics calculations (48) were performed on one (randomly chosen) of the six most refined models using QSite module from Schrödinger. Four minimization runs were performed on the model using the prototype agonist (GABA), the selective antagonist (TPMPA) and the conformationally restricted partial agonist (CACP). In the case of the partial agonist (CACP), two conformations were used, one with the amino group in a conformation similar to the agonist-bound conformation, while the other resembled the amino group in a conformation similar to the TPMPA (antagonist) bound conformation. The two conformations of (CACP) used assume that partial agonism is a result of equilibrium between agonist and antagonist conformations of the receptor rather than the ligand only partially opening the channel.
The OPLS_2001 force field (49) was used for the MM minimization of the model residues excluding the QM region, the QM region contained the ligand and the binding residues; S168, Y198, R104 and S243 as well as the loop C residues Y241, T244 and Y247, that were subjected to minimization using the density-functional theory using DFT-B3LYP hybrid density function and the 6–31 + G* basis set in the gas phase (50,51). The validity of the calculations was established by exploring the conformational space of GABA and TPMPA molecules without the model subunits. This conformational exploration was carried out using the same force fields and density function as the ligand-receptor calculations but with alternating solvation conditions. The Continuum Solvation using the Poisson–Boltzmann Solver (52) was used in the first run, while the second run was carried out at the gas phase with a dielectric constant of $\varepsilon = 78.39$ that mimics the dielectric constant of water without applying any continuum solvation function.

To establish whether the conformations of the new models are valid, new runs of flexible docking were performed. Each model was prepared and refined and three new grids were generated. The same ligands used for the docking runs were docked into the binding site of each of the generated grids using the extra-precision module.

**Results**

There are significant challenges in establishing an accurate, reliable $\rho 1$ GABA$_C$ receptor model that can be used as a starting point for structure-based drug design. These include: (i) low-sequence homology between $\rho 1$ GABA$_C$ receptors and the $L$-AChBP. With sequences of structural homology less than 30%, a marked decrease in structural similarity within the same family is observed (53,54). This implies that $L$-AChBP may be considered as a low homology template for the $\rho 1$ GABA$_C$ receptor $N$-terminal domain (54); (ii) the limited number and lack of structural diversity of known $\rho 1$ GABA$_C$ receptor ligands; (iii) $\rho 1$ GABA$_C$ receptor binding site is mainly formed from loop regions (4). Loops are short protein segments, thus they are less likely to provide enough data to reveal their local fold, and the conformation of the loop is influenced by the structure of the surrounding protein regions (55,56). Loop modelling accuracy is one of the major limiting factors in protein structure prediction and affects the overall accuracy of the resulting comparative models (55,56); (iv) the binding site of the LGICs is at the interface between two facing separate subunits (4). This implies the importance of the size of the binding site cavity that is dictated by the distance between the two adjacent subunits (46). This distance along with the specific conformation of the ligand-binding residues play a major role in determining the activity of the ligands and therefore their role in activation or inhibition (46,57).

Consequently, a multi-step methodology utilizing different modelling techniques was developed to identify the structure of the $\rho 1$ GABA$_C$ receptors binding site. Our methodology consists of four main steps: (i) the generation of a large number of models with differences in the orientation of the binding site residues; (ii) initial model validation; (iii) automated flexible docking of the different ligands into the binding site of all of the generated models; and (iv) selection of the best model based on agreement with the experimental data.

**Comparative modelling**

Models were developed based on the alignment shown in Figure 1. Sequence identity of 9.3% and similarity of 26.3% was found between the sequence of a single $L$-AChBP subunit and the $N$-terminal domain of a single $\rho 1$ GABA$_C$ receptor subunit. The models were generated using multi-subunit modelling; all five subunits were modelled in a single run based on the 3D structure of $L$-AChBP. This approach was used to account for the complementarity expected at the interface between the two adjacent subunits (4,57) and to preserve the pseudo-symmetrical arrangement of the receptor (4). The $\rho 1$ GABA$_C$ receptors binding site is formed from buried uncharged amino acid residues (4); therefore, different orientations are expected to be obtained if these residues were modelled in a solvent accessible environment (57). Fifty models were generated from two modelling runs; model building and loop refinement runs. An example of the models generated for the $\rho 1$ GABA$_C$ receptors $N$-terminal domain is shown in Figure 2.

**Model validation**

The most important feature for model validation was the presence of known binding site residues lining the interface between the two adjacent subunits. Site-directed mutagenesis and electrophysiological studies have identified key amino acids that form the GABA binding site in the $\rho 1$ GABA$_C$ receptor (14–18). Residues that were found to be important for ligand binding and/or gating are distributed among the different loops. These consist of loop A residues: aspartate 136 (D136), phenylalanines (F138, F139 and F146), tryptophan 133 (W133), proline 135 (P135), valine 140 (V140) and serine 142 (S142). Loop B residues: tyrosine 198 and 200 (Y198 and Y200). Loop C residues: tyrosine 241, serine 243 (S243), threonine 244 and tyrosine 247 (Y241, S243, T244 and Y247, respectively). The main residues that are believed to contribute to the binding site from loop D are tyrosine 102 (Y102) and arginine 104 (R104) while serine (S168) and arginine 170 (R170) are the main residues in loop E (14–17).

All generated models were found to have the known binding site residues lining the interface between the two adjacent subunits. Figure 3 shows two adjacent subunits from one of the $\rho 1$ GABA$_C$ receptor models, highlighting the binding site loops and the main residues lining the binding site between the two subunits.

Diversity of the binding site occurs because of conformational flexibility in the binding site residues (Figure 4). This structural diversity in the binding site is expected to provide variations in conformational space that can be evaluated in the search for the most accurate $\rho 1$ GABA$_C$ receptor binding site conformation. Accordingly all models were used in the validation steps. The RMSD values, based on all model residues, among the different
models ranged from 1.62 to 2.56 for models from the first run and from 1.62 to 4.1 for models generated from the second loop refinement run.

Additionally, Ramachandran plots (58) were used for further model validation. Ninety-six to ninety-seven per cent of the backbone dihedral angles were found to cluster within the sterically-allowed regions. However, the 3–4% of the residues that lay outside these regions were found to be located in areas of the model that are less likely to contribute to ligand binding. Therefore, such models were included and further validated. Out of 50 models, 20 models were disregarded and not considered further in the next step. These models showed an increase in the number of residues outside the sterically-allowed regions and particularly the binding site residues.

**Ligand docking and model selection**

A large number of docked poses were generated from the initial ligand docking runs using the standard precision module. An average of 70 poses per ligand was generated. This large number of poses was not unexpected as \( \rho1 \) GABA\(_C\) receptor ligands are, in general, small, highly flexible molecules that were being docked into a large binding pocket, the average binding pocket volume of all generated models was found to be 488 Å\(^2\). The high diversity of poses is expected to provide an indication about the possible ligand-binding conformations and their interactions with their respective binding site residues.

All ligands studied were docked into the same region of the binding site for the remaining 30 models, allowing the docking data to be exhaustively analysed to identify the conformation(s) of each ligand and the residues to which it was bound. The
models with binding sites that were more likely to provide comparable binding modes for agonists and antagonists as well as providing an insight for the molecular bases for their respective pharmacological effects were considered for further validation. Of the 30 models, six showed very high agreement with reported experimental data and revealed similar types of binding interactions both for agonists and antagonists. Interestingly, in these six models, a consensus in the orientation of the main residues found to be involved in ligand binding was observed. RMSD values were found to be less than 0.4 Å among all the experimentally determined binding site residues for the six models. For the reasons mentioned earlier, only one of the six models was selected for subsequent steps.

The initial docking experiments allowed the determination of the best model in reference to the experimental data. In order to further establish the validity of the selected model, a second flexible docking run using the extra-precision module was carried out. Analysis of the docking runs showed that the docked poses of active ligands were found to have common H-bond interactions with tyrosine 198 (Y198) and arginine 104 (R104). In addition to Y198 and R104, docking results showed that other residues can contribute in GABA binding. These included serine 168 (S168), tyrosine (Y241), serine 243 (S243), threonine 244 (T244) and tyrosine 247 (Y247) (18). Figures 5 and 6 show examples of the docking studies using the agonist (GABA) and antagonist (TPMPA) with the different binding site residues, respectively. Similar modes of interactions were observed for all other docked ligands.

The different interactions with the binding residues were determined using the H-bond analysis tool (HBAT) and all possible hydrogen bonds formed were determined (59). Table 2 summarizes the interactions of the docked GABA (+)-CACP and TPMPA molecules with the binding residues of the protein and which are explained in detail below.

Tyrosine 198 (Y198)
Among all the determined binding site residues (14,15,17), tyrosine (Y198), found on loop B ([+]) side, is believed to be one of the major contributors to H-bonding within the binding site. Our model shows that all ligands (both agonists and antagonists) have H-bond interactions with this residue. Specifically, we show that H-bonds are formed between the carbonyl group of Y198 and the amine group of all ligands. An additional H-bond was identified between the hydroxyl group of Y198 and the acidic moiety of antagonists but not the agonists (Figures 5 and 6).
Arginine 104 (R104)
In our model, R104, found on loop D (| side), is in a conformation, whereby the guanidinium group points towards the binding site. This residue was found to interact with the acidic moiety of all docked ligands most likely via the formation of a salt bridge (Figures 5 and 6). Although the exact role played by R104 is not completely clear, mutagenesis studies indicate that this residue may play a role in both ligand binding and channel gating (16).

Serine 168 and 243 (S168 and S243)
S168, found on loop E (| side) and S243, found on loop C (|+ side), are expected to be involved in ligand binding (14). Both residues are believed to stabilize GABA via H-bond formation (14). From our docking studies, an H-bond was found to form between S168 and the agonist through the interaction of the acidic moiety of the agonist and the hydroxyl group of S168 (Figure 5). Additionally, docking studies showed that an H-bond may be formed between the acidic moieties of most of the docked ligands (both agonists and antagonists) and the side chain hydroxyl group of S243.

Tyrosine (Y241), threonine 244 (T244) and tyrosine 247 (Y247)
Y241, TS244 and Y247 are the main residues in loop C and are most likely to interact with the bound ligands. Hansen and colleagues proposed that the different types of interactions with these residues will govern loop C conformations and thereby channel activation or inhibition (35).

Ligand-receptor cation-κ interactions
Cation-κ interactions are believed to play an important role in stabilizing the ligands’ cation within the binding site of all members of the Cys-loop family (40,60,61). Thus, we expected from our model to identify similar interactions between GABA<sub>C</sub> receptor ligands and the aromatic residues known to be involved in this type of interaction. An analysis of the ligand-docked poses indicates that Y198, Y241 and Y247 are in close proximity to the ammonium ions of all ligands, which favour such interactions. The important roles played by these residues in ligand binding have been previously established (15,60). Y198 in the q<sub>1</sub> GABA receptor is equivalent to the A-AChBP tryptophan 147 (W147), which is believed to be a major

### Table 2: The interactions between the docked GABA, (+)-CACP and TPMPA with the binding residues of the q<sub>1</sub> GABA<sub>C</sub> receptor model

<table>
<thead>
<tr>
<th></th>
<th>Docked GABA</th>
<th>Minimized GABA</th>
<th>Docked (+)-CACP</th>
<th>Minimized (+)-CACP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arginine 104</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N–H…O</td>
<td>ARG (NE-104)</td>
<td>GABA (O1)</td>
<td>2.98</td>
<td>1.83</td>
</tr>
<tr>
<td>N–H…O</td>
<td>ARG (NH2–104)</td>
<td>GABA (O1)</td>
<td>1.78</td>
<td>1.94</td>
</tr>
<tr>
<td><strong>Serine 168</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O–H…O</td>
<td>SER (OH-168)</td>
<td>GABA (O2)</td>
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<td>1.84</td>
</tr>
<tr>
<td><strong>Serine 243</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O–H…O</td>
<td>SER (OH-243)</td>
<td>GABA (O1)</td>
<td>1.89</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>SER (OH-243)</td>
<td>GABA (O2)</td>
<td>2.77</td>
<td></td>
</tr>
<tr>
<td><strong>Tyrosine 198</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N–H…O</td>
<td>GABA (N1)</td>
<td>TYR (O-198)</td>
<td>1.66</td>
<td>2.16</td>
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<tr>
<td><strong>Tyrosine 247</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N–H…O</td>
<td>GABA (N1)</td>
<td>TYR (O-247)</td>
<td>2.67</td>
<td>2.15</td>
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</table>

**GABA-oriented (+)-CACP conformation**

<table>
<thead>
<tr>
<th></th>
<th>Docked (+)-CACP</th>
<th>Minimized (+)-CACP</th>
<th>Docked (+)-CACP</th>
<th>Minimized (+)-CACP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arginine 104</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N–H…O</td>
<td>ARG (NE-104)</td>
<td>(+)-CACP (O1)</td>
<td>1.92</td>
<td>2.81</td>
</tr>
<tr>
<td>N–H…O</td>
<td>ARG (NH2–104)</td>
<td>(+)-CACP (O1)</td>
<td>1.99</td>
<td></td>
</tr>
<tr>
<td><strong>Serine 168</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O–H…O</td>
<td>SER (OH-168)</td>
<td>(+)-CACP (O1)</td>
<td>1.63</td>
<td>1.74</td>
</tr>
<tr>
<td><strong>Serine 243</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O–H…O</td>
<td>SER (OH-243)</td>
<td>(+)-CACP (O1)</td>
<td>1.63</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>SER (OH-243)</td>
<td>(+)-CACP (O2)</td>
<td>2.05</td>
<td>1.94 and 2.00</td>
</tr>
<tr>
<td><strong>Tyrosine 198</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N–H…O</td>
<td>(+)-CACP (N1)</td>
<td>TYR (O-198)</td>
<td>2.90</td>
<td>2.30</td>
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<tr>
<td>O–H…O</td>
<td>TYR (OH-198)</td>
<td>(+)-CACP (O2)</td>
<td>1.87</td>
<td>2.39</td>
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**TPMPA-oriented (+)-CACP conformation**

<table>
<thead>
<tr>
<th></th>
<th>Docked (+)-CACP</th>
<th>Minimized (+)-CACP</th>
<th>Docked (+)-CACP</th>
<th>Minimized (+)-CACP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arginine 104</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N–H…O</td>
<td>ARG (NE-104)</td>
<td>(+)-CACP (O1)</td>
<td>1.92</td>
<td>2.81</td>
</tr>
<tr>
<td>N–H…O</td>
<td>ARG (NH2–104)</td>
<td>(+)-CACP (O1)</td>
<td>1.99</td>
<td></td>
</tr>
<tr>
<td><strong>Serine 168</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O–H…O</td>
<td>SER (OH-168)</td>
<td>(+)-CACP (O1)</td>
<td>1.63</td>
<td>1.74</td>
</tr>
<tr>
<td><strong>Serine 243</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O–H…O</td>
<td>SER (OH-243)</td>
<td>(+)-CACP (O1)</td>
<td>2.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SER (OH-243)</td>
<td>(+)-CACP (O2)</td>
<td>2.09</td>
<td>1.94</td>
</tr>
<tr>
<td><strong>Tyrosine 198</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N–H…O</td>
<td>(+)-CACP (N1)</td>
<td>TYR (O-198)</td>
<td>2.05</td>
<td>2.76</td>
</tr>
</tbody>
</table>

**Tyrosine (Y241), threonine 244 (T244) and tyrosine 247 (Y247)**
Y241, TS244 and Y247 are the main residues in loop C and are most likely to interact with the bound ligands. Hansen and colleagues proposed that the different types of interactions with these residues will govern loop C conformations and thereby channel activation or inhibition (35).
contributor to agonist binding in the $\alpha$-AChBP (35). On the contrary, van der Waals interactions with the loop C residues of the $\alpha$-AChBP; tyrosine 188 and 195 (Y188 and Y195) have been established (35). Similar interactions are expected to contribute to GABA binding with the equivalent loop C residues in the $\rho$1 GABA$_C$ receptor, namely, Y241 and Y247. Furthermore, S243, the residue equivalent to $\alpha$-AChBP cysteine 190 (C190), was found to form an H-bond with the carboxylate group of GABA.

The binding site cavity

The $\rho$1 GABA$_C$ receptor binding site is expected to have a cavity large enough to accommodate all the known and active GABA$_C$ ligands. The volume of the binding site of the selected model was found to be 418 $\AA^3$. The average distance between the ligand-binding hydroxyl group of Y198 and the ligand-binding guanidinium group of R104 was found to be 6.9 $\AA$.

In a previous study, Crittenden and colleagues found that the distance between the carboxyl and amine moieties (C–N) of TACA to be 4.775 $\AA$ using conductor-like screening solvation model (COSMO) carried out within a dielectric continuum (62). This was found to be in agreement with our docking studies in which the range of (C–N) distances for a variety of docked TACA conformers was found to be 4.88–5.02 $\AA$.

Our model has also assisted us in identifying two different cavities that accommodated different functionalities of the $\rho$1 GABA$_C$ receptor ligands. The first cavity accommodated the P-alkyl substituents of the different antagonists. Figure 6 shows TPMPA bound within the binding site of the $\rho$1 GABA$_C$ receptor model highlighting the cavity accommodating the methyl substituent. This cavity appears to accommodate the hydrophobic substituents found on all phosphinic acid analogues docked. An example is highlighted in Figure 7, whereby the butyl-phosphinic acid analogue of GABA, SGS742 (CGP36742), is docked showing that the butyl group is lying in this cavity.

In addition, a second cavity was found to extend behind the binding site and accommodate the aromatic ring of the agonists, 5-Ph, 5-p- Me-Ph, and 5-p-F-Ph imidazole-4-acetic acid analogues, and the non-selective antagonist; SR-95331 (gabazine).

Inactive compounds

Compounds known to lack activity on $\rho$1 GABA$_C$ receptors were also docked into the binding site. Some of the molecules failed to be docked indicating their lack of their ability to bind within the determined binding site. However, the inactive compounds, 2-Me-TPMPA, (±)-3-Me-TPMPA and (±)-5-Me-TPMPA, were found to be docked in a manner similar to TPMPA and thus allowed us to study the interactions of the ligand with the binding residues of the protein (Figure 8).

Quantum mechanics/molecular mechanics calculations

To overcome the computational expense of calculations performed on the large receptors and the bound ligand, we validated the calculations performed in the gas phase by utilizing different minimizations under different solvation preferences. The calculations performed on the ligands showed very minor differences in the conformations observed from the different runs using different solvation preferences which justified the use of the gas phase for the overall QM/MM calculations. Therefore, we decided to proceed with the ligand–receptor complex calculations under the more computationally economic gas-phase conditions.

Different conformational changes were observed in the minimized receptor models of different ligands bound in the binding site. The GABA-bound model showed a smaller binding site in contrast to the larger cavity observed with the TPMPA-bound model as highlighted by the respective volumes of 342 and 416 $\AA^3$. The (±)-CACP optimized models also identified two cavity volumes: 389 $\AA^3$ for the GABA-oriented and 414 $\AA^3$ for the TPMPA-oriented (±)-CACP conformations, respectively.
The RMSD values, based on all receptor residues, between the free-unbound model and the GABA-minimized model was found to be 3.06 Å, while the RMSD value between the free-unbound model and the TPMPA-minimized model was found to be 2.87 Å. These values were in agreement with the RMSD of 3.61 and 2.93 Å between the free-unbound model and the agonist and antagonist minimized (+)-CACP models, respectively.

During the simulation, the main residues that showed significant structural rearrangements were R170, aspartate 164 (D164), Y102 and histamine 141 (H141). The H-bond interactions of different residues in the apo and the four minimized models were further analysed using the HBAT software and all possible H-bonds interactions were determined (59). Distinctive patterns of H-bond interactions were found to form between the different residues in the five models. Table 3 highlights the changes in the distances and the H-bond interactions between the main residues in and around the binding site.

New runs of flexible docking were performed on the minimized models. The docking results confirm the validity of the new binding site orientations. All full agonists were able to be docked into the GABA-optimized binding site indicating a possible cavity dimension, where all the agonists may fit and induce the conformational changes required for channel activation. In contrast, the partial agonists, (+)-CACP, (-)-CACP, (+)-TACP, were not able to be docked into the narrow binding site of the agonist but were able to be docked into the two (+)-CACP minimized models. Antagonists, however, failed to be docked into the smaller binding site of both the agonists and partial agonist minimized models.

**Table 3:** The changes in the distances and the H-bond interactions between the main residues in the binding site and the bound ligands, GABA, (+)-CACP and TPMPA

<table>
<thead>
<tr>
<th>Residue (1)</th>
<th>Residue (2)</th>
<th>Distance (Å)</th>
<th>Parent model</th>
<th>GABA minimized</th>
<th>TPMPA minimized</th>
<th>GABA-oriented (+)-CACP minimized</th>
<th>TPMPA-oriented (+)-CACP minimized</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP (NE1-133)</td>
<td>TYR (OH-200)</td>
<td>1.85</td>
<td>1.88</td>
<td>1.74</td>
<td>1.87</td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>TYR (OH-247)</td>
<td>TYR (O-200)</td>
<td>5.00</td>
<td>4.13</td>
<td>1.75</td>
<td>3.96</td>
<td>3.27</td>
<td></td>
</tr>
<tr>
<td>TYR (OH-102)</td>
<td>ASP (OD2-153)</td>
<td>7.81</td>
<td>8.91</td>
<td>1.58</td>
<td>10.84</td>
<td>8.20</td>
<td></td>
</tr>
<tr>
<td>TYR (OH-198)</td>
<td>TYR (O-198)</td>
<td>4.31</td>
<td>2.76</td>
<td>3.25</td>
<td>1.69</td>
<td>2.08</td>
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</tr>
<tr>
<td>ARG (NH1-170)</td>
<td>ASP (OD2-153)</td>
<td>6.69</td>
<td>1.56</td>
<td>6.00</td>
<td>1.75</td>
<td>2.66</td>
<td></td>
</tr>
<tr>
<td>SER (N-243)</td>
<td>ASP (OD2-164)</td>
<td>5.33</td>
<td>1.88</td>
<td>3.89</td>
<td>1.85</td>
<td>3.06</td>
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<tr>
<td>TYR (OH-102)</td>
<td>HIS (ND1-141)</td>
<td>3.02</td>
<td>1.69</td>
<td>5.06</td>
<td>2.07</td>
<td>2.323</td>
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</tbody>
</table>

*H-bond distance ≤2.9 Å.

Discussion

Here, we provide a comprehensive model that attempts to explain the mutagenesis and SAR data known to date about the GABA$_C$ receptor. Our study has identified four conformations of the GABA$_C$ receptor binding site: (i) agonist bound, (ii) antagonist bound, (iii) partial agonist bound and (iv) free-unbound conformations. The conformational differences between the models have provided insights on the molecular mechanisms by which agonists activate, antagonists inhibit and some related analogues are inactive at this receptor. The antagonists studied in this work are competitive and thus were assumed to have comparable binding modes to those of agonists (14,46), and thus residues implicated in agonist binding were hypothesized to also be involved in the antagonist binding (14,35,46), but may have different binding modes that induce distinct conformational changes in the structure of the receptor (14,35,46). The QM/MM calculations provided an insight into the different conformations obtained by the receptor upon binding to agonists, antagonists and partial agonists.

Agonist-bound conformation

Figure 9 shows GABA bound to the agonist-bound conformation of the binding site. GABA was found to adopt a folded conformation which resulted in the formation of an internal H-bond between the ammonium and carboxylate groups. In addition H-bonds were formed between the ammonium ion of GABA and Y198 and S243 on the (+) face and the carboxylate group of GABA with R104 and S168 from the (−) face, similar to those observed in the unminimized conformation. The close proximity of the ammonium ion of GABA to Y198 and Y247 (3.461 and 3.227 Å, respectively) may result in cationic-π interactions. Such interactions are believed to play a key role in stabilizing the ammonium ion of GABA and other ligands upon binding (60). Furthermore, S243 was found to form an H-bond with the carboxylate group of GABA (Figure 9).

As shown in Figure 9, R104 was found to bind the acidic moiety of GABA via the formation of a salt bridge. Evidence of such interactions can be seen from the recently revealed crystal structures of glutamic acid decarboxylases, GAD67 (PDB: 2OKJ) and GAD65 (PDB: 2OKK), that showed salt bridge formation between the carboxylic acid group of GABA and the guanidinium group of arginine residues (63).

The second residue which was found to contribute to GABA binding from the (−) face was S168. A hydrogen bond was found to be formed between the acidic moiety of GABA and the side chain hydroxyl group of S168 (Figure 9).

Antagonist-bound conformation

The interactions between TPMPA with the minimized receptor are expected to give an insight into the mechanism by which competitive antagonists act to inhibit the channel and showed marked differences to the GABA-bound conformation (Figure 10). Figure 10 shows that upon antagonist binding, there is a more open loop C when compared with the agonist-bound model. In addition,
Partial agonist-bound conformations

(+)-CACP was found to be docked into the apo model in two distinct conformations; one in which the amino group acquired a conformation similar to the amino group of the docked GABA molecule, while the other one was in an orientation similar to that of TPMPA. This is believed to explain the partial agonistic effect, i.e., the molecule was able to bind into the binding site in two different conformations (Figures 11A and 11B). Similar interactions between (+)-CACP and the binding site residues were observed when compared with GABA and TPMPA (Table 2). Loop C in the agonist minimized (+)-CACP conformation model showed a similar 'closed' orientation to that of the GABA-minimized model, yet a more 'open' loop C was observed in the TPMPA oriented minimized (+)-CACP conformation model. In addition, a smaller binding site was observed for the (+)-CACP minimized model compared with that of the TPMPA-minimized model.

Conformational changes for \( \beta \)1 GABA\(_\text{C} \) receptor agonists, partial agonists and antagonists

In the agonist-bound model, key amino acid residues were found to 'wrap' around GABA, while in contrast to the more open conformation acquired to accommodate the antagonist TPMPA. It is proposed that upon GABA binding, a narrowing of the binding site occurs that leads to the repositioning of Y247 in loop C. Y247 moves closer to the ammonium ion of GABA favouring an additional cation-π interaction with Y198. Thus, our model suggests that two possible cation-π interactions occur: one with Y198 and one with Y247. These interactions are expected to promote loop C closure in order to enclose or engulf GABA tighter into the binding site. These findings are consistent with loop C behaviour observed upon agonist binding in the -AChBP (35). The new bonds formed will result in tighter binding and may explain the slow dissociation rates of agonists from the receptor (64).

It is proposed from our model that the tighter the binding of the agonist, the slower the dissociation. In GABA-minimized model we observed additional H-bonds between aspartate 164 (D164) and S243 to further enclose loop C around the ligand.

The presence of an H-bond between GABA and S243 also provides a possible explanation as to why there is a 30-fold reduction in GABA activity when this amino acid is mutated to threonine. The increased chain length found in threonine may contribute to adverse steric interactions at this position. Despite the observed H-bond between GABA and S243 in our model, it is proposed not to be crucial for activity as there is only a threefold decrease in GABA activity when the amino acid is mutated to alanine (14).

Studies show that when S168 is mutated to cysteine, threonine and alanine, there is a 400-, 10- and 50-fold increase in GABA \( EC_{50} \), respectively (14), indicating that the S168 hydroxyl group has a role in the binding of GABA and most likely via H-bond with this residue. Furthermore, when S168 was mutated to a valine, the activity of GABA was lost indicating that steric interactions may be occurring (14). In our model, we show that S168 H-bonds to the acidic moiety of GABA providing a possible explanation for the mutagenesis data.

There were a number of differences between the GABA- and TPMPA-bound models. In the docked TPMPA model, there was the formation of an additional H-bond between its acidic moiety and
The methyl substituent of the TPMPA analogues appeared to sterically interfering with the binding site residues. Figure 8 shows the methyl group of (+/-)-5-Me-TPMPA facing Y198. We propose that this steric interaction may hinder the binding of the ligand to this site.

**Homology Model of the GABA<sub>C</sub> Receptor Binding Site**

The interaction between agonist- and antagonist-bound conformations of the receptor leads to the repositioning of key amino acid residues. The repositioning of the amino acids in the agonist-bound conformation may provide a mechanism by which agonists activate the channel. In addition, the positions of the amino acids in the antagonist-bound conformation may provide a mechanism by which antagonists block the channel.

A number of mutagenesis studies identify amino acids important for binding and/or gating. The experimental data do not differentiate between these events. Our model appears to differentiate between residues involved in binding versus gating. It has been proposed that the gating mechanism is a net result of a series of conformational changes propagated through a network of residue interactions all the way from the N-terminal domain and ending in the transmembrane pore leading to channel opening (7,46). Rearrangement of amino acids results in the reorientation of the two adjacent subunits without prominent changes in the structure of each subunit that leads to the opening of the receptor.

Furthermore, it has been proposed that GABA antagonists do not sterically interfere with GABA binding, but rather they induce different conformational changes to those of agonists (65). Thus, it is expected that the conformational changes induced upon agonist and antagonist binding should lead to different amino acid interactions that lead to either the channel opening or remaining shut. Minimal conformational changes are expected to occur in the binding site upon ligand binding as channel opening is rapid and requires only small differences in energy states (66).

The analysis of the structural rearrangements obtained upon GABA- and TPMPA-bound minimized protein structures revealed unique architecture of the two binding sites and new patterns of interactions occurring between the residues and bound ligand (Table 3). Accordingly, we can propose that such conformational rearrangements may provide insights into channel gating and inhibition mechanisms (33,44,67,68).

**Mutagenesis studies** have proposed a number of amino acids as being involved in channel gating. These included tyrosine 102 (Y102), tryptophan 133 (W133), proline 135 (P135), phenylalanine 139 (F139), arginine 170 (R170) and tyrosine 200 (Y200) (33,44,67,68). We propose that for a residue to be involved in channel gating (i.e., signal conveying residues), it must be in close proximity to the ligand-binding residue(s). Consequently, they may play a role in the conformational changes occurring upon ligand binding that will lead to structural rearrangements of these residues affecting the channel function. In our model, a number of residues were

**Inactive compounds**

The docking of inactive-substituted TPMPA analogues provided us with a hypothesis as to why these compounds had no effect.
found to surround the binding site and are in close proximity to the ligand-binding residues.

**Tyrosine 102 (Y102)**
The role of Y102 is not clear as it has been proposed to be involved in ligand binding and/or channel gating (15). Our model does not indicate that Y102 is involved in ligand binding, and we propose that it may be involved in channel gating. Y102 was found to be surrounded with a number of aromatic residues, namely, the agonist binding Y198 and phenylalanine 138 (F138) which indicates the possibility of \( \pi-\pi \) stacking interactions leading to channel activation upon agonist binding. This is supported by the fact that a series of Y102 mutations resulted in a marked decrease in GABA activity with the exception of a phenylalanine mutation (17). In addition, when Y102 was mutated to serine a spontaneously open channel was formed, suggesting the possible role of this residue in channel gating (17).

Furthermore, the generated model shows that Y102 protrudes in the space between Y198 and R104. Y102 and R104 are the main residues of loop D, and we hypothesize that the conformational changes observed upon ligand binding to R104 may affect the orientation of Y102. As a result of the strong interaction between the two residues, there will be an effect on the conformation of Y102 as a consequence of any conformational changes of R104 induced upon ligand binding.

The mutual interactions between the two ligand-binding residues (Y198 and R104) and the Y102 residue may support the fact that the gating mechanism is due to the rearrangements of the N-terminal domains of two adjacent subunits with respect to each other with minor structural changes within each individual subunit (17).

**Phenylalanine 139 (F139)**
This amino acid appears to be important in channel gating as non-functional receptors are obtained upon mutating F139 to cysteine (14). In our model, F139 in its different proposed conformations points towards the Cys-loop and thus may provide the required interaction that conveys all the conformational changes that occur from binding site leading to channel gate.

In addition, we find the amino acids, valine 140 (V140), serine 142 (S142) and histidine 141 (H141) from loop A (14), form a link between Y102 of loop D and F139 on loop A. As a consequence, we propose a role for these residues in channel gating.

**Tryptophan 133 (W133) and proline 135 (P135)**
Studies show that when W133 and P135 are mutated to cysteine, non-functional receptors are formed. It has been proposed that these mutations directly affect the conformation of the binding site required for GABA binding. However, these studies do not rule out the possibility that these amino acids have a role in channel gating (14). In our model, we find that W133 and P135 do not form part of the binding site but are in close proximity to the binding site residues. The distance separating them from the binding site suggest a role for these residues in channel gating.

**Tyrosine 200 (Y200)**
When Y200 was mutated to phenylalanine and serine, there was a 10- and 3000-fold increase in the GABA EC\(_{50}\), respectively, indicating a possible role in the gating mechanism (15). In our model, Y200 is in close proximity to the loop C residue, Y247, and an H-bond can be formed between the two residues. We propose that any change in the Y247 conformation is dependent on the orientation of the nearby Y200. Interestingly, Y200 is also in a close proximity to W133 suggesting possible \( \pi-\pi \) stacking occurring between the two residues that may lead to channel activation. This hypothesis is consistent in explaining the dramatically reduced GABA activity observed with the serine mutation but not the phenylalanine mutation of Y200 and the loss of the receptor function upon W133 mutations (14).

**Arginine 170 (R170)**
Our model suggests a critical role for R170 in the channel gating mechanism. Ionic interactions were found to occur between R170 and aspartic acid 153 (D153) in the GABA and (+) CACP minimized models but not in the TPMPA-minimized model (Table 3), indicating a key role for these residues in channel gating. The importance of a positively charged residue was previously proposed as mutations of these residues result in non-functional receptors except when arginine is mutated to lysine (16).

The observed location of the different residues and the differences in their orientations with respect to the agonist and antagonist-bound conformations have lead us to propose a mechanism of channel activation and inhibition.

**Minimized models and docking**
The ability of full agonists to be docked into the GABA-minimized model indicates similar binding mechanisms for agonists that lead to channel gating. However, not all partial agonists were able to bind to this model indicating that the binding of partial agonists is different leading to conformational changes in the receptor. Two possibilities arise: (i) either the receptor is partially open or (ii) there exists a mixture of agonist- and antagonist-bound conformations. In contrast, antagonists failed to be docked into the smaller binding site of the agonist- and partial agonist-minimized models. We propose that this binding conformation is not suitable for antagonists. As expected, all agonists, partial agonists and antagonists were able to be docked into the TPMPA-optimized binding site.

**Proposed mechanism of channel gating**
From our model, we propose a sequence of events that will lead to channel activation. When GABA binds, a series of conformational changes will occur that narrows the binding site, reducing the distance between Y198 (loop B) and Y102 (loop D) resulting in possible \( \pi-\pi \) interactions. This narrowing results in the closure of loop C. Y247 then undergoes a conformational change that will affect the orientation of the nearby Y200 which in turn interacts with W133 on loop A.

As a consequence of the binding site narrowing, the next line of amino acids, namely, V140, S142, H141 from loop A become
important resulting in the formation of H-bond between Y102 and H141. As H141 moves, it interacts with F139 which in turn interacts with the Cys-loop and provides the required interactions that will lead to channel gating.

The interaction between Y102 and H141 as a result of GABA binding enlarges the space available for R170 (loop E). This extra space allows R170 to re-orientate and form a salt bridge with D153. The coordinated changes in the orientation of the different residues, albeit being part of different loops is believed to be induced by agonist binding that leads to channel gating.

In contrast, a different series of events are proposed to occur when the antagonist binds. It is postulated that upon TPMPA binding, loop C remains in the open conformation resulting in a larger binding cavity. The larger cavity allows Y247 to acquire a more relaxed conformation. This allows H-bonds to be formed between the antagonist, TPMPA, and Y247 and between Y247 and Y200 preventing Y200 from interacting with W133 and thus interfering with the possible gating mechanism. Such interactions are not observed in the GABA and (+) CACP minimized models which allow free movement of Y200 (Table 3).

Furthermore, there is a repositioning of Y198, Y102 and F139 in the presence of TPMPA that interferes with the proposed interactions that occur when an agonist or GABA binds. In contrast, it is proposed that in the TPMPA-minimized model, the hydroxyl group of Y102 H-bonds with D153 preventing its interaction with R170. This interaction may lock R170 in the ‘antagonist’ bound state and thus prevents channel activation. In addition, an H-bond was found to form between the antagonist, R104 (loop D) and D164 in the TPMPA-minimized model but not in the GABA-minimized model (Table 3). This bond may also hinder R104 from adopting the conformation required for propagating the channel-activating signal.

**Conclusion**

In this paper, we present the most comprehensive model to date for the extracellular ligand-binding domain of p1 GABA<sub>c</sub> receptors. The model was rigorously validated to overcome the problems associated with LGICs homology modelling and was found to agree with all current experimental data, providing a better understanding of the different pharmacological activities observed among different ligands. Different cavities were located within and near the binding site which will be further explored in future studies.

QM/MM calculations confirmed that different conformations of the receptor exist; one upon agonist binding, one upon antagonist binding and the unbound free conformation that we expect to occur between the agonist- and the antagonist-bound conformations. In addition, a number of partial agonist-bound conformations were identified. Agonist binding elicits loop C closure and induces the conformational changes leading to channel opening, while a more open loop C may be observed upon competitive antagonists binding.

The rigorous selection and validation of the model in addition to its total agreement with the experimental data suggest its validity to be used in target-based drug design. This lead structural model will be used in search for new selective p1 GABA<sub>c</sub> receptor ligands and future studies will explore the effects on activity of ligands that occupy the different cavities located within and near the binding site.

**References**


Notes

