Theoretical investigation of selective ligand binding mode of galanin receptors

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ABSTRACT
The Galaninergic system consists of Galanin and its receptors, involved in neuromodulation and neurotransmission. Galanin regulates its physiologic and pathologic functions by interacting with three G-protein coupled receptors; GalR1, GalR2 and GalR3. The widespread distribution of Galanin and its receptor subtypes in central and peripheral nervous systems makes them an attractive drug target for the treatment of neurological diseases. However, subtypes selective ligands paucity and little structural information related to either Galanin receptors and Galanin receptor-ligand complexes hampered the structure-based drug design. Thus computational modeling characterization strategy was utilized for Galanin receptor 3D structure prediction and subtypes ligands binding selectivity. Reported ligands with experimental activity were docked against the homology model of Galanin receptors. Further, the MD simulation and binding free energy calculation were carried out to determine the binding interactions pattern consistency and selectivity towards receptor subtype. Results of binding free energy of per residue indicate key contribution of GalR1 Phe115 and His267 in the selective binding of ligands while Tyr103, Tyr270 and His277 play major role in the selective binding of GalR3 ligands. Our study provide rationale for further in silico virtual screening of small molecules for the development of selective ligands against Galanin receptor subtypes.

Introduction
GPCRs (G-protein-coupled receptors) the largest superfamily of membrane proteins among metabotropic receptors with more than 800 members encode by human genome. GPCRs are pharmacologically targeted by almost 30% prescribed drugs due to their involvement in diverse pathophysiological process (Fredriksson et al., 2003).

They represent the 40% of therapeutic target in human because of their diverse physiological functions including growth, homeostasis, reproduction, sleep, appetite, mood behavior and others (Veulens & Rodríguez, 2009). Galanin, a neuropeptide widely expressed in central and peripheral nervous systems, involved in neuromodulation and neurotransmission. It is a post-translational product of prepro-Galanin (121 amino acid) and comprises of 30 amino acid long peptide (Torres & Polymeropoulos, 1998). Galanin regulates its physiological and pathological functions by interacting with three G-protein-coupled receptors; GalR1, GalR2 and GalR3 (Šipková et al., 2017). Galanin receptors are Heptahelical GPCRs each helix from N-terminus to C-terminus connected by three intracellular and extracellular loop regions (Rinne et al., 2019).

The neuroanatomical localization and neurological manifestations of Galanin and its receptors demonstrated in the brain hippocampus which is often involved in epileptic seizures, depression-like behavior and Alzheimer disease (Counts et al., 2008; Demsie et al., 2020; Freimann et al., 2015; Guipponi et al., 2015; Lundström et al., 2005; Mazarati, 2004; McMillan et al., 2004; Pérez et al., 2002). Owing to the involvement of Galanin in various pathophysiological states, the Galanin receptors are attractive drug targets.

GPCRs predominantly demonstrate a key ‘ligand binding specificity’ property and it is a great challenge in pharmaceutical research to design selective drugs that are intended to reduce side effects. Galanin receptors are potential therapeutic targets for neurological disorders. However, a high degree of conservation in the endogenous ligand binding site, makes it difficult to develop receptor subtype selective ligands. Over recent years, some progress has been made towards the design and development of Galanin receptors selective and non-selective ligands to allocate the association of three receptors in various pathophysiological processes. Many non-peptide ligands have been discovered against Galanin receptor subtypes (Table 1). Galnon, an agonist of Galanin receptors was identified by the screening of peptidomimetic library and evaluated in animal models of depression, anxiety, pain and feeding (Abramov et al., 2004; Rajarao et al., 2007; W. P. Wu et al., 2003). Galmic, another non-peptide agonist with a higher affinity towards GalR1 as compared to GalR2 was identified from small a synthetic library
Table 1. Galanin receptor subtypes ligands with their experimental activities and binding site residues.

| Receptor | Binding site residues | Ligands | Experimental activity (nM) |
|----------|-----------------------|---------|---------------------------|
| GalR1    | Phe115, Phe186, His264, Phe282, Glu271, His267 (Berthold et al., 1997; Church et al., 2002) | [I] | 190 |
|          |                       | [II]    | 1700 |
|          |                       | [III]   | >1000 |
| GalR2    | His252, His253, Ile256, Phe264, Tyr271 (Lundström et al., 2007) | [I] | 30,000 |
|          |                       | [II]    | Not tested |
|          |                       | [III]   | >1000 |
| GalR3    | Tyr103, His251, Phe263, Tyr270, Arg273, His277 (Runesson et al., 2010) | [I] | Not tested |
|          |                       | [II]    | Not tested |
|          |                       | [III]   | 5.3 |

and tested in vitro and in vivo (Bartfai et al., 2004; Ceide et al., 2005). Later a series of several 1,4-dithiins and dithiipine-1,1,4,4-tetroxides were identified as inhibitors of GalR1. The compound 2,3-dihydro-2-(4-methylphenyl)-1,4-dithiipine-1,1,4,4-tetroxide [I] (Figure S1) was shown to inhibit the activity of GalR1 with IC50 value of 190 nM to date (Scott et al., 2000). Sch 202596 [II] (Figure S1), a natural product isolated from Aspergillus sp. culture was identified as GalR1 ligand with moderate affinity (Chu et al., 1997). Moreover, analogues of 3-imino-2-indolones were identified as inhibitors of GalR3 among which one of the analogues known as SNAP-398299 [III] (Figure S1), showed lowest Ki value (17 nM) against GalR3 in comparison of GalR1 and GalR2 (Swanson et al., 2005). Besides, progress has also been made to identify residues, crucial for the pathological and physiological functions of Galanin receptors. Site-directed mutagenesis of GalR1 has identified Phe115, Phe186, His264, Phe282, Glu271 and His267 as crucial determinants of receptor function (Table 1). In another study, Lundström and coworkers performed mutagenesis study on GalR2 and identify His252, His253, Ile256, Phe264 and Tyr271 as crucial residues for the activation of the receptor (Table 1). Likewise, Runesson and coworkers produced the seven mutated GalR3 constructs and identify Tyr103, His251, Phe263, Tyr270, Arg273 and His277 as essential determinants of the receptor, responsible for the ligand binding (Table 1). Despite of such extensive research, no Galaninergic therapeutic discovered beyond the animal model stage. Thus development of high affinity subtype-selective ligands is highly desirable.

The 3D information of GPCR bound with agonist/antagonist is of great importance in term of understanding its function and rational designing of drug (Goddard & Abrol, 2007). Galanin receptors are integral membrane proteins; their high-resolution 3D structural characterization is still a challenging task due to the major experimental challenges and difficulties in expression, purification and crystallization (Parker & Newstead, 2016). In contrast, computational modeling techniques can provide useful structural information and models for generating hypotheses about a function of the protein of interest and directing further experimental work for the design and development of effective and selective pharmaceuticals (Kolodny & Kosloff, 2013). Thus, in this study, homology modeling was used to build a 3D model of all three Galanin receptors to provide the structural insights. Cross docking of Galanin receptor ligands with their selectivity, profile towards receptor subtypes was performed to insight the ligand binding properties. Moreover, receptor-ligand complexes were subjected to MD simulation to study the dynamic behavior of Galanin receptors upon binding of selective and non-selective ligands. Further energy contribution of per residue was determined to find out the crucial residues involved in the selective binding of the ligands.

Methodology

Homology modeling

The primary sequence of GalR1, GalR2 and GalR3 were retrieved from UniProtKB (https://www.uniprot.org/), accession code: P47211, O43603 and Q60755, respectively, and subjected to Basic Local Alignment Search Tool (Blast) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for the identification of homologous or template structures in the protein databases. After template search, an iterative threading assembly refinement server (I-TASSER) was used for 3D model generation (Zhang, 2008). The generated models were optimized by energy minimization using molecular mechanics force fields. Moreover, the models were subjected to MD simulation for further refinement. Topologies of the models were processed by pdb2gmx module of GROMACS v.2018.4 using GROMOS96 54a7 force field. Afterwards the models were placed in a cubic box solvated with SPC water model followed by the addition of counter ions for neutralization. The neutralized system were minimized by using steepest descent algorithm followed by the NVT and NPT ensemble of 100 ps by using Berendsen barostat and thermostat algorithms. LINCS algorithm and Particle Mesh Ewald method was used to constraining the bond length and to calculate electrostatic long range interactions, respectively. Finally the MD production of 20 ns was run for all the equilibrated systems with the time step of 2.0 fs. The stereochemical quality of the optimized models were evaluated by many statistical tools, including Ramachandran plot, ERRAT plot and Verify 3D (Colovos & Yeates, 1993; Eisenberg et al., 1997; Hooft et al., 1997). Taking into account all these parameters, one of the best model for each Galanin receptor was selected for molecular docking studies.

Molecular docking

Molecular docking simulation is commonly used to determine the binding affinity and preferred orientation of small molecules in the binding pocket of the target of interest. Herein, we performed the cross docking studies of two of the Galanin receptors ligands; [I] and [III] selective towards GalR1 and GalR3, respectively. The compounds were docked
into the binding site of all three Galanin receptors using same docking parameters. The ligands were sketched using Builder module in Molecular Operating Environment v.2019 (MOE) (Toberg¢te & Curtis, 2013) and subjected to structure corrections, that is, correction of atom type, residues, formal charge and bond order followed by protonation using GBVI/WSA as electrostatic function with the dielectric value of 2. Further, the ligands were minimized using MMFF94x force field (Wang et al., 2004). The modeled structures of all three Galanin receptors were prepared using Structure Preparation module implemented in MOE. Hydrogens were added using Protonate 3D module followed by energy minimization using Amber99 force field.

To define the docking grid, the binding sites of Galanin receptors were studied by using SiteFinder module of MOE, which computes the possible binding sites from the 3D atomic coordinates of the receptor. Calculated binding regions from the SiteFinder were further validated by using DoGSiteScorer (Volkamer et al., 2012). Further, the dummy atoms were assigned to the binding site with the highest score and used as a docking grid during docking simulation. For docking, Triangular Matcher was used as a placement method with London dG and GBVI/WSA as scoring and rescoring functions, respectively. For each ligand thirty conformations were generated using Induce Fit method and conformations with the highest binding affinity were analyzed for protein-ligand interactions.

Molecular dynamics simulation

Molecular dynamics simulation was used to study the dynamic behavior of receptors upon binding of ligands and to determine the time dependent interactions between protein-ligand complexes. Six protein-ligand complexes (GalR1-[I], GalR2-[I], GalR3-[I], GalR1-[III], GalR2-[III] and GalR3-[III]) were subjected to dynamic study using GROMACS v.2018.4 (Van Der Spoel et al., 2005). Initially OPM server (https://opm.phar.umich.edu/) generated the orientation of three receptors within the membrane. The membrane-oriented receptors were embedded in a POPC lipid bilayer by using CHARMM-GUI (E. L. Wu et al., 2014). The generated structure of three receptors consisted of 256 POPC molecules with 329 molecules of TIP3 water model in a rectangular box size of 90 Å × 90 Å × 90 Å. For neutralization, 0.15 M NaCl was added to provide the physiological ion concentration. CHARMM36m force field was used to assess the conformational changes in the three receptors. To remove the bad clashes and steric hindrance, the energy of the systems was minimized by performing 5000 steps using steepest descent algorithm by keeping the maximum force < 1000 kJ/mol/nm. The minimized systems were relaxed to reach the 303 K temperature in NVT and NPT ensemble equilibration. During equilibration, LINCS algorithm was used with the time step of 2 fs. The van der Waals and electrostatic interactions were treated using Verlet scheme. To keep the pressure at 1 bar, Parrinello-Rahman barostat was used during production run. For each equilibrated system 100 ns production was run at time step of 2 fs. VMD v.1.9.3 (Humphrey et al., 1996) was used to analyze the simulated trajectories. To evaluate the stability or dynamic behavior of the simulated systems RMSD and RMSF were calculated.

Per residue free energy contribution

Binding free energy calculation is the commonly used method to calculate the relative binding affinity of small molecule with the target protein by using the Molecular Mechanics Poisson–Boltzmann Surface Area (MMPBSA) method (Genheden & Ryde, 2015). Herein, we used the g_mmpbsa tool of GROMACS to execute the total binding free energy and energy contribution of per residue to determine the role of crucial residues in the selective binding of [I] and [III] with Galanin receptors. The binding free energy of protein-ligand complexes was calculated by the following equation:

$$\Delta G_{\text{bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{PB}} + \Delta G_{\text{SA}} - T\Delta S$$

Where $\Delta E_{\text{MM}}$ is the difference of energy of electrostatic and van der Waals in a vacuum. $\Delta G_{\text{PB}}$ represents the difference of solvation free energy of polar solvents while $\Delta G_{\text{SA}}$ represents the difference of solvation free energy of polar solvents and $T\Delta S$ represents the entropic contribution. For identification of per residue contribution in the binding of ligands, the total binding free energy was decomposed into per residue energy contribution. The residue with energy contribution higher than −1.00 kcal/mol considered as a key contributor in the binding.

Results and discussion

Homology modeling

Model construction

The X-ray crystal structures of most of the GPCR has not been resolved, due to the major experimental challenges and difficulties in expression, purification and crystallization. Thus, computational modeling approaches are commonly used in hit to lead optimization during the drug development process. In this study, 3D structure of all three Galanin receptor subtypes were constructed by homology modeling approach using i-TASSER. The primary sequences of the three receptors were retrieved from UniProtKB and align to identify the sequence similarity using ClustalW (Figure S2). The sequences were further subjected to protein Blast for the search of homologous proteins. Blast search revealed many homologous proteins for all three receptors with the highest sequence identity of 35% (Table S1). All the homologous proteins were belong to GPCR class A. The sequences were submitted to i-TASSER for 3D model generation. Initially, five models were built for each receptor, based on the homologous structures. The templates used by i-TASSER for model generation of Galanin receptors were opioid receptors (Table S1 and Figure S3). The resulting models were ranked using C-score and the models with the highest C-score were further optimized and validated.
Model optimization and validation

The constructed models were initially refined by consecutive energy minimizations to remove the bad clashes, improper contacts and allow loop regions (intracellular and extracellular) to achieve stability. Afterwards, the final simulation of 20 ns was run for all the three receptors at 300 K. The optimized structures from MD simulation were evaluated to check the stereochemical and geometrical quality by plotting Ramachandran plot, Verify 3D and ERRAT plot. The Ramachandran plot analysis of GalR1 model revealed that psi and phi backbone dihedral angles were significantly accurate as 89% residues were occupying the most favored regions and 9.7% were located in additional allowed regions (Figure S4(A)). Only 1% and 0.3% residues were inside the generously allowed and disallowed regions, respectively. The residue Trp19 was found in the disallowed region, however none of binding site residue was found in this region. In case of GalR2 the analysis of Ramachandran plot revealed that 82.1%, 14.9% and 1.5% residues were inside the most favored, additional allowed and generously allowed regions, respectively (Figure S4(B)). However, 1.5% residues were inside the disallowed regions including amino acid residues Glu24, Ala16, Phe264, Gln373 and Ala377. The Ramachandran plot of GalR3 optimized model demonstrated that 84.8%, 13.3% and 1% residues were inside the most favored, additional allowed and generously allowed regions, respectively (Figure S4(C)). Only 1% residues lie in the disallowed region including Ala4, Leu9 and Arg365 which were also not from the binding site. Considering the psi and phi distribution of the amino acid residues, the constructed models were reasonably accurate.

The additional assessment of homology models of Galanin receptors was done by Verify 3D; which provides the inter-amino acid contacts and local environment compatibility of 3D model and 1D amino acid sequence. The constructed model is said to be accurate if the Verify 3D score \( \geq 0.2 \), which mean 80% of the amino acids in the structures have a score \( \geq 0.2 \) in the 3D/1D profile. The resulting Verify 3D values for GalR1, GalR2 and GalR3 were found to be 78%, 81% and 82%, respectively. As indicated, only the GalR1 model did not pass the assessment score (Figure S5(A–C)). Moreover, the quality of the constructed models based on interactions of non-bonded atoms was evaluated by plotting the ERRAT plot. The ERRAT score for GalR1, GalR2 and GalR3 was found to be 82.4%, 94.6% and 96.6%, respectively (Figure S6(A–C)). Based on the statistical analysis, the constructed models were found accurate to be used for additional steps (Figure 1). Taken together the results suggested the overall refinement of modeled structures as the stereo chemical parameters of all the models were improved after MD simulation (Figures S4–S6).

The refined models were visually analyzed to compare the binding site of the three receptors (Figure 2). The alignment of binding site of all three receptors showed that most of the Histidine residues are in good agreement. His264 in GalR1 correspond to His253 in GalR2 while His252 correspond to His251 in GalR3. However, His264 in GalR1 and His277 in GalR3 does not have any corresponding Histidine residue. Similarly, Phe282 in GalR1 correspond to Phe264 in GalR2 and Phe263 in GalR3 while Phe115 in GalR1 does not have any corresponding Phenylalanine residue. Tyr270 in GalR3 may play similar role in binding as Phe186 in GalR1 while Tyr103 does not have any corresponding residue.
Taken together cluster of hydrophobic residues may constitute the binding pocket of all the three receptors. The hydrophobic binding pocket in all receptors may attract ligands to bind to the inner part of the binding pocket. The final models for the three receptors were utilized to perform molecular docking studies to determine the binding affinity and ligand binding properties of the reported Galanin receptor ligands.

**Molecular docking simulation**

In order to investigate the ligands selectivity among Galanin receptors, two ligands were selected with their selective inhibitory profile against Galanin receptors and cross docked into the binding site of all three receptors. For prediction of binding site, SiteFinder module of MOE and DoGSiteScorer were utilized. It was interesting to note that SiteFinder and
DoGSiteScorer predicted the same binding site for the three receptors. The predicted binding site for the three receptors was used as docking gird during docking simulation. The experimental activity of the selected ligands is summarized in Table 1. From the observed experimental values, it was found that [I] inhibited the GalR1 with the highest biological activity to date while [III] is selective towards GalR3 as compared to GalR1 and GalR2. Molecular docking studies revealed that [I] showed the highest binding score with GalR1 as compared to GalR2 and GalR3, which is in good agreement with experimental activities (Tables 2 and S2). Binding mode analysis of [I] in the binding site of GalR1 showed that it well resides in the binding site by mediating several hydrophobic and hydrogen bond contacts with binding site residues (Figure 3(A)). Consequently, the docked pose of [I] in the binding site of GalR2 (Figure 3(B)) and GalR3 (Figure 3(C)) showed fewer number of hydrophobic and hydrogen bonding interactions as compared to GalR1. Similarly, [III] displayed the highest docking score against

![Graph of GalR1, GalR2, and GalR3](image1.png)

**Figure 5.** Time dependent Root Mean Square Deviation of GalR1, GalR2 and GalR3 upon binding of [III].

![Graph of GalR1, GalR2, and GalR3](image2.png)

**Figure 6.** Time dependent Root Mean Square Fluctuation of amino acid residues of Galanin receptor subtypes upon binding of [I].

| Receptor | GalR1 | GalR2 | GalR3 |
|----------|-------|-------|-------|
| Ligand ID | I     | II    | III   |
| Score (kcal/mol) | -8.7  | -6.4  | -7.1  |
|                    | -10.4 |       | -7.2  |
|                    |       |       | -9.0  |

**Table 2.** Binding scores of [I] and [III] with Galanin receptor ligands.

![Graph of Galanin receptor](image3.png)
GalR2 as compared to GalR1 and GalR3. However, the stable profile of [III] at the binding site of GalR3 (Figure 3(F)) could be explained due to the high number of hydrogen bonds and van der Waals interactions in comparison of GalR1 and GalR2 (Figure 3(D, E)). Further, the docked complexes were subjected to MD simulation studies.

**MD simulation**

MD simulation studies are routinely used to study dynamics of G-protein couple receptors upon binding of agonist/antagonist (Kaushik et al., 2018; Nath et al., 2019). Thus, to investigate the dynamics of receptor complexes in membrane and time dependent persistence of binding interactions between receptor and ligand to address selectivity, molecular dynamics simulation studies were carried out. Overall, the stability of the simulated systems was evaluated by plotting Root Mean Square Deviation (RMSD) of backbone carbon atoms with respect to their initial structures. GPCRs are dynamic in nature, their extracellular and intracellular loops show fluctuations. Thus, to reduce the fluctuation effect of loops region, RMSD of backbone carbon atoms was calculated only for binding pocket residues. The analysis of RMSD plot of [I] in complex with GalR1 revealed that system was converged during the 10 ns of simulation and remain stable throughout the 100 ns of simulation and prompted the RMSD of less than 0.18 nm (Figure 4). The RMSD fluctuation analysis of [I] in complex with GalR2 and GalR3 showed variable fluctuation as compared to GalR1 during the course of simulation (Figure 4). Upon examining the RMSD plot of [III] in complex with GalR3 it was observed that GalR3 was least deviated from its initial structure and showed inconsiderable fluctuations between 0.05 to 0.2 nm during the whole simulation (Figure 5). However, the RMSD start increasing after 80 ns. Similarly, GalR1 and GalR2 fluctuated between 0.12 nm to 0.25 nm with significantly stable RMSD (Figure 5).

In addition, backbone atom deviation in the targeted systems during initial and final time period were compared and are illustrated in Figure S8.

To investigate the mean fluctuation of per residues upon binding of ligands, Root Mean Square Fluctuation (RMSF) was calculated. The RMSF analysis of simulated trajectories demonstrated that binding of [I] considerably stabilized the three receptors (Figure 6). However, the binding site residues (Phe115, His264, His267 and Phe282) of GalR1 seems to be more rigid and less fluctuated as compared to Apo, GalR2 and GalR3 binding site residues (Table 3). Similarly, the RMSF plot of [III] in complex with three receptors showed the considerable stability for three receptors (Figure 7). However, the analysis of binding site residues of three receptors reveal that the residues (Tyr103, His251, Tyr270 and His277) of GalR3 exhibited least fluctuation as compared to Apo, GalR1 and GalR2 (Table 3).

**Binding mode analysis**

To get detailed insight into the binding mode of [I] and [III] in the binding site of all three Galanin receptors, the simulated trajectories were visually analyzed. The [I] was tightly buried in the hydrophobic cavity of the GalR1 binding site. The methylated benzene ring of [I] stack between Phe115, His267 and Phe282 producing hydrophobic effect (Figure 8(A)). It was observed that after 30 ns, Phe282 moves perpendicular to the methylated benzene ring of [I] and remain there till the end of the simulation. However, aromatic ring of Phe115 and His267 mediate parallel π-π and π-alkyl interaction, respectively, with methylated benzene ring during the whole simulation, which facilitated the tight binding of [I]. Similarly, Trp101, Arg285 and Ile286 were observed to mediate hydrophobic interactions. In addition to
hydrophobic interactions, hydrogen bonds contact with Gln92 and Tyr106 were also observed. Likewise, \( \pi \)-system of [I] mediate \( \pi \)-\( \pi \) stacking interaction with the aromatic ring of Tyr271 of GalR2 (Figure 8(B)). The hydrocarbon side chain of Ile256 of GalR2 establishes \( \pi \)-alkyl interactions with thiepine ring of [I] which persist during the whole simulation. However, compound accommodate in the binding site in such a way that it did not make any interaction with other crucial binding site residues during the course of simulation. The residues other than binding site involved in mediating hydrophobic interaction with [I] include, Trp91, Phe106, Ile105 and Val259. Similarly, in case of [I] bound to GalR3, the methylated benzene ring of [I] perpendicularly stack with Tyr103 by mediating \( \pi \)-\( \pi \) interactions (Figure 8(C)). However, other residues of binding site of GalR3 did not mediate any interaction, which indicated that most of the time [I] remain outside the binding site during the simulation. The residues other than binding site that were involved in the binding of [I] to GalR3 include, Tyr107, Leu256, Trp259, Tyr157 and Thr188.

The analysis of simulation trajectories of [III] in the binding site of three Galanin receptors suggested that [III] tightly buried in the hydrophobic pocket of GalR3. Binding mode of [III] in the binding site of GalR1 mediate \( \pi \)-\( \pi \) stacking with the binding site residues, Phe186 and Phe282 (Figure 8(D)). However, Phe186 and Phe282 after 70 ns moves away from the [III] and remain far till the end of the simulation. Similarly, Leu26, Ile111, Trp188, Arg285 and Ile286 were involved in the hydrophobic interactions with [III]. Likewise, the binding mode of [III] in the binding site of GalR2 demonstrated the \( \pi \) stacking interaction with His252, His253 and Ile256 while Phe264 and Tyr271 did not mediate any interaction with [III] (Figure 8(E)). In contrast, [III] was reside well in the binding site of GalR3 during the course of simulation and stabilized the receptor. The aromatic rings of [III] mediate \( \pi \)-\( \pi \) interaction with the aromatic ring of Tyr103, Tyr270 and His277 and remain in the same conformation till the end of simulation, that attribute the tight binding of S1 to GalR3 (Figure 8(F)). Moreover, Ile82, Ile102, Leu256, Ile255, Tyr161 and Pro174 also facilitated the binding of [III] to GalR3 by mediating hydrophobic interactions.
Per residue energy contribution

To further insight into the contribution of crucial residues in the binding of the ligands, the binding free energy of per residue was calculated. In case of [I] in complex with GalR1, Phe115 and His267 contribute more significantly by mediating hydrophobic interactions with the binding energy of \(-3.93\) kcal/mol and \(-1.95\) kcal/mol as compared to other binding site residues (Figure 9). The hydrophobic and \(\pi-\pi\) stacking interaction of methylated benzene with the aromatic ring of Phe115 and His267 may contribute significantly to the binding energy. Other binding site residues, including Phe186, His264, and Glu271 contribute less significantly with energy values of \(-0.09\) kcal/mol, \(-0.32\) kcal/mol, \(-0.59\) kcal/mol and \(-0.24\) kcal/mol, respectively, in the binding of [I] to GalR1. Similarly, in case of [I] in complex with GalR2, the major contribution provided by Ile256 with value of \(-2.92\) kcal/mol while other binding site residues including, His253, Phe264 and Tyr271 contribute less significantly with values of \(-0.58\) kcal/mol, \(-0.10\) kcal/mol and \(-0.72\) kcal/mol to the binding (Figure 10). His252 which is crucial for the activity of GalR2 showed no contribution with the value of \(-1.68\) kcal/mol in the binding. Whereas in case of [I] in complex with GalR3, the major contributor is Phe282 with the value of \(-6.43\) kcal/mol (Figure 9). Similarily, other residues including, Phe115, Phe186, His264, His267, and Glu271 contribute less in the binding with the energy values of \(-0.43\) kcal/mol, \(-0.12\) kcal/mol, \(-0.05\) kcal/mol, \(-0.004\) kcal/mol and \(-0.06\) kcal/mol. Likewise, in case of [III] in complex with GalR2, it was observed that the key residues that contribute significantly to the binding were Ile256 and Phe264 with the values of \(-3.08\) kcal/mol and \(-2.30\) kcal/mol, respectively (Figure 10). However, His252, His253 and Tyr271 with the values of \(-0.84\) kcal/mol, \(-3.40\) kcal/mol and \(-1.34\) kcal/mol, respectively, showed no contribution in the binding. Similarly, in the binding of [III] to GalR3, Tyr103, Tyr270 and His227 were found as key contributors with the values of \(-1.43\) kcal/mol, \(-1.09\) kcal/mol and \(-0.86\) kcal/mol, respectively, and attributed towards the selectivity (Figure 11). However, His251, Phe263, Arg273 with the affinity values of \(-0.08\) kcal/mol, \(-0.08\) kcal/mol and \(-1.36\) kcal/mol contribute nothing or very less in the binding. The results of per residue decomposition suggested that in the selective binding of [III] to GalR3, Tyr103, Tyr270 and His227 play major role and could be used as key residues in the design of selective ligands against GalR3. Likewise, in previous experimental studies these residues were found as crucial determinants of receptor function.
Conclusion

Galanin receptor subtypes are important drug targets in the treatment of neurodegenerative diseases. Exploration of structural and dynamic behavior of Galanin receptor subtypes thus provides a clear understanding of Galanin receptors structural detail and their binding mechanism, thus help in providing the remedial solutions of neurodegenerative disorders, effective treatment of cancer and other diseases caused by malfunctioning of these target proteins. So far, the X-ray structure of these Galanin receptors has not been resolved yet due to the major experimental challenges and difficulties in crystallization, expression and purification. Thus, in this study, we utilized computational modeling to identify the 3D structure of Galanin receptors followed by MD simulation and binding free energy calculation to explore the specific subtype ligand binding properties. Taken together the results indicated that Phe115 and His267 of GalR1 and Tyr103, Tyr270 and His277 of GalR3 play a vital role in the selective binding of ligands. This study will be helpful in future for identification and characterization of new selective inhibitors against Galanin receptors subtypes.

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