Ind. J. Pharm. Edu. Res., 2025; 59(2s):s641-s655.
https://www.ijper.org

Original Article

Optimizing 3D Structural Predictions of Murine β2-Adrenergic Receptor: Swiss-Model Outperforms AlphaFold

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ABSTRACT

Background and Aim: The β2 adrenergic receptor is vital in physiological processes and a key target for metabolic syndrome. Understanding the structural attributes of the murine β2-AR is crucial for comprehending metabolic regulation due to its close resemblance to the human β2-AR. Our study aimed to model the 3D structure of murine β2-AR using molecular simulation techniques to bridge gaps in structural understanding. **Materials and Methods:** In this study, we utilized *in silico* approaches to predict the 3D structure of murine β2-AR, employing 4 distinct modelling servers: SWISS-MODEL, Phyre2, I-TASSER and AlphaFold. Primary sequence analysed using Expasy's Protparam provided insights into charge distribution, stability and hydrophobic properties. Stereochemical analysis was performed using Ramachandran plot. **Results:** The primary sequence analysis of murine β2-AR revealed important characteristics, including an isoelectric point of 7.07 and an instability index score of 42.24. The high aliphatic index of 94.86 suggests thermal stability, while the GRAVY score of 0.145 indicates mild hydrophobicity. SWISS-MODEL emerged as the most reliable predictor, producing a highly promising structure with the maximum number of residues in favoured regions of the Ramachandran plot (93.1%) and no residues in disallowed regions. Additionally, the predicted structure exhibited a substantial number of helices (19) and a moderate number of turns (22) and strands (2), indicating its robust conformation. A 50 ns MD simulation demonstrated the consistent stability and integrity of the β2-AR protein. **Conclusion:** The homology model predicted by SWISS-MODEL outperformed those generated by AlphaFold. Overall, our findings underscore the significance of integrating computational modelling with experimental validation to unravel the intricate structural information of murine β2-AR and its implications in translational research.

Keywords: β2 adrenergic receptors, Metabolic syndrome, AlphaFold, SWISS-MODEL, Computational modelling.

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Received: 26-02-2024;
Revised: 18-07-2024;
Accepted: 07-12-2024.

INTRODUCTION

Metabolic Syndrome (MetS) represents profound challenges to global health, characterized by dysregulated glucose and lipid metabolism, insulin resistance and cardiovascular complications. In complex metabolic systems the β2 Adrenergic Receptor (β2-AR), plays a crucial role in maintaining metabolic balance and regulating glucose metabolism. Among the extensive G Protein-Coupled Receptor (GPCR) family, β2-AR exhibit a complex structural composition, featuring seven transmembrane alpha helices intricately linked by extracellular and intracellular loops, ultimately terminating in distinct C and N terminals.[1] Among these receptors, β2-AR emerges as central players in thermogenesis, lipolysis and glucose metabolism, exerting profound influence on metabolic regulation.[2] Genetic variants within β2-AR have been implicated in metabolic disorders, notably the ADRB2 Gln27Glu polymorphism, associated with alterations in triglyceride and HDL levels, predisposing individuals to type 2 diabetes mellitus and influencing visceral fat accumulation.[3] Numerous drugs targeting β2-AR were developed with the aim of potentially treating MetS. However, their effectiveness in clinical trials produced inconsistent results due to several factors, including the syndrome's complexity, incomplete understanding of β2-AR function, potential off-target effects, patient heterogeneity and trial design considerations.[4]

The elucidation of the crystallized structure of human β2-AR in 2007 marked a significant milestone in structural biology,

DOI: 10.5530/ijper.20256524

providing unprecedented insights into receptor-ligand interactions and guiding drug discovery efforts.[5-7] While studying the human β2-AR structure is indeed valuable for drug development, understanding the murine β2-AR structure is also essential as the murine receptor closely resembles the human version and is relevant for preclinical research. Murine models provide a tractable system for *in vivo* experimentation, allowing investigation of physiological responses and disease pathogenesis within a genetically manipulable organism.[8] Moreover, the conservation of β2-AR function across species enables extrapolation of findings from murine studies to human biology, facilitating translation of preclinical discoveries into clinical applications. Predicting the structure of murine β2-AR grants a comprehensive understanding of receptor-ligand interactions, allosteric modulation and signal transduction pathways specific to the murine system.[9,10] Moreover, such insights enable the design of tailored pharmacological interventions aimed at restoring metabolic balance and mitigating disease progression.

Additionally, structure prediction of murine β2-AR offers a promising avenue for rational drug design and targeted therapeutics.[11] By harnessing computational techniques and bioinformatics tools, elucidating the three-Dimensional (3D) architecture of murine β2-AR can facilitate identification of novel ligands and allosteric modulators with enhanced selectivity and efficacy. Our study focused on modeling the 3D structure of the murine β2-AR to bridge gaps in our structural knowledge and gain insights into how species-specific differences might affect drug responses in preclinical models.

MATERIALS AND METHODS

β2-AR Protein Sequence Retrieval and Primary Sequence Assessment

The murine β2-AR amino acid sequence (Accession No: NP_031446.2) was retrieved from GeneBank in FASTA format (https://www.ncbi.nlm.nih.gov/protein/NP_031446.2?report=fasta). Physicochemical properties were analysed using ExPASy's ProtParam server (http://web.expasy.org/protparam/). Parameters including molecular weight, isoelectric point (pI), extinction coefficient (EI), aliphatic index (AI) and +R and -R counts were determined. Grand average of hydropathicity index (GRAVY) parameters were also computed for the β2-AR protein in Mus musculus. To assess the sequence similarity of murine β2-AR, we conducted a comparative analysis. This involved using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to search the Protein Data Bank (PDB) for closely related template sequences. Subsequently, we aligned these templates with the murine β2-AR sequence using the EMBL-Clustal W multiple sequence alignment web server (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). This systematic alignment process revealed commonalities and variations, enabling a comprehensive examination of conserved regions relevant to β2-AR across species.

Transmembrane Helix Analysis of Murine β2-AR

We utilized the TMHMM search tool (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) to predict transmembrane regions within the protein sequence of Murine β2-AR. TMHMM designed specifically for predicting transmembrane helices in integral membrane proteins.

Investigating motifs, domains, and analysing Protein-Protein Interaction Networks

Motif discovery in the murine β2-AR protein sequence aims to unveil conserved patterns specific to this receptor, shedding light on its distinct biological roles. Using MEME (https://meme-suite.org/meme/tools/meme) in 'Classic mode' with the sequence alphabet set to 'Protein', we analysed β2-AR protein sequences from Homo sapiens obtained from PDB entries. Parameters were set to identify three distinct motifs capturing conserved features across species. Additionally, we used ScanProsite (https://prosite.expasy.org/cgi-bin/prosite/scanprosite) to explore potential functional features within the murine β2-AR amino acid sequence. For functional network analysis, β2-AR was examined using the STRING database search (https://string-db.org/). Interactions were filtered for medium confidence (0.4), ensuring reliability. The full STRING network type was chosen to represent comprehensive protein associations.

Homology modelling for murine β2-AR

The SWISS-MODEL (https://swissmodel.expasy.org) was used to predict the homology model for the murine β2-AR. This involved steps like identifying the template structure, aligning the target sequence, building the model and assessing its quality, focusing on criteria like sequence coverage and percentage identity for accuracy.[12] Alternatively, the Phyre2.0 (http://www.sbg.bio.ic.ac.uk/phyre2) server offers another method for generating models, utilizing predictive algorithms such as homology modelling and ab initio modelling to predict protein secondary structure and compare it to a database of known structures.[13] Threaded ASSEmbly Refinement (TASSER) (https://zhanggroup.org/I-TASSER/)threads the β2-AR sequence through a database to uncover potential structural templates, forming initial models via ab initio modelling, which are then refined based on chosen templates.[14] The AlphaFold (AF) protein structure prediction database (https://alphafold.ebi.ac.uk/) enhances this strategy by providing a comprehensive analysis of the protein structure,[15] using various prediction tools and servers to ensure accuracy and reliability, including comparison with AF for assessment.

Murine β2-AR structure validation

The model quality of the murine β2-AR structures was evaluated using the Ramachandran plot generated by PDBSUM (https://

www.ebi.ac.uk/thornton-srv/databases/pdbsum/), along with stereochemical analyses using SAVES structure validation server (https://saves.mbi.ucla.edu/). The structure was further refined using ModRefiner (https://bio.tools/modrefiner).

Determination of Binding Site

A Computed Atlas of Surface Topography of proteins (CASTp) (http://sts.bioe.uic.edu/castp/) was used to visualize the annotated functional residues. The active sites of β2- AR proteins have been predicted using CASTp. The predicted β2-AR protein was submitted on the server and the necessary amino acids for binding interactions were predicted.

Molecular Dynamic Simulation

Molecular Dynamics (MD) simulations were conducted using GROMACS on the WEBGRO server with the GROMOS96 43a1 forcefield. The SPC water model and a triclinic box were used, with sodium chloride added at 0.15M to ensure system neutrality. An energy minimization step with the Steepest Descent integrator over 5000 steps was performed before production simulations. NVT (constant Number of particles, Volume and Temperature) and NPT (constant Number of particles, Pressure and Temperature) ensembles were used for equilibration and MD production runs, maintaining 300 K and 1.0 bar. The Leap-frog integrator tracked system behavior over a 50 ns simulation, collecting approximately 1000 frames for comprehensive analysis.

RESULTS

In our study, we obtained the FASTA format of the murine β2-AR amino acid sequence from NCBI. It comprised 418 amino acid residues, associated with accession number NP_031446.2.

Physico-chemical analysis and trance membrane prediction

For murine β2-AR, the theoretical pI, was found to be 7.07, placing it on the neutral side of the pH spectrum. ProtParam employs this rule to predict protein half-life across three model species: human, yeast and *E. coli*. The estimated half-life was notably 30 hr for mammalian reticulocytes, greater than 20 hr for yeast and exceeding 10 hr for E. coli. The Instability Index (II), a measure of *in vitro* protein stability based on the N-end rule, revealed a value of 42.24 for murine β2-AR, indicating structural instability. The AI, representing the proportion of volume occupied by aliphatic side chains (Ala, Val, Iso and Leu) and influencing globular protein thermostability, was found to be 94.86 for β2-AR. This high AI suggests thermal stability, indicating that the protein is likely to maintain structural integrity at elevated temperatures. The GRAVY score for β2-AR was 0.145, indicating mild hydrophobicity.

Homology modelling was employed to analyze the 3D structure of murine β2-AR. Sequence similarity was evaluated by comparing it with protein databases using NCBI BLASTp to find suitable templates for comparative structural analysis. BLASTp analysis identified numerous potential hits and from these, the top five hits were chosen for further investigation via multiple sequence alignment. These selected hits include the following proteins: Chain A of β2-AR from Homo sapiens (2R4R_A) having 89.92% sequence identity with 87% query coverage, Chain A of β2-AR from Homo sapiens (3KJ6_A) having 90.16% sequence identity with 87% query coverage, Chain A of β2-AR from Homo sapiens (2R4S_A) having 90.46% sequence identity with 82% query coverage, Chain A of β2-AR from Homo sapiens (6KR8_A) having 77% sequence identity with 88.27% query coverage and Chain R of β2-AR from Homo sapiens (7DHI_R) having

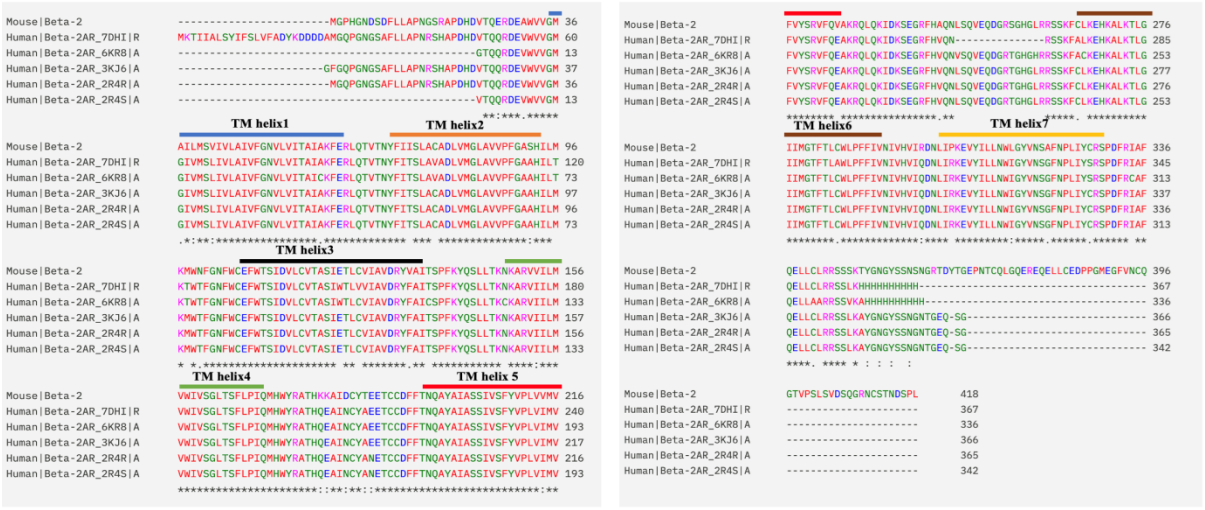


Figure 1: Multiple Sequence alignment for murine β2-AR, with reference structure of human β2-AR (pdb ID: 2R4R_A, 3KJ6_A, 2R4S_A, 6KR8_A and 7DHI_R) Amino acid sequence: amino acid positions are numbered on right, (*) indicates the conserved regions, (:) indicates the conservation between groups of strong similar property, (.) indicates the conservation between groups of weakly similar and (–) indicates the deletion. The conserved domains are indicated in different colors. TMh: Transmembrane helix.

83% sequence identity with 85.34% query coverage. A notable sequence similarity was observed among the templates (Figure 1). Among the extensive GPCR family, β2 adrenergic receptors exhibit a complex structural composition, featuring seven transmembrane alpha helices intricately linked by extracellular and intracellular loops, ultimately terminating in distinct C and N terminals (Figure 2).

Motif, domain prediction and Protein-Protein Interaction (PPI)

The TM prediction using TMHMM revealed 7TMs (Figure 3), the conserved domain analysis indicated the presence of 7TMs (TMh1, TMh2, TMh3, TMh4, TMh5, TMh6 and TMh7). The first motif, with an E-value of 9.5e-222, spans 50 amino acids. Similarly, the second motif, with an E-value of 6.2e-210, also spans 50 amino acids and is found in six sites. The third motif, with an E-value of 2.1e-205, covers 50 amino acids and is distributed across six sites within the protein sequences (Figure 4A). Additionally, the motif locations for each sequence were provided, underscoring the importance of these motifs within their respective protein sequences (Figure 4B). The domain analysis identified profile for the GPCR family 1 (Accession No: PS50262) spanned residues 50 to 326 within the receptor. The computed overall score for this profile was 48.918, indicating a high likelihood of the protein's association with the specified family. Notably, predicted features revealed the presence of two disulfide bonds in the receptor's structure. The first disulfide bond was predicted between residues Leu 106 and Ser 191, while the second disulfide bond was anticipated between residues Cys 184 and Leu 190. These findings suggest potential structural stabilization facilitated by disulfide linkages in the murine β2-AR (Figure 4C).

The Protein-Protein Interaction (PPI) network analysis of murine β2-AR (ADRB2) protein revealed a complex network of interactions with various proteins in the murine genome. ADRB2 displayed strong connections with proteins such as ARRB1, ARRB2, GNAS and ADRB3 (Figure 4D). The combined score from the STRING database indicated a high confidence level in these interactions. Network analysis unveiled an 11-node network with 44 edges, indicative of a well-connected system. A high average local clustering coefficient (0.891) suggested a dense and highly clustered network.[16] Remarkably, the protein-protein interaction enrichment p-value was notably low at 3.74e-07, indicating a statistically significant enrichment of interactions in the network.

Predicting and comparing the 3D structure of murine β2-AR protein

In our study, we analysed and compared different models, selecting the best SWISS-MODEL based on Global Model Quality Estimation (GMQE) and Qualitative Model Energy Analysis (QMEAN) values. Constructed using the 2rh1.1.A template, it showed a percentage identity of 83.63%, a GMQE value of 0.64 and a QMEAN score of 0.72±0.05. Phyre used the c3pdsA template to construct the structure. The target and template

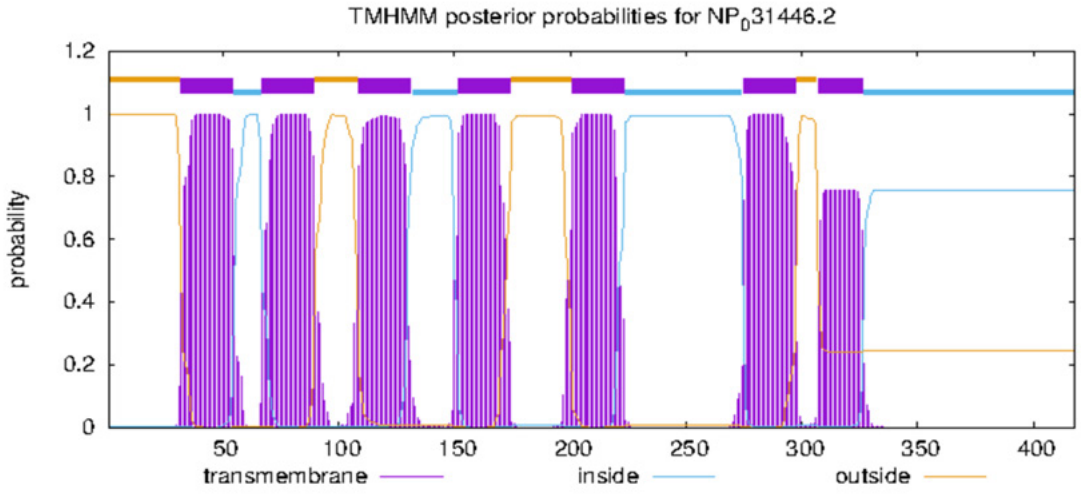


Figure 2: Transmembrane region prediction of murine β2-AR protein.

Table 1: Best-predicted models of β2-AR constructed using four software tools.

Sl. No.	Tool	Template	Identity (%)	PDB Id
1	SWISS MODEL	2rh1.1.A	83.63%	2RH1
2	PHYRE	c3pdsA	87	3PDS
3	iTASSER	2rh1A	48	2RH1
4	Alpha fold	Not available	Not available	Not available

Table 2: Structural properties of best-predicted 3D structures of β2-AR constructed using four software tools.

Structural properties					
		β2adr_SWISS	β2adr_iTASSER	β2adr_Phyre	β2adr_Alphafold
1	Number of amino acids	317	418	313	418
2	Number of helices	19	16	12	15
3	Number of strands	2	4	0	0
4	Number of turns	22	39	25	18
Ramachandran plot validation					
	Ramachandran plot parameters	**β2adr_SWISS**	**β2adr_iTASSER**	**β2adr_Phyre**	**β2adr_Alphafold**
1	Residues in most favoured regions [A,B,L]	271 (93.1%)	269 (77.1%)	266 (92%)	311 (82.9%)
2	Residues in additional allowed regions [a,b,l,p]	18 (6.2%)	81 (21.6%)	22 (7.6%)	45 (12.0%)
3	Residues in generously allowed regions [~a,~b,~l,~p]	2 (0.7%)	16 (4.3%)	0 (0.0%)	10 (2.7%)
4	Residues in disallowed regions	0.0	9 (2.4)	1 (0.3%)	9 (2.4%)
5	Number of non-glycine and non-proline residues	291 (100%)	375 (100%)	289 (100%)	375 (100%)
6	Number of end-residues (excl. Gly and Pro)	5	2	3	2
7	Number of glycine residues (shown as triangles)	13	25	13	25
8	Number of proline residues 28	8	16	8	16
9	Total number of residues	317	418	313	418

sequences shared 87% of amino acids, with sequence coverage of 74%. These values are crucial for predicting model accuracy, with higher percentages typically indicating greater accuracy.[13] The best structure from iTASSER had a C Score of 1.18, indicating high confidence. The estimated RMSD for model was 9.6±4.6Å, with lower values suggesting better agreement. The Estimated TM Score for model was 0.57±0.15, indicating significant structural similarity to the actual target protein. The AF predicted model exhibited a pLDDT (probability of local distance difference test) value of 94.56%, indicating precise predictions and well predicted structures. The pLDDT score is a measure of confidence in the predicted structure, with higher values suggesting better accuracy.

Further we evaluated the stereochemical characteristics and accuracy of predicted murine β2-AR structure (Table 1). PROCHECK's Ramachandran plot analysis assessed the overall stereochemical quality of the models and identified potential outliers, which could indicate errors or inaccuracies. Additionally, ERRAT evaluated the model's compatibility with X-ray crystallography or NMR experimental data, with a higher ERRAT score indicating a better fit between the model and experimental data.[17]

For structure from SWISS-MODEL, PROCHECK's Ramachandran plot analysis revealed that (93.1%.) of residues were within favoured regions, indicating good structural quality, with lower percentages in allowed (6.2%) and generously allowed (0.7%) regions and a minimal proportion in disallowed regions (0.0%). The ERRAT value for the same model was 94.2373, suggesting good accuracy (Figure 5A; 4B). For the β2-AR protein, models generated by Phyre and iTASSER were assessed. Phyre showed (92%) of residues in favoured regions on the Ramachandran plot, lower percentages in allowed (7.6%) and generously allowed (0.0%) regions and a minimal proportion in disallowed regions (0.3%) with ERRAT value of 79.6552 indicating room for improvement. The iTASSER model displayed 77.1 %of residues in favoured regions, lower percentages in allowed (21.6%) and generously allowed (4.3%) regions and a minimal proportion in disallowed regions (2.4%) with an ERRAT score of 87.0416, suggesting good quality with moderate deviations from known structures. Comparatively, AF analysis of murine β2-AR revealed 82.9% of residues in favoured regions, lower percentages in allowed (12.0%) and generously allowed (2.7%) regions and a minimal proportion in disallowed regions (2.4%) with an ERRAT value of 97.4522, indicating reliable structure prediction. Secondary structure elements, including helices, strands and turns, were also analysed to assess the overall fold of the protein models (Table 2). The SWISS-MODEL and Phyre models consisted of 317 and 313 amino acids, respectively, while

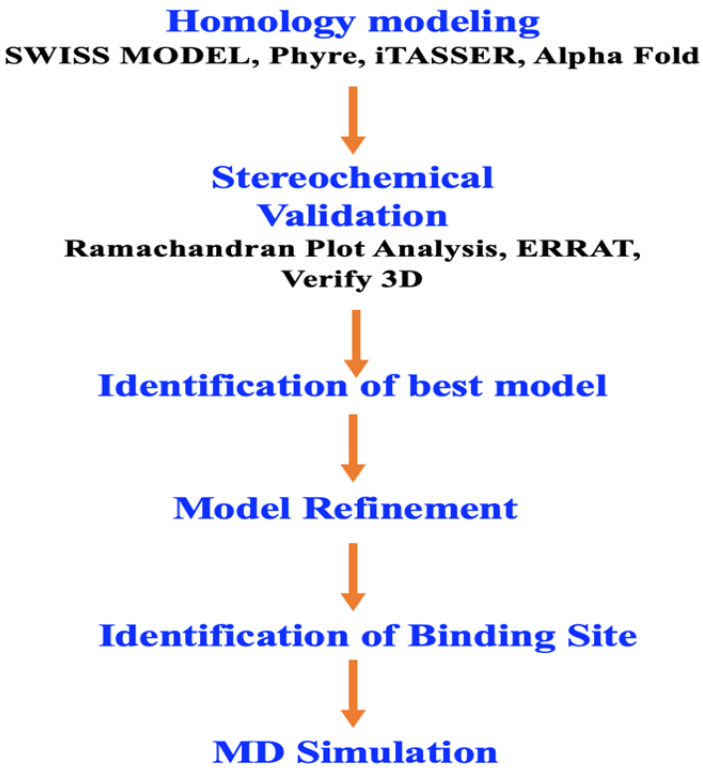
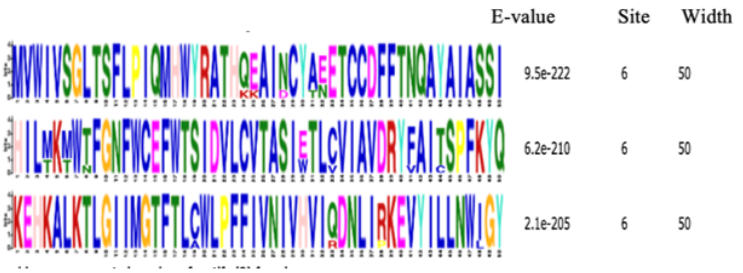
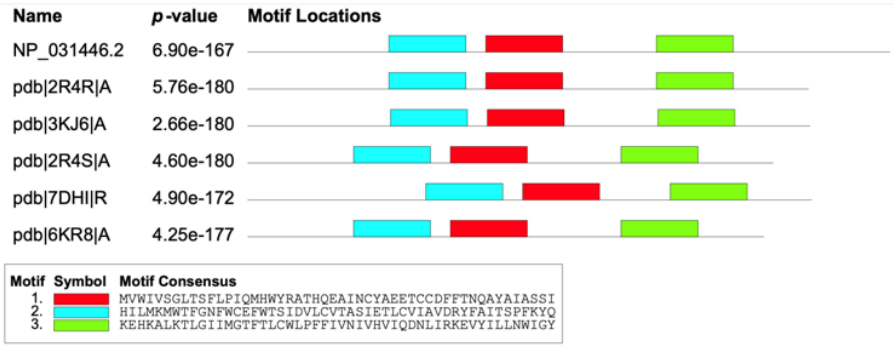


Figure 3: Schematic representation of developing a 3D model for murine β2-AR.



A



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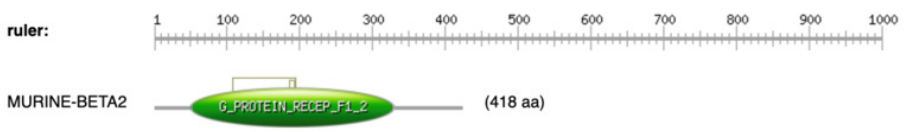

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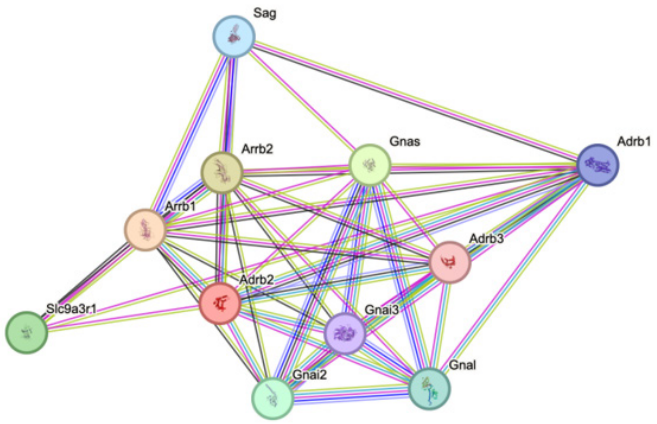

D

Figure 4: Motif, Domain Prediction and Protein-Protein Interaction (PPI) Analysis. A) Predicted motif logos in the β2-AR protein and five other proteins. B) Identified locations of predicted motifs in the β2-AR protein and the selected five proteins. C) Predicted functional domain of murine β2-AR protein. D) Protein-protein interaction network associated with murine β2-AR protein. The thickness of edges is level of edge confidence.

the iTASSER and AF models contained 418 amino acids each. The SWISS-MODEL exhibited the highest number of helices-19, followed by iTASSER-16, AF-15 and Phyre-12. Similarly, iTASSER and SWISS-MODELs contained the most significant number of strands i.e. 4 and 2, respectively, while Phyre and Alpha Fold models lacked strands entirely. The number of turns varied among the models, with iTASSER exhibiting the highest count of 39, followed by Phyre-25, SWISS-MODEL-22 and AF-18 (Table 2).

Identification of a binding site in β2-AR

The refined structure of murine β2-AR used for binding site identification. Using the CASTp 3.0 tool, the top three binding pockets were selected based on area and volume (Figure 6). The active sites within murine β2-AR have three distinct pockets: Pocket 1 (872.500 Å² area, 572.313 Å³ volume), Pocket 2 (346.416 Å² area, 307.582 Å³ volume) and Pocket 3 (143.398 Å² area, 158.627 Å³ volume). These differences suggest varying binding capabilities or functional roles among the pockets. The phenyl ethanolamine moiety of mirabegron binds similarly to adrenaline in β2-AR, indicating structural similarities with human β2-AR and providing insights into their roles in insulin secretion and other physiological processes.

Molecular Dynamics Simulation of Murine β2-AR Structure

Molecular Dynamics (MD) simulations were employed to investigate the stability of the predicted structure of murine β2-AR, assessing its structural integrity and dynamic behavior over time. During a 50 ns simulation, the murine β2-AR protein exhibited a consistent level of stability, as indicated by the Root Mean Square Deviation (RMSD) analysis (Figure 7A). The average RMSD value throughout the simulation was approximately 0.8 nm, signifying that the protein reached an equilibrium state early in the simulation. This suggests the homology model retained its overall structural integrity, with only moderate deviations from the initial conformation.

Furthermore, the analysis of protein fluctuations during the simulation provided insights into the dynamic behavior of the

murine β2-AR model. These fluctuations, characterized by Root Mean Square Fluctuation (RMSF), revealed regions with higher flexibility, particularly around residues 361-370 (Figure 7B). This localized flexibility may have functional implications, suggesting that this region could play a role in ligand binding, conformational changes, or interactions with other biomolecules.

To further assess the compactness and spatial distribution of the murine β2-AR protein, we examined the Radius of gyration (Rg), a crucial parameter for understanding molecular folding and conformational dynamics. The average Rg value for the protein was determined to be approximately 2.4 nm, indicating that the murine β2-AR maintained a reasonably compact structure throughout the simulation (Figure 7C). This observation aligns with the notion that the protein remained stable and well-folded during the 50 ns trajectory.

DISCUSSION

The structure of the murine β2-AR is crucial for drug discovery as it provides insights into the receptor's topology, conformational changes and ligand recognition.[18] The theoretical pI obtained from the primary sequence analysis signifies the pH at which a molecule or surface carries no net electrical charge, offering insights into protein charge stability.[19] For murine β2-AR, the theoretical pI was found to be 7.07, placing it on the neutral side of the pH spectrum. This information is crucial as it helps predict the protein's behaviour under different pH conditions, which

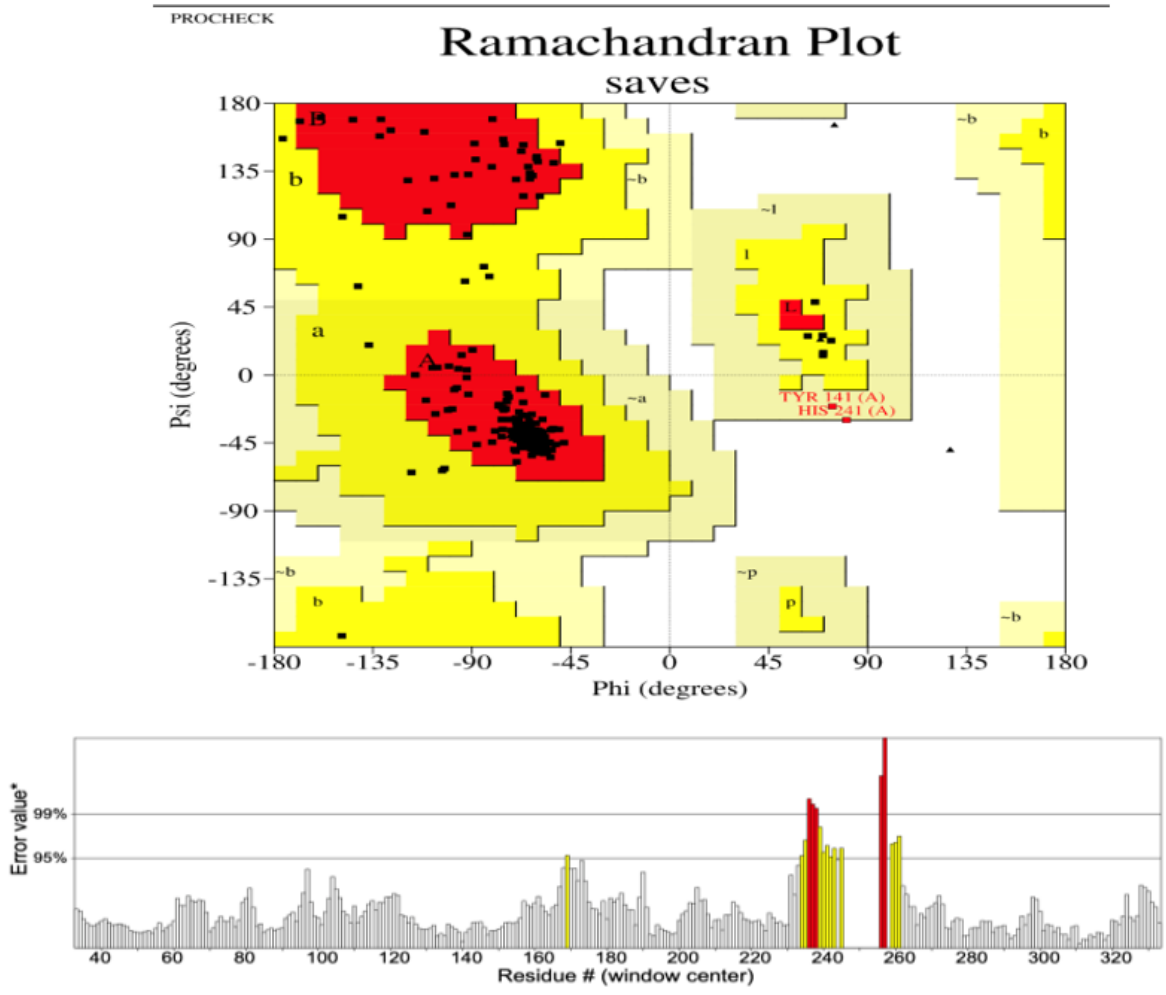

Figure 5: Homology model created for β2-AR from SWISS Model server. A) A Ramachandran plot analysis displaying the presence of residues within favoured (red), allowed (yellow), generously allowed (light yellow) and disallowed (white) regions. B) The model's overall quality, as determined by the ERRAT program. On the error axis, two lines are drawn to indicate the confidence with which it is possible to reject regions that exceed that error value. Two lines (95 and 99%) on the error axis demonstrated the confidence with which regions that are larger than that error value can be discarded.

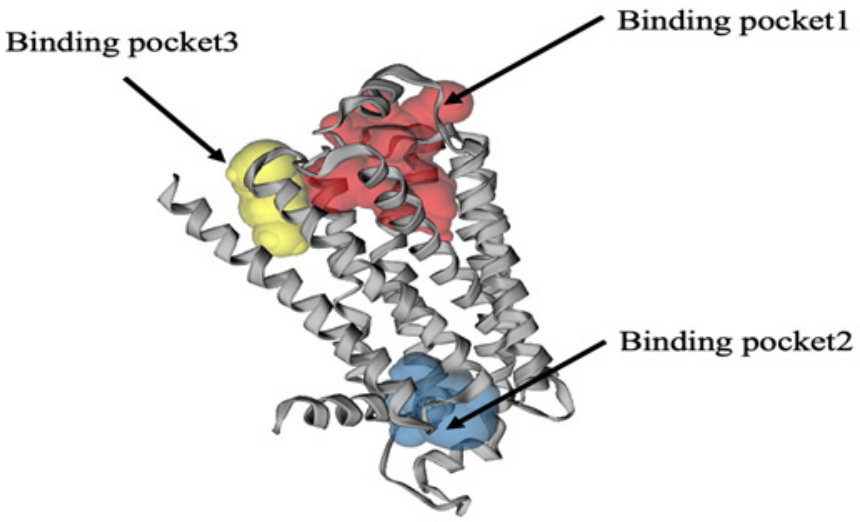

Figure 6: The surface of the binding pocket of the modeled murine β2-AR protein as computed using CASTp 3.0. The top three binding sites are highlighted in red, blue and yellow, respectively.

is especially relevant for understanding its function in various cellular environments.

The estimated half-life of the murine β2-AR in our study is notably 30 hr for mammalian reticulocytes, greater than 20 hr for yeast and exceeding 10 hr for *E. coli*. This extended half-life in different organisms suggests the stability of the receptor, which is significant for its functional regulation and turnover. The prolonged half-life may influence the receptor's signalling and desensitization, as well as its regulation by processes such as ubiquitination and lysosomal degradation.[20,21]

The instability index serves as a predictor of a protein's stability based on the N-end rule under experimental conditions. Proteins with an instability index below 40 are typically anticipated to be stable.[22] For murine β2-AR, an II value of 42.24 was obtained, indicating structural instability. This insight is crucial for understanding the protein's folding dynamics and susceptibility to degradation, which can influence its biological activity and regulation. Additionally, the AI of β2-AR was found to be 94.86, suggesting thermal stability. The AI represents the proportion of volume occupied by aliphatic side chains, such as Ala, Val, Iso and Leu and influences the globular protein's thermostability.[23] A high AI value implies that the protein is likely to maintain its structural integrity even at elevated temperatures, which is essential for its proper functioning in biological systems. The GRAVY value of a peptide or protein is calculated by summing the hydropathy values of its amino acids and dividing by the total number of residues. Negative GRAVY values suggest hydrophilicity, while positive values suggest hydrophobicity, usually falling within the range of -2 to +2.[24] In our study, the murine β2-AR had a GRAVY score of 0.145, indicating mild hydrophobicity which can influence its interactions with other molecules and cellular membranes.

The BLASTp analysis revealed significant sequence similarity between murine β2-AR and several β2-AR isoforms from Homo sapiens, indicating evolutionary conservation of the receptor across species. β2-AR are known for their complex structural composition, characterized by seven transmembrane alpha helices interconnected by extracellular and intracellular loops.[25] This intricate arrangement plays a pivotal role in ligand binding, signal transduction and receptor activation. The conservation of key structural motifs and domains among β2-AR isoforms suggests their functional significance in mediating cellular signalling pathways and physiological responses to catecholamines. The presence of three distinct motifs, each spanning 50 amino acids and distributed across multiple sites within the protein sequence, underscores their significance in mediating cellular signalling pathways. The remarkably low E-values associated with these motifs indicate their high statistical significance, suggesting their conservation and functional importance. These findings lay the groundwork for further exploration of the roles played by these motifs in regulating β2-AR function and their implications in cellular signalling cascades. Studies report that β2-AR, has a highly conserved DRY motif in its Intracellular Loop 3 (ICL3), which is crucial for G-protein coupling and signalling. Additionally, the β2-AR's intrinsically disordered Carboxyl Terminus (CT) is a substrate for GPCR kinases and binds β-arrestins to regulate signalling.[26,27]

Domain analysis of β2-AR revealed the presence of a profile corresponding to the G-protein coupled receptors family 1, spanning residues 50 to 326 within the receptor. The high overall score for this domain profile indicates a strong likelihood of β2-AR's association with the GPCR family, corroborating its classification as a member of this receptor family. Furthermore, the prediction of two disulfide bonds within the receptor's structure

suggests potential mechanisms for structural stabilization. Disulfide linkages, formed between specific cysteine residues, have a key role in maintaining the tertiary structure of proteins and are often involved in stabilizing extracellular domains of membrane proteins like GPCRs. The predicted disulfide bonds between residues 106-191 and 184-190 highlight regions of potential structural importance within β2-AR, providing insights into its folding and stability. Noda (1994) identified four Cys residues within the β2-AR extracellular loop 2 (EL2). Specifically, 2 of these residues (Cys184 and Cys190) were noted to form an internal disulfide bridge within EL2, while the remaining residue (Cys191) was found to form a disulfide bridge with a Cys residue located extracellularly in TM3, a characteristic conserved across most GPCRs.[28] The PPI network analysis of murine β2-AR revealed a complex network of interactions with various proteins encoded in the murine genome. Notably, β2-AR displayed strong connections with proteins such as ARRB1, ARRB2, GNAS and ADRB3, indicative of its involvement in diverse physiological processes and cellular signalling pathways.[29] The dense and highly clustered network observed, characterized by a high average local clustering coefficient, suggests a tightly interconnected system of protein interactions centred around β2-AR.[30]

In our study, the SWISS-MODEL generated a 3D model of the murine β2-AR protein based on its sequence alignment with the most appropriate structural template available in the PDB library. The GMQE score, which represents the overall quality estimate of the predicted, was calculated based on the alignment of the target sequence with the selected template structure. A higher GMQE value indicates increased reliability of the alignment and, consequently, the predicted model. In the current study, the model was constructed using the 2rh1.1.A template exhibited a GMQE value of 0.64, signifying a relatively good level of confidence in the accuracy of the predicted structure.[31]

Additionally, the QMEAN score provided further insights into the quality of the generated model by assessing its geometrical features in comparison to experimental structures. A QMEAN score approaching zero indicates a good fit between the predicted and experimental structures, suggesting high quality. In our study, the QMEAN score of the generated structure was 0.72±0.05, indicating good structural quality and alignment with experimental data.[32] Furthermore, the percentage identity between the target sequence and the selected template was considered, with a higher percentage indicating a closer match between the two sequences. Our analysis revealed a percentage identity of 83.63%, indicating significant similarity between the target and template sequences and further supporting the reliability of the predicted structure.

Phyre, employing an ab initio approach, relies on various metrics to assess protein similarity. One such metric is sequence identity, which quantifies the percentage of identical amino acid residues shared between the protein of interest and its closest homolog in the database. A higher sequence identity generally suggests a more reliable structure. Additionally, Phyre assigns a confidence score to each residue in the predicted structure, indicating the degree of confidence in its position. This score ranges from 0 to 1, with 1 representing a high confidence level. Furthermore, in our study, Phyre utilized the c3pdsA template to construct the 3D structure of the target protein. The high sequence identity of 87% and sequence coverage of 74% between the target and template sequences are indicative of the 3D structure's accuracy, with higher percentages typically associated with greater reliability.

iTASSER (Threaded ASSEmbly Refinement) relies on threading a protein sequence through a comprehensive database of known protein structures, by employing a combination of ab initio modelling and refinement techniques. In our study, iTASSER was employed to estimate the protein structure of the target protein, resulting in structure with a significant degree of accuracy. Here, the C score is used to assess the confidence of a predicted murine β2-AR protein structure. The C Score range is typically between −5 and 2, with higher values implying higher confidence. The predicted structure with the highest C score is considered the best model. In our study, the best predicted structure from iTASSER had a C Score of 1.18, indicating high confidence. Estimated RMSD is an estimate of the structural similarity between the predicted and experimental structures. In our study, the estimated RMSD for the model was 9.6±4.6Å, with lower values suggesting better agreement. AF, a deep learning-based protein structure prediction method, has been successfully used to predict structural features of GPCRs, including the β2-adrenergic receptor.AF has demonstrated reasonable correspondence to experimental structures and its ability to predict structural features has been evaluated for various receptors, including the β2-AR.[33]

We observed variations in the number of amino acids among the generated models. While the number of amino acids alone does not determine the quality of a model, it is noteworthy that the SWISS-MODEL model contained a relatively lower number of amino acids i.e. 317 compared to the iTASSER and AF predicted structure (i.e. 418 each). This may suggest a more concise representation of the protein structure without unnecessary extensions. The variation in the length of amino acids in different predicted structures is primarily due to the sequence identity to the templates used, rather than the algorithms themselves. When a template with high sequence identity to the target protein is used, the predicted structure is more likely to have a similar length and overall conformation. Conversely, templates with lower sequence identity may result in structures with differing lengths due to insertions, deletions, or differences in loop regions. Therefore, the choice of template significantly influences the predicted amino acid length, highlighting the importance of sequence identity in structural modeling. The presence or absence of specific structural elements, such as strands, can significantly influence the

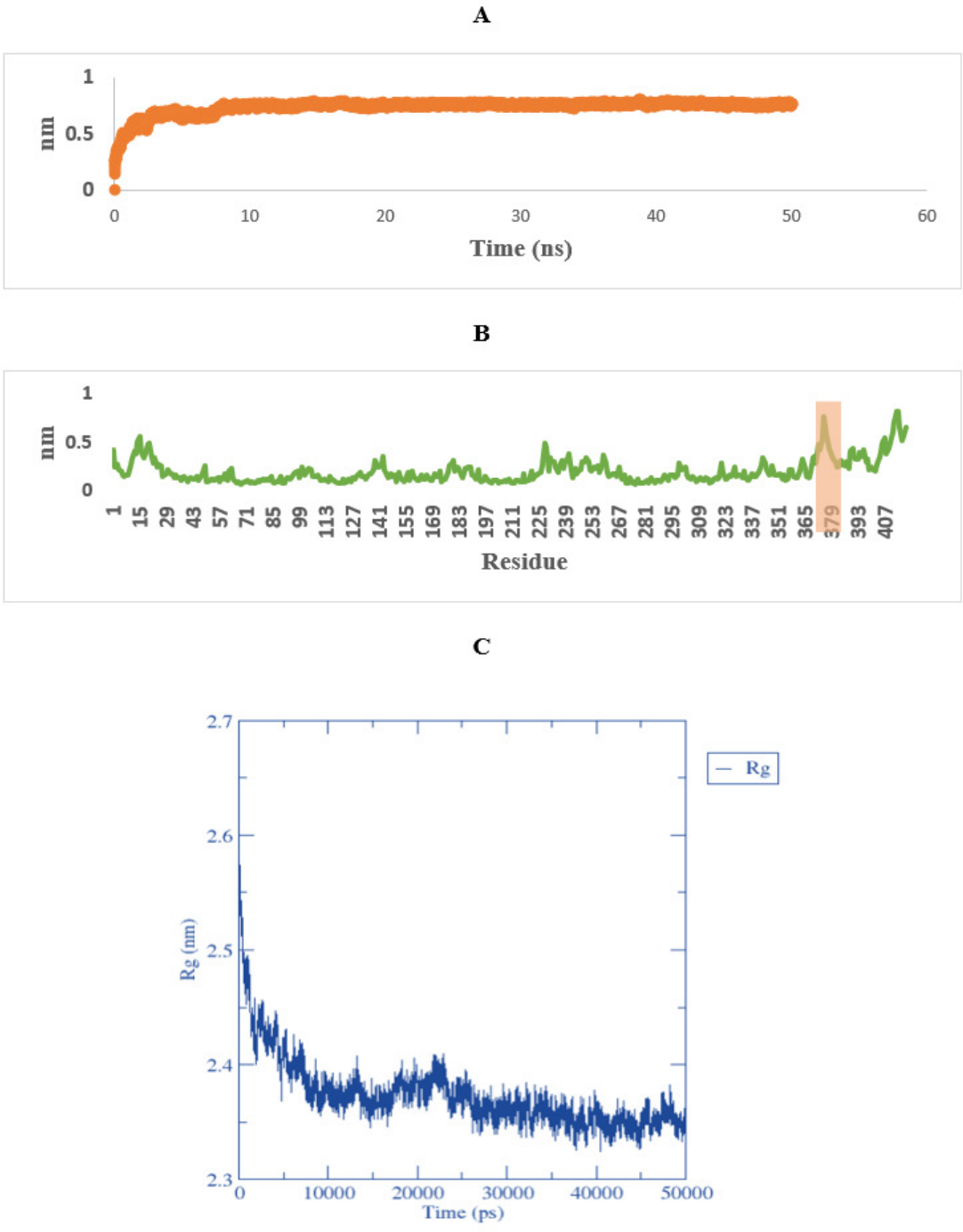

Figure 7: MD simulations analysis for murine β2-AR protein. (A) RMSD study plot of murine β2-AR protein over 50 ns, (B) RMSF plot depicting the assessment of murine β2-AR, (C) Radius of Gyration (Rg) plot reflecting the changes observed in the conformational behavior of murine β2-AR.

overall topology and functional properties of a protein. Strands are essential components of β-sheets, a common secondary structure motif found in proteins, and they play crucial roles in protein folding, stability and interactions with other molecules.[34] Therefore, the absence of strands in the AF structure may impact its overall structural integrity and functional characteristics compared to the SWISS-MODEL predicted structure.

The stereochemical characteristics and accuracy of homology models for murine β2-AR were evaluated using several widely employed computational tools available on the SAVES server. Specifically, Ramachandran plot analysis was utilized to evaluate the stereochemical quality of models by examining the distribution of phi (ϕ) and psi (ψ) dihedral angles for amino acid residues.[35] The structural quality of protein models is a critical aspect of computational protein modelling and has a direct impact on the

reliability of the predicted structures, thereby determining their biological functions and interactions. In our study, we evaluated the structural characteristics of murine β2-AR structure generated by SWISS-MODEL, iTASSER, Phyre and AF, focusing on the amino acids number, secondary structure elements and validation using Ramachandran plot analysis. Overall, the models generated by SWISS-MODEL, iTASSER, Phyre and AF exhibited variations in their Ramachandran plot parameters.

SWISS-MODEL predicted a model with 93.1% of residues in the most favoured regions, suggesting good structural quality. However, iTASSER exhibited a lower percentage (77.1%) of residues in favoured regions, suggesting deviations from optimal backbone conformations. Phyre and AF models showed intermediate values, with 92% and 82.9% of residues in favoured regions, respectively. Furthermore, iTASSER and AF models displayed a higher proportion of residues in additional allowed and generously allowed regions compared to SWISS-MODEL and Phyre. This discrepancy may indicate variations in the accuracy and refinement of the predicted structure.

Based on structural properties and Ramachandran plot analysis, the SWISS-MODEL-generated structure for murine β2-AR appears to be the most promising. It exhibited the highest percentage of residues in favoured regions and minimal presence in disallowed regions. The presence of secondary structure elements and its concise representation suggests a robust structural prediction compared to other models. In our study, the predicted 3D murine β2-AR model, generated using SWISS-MODEL, utilized the high-Resolution Human β2-AR template (2RH1), which has 97.12% of residues in the favored regions of the Ramachandran plot. The murine model itself shows 93.1% of residues in these favored regions, indicating high structural quality and similarity. This close alignment suggests that the murine model reliably mimics the human β2-AR structure, ensuring that key structural and functional features are preserved.

However, it is important to note that AF has gained recognition for its high accuracy in predicting protein structures and its performance has been validated in various benchmarks. Therefore, while the SWISS-MODEL model may exhibit slightly better structural properties in this specific analysis, the AF model remains a strong contender and may offer valuable insights into the structural characteristics. Binbay (2023) demonstrated superior predictive performance of homology modelling compared to AF in assessing the overall quality of heme-binding proteins APC, Hx, HSA and IL-36α. Notably, AF exhibited inconsistencies in predicting heme-binding motifs, underscoring the importance of accurate modelling for understanding structural changes during heme binding.[35] Furthermore, homology modelling successfully anticipated heme-binding to hemopexin, an accomplishment beyond the capability of AF.[36] While computational approaches yielded high-quality predictions of the overall protein structures, minor modelling issues were observed. Specifically, the lack of strands in the structure was notable in the models generated by the AF. However, the homology models predicted by SWISS-MODEL outperformed those generated by AF in this aspect by incorporating strands into the models more effectively.

AF's limitations have been reported, revealing that it faces challenges in predicting intrinsically disordered proteins/regions. These regions are crucial for drug screening and design as they are exposed on the protein surface and interact with solvents and other proteins. Studies have found that low-accuracy predictions by AF overlap with intrinsically disordered regions,[37] and that AF accurately predicts only short loops (<20 amino acids) while tending to over-predict secondary structures in loop regions.[38] These regions are highly variable and flexible across orthologs, complicating the identification of evolutionary constraints from MSA.

AF predicts only a single conformer, not distinguishing between apo and holo forms. Saldaño *et al.* (2022) found that in 67% of cases, AF's predictions resembled the holo form, with lower predictability when conformational differences between apo and holo forms increased.[39] Azzaz *et al.* (2022) demonstrated that AF's predictions for membrane proteins are unreliable due to inconsistencies in transmembrane domain locations.[40] These studies, along with AF's inability to predict structures with metal ions, cofactors, DNA/RNA complexes, or post-translational modifications,[41] highlight areas needing improvement for drug screening and design. It has been shown that AF models underperformed in high-throughput docking compared to experimental PDB structures and demonstrated weak performance in reverse docking for binding targets of bacterial compounds, indicating a need for more accurate protein-ligand interaction models for drug discovery.[42,43] Additionally, AF struggles to predict defects in protein folding due to point mutations. Studies have found minimal differences between mutated and wild-type models predicted by AF with backbone RMSD values lower than 1Å.[44] There is also no correlation between AF's accuracy metrics (pLDDT) and the impact of mutations on protein stability change (ΔΔG) or side chain size changes.[45]

The identification of three distinct binding pockets in murine β2-AR provides a structural basis for understanding the receptor's complex pharmacology and function. This structural diversity suggests that the murine β2-AR may accommodate a range of ligands with different sizes and chemical properties, potentially contributing to its versatile role in various physiological processes. These findings align with the growing understanding of the dynamic nature of β2-AR and its complex activation mechanisms. Previous studies have shown that β2-AR can adopt multiple conformational states, allowing for diverse ligand interactions and signaling outcomes.[46] The presence of multiple binding pockets could contribute to this conformational flexibility and the receptor's ability to interact with a wide range of ligands, including both agonists and antagonists. The largest

pocket (Pocket 1) may correspond to the orthosteric binding site, where endogenous ligands like epinephrine typically bind. This is supported by previous structural studies of β2-AR, which have revealed a spacious binding pocket capable of accommodating various ligands. The smaller pockets (Pockets 2 and 3) could potentially serve as allosteric binding sites or play roles in receptor activation and signal transduction. Recent molecular dynamics simulations have highlighted the importance of specific residues and regions in β2-AR activation and ligand binding. For instance, the upward movement of certain residues (e.g., F193) has been observed to play a role in receptor activation.[47] The multiple binding pockets identified in our study could be involved in these dynamic processes, potentially influencing the receptor's conformational changes upon ligand binding. Furthermore, the presence of multiple binding pockets could explain the diverse pharmacological profiles observed with different β2-AR ligands. Some ligands might preferentially interact with specific pockets, leading to distinct functional outcomes. This structural complexity could contribute to the receptor's involvement in various physiological processes, including its roles in immune regulation.[48]

The RMSD analysis indicates that the murine β2-AR model reached a stable equilibrium state quickly, consistent with previous MD studies on human β2-AR, which also demonstrated rapid equilibration and overall structural stability.[49] The stability of our murine model suggests that the homology modeling approach used to generate the structure was robust and produced a physiologically relevant conformation. The RMSF analysis revealed higher flexibility in the region spanning residues 361-370, which may have functional significance. In human β2-AR, the corresponding region is involved in G protein coupling and β-arrestin binding.[50] The observed flexibility in our murine model could indicate a conserved mechanism for receptor activation and signal transduction across species. Future studies could focus on this region to explore its role in ligand-induced conformational changes and protein-protein interactions specific to the murine β2-AR. The Rg analysis demonstrates that the murine β2-AR maintained a compact structure throughout the simulation, characteristic of well-folded GPCRs, which is crucial for their function in signal transduction across cell membranes.[51] The stability in Rg further supports the validity of our homology model and suggests that the overall fold of the murine β2-AR is like its human counterpart.

Comparative analysis with existing literature on human β2-AR shows that despite some sequence differences, the overall structure and function of β2-AR are highly conserved between mice and humans. Our MD simulations support this conservation, as the observed stability and dynamic behavior of the murine model align well with known characteristics of human β2-AR.

CONCLUSION

Our study provides a comprehensive analysis of the structural properties of the murine β2-AR using advanced computational modelling techniques. Through theoretical assessments and comparative analyses of different modelling servers, we have gained valuable insights into the receptor's stability, conservation and functional implications. Notably, SWISS-MODEL emerges as a superior tool for accurately predicting the β2-AR's 3D structure, showcasing its ability to capture essential structural features with high fidelity. While other modelling servers also demonstrate potential, SWISS-MODEL's excellence in incorporating key structural elements underscores its significance in drug discovery and structural biology research. Our findings contribute to a deeper understanding of the murine β2-AR's structural architecture and lay the groundwork for future investigations aimed at unravelling its role in physiological processes and disease mechanisms. MD simulations provide strong evidence for the structural stability and physiological relevance of the predicted murine β2-AR model. The identified regions of flexibility and the overall compact structure offer new insights into the molecular basis of β2-AR function in mice. These findings not only validate our homology model but also provide a foundation for future studies investigating species-specific differences in adrenergic signaling and for the development of more targeted therapeutic approaches.

ACKNOWLEDGEMENT

We express our gratitude to Principal Rev. Dr. Praveen Martis S J, St Aloysius (Deemed to be University) in Mangalore for providing the necessary lab facilities for carrying out the project. Dr Asha Abraham and Ms. Vijayalakshmi Gangadhara would like to thank the Mangalore Jesuits Educational Society (MJES) for their support through an intramural seed grant for research. Ms. Vijayalakshmi Gangadhara also extends her thanks to Rev. Dr Leo D'Souza S J for providing the Bartena Fellowship. Additionally, we are thankful to DST FIST, the Government of India, the Vision Group on Science and Technology (VGST) and KBITS, the Government of Karnataka for their infrastructural support.

FUNDING INFORMATION

Mangalore Jesuits Educational Society-Intramural Research Grant (Sanction no:1/MJES- MAJOR/2019 dated 09.02.2019).Vision Group on Science and Technology (VGST) Government of Karnataka -Minor research grant (Sanction No. KSTePS/VGST-RFTT/2016-17/279/5 dated 27.11.2017).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

β2 AR: β2 adrenergic receptor; **3D:** Three dimensional; **MetS:** Metabolic syndrome; **GPCR:** G protein-coupled receptor; **pI:** Isoelectric point; **EI:** Extinction coefficient; **AI:** Aliphatic index; **GRAVY:** Grand average of hydropathicity index; **PDB:** Protein Data Bank; **AF:** AlphaFold; **CASTp:** Computed Atlas of Surface Topography of proteins; **MD:** Molecular Dynamics; **TM:** Transmembrane helix; **PPI:** Protein-protein interaction; **GMQE:** Global Model Quality Estimation; **QMEAN:** Qualitative Model Energy Analysis; **RMSD:** Root mean square deviation; **pLDDT:** Probability of local distance difference test; **RMSF:** Root Mean Square Fluctuation; **Rg:** Radius of Gyration; **ICL:** Intracellular loop; **EL:** Extracellular loop; **MSA:** Multiple sequence alignment.

SUMMARY

This study explores the structural attributes of the murine β2-adrenergic receptor (β2-AR), a key regulator of physiological processes and a vital target in MetS research. Due to its close similarity to the human β2-AR, understanding the murine β2-AR structure provides insights into metabolic regulation. Using molecular simulation techniques, the 3D structure of murine β2-AR was predicted through four modelling servers: SWISS-MODEL, Phyre2, I-TASSER and AlphaFold.

Primary sequence analysis revealed notable properties, including an isoelectric point of 7.07, thermal stability suggested by an aliphatic index of 94.86 and mild hydrophobicity with a GRAVY score of 0.145. Among the modelling servers, SWISS-MODEL produced the most accurate structure, with 93.1% of residues in favored regions and none in disallowed regions of the Ramachandran plot. The predicted structure exhibited 19 helices, 22 turns and 2 strands, indicating a robust conformation. A 50 ns molecular dynamics simulation further confirmed the stability and integrity of the modelled receptor.

These findings highlight the advantages of integrating computational modelling with experimental approaches, emphasizing SWISS-MODEL's reliability. The results not only enhance our understanding of murine β2-AR structure but also support its translational relevance in studying metabolic regulation and developing targeted therapeutic strategies.

AUTHORS' CONTRIBUTIONS

V.G.: Writing-Original Draft, Methodology, Data Analysis and Validation. A.A.: Conceptualization, Review and Editing, Supervision, Funding Acquisition and Resources.

ETHICAL APPROVALS

The research work does not contain testing on animals or human subjects.

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Cite this article: Gangadhara V, Abraham A. Optimizing 3D Structural Predictions of Murine β2-Adrenergic Receptor: Swiss-Model Outperforms AlphaFolds. Indian J of Pharmaceutical Education and Research. 2025;59(2s):s641-s655.