

Targeting the multidrug efflux pump; Tap protein to reduce survival of *Mycobacterium tuberculosis*

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Abstract

Tuberculosis (TB) is a serious communicative disease caused by a bacterium named *Mycobacterium tuberculosis*. Albeit there are vaccines and drugs available to treat the disease, Multidrug-resistant TB (MDR-TB) is still one of the most critical challenges for the investigators where the development of efflux systems makes them resistant to drugs. Tap is a multidrug efflux pump and proposed to have a significant role in the survival of *M. tuberculosis* making it drug-resistant. In the present study, we have utilized various *In silico* approaches to predict the applicability of FDA approved ion channel inhibitors and blockers as therapeutic leads against Tuberculosis. We have analysed 18 inhibitor compounds and eventually screened three ligands as drugs, Glibenclimide, Lubiprostone and Flecainide that have displayed the novel stable binding with Tap protein aiming to affect or inhibit its activity. Structure of Tap protein is predicted by Phyre2 server followed by its characterization by 10ns MD simulations using the CABS-flex 2.0 server and validation by Ramachandran plot. PyRx software presented the binding affinity varied in the range of -8.00 kcal/mol to -9.8 kcal/mol, implies that the drug molecules can spontaneously interact with the target protein. Amongst them, Glibenclimide shows the highest binding affinity with ΔG of -9.8 kcal/mol. This study proposed Tap protein as an interesting drug target and investigated drugs may show considerable effects on the target protein showing a novel therapeutic lead against Tuberculosis.

Introduction

Tuberculosis aka TB is an infectious disease caused by a bacterium named *Mycobacterium tuberculosis*, which mostly affects lungs. According to World Health Organisation (WHO), in the year of 2018, a total of 1.5 million people died from Tuberculosis (including 251000 people with HIV) and an estimated 10 million people felt ill due to tuberculosis (TB) worldwide which includes 5.7 million men, 3.2 million women and 1.1 million children. Globally, it is one of the top 10 causes of death and also the leading cause from a single infectious agent (above HIV/AIDS). As of 2018, most of the TB cases occurred in the South-East Asian region (44%), followed by African region (24%) and the Western Pacific (18%) region [1] [2].

Multidrug-resistant TB (MDR-TB) is still one of the biggest threats to the public health. According to WHO, there were 484000 new cases with resistance to rifampicin – the most effective first-line drug, of which 78% had MDR-TB [1]. In recent years, effort has been given to design new drugs against multidrug-resistant (MDR), extensively drug-resistant (XDR) and total drug resistant (TDR) strains of *Mycobacterium tuberculosis* is significant [3].

In previous decades, significant work has been done to reveal the properties of the genes indulged in mycobacteria to provide acquired drug resistance. This work got speed up when various clinical strains and isolates of multiple drug resistant *Mycobacterium tuberculosis* get characterized in 1995 [4]

Characterization and findings on molecular basis of resistance was presented by Musser, 1995, revealed that the structural or metabolic genes determine a high level of resistance to a single drug when altered. Most of multiple drug resistant isolates shows the collective independent mutation in various genes [5]. There are various other bacterial strains where the single gene product is a determinant of multidrug resistance phenotype [6]. These gene product basically includes the membrane transport proteins which facilitate the excretion of toxic or unwanted compound by active transport which have introduced to the bacterial cells by diffusion. These bacterial membrane efflux pumps / systems belong to the heterogeneous and huge family of active membrane transporter proteins. Moreover, mutation on these pumps may assist bacteria to cover wide range of components transported and to acquire the higher level of drug resistance [7].

Now investigators are working on these efflux pumps to regulate the pathogens because blockers or inhibitors of these efflux systems can make these pathogens more susceptible to antimicrobial compounds [8].

Therefore, novel therapeutic strategies may be developed on the basis of characteristics of these efflux pumps. In some investigations efflux pump LfrA, Tet(V), and Emb were found in non-pathogenic *M. smegmatis* that confers to low-level resistance to fluoroquinolones and other compounds, resistance to tetracycline and resistance to ethambutol respectively [9-11].

In order to prevent the further development in multidrug resistant strain, inhibition or blocking of such efflux pumps may provide effective strategy. In recent studies several compounds have been proposed as an excellent putative efflux pump inhibitor e.g., Verapamil as Calcium channel blocker of eukaryotic cells, thioridazine; a class of antipsychotics drugs as well as some natural-derived compounds such as reserpine. These compounds have shown the antimicrobial activity against susceptible *M. tuberculosis* in vitro and in the macrophage model [12-15].

In the present study, we performed *In silico* investigation on putative multidrug efflux pump that is Tap efflux protein from *Mycobacterium tuberculosis* (H37Rv), Tap_{tub} (Rv1258). Tap contributes to the intrinsic antibiotic resistance of *M. Tuberculosis* [16] and involved in transport of undetermined substrate (possibly macrolide) across the membrane (export) [1, 2]. This efflux protein has sequence similarities to other proteins associated with multidrug-resistance phenotypes, especially tetracycline and macrolide proton-dependent efflux pumps, and a sequence motif characteristic of drug export proteins. Drug screening is done to explore the applicability of selected compounds against the putative efflux pump. *In silico* approach provides a crucial information on Tap protein and compound which shows efficient interaction and binding with Tap in order to inhibits its activity. Being a potent drug target, three-dimensional structure of the protein, Tap, had been generated and validated followed by molecular docking studies with some known drug molecules [3]. These compounds may be proposed to have considerable properties to be presented as a drug or model study for drug discovery against Tuberculosis.

There are a number of ion channels and transporter proteins present in *Mycobacterium tuberculosis* which are likely to be responsible for its survival and virulence, e.g. Tap, EfpA, Amt, chaA, DMT etc [3]. Tap proteins are the putative multidrug efflux pump in *Mycobacterium tuberculosis*. This is responsible for the transportation of undetermined substrate (possibly macrolide) across the membrane (export) [16-19].

Present work shows the significant outcomes on Tap protein by *In Silico* investigation. Three-dimensional structure of the protein, has been generated by homology modelling and validated followed by molecular docking studies with some known drug molecules, that could be considered as drug repurposing or drug repositioning. Now a days, Drug repurposing is a promising area where new therapeutic purposes of approved drugs have to be explored. This is a beneficial route for treating diseases in a short time span and reduced risks for the patients [20]. Through this study we propose the predicted ligands or compounds as promising drugs or inhibitors that may reduce or block the activity of TAP transporter proteins and eventually cause the reduction in *M. tuberculosis* survival and chances of MDR development.

Materials And Methods

2.1 Sequence Retrieval and Structure Prediction:

The structure of Multidrug efflux pump; Tap of *M. tuberculosis* has not been determined yet and do not have any PDB entry therefore, the route of homology modelling was employed to construct the three-dimensional protein structure using Phyre2 server [21]. Phyre2 (Protein Homology/AnalogY Recognition Engine) is a free web server for protein structure prediction and extensively being applied to predict and analyse various protein structure, function and mutations. This platform provides biologists with a simple and intuitive interface to state-of-the-art protein bioinformatics tools [21]. The protein sequence was retrieved in FASTA format from UniProt website [22]. To perform the protein modelling through Phyre2 web server, FASTA sequence of TAP protein was provided as an input and modelling was done using intensive mode.

2.2 MD Simulations:

The structural stability of the constructed model of the protein had been confirmed by running a 10ns MD simulations using the CABS-flex 2.0 server [23]. CABS-flex is an efficient modelling procedure for fast simulations of protein structure flexibility and implements an efficient simulation engine that allows for modelling of large-scale conformational transitions of protein systems. For this experiment, protein structure in PDB format has been provided as input. PDB format of the protein structure was generated by the protein modelling tools, Phyre2.

2.3 Model Validation:

After getting the simulation results, the resulting model was validated by analysing the Ramachandran plot [Fig. 7] with the help of PROCHECK web server [24, 25]. This server checks the stereochemical quality

of a protein structure, producing a number of PostScript plots analysing its overall and residue-by-residue geometry.

2.4 Binding Site Prediction:

Using CASTp 3.0 web server, the active sites of the modelled protein had been determined. CASTp 3.0 (Computer Atlas of Surface Topography of Proteins) provides comprehensive and detailed quantitative characterization of topographic features of protein [26]. This works based on recent theoretical and algorithmic results of Computational biology.

2.5 Molecular Docking:

Previously Saumya Bajaj *et al* (2020) has mentioned several ion channel drugs approved by the Food and Drug Administration (FDA) during the last two decades [27]. Various Properties of ligands selected for this study is explained in table 1. All the docking studies have been done using PyRx, autodock embedded virtual screening tool. [28, 29]. PubChem website was used to find out the appropriate ligands and 3D .sdf file format of the selected ligands were downloaded [30] and entry has been docked individually. Further, energy minimization was performed for each ligand and converted into .pdbqt format. A grid box of specific dimensions (25x25x25 Angstrom) automatically generated around the target protein. After virtual screening, docking poses were visualized using Discovery Studio and Pymol respectively. 2D protein-ligand interaction plot was also made for better understanding. [31].

Results

3.1 Sequence Retrieval and structure prediction:

The Mycobacterial Tap protein sequence (Locus tag: Rv1258, Uniprot ID: P9WJX9) was downloaded from Uniprot (<https://www.uniprot.org/uniprot/P9WJX9>) and used to perform structure prediction and for other purposes. This sequence was retrieved in FASTA format [22] for further studies. This protein comprised of 419 residues having the size of 43.287 kDa. TAP protein sequence in FASTA format is shown below;

```
>sp|P9WJX9|TAP_MYCTU Multidrug efflux pump Tap OS=Mycobacterium tuberculosis  
(strain ATCC 25618 / H37Rv) OX=83332 GN=tap PE=1 SV=1
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MRNSNRGPAFLILFATLMAAAGDGVSIVAFPWLVLQREGSAGQASIVASATMLPLLFLATLVA  
GTAVDYFGRRRVSMVADALSGAAVAGVPLVAWGYGGDAVNVLVLAFLAALAAAFGPAG  
MTARDSMLPEAAARAGWSLDRINGAYEAILNLAFLVGAIGGLMIATVGGITTMWITATAFG  
LSILAIAALQLEGAGKPHHTSRPQGLVSGIAEGLRFVWNLRLRTLGMIDLTVTALYLPMES  
VLFPKYFTDHQQPVQLGWALMAIAGGGLVGALGYAVLAIRVPRRVTMSTAVLTLGLASMV
```

IAFLPPLPVIMVLCVVGLVYGPIQPIYNYVIQTRAAQHLLRGRVVGVM TSLAYAAGPLGLLL
AGPLTDAAGLHATFLALALPIVCTGLVAIRLPALRELDLAPQADIDRPVGS AQ

In the final structure predicted by Phyre2, 100% residues were modelled at >90% confidence [Fig. 1 and 2]. 82% of the sequence acquires alpha-helical structure and 12% are disordered whereas 59% of the sequence is a part of TM helix. Surprisingly, the predicted model did not show any β -strand. According to Phyre2, six templates were selected to model TAP protein based on heuristics to maximise confidence, percentage identity and alignment coverage; only two residues were modelled by *ab initio*. Transmembrane helices have also been predicted by Phyre2 in our sequence to adopt the best topology as shown in figure 3 [21]. Eventually, based on these parameters, a suitable model of TAP protein was utilized to conduct the further *In silico* analyses and validation.

3.2 MD Simulations and selection of the best model:

The dynamics of biological systems are crucial for their structure and functions, but using the experimental setup, deciphering the dynamics is difficult or even sometimes impossible; so computational tools are the saviour here. Molecular dynamics simulation was implemented for analysing the dynamics of the modelled TAP protein. As all-atom simulations are computationally expensive, the route of coarse-grained simulation is preferred sometimes. CABS-Flex is a web-server for fast simulations of proteins. Using CABS-Flex server, 10 ns MD simulation was performed and the best model was selected after clustering [Fig. 4]. The contact map of the residues [Fig. 6] and Root Mean Square Fluctuation (RMSF) plot [Fig. 5] were also generated using this server. RMSF plot is the residue-wise fluctuations recorded throughout the MD simulation while the Contact map provides a detailed view of the protein's residue-residue interaction pattern. Based on the generated trajectory, the residue fluctuation profile is calculated as,

$$\langle(\Delta R)_i^2\rangle = \frac{1}{N} \sum_j^N (x_i(j) - \langle x_i \rangle)^2$$

where $\langle \rangle$ denotes the average over a whole trajectory, and x is the position of particle i in the frame j [23, 32].

3.3 Validation of the TAP protein model:

The quality of the structure of the predicted model of TAP protein was verified by Ramachandran Plot (in PROCHECK server) [24]. This experiment suggested that 79.3% of residues were in the favoured regions, 14.1% in the additional allowed regions, 4.3% in the generously allowed regions and 2.3% in disallowed regions [Fig. 7].

3.4 Binding Site Prediction:

The active site can be represented as a part of the protein surface to which a specific substrate (ligand) or set of substrates (ligands) binds. We predicted the active sites in the modelled protein by CASTp 3.0 server. CASTp server facilitates the identification and measurements of surface accessible pockets for proteins with the active site residues. In **Fig. 8**, top two pockets are shown in accordance with their Area (SA) and Volume (SA). In the Table 2, the red coloured pocket has the highest Area (SA) and Volume (SA), 3132.679 and 2572.874 respectively. Whereas, the second-best pocket (green coloured) has a much lesser Area and Volume compared to the first one. Table 2 is representing the values and colour ID of the pockets. The best two active site residues with pocket volume are shown in Fig. 8 in red and green color.

3.5 Molecular Docking:

Docking is a powerful computational tool for estimating binding affinities of the ligands with the target protein.

From the molecular docking studies with Tap protein in PyRx software, the ligands are ranked in terms of binding affinity. Greater the negative value of score indicates better binding. Top three (3) drugs have been selected for further investigation, 1) Glibenclimide, 2) Lubiprostone, 3) Flecaicnde demonstrating the significant negative change in free energy (ΔG) -9.8, -8.4 and -8.1 respectively. Docking score of the all-tested ligands are mentioned in table 3. Other details of these compounds such as 2D structure, H-Bond donor and acceptor, molecular weight etc. are provided in table 1. Docking poses are visualized using Discovery Studio [**Fig. 9**] [33-35].

Discussion

In present condition, studies on interaction between drugs and their targets, development of new drugs and screening of druggable compound using *In silico* approaches like molecular docking, modeller etc are attracting vast attention from researchers due to the its requirement of favourable time and cost effective method of *In silico* drug screening compared with traditional laboratory based wet lab experiments. In present study, we have employed phyre2 for modelling the protein, MD simulation by CABS-Flex server whereas protein-ligand docking studies were carried out by PyRx software which are open-source software. The interaction between the modelled TAP protein and selected ligands Glibenclimide, Lubiprostone and Flecaicnde are carried out spontaneously when change in free energy (ΔG) is negative showing the inhibitory action during the function of TAP protein that would be significant to reduce the survival of the *Mycobacterium tuberculosis*. At this condition the stability of the TAP protein–ligand interaction is directly proportional to the difference in levels of free energy change of complexed molecules and molecules in unbound free states. This process is supported by the principle that protein-ligand interaction and binding as well as protein folding event happen when the system has low free

energy change (ΔG) [36-37]. Hence, negative values of ΔG play substantial part in the stable interaction between the protein complex and ligands which is the one of crucial properties of effective and successful drug compounds [38]. During this study we found that the predicted three ligand has the largest negative ΔG among all. MD simulation was performed using CABS-Flex server with 10 ns that showed the RMSF plot recorded in residue-wise fluctuations while the Contact map of the residues provided a detailed view of the protein's residue-residue interaction pattern. These results and considerable change in free energy represented the high binding affinity between TAP protein and selected ligands. Thus, we are proposing these ligands as potential compounds / inhibitors to form stable complex and thereby causing the reduced activity of TAP protein. Along with important role of free energy to define stable protein-ligand binding, the types of the molecular interaction between the amino acid residues in protein's active pocket/ binding site and ligands are determining factors during the binding of ligands in favourable conformations [39]. These interactions may encompass the hydrogen bond, hydrophobic, and electrostatic interactions. The construction of stable protein complex includes hydrophobic interaction as a major contributor whereas hydrogen bonding is also involved in protein stability but in lesser degree compare to the hydrophobic interaction. It has been proposed that the various protein folding configuration equilibria in native condition is determined by the hydrophobic bindings [40]. The current study has shown that the during interaction between the TAP protein and selected ligands, various amino acid residues bring about the hydrophobic and electrostatic interactions. Glibenclimide is a second-generation (2G) sulfonylurea (SU) drug class used for the treatment of type 2 diabetes mellitus (T2DM) to improve glycemic control. Lubiprostone is a bicyclic fatty acid derived from prostaglandin E1; it is a chloride channel activator with laxative activity. Flecainide is a synthetic agent derived from trifluoroethoxy-benzamide showing antiarrhythmic and local anesthetic activity and effective against sodium channels.

Among the three drugs, Glibenclimide has the best binding affinity score against tap. There are following non-covalent interactions: one pi-sigma (Leu 150), one pi-sulfur (Tyr 239), three alkyl/ pi-alkyl interactions (Ile 27, Phe 30, Tyr 325). But surprisingly, there's no conventional hydrogen bond exists between the protein and ligand, which arises question about the effectivity of this drug against target protein. Whereas, in the case of Flecainide, there are two unfavourable donor-donor interactions (Thr 59, Arg 345); most probably these unfavourable interactions are responsible for the comparatively low binding affinity of the ligand against the protein even after having three hydrogen bonds (Leu 53, Leu 56, Asp 125), three halogen (Fluorine) bonds (Met 121(2), Asp 125) and multiple alkyl/ pi-alkyl interactions. Lubiprostone seems to be the most efficient drug against the protein, having three conventional hydrogen bonds (Thr 16, Ala 19), one halogen bond (Ile 267), one pi-sigma interaction (Tyr 325) and multiple alkyl/ pi-alkyl interactions.

In the view of said findings through the *In silico* approaches, we have shown here the compound Glibenclimide, Lubiprostone and Flecainide have potential as ligands that may inhibit activity of multi drug efflux protein, TAP to reduce the survival of *Mycobacterium tuberculosis*. *In vivo* or *In vitro* validation would be the next step to present its applicability within biological system.

Conclusions

From the docking studies, top five most promising drugs has been reported against Tap protein of *Mycobacterium Tuberculosis*. These drugs are effective against various ion channels and already approved by FDA. In terms of the ΔG predicted by PyRx software, the top three compounds are showing decent docking score (< -8.0) with the target protein. The estimated ΔG of binding varied in the range of -8.00 kcal/mol to -9.8 kcal/mol, implies that the drug molecules can spontaneously interact with the target protein. Glibenclimide is showing minimum ΔG , -9.8 kcal/mol which is a second-generation (2G) sulfonylurea (SU) drug class used for the treatment of type 2 diabetes mellitus (T2DM) to improve glycemic control.

MDR-TB is still a challenge against the humankind and considering the fact, this computational study was performed to find a way to cure it. This study revealed that Tap protein can be an interesting drug target and the proposed drugs may show considerable effects on the target protein. Other similar proteins can be considered as drug-target can be EfpA and DMT. Being a purely computational study, the effectiveness of the proposed drugs against the target protein and other pharmaceutical queries cannot be quenched by this this study. So, Further validations and investigations must be done with *In Vitro* and *In Vivo* experiments to get a better understanding and a practical cure against the disease [41-43].

Declarations

5. **Acknowledgment:** MD acknowledges the DST-INSPIRE Faculty award (2017), Govt. of India and SM acknowledges the DST-INSPIRE SHE award (2016) by Govt. of India.
6. **Author contribution:** MD have designed and conceptualized the work. Experiments were performed by SM. Manuscript was written and results were analysed by MD and SM.
7. **Conflict of Interest:** Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Tables

Please see the supplementary files section to view the tables.

Figures

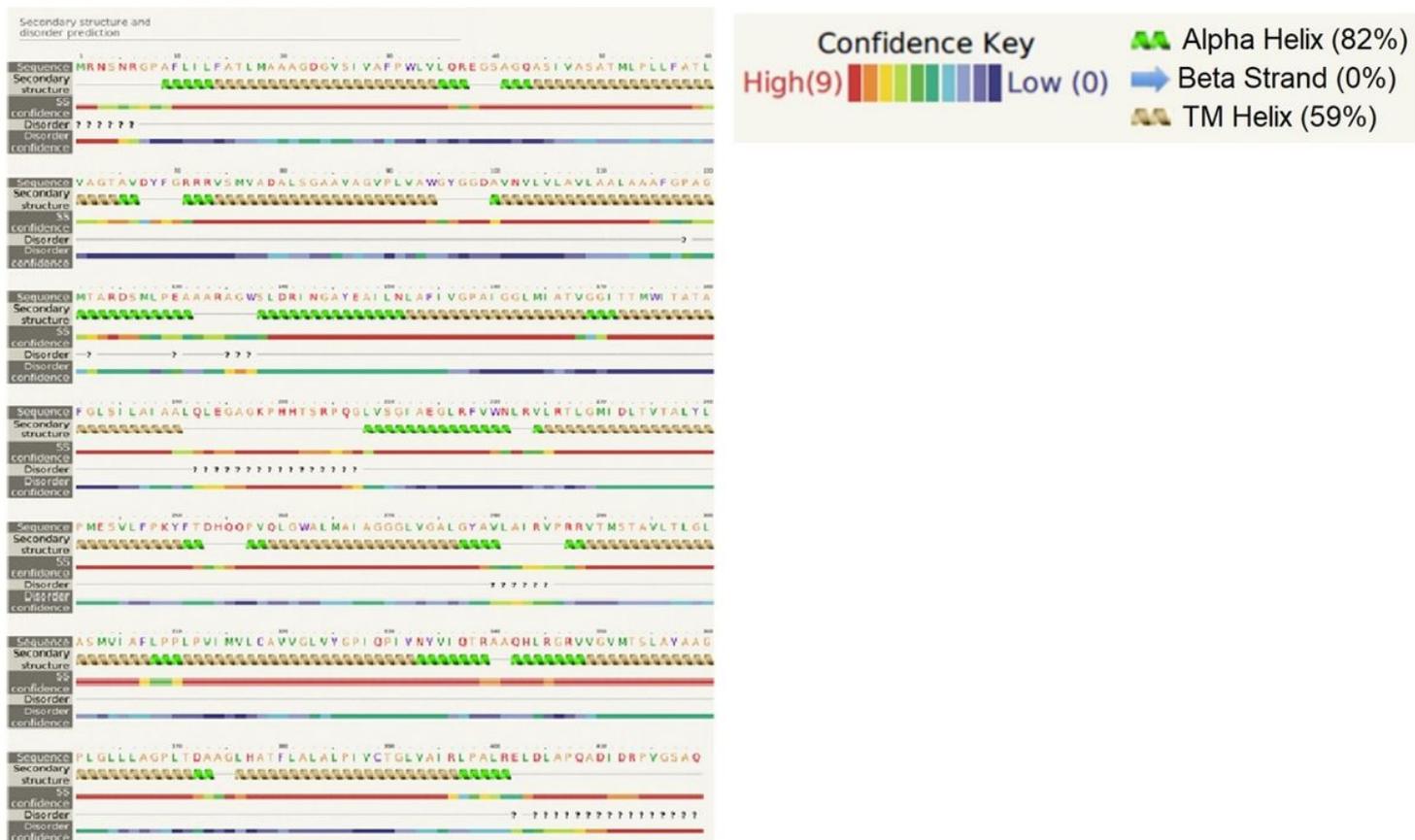


Figure 1

Secondary Structure and Disorder prediction by Phyre2 Server

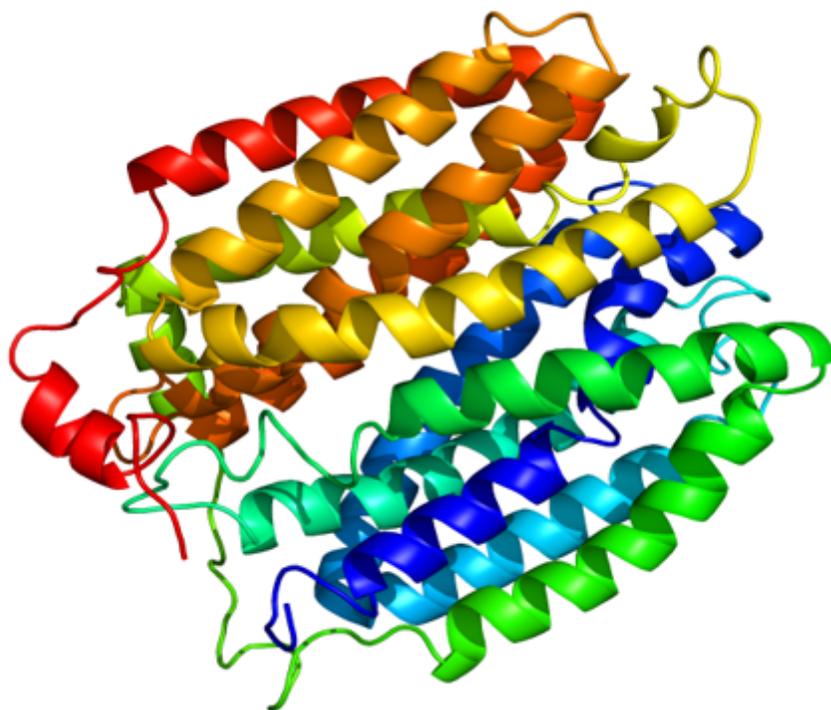


Figure 2

Model of Tap protein constructed by Phyre2 web server

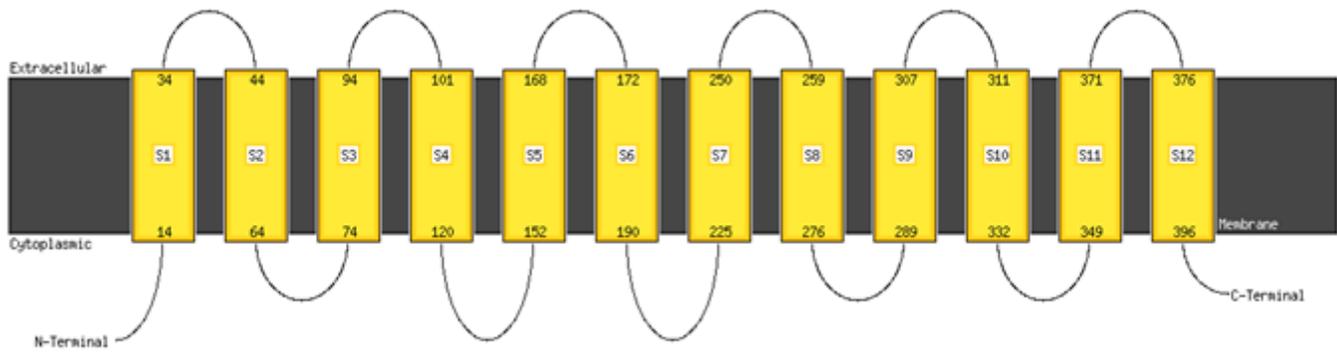


Figure 3

Transmembrane helices predicted by Phyre2server in our sequence to adopt this topology

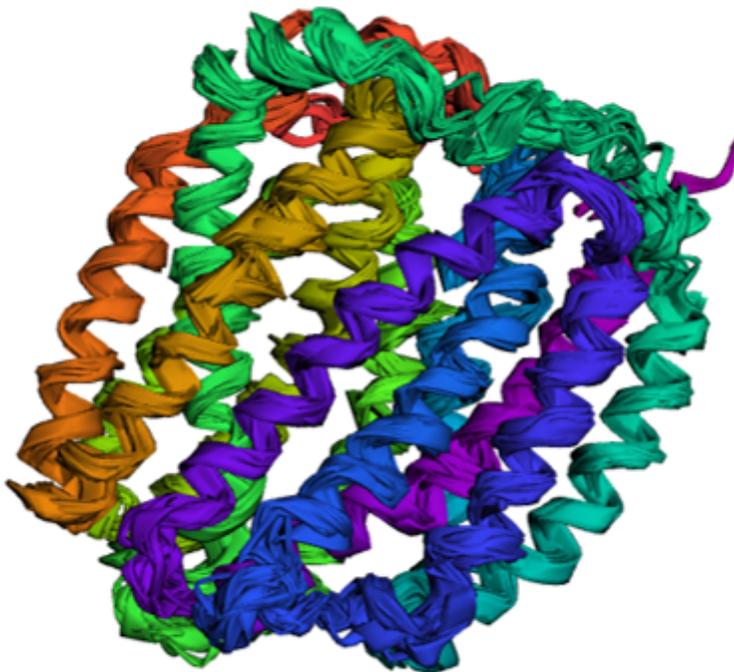


Figure 4

Superimposition of the top ten(10) best models obtained from the clusters after performing the 10 ns MD simulations using CABS-flex 2.0 server

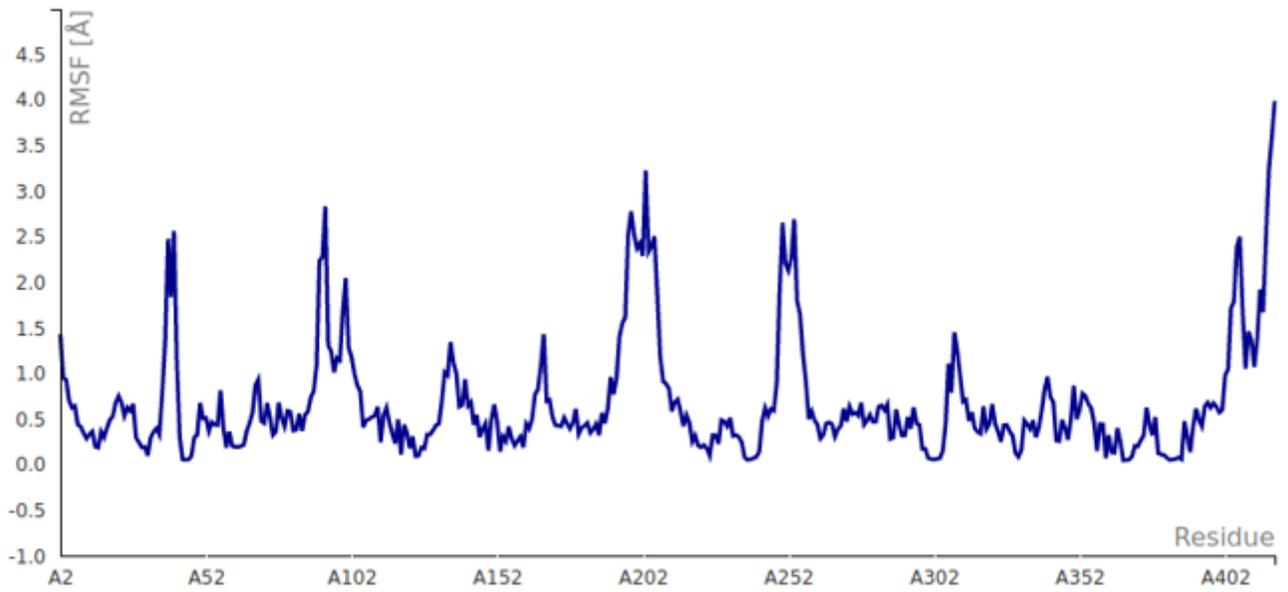


Figure 5

Root Mean Square Fluctuation (RMSF) plot of the constructed model over the 10 ns simulation trajectory

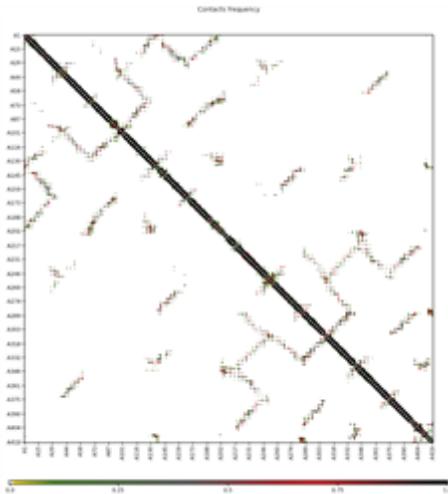


Figure 6

The residue contact maps of the constructed model of Tap protein

Ramachandran Plot

5633236

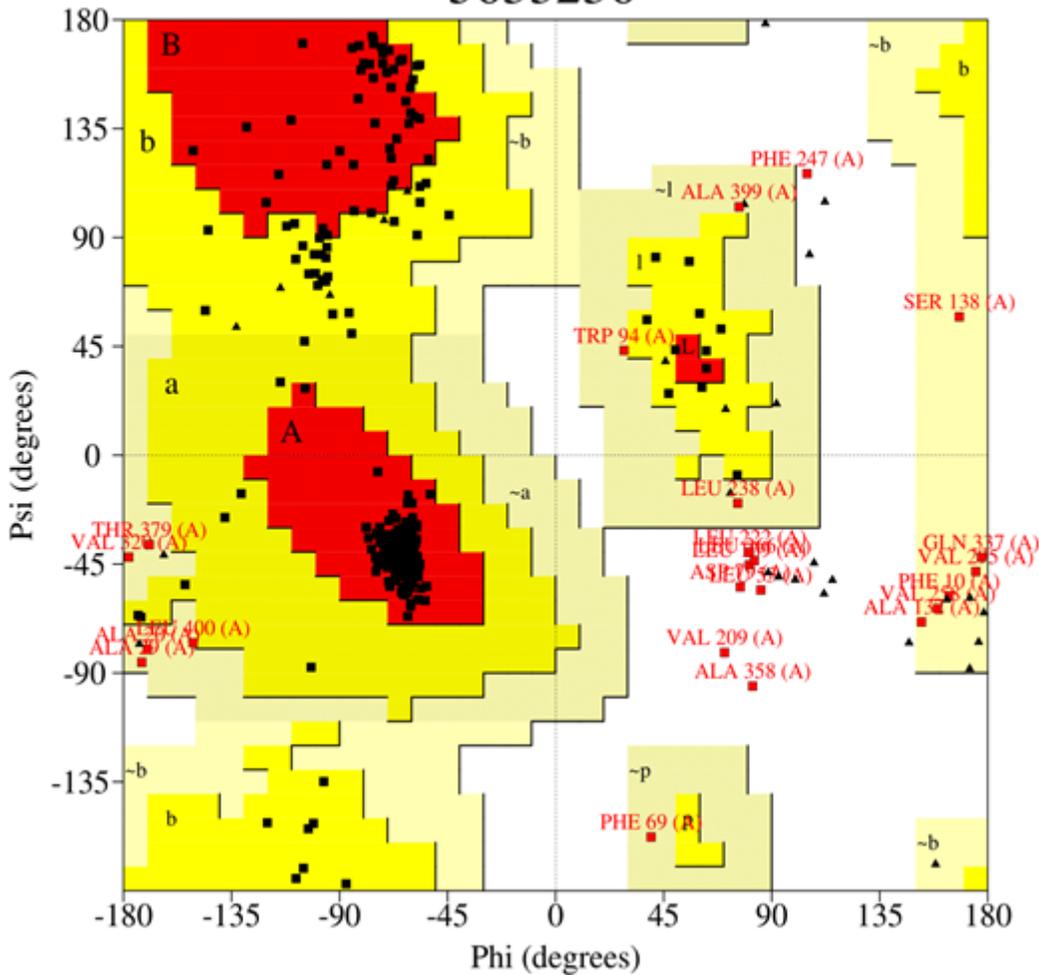


Figure 7

Ramachandran Plot of the modelled TAP protein generated by PROCHECK server. This plot shows the 79.3% residues (276 amino acids) in most favoured region, 14.1% amino acids (49) are in additional allowed region, 4.2% residues (15 amino acids) in generously allowed region whereas 2.3% residues (8 amino acids) are presented in disallowed region.

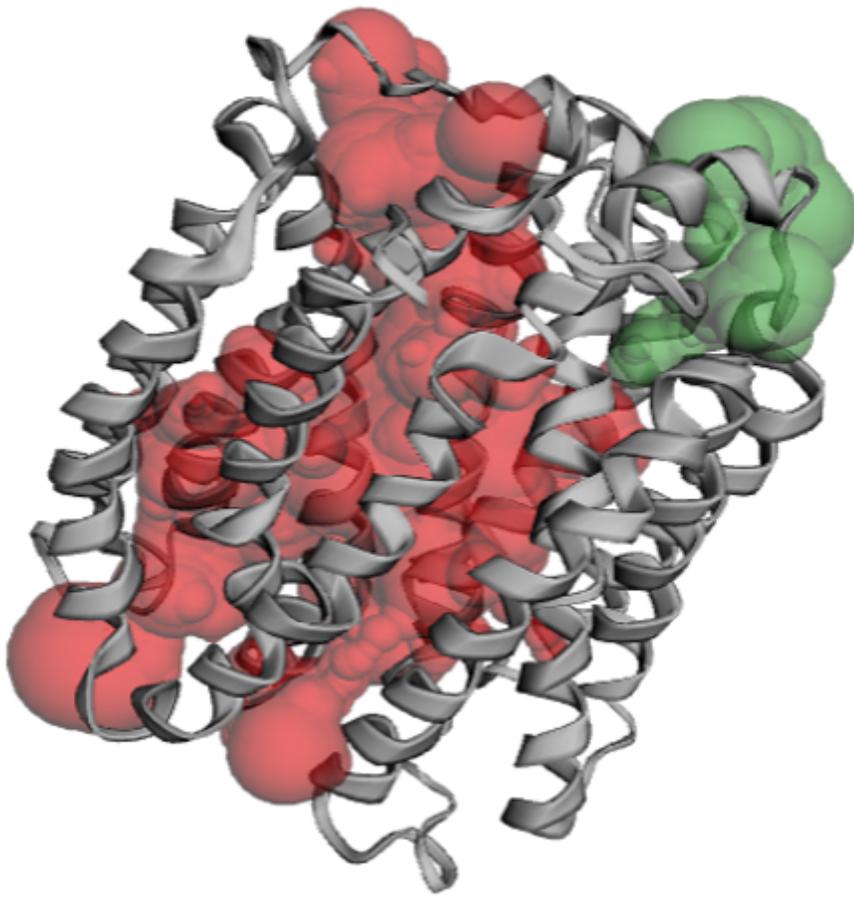


Figure 8

Binding Pockets (top 2) of the TAP Protein predicted by CASTp server. For detail refer to text.

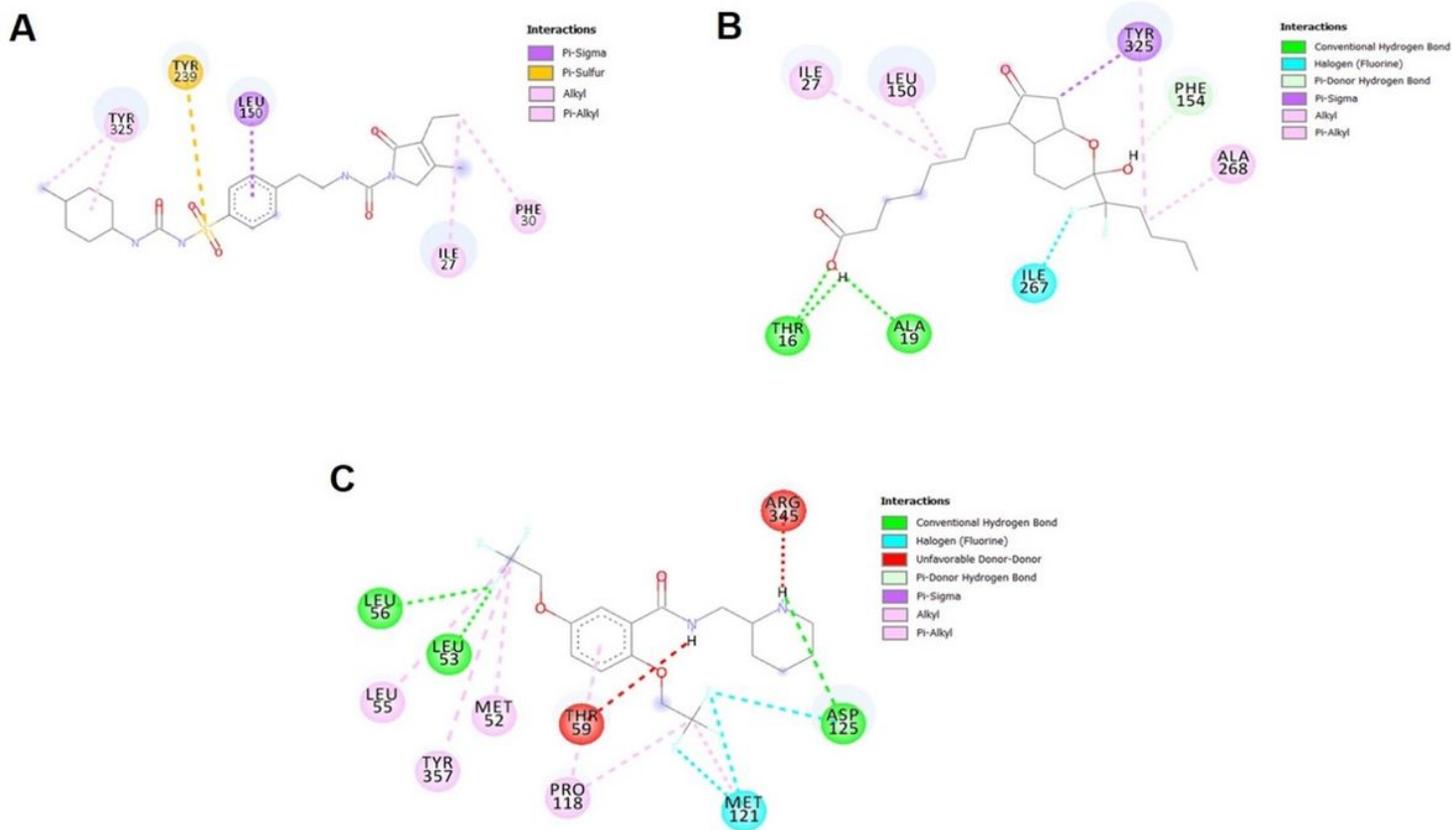


Figure 9

Docking Pose of proposed compounds with TAP protein. For the details of interaction studies refer to text part.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Tables.docx](#)