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Docking and Dynamic Simulation Analysis of P-glycoprotein pumps - Responsible for Chemotherapeutic Resistance post-treatment with Urea and β–mercaptoethanol

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Abstract
The designing of cancer chemotherapy has become increasingly sophisticated over years, against disseminated cancer. With increasing advances in research, resistance to these therapies has also been reported over years. Resistance to treatment with anticancer drugs results from a variety of factors including polymorphic variations in patients and genetic differences in tumours. The most common reason for resistance to a broad range of anticancer drugs is influenced by the expression of one or more energy-dependent transporters (p-glycoprotein pumps) that detect and eject anticancer drugs from cells. Deactivating these pumps can help to overcome such resistance. Thus in this current study lead compounds urea and β–mercaptoethanol has been used to alter the structural confirmation of these P-gp (pump proteins) by using molecular docking and dynamic simulation analysis. Urea & β–mercaptoethanol can bind to the target protein with best docking scores of -15.5995 & -10.0501 respectively. Binding of β – mercaptoethanol caused a considerable perturbation in the backbone of the target protein with RMSD value eventually deviating to approximately 1.3 and urea further deviate the value to approximately 1.6. Furthermore decrease in the intra-molecular hydrogen bonds over the simulation period confirms the secondary structural change thus ceasing the biological activity of the target protein.

Keywords: P-glycoprotein pumps, Urea, β–mercaptoethanol, Molecular Docking, Dynamic Simulation Analysis

INTRODUCTION

A standout amongst the most contemplated components of cancer drug resistance includes reduced accumulation of the drug inside the cell due to enhanced efflux action. Membrane transporters especially the members of ATP-binding cassette (ABC) transporter family are highly involved in this process. 49 members of this family have been identified in humans. These transporters are defined by two functional domains – nucleotide binding domain which is highly conserved and transmembrane domain which is comparatively variable [1]. Substrate binding sites of these pumps are present towards the cytosolic face. When the substrate binds hydrolysis of ATP at the nucleotide binding site induces a conformational change in these pumps which drives the substrate from the cytosolic face towards the extracellular matrix. Under normal condition these pumps are highly essential as they prevent accumulation of toxins within the cell [2]. These pumps are mostly dominant in our liver epithelium and intestinal epithelium where they pump out the drugs and harmful molecules into bile duct and intestinal lumen thus protecting our body from any adverse effect. Maintenance of blood brain barrier also comes under the influence of these pumps [3, 4].

Efflux actions of these pumps are a part of their normal physiological process. In cancer cell these pumps are over expressed and are responsible for pumping the chemotherapeutic drugs outside the cell and thus inducing multi drug resistance. There are three transporters—multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP) — who all are involved in drug resistance. All the three transporters are highly specific for their substrate which includes vinca alkaloids, epipodophyllotoxins, anthracyclines, taxanes, and kinase inhibitors (chemotherapeutic drugs). MDR1 gene produces p-gp which is expressed in colon, liver and kidney [5-7]. Expression of p-gp increases when the tissues turn cancerous. The over expression is induced by intrinsic factors and acquired mechanism [8]. The tissues which do not express MDR1 (lung, breast and prostate cells) become resistant due to over expression of MRP1 and BCRP [9, 10]. Deactivating the expression of energy dependent drug efflux pump (P-glycoprotein pump) or the multidrug transporter by treating the cells with urea in the presence of the reducing agent β–mercaptoethanol can be used as a novel technique to overcome multidrug resistance. Urea is used to break non covalent bonds i.e. hydrogen bonds. β–mercaptoethanol, a disulfide reducing agent can covalently interact with specific protein functional groups [11]. This chemical cocktail is ideally used for unfolding the proteins. The misfolded protein does not reach the cell surface and is retained in the endoplasmic reticulum (ER) followed by rapidly degraded. Unfolded pump proteins which cannot be expressed in the cell surface, will allow the chemotherapeutic drug retention in cancer cells which otherwise pump out the drugs from

the cell into the matrix thus gaining chemotherapeutic resistance.

MATERIAL and METHODS

Protein Preparation: 3D structure of p-glycoprotein pump was retrieved from Protein Data Bank (PDB) using query ID 2CBZ [12]. The PDB ID, source and detail of PDB structure used for the study are listed in Table 1. Co-crystallized ligands (ATP & Mg^{2+}) Fig 1(a) & (b) were identified and removed from the target protein and then crystallographic water molecules were eliminated from the 3D coordinate file [13]. The structure thus obtained was stabilized using CHARM force field [14]. Active site residues (TYP653, THR660, LEU661, VAL680, GLY681, CYS682, GLY683, LYS684, SER685, SER686, LEU687, SER689, MET695, GLN713 and ASP792) were selected for molecular docking studies.

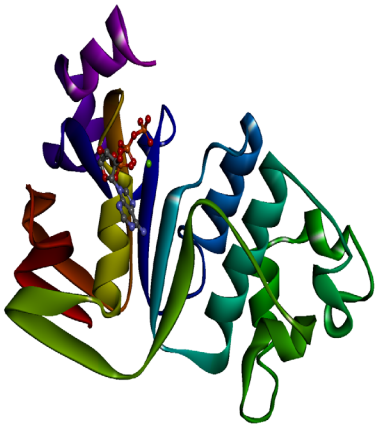


Figure 1. (a): Co-crystallized ligands (ATP & Mg^{2+}) with 2CBZ

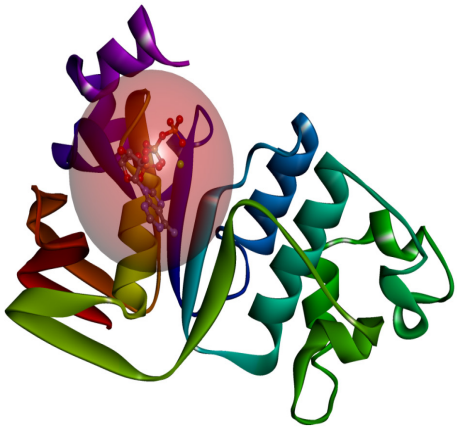


Figure 1. (b): Active site interaction

Ligand Preparation (Urea and β – mercaptoethanol): The structures of Urea and β – mercaptoethanol were obtained from the NCBI PubChem Compound database the details of which are listed in Table 2. Energy minimization before docking is one of the major steps as it removes clashes among atoms of the ligand and develops a reasonable stable confirmation to initiate docking with macromolecules (target protein). Ligand optimization was carried out using Chemistry at Harvard Molecular Mechanics (CHARMm) force field followed by energy minimization protocol [15]. Several stereo isomers were generated (same structure with different orientation or confirmation) and the one with most stable configuration is chosen for docking. These ligand conformations were generated based on bond energy, CHARM energy, dihedral energy, electrostatic energy, initial potential energy, and initial RMS gradient values. The CHARMm energy value, angle energy, dihedral angle, electrostatic energy, potential energy, van der waals energy, RMS Gradient and bond energy values were listed in Table 3 for Urea and β – mercaptoethanol.

Molecular Docking: Molecular docking studies were carried out using the LeadIT software which uses flexible docking approach i.e. it considers ligand flexibility by changing the conformations of the ligand in the active site while making the protein rigid [14]. 2CBZ was considered as a receptor protein to be more specific it's a membrane transported involved drug efflux. Urea and β – mercaptoethanol were the potential ligands. The docking results in receptor-ligand complex which comprises of intermolecular interaction energies. These interactions include hydrogen bonds and hydrophobic and electrostatic interaction. Receptor-ligand complex with least binding energy are considered to be best binding compound.

Docking consists of 2 parts (i) pose generation and (ii) scoring i.e. numerical value to docking. Translation (movement of the ligand into the binding pocket) and rotation of the ligand (once the ligand reaches the binding pocket it rotates around the rotatable bonds to accommodate in the pocket) are required for pose generation they are designated as optimization problem in T and R space. Bond distance and angle are left at their original values. Torsion will have to adopt proper configuration so that the ligand fits the pocket which is designated as optimization problem in ϕ space.

Scoring depends on H- bonds, Pi-Pi interaction and freezing of rotatable bonds. Affinity of the ligand increases with additional torsional strain, desolvation energy, metal interaction and reduce intra and intermolecular clashes.

Molecular dynamics simulation: MD simulations were conducted for the protein–ligand complex (2CBZ-Urea and 2CBZ - β – mercaptoethanol) as well as for the standard protein (2CBZ) without docking it with chemical compounds in "Discovery Studio". For dynamics study, the following parameters are considered: H_2 atoms are added, CHARM force field was applied, potential energy calculations with respect to bond length, bond angle, torsion angle and vander waals energy, H_2O molecules are added (salvation) finally, 6 ns MD was performed in order to analyze the stability of each system.

Root-mean-square-deviation (RMSD) and H-bonds are used to check the stability of the complexes as well as the standard protein. Secondary structure analysis is done to differentiate the native form of the protein from the unfolded form (post treatment with urea and β – mercaptoethanol).

Root mean square deviation: The RMSD is a crucial parameter to analyze the equilibration of MD trajectories. RMSD of the protein backbone atoms are plotted as a function of time to check the stability of each system throughout the simulation [13].

Table 1. Crystal Structure of p-glycoprotein

PDB ID	Details	Gene	Chain A	Length (aa)	Source	References
2CBZ	Structure of the Human Multidrug Resistance Protein 1 Nucleotide Binding Domain 1	ABCC1 - ATP binding cassette subfamily C member 1	fragment of the protein with cytoplasmic localization	237 amino acid	*Homo sapiens*	Ramaen. O et. al. (2006) [12]

Table 2. Structure of ligands with their molecular properties

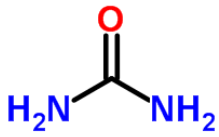
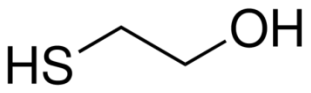
Name	Compound summary	2D image
Urea	PubChem CID: 1176 Molecular Formula: CH_4N_2O Molecular Weight: 60.056 g/mol Hydrogen Bond Donor Count: 2 Hydrogen Bond Acceptor Count:1 Rotatable Bond Count : 0	
β – mercaptoethanol	PubChem CID: 1567 Molecular Formula: C_2H_6OS Molecular Weight: 78.129 g/mol Hydrogen Bond Donor Count: 2 Hydrogen Bond Acceptor Count:2 Rotatable Bond Count : 1	

Table 3. CHARMm energy value, angle energy, dihedral anagle, electrostatic energy, potential energy, van der waals energy, RMS Gradient and bond energy values for Urea and β – mercaptoethanol.

Compound	CHARMm energy	Angle Energy	Dihedral Energy	Electrostatic Energy	Potential Energy	Van der Waals Energy	RMS Gradient	Bond Energy
Urea	-39.3273	0.93818	0	-40.0195	-39.3273	-0.39329	0.00807	0.14724
β – mercaptoethanol	4.95897	0.66122	0.00547	3.07788	4.95854	1.0854	0.00781	0.12857

Table 4. Ligand-protein interaction with docking scores

Complex	Docking Score	H-Bonds	Amino acids	Amino acid atom	Ligand atom
Urea - 2CBZ	-15.5995	4	Cys682 Gly683 Lys684 Ser685	HN HN HN OH	O O O HN
β – mercaptoethanol - 2CBZ	-10.0501	6	Cys682 Gly683 Lys684 Ser685 Ser686	SH, HN HN HN HN HN	OH, O O O SH SH

Table 5. Bond Lengths

Complexes	Interacting Atoms	H- bond Lengths
Urea - 2CBZ	O1 - C4	1.21804
	N2 - C4	1.33273
	N2 - H5	0.999024
	N2 - H6	0.996921
	N3 - C4	1.3327
	N3 - H7	0.99905
	N3 - H8	0.99685
β – mercaptoethanol - 2CBZ	S1 - C3	1.81004
	S1 - H9	1.33504
	O2 - C4	1.42052
	O2 - H10	0.954285
	C3 - C4	1.52989
	C3 - H5	1.09015
	C3 - H6	1.09025
	C4 - H7	1.09521
	C4 - H8	1.09522

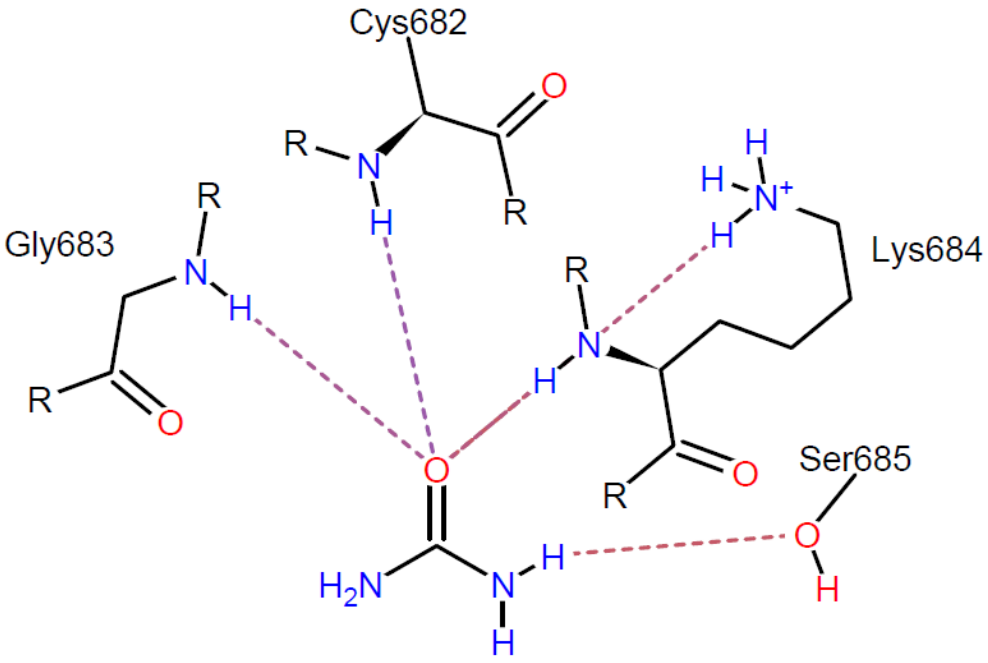


Figure 2. Docking of Urea with 2CBZ (Urea is forming H- bond with Cys682, Gly683, Lys684 and Ser685)

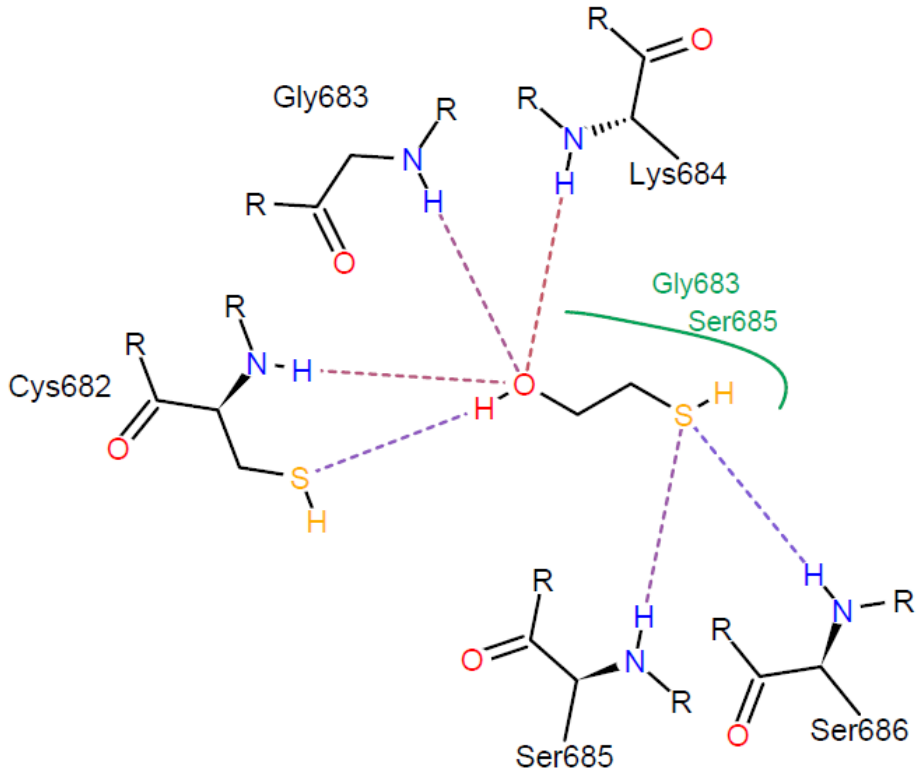


Figure 3. Docking of β – mercaptoethanol with 2CBZ (β – mercaptoethanol is forming H-bonds with Cys682, Gly683, Lys684, Ser685 and Ser686)

H-bonds: The intermolecular hydrogen bonding between the protein and the ligand plays an essential role in stabilizing the protein–ligand complexes. The stability of the hydrogen bond network formed between 2CBZ-Urea and 2CBZ - β – mercaptoethanol is calculated throughout the simulation for the two complexes respectively. Simultaneously the intra molecular hydrogen bond i.e. connecting the sheets and helixes of the protein 2CBZ are essential for maintaining the native state of the protein. The integrity of such bonds are calculated for the standard protein (without treating with Urea and β – mercaptoethanol) and the docking complexes (2CBZ-Urea and 2CBZ - β – mercaptoethanol) [13].

Secondary structure: In a protein large number of amino acids has hydrophobic side chains, while the primary chain, or spine, is hydrophilic. The harmony between these restricting strengths is expert through the formation of discrete secondary structural elements (Pauling and Corey, 1951) [16].

A α - helix is characterized as a corkscrew-sort structure with the fundamental chain framing the spine and the side chains of the amino acids anticipating outward from the helix. The spine is balanced out by the development of hydrogen bonds between the CO of every amino acid and the NH group at four positions C-terminal. Interestingly, the β-strand is a considerably more expanded structure. As opposed to hydrogen bonds shaping inside the optional basic unit itself, adjustment happens through holding with at least one contiguous β - strands. The general structure framed through the association of these individual β - strands is known as a β - pleated sheet (parallel or ant-parallel). Thus by disrupting these hydrogen bonds one can unfold the protein structure [16].

Over the simulation period the stability of such secondary structure has been checked for the standard protein as well as for the docked complexes.

RESULT and DISCUSSIONS

Molecular docking: Molecular docking is a method to foresee the preliminary binding sites of small molecules with the protein of solved three-dimensional structure. Binding poses are basic to illustrate key communications between the small molecules and receptors also; they give accommodating information to outlining viable inhibitors. In this current study flexible docking method was used, using Biosolve LeadIT to dock Urea and β – mercaptoethanol into the active site of the protein (p-gp). The reason of utilizing flexible docking is to give compounds enough adaptability to accomplish all the conceivable 3D space adaptation and not to limit as it were certain rigid structures. Docking results showed that Urea and β – mercaptoethanol has used Mg^{2+} and ATP (natural ligands) binding site of 2CBZ for docking. The ligand receptor interactions are depicted in the Fig 2 and 3. The detailed overview of the binding scores and interacting residues are shown in Table 4 and H-bond lengths (A˚) are listed in Table 5. Urea binds the native protein 2CBZ with a Lead-IT score of -15.5995 and interacts with four amino acid residues namely Cys682, Gly683, Lys684 and Ser685. Similarly β – mercaptoethanol has a docking score of -10.0501 and forms 6 hydrogen bonds with the following amino acid residues Cys682, Gly683, Lys684, Ser685 and Ser686. Negative docking score confirms a better fit. The results thus show that Urea has better binding ability with the protein 2CBZ than β – mercaptoethanol.

Molecular dynamics simulation: Molecular Dynamic Simulation studies were conducted using discovery Studio in order to compare the structural behavior and flexibility of the standard protein with the docked complexes with the lead compounds Urea and β – mercaptoethanol. The studies were performed for 60 ns for each complex. The dynamic simulation runs create a virtual system that tries to mimic physiological environment of t5he cell membrane to check if the ligands (Urea and β – mercaptoethanol) are really stable

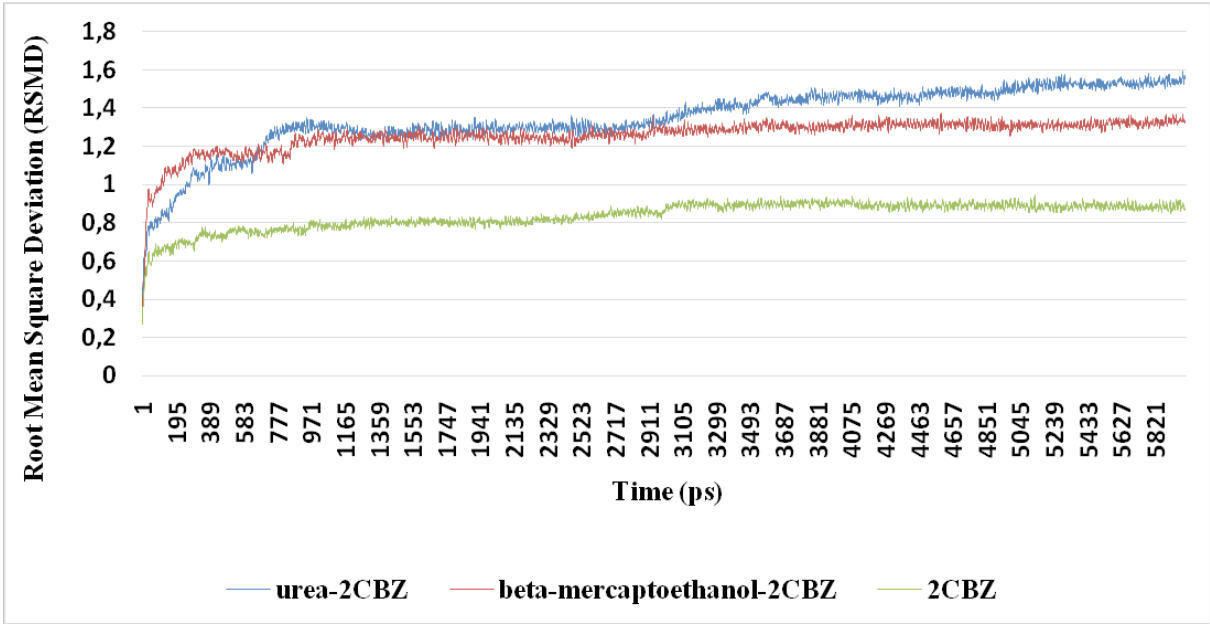


Figure 4. Protein backbone RMSD calculation plots for the standard protein (2CBZ) and ligand bound complexes. Green curve indicates the standard protein without out any bound ligand. Blue curve indicates the 2CBZ – Urea complex with RMSD value of 1.6. Red curve indicates 2CBZ - β- mercaptoethanol complex with RMSD value 1.3.

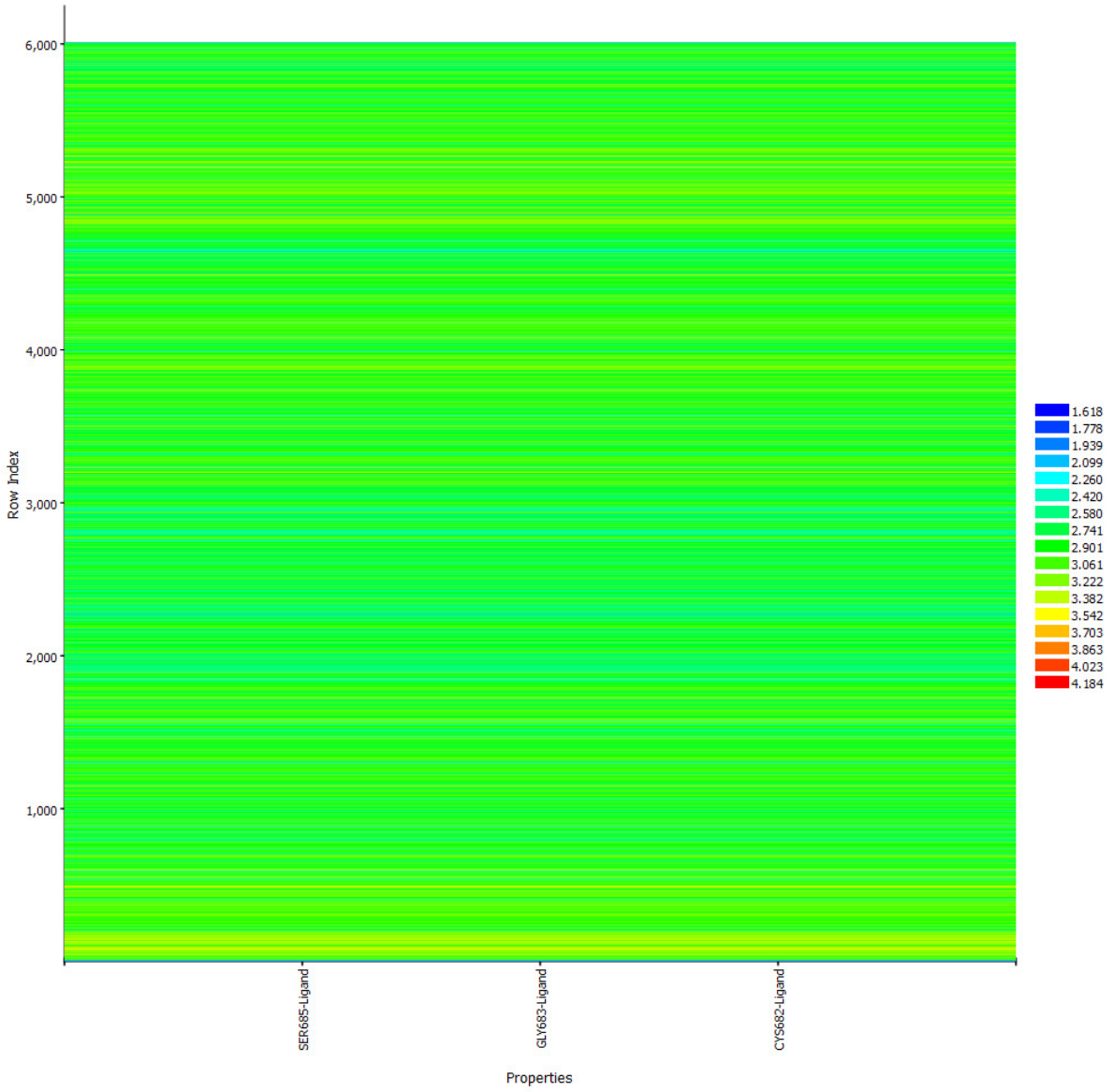


Figure 5. Intermolecular hydrogen bond between 2CBZ and Urea. The majority of green lines in the graph indicate that the intermolecular hydrogen bonds during the simulation period ranged from 2.2 to 2.9 A^0 which is considered fairly stable.

within the binding pocket of the target protein, maintain bonds, and be able to inhibit the activity i.e. unfold the target protein by disrupting its hydrogen bonds and disulphide bonds which will result in therapeutic actions.

Root Mean Square Deviations (RMSD): Root mean square deviations (RMSD) of the standard protein and the complexes were calculated against their initial structure in the protein-ligand complexes and graphs were generated to compare the flexibility of the protein once the ligand is bound to the structure. Over the simulation period, the backbone of the standard protein (2CBZ) remained fairly stable. Binding of β – mercaptoethanol caused a considerable perturbation in the backbone with RMSD value eventually deviating to approximately 1.3 and urea further deviate the value to approximately 1.6 (Fig 4). Increased RMSD value confirms change in the protein structure and compare to β – mercaptoethanol, urea is a more potential compound inducing such structural change.

H-bonds:

The intermolecular hydrogen bonding between the protein and the ligand plays an essential role in stabilizing the protein–ligand complexes. The stability of the hydrogen bond network formed between 2CBZ – Urea and 2CBZ - β- mercaptoethanol are calculated throughout the simulation period for 6 ns. The results indicate that urea shows stable and strong H-bonds with 2CBZ compare to β – mercaptoethanol (Fig 5 and 6).

Urea is used to break non-covalent bonds such as hydrogen bonds and ionic bonds that hold together the secondary and tertiary structure of protein. Disulfide bridges can be disrupted by treating a protein with β-mercaptoethanol. The intra-molecular hydrogen bonds remain stable over the simulation period for the standard protein (2CBZ) but for the complexes with urea and β-mercaptoethanol had shown decrease in the number of hydrogen bonds thus making the protein unstable (Fig 7).

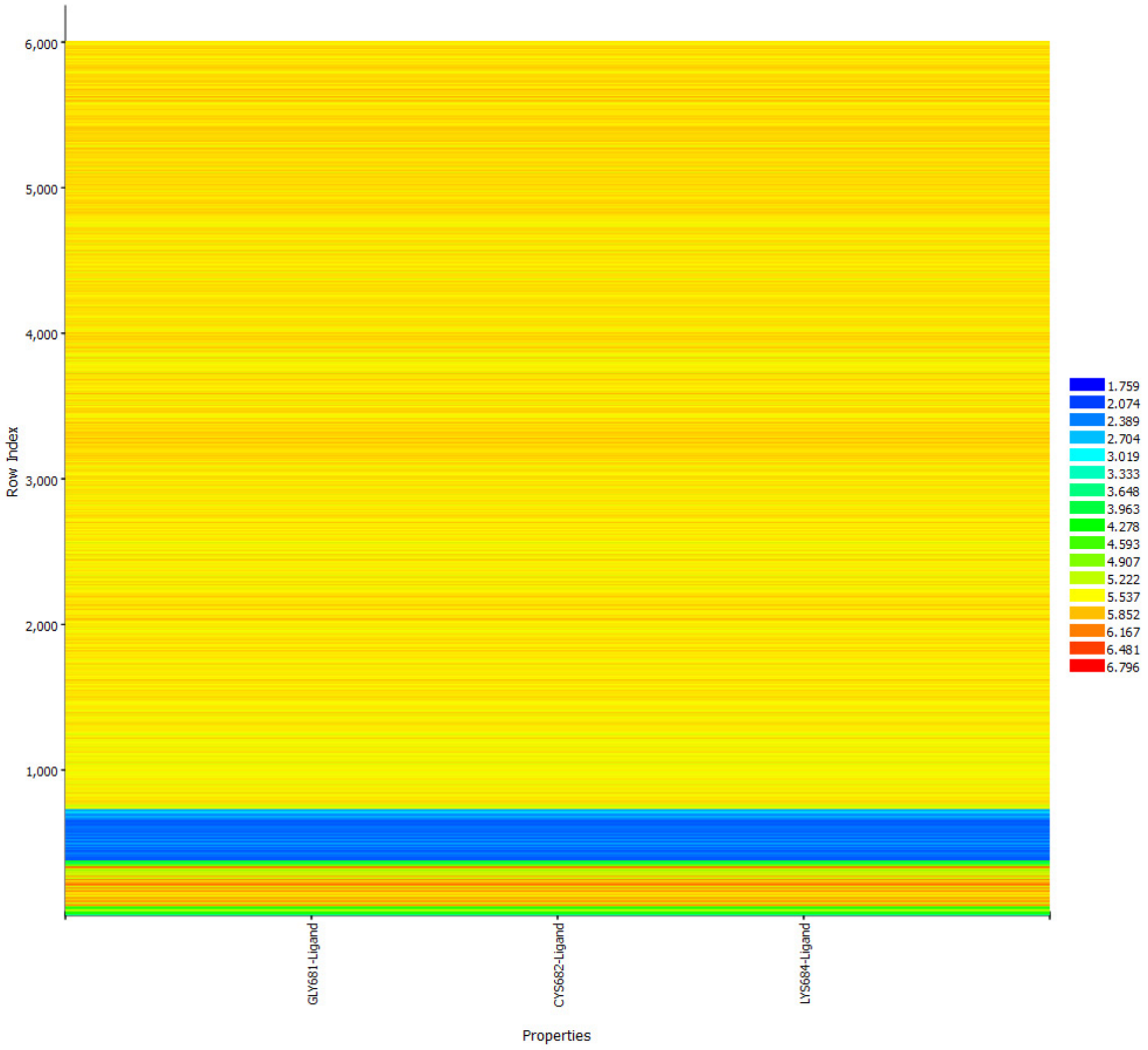


Figure 6. Intermolecular hydrogen bond between 2CBZ and β – mercaptoethanol. In the beginning of the simulation period few blue lines in the graph indicate that the hydrogen bonds were stable ranging from 1.7 – 2.7 A^0. But over the time period majority of yellow lines indicate unstable bonding which is approximately more than 5.5A^0.

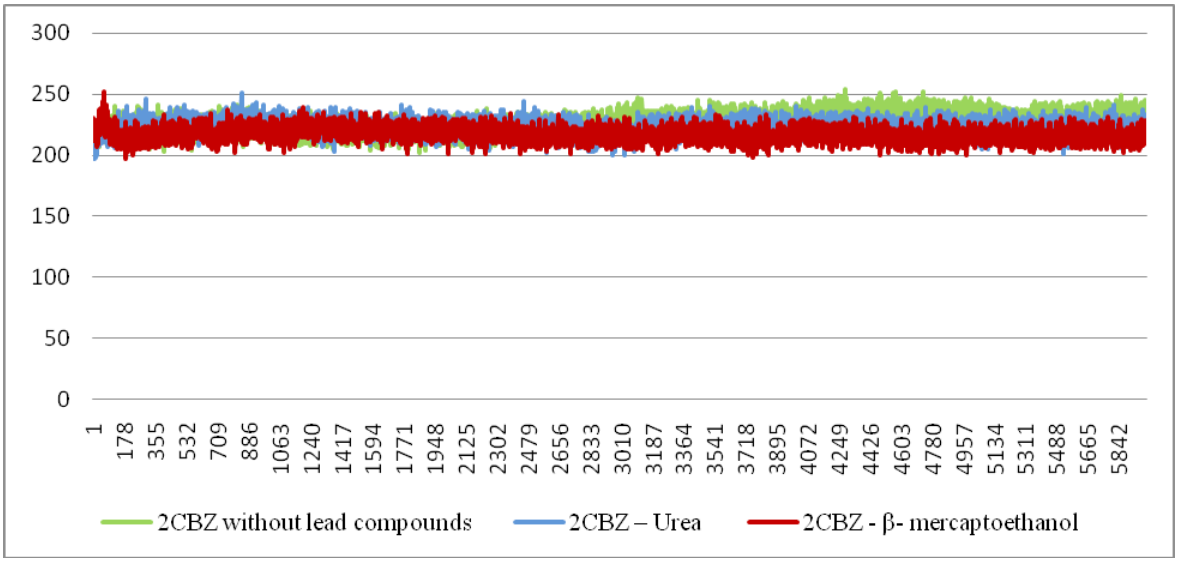


Figure 7. Intra-molecular hydrogen bonds: The green peaks of the graph shows the number of hydrogen bonds continues to be more than 250 over the simulation period. But for the complexes with urea (blue curve) and β-mercaptoethanol (red curve) shows reduction in the number of intra-molecular hydrogen bonds as the peaks drop below 200.

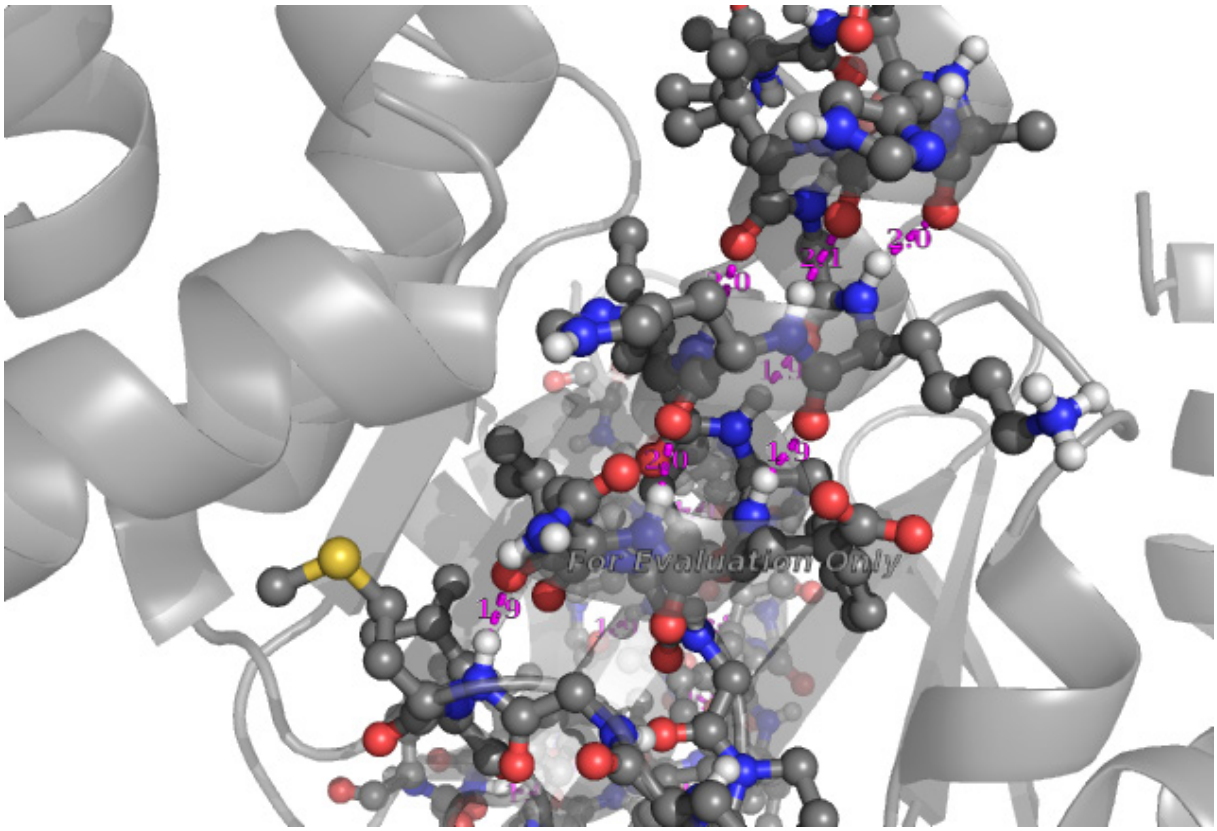


Figure 8. (a): Hydrogen bonds connecting H_2 – O_2 atom holding the alpha – helixes of the target protein 2CBZ (generated by PyMol)

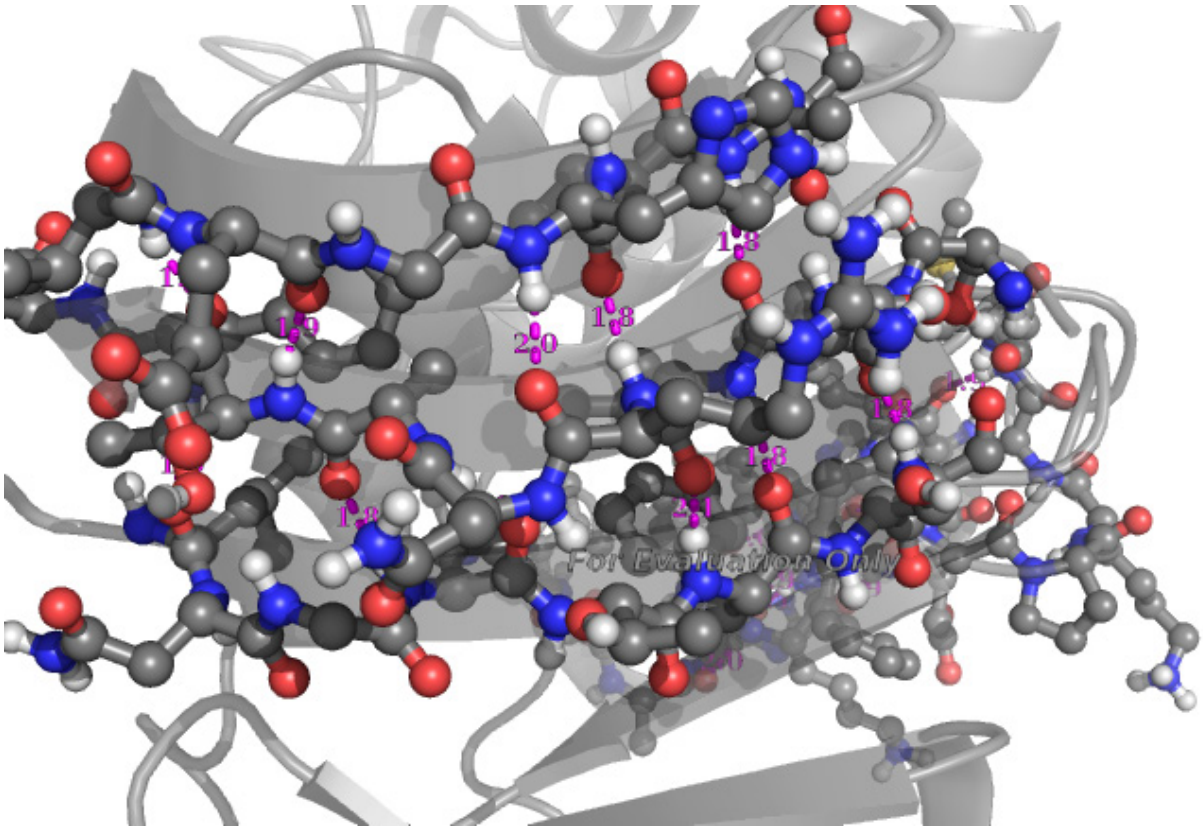


Figure 8. (b): Hydrogen bonds connecting H_2 – O_2 atom holding the β - sheets of the target protein 2CBZ (generated by PyMol)

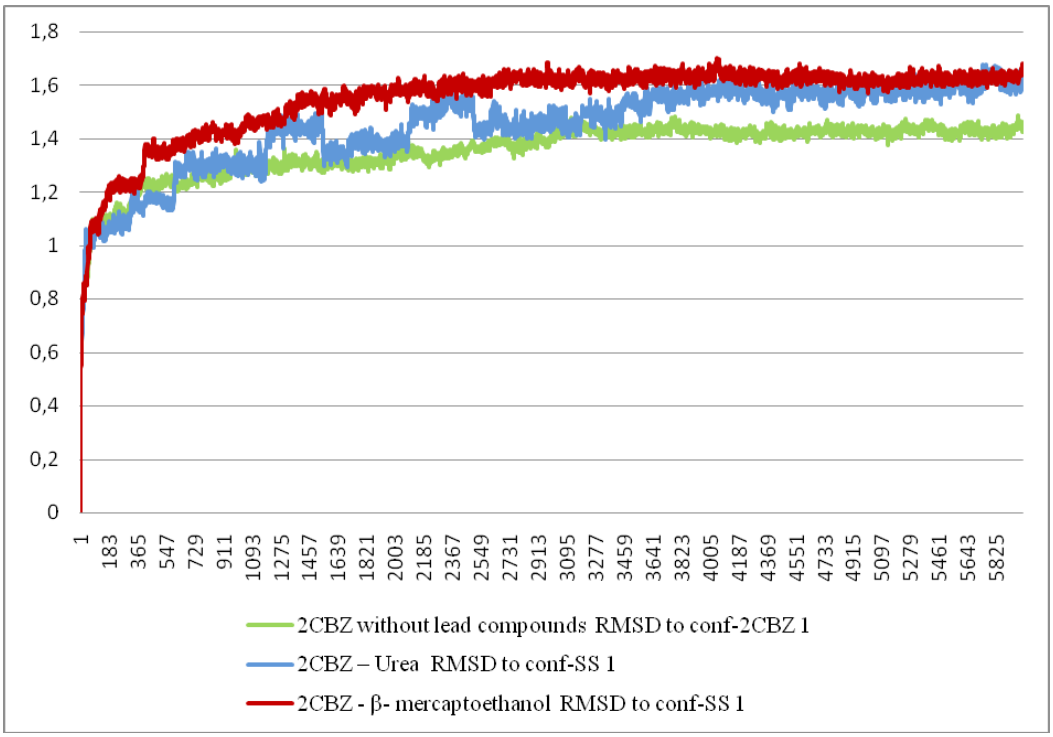


Figure 9. Secondary structure prediction graph: Green curve indicates the standard protein 2CBZ without any docked compound whose simulation values are nearly constant indicating a fairly stable secondary structure over the simulation period. Blue curve (2CBZ – Urea complex) and red curve (2CBZ - β – mercaptoethanol complex) showed significant deviation in the simulation value (1.8) thus indicating a change in the secondary configuration of the protein.

Secondary structure: Stability of a secondary structure depends upon its hydrogen bonds which hold the helix and sheets together thus maintaining the native state of the protein as shown in the Fig 8(a) and (b). Disruption of these bonds can unfold the protein and alter its biological activity. In the previous section we have seen urea and β-mercaptoethanol are the potential chemicals which can reduce the number of intra – molecular hydrogen bonds of the target protein. Thus over the simulation period of 6 ns these chemical cocktail can alter the secondary structure of the protein 2CBZ as depicted in the graph (Fig 9) and are capable of ceasing its biological activity as a membrane pump.

CONCLUSIONS

In the present study molecular docking has been performed to find out the binding sites of urea and β – mercaptoethanol to p-gp pumps (2CBZ). It has been observed that these lead compounds are using ATP and Mg^{2+} binding sites of the pump protein for interaction. The best docked confirmation has been selected based on binding energy score and hydrogen bonding. Urea has better binding ability than β – mercaptoethanol. Furthermore MD simulation has been performed to check the stability of 2CBZ – Urea complex and 2CBZ - β – mercaptoethanol complex in comparison to the standard protein (untreated protein). RSMD, rg, H-bonds and secondary structure prediction shows urea and β – mercaptoethanol are potential compounds altering the protein folding.

Unfolding the pump proteins with urea and β – mercaptoethanol will prevent them from getting transported to the membrane. They will be retained in the endoplasmic reticulum followed by degradation. Without the active pump the cell (cancer) will retain the macromolecules especially the anti cancer drugs which are otherwise being thrown out of the cell. Thus a cancer cell can overcome the property of multi-drug resistance and eventually it will undergo programmed cell death i.e. apoptosis without causing damage to the normal cells. The present study could be the basis for development of novel methods for prevention of multidrug resistance in cancer cell.

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