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Investigation of Paclitaxel Resistant R306C Mutation in β-Tubulin—A Computational Approach

Kanika Verma and Ramanathan K.*

Industrial Biotechnology Division, School of Bio Sciences and Technology, VIT University, Vellore 632014, Tamil Nadu, India

ABSTRACT

Paclitaxel is the most effective chemotherapeutic agent used for the treatment of a broad spectrum of solid tumors. However, observed paclitaxel resistance in clinical trials presents one of the major obstacles for cancer chemotherapy. Most importantly, resistance due to β-tubulin mutations (R306C) has been intensely debated in recent years. Despite all efforts, mechanism of resistance is still not well understood. In this study, computational techniques were employed to uncover the effect of R306C mutation in the β-tubulin structure and its function. The tools such as I-Mutant, CUPSAT and Fold-X were employed to address the consequence of R306C mutation in the structural stability of β-tubulin. Further, molecular docking and molecular dynamics study was employed to understand the functional impact of β-tubulin mutation. Our results suggest that the R306C mutation causes a significant reduction in the binding affinity between β-tubulin and paclitaxel. Further, docked complex analysis indicates that destruction of conservative hydrogen bond maintained by the residues Arg282 and Gly360 should be responsible for the large conformation changes of the binding pocket in R306C mutant. Finally, molecular dynamics simulations study confirms the stable binding of paclitaxel with native type β-tubulin structure rather than mutant (R306C) type. We certainly believe that this study will provide useful guidance for the development of novel inhibitors that are less susceptible to drug resistance. J. Cell. Biochem. 116: 1318–1324, 2015.

KEY WORDS: PACLITAXEL; R306C MUTATION; MOLECULAR DOCKING; MOLECULAR DYNAMICS

Microtubules are major dynamic structural components in cells [Wang et al., 2005] that have a central role in cell division and they are major target in cancer chemotherapy [Shing et al., 2014]. Tubulin (globular protein) is a heterodimer composed of α- and β-tubulin subunits that form the microtubules. Microtubules function is strongly connected with their stability. In the mitotic period of the cell cycle, microtubules are in dynamic equilibrium between their polymerized microtubule structure and depolymerized α- and β-tubulin dimer structure. The disruption in the dynamic equilibrium blocks cells in mitosis at the metaphase/anaphase transition and eventually leads to apoptosis. Therefore, the compounds that interrupt the dynamics of β-tubulin polymerization and microtubule depolymerization would be useful in the cancer treatment [Jordan et al., 1998]. Recently, paclitaxel has come out as a potential drug for treating several solid tumors including breast, ovarian and non-small cell lung carcinomas (NSCLC) by the disruption of microtubule dynamics [Yin et al., 2010]. It is strongly believed that microtubule's stability is based on the conformational change induced by paclitaxel binding to β-tubulin. This, in order influences the normal formation of mitotic spindles, chromosome segregation and subsequently leads to mitotic arrest [Gascoigne and Taylor, 2009]. However, as like with numerous malignancy chemotherapeutic agents, clinical efficacy of paclitaxel has greatly constrained due to the emergence of β-tubulin mutations [Dumontet and Sikic, 1999; Drukman and Kavallaris, 2002]. Several mechanisms of resistance were proposed by utilizing cell culture techniques [Orr et al., 2003]. The overexpression of ATP-binding cassette (ABC) transporters is one of the reported resistance mechanism. Drug resistance is caused by actively pumping out variety of drugs from the cell via ABC transporters, including microtubule agents such as taxanes and vinca alkaloids thus rendering the cells resistant to these drugs [Hari et al., 2006]. The other explored mechanisms of drug resistance with known clinical importance are: (a) effluxing of different chemical substance from the cells on activation of transmembrane proteins, (b) the activation of the enzymes of the glutathione detoxification system, and (c) alterations of the genes and the proteins involved in the control of apoptosis [Choi, 2005]. In addition, the microtubule dynamics could also play a major role in the

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*Correspondence to: Ramanathan K., Industrial Biotechnology Division, School of Bio Sciences and Technology, VIT University, Vellore 632014, Tamil Nadu, India. E-mail: kramanathan@vit.ac.in
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paclitaxel resistance [Wang and Nogales, 2005]. Recent evidence indicates that resistance of cancer cells to paclitaxel is due to overexpression of heat shock transcription factor 1 [Vydra et al., 2013]. However, one reported paclitaxel resistance mechanism that has received huge attention in recent years involves β-tubulin mutations [Berrieman et al., 2004]. Most importantly, literature evidence indicates that microtubule stability and drug-target binding is significantly affected by R306C mutation in β-tubulin [Yin et al., 2010]. In fact, when patients are treated with paclitaxel they are facing clinical failure or reduced susceptibility of drug. This is mainly due to significant increase of resistance towards paclitaxel. To overcome these obstacles and to improve cancer therapy, the pharmaceutical industries have focused their efforts on developing lead compounds that target not only the native type but also the mutant type β-tubulin. These efforts could be achieved only by the complete understanding of the structural alterations in its intracellular target, β-tubulin. Recent literature evidences also highlights that molecular dynamics simulation study is one of the key techniques and could produce significant impact both in the analysis and understanding of the drug resistance pattern of the target protein [Rajendran et al., 2012; Purohit, 2014; Rajendran and Sethumadhavan, 2014]. Hence, molecular dynamics simulation study was initiated in order to understand the behavior of β-tubulin and to analyze the physical basis for drug resistance with the help of available structures. We sincerely hope that results obtained from our analysis helps not only in understanding of the active pocket for paclitaxel but also helpful to gain a clear picture of the key residues in β-tubulin.

MATERIALS AND METHODS

DATA SET

The crystal structures of β-tubulin used in our analysis were obtained from the Protein Data Bank (PDB). The corresponding PDB code is 1TVK [Xu et al., 2012] and the mutant structure was generated using Swiss-PDB viewer [Guex and Peitsch, 1997]. Paclitaxel was used as the drug molecule for our studies. The SMILES notation for paclitaxel was retrieved from PubChem (NCBI) [Feldman et al., 2006] and submitted to CORINA for deducing the 3D structure of paclitaxel molecule [Gasteiger et al., 1990]. All the water molecules and the heteroatoms were removed before performing molecular dynamics (MD) simulation.

RECOGNITION OF LIGAND BINDING SITE RESIDUES OF β-TUBULIN

Protein–ligand interactions are the most fundamental to all biological mechanisms. These interactions are highly specific and are the consequence of distinct molecular interaction properties of the binding sites. Therefore, the analysis of binding site residues is certainly important for the understanding of ligand affinity and ultimately for the molecular underpinnings of protein functions. In the present investigation, we employed SITECOMP server [Lin et al., 2012] to obtain the binding site locations in β-tubulin structure. The SITECOMP program uses molecular interaction fields (MIFs) for the analysis. The PDB Code 1TVK was submitted into the program to get the binding residues information in the β-tubulin structure. Moreover, literature evidence [Ganesh et al., 2004] was also used to validate the obtained results.

PROTEIN STABILITY ANALYSIS

First, we studied the impact of R306C mutation in the β-tubulin structural stability. The protein stability change upon R306C mutation was analyzed using three different approaches with the increasing resolution: FoldX, which allows the computation of the free energy of a macromolecule based on its high-resolution three-dimensional structure. FoldX analysis of protein stability is based on the empirical force field, which was developed for the quick evaluation of the effect of mutations on the stability, folding, and dynamics of nucleic acids and proteins [Schymkowitz et al., 2005]. In this approach the free energy of folding is calculated from the difference in Gibbs free energy between the crystal structures of the protein hypothetical unfolded reference state of which no structural details are known. Subsequently, Cologne University Protein Stability Analysis Tool (CUPSAT) was employed for the prediction and analysis of protein stability changes upon point mutations. In the CUPSAT approach, coarse-grained atom potentials and torsion angle potentials were used to predict protein stability upon point mutations [Parthiban et al., 2006]. Finally, the I-Mutant program was also employed for investigating the impact of R306C mutation in β-tubulin stability. It is an artificial neural network based system to predict the direction towards which the mutation shifts the stability of the protein instead of directly estimating the relative stability changes upon protein mutation [Raghav and Sharma, 2013].

PROTEIN LIGAND DOCKING

Gromacs package 4.5.3 was used to generate energy minimized structures of native and mutant β-tubulin [Van Der Spoel et al., 2005; Hess et al., 2008]. One thousand steps of steepest descent energy minimization were carried out for the native and mutant structures. Consequently, flexible Protein–Ligand docking studies were carried out by using Autodock program. It is believed that autodock is a widely used program with several examples of validation [Ragno et al., 2005; Kumar and Ramanathan, 2014]. Autodock examines ligand conformations comprehensively and estimates the binding affinity of the drug molecule. Initially, polar hydrogen was added to the structure of β-tubulin. Subsequently Kollman charges and Gasteiger charges were assigned to all atoms and rotable bonds were assigned using Autodock Tools. The free binding energies between β-tubulin and paclitaxel were estimated using atom affinity potentials pre-calculated using AutoGrid4 on grid maps. Grid center was focused on the active site and $60 \times 60 \times 60$ frame lattice with grid positioning of 0.375 Å [Rathinasamy et al., 2010] were determined. For molecular docking Global and Local Search (GA-LS) method was used [Iman et al., 2011]. For GA different parameters are followed: a maximum number of 250,000 energy evaluations; a maximum number of generations of 27,000; mutation and crossover rates of 0.02 and 0.8, respectively. Pseudo-Solis & Wets parameters were used for local search and 300 iterations of Solis & Wets local search were imposed. Both Autogrid and Autodock computations were performed on Cygwin [Iman et al., 2013] and 10 independent docking runs were performed for each native and mutant β-tubulin protein. Autodock evaluates the ligand binding through the conformational search

space using a Lamarckian genetic algorithm. Final Autodock result was analyzed using Autodock tools, graphical user interface of Autodock.

MOLECULAR DYNAMICS SIMULATION

The native and mutant type′s β-tubulin and paclitaxel docked complexes were used as starting structure for performing MD study. The GROMACS package 4.6.3 [Elengoe et al., 2014] implemented with the GROMOS43a1 force-field was used to run MD simulation [Van Der Spoel et al., 2005]. The system was solvated in cubic 0.9 nm, using periodic boundary conditions and the SPC water model [Meagher and Carlson, 2005]. The PRODRG server [Schuttelkopf and Van Aalten, 2004] was utilized to prepare ligand topology file. One thousand steepest descent energy minimization steps were carried out with no constraints to minimize the systems energy. After energy minimization, the system was equilibrated at constant temperature and pressure. The equilibrated complex structures were then exposed to MD simulations for 50,000 ps and the integration time was set to 2 fs. The atom-based cutoff of 8 Å method was used to treat the van der Waal′s interactions. The particle-mesh Ewald algorithm was used to treat the long-range electrostatic interactions [Darden et al., 1999]. For Lennard–Jones interaction 0.9 nm cutoff was employed. During the stimulations, all bond lengths containing hydrogen were controlled by using Lincs algorithm [Lindahl et al., 2001]. For structural analysis the trajectory snapshots were stored at every picosecond. Root mean square deviation (RMSD), root mean square fluctuation (RMSF), and intramolecular H bonds details were analyzed using GROMACS conveniences g_rms, g_rmsf, and g_hbond, respectively. Graphing, Advanced, computation and exploration (GRACE) program was utilized in the molecular dynamics data analysis.

RESULTS AND DISCUSSION

PROTEIN STABILITY ANALYSIS

Identifying the change in the stability of the protein structure allows the biologist to quickly annotate the functions of the protein. Hence, initially we investigated the stability of β-tubulin protein upon mutation (R306C) using I-Mutant, CUPSAT and FoldX, The results of I-Mutant and CUPSAT is shown in (Table I) and the results of FoldX is given in Figure 1. These results clearly depicts that the mutation (arginine–cysteine at 306 position) significantly affects the structural stability of the β-tubulin. It is mainly because the native type residue forms a salt bridge with the Aspartic acid on position 295 and on position 304. The difference in charge will disturb the ionic

TABLE I. Protein Stability Analysis of Mutant β-Tubulin Using I-Mutant and CUPSAT

S. no.	Mutation	I-Mutant DDG value (kcal/mol)	CUPSAT DDG value (kcal/mol)	Overall stability
1.	R306C	−0.42	−1.04	Destabilizing

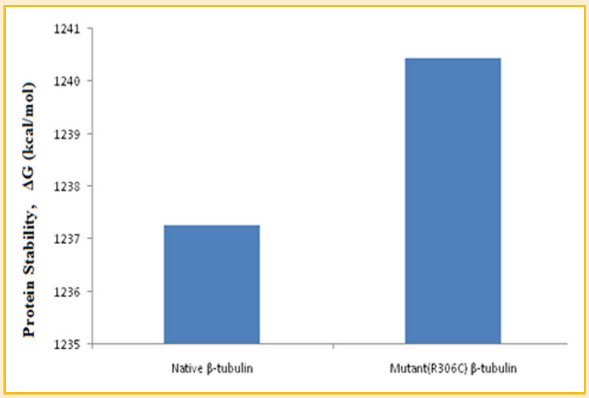

Fig. 1. Protein stability analysis using FoldX algorithm.

interaction made by the original, native-type residue [Venselaar et al., 2010]. Moreover, the conservation score of the native-type residue is very high. Thus, mutation at this conserved position damages the protein structure and its function as well.

MOLECULAR DOCKING STUDIES

The docking procedure implicates the prediction of ligand conformation and orientation within a targeted binding site. In general, the docking study is essential for the precise prediction of activity of drug molecule against the target structure. In this study, GROMACS package 4.6.3 was used to minimize the structure of native and mutant protein. One thousand steps of steepest descent energy minimization were carried out for the β-tubulin proteins. Initially, the binding site residues information (Fig. 2) in the structure of β-tubulin were obtained, using SITECOMP server and the results are validated using available literature evidence [Ganesh et al., 2004]. Subsequently, flexible docking studies were performed using Autodock program. The docked complex is shown in Figure 3. The binding free energies of the native and mutant types of β-tubulin–paclitaxel complex determined by Autodock were −8.11 and −4.44 kcal/mol, respectively (Table II). The binding energy difference indicates that the

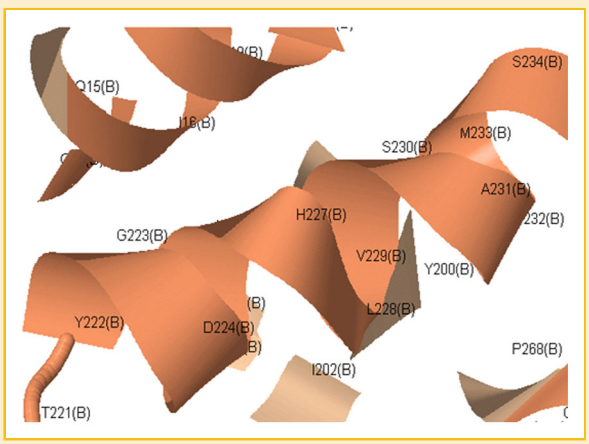

Fig. 2. Binding site residues obtained from SITECOMP server.

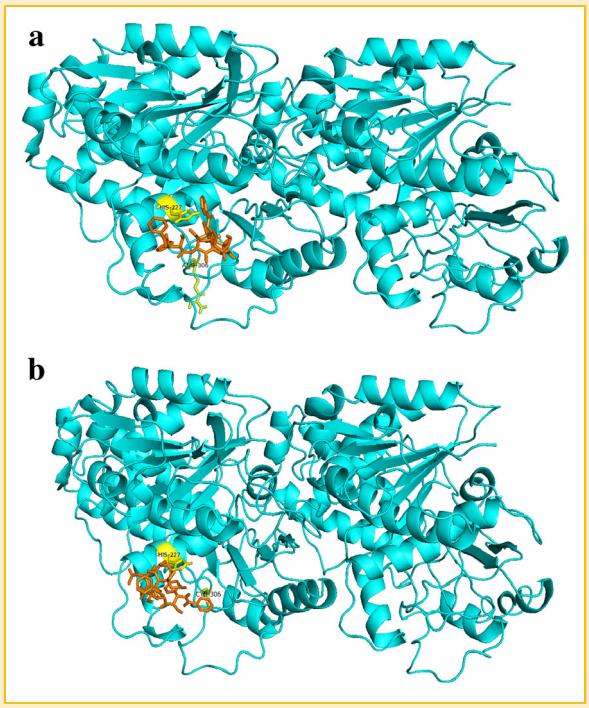

Fig. 3. The binding conformations of paclitaxel interacting with the native (a) and mutant (R306C) (b) β-tubulin structure.

TABLE II. Analysis of Free Binding Energy of Paclitaxel With Native and Mutant Type β-Tubulin Protein

S. no.	β-Tubulin–paclitaxel complex	Inter-molecular energy (kcal/mol)	Total internal energy (kcal/mol)	Torsional free energy (kcal/mol)	Unbound systems energy (kcal/mol)	Binding free energy (kcal/mol)
1.	Native	−13.18	−5.82	+5.07	−5.82	−8.11
2.	Mutant	−9.51	−7.07	+5.07	−7.07	−4.44

incompetent binding of paclitaxel with mutant (R306C) protein. The available computational analysis highlights that mutation could bring the decrease in flexibility of binding residues and produce conformational change in the protein structure [Purohit et al., 2011]. Moreover, the computational results reported in the literature also indicate that there is a possible lost of intermolecular contacts when there are conformational changes in the binding pocket residues [Purohit et al., 2011a]. Therefore, we have also examined the existence of intermolecular interactions with help of docked complex structures. Moreover, LIGPLOT analysis was carried out to study the possible reasons for the decreased binding affinity of paclitaxel with mutant structure. Thus results are shown in Figure 4. The dotted lines and red arc represents the hydrogen and hydrophobic contacts in the complex structures, respectively. It is evident from the figure that five residues in native type protein such as Arg282, Gly360, Arg276, His227, and Asp224 are involved in the hydrogen bonding with paclitaxel. On the other hand, only three hydrogen bonding

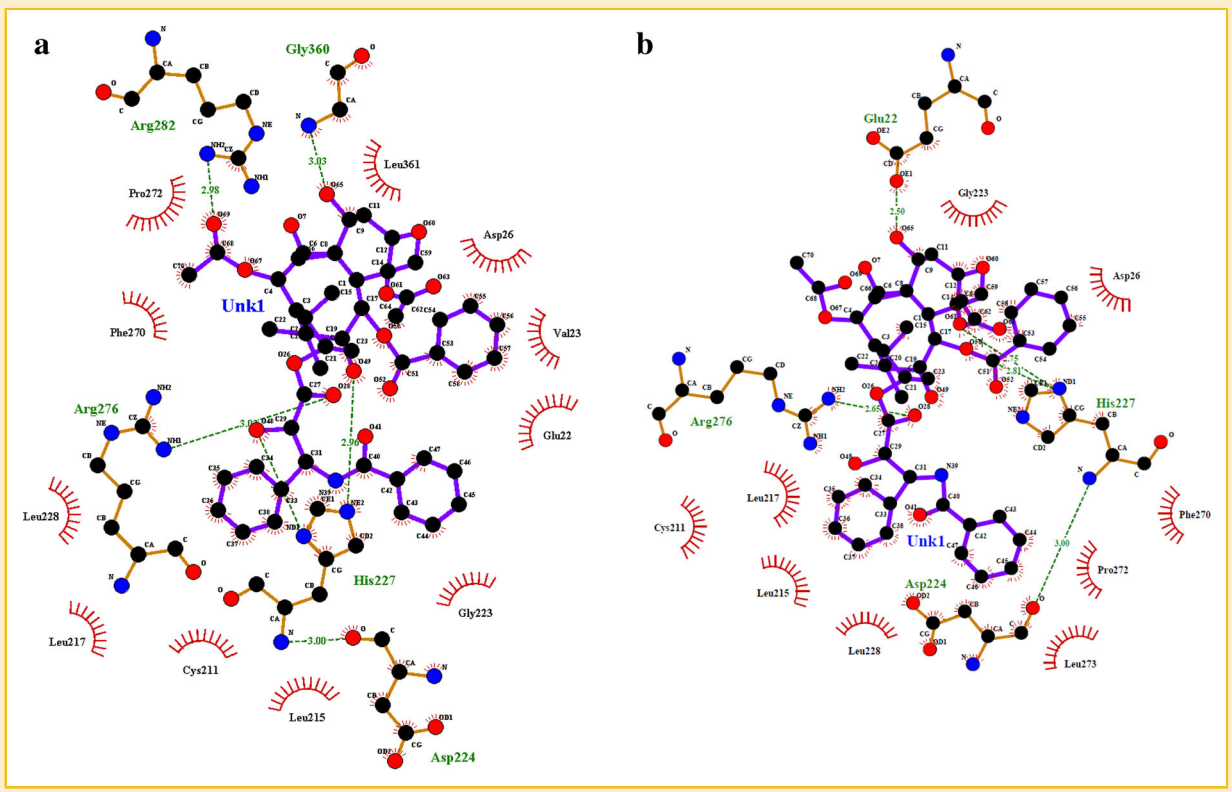

Fig. 4. Representation of intermolecular interactions in the native complex (a) and mutant (R306C) (b) complex.

interactions were observed in the mutant structure. For instance, Arg282 and Gly360 did not involve in the drug binding process. This is mainly because of the difference in charge between the native-type and mutant amino acid. The charge of the native-type residue will be lost; this can cause loss of interactions with other molecules or residues. In addition, the native-type and mutant amino acids differ in size. In fact, the cystein is smaller than arginine and this might lead to the loss of interactions. Furthermore, the hydrophobicity of the native-type and mutant residue is also differs significantly. The mutation introduces a more hydrophobic residue at this position. This can result in loss of hydrogen bonds and disturb correct folding. These evidences highlight that interaction formed by the residues Arg282 and Gly360 is certainly important for the effective binding of paclitaxel with β-tubulin. It is noteworthy to mention that the results obtained from this study correlates well with the available experimental evidence [Huzil et al., 2008; Contini et al., 2012].

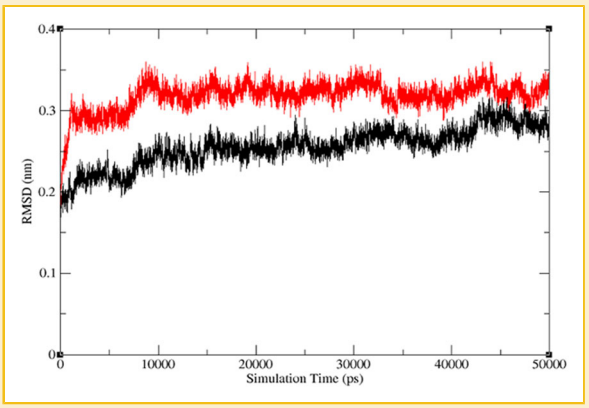

Fig. 5. RMSD analysis of complex structure during molecular dynamics simulation. Native complex (black) and mutant complex (red).

MOLECULAR DYNAMICS ANALYSIS

As ligand binding is a microscopic event that take place in mere millionths of a second, a complete understanding of the energetic and mechanics of binding is unattainable using current experimental techniques. This issue could be addressed with the aid of molecular dynamics simulations study [Durrant and McCammon, 2011]. In the present study, MD simulations were performed for native and mutant β-tubulin protein and paclitaxel complexes, using GROMACS package that execute the GROMOS96 force field parameters. Initially, the protein drug complex system is neutralized by adding counter ions using the "genion" tool incorporated into the Gromacs package. For instance, 15 Na+ and 16 Na+ counter ions were added to the native and mutant (R306C) type β-tubulin–paclitaxel complex structures respectively to neutralize the net charge of the protein–drug complex. Subsequently, MD simulation is initiated for a period of 50,000 ps. The data such as RMSD, RMSF, and Hydrogen Bond details were analyzed from the MD trajectory file. RMSD analysis can give an idea of how much the three-dimensional structure has fluctuated over time. We observed a significant structural deviation in the mutant protein (R306C) when compared to native protein structure from the starting till 50,000 ps (Fig. 5). It is also clear from figure that native structure showed less deviation in starting of the simulation period (0–10,000 ps) and attained an RMSD value of ~0.19 nm. On the other hand, mutant structure deviated to a great extent from their original position and reached the backbone RMSD of ~0.21 nm at 10,000 ps. From 10,000 to 30,000 ps RMSD value maintained in constant level for both native and mutant structure. Between the 30,000 and 45,000 ps, significant deviation observed in native type and attained the RMSD of ~0.25 nm whereas mutant structure attained ~0.35 nm. The deviation in RMSD is significantly lesser after 45,000 ps for the native and mutant type and attained a value of ~0.28 and ~0.34 nm, respectively, in the end of simulation period. These data indicate that the deviation of the native structure was minimal when compared with mutant type throughout

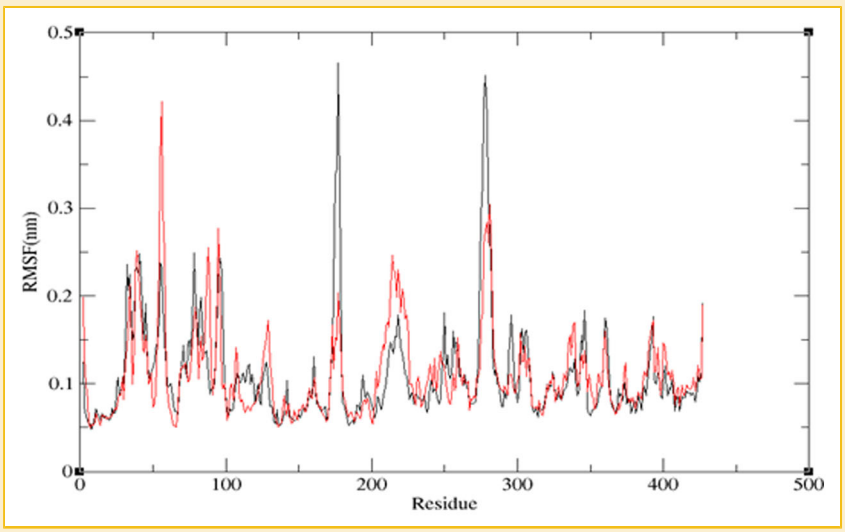

Fig. 6. RMSF analysis of complex structure during molecular dynamics simulation. Native complex (black) and mutant complex (red).

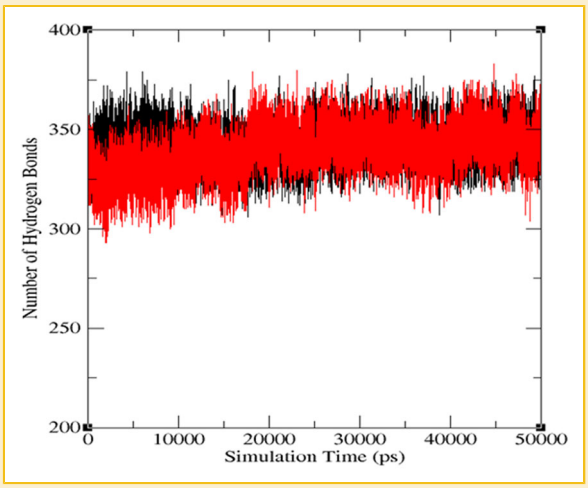

Fig. 7. Hydrogen bond analysis of complex structure during molecular dynamics simulation. Native complex (black) and mutant complex (red).

the simulation period. This highlights the stability of native type-paclitaxel complex than R306C paclitaxel complex. Furthermore, RMSF analysis was initiated to understand the cause of inefficient binding of paclitaxel with mutant protein. In particular, only binding site region were examined in the present study with the aid of molecular dynamics trajectory file. The result is shown in Figure 6. It is evident from the figure that flexibility of mutant β-tubulin–paclitaxel complex is more than native type complex. The higher flexibility of mutant complex is mainly because of minimum number of interactions of paclitaxel with the target protein, β-tubulin. This inherent flexibility of amino acid residues is likely to play an important role in the ligand binding. Finally, the intramolecular hydrogen bond details were analyzed during the molecular dynamics simulation study. The result is shown in Figure 7. It is clear from the figure that native type protein can maintain maximum of 375 intramolecular hydrogen bonds whereas the mutant type protein can maintain only 352 hydrogen bonds. Further, the frequency of occurrence of hydrogen bonding interaction was significantly higher for the native type β-tubulin than mutant type β-tubulin throughout simulation time. These evidences undoubtedly indicate that mutation at position 306 significantly alters the conformations of the amino acid and thus confers resistance for paclitaxel binding.

In conclusion, we suggest that despite recent advances in treatment modalities, cancer remains a major source of morbidity and mortality throughout the world. The reasons for this failure are certainly diverse but the key unresolved issue in anticancer chemotherapy is resistance against anticancer drugs. In particular, the involvement of tubulin mutations as a cause of paclitaxel resistance is the major concern in cancer chemotherapy. In the present study, we have investigated the mechanism of palictaxel resistance with the aid of computational techniques. The result of the stability analysis signifies that R306C mutation alters the structural stability of the tubulin protein. The binding affinity data obtained from the docking study explains the ineffectual binding of palitaxel with mutant (R306C) type tubulin. The results of RMSD analysis undoubtedly indicate the stable binding of paclitaxel with native type protein rather than mutant type. The results from our analysis also indicate that mutation significantly alters the flexibility of the binding pocket residues which results in the marked decrease in the existence of hydrogen bonding network in the mutant (R306C) protein. Finally, based on all our results we conclude that Arg282 and Gly360 are needed for the effective binding of palitaxel with tubulin protein. Hopefully, the results obtained from this study may be of valuable guidance for the management of paclitaxel resistance in the near future.

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