

An *In silico* Based Comparison of Drug Interactions in Wild and Mutant Human β -tubulin through Docking Studies

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Abstract

Background: Tubulin protein being the fundamental unit of microtubules is actively involved in cell division thus making them a potential anti-cancer drug target. In spite of many reported drugs against tubulin, few of them have started developing resistance in human β -tubulin due to amino acid substitutions.

Methods: In this study we generated three mutants (F270V, A364T and Q292E) using Modeller9v10 which were targeted with compounds from higher and lower plants along with marine isolates using iGEMDOCK2.0 to identify their residual interactions.

Results: The mutant F270V does not bring in any increase in the binding affinity in comparison with the taxol-wild type due to their conservative substitutions. However, it increases the volume of the active site. A364T mutant brings a better binding among few of the marine and higher plants isolates due to the substitution of the non-reactive methyl group with the polar residue. But this leads to reduced active site volume. Finally the mutant Q292E from epothilone binding site brings a remarkable change in drug binding in the mutants in comparison with the wild type due to the substitution of uncharged residue with the charged one. But as such there was no change in the volume of the active site observed in them.

Conclusion: Lower plants extracts were reported to exhibit better interactions with the taxol and epothilone binding sites. Whereas marine and higher plants isolates shows significant interactions only in the wild type instead of the mutants. In addition to this, the residual substitutions were also found to alter the conformations of the active sites in mutants.

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Introduction

Tubulin being the fundamental unit of microtubules is critically involved in chromosomal segregation, cell division, motility and intracellular transportation¹. They are made up of α and β subunits alternatively arranged in a lateral and longitudinal manner. The lateral contacts involve the interactions of H1-S2 loop and helix H3 with the M-loop of the

adjacent protofilament. Thus, 13 protofilaments associate laterally and are found to be more electrostatic and less hydrophobic than the longitudinal contacts²⁻⁶. The alternative arrangement of α and β subunits results in longitudinal interactions which are classified into intra- and interdimer interfaces. The intradimeric interface is observed between β

and α subunits whereas the interdimeric interface is found between α and β subunits⁷⁻⁹. Further, the longitudinal contact involves the interaction of H8 of α tubulin with H11-H12, T5, T3 and γ -phosphate of the adjacent subunit. Similarly, T7 of α -tubulin shows interactions with phosphates T2, T1, H7 and Guanine¹⁰.

β -tubulin subunit comprises three distinct domains which include the N-terminal domain, intermediate domain and C-terminal domain. The N-terminal domain harbors the nucleotide and has 6 parallel beta strands (S1-S6) along with the same number of alpha helix (H1-H6). The intermediate domain has strands S7-S10 with three helices H8-H10 which accommodates taxol. The C-terminal is made up of two antiparallel helices H11-H12 which interacts with Microtubule Associated Protein (MAP)¹¹.

Even though the overall domain architecture remains the same in α - and β -tubulin, still they differentiate themselves both sequentially and structurally. Sequentially they share 40% residual identity, while structurally two positional gaps were observed in β -tubulin at H1-S2 loop (45-46) and S loop (361-368). The larger gap in β -tubulin gets well accommodated by taxol¹². Both the subunits get well associated with GTP; however, hydrolysis is restricted to β -subunit resulting in GTP formation at the exchangeable site. However, it gets sequestered in non-exchangeable site in α subunit¹³⁻²³.

Tubulin with its active role in cell division has been considered as a potential anticancer drug target²⁴. It comprises three distinct drug binding domains which include taxol, vinca-alkaloid and colchicine binding sites. Drugs associated with these sites were religiously involved in arresting the mitotic spindle formation²⁵. Further, taxol and colchicine share overlapping residual interactions towards the inner surface of the microtubules²⁶. Paclitaxel being the powerful drug for treating several solid tumors including breast, ovarian and non-small cell lung carcinomas²⁷ prefers to bind with M-loop proximal to S loop resulting

in stabilization of lateral interactions of two adjacent protofilaments²⁸. The important residues associated with taxol binding include V23, D226, H229, T276, R278, R369 and Gly370. These residues were scattered around H1-S2 loop, H7, M-loop and S-loop²⁰. Apart from paclitaxel, epothilones A and B, eleutherobin and discodermolide have also been reported to bind to the taxol binding site²⁹.

In spite of all these drug interactions, there is a report of drug resistance among these drugs. The main reason being cited is the residual substitutions associated with the drug resistance. Even though literature supports the correlation between the residual substitutions and drug resistance, but still there is a controversy over their role because of the inclusion of pseudogenes during drug resistance analysis³⁰. Thus, the debate on the role of residues on drug resistance remains elusive. The reported residual mutations in human β -tubulin include D26E, V60A, S172A, P173A, D197N, E198G, A231T, L240I, F270V, T274P, R282Q, Q292E, R306C, K350N, A364 and Y442C. Several types of resistance have been observed for the drugs like taxol, epothilone, hemiasterlin, 2-methoxyestradiol, vinca alkaloids and indancocine³¹⁻³⁷.

Previous studies report about drugs resistance and the reduced binding affinity of the available drugs, but still not much has been discussed about the drugs targeted against the mutant proteins. In this paper, we tried to target the available chemical compounds from higher plants, lower plants and seaweed secondary metabolites against wild and the mutants of human β -tubulin to investigate their level of interactions.

Materials and Methods

To begin with, human β -tubulin sequence was downloaded from SwissProt database (accession number: Q9BVA1.1)³⁸. With no reported crystal structure of human β -tubulin till date, a BLAST-PDB based³⁹ search was carried out to identify suitable templates. Out of the reported hits, 1JFF was downloaded from Protein Data Bank (www.rcsb.org)⁴⁰

which was further considered as a template (1JFF-B chain) for the modeling of the query sequence. Pairwise alignment of the template and the query sequence were generated using Modeller9v10⁴¹ (Figure 1). Using single protocol template from Modeller, 30 structures were generated. Of these generated structures, the model with the least DOPE score (Discrete Optimized Protein Energy) was considered for energy minimization with 100 iterations using steepest descent in SwissPdbViewer⁴². The template and the modelled structures were superimposed with each other (Figure 2A-C). The DOPE score of the template was -49714.55 and the modelled structure was -55870.25. The optimized structure was further validated using PROCHECK of SAVES server (<http://nihserver.mbi.ucla.edu/SAVES/>)⁴³. Similar methods were followed for the generation of mutants F270V, A364T and Q292E, respectively.

Conversely, chemical compounds needed for docking against wild and the mutants of human β -tubulin were obtained from marine flora. The chemical compounds were obtained from seaweed secondary metabolite database (www.swmd.co.in)⁴⁴. All these available compounds were isolated from red algae *Laurencia obtuse* (RL) and *Galaxaura marginata* (RG type). Some of these compounds were reported to be cytotoxic against cancer cell lines⁴⁵⁻⁴⁹. During our previous study, out of the 517 compounds, we could identify four lead compounds named RG012 (6 β , 24 ϵ -Dihydroxycholesta-4, 25-dien-3-one), RL381 (diterpenes containing bromine), RL366 (15-

1jffb	-REIVHIQAGQCGNQIGAKFWEVISEDHGDPTGSYHGSDQLQLERINVYYNEAAGNKYVPRAILV
humanbeta	MREIVHIQAGQCGNQIGAKFWEVISEDHGDPTGTYHGSDQLQLDRISVYYNEATGGKYVPRAILV
1jffb	DLEPGTMDSVRSRSGPFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESESCDCLQG
humanbeta	DLEPGTMDSVRSRSGPFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKEAESESCDCLQG
1jffb	FQLTHSLGGGTGSGMGTLIIISKIREEYPDRIMNTFSVVPSPKVSdTWEPEYNATLSVHQLVENTDE
humanbeta	FQLTHSLGGGTGSGMGTLIIISKIREEYPDRIMNTFSVVPSPKVSdTWEPEYNATLSVHQLVENTDE
1jffb	TYCIDNEALYDICFRTLKLTTPYGDNLNHLVSATMSGVTTCLRFPGQLNADLRKLAVNMPFPRRLH
humanbeta	TYCIDNEALYDICFRTLKLTTPYGDNLNHLVSATMSGVTTCLRFPGQLNADLRKLAVNMPFPRRLH
1jffb	FFMPGFAPLTSRGSQQYRALTVPELTQQMFDANKMMAACDPRHGRYLTVAAVFRGRMSMKEVDEQM
humanbeta	FFMPGFAPLTSRGSQQYRALTVPELTQQVFDANKMMAACDPRHGRYLTVAAVFRGRMSMKEVDEQM
1jffb	LNVQNKNSSYFVEWIPNNVKTAVCDIPPRGLKMSATFIGNSTAIQELFKRISEQFTAMFRRKAFLH
humanbeta	LNVQNKNSSYFVEWIPNNVKTAVCDIPPRGLKMAVTFIGNSTAIQELFKRISEQFTAMFRRKAFLH
1jffb	WYTGEGMDEMEFTEAESNMNDLVSEYQQYQD-----
humanbeta	WYTGEGMDEMEFTEAESNMNDLVSEYQQYQDATAEEEEEDFGEEAEEEA

Figure 1. Pairwise sequence alignment of template (1JFF-B chain) with the query sequence of human β -tubulin

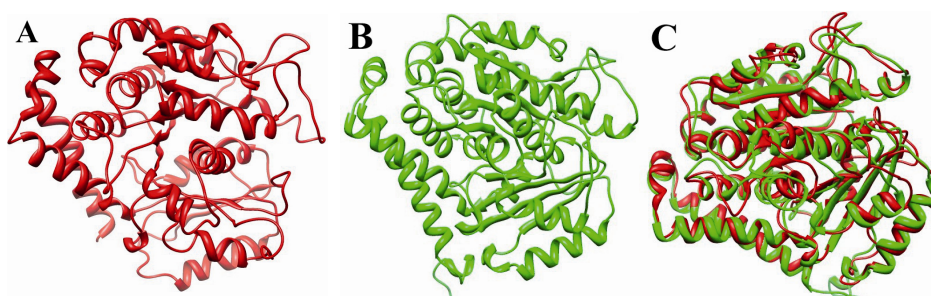


Figure 2. 3D structure of the template; A) the modelled structure of human β -tubulin; B) and its structural superimposition C)

epi-prostaglandin A2 diester), and RL376 (diterpenes containing bromine) which exhibit better binding to wild type human β -tubulin protein⁵⁰. All these selected compounds were taken into consideration for docking against the wild and mutant human β -tubulin proteins in the current study.

Next, through literature survey, we identified chemical compounds both from higher and lower plants. To begin with, the compounds from higher plants include Berberamine, Butulinic acid, Camptothecin, Cucurbitacin, Ellipticine, Flavopiridol, Homoharringtonine, Silvestrol, Berberine, Daphnoretin and Podophyllotoxin. Regarding their sources, Berberamine is extracted from *Berberis vulgaris* with a reported apoptosis in human myeloma cells^{51,52}. Betulinic acid is a pentacyclic triterpenoid with reported antiretroviral, anti-malarial and anti-inflammatory activity and anticancer properties extracted from the bark of *Betula pubescens*^{53,54}.

Camptothecin isolated from the bark and stem of *Camptotheca acuminata* is a cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase I⁵⁵. Cucurbitacins are from the family of Cucurbitaceae with anticancer and anti-inflammatory activities⁵⁶. Podophyllotoxin is a non-alkaloid toxin lignan extracted from the roots and rhizomes of *Podophyllum* species which is again an antitumor agent⁵⁷⁻⁵⁹. Silvestrol is isolated from *Aglaia foveolata* and results in apoptosis in the cell lines of hormone-dependent human prostate cancer⁶⁰. Homoharringtonine is extracted from *Cephalotaxus harringtonia* which is identified to be a cytotoxic alkaloid and is generally reported to block the progression of cells from G1 phase into S phase and G2 phase into M phase⁶¹. Flavopiridol is an indigenous plant from India, which can arrest cell cycle progression at the G1/S and G2/M boundaries⁶². Daphnoretin obtained from *Wikstroemia indica* exhibits strong antiviral and anti-tumor activities with a report of cell cycle arrest in the G2/M phase⁶³. Ellipticine is isolated from Apocyanaceae plants with established antitumor and anti-HIV activities

with their limited toxic side effects and their lack of hematological toxicity⁶⁴.

Regarding lower plants, Aclarubicin is produced by *Streptomyces galilaeus* actively used in the treatment of cancer⁶⁵. Daunorubicin and its derivative, doxorubicin are antitumor anthracycline antibiotics produced by *Streptomyces peucetius*⁶⁶. Blasticidin is a potent antifungal and cytotoxic peptidyl nucleoside antibiotic from *Streptomyces griseochromogenes* which plays a significant role in controlling prokaryotic and eukaryotic cell growth⁶⁷. Chartreusin is a potent antitumor agent with a mixed polyketide-carbohydrate structure produced by *Streptomyces chartreusis*⁶⁸. Neothramycin has been isolated from *Streptomyces* MC916-C4 which is a potent antitumor antibiotic of the pyrrolo (1, 4) benzodiazepine group^{69,70}. Pirarubicin is an anthracycline drug which has a diversified antitumor activity^{71,72}. All these chemical compounds were downloaded from Pubchem database. The chemical structures were obtained from chemical book (www.Chemical-book.com) (Table 1). The summary of the chemical compounds of lower and higher floras along with marine derivatives are tabulated in table 2.

All these selected compounds were charged using gasteiger charges available in CHIMERA software⁷³. Energy was minimized using PRODRG and hydrogen atoms were added to them. Then these compounds were considered for docking using iGEMDOCK software⁷⁴. The active sites of taxol (V23, D226, H229, T276, R278, R369 and Gly370) and epothilone (H227, A231, T274, R276, R282 and Q292)⁷⁵ were selected for docking (Figure 3). Next, only the residual mutants with deleterious effects were considered for homology modeling. This was identified using amino acid substitution (AAS) tools like Polyphen2 (polymorphism phenotyping)^{76,77}, PANTHER (protein analysis through evolutionary relationship)⁷⁸ and I-Mutant 2.0⁷⁹. The list of residual substitutions which bring in deleterious effects are tabulated (Table 3) (for detailed report refer)⁸⁰. Out of ten deleterious sites iden-

Table 1. Chemical structures of higher and lower plants along with marine compounds

Chemical compounds	Structure
Aclarubicin	
Blasticidin	
Chartreusin	
Daunorubicin	
Neothramycin	
Pirarubicin	
RL376	
RL366	
RL381	
RG012	
Berberine	
Butulinic acid	
Camptothecin	
Cucurbitacin	
Ellipticine	
Flavopiridol	
Homoharringtonine	
Silvestrol	
Berberine	
Daphnoretin	
Taxol	
Epothilone	
Podophyllotoxin	

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Table 2. Summary of chemical compounds of lower and higher floras along with marine derivatives

Chemical compounds	Molecular weight (g/mol)	xlogP3	H-bond donor	H-bond acceptor
Aclarubicin	811.86	3.8	4	16
Blasticidin	422.43	-5.2	6	7
Chartreusin	640.58	2.1	5	14
Daunorubicin	527.51	1.8	5	14
Neothramycin	262.26	0.2	2	5
Pirarubicin	627.63	2.7	5	13
RL376	418.52	3.1	1	7
RL366	695.81	8.6	2	7
RL381	478.58	2.6	2	9
RG012	414.62	4.6	2	5
Berbamine	608.72	6.1	1	8
Butulinic acid	456.70	8.94	2	3
Camptothecin	348.35	1	1	5
Cucurbitacin	514.65	2.7	4	7
Ellipticine	246.30	4.8	1	1
Flavopiridol	401.84	3.3	3	6
Homoharringtonine	545.62	0.8	2	10
Silvestrol	654.65	1.6	4	13
Berberine	336.36	3.6	0	4
Daphnoretin	352.29	3.3	1	7
Taxol	853.90	2.5	4	14
Epothilone	493.65	4	2	8
Podophyllotoxin	414.40	2	1	8

Table 3. Predicting the effect of residual substitutions through amino acid substitutions tools Polyphen2, PANTHER and I-Mutant2.0

Residual position	Polyphen2		PANTHER	I-Mutant 2.0
	Prediction score		Pdeleterious	(in kcal/mol)
V60A	0.88	Possibly damaging	0.81	-2.2
P173A	NA	NA	0.67	-1.9
D197N	0.99	Probably damaging	0.84	-0.37
L240I	0.95	Probably damaging	0.68	-1.12
F270V	0.9	Possibly damaging	0.5	-1.66
Q292E	0.99	Probably damaging	0.6	0.28
R306C	1	Probably damaging	0.9	0.1
K350N	0.99	Probably damaging	0.79	-0.75
A364T	0.88	Probably damaging	0.76	-1.3
Y422C	1	Probably damaging	0.92	1.54

tified, only three were selected as these substitutions were found to be proximal to the drug binding sites. Unfortunately, the rest of the positions were located at distal sites of both taxol and epothilone binding sites. Docking of the lower and higher plants along with marine isolates were carried out using iGEM

DOCK for taxol-wild, taxol-mutants (F270V, A364T), epothilone-wild and epothilone-mutant (Q292E). The docking study followed the accurate docking protocol which was very slow with population size of 800 and the number of solution equal to 10. The generation number was maintained at 80. Similar

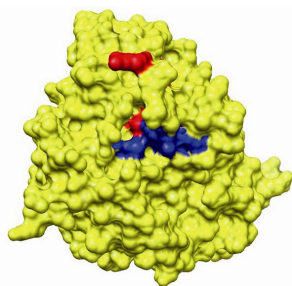


Figure 3. Drug binding sites in human β -tubulin for epothilone (Red) and taxol (Blue)

protocol was followed for taxol and epothilone binding pockets. Finally, we measured the size of the normal grooves for wild and the mutants of taxol and epothilone binding sites using SwissPdbViewer.

Results

In the present study, the chemical compounds obtained from different resources like lower plants, higher plants and marine isolates were docked against the wild and the mutant

human β -tubulin proteins. This study was mainly instigated to identify the level of interactions exhibited by both the wild and the mutants for taxol resistance (F270V, A364) and epothilone resistance (Q292). For this study, we considered the results of our previously reported lead molecules from seaweed secondary metabolites. Simultaneously, the chemical compounds from lower and higher plants were also taken into consideration.

First of all, the wild tubulin when docked with these compounds exhibited better binding to Aclarubicin. Next, the compounds from higher plants like Berbamine, Butulinic acid, Cucurbitacin, taxol and Podophyllotoxin exhibited better interactions in the wild type in the taxol binding site similar to the marine compounds. Next, the mutants were considered for docking with all the available compounds. Here, Camptothecin, Flavopiridol and Berberine exhibited better interactions in mutant1 (F270V). This site is proximal to M-

Table 4. Docking of chemical compounds from lower, higher and marine resources against taxol and epothilone binding sites in wild and mutant proteins

Origin	Compounds	Taxol binding site (binding energy in kcal/mol)			Epothilone binding site (binding energy in kcal/mol)	
		Wild	Mutant1 (F270V)	Mutant2 (A364T)	Wild	Mutant (Q292E)
Lower plants	Aclarubicin	-125.84	-121.20	-96.84	-69.66	-69.68
	Blasticidin	65.17	13.35	88.07	-33.98	72.76
	chartreusin	319.11	38.04	388.28	221.045	54.33
	Daunorubicin	207.93	-4.9	46.49	0.45	-11.00
	Neothramycin	-66.27	-66.98	69.74	-28.26	-31.36
	Pirarubicin	90.65	95.46	50.78	332.44	66.66
Marine compounds	RL376	-86.97	-83.51	-81.81	-45.38	-33.72
	RL366	-82.61	-79.19	-81.44	-30.86	-37.99
	RL381	-78.16	-75.38	-79.92	-28.33	-30.66
	RG012	-77.55	-76.07	-65.17	-38.40	-38.49
	Berbamine	-19.55	235.23	129.56	26.44	-33.49
	Butulinic acid	-79.00	-17.77	-15.62	-20.91	2.02
	Camptothecin	-82.97	-79.27	-86.75	-44.01	-43.85
	Cucurbitacin	-86.92	-84.52	-78.20	-24.33	-24.36
	Ellipticine	-66.21	-64.62	-75.40	-39.95	-15.65
	Flavopiridol	-67.46	-69.65	-74.93	-4.32	-39.29
Higher plants	Homoharringtonine	107.36	145.86	13.95	181.80	7.39
	Silvestrol	133.96	153.26	57.28	43.01	25.84
	Berberine	-75.72	-81.20	-82.74	-29.36	-36.08
	Daphnoretin	-80.37	-79.21	-82.75	-34.01	-37.03
	Taxol	-82.60	-79.20	-81.48	--	--
	Epothilone	--	--	--	-37.10	-21.84
	Podophyllotoxin	-83.00	-80.23	-75.71	-40.19	-38.18

loop and is also the taxol binding site. With respect to mutant2 (A364T), the chemical compounds like Camptothecin, Ellipticine, Flavopiridol, Berberine, Daphnoretin showed better chemical interactions. In epothilone binding site, lower plant compounds like Aclarubicin and Blasticidin showed better interaction in the wild type. The compounds like Neothramycin, Daunorubicin and Aclarubicin showed slightly better interaction with the mutation of glutamate with Glu at residual position 292. Thus, our overall study confirms that marine compounds exhibit better interaction in this mutant protein except RL376. Among higher plant products, Camptothecin, Ellipticine, Epothilone, Podophyllotoxin exhibit better interaction in the wild type of epothilone binding site. But compounds like Berbamine, Flavopiridol, Berberine and Daphnoretin conveyed better interactions again in the mutant (Table 4). General survey confirms a similar type of interactions exhibited both by wild and the mutant proteins. Thus, a closer inspection of the pocket was much needed for both sets of proteins. Therefore, we measured the groove size for wild, F270V and A364T of the taxol binding site. The volume was 550, 607 and 220 \AA^3 respectively. However, for epothilone binding pockets, the wild and the mutant (Q292E) maintained same volume of 8 \AA^3 . There was an increase in pocket volume observed for mutant1 in com-

parison with the wild type due to non-conservative residual substitution. Similarly, the volume of mutant2 decreases in comparison with the wild type in spite of conservative substitution. However, in epothilone binding site, the conservative substitution does not bring in any change in the volume both in wild and the mutant and the grooves were found to be discontinuous with the maximum volume size of 8 \AA^3 (Figure 4).

Discussion

During the docking of the wild and the mutants against the available compounds, only three mutants were generated which were proximal to the drug binding site, and were also reported to be lethal. Thus, the generated mutants were christened as mutant1 (F270V) mutant2 (A364T) for the taxol binding site. Similarly, mutant (Q292E) was selected for epothilone binding site. Docked structures were separately investigated for their interactions using CHIMERA software. We observed that Neothramycin exhibits interactions with the taxol binding site through P272 and R276 (Figure 5A). Similar interactions were exhibited by mutant1 (Figure 6A). Also mutant2 exhibited interaction with L361 (Figure 6B). Likewise, RL381 displayed interactions in the wild type of taxol binding site with H227, R282, and R359 (Figure 5B). Camptothecin shows interaction with T274 (Figure 5C)

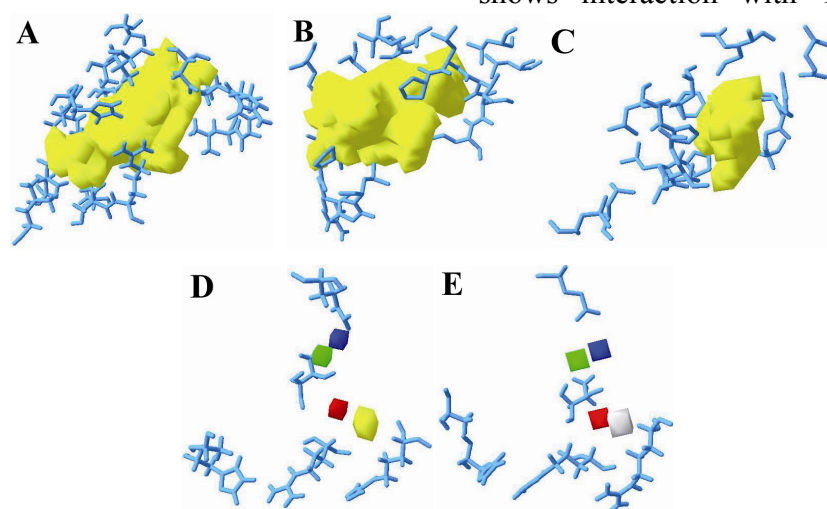


Figure 4. The groove of the active site pockets in wild and the mutant human β -tubulin; A) wild-taxol binding site; B) mutant1-taxol binding site; C) mutant2-taxol binding site; D) wild-epothilone binding site; E) mutant-epothilone binding site

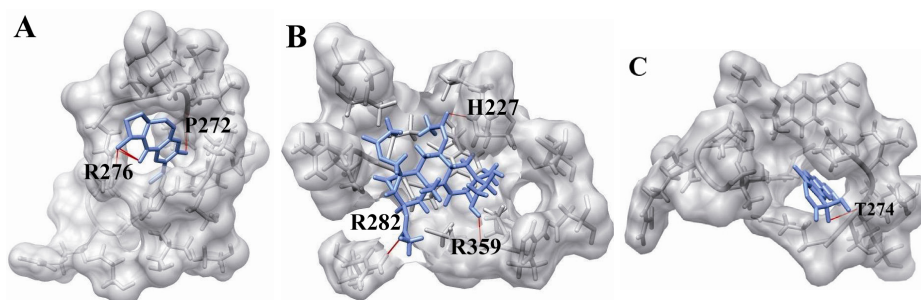


Figure 5. The docking of chemical compounds in the taxol binding sites of wild human β -tubulin; A) Neothramycin-taxol-wild; B) RL381-taxol-wild; C) Camptothecin-taxol-wild

while taxol based mutants, irrespective of their higher binding energy, do not display any interactions. Ellipticine also confirms no significant binding in mutant2. Flavopiridol does not show any significant interactions in the wild taxol binding site. But mutant1 shows interaction with H227 (Figure 6C). Also mutant2 shows interactions with T274 and A275 (Figure 6D). Berberine also shows no significant interaction in the wild type and mutant2 of taxol binding site but mutant1 confirms their interaction with R276 (Figure 6E). Again with Daphnoretin, both wild and mutant2 show no interactions, but mutant1 interacts with R276 (Figure 6F). Next, regarding the epothilone binding site, Neothramycin binds with mutant at T274 (Figure 7A). RL366 interacts in the mutant with R276

(Figure 7B). However, the rest of the compounds like RL381, Flavopiridol, Berberine and Daphnoretin exhibited no significant interactions. Since no remarkable change in the pattern of interactions both in wild and the mutant was observed, we considered the groove analysis for the taxol and the epothilone binding site. This study confirms an increase in the channel size for mutant1 and a decrease in the channel size for mutant2 in comparison with wild type. Moreover, epothilone binding site remains undisturbed with the residual substitution. We hypothesized that the drug resistance especially for taxol binding drugs could be related to the conformational changes in the active site pocket due to residual mutations.

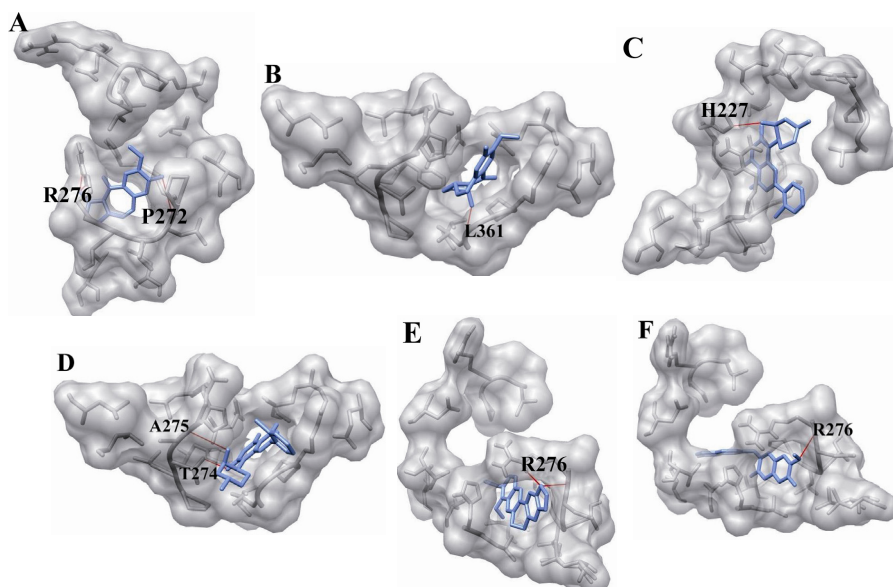


Figure 6. The docking of chemical compounds in the taxol binding sites of mutant human β -tubulin. A) Neothramycin-taxol-mutant1; B) Flavopiridol-taxol-mutant1; C) Berberine-taxol-mutant1; D) Daphnoretin-taxol-mutant1; E) Neothramycin-taxol-mutant2; F) Flavopiridol-taxol-mutant2

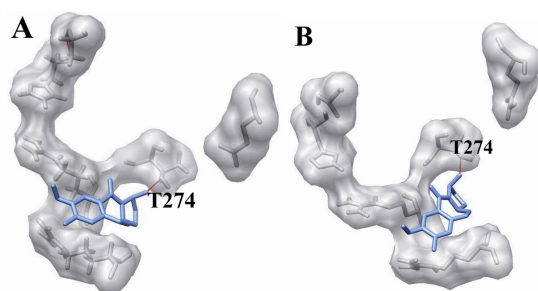


Figure 7. The docking of chemical compounds in the epothilone binding sites of mutant (Q292E) human β -tubulin. A) Neothramycin-epothilone-mutant; B) RL366-epothilone-mutant

Conclusion

The residual interaction analysis in wild and the mutants of taxol and epothilone binding sites reveals a better drug binding with the lower plants in the wild types of taxol and epothilone. However, the marine compound and the higher plants in spite of their increase in binding score could not establish a better contact with the residues of both the binding sites. Further, the chemical compounds showed interactions with the charged residues like arginine and histidine for better drug binding.

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