1. INTRODUCTION

Cancer has been one of the most serious diseases endangering human life and health.\(^1\) How to prevent and treat various malignant tumors effectively is a great challenge for science and medicine. Surgery, chemotherapy, and radiotherapy are the main means to treat cancers. Chemotherapy can be used to treat systemically and throughout the disease,\(^2,3\) at the same time, facing drug resistance problems and serious side effects. Therefore, it is necessary to study new drugs with strong anticancer cytotoxicity and low toxicity. An important target in this field is the tubulin system\(^4\) because the cells entering the dividing phase, that is, the M phase, and their mitotic process is frequent and the cell cycle is significantly shorter than normal cells. Therefore, if the above dynamic cycle is disrupted, the mitotic process of the tumor cells is affected, and the growth thereof is inhibited or the apoptosis of the tumor cells is induced. Tubulin and microtubules are important targets for anticancer drugs because of their important role in mitosis.

Tubulin is mainly composed of \(\alpha\) and \(\beta\)-tubulin. Both \(\alpha\)- and \(\beta\)-tubulin contain about 450 amino acid residues, have 42% homology in sequence, and can form heterodimer.\(^9\) As a target at \(\alpha\)- and \(\beta\)-tubulin, several drugs capable of disrupting the homeostasis of tubulin have been developed. According to different mechanisms, drugs targeting microtubulins can be divided into two categories: ones that promote microtubulin polymerization, belonging to taxol compounds,\(^10\) and others the mitotic phase will stop dividing and then apoptosis will be induced. Malignant tumor cells have rapid proliferative capacity, and their mitotic process is frequent and the cell cycle is significantly shorter than normal cells. Therefore, if the above dynamic cycle is disrupted, the mitotic process of the tumor cells is affected, and the growth thereof is inhibited or the apoptosis of the tumor cells is induced. Tubulin and microtubules are important targets for anticancer drugs because of their important role in mitosis.

Structural Basis and Mechanism for Vindoline Dimers Interacting with \(\alpha,\beta\)-Tubulin

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ABSTRACT: Vinblastine and its derivatives used in clinics as antitumor drugs often cause drug resistance and some serious side effects; thus, it is necessary to study new vinblastine analogues with strong anticancer cytotoxicity and low toxicity. We designed a dimer molecule using two vindoline-bonded dimer vindoline (DVB) and studied its interaction with \(\alpha,\beta\)-tubulin through the double-sided adhesive mechanism to explore its anticancer cytotoxicity. In our work, DVB was docked into the interface between \(\alpha\)-tubulin and \(\beta\)-tubulin to construct a complex protein structure, and then it was simulated for 100 ns using the molecular dynamics system to become a stable and refined complex protein structure. Based on such a refined structure, the quantum chemistry at the level of the MP2/6-31G(d,p) method was used to calculate the binding energies for DVB interacting with respective residues. By the obtained binding energies, the active site residues for interaction with DVB were found. Up to 20 active sites of residues within \(\alpha,\beta\)-tubulin interacting with DVB are labeled in \(\beta\)-Asp179, \(\beta\)-Glu207, \(\beta\)-Tyr210, \(\beta\)-Asp211, \(\beta\)-Phe214, \(\beta\)-Pro222, \(\beta\)-Tyr224, and \(\beta\)-Leu227 and \(\alpha\)-Asn249, \(\alpha\)-Arg308, \(\alpha\)-Lys326, \(\alpha\)-Asn329, \(\alpha\)-Ala333, \(\alpha\)-Thr334, \(\alpha\)-Lys336, \(\alpha\)-Arg339, \(\alpha\)-Ser340, \(\alpha\)-Thr349, and \(\alpha\)-Pro222. The total binding energy between DVB and \(\alpha,\beta\)-tubulin is about \(-251.0\) \(\text{kJ mol}^{-1}\). The sampling average force potential (PMF) method was further used to study the dissociation free energy (\(\Delta G\)) along the separation trajectory of \(\alpha,\beta\)-tubulin under the presence of DVB based on the refined structure of DVB with \(\alpha,\beta\)-tubulin. Because of the presence of DVB within the interface between \(\alpha\)- and \(\beta\)-tubulin, \(\Delta G\) is 252.3 \(\text{kJ mol}^{-1}\). In contrast to the absence of DVB, the separation of pure \(\beta\)-tubulin needs a free energy of 196.9 \(\text{kJ mol}^{-1}\). The data show that the presence of DVB adds more \(35.4\) \(\text{kJ mol}^{-1}\) of \(\Delta G\) to hinder the normal separation of \(\alpha,\beta\)-tubulin. Compared to vinblastine existing, the free energy required for the separation of \(\alpha,\beta\)-tubulin is 220.5 \(\text{kJ mol}^{-1}\). Vinblastine and DVB can both be considered through the same double-sided adhesive mechanism to give anticancer cytotoxicity. Because of the presence of DVB, a larger free energy is needed for the separation of \(\alpha,\beta\)-tubulin, which suggests that DVB should have stronger anticancer cytotoxicity than vinblastine and shows that DVB has a broad application prospect.
that inhibit microtubulin polymerization, belonging to colchicine compounds and vinblastine compounds.12,13 Vinblastine was isolated from the periwinkle. Because its antitumor activity had been verified, a variety of alkaloids have been isolated from the periwinkle, and artificial semisynthetic analogues have also been developed. Among them, vinblastine (VLB),14 vincristine (VCR),13 vindesin (VDS),16 and vinorelbine (NVB)17 have good antitumor activity and are formally applied in clinical practice.

Vinblastine and its derivatives as anticancer drugs have the same binding sites to get activity against cancer cells by double-sides sticking mechanism interacting with α-β-tubulin.19 The combination of vinblastine with tubulin inhibits the dynamics of microtubule polymerization, resulting in the inability to form a spindle and termination of cells in the metaphase.19 However, after long-term use of vinblastine as an anticancer drug, patients are prone to drug resistance and side effects such as leukopenia, bone marrow suppression, and neurotoxicity to varying degrees. Therefore, new vinblastine derivatives with high anticancer cytotoxicity need to be discovered and developed.

At present, most studies on vinblastine derivatives focus on the modification of the vinca alkaloid ring.20,21 One of the results was that vinflunine was obtained by modifying the vinca alkaloid ring.24 It is generally believed that the affinity of vinblastine using the vindoline ring is stronger than that using the vinca alkaloid ring.25 Therefore, the vindoline ring of vinblastine has a relatively few structural modifications. We hypothesized that if the vinca alkaloid ring is replaced by the vindoline ring, it should be a very ideal structural modification, which can evenly act as a real double-sided adhesive mechanism, capable of symmetry interactions with α- and β-tubulin, giving rise to a high activity of anticancer cytotoxicity. Therefore, we design a dimer molecule from double vindoline-bonded DVB shown in Figure 1.

![Figure 1. Molecular structure of DVB bonded by a C–C bond.](image)

We first optimized the structure of DVB using quantum chemistry and then docked it into the α-β-tubulin interface to construct a complex structure. Using molecular dynamics technology, the complex structure was optimized to obtain a stable and refined structure. Based on the fine structure, we not only studied the active site residues forming an active pocket for interaction with DVB but also investigated the free energy along the separation trajectory of α-β-tubulin under the presence of DVB. We explored the mechanism for DVB interacting with α-β-tubulin to get its antitumor cell activity for the development and application of DVB.

2. RESULTS AND DISCUSSION

2.1. Results of Docking DVB to α-β-Tubulin. The DVB molecular structure is formed through the C–C bond at the same position’s carbons in the benzene ring of the vindoline ring. The DVB structure was optimized at the level of MP2/6-31G(d,p) to get its minimum structure without negative frequency shown in Figures 1 and 12. Figure 2, formed by Chimera program,24,25 shows the complex structure docked for DVB with α-β-tubulin. The docking energy between DVB and α-β-tubulin is −124.7 kJ mol⁻¹, composed of −103.8 kJ mol⁻¹ from van der Waals interaction and −20.8 kJ mol⁻¹ from electrostatic interaction. In this figure, the small blue molecule is DVB near guanosine 5'-diphosphate (GDP) which is shown in red.

2.2. Molecular Dynamics Simulation for the DVB-α,β-Tubulin System. As usual, to characterize the stability using root-mean-square deviation (rmsd),26–28 the values of rmsd for the total system and each components are shown in Figure 3. In the total system, the rmsd of α,β-tubulin is divided into two stages. In the first stage, a quick increment of rmsd values takes place between 0 and 14 ns to arrive at a value of 0.33 nm. During the period of 14–100 ns, rmsd is fluctuating between 0.286 and 0.364 nm with a range of 0.081 nm at the center around 0.320 nm, which indicates the structure of α,β-tubulin to be a stable equilibrium state after 14 ns. The rmsd value of DVB quickly reached to about 0.320 nm and fluctuated at the center of 0.320 nm. Because the component of water is relatively stable, their rmsd values remained stable with a smooth line after 2 ns. The DVB-α,β-tubulin-H₂O total system affected by the component of water reached a stable state after 3.8 ns, and its rmsd value was fluctuating slightly around 6.617 nm. After 100 ns of MD simulation, all the results of rmsd showed the total system and respective components to be stable.

2.3. Active Site Residues for Binding with DVB within α,β-Tubulin. We determine the active site residues using the binding energy (E_b) between DVB and residues59 from the system obtained after performing MD simulation for 100 ns with an additional energy minimization. The MP2 method with the 6-31G(d,p) basis was used to calculate E_b. The overlapping error of basis was corrected by the Boys and Bernardi method. Table 1 shows the values of E_b for DVB interacting with the residues within a sphere of 6 Å radius around DVB. In Table 1, E_b(BSSE) includes the correction of basis overlap error (BSSE),30,31 and E_b is the values without correcting basis overlap error. Similar to the previous studies,32–34 the active site residues are defined according to the value of E_b(BSSE) less than −4.0 kJ mol⁻¹.

| Residue | Interaction | E_b (kJ mol⁻¹) | E_b(BSSE) (kJ mol⁻¹) |
|---------|-------------|---------------|---------------------|
| Asp179  |             |               |                     |
| Glu207  |             |               |                     |
| Tyr210  |             |               |                     |
| Asp211  |             |               |                     |
| Phe214  |             |               |                     |

Figure 2. Complex structure of DVB docked with α-β-tubulin.
Interacted by the active site residues. The more specific residues interacting with DVB are shown in Figure 4. Using DVB as a Center, the relative position image for DVB compared to the all active residues is shown in Figure 4.

Table 1. Binding Energy ($E_b$) (kJ·mol$^{-1}$) of Residues within αβ-Tubulin with DVB in a Sphere Formed by a Radius of 0.6 nm

| Residue | $E_b$(BSSE) | $E_b$ | BSSE | Residue | $E_b$(BSSE) | $E_b$ | BSSE |
|---------|-------------|-------|------|---------|-------------|-------|------|
| β-Gln11 | 0.17        | 0.17  | 0.00 | α-Leu317 | −0.35       | −0.35 | 0.00 |
| β-Cys12 | −0.70       | −0.70 | 0.00 | α-Tyr319 | −0.40       | −0.40 | 0.00 |
| β-Ser174| −1.05       | −1.05 | 0.00 | α-Pro325 | 0.09        | 0.09  | 0.00 |
| β-Pro175| −0.82       | −0.83 | 0.00 | α-Lys326 | −4.73       | −5.03 | 0.30 |
| β-Lys176| 2.99        | 2.01  | 0.98 | α-Val328 | −1.18       | −1.20 | 0.02 |
| β-Val177| −3.49       | −14.38| 10.89| α-Asn329 | −28.00      | −51.37| 23.37|
| β-Ser178| −2.62       | −8.99 | 6.37 | α-Ala330 | −3.24       | −10.71| 7.47 |
| β-Asp179| −26.51      | −41.68| 15.16| α-Ala331 | −0.40       | −0.42 | 0.02 |
| β-Thr180| −0.58       | −0.61 | 0.02 | α-Ile332 | 4.17        | 1.36  | 2.82 |
| β-Asn206| −0.57       | −0.58 | 0.01 | α-Ala333 | −8.14       | −30.34| 22.20|
| β-Glu207| −14.06      | −14.06| 0.00 | α-Thr334 | −4.11       | −6.05 | 1.94 |
| β-Leu209| −0.89       | −0.89 | 0.00 | α-Ile335 | −0.94       | −0.95 | 0.00 |
| β-Tyr210| −18.12      | −42.60| 24.49| α-Lys336 | −31.18      | −46.65| 15.47|
| β-Asp211| −19.58      | −19.58| 0.01 | α-Thr337 | −3.52       | −20.53| 17.01|
| β-Cys213| 0.03        | 0.03  | 0.00 | α-Lys338 | −13.03      | −13.04| 0.01 |
| β-Phe214| −7.56       | −14.84| 7.28 | α-Arg339 | −8.92       | −8.92 | 0.00 |
| β-Thr220| −1.93       | −2.15 | 0.22 | α-Ser340 | −5.53       | −15.18| 9.66 |
| β-Thr221| −3.62       | −6.83 | 3.21 | α-Ile341 | −2.29       | −2.36 | 0.07 |
| β-Pro222| −6.57       | −20.79| 14.23| α-Phe342 | −1.27       | −1.27 | 0.00 |
| β-Thr223| 0.19        | −4.52 | 4.71 | α-Thr349 | −4.59       | −7.80 | 3.21 |
| β-Tyr224| −8.44       | −20.03| 11.59| α-Gly350 | −0.09       | −0.09 | 0.00 |
| β-Gly225| −0.36       | −0.36 | 0.00 | α-Phe351 | −9.18       | −15.80| 6.63 |
| β-Leu227| −4.97       | −10.69| 5.72 | α-Val353 | 9.86        | 5.80  | 4.07 |
| α-Asn249| −4.71       | −4.90 | 0.20 | α-Ile355 | −0.26       | −0.26 | 0.00 |
| α-Arg308| −10.02      | −10.02| 0.00 | sum      | −250.98     | −470.34| 219.36|

β-Pro222, β-Tyr224, β-Leu227, and from α-tubulin, α-Asn249, α-Arg308, α-Lys326, α-Asn329, α-Ala333, α-Thr334, α-Lys336, α-Lys338, α-Arg339, α-Ser340, α-Thr349, and α-Phe351. The total $E_b$(BSSE) is −250.98 kJ·mol$^{-1}$, which shows a strong interaction between DVB and αβ-tubulin. The relative position image for DVB compared to the all active residues is shown in Figure 4.

From Figure 4, we can recognize all sides of DVB to be interacted by the active site residues. The more specific detailed active residues interacting with DVB are shown in Figure 5. β-Glu207 is a negatively charged amino acid to have an electrostatic interaction of −14.06 kJ·mol$^{-1}$ from the carbonyl carbon of −COOCH$_3$ in DVB interacting with the methyl carbon in −OCH$_3$ by the distances of 0.4420 and 0.4721 nm. β-Leu227 methyl carbon has a weak electrostatic interaction of −4.97 kJ·mol$^{-1}$ with nitrogen of C$_4$NH (5-membered ring) due to a distance of 0.3859 nm. The positively charged α-Lys326 has an electrostatic interaction of −4.73 kJ·mol$^{-1}$ with the nitrogen of C$_4$NH (5-membered ring) because of the distance of 0.3360 nm. The carbonyl carbon of α-Asn249 interacts with two O atoms in the −OCOCH$_3$ group from DVB shown by the distances of 0.2771 and 0.4300 nm.
respectively. The N atom of $\alpha$-Asn249 has an electrostatic interaction of $-4.71$ kJ·mol$^{-1}$ with the carbonyl carbon of $\text{COOCH}_3$ because of the distance of 0.4210 nm. $\alpha$-Thr334 has a weak hydrogen bonding, with the O atom of DVB responsible for a binding energy of 4.11 kJ·mol$^{-1}$ because of the distance of 0.2154 nm. The positively charged $\alpha$-Arg308 also has an electrostatic interaction of $-10.02$ kJ·mol$^{-1}$ with the carbonyl oxygen of $\text{COOCH}_3$ because of the distances of 0.4504 and 0.2381 nm, respectively. The near distance of 0.2512 nm between $\beta$-Phe214 and DVB generates a binding energy of $-7.56$ kJ·mol$^{-1}$. The positively charged $\alpha$-Lys338 has an electrostatic interaction of $-13.03$ kJ·mol$^{-1}$ with the carbonyl oxygen of $\text{OCOCH}_3$ from DVB because of the distance of 0.1534 nm. Similarly, $\alpha$-Thr349 has a hydrogen bonding with the carbonyl oxygen of $\text{OCOCH}_3$ in DVB to have an energy of $-4.59$ kJ·mol$^{-1}$ because of the distance of 0.2778 nm. The phenolic OH from $\beta$-Tyr210 and $\beta$-Tyr224 forms two hydrogen bonds with the nitrogen of C$_5$N (6-member ring) to have the binding energies of $-18.12$ and $-8.44$ kJ·mol$^{-1}$ because of the distances of 0.1695 and 0.2643 nm, respectively. $\alpha$-Ala333 has a weak hydrogen bonding of $-8.14$ kJ·mol$^{-1}$ with the carbonyl O in the $\text{COOCH}_3$ group of DVB because of the distance of 0.2432 nm.

In addition, $\alpha$-Lys336 has a strong electrostatic interaction of $-31.18$ kJ·mol$^{-1}$ with the carbonyl O of $\text{COOCH}_3$ because of the distances of 0.4550 and 0.4655 nm. C-Arg339 also has an electrostatic interaction of $-8.92$ kJ·mol$^{-1}$ with the carbonyl O of $\text{OCOCH}_3$ because of the distance of 0.4418 nm. $\alpha$-Asn329 interacts with two O atoms of $\text{OCOCH}_3$ to give a binding energy of $-28.00$ kJ·mol$^{-1}$ because of the distances of 0.4275 and 0.2256 nm. $\beta$-Pro222 has an electrostatic interaction of $-6.57$ kJ·mol$^{-1}$ with oxygen of

**Figure 4.** Relative positions between DVB and the active site residue located in the sphere with the radius of 0.6 nm around DVB, B means the B chain ($\beta$-tubulin), C means the C chain ($\alpha$-tubulin) originating from the crystal structure of protein code number: 1Z2B.

**Figure 5.** Structures of the more specific detailed active site residues interacting with DVB, the distances labeled in a unit of 0.1 nm, B instead of $\beta$, C instead of $\alpha$. (a) C-Asn249, C-Arg308, C-Lys326, B-Glu207, and B-Leu227; (b) C-Ser340, C-Thr349, B-Tyr210, B-Phe214, and B-Tyr224; (c) C-Lys336, C-Lys338, C-Ala333, C-Thr334, and C-Arg339; (d) C-Phe351, B-Asp179, C-Asn329, B-Asp211, and B-Pro222. B means the B chain ($\beta$-tubulin) and C means the C chain ($\alpha$-tubulin) originating from the crystal structure of protein code number: 1Z2B.
2.4. Free Energy of αβ-Tubulin Moving along Its Separation Trajectory. 2.4.1. Free Energy of Pure αβ-Tubulin Separation. The free energy of pure αβ-tubulin separation was reported. However, for the sake of better understanding in comparison to the presence of DVB, we briefly introduce the free energy for pure αβ-tubulin moving along its separation trajectory.

On the basis of the optimized pure αβ-tubulin structure, α-tubulin was set as the reference group, β-tubulin was set as the moving group, and GDP was set as a free group. An external force of no more than 2000 kJ/mol was applied to the x+ axis, thus to push β-tubulin moving along the x+ direction. In the actual process, the program provided an appropriate external force less than 2000 kJ/mol to make β-tubulin move and generate its separation trajectory. The configurations of four points along the trajectory are shown in Figure 6, where (o) is the starting point of the system, passing through the trajectory systems of points (a) and (b), and reaching the trajectory system of point (c), which shows that β-tubulin leaves from α-tubulin, while GDP is moving with β-tubulin.

The separation trajectory contained 400 sampling configurations, from which, 81 umbrella sampling configurations were selected and are listed in Table 2. The sampling system was rebalanced under the condition of a fixed distance between the moving and reference groups after performing MD simulation for 800 ps, during which the values of rmsd were used to characterize the equilibrium of the sampling system (data not given). The values of rmsd for the respective sampling systems were fluctuating within a small range of 0.03 nm to indicate the sampling systems to be rebalanced after 400,000 steps (800 ps) of MD simulation.

Then, the weighted histogram analysis method (WHAM) from the Gromacs 4.5 program was used to convert the biased sampling into the unbiased statistical results and obtain the free energy shown in Figure 7. For the sake of simplicity, only key four configurations for β-tubulin moving along its separation trajectory are given in Figure 6. Considering the distance and sampling number together listed in Table 2, the position of β-tubulin along the protein system can be determined to get the free-energy variety of β-tubulin along the separation trajectory. The β-tubulin trajectory starts from the starting point (o) (3.97, 0.00) corresponding to sampling 1, then point (a) (5.01, 142.54) corresponding to sampling 147 and (b) (6.43, 194.82), corresponding to sampling 260, and reaches point (c) (7.92, 196.85) corresponding to sampling 400. The free energy of β-tubulin along its trajectory goes up to 196.85 kJ/mol, as shown in Figure 7.

2.4.2. Free Energy of αβ-Tubulin with DVB along the Separation Trajectory. Using the optimized DVB-αβ-tubulin structure, the separation trajectory of β-tubulin was recorded, during which β-tubulin was set as the moving group and the reference group was α-tubulin, while DVB and GDP were set free. Four key configurations along the trajectory are shown in Figure 8. Point (o) is the starting system, after passing through points (a,b) and arriving at point (c), to show β-tubulin leaving away from α-tubulin. DVB was following α-tubulin, while GDP was moving with β-tubulin. Ninety-five sampling systems were selected from the file of trajectory coordinates recorded for β-tubulin to separate and are listed in Table 3. All the selected sampling systems were re-balanced after 800 ps of MD simulation under the same conditions and characterized with rmsd fluctuating within a small range of 0.03 nm.

Similarly, WHAM was used to convert the biased sampling into the unbiased statistical results and obtain the free energy shown in Figure 9. In combination with the separation trajectory of αβ-tubulin shown in Figure 8, point (o) is the initial state corresponding to sampling 1 with a distance of 3.98 nm between α- and β-tubulin. After point (a) (4.89, 162.23) corresponding to sampling 131 and point (b) (6.46, 249.00) corresponding to sampling 257, the system reaches point (c) (7.95, 250.10) corresponding to sampling 398. Along the trajectory of αβ-tubulin separation, the free-energy variation (ΔG) reaches 252.27 kJ/mol under the presence of DVB in Figure 9.

In Figure 10, the weighted histograms of umbrella sampling are presented. The left panel is for the separation trajectory of pure αβ-tubulin, and the right panel is for the separation trajectory of αβ-tubulin under the presence of DVB. Both weighted histograms of umbrella sampling show good overlap to indicate that the calculated free energy is of high reliability for αβ-tubulin separated along the trajectories.

The interaction between DVB and αβ-tubulin, calculated by the quantum chemistry at the level of MP2/6-31G(d,p), is −250.98 kJ/mol including the basis overlap error. If no basis overlap error is included, the interaction is up to −470.34 kJ/mol. Because the calculation is performed by the quantum chemistry, this interaction naturally includes the hydrogen bonding and molecular interactions. However, our work did not apply the quantum theory of atoms in molecules (QTAIM) to calculate the independent hydrogen bonding energies between DVB and respective residues, although a lot
of very interesting work in relation to QTAIM were published. Furthermore, the interaction of \(-250.98 \text{ kJ}\cdot\text{mol}^{-1}\) is very consistent with the separated free energy of \(-252.27 \text{ kJ}\cdot\text{mol}^{-1}\) between \(\alpha\) and \(\beta\)-tubulin at the presence of DVB molecule. This result shows that the calculated interaction between DVB and \(\alpha\beta\)-tubulin is reliable.

The free energy above along the trajectories of \(\alpha\beta\)-tubulin separation was given under the presence and absence of DVB. Because DVB is a vindoline dimer, its active sites are believed to be the same as those of vinblastine because the same active sites are located within the interface of \(\alpha\) and \(\beta\)-tubulin. It is known that the essence of vinblastine for the treatment of various cancers comes from its interaction with the active site residues within the interface of \(\alpha\) and \(\beta\)-tubulin. Other factors have a little effect. For reducing deviation like analytical chemistry, a relatively pure sample is needed for obtaining the reliable results. Otherwise, the results may be affected by some uncontrolled factors. Therefore, we chose an interface system that is only associated with \(\beta\)- and \(\alpha\)-tubulin, which is directly related to DVB. Other structures, which are not clearly associated with this interface, are not included in our research system. In order to study the effect of DVB on the interface of \(\beta\) and \(\alpha\)-tubulin, we specifically gave the separation from pure \(\beta\) and \(\alpha\)-tubulin in the absence of DVB. This investigation is like a blank experiment. The effects of all backgrounds are included in the blank experiment. Under the same experimental conditions, when DVB is present at the interface of \(\beta\) and \(\alpha\)-tubulin, after considering the blank experiment, the results of DVB interacting with \(\alpha\) and \(\beta\)-tubulin are readily available. Therefore, we can obtain the significant results about the separation trajectory and free energy of \(\alpha\beta\)-tubulin under the presence of DVB compared with its absence. Furthermore, many experiments have proved vinblastine and its derivatives to have strong anticancer cytotoxicity, and then, vinblastine and its derivatives are currently still used as antitumor drugs in clinics. Based on such experimental and clinical basis from the vinblastine practice, the same method is used to study the double-sided adhesive mechanism within \(\alpha\beta\)-tubulin for the vindoline dimer compared with vinblastine to get anticancer cytotoxicity; we can believe that the obtained result about DVB to get better anticancer cytotoxicity also possesses the experimental basis.

### 3. CONCLUSIONS

We dock DVB to the \(\alpha\beta\)-tubulin interface to form the DVB-\(\alpha\beta\)-tubulin system and get a docking energy of \(-124.7 \text{ kJ}\cdot\text{mol}^{-1}\). After 100 ns of MD simulation, the stable and refined structure of DVB-\(\alpha\beta\)-tubulin was obtained. Based on such a structure, we obtain as many as 20 active site residues within \(\alpha\beta\)-tubulin interacting with DVB, which are \(\beta\)-Asp179, \(\beta\)-Asp179, etc.
Glu207, β-Tyr210, β-Asp211, β-Pro222, β-Tyr224, and β-Leu227 and α-Leu228, α-Asn249, α-Arg308, α-Lys326, α-Asn329, α-Ala333, α-Thr334, α-Lys336, α-Lys338, α-Arg339, α-Ser340, α-Thr349, and α-Phe351. The binding energy of DVB with α, β-tubulin is up to $-251.0 \text{ kJ} \cdot \text{mol}^{-1}$. The free energy required for the dissociation of pure α, β-tubulin is $196.85 \text{ kJ} \cdot \text{mol}^{-1}$. Under the presence of DVB, the dissociation of α, β-tubulin requires $252.27 \text{ kJ} \cdot \text{mol}^{-1}$ ($\Delta G$). The data show that DVB increases the free energy required for the separation of α, β-tubulin through the double-sided adhesive mechanism, which disrupts the normal dynamics between polymerization and depolymerization of α, β-tubulin. Compared with vinblastine, the free energy required for the separation of α, β-tubulin is going up $220.5 \text{ kJ} \cdot \text{mol}^{-1}$. Vinblastine and DVB can be considered to acquire anticancer cytotoxicity using the same double-sided adhesive mechanism. In the presence of DVB, larger free energy for the separation of α, β-tubulin is required, which suggests that DVB would possess a stronger anticancer cytotoxicity than vinblastine, thus theoretically to imply that

Table 3. Umbrella Sampling Selected from the System of α, β-Tubulin Moving along Its Separation Trajectory under the Presence of DVB

| $N$ | $D$ (nm) | $N$ | $D$ (nm) | $N$ | $D$ (nm) | $N$ | $D$ (nm) |
|-----|----------|-----|----------|-----|----------|-----|----------|
| 13  | 4.47     | 161 | 5.20     | 219 | 5.90     | 268 | 6.56     |
| 32  | 4.50     | 164 | 5.24     | 221 | 5.93     | 272 | 6.60     |
| 49  | 4.54     | 170 | 5.28     | 223 | 5.97     | 275 | 6.64     |
| 59  | 4.58     | 173 | 5.32     | 226 | 6.01     | 277 | 6.69     |
| 84  | 4.62     | 175 | 5.35     | 229 | 6.04     | 280 | 6.74     |
| 91  | 4.66     | 178 | 5.39     | 231 | 6.08     | 283 | 6.78     |
| 102 | 4.70     | 180 | 5.42     | 233 | 6.11     | 286 | 6.82     |
| 111 | 4.74     | 182 | 5.45     | 236 | 6.15     | 290 | 6.86     |
| 121 | 4.79     | 184 | 5.49     | 238 | 6.18     | 292 | 6.89     |
| 124 | 4.82     | 186 | 5.53     | 242 | 6.22     | 295 | 6.92     |
| 128 | 4.85     | 189 | 5.57     | 244 | 6.25     | 297 | 6.95     |
| 131 | 4.89     | 193 | 5.62     | 245 | 6.28     | 299 | 6.98     |
| 135 | 4.93     | 197 | 5.65     | 248 | 6.32     | 302 | 7.02     |
| 138 | 4.96     | 198 | 5.68     | 249 | 6.35     | 306 | 7.05     |
| 143 | 5.00     | 200 | 5.72     | 251 | 6.39     | 309 | 7.10     |
| 146 | 5.04     | 206 | 5.76     | 254 | 6.42     | 316 | 7.14     |
| 148 | 5.08     | 210 | 5.80     | 257 | 6.45     | 322 | 7.18     |
| 152 | 5.12     | 216 | 5.84     | 259 | 6.49     | 326 | 7.22     |
| 157 | 5.16     | 217 | 5.87     | 263 | 6.52     | 328 | 7.26     |

*N*: sampling number, $D$: the distance between two groups.

Figure 9. Free energy variety of α, β-tubulin along its separation trajectory under the presence of DVB.

Figure 10. Weight histograms of umbrella sampling for pure α, β-tubulin along its separation trajectory (left panel) and for α, β-tubulin along its separation trajectory under the presence of DVB (right panel).
DVB has a broad application and a clear development prospect.

4. MATERIALS AND METHODS

The basic material of this paper is \( \alpha \beta \)-tubulin from the protein crystal structure (CN: 1Z2B).\(^{40}\) In order to eliminate the influence of the external structure for reducing errors and highlight the essence of interaction between DVB and \( \alpha \beta \)-tubulin, we just extract the B chain (\( \beta \)-tubulin) and C chain (\( \alpha \)-tubulin) from the crystal structure (1Z2B). In Figure 11, the extracted structure of \( \alpha \beta \)-tubulin contains a GDP molecule upon the surface of the \( \beta \)-tubulin structure. In the end of \( \alpha \beta \)-tubulin, some residues are missed; thus, the \( \beta \)-tubulin structure (B chain) contains 427 residues and 1 GDP molecule and the \( \alpha \)-tubulin structure (C chain) contains 436 residues. The molecular structure of DVB (Figure 12) labeled in atomic numbers is optimized using the MP2/6-31G(d,p) method from the Gaussian 03 program.\(^{41}\) The optimized structure of DVB is determined without imaginary vibration frequency calculated at the same method.

4.1. DVB Docked with \( \alpha \beta \)-Tubulin. To prepare the docking experiment, Sybyl 7.2 program was used to give the correct atomic type of DVB,\(^ {42}\) add H atoms and Gasteiger—Hückel charge for DVB, and save it as a file of MOL2 format. Meanwhile, H atoms and the charge of Amber77 FF99 type were added to \( \alpha \beta \)-tubulin using the Sybyl 7.2 program. The newly added H atoms need to be optimized. The Powell gradient method was used as an optimization method, and the convergence standard was set as 0.04 \( \text{kJ·mol}^{-1} \), and the maximum number of steps was set as 10 000 with Tripos force field applied and current charge used.\(^ {43}\) It is the first step using the Dock6 program to construct the Sphgen and then to select the stable DVB conformation obtained by \( \alpha \beta \)-tubulin-H\(_2\)O system containing 863 residues, a DVB, a GDP, 24 463 H\(_2\)O, and 61 Na\(^+\). The force field parameters of DVB were set according to gmx field format on its optimized structure.\(^ {49−51}\) Some basic data were generated by the PRODRG program.\(^ {52}\) The atomic charges of DVB were obtained from the calculation by MP2/6-31G(d,p).

To eliminate possible particle overlap, the energy minimization simulation (em step) using the Steep algorithm was performed for the total system at the periodic boundary condition. The convergence standard was set as 500.0 \( \text{kJ·mol}^{-1} \)·\( \text{nm}^{-1} \), and the step length was given at 2 fs. The atomic electrostatic interactions were calculated by the particle-mesh-ewald (PME) method,\(^ {3,54}\) while the Coulomb threshold (rcoulomb) was set as 1.0 nm and the van der Waals threshold (rvdw) was set as 1.0 nm too. When the system temperature was increased to 310 K,\(^ {55,56}\) the system temperature balance (nvt step) was performed for 100 ps with a step length of 2 fs at the conditions of \( V \)-rescale used for temperature coupling and the coupling time constant set to 0.1 ps.\(^ {57}\)

Then, the pressure balance simulation (npt step) was carried out for 3 ns using the Parrinello—Rahman method at the conditions of 10 ps coupling time constant and a step length of 2 fs. The temperature coupling was Nose—Hoover to increase the pressure to 1 atm. After balancing the system pressure, MD simulation of 100 ns was performed for the total system. During this simulation, the temperature coupling time constant was set as 0.5 ps and pressure coupling time constant was set as 10 ps. The coupling temperature was 310 K, and the coupling pressure was 1 atm. The Coulomb force threshold was set to

\[ \text{Figure 11.} \text{ } \alpha \text{-Tubulin on the left, } \beta \text{-tubulin on the right, and a GDP molecule in the red from the crystal structure of } \alpha \beta \text{-tubulin (1Z2B).} \]

\[ \text{Figure 12. Atomic number of DVB optimized at the level of MP2/6-31G(d,p).} \]

\[ \text{Figure 13. Structural picture of the DVB-} \alpha \beta \text{-tubulin-H}_2\text{O complex system.} \]
1.2 nm, van der Waals threshold (vdw) was set as 1.2 nm too, and simulation step size was set as 2 fs using a periodic boundary condition.

4.3. Active Site Residues for Binding with DVB within Tubulin. The interaction of amino acids used as active sites with small drug molecules directly affects the function of protein. We used the stable DVB-αβ-tubulin-H2O system after the dynamics simulation and further energy minimization to study the active site residues. During the energy minimization, the Steep algorithm and PME method was used to calculate the atomic electrostatic interaction. The energy convergence was set as 80 kJ mol⁻¹ nm⁻¹. After the energy minimization for the system, water and sodium ions were removed to remain energy minimization for the system, water and sodium ions were added with H-bonding hydrogen and Gasteiger Hückel charges for the DVB molecule and Amber7 FF99 for αβ-tubulin. Tripos field was selected to optimize all added hydrogen atoms, and Powell mode was selected as the optimization method. The gradient parameter was 0.04 kJ mol⁻¹ with step numbers of 10 000. The optimization structure was saved in a file of MOL2 format. The residues within the sphere of 6 Å radius around DVB as a center were extracted to form a pair of DVB and respective residue, while the carbonyl end and amino end of the residues were fixed with hydrogen atoms. The binding energies of individual residues with DVB were calculated using MP2/6-31G(d,p) and the overlapping error of basis was corrected by the Boys and Bernardi method.

4.4. Free-Energy Surface along the Trajectory of αβ-Tubulin Separation. Because αβ-tubulin moves to separate and generate a trajectory, a large box needs to ensure that the entire system has enough room for the movement. Therefore, the simulation system box was reset to 18, 10, and 9 nm along the x, y, and z axis directions. The number of water molecules filled in the system was changed to 49 733. Similarly, the constructed system was simulated by MD simulation, and the energy minimization step (em step) was used to balance the system and to eliminate particle overlap for promoting a reasonable structure. After the completion of the energy minimization, the equilibrium of the system temperature (nvt step) and pressure (npt step) under the condition of allowing the water molecules to move freely was performed. After the system pressure was equilibrated, an MD simulation of 400 ps was performed as an addition. The parameter settings for all steps were the same as those in the previous section.

Based on the simulated DVB-αβ-tubulin-H2O system, a trajectory was generated by the Gromacs 4.5 program. During this process, a protein chain was moving; thus, the complex system is grouped in an ndx file. Chain B (β-tubulin) was grouped into chain_A, and chain C (α-tubulin) was assigned to chain_B. DVB was set as chain_C, and SOL and Na⁺ were used as a component. The structural file obtained after the optimization of MD simulation was used as the input file of the initial structure for the trajectory dynamics with the same topology.

To study the separation trajectory of αβ-tubulin under the presence of DVB within the interface of αβ-tubulin, it is necessary to apply an external force to a protein chain to move away from another chain. In this work, external force was applied to the B chain (β-tubulin) as a moving group, while chain_A (α-tubulin) was used as a reference system. An external force of no more than 2000 kJ mol⁻¹ along the X-axis direction forced β-tubulin to move and generate its trajectory. The maximum movement speed was 10 nm ns⁻¹, and the step length was 2 fs with the number of steps set as 400 000. The structural conformation was output once per 1 ps. The Coulomb force threshold was set as 1.2 nm, and the van der Waals threshold was 1.2 nm. The PME calculation was used, and the energies were divided into five groups of DVB, protein, GDP, SOL, and Na. Temperature coupling was Nose–Hoover with a coupling time constant of 0.5 ps. The pressure coupling adopted Parrinello–Rahman with the coupling time constant of 2 ps. Then, the system temperature was set to 310 K, and the pressure was set to 1.0 atm. We used VMD and Chimera programs to do the visual analysis for the output trajectory data.

The umbrella sampling method from the Gromacs 4.5 program was used to obtain the free-energy potential of αβ-tubulin separation under the presence of DVB. The theoretical basis is on the formula: ΔG = −RT ln K, where ΔG is free-energy variety, R is ideal gas constant, T is kelvin temperature, and K is the equilibrium constant of the system obtained by MD simulation.

Using the g_dist program from the Gromacs 4.5 program, the tpr file generated in the previous step was calculated to give the umbrella sampling recorded in the xvg file to contain 400 conformation data. Starting from the minimum centroid distance between the reference and moving groups, according to the 0.04 nm rule, a series of sampling conformations was selected to rebalance the conformation systems. The external forces to generate the movement of β-tubulin caused the original complex system deviating from the equilibrium. Therefore, before calculating the free-energy potential surface, it is necessary to perform MD simulation on the selected umbrella sampling at the defined centroid distance between the moving and reference groups, so that each umbrella sampling system reached the equilibrium state again. The molecular dynamics rebalancing umbrella sampling system requires some files, including the umbrella sampling file, same topology file, same pointer file, and parameter file. In the parameter file, the centroid distance was set to be constant, and the motion speed is set to 0 nm ns⁻¹, indicating that β-tubulin did not move along the trajectory, except for vibration and rotating at a given centroid distance for two groups. The number of simulation steps was set to 400 000 (800 ps) with a step length of 2 fs. rmsd was used to measure the balance of the sampling system.

WHAM (g_wham) in the Gromacs 4.5 program was adopted to convert the biased sampling above into unbiased results. The potential of mean force was calculated from a series of umbrella sampling, and the calculated deviation was below at 10⁻⁶ kJ mol⁻¹. By using average force potential (PMF), the free-energy variety (ΔG) can be obtained. The g_wham program also outputs histogram data. The weighted histogram can determine the reliability of free-energy results. If the histogram has a high degree of overlap, the generated PMF potential energy surface will be smooth and the free energy is reliable.

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