Increasing the distance between two monomers of topoisomerase IIβ under the action of antitumor agent 4β-sulfur-(benzimidazole) 4′-demethylepipodophyllotoxin

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Topoisomerases II (Top2s) are a group of essential enzymes involved in replication, transcription, chromosome condensation, and segregation via altering DNA topology. The mechanism of the Top2s poisons such as etoposide (VP-16) was reported as stabilizing the Top2-DNA complex and engendering permanent DNA breakage. As the structurally similar compound of VP-16, a novel 4β-sulfur-substituted 4′-demethylepipodophyllotoxin (DMEP) derivative (compound C-Bi) with superior antitumor activity was developed in our previous study. To understand the structural basis of the compound action, the crystal structure (2.54 Å) of human Top2β isoform (hTop2β) cleavage complexes stabilized by compound C-Bi was determined. However, compound C-Bi was not visible in the crystal structure. Through the comparison of the structures of hTop2β-DNA-etoposide ternary complex and hTop2β-DNA binary complex, it could be observed that the distance between drug-binding sites Arg503 of the two monomers was 26.62 Å in hTop2β-DNA-etoposide ternary complex and 34.54 Å in hTop2β-DNA binary complex, respectively. Significant twist were observed in the DNA chains of binary complex. It suggested that compound C-Bi played antitumor roles through increasing spacing of hTop2β monomers. The changes in hTop2β structure further caused double changes in the torsional direction and migration distance of the DNA chains, resulting in impeding religation of DNA.
activity, the inhibition activity of target proteins Top2s, and apoptosis induction to that of VP-16.

However, the molecular mechanism underlying the difference is not well understood. Herein, the crystal structure of the catalytic core of hTop2β in complex with DNA and DMEP derivative was determined. The results can provide useful information for better understanding of the action mechanisms of Top2s-targeting antitumor agents.

Results
Detecting hTop2β-DNA covalent complexes using the ICE (in vivo complex of enzyme) bioassay. The ICE bioassay detected the hTop2β-DNA covalent complex formation in vivo with HeLa cells. Exponentially growing cultures were either treated with drugs (100 μM) or without drug. And then processed for covalent complexes. In the CsCl gradients (Fig. 1A), DNA mainly distributed at the expected density in fractions 9–12. Then, immunoblotting of the gradient fractions using an appropriate antibody measures hTop2β. As shown in Fig. 1B, free proteins were in the top fractions 1–8. No stabilized hTop2β-DNA intermediates were detected in the absence of drug, indicating a lack of cross-reactivity between hTop2β and DNA. In the presence of VP-16 or compound C-Bi, hTop2β was detected in the DNA peak fractions. It clearly suggested that the formation of hTop2β-DNA covalent complexes could be promoted under the action of VP-16 or compound C-Bi.

DNA cleavage assay. To determine whether the DNA cleavage induced by compound C-Bi is associated with the interruption of topoisomerase II activity, a cell-free DNA cleavage assay using an enzyme-mediated negatively supercoiled pHOT1 plasmid DNA was applied. A representative gel image of the relaxation assay for determining the catalytic inhibition on hTop2β was presented in Fig. 2A. Topoisomerase IIβ poisons such as VP-16 were known to stabilize the topoisomerase IIβ-DNA complex that lead to DNA breaks and generate linear DNA. In agarose gel, linear DNA (L) was difficult to enter and appeared at the top; whereas the relaxed DNA (R), and supercoiled DNA (S) move easily into the gel. As shown in Fig. 2A, compound C-Bi stabilized hTop2β cleavage complexes and exhibited the formation of linear DNA. This result shows that compound C-Bi acted as hTop2β poison. To more clearly describe the concentration-dependent effect on linear DNA, a gray-scale analysis was performed (Fig. 2B). It was observed that the DNA cleavage increased with an increase in the concentration of VP-16 and compound C-Bi. It indicated that VP-16 and compound C-Bi stabilized or trapped the Top2-cleaved DNA complex in a concentration-dependent manner.

Binding of DMEP derivatives to hTop2β-DNA complex. To further investigate the binding affinity of compound C-Bi and VP-16 to hTop2β-DNA complex, surface plasmon resonance (SPR) was employed. As shown in Fig. 3, the response unit increased in a concentration-dependent manner. The equilibrium dissociation constant (Kₐ) of compound C-Bi (45.9 ± 3.6 μM) were approximately 2.5 folds lower than that of VP-16 (113.6 ± 6.4 μM). These data indicated DMEP derivative exhibited higher affinity for hTop2β-DNA complex than VP-16.

Structure basis of the complex. To provide insights into the interactions of compound C-Bi with hTop2β at the molecular level, we initially sought to investigate the molecular mechanism of action of compound C-Bi. Using the well-established system for hTop2β crystal growth, the crystal of hTop2β cleavage complex containing two monomers of hTop2β and cleaved DNA chain was obtained under the action of compound C-Bi with the
resolution of 2.54 Å (Table 1, Fig. 4A,B). The DNA was embedded in the hTop2β (Fig. 4C,D). All DNA base pairs were visible in the electron density maps (Supplementary Fig. S2). However, no molecule of compound C-Bi was observed in the stabilized hTop2β cleavage complex structures.

In order to understand the mechanism of compound C-Bi, the structures of hTop2β-DNA binary complex and hTop2β-DNA-etoposide ternary complex were compared. The RMSD value for this alignment was 3.604 Å (1304 to 1304 atoms). The structure of the hTop2β-DNA binary complex revealed a looser assembly pattern than in hTop2β-DNA-etoposide ternary complex (Fig. 5A,B) and other DNA-bound structures (Supplementary

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**Figure 2.** Concentration dependent effect of compound C-Bi and VP-16 on the hTop2β-mediated cleavage of supercoiled pHOT1 plasmid DNA to produce linear DNA. (A) The linear DNA detection of different reaction systems. The concentration series were 0.1, 1, 5, 10, 20, 40, 80, 100μM for compound C-Bi and VP-16. L, linear DNA; S, supercoiled DNA; R, relaxed DNA. (B) The gray scale value of the linear DNA amount. Data plotted are the mean ± SD of three separate experiments.

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**Figure 3.** The interaction between compound C-Bi and VP-16 with immobilized hTop2β-DNA complex was measured by surface plasmon resonance (SPR). (A) VP-16; (B) Compound C-Bi. In each case, the signal is proportional to the compounds concentration (For complete kinetics parameters we refer to Supplementary Table S1). The largest signal corresponds to the highest compound concentration and vice versa.
Table S2). However, the changes of structure were mainly located in the DNA-gate, and there was no obvious change in the C-gate. The distance between the main DNA-contacting domains of the two monomers was significantly increased under the action of compound C-Bi (Fig. 5C).

| Structure  | 5ZAD |
|------------|------|
| Data Collection |      |
| Wavelength  | 0.97845 |
| Space group  | P32   |
| Cell dimensions |  |
| a, b, c (Å)  | 95.006, 95.006, 230.289 |
| α, β, γ (°)  | 90, 90, 120 |
| Resolution range (Å) | 47.50–2.20 |
| No. of unique reflections | 118178 |
| Completeness (%) | 99.5 |
| Rsym        | 0.083 |
| Refinement |      |
| Resolution range (Å) | 28.06–2.54 |
| Rwork/Rfree (%) | 18.0/22.7 |
| No. of reflection | 76508 |
| Bond lengths (Å)  | 0.009 |
| Bond angles (°)  | 1.25 |
| Ramachandran statistics | |
| Favored regions (%) | 94.54 |
| Outliers (%) | 0.21 |

Table 1. Summary of crystallographic analysis.

Figure 4. Structure of hTop2β–DNA complex. (A,B) Orthogonal views of the hTop2β–DNA complex from front view (A) and top view (B). (C,D) The cartoon representation of the hTop2β–DNA complex from front view (C) and top view (D). The dimeric hTop2β protein was shown as a surface representation and colored according to polypeptide chain. DNA was shown in yellow.

Table S2). However, the changes of structure were mainly located in the DNA-gate, and there was no obvious change in the C-gate. The distance between the main DNA-contacting domains of the two monomers was significantly increased under the action of compound C-Bi (Fig. 5C). The distance between drug-binding sites Arg503
of the two monomers was 26.62 Å in hTop2β-DNA-etoposide ternary complex and 34.54 Å in hTop2β-DNA binary complex, respectively. The distance between DNA-intercalating sites Ile872 of the two monomers was 52.65 Å in the ternary complex, and 58.71 Å in the binary complex.

Arg503 is a major drug-contacting residue. In hTop2β-DNA-etoposide ternary complex, for interacting with VP-16, the +5 guanine base could form a hydrogen bond with the main-chain carbonyl of Arg503. In this work, perhaps because of the loose assembly of monomers, the distance between Arg503 and +5 guanine base was increased, and no hydrogen bond was observed (Fig. 6). Similarly, the phosphotyrosyl linkage that should existed between +1 nucleotide and active site residue Tyr821 was weaken. DNA-intercalating isoleucine (Ile872) still bound between the +8 and +9 bases.

To compare the differences between DNA molecules, the superimposition of DNA molecules from hTop2β-DNA binary cleavage complex and hTop2β-DNA-etoposide ternary cleavage complex were shown. The insertion of VP-16 abolished the stacking interaction between the +1/+4 and −1/+5 base pairs. Thus, a drug-binding site for compound C-Bi between the same base pairs in hTop2β-DNA binary complex was assumed. More obvious twist have been observed in the DNA chains of binary cleavage complex (Fig. 7). Compared with that of VP-16, the DNA chain configuration was changed by approximately 40.3° of the angle and 3.9 Å of distance under the action of compound C-Bi.
Discussion

VP-16 is one of the most effective anticancer drugs in clinical use, the mechanism of which was reported as stabilized the hTop2β-DNA complex by bonding at the two scissile bonds of a hTop2β-DNA cleavage complex. In our previous work, a novel 4β-sulfur-substituted DMEP derivative was developed, which exhibited superior antitumor activity to that of VP-16. As the structurally similar compound of VP-16, the antitumor mechanism of compound C-Bi remains largely unknown.

Through ICE assays, DNA cleavage assay and kinetic analysis, we have identified compound C-Bi could bind to hTop2β, and stabilize the formation of ternary complex. To further understand the mechanism, the crystal structure (2.54 Å) of hTop2β cleavage complexes stabilized by compound C-Bi was determined. However, no molecule of compound C-Bi was observed in the hTop2β cleavage complex structures. In our study, the stable hTop2β-DNA complex cannot be formed without the action of DMEP derivative (data not shown). In the previous work, the hTop2β-DNA binary complex can only be obtained through soaking out of VP-16 from the hTop2β-DNA-etoposide ternary complex. By comparison of the structure of hTop2β-DNA-VP-16 ternary complex (PDB: 3QX3) and hTop2β-DNA binary complex (PDB: 4J3N), the difference was only the absence of VP-16. It indicated that although there was no compound C-Bi in the complex structure, the crystal structure could still provide information for explaining the mechanism of compound C-Bi action.

To assess the possible mechanism of compound C-Bi, we compared the structure mode of hTop2βcore-DNA binary complex (PDB: 5ZAD) and hTop2βcore-DNA-etoposide ternary complex (PDB: 3QX3). In the crystal structure of hTop2βcore-DNA complex under the action of compound C-Bi, with the two monomers moving away from each other, the distances between the drug-contacting sites (Arg503 and Arg503') and between the two DNA-intercalating isoleucines (Ile872 and Ile872') are longer than those observed in VP-16-stabilized structures (Fig. 5, Supplementary Table S2). Increased spacing may suggest an intermediate state between a closed state and the open conformation. It indicated the action of compound C-Bi needed a looser packing between the two monomers. Therefore, the intermediate states of the movement of two monomers could be easily captured.

The change of cleavage DNA was also observed with more significant twist of DNA chains (Fig. 7). Compared with VP-16, the action of compound C-Bi led to further migration of the two DNA chains. That may be caused by the increased distance between the two monomers. The interaction between the residues and the base was weakened or eliminated by the migration of the monomers. DNA chains were more significantly twisted, and some bases could not be paired with each other (+1/+4). Lack of base-pairing and torsion of the DNA chains leads to an inability to re-ligate the DNA break, thus preventing repair of the DNA damage. When Top2s play roles in replication, transcription, chromosome condensation, and segregation, Top2 alters DNA topology via the formation of a Top2-DNA cleavage complex. Top2 poisons (e.g. VP-16) inhibit the relocation of cleaved DNA resulting in

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**Figure 7.** The superimposition of DNA molecules from hTop2β-DNA binary cleavage complex (PDB ID 5ZAD: yellow) and hTop2β-DNA-etoposide ternary cleavage complex (PDB ID 3QX3: grey). VP-16 was shown in blue stick representation. The red arrow represented the conjectural binding site of compound C-Bi. The comparison of the changes in configuration by calculating the angle and the distance for the structure of the enclosed region were shown from close-up view. The angle calculation was based on the overlapping point of the top view projection of DNA chains. This overlapping point was approximately +6 base of hTop2β-DNA-etoposide ternary cleavage complex. The labels of base were colored orange (PDB ID 5ZAD) and black (PDB ID 3QX3), respectively.
DNA damage\(^{27}\). Accumulation of Top2-mediated DNA damage leads to cell death. It suggested that compound C-Bi played antitumor roles through increasing spacing of hTop2\(^{\beta}\) monomers. The changes in hTop2\(^{\beta}\) structure further caused double changes in the torsional direction and migration distance of the DNA chains, resulting in impeding religation of DNA.

In summary, to understand the structural basis of compound C-Bi action, we determined the interaction of compound C-Bi with hTop2\(^{\beta}\) and DNA using X-ray crystallography. By comparing the structures of the complexes, the distance between the two monomers of hTop2\(^{\beta}\) was increased, and significant twist of DNA chains was found under the action of compound C-Bi. It suggested that compound C-Bi played antitumor roles through increasing the change of hTop2\(^{\beta}\) monomers structure and structural changes will further generated DNA damage. The structure information reported here advanced the understanding of the inhibitory mechanisms of Top2-targeting anticancer compounds.

**Methods**

**Construction and expression of hTop2\(^{\beta\text{core}}\).** The hTop2\(^{\beta\text{core}}\)-PET51b plasmid was kindly provided by Dr. Nei-Li Chan at National Taiwan University\(^{20}\). The plasmid was transformed into *Escherichia coli* BL21 (DE3) pLysS cells. For expression, the transformed strain was grown in LB medium at 37 °C to an OD\(_{600}\) = 1.0. Isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) (BioSSharp, Seoul, Korea) was added to a final concentration of 0.3 mM, and protein expression was induced at 20 °C for 16 h.

**hTop2\(^{\beta\text{core}}\) purification.** The cells were centrifuged at 5000 rpm for 15 min (4 °C), and the pellets were resuspended in lysis buffer containing 50 mM NaPi (pH 7.4), 10% glycerol, 500 mM NaCl, 5 mM \(\beta\)-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, and 10 mM imidazole\(^{20}\). And then the cells were disrupted using a high pressure cell crusher (AH-1500, ATS, Canada) at 4 °C. Unbroken cells were removed by centrifugation at 15,000 rpm for 30 min. The hTop2\(^{\beta\text{core}}\) protein was isolated from the cell lysate supernatant using Ni-NTA column (Clontech, USA) and eluted with elution buffer containing 250 mM imidazole without NaCl. The resulting protein was loaded onto a HiPrep 16/10 Heparin FF column (GE Healthcare). The protein was eluted in a linear gradient over 10 column volumes with buffer A (30 mM Tris-HCl pH 7.5, 15 mM NaCl, 2 mM \(\beta\)-mercaptoethanol, and 1 mM EDTA) and buffer B (buffer A containing 1 M NaCl). The eluted fractions were pooled and purified on a gel filtration column (Superdex 200, GE Healthcare) in buffer C (buffer A containing 70 mM NaCl). The hTop2\(^{\beta\text{core}}\) protein (with molecular weight about 180 kDa) was collected and concentrated to 6.5 mg/ml for crystallization.

**DNA substrate for crystallography.** The DNA sequence 5\'-AGCCGAGCTCGAGCTCGCCTG-3' of the double-stranded DNA substrate was prepared as previously described\(^{20}\). Synthesis and purification of DNA were performed by a commercial company (GenScript, Nanjing, China). The oligonucleotides were dissolved in buffer containing 30 mM Tris-HCl (pH 7.5), 70 mM NaCl, 2 mM \(\beta\)-mercaptoethanol, and 1 mM EDTA) at 55 °C to generate double-stranded DNA for crystallization.

**ICE assay.** The ICE bioassay was performed as previously described\(^{28,29}\). HeLa cells were cultured in 25 cm\(^2\) cell culture dishes at 37 °C, and a single Petri dish was used per treatment (90% confluent, approx 1 \(	imes\) 10\(^7\) cells). The medium was removed and replaced with serum-free medium (1 mL) containing topoisomerase inhibitors (e.g., 100 \(\mu\)M etoposide or compound C-Bi, final concentration) and cells were incubated at 37 °C for 60 min. The negative control containing no drug (solvent only) was prepared in parallel. Then the drug containing medium was removed, and cells were lysed by the 1% sarkosyl equilibrated to 37 °C (2 mL/plate). The sarkosyl was squirted over 10 column volumes with buffer A (30 mM Tris-HCl pH 7.5, 15 mM NaCl, 2 mM \(\beta\)-mercaptoethanol, and 1 mM EDTA) and buffer B (buffer A containing 1 M NaCl). The eluted fractions were pooled and purified on a gel filtration column (Superdex 200, GE Healthcare) in buffer C (buffer A containing 70 mM NaCl). The hTop2\(^{\beta\text{core}}\) protein (with molecular weight about 180 kDa) was collected and concentrated to 6.5 mg/ml for crystallization.

**DNA cleavage assay.** The effect of compounds C-Bi on hTop2\(^{\beta}\) was measured using a topoisomerase II drug screening kit (TopoGEN, USA). The cleavage reactions were carried out in a final volume of 20 \(\mu\)L containing 100 ng of supercoiled pHOT1 plasmid DNA, 3 units of hTop2\(^{\beta}\), 0–100 \(\mu\)M compound C-Bi (dissolved in 1% DMSO), and complete assay buffer (3 \(\times\)). After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 2\(\mu\)L sodium dodecyl sulphate (10%). Then the reaction mixtures were incubated with 2\(\mu\)L of proteinase K (50 \(\mu\)g/mL) at 37 °C for 15 min to digest hTop2\(^{\beta}\). Final samples were mixed with 2\(\mu\)L loading buffer (10 \(\times\)) and subjected to electrophoresis in 1% agarose.

**Surface plasmon resonance.** The SPR assay was performed at 25 °C using a BI2000 SPR system (Biosensing Instrument, USA). Before determination, 1 \(\times\) cysteamine hydrochloride (Sigma-Aldrich) was covered onto the sensor chip in a dark. After 12 h incubation, the chip was further covered by the mixture solution of dextran sodium salt, N-hydroxysuccinimid and N-(3-dimethylaminopropyl)-N\'-ethylcarbodiimide hydrochloride (All from Sigma-Aldrich) for 3 h. hTop2\(^{\beta}\) and DNA (in a 1.2-fold molar ratio to protein) were incubated at 4 °C for 1 h, and then the hTop2\(^{\beta}\)-DNA binary complex was immobilized onto the sensor chip. The running
buffer was PBS (pH 6.5) containing 5% DMSO. 1 M ethanolamine hydrochloride solution (30μL/min, 100 μL) was then injected to block the unreacted ester group. After that, compound C-Bi was injected to flow across the chip surface with the concentrations from 50 to 800 μM at a flow rate of 60 μL/min. Each analyte was in triplicate
determination. Between measurements, a regeneration cycle was completed by applying 20 mM NaOH over the sensor chip to dissociate the compound. The kinetic parameters provided estimates of both association and
dissociation rate constants (k_a and k_d), and from these values the equilibrium parameter (K_D) for evaluating binding affinity of hTop2α-DNA for DMEP derivative was calculated using the relationship K_D = k_a/k_d.

Cry stallization. The protein sample was mixed with 2 mM compound C-Bi (in DMSO) and DNA substrate (in a 1.2-fold molar ratio to protein). Initial crystallization trials for the hTop2α-DNA-compound C-Bi complex were performed as previously reported. 1 μL of concentrated hTop2α-DNA- compound C-Bi was mixed with an equal amount of reservoir solution and equilibrated against 500 μL of reservoir solution at 4°C. The complex was crystallized by the hanging-drop vapor diffusion method using 100 mM magnesium chloride, 50 mM sodium citrate (pH 5.3), and 20% 2-methyl-2,4-pentanediol (MPD) as the precipitating agent, and 0.05% ethyl acetate (Hampton Research, USA) was used as additive. Crystals reached the maximum dimensions in around two weeks. Crystals were harvested by transferring into mother liquor containing 30% MPD and 1 mM corresponding compound before looping and flash-freezing in liquid nitrogen for data collection.

Structure determination. The X-ray diffraction data on the hTop2α-DNA complex were collected at the Shanghai Synchrotron Radiation Facility (SSRF) (Shanghai, China) beamline BL18U and BL19U and were processed using the HKL3000 program suite. The structure was solved by molecular replacement with the PHENIX MR (using the structure of hTop2α-DNA-VP-16 (PDB: 3QX3) as the search model). Then the structure was built and refined by Coot and PHENIX and analyzed by PyMOL.

Accession code. The crystal structure of the complex of hTop2α-DNA, has been deposited with the RCSB Protein Data Bank under the accession code 5ZAD.

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Acknowledgements
We thank Huan Zhou at Shanghai Synchrotron Radiation Facility (SSRF) beamline BL17U for their on-site assistance. We thank the research associates, Delin Zhang, at the Center for Protein Research (CPR), Huazhong Agricultural University, for his technical support. Financial supports from the National Natural Science Foundation for Distinguished Young Scholars (Grant No. 21625602), the National Natural Science Foundation of China (Grant Nos 21838002, 81503112, 21506049, and 31570054), and Hubei Provincial Science and Technology Innovation Major Project (2017ACA173) are gratefully acknowledged.

Author Contributions
Y.J.T. conceived the project. L.W.Z. designed the experiments; L.Y.S. implemented the analysis workflow and conducted the experiments. L.Y.S. and L.W.Z. contributed to the analysis with constructive discussions, L.Y.S. prepared all figures and tables, L.Y.S., L.W.Z. and Y.J.T. wrote the manuscript. All authors reviewed, commented on, and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-33366-2.

Competing Interests: The authors declare no competing interests.

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