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Benz[f]indole-4,9-dione 유도체의 Topoisomerase I-DNA에 대한 결합형태와 저해작용 간의 상관관계

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Correlation between the Binding Mode and Inhibitory Activity of Benz[f]indole-4,9-dione Analogs with Human Topoisomerase I-DNA

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요 약. 새로 합성된 benz[f]indole-4,9-dione 유도체와 topoisomerase I(TOP1)-DNA 복합체의 결합형태를 규명하기 위해 세포독성과 TOP1 저해작용이 검사된 15개의 유도체와 camptothecin을 TOP1-DNA 복합체의 X-선 구조에 FlexiDock을 사용하여 docking하였다. 그 결과 N_1에 할로겐이 결합된 두 화합물은 -1/+1 염기 사이에 끼어들어 깊숙이 결합되었고 각각 3개의 수소결합과 다수의 소수성 상호작용으로 안정화되었다. 활성검사에서 가장 강한 작용이 나타나 고유한 docking 결과와 잘 부합되었다. 염기사이에 끼어들지 않고 수소결합도 형성하지 않은 6개의 화합물에서는 활성이 거의 나타나지 않았다. 한 개 내지 4개의 수소 결합을 형성하면서 염기 사이에 끼어들었으나 TOP1에 의해 잘라지는 위치에 깊게 결합하지 못한 7개의 화합물은 중간 정도의 활성을 나타내어 구조-활성 관계가 실험 치와 잘 일치하였다. 이 결과는 TOP1을 목표로 하는 새로운 항암제의 구조를 고안하는데 합리적인 근거를 제공할 것이다.

주제어: Benz[f]indole-4,9-diones, Topoisomerase I, 결합형태, 저해작용, FlexiDock

ABSTRACT. We present binding modes of newly synthesized benz[f]indole-4,9-dione analogs with topoisomerase I (TOP1)-DNA complex. The compounds were tested for cytotoxicity and TOP1 inhibitory activity in our previous study. Camptothecin (CPT) and each of the 15 analogs were docked into the X-ray crystal structure of human TOP1-DNA binary complex to develop the ternary complex of TOP1-DNA-ligand using FlexiDock. The results demonstrated that two compounds with N_1-attached halogens intercalated exactly between the -1 and +1 DNA bases, deeply toward the cleavage site and were stabilized by three H-bonds each and an array of hydrophobic interactions with both the enzyme and the DNA. The most potent inhibitory activities they showed in the test agree well with the proper docking results. Six compounds which did not intercalate and formed no H-bond showed no or very low inhibitory activities. Seven compounds that intercalated between the bases with one to four H-bonds but not deeply enough toward the cleavage site showed intermediate activities. The structure-activity relationship was consistent with the experimental data. The ternary complex presented potential binding mode of each compound and thus could provide a rational basis for novel inhibitor design to develop potent anticancer drugs targeting TOP1.

Keywords: Benz[f]indole-4,9-Diones, Topoisomerase I, Binding Mode, Inhibitory Activity, FlexiDock

INTRODUCTION

Topoisomerase I (TOP1) has been investigated as a new target for antineoplastic drugs.[1] Human TOP1 is an essential enzyme that relaxes DNA supercoiling and relieves torsional strain during DNA processing including replication, transcription, and repair.[1,2] TOP1 transiently relaxes DNA by cleaving one strand of DNA by attacking a backbone phosphate with a catalytic tyrosine, resulting in a 3'-phosphate-tyrosine (PTR723) bound to the 3' end of the -1 cleavage site nucleoside (T10), and a free 5'OH on the +1 cleavage site nucleoside (G11). TOP1 subsequently religates the cleaved single strand DNA without an energy cofactor.[2,3]

Most of the currently used TOP1 inhibitors are DNA intercalators. They show antitumor effects by intercalating between DNA bases, eventually blocking the replication and transcription of DNA. They also interact with TOP1 residues inducing cell death in the end.[4] The representative inhibitor of TOP1, camptothecin (CPT), is a planar molecule with an arc shape and inhibits the religation step of TOP1-DNA by binding to the binary complex at or near the site of DNA cleavage.[3,5,6] This intercalative binding mode of CPT between the +1 and −1 bases makes it interact favorably with both the enzyme and the DNA.[7] However, in recent years, attempts to synthesize novel TOP1 inhibitors have been actively going on because CPT derivatives have major limitations: (1) they are in equilibrium with their inactive carboxylate form at physiological pH, (2) the CPT-trapped cleavage complexes reverse within minutes after drug removal, which imposes long infusion for cancer treatment,[5] and (3) the clinically relevant analogs are substrates for efflux transporters leading to resistance.[8]

Benz[f]indole-4,9-dione analogs were synthesized as novel antitumor agents based on the antitumor activity of 1,4-naphthoquinone.[9] It is known that 1,4-naphthoquinone derivatives possess versatile pharmacological effects such as antineoplastic, carcinostatic, antibacterial, fungicidic, and anticoagulant actions.[10] The structure of benz(f)indole has a planar tri-heterocyclic ring of 7.161Å width and a *p*-conjugated ketone group, therefore, is fully consistent with the conditions of intercalators.[11] The ability to inhibit TOP1 has been evaluated with DNA relaxation assay using supercoiled pHOTI DNA as a substrate in our previous study.[12]

We report here the structural models of the human TOP1-DNA-ligand ternary complexes formed between the X-ray crystal structure of human TOP1-DNA binary cleavable complex and the ligands, CPT and 15 benz[f]indole-4,9-dione analogs. Previously, Kerrigan *et al.* carried out docking studies of CPT and 8 other compounds with different degrees of TOP1 inhibition activities into the X-ray structure of human TOP1-DNA complex using the docking program, AutoDock.[6,8] Laco *et al.* docked CPT and its derivatives into a structure-based active site model[2] and Fan *et al.* did molecular modeling studies of the ternary CPT-TOP1-DNA cleavable complex with a "rigid docking procedure" of SYBYL 6.2.[7] In this study we used a flexible docking program, FlexiDock, from SYBYL 6.9 package which is programmed based on genetic algorithm. The binding modes of the novel 15 non-CPT TOP1-targeting compounds with the TOP1-DNA complex were compared with the experimental activity data for the respective compounds to see if a correlation existed between the binding modes and the TOP1 inhibitory activities.

MATERIALS AND METHODS

Calculations were performed on a Silicon Graphics Octane Workstation using molecular modeling software package, SYBYL 6.9, (Tripos Co., St. Louis, MO).

Preparation of TOP1-DNA cleavable complex for docking

Among several human X-ray crystal structures of TOP1-DNA covalent complex in Protein Data Bank (PDB), the wild type structure with the resolution of 2.10 Å (1A31.PDB) was selected. The catalytic residue, Tyr723, is intact and covalently bonded to the -1 DNA base of the scissile strand by a phosphotyrosyl linkage.[13] DNA sequence modifications were made to this structure because CPT and its

derivatives were known to be sensitive to specific sequence of DNA bases.[14] All of iodouracils in the X-ray structure were converted to thymines, so that the iodouracil at the -1 site of the scissile strand was changed to thymine (T10). The thymine at the +1 site and its complement base pair adenine (A112) were converted to guanine (G11) and cytosine (C112), respectively. These modifications resulted in the DNA sequence shown below[6]:

-1+1

Before 5'-AAAAAGACTUU TGAAAAATTUUU-3' Scissile
3'-TTTTTCTGAAA--ACUUU TAAAAA-5' Intact
After 5'-AAAAAGACTTT GGAAAAATTTTT-3' Scissile
3'-TTTTTCTGAAA--CCTTTTTAAAAA-5' Intact

All the crystallographic water atoms were removed and hydrogen atoms were added to both the enzyme and the DNA. Minimization of the modified structure was carried out by using Tripos force field and recalculating atomic charges with Kollman-all-atom and Gasteiger-Hückel charges for the enzyme and the DNA, respectively, and terminating when the conjugate gradient reached 0.1 kcal/mol·Å.

Preparation of ligands for docking

The structures of the ligands, CPT and 15 benz[f]indole-4,9-dione analogs, were constructed using the sketch module of SYBYL program (*Table* 1). Hydrogen atoms were added to all structures. To obtain thermally stable conformations, full minimizations were carried out for all structures using Tripos force field and Gasteiger-Hückel charge until the conjugate gradient reached 0.001 kcal/mol·Å. Then, a random conformational search for each minimized structure was performed using Tripos force field. The lowest energy conformation for each compound was selected for docking.

Docking ligands into the TOP1-DNA cleavable complex

Each ligand was first manually positioned in the refined crystal structure of the human TOP1-DNA cleavable complex. The location was somewhere between the −1 and −1 DNA bases of the complex. Docking simulations were then performed using the FlexiDock, which allows low-temperature flexible bond rotations for both ligand and protein. Hydrogen bond donors and acceptors for both the TOP1-DNA complex and each ligand were assigned. The side chain structures of the active site residues and rotatable bonds of ligands were allowed to rotate during docking simulations. FlexiDock routine was executed at 30,000 generation. The best TOP1-DNA-ligand ternary complex structure for each compound was chosen based on the orientations and binding energies. The selected complexes were simulated again at 230,000 generation. The conformation obtained with the lowest energy value was chosen and minimized for optimization using anneal function by assigning the hot and interesting regions within the radius of 6 Å and 12 Å, respectively, from the ligand. Tripos force field was used until the conjugate gradient reached 0.05 kcal/mol·Å.[15] The resulting conformation was accepted as a final model.

Evaluation methods

Docking results were evaluated first based on energy. The structure with the lowest total binding energy was chosen among the simulated models presented for each compound. Since each ligand has different potential energy, the total binding energy of the complex could not be used to compare the stability of the ternary complexes with different ligands. The structure-activity relationship was evaluated based on the TOP1 inhibition mechanism of CPT, that is, the inhibition of the religation step of TOP1-DNA complex.[16,17] The most important criterion was the intercalative binding mode of each compound between the -1 and +1 bases of DNA, the cleavage site. The orientation and location of the ligand should be such that the religation of the scissile strand of DNA must be prevented. Hydrogen bond formation with important TOP1 residues such as Arg364, Lys532, Asp533, Asn722 and PTR723 was considered valuable in judging the stability of the ternary complex formed. Hydrophobic and/or van der Waals interactions with both TOP1 residues and DNA bases were also examined.

Table 1. Relaxation activity and IC_{50} of CPT and 15 benz[f]indole derivatives with structures

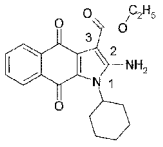
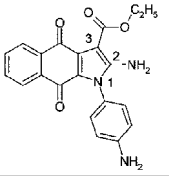
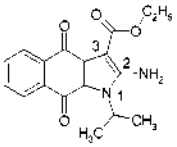
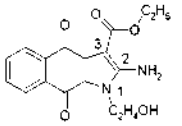
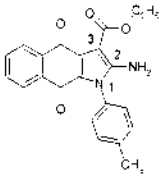
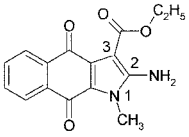
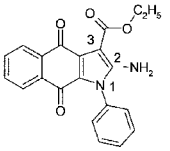
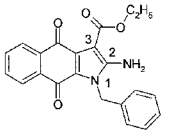
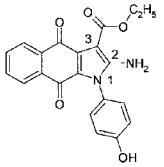
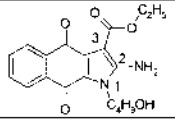
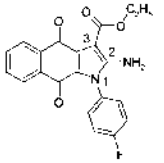
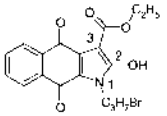
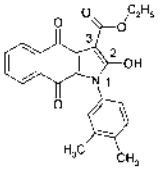
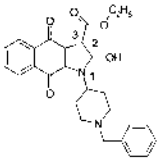
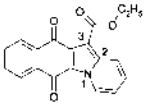
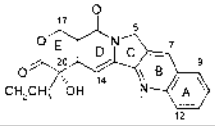
Compound	Relaxation activity (% Inhibition)[a]	Human cancer cells[a] (IC_{50}, μg/ml)			Structure
		A549	Col2	SNU-638	
1	12.2	16.8	>20	>20	
2	9.4	>20	>20	>20	
3	8.6	14.1	>20	>20	
4	27.3	3.6	4.9	7.8	
5	4.3	>20	>20	>20	
6	5.8	0.2	0.1	0.5	
7	2.9	>20	>20	>20	
8	15.7	>20	>20	>20	
9	0	>20	>20	>20	

Table 1. Continued

Compound	Relaxation activity (% Inhibition)[a]	Human cancer cells[a] (IC_{50}; μg/ml)			Structure
		A549	Col2	SNU-638	
10	0	0.3	1.2	3.0	
11	67.8	>20	>20	>20	
12	58.7	3.0	3.3	4.4	
13	6.6	0.4	>20	>20	
14	4.1	>20	>20	>20	
15	4.1	16.3	15.4	0.2	
CPT	50[b]	NA[c]	NA	NA	

[a]Data from Park *et al.*, 2003.
[b]Data from Park *et al.*, 2004.
[c]Not available

RESULTS

Docking interactions of CPT with the TOP1-DNA complex

The ternary complex of TOP1-DNA-CPT was constructed by docking 20(S)-CPT into the TOP1-DNA binary complex, the X-ray crystal structure used in this study (*Fig.* 1). In our model, the CPT intercalated between the −1 and +1 bases, perpendicular to the plane of the base pairs. The A-ring protruded into the major groove and the E-ring with 20-ethyl group was positioned into the minor groove. The CPT stabilized the cleavable complex through hydrogen bonding interactions between the O22 atom of E-ring and Arg364, the residue in the minor groove. The mutation of Arg364 is known to cause resistance to the drug, suggesting the importance of the H-bond interaction between the lac-

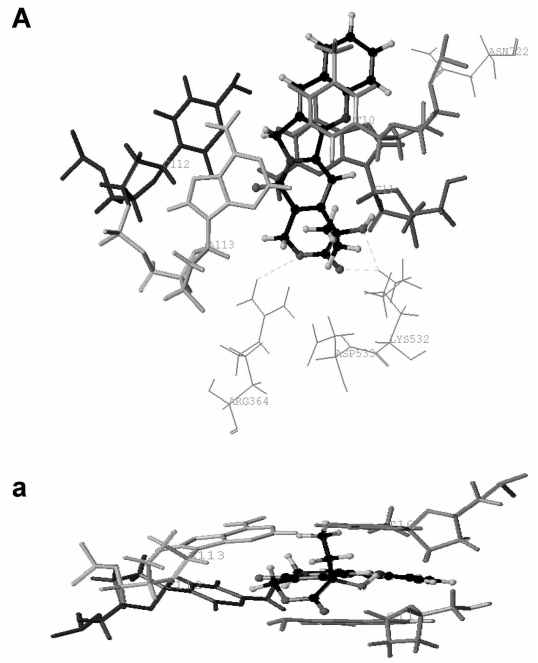


Fig. 1. Docking of CPT into the human TOP1-DNA cleavable complex. A: A view looking down the DNA helix axis. a: A sideview perpendicular to the DNA helix axis. Thin line: amino acid residues. Grey to black stick: DNA nucleotides. Black ball-stick: 20(S)-CPT. Black dotted lines: hydrogen bonds.

tone moiety of E-ring and Arg364.[2] In addition, both the O of 21-carbonyl and the O of 20-hydroxyl of the E-ring made multiple hydrogen bonding contacts with Lys532. The H of the 20-hydroxyl of E-ring also formed one H-bond with DNA furanose ring oxygen atom of -1 guanine, in accordance with the report that this interaction is essential for the activity of CPT.[14]

Our model presented the possibilities of additional interactions with Asp533 and Asn722 which are located at close distances from the CPT. Interactions with these residues could be very important because the mutation of both residues were known to cause resistance to CPT.[18] Although the CPT didn't form direct hydrogen bonds with Asp533 and Asn722, the model suggested that water molecules could act to form H-bond bridges between these residues and the CPT.[6] The stacking and charge transfer interactions of the B and C-rings of the CPT with both the pyrimidine ring of T10 and the purine ring of G11 contributed to the stability of the ternary complex.

The binding modes of this model were compared with the model derived from AutoDock study. The intercalative mode was similar but the sites of intercalation and H-bonds were slightly different. In both models, the A-ring is pointed into the major groove, while the E-ring into the minor groove. However, the CPT in AutoDock model was located slightly toward the bases of the nonscissile strand in comparison with the position in our model which is in between the bases of the scissile strand. In both models, the CPT formed H-bonds with Arg364 but not with either Asp533 or Asn722 directly. In our model, the CPT formed H-bond contacts with Lys532, unlike the AutoDock model, and stacking interactions between the rings of the CPT and T10 and G11 bases stabilized the ternary complex.

Binding modes of 15 benz[f]indole-4,9-dione analogs

Compounds docked deeply into the cleavage site:

The template structure, benz[f]indole-4,9-dione, of compound **11** intercalated exactly between the –1 and +1 bases and the N_1-*p*-fluorophenyl group was docked deeply towards the cleavage site near PTR723 of TOP1 (*Fig.* 2A,a). Fluorine, the most electronegative element, formed one H-bond with the OH of PTR723, therefore, trapping the cleavable complex at the site of DNA cleavage, preventing the religation of DNA. There were two other H-bonds formed with COO^- of Asp533, which is an important residue, and the 2-NH_2 of G11. The three rings of the template formed strong hydrophobic and van der Waals interactions with the residues of the enzyme and the DNA bases. Compound **12** showed almost identical binding modes in that the template intercalated exactly between the –1 and +1 bases and the N_1-bromopropyl group was positioned deep in the cleavage site, blocking the religation of DNA sufficiently (*Fig.* 2B,b). Three H-bonds were formed: the H of 2-hydroxyl and COO^- of Asp533, the O of 3-ethoxy and -NH_3^+ of Lys532, and the O of carbonyl and 2-NH_2 of G11. Besides,

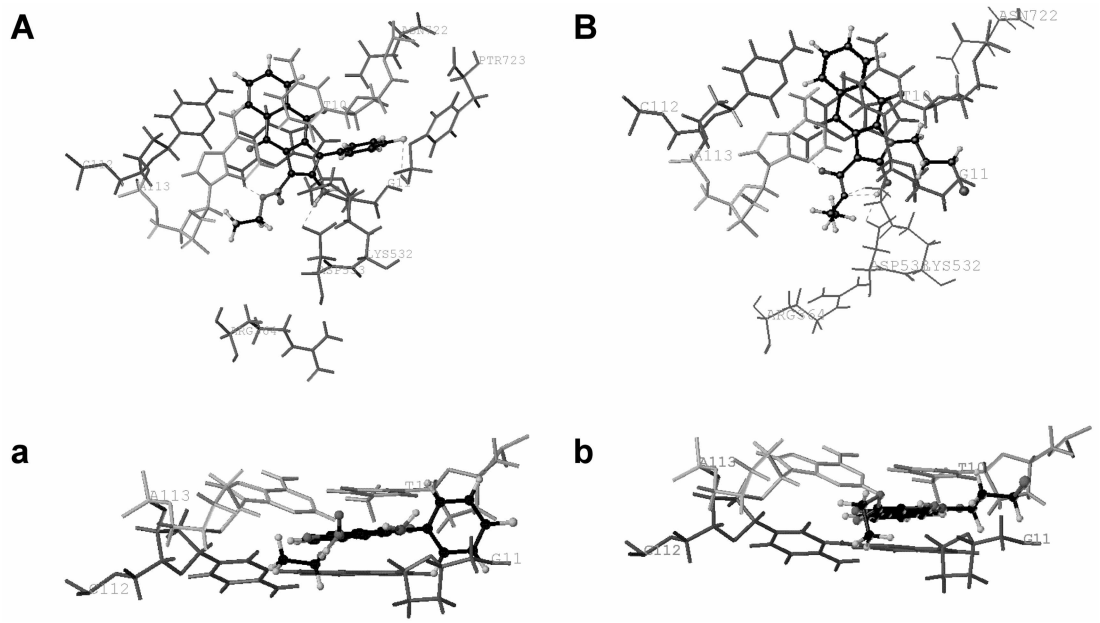


Fig. 2. Docking of compounds **11** (A and a) and **12** (B and b) into the human TOP1-DNA cleavable complex. A and B: Views looking down the DNA helix axis. a and b: Sideviews perpendicular to the DNA helix axis. Thin line: amino acid residues. Grey to black stick: DNA nucleotides. Black ball- stick: 20(S)-CPT. Black dotted lines: hydrogen bonds.

stacking and charge transfer interactions between the compound and the DNA bases contribute greatly to the stability of the complex.

The proper orientations and intercalative modes of **11** and **12** with three H-bond interactions each could be an indication of the strong inhibition effects of 67.8% and 58.7%, respectively, more potent than that of the CPT.[12] Substitutions with electron-withdrawing groups such as halogens at proper distance seem to potentiate the activity.

Compounds docked at intermediate location:

The template moiety of compound **1** intercalated between the −1 and +1 bases of DNA (*Fig.* 3A). However, the N_1-cyclohexane ring is located outside the G11, not being able to block religation of DNA effectively. One H-bond between the 2-NH_2 of G11 and the O of 3-ethoxycarbonyl was observed. Having no H-bond with any of the enzyme residues but only with DNA and improper intercalative position of the molecule might account for the moderately low inhibitory activity of 12.2%. The N_1-cyclohexane ring formed a strong hydrophobic interaction with the ribosyl group of G11 which pulled the compound towards the minor groove eventually causing the 3-ethoxycarbonyl moiety to push away the side chains of the minor groove residues.

Compound **3** also intercalated between the −1 and +1 bases, but not deep enough to block religation of DNA properly as shown in the side view of *Fig.* 3b. Two H-bonds are formed between the N of 2-NH_2 and the side chain -NH_3^+ of Lys532, and the O of 3-ethoxycarbonyl and the side chain NH_2 of G11. Part of the template 3-membered rings had van der Waals interaction with A113 and the hydrophobic interactions between the ribosyl group of G11 and the N_1-isopropyl group may have caused the compound to be located in the minor groove. The low activity of 8.6% agrees with the improper intercalative mode shown here.

The template of compound **4** intercalated stably between the −1 and +1 bases of DNA (*Fig.* 3C). Four H-bonds between the compound and the three important TOP1 residues of Arg364, Lys532 and Asp533 in the minor groove were formed. The O of 3-ethoxycarbonyl formed two H-bonds with the

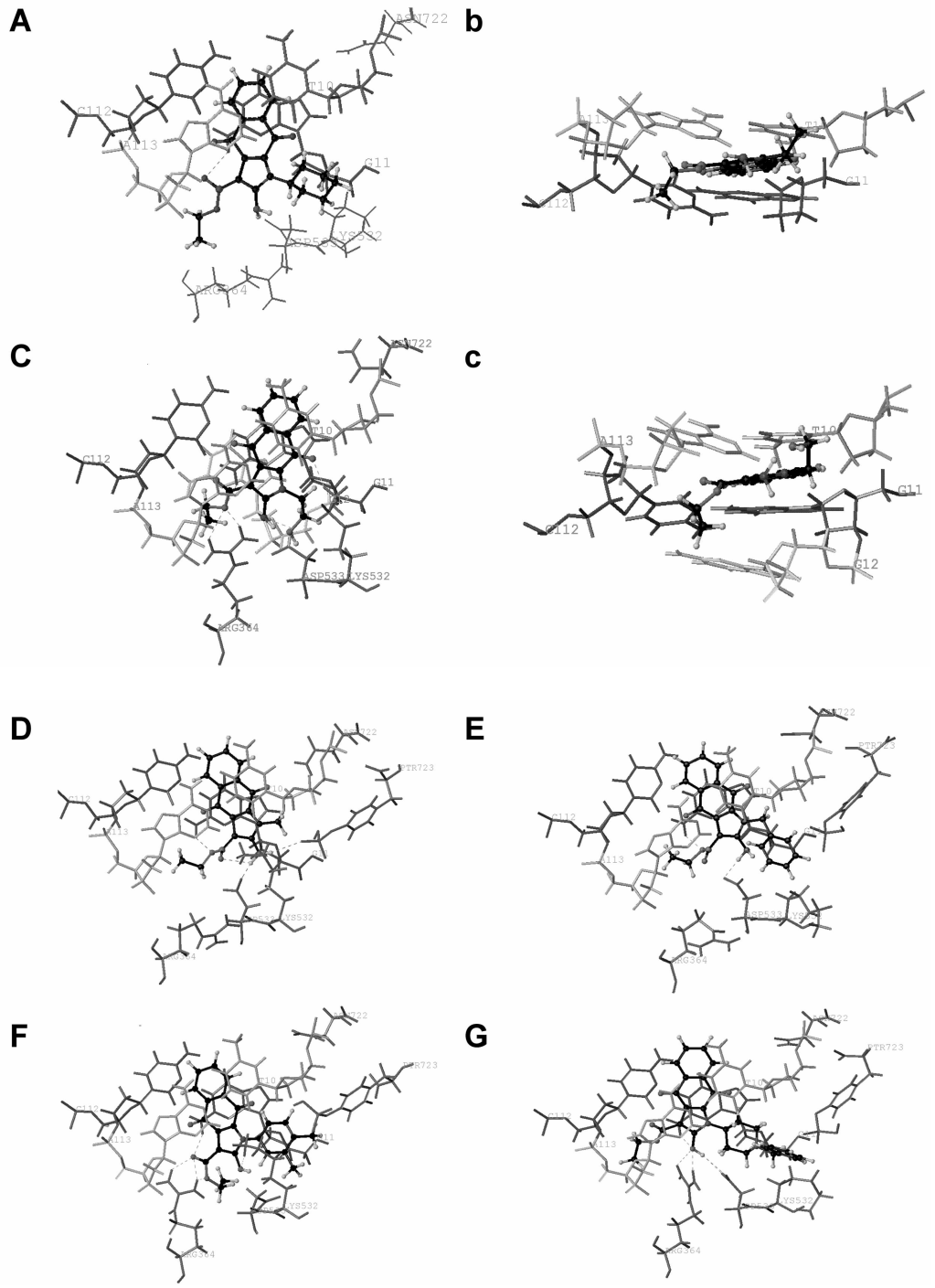


Fig. 3. Docking of compounds 1 (A), 3 (b), 4 (C and c), 6 (D), 8 (E), 13 (F), and 14 (G) into the human TOP1-DNA cleavable complex. A, C, D, E, F and G; Views looking down the DNA helix axis. b and c; Sideviews perpendicular to the DNA helix axis. Thin line: amino acid residues. Grey to black stick: DNA nucleotides. Black ball- stick: 20(S)-CPT. Black dotted lines: hydrogen bonds.

side chain NH_2 of Arg364 and the O of 4-ketone group formed one H-bond with the side chain -NH_3^+ of Lys532. The H of 2-NH_2 formed one H-bond with the side chain COO^- of Asp533. Strong hydrogen bonding interactions suggest stable binding of the compound to the complex. However, the stability alone does not seem to correlate with activity. The TOP1 inhibition activity was shown as 27.3% and IC_{50} on A549, Col2 and SNU638 cells were 3.6, 4.9, and 7.8 μg/ml, respectively.[12] These experimental data imply moderate activity even though it was held strongly by four hydrogen bonds. As the side view of the docked structure shows (*Fig.* 3c), the N_1-hydroxyethyl moiety is facing outward from the plane of DNA base pairs, thereby not being able to block the cleavage site of the scissile strand sufficiently. DNA religation could be hindered but not much. The result strongly demonstrates that the intercalative binding mode is more important parameter than the number of H-bonds in judging the structure-activity relationship.

Compound **6** (*Fig.* 3D) intercalated between the −1 and +1 DNA bases but the N_1-methyl group was located close to the T10, allowing the cleavage site open. The two H of 2-NH_2 formed two H-bonds with COO^- of Asp533 and the O of phosphate group of PTR723, respectively. The O of 3-ethoxycarbonyl formed another H-bond with NH_2 of G11. The ethyl moiety of the 3-ethoxycarbonyl group formed hydrophobic interactions with the ribosyl group of A113. Even though it formed three H-bonds, the TOP1 relaxation assay showed 5.8% inhibition, confirming again that the intercalative mode that prevents religation of DNA is the most important factor in the evaluation parameters.

The compound **8** intercalated between the bases with the benzyl group stacking with G11 (*Fig.* 3E), inhibiting the enzyme by 15.7%. The cleavage site was not blocked enough to give higher inhibition activity. Two H-bonds were formed between the H of 2-NH_2 and the COO^- of Asp533, and the O of 3-ethoxy and NH_2 of G11.

The compound **13** (*Fig.* 3F) bound to the complex with three H-bonds but slipped down to the minor groove, leaving the cleavage site of the DNA open. The H-bonds were formed between the compound and Arg364, Lys532, and G11. The activity was very low as expected (6.6%).

Compound **14** was located in between DNA bases but closer to the complement bases rather than the -1 and +1 bases (*Fig.* 3G), leaving the cleavage site wide open. Three H-bonds were formed with residues in the minor groove, not being able to contribute to the activity of the compound (4.1%).

Compounds docked at wrong location with no H-bond:

Compounds **2** (not shown), **7** (*Fig.* 4A) and **9** (not shown) did not intercalate between the DNA bases. Instead, they were located towards the major groove and there was no H-bond formed. Since the compounds could not intercalate, it seems impossible to block the religation of the DNA strand. Accordingly the TOP1 relaxation activities were 9.4, 2.9 and 0% for **2**, **7** and **9**, respectively, and IC_{50} on four different cells[12] were all over 20 μg/ml for these three compounds.

The three-ring templates of compounds **5** (*Fig.* 4B) and **15** (not shown) were located in the minor groove also not intercalating between the DNA bases, making the position far from the cleavage site. There was no H-bond between the compounds and the enzyme. The negative docking results agree with the negligible activity of 4.3 and 4.1% for **5** and **15**, respectively.

Although compound **10** (*Fig.* 4C) bound to the complex, the location was not between the -1 and +1 bases, but between the DNA bases of the non-scissile strand, C112 and A113. No activity was shown.

DISCUSSION

We have determined the binding modes of 20(S)-CPT and 15 benz[f]indole-4,9-dione analogs with the human binary TOP1-DNA cleavable complex by means of docking study using FlexiDock program. TOP1-DNA-ligand ternary complexes were formed and their docking energies were very low suggesting the stability of the complexes. The binding modes obtained correlated well with the experi-

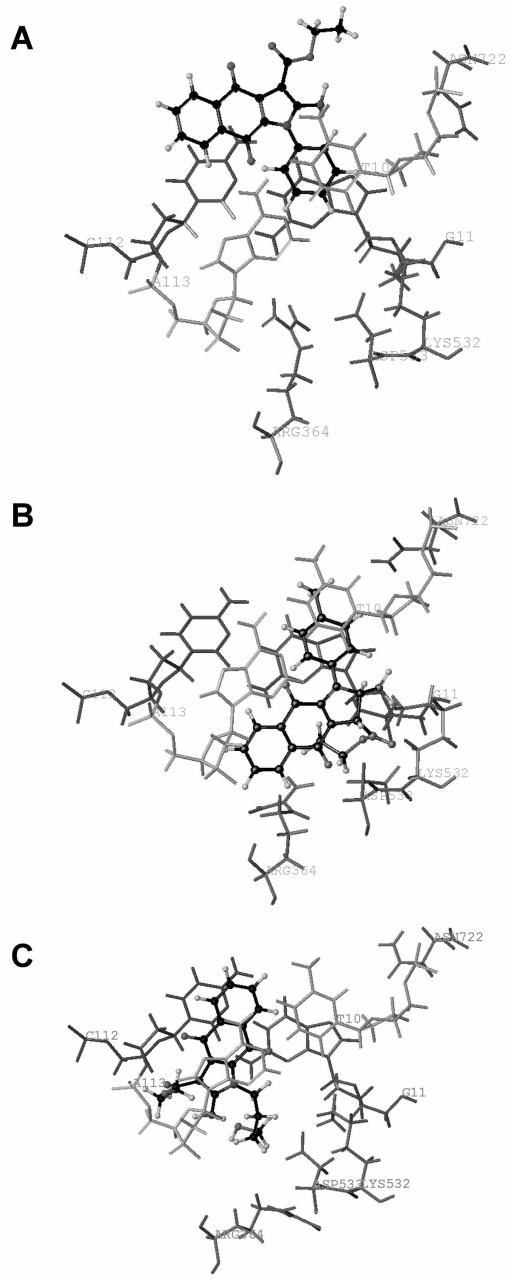


Fig. 4. Docking of compounds 7 (A), 5 (B) and 10 (C) into the human TOP1-DNA cleavable complex. A, B, C: Views looking down the DNA helix axis. Thin line: amino acid residues, Grey to black stick: DNA nucleotides, Black ball-stick: 20(S)-CPT.

mental activity data.

In general, a network of potential hydrogen bonds and van der Waals interactions contribute to the stability of the ternary complex. The stacking interactions between the compound and the DNA bases are also important.[2-6] However, the specific intercalation site of the ligand seems to have the most important impact on the inhibitory activity of the compounds. Compounds 11 and 12 that deeply intercalated toward the cleavage site exerted high inhibitory activities. As the benz[f]indole-4,9 dione moiety of these two compounds fully intercalated between the -1 and +1 bases, the functional side chains of each compound interacted with the catalytic residue, PTR723. This interaction is important as it prevents the free 5'-hydroxyl group at the nick from religation.[17]

Binding modes of compounds 1, 3, 4, 6, 8, 13 and 14 showed that either the template or the side chains of these compounds did not intercalate fully into the cleavage site. Their inhibitory activities are moderately low, despite of the H-bond formation. Compounds 2, 7, 9, 5, and 15 that were located either in the major or minor grooves and compound 10 that intercalated between the DNA bases of nonscissile strand showed the lowest or no activities.

Hydrogen bond formation with the +1 guanine may account for the good inhibitory activities since the binding of DNA intercalators is known to depend on the presence of guanine; the 2-amino group of guanine is important for the formation of a stable drug-DNA complex.[19] Many compounds (1, 3, 4, 6, 8, 11, 12 and 13) formed hydrogen bonds specifically with the 2-amino group of the +1 guanine. A hydrogen bond between the CPT and the furanose ring of +1 guanine has also been observed in the docked structure. This result agrees with the study that CPT preferentially enhances TOP1-mediated DNA cleavage when the +1 base is a guanine.[5,13] Moreover, studies by Pommier *et al.* have shown that chemically reactive analogs of CPT that contain a chloromethyl group to the C-7 position can alkylate the N-3 nitrogen of the −1 guanine after cleavage. It has been inferred from these and other studies[20] that upon cleavage and covalent attachment, the +1 guanine can assume a conforma-

tion in which it is stacked against the five-membered CPT ring system such that the guanine N-3 is in close proximity to the C-7 of CPT.[13]

Observed H-bonds with the minor groove residues in our data suggested the importance of these residues for TOP1 poisoning by drugs.[13,21] Arg364 and Asp533 in the minor groove are known to lead to resistance to CPT when mutated.[1,6,13,21] Many researchers have discussed the importance of forming H-bonds with Arg364 rather than Asp533 because its mutation would cause the loss of the critical H-bond with the CPT E-ring lactone moiety.[1] Our results strongly indicate that H-bonds with Asp533 might have more influence than that of Arg364 in the activity as compounds 4, 8, 11 and 12 having H-bonds with Asp533 showed higher activities. Compounds 11 and 12 that formed H-bonds with Asp533 showed higher activities than the CPT that formed a H-bond with only Arg364.

Lys532 functions as a general acid during cleavage to protonate the leaving 5'-oxygen. One base-specific contact, a 2.6 to 2.9 Å H-bond in the minor groove between Lys532 and the O-2 carbonyl oxygen of the -1 thymine of the scissile strand, has been observed.[13] It is assumed that this interaction is important during catalysis because it provides spatial rigidity to the active site of the enzyme.[17] Thymine is the only nucleotide base in which the two lone electron pairs of the O-2 atom are freely available to accept a H-bond from the δ-amino group of Lys532. In our docking results, CPT, 4 and 12 that formed H-bonds with Lys532 were stably stacked under the -1 thymine, so that the spatial rigidity of the active site might destabilize the enzyme inducing TOP1 inhibition.

In terms of structure-activity relations, CPT derivatives have been indicated that substitutions at the 12-position on the A-ring will decrease the activity.[22] Our results support the indication by showing the steric clash between the substituent at the 12-position and the DNA backbone (*Fig.* 1A). However, substitutions at the 7, 9, and 10-positions of the drug have been known to increase activity. Since the A and B-rings are directed out toward the DNA major groove, these positions could be substituted without steric penalty (*Fig.* 1A). Substitutions with electron-withdrawing groups including halogens as in compounds 11 and 12 seem to potentiate the activity by trapping the complex strongly. Among alkyl groups attached to N1, the ethyl of compound 4 (27.3%) and propyl of compound 12 (58.7%) showed good inhibitory activities but butyl of compound 10 (0%) seemed to be too flexible and bulky to dock in the minor groove area around Lys532 and Asp533.

Not all compounds showed obvious correlation between the binding mode and the inhibitory activity measured *in vitro*. In these cases, the cytotoxicity of the individual compound as well as the differences in uptake rate into cells, or different ways of metabolism in cells or even different mechanisms of actions might be involved.[12]

CONCLUSION

Our docking results and the TOP1 relaxation assay data have correlated well. In order for these compounds to have high inhibitory activities, they should stabilize the TOP1-DNA cleavable complex through interactions with both the enzyme and the DNA, and intercalate deeply into the cleavage site. These results are important in the future development of novel drugs as they present the binding modes that suggest the structures of ideal compounds to be developed to effective drugs. The flexible protein-ligand docking program, FlexiDock, is proven to be very useful in determining the precise binding modes of protein-ligand interactions. Until now, the only class of TOP1-targeting inhibitors approved for clinical use has been the CPT derivatives. However the present study demonstrates the possibility of benz[f] indole-4,9-dione analogs being a novel class of clinical agents that target TOP1. Further study on the mechanism of action of these compounds should be carried out based on the binding modes presented in this study.

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