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Assessment of Molecular Binding of Podophyllotoxin Analogues into ATPase Domain of Topoisomerase II Using Docking-MM-GB/SA Approach

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Abstract: Synthetic analogues of Podophyllotoxin have been used to create efficient safer anticancer drugs. One hundred twenty six analogues using combinatorial design with structural modifications of the scaffold structure of podophyllotoxin are herein described. Molecular interaction and binding affinities with ATPase domain of human DNA Topoisomerase II (TP-II) using docking-MM-GB/SA screening are illustrated. Results showed that these analogues docked in a similar position and orientation on the ATPase domain of TP-II. A linear correlation (r^2 = 0.5707) was observed between the calculated free energy of binding (FEB) and experimental IC_{50} for the inhibitors, suggesting that theses inhibitors bind weakly with TP-II. Three H-bonds between podophyllotoxin analogues (*trans* lactones) and DNA topoisomerase II were observed. The vdW energy estimated by generalized born/surface area (GB/SA) plays an important role in the binding affinity of podophyllotoxin analogues. Out of 126 derivatives, lactones tetralines were found to be the most potent in general in comparison with the *non*-lactones tetralines and non-lactones cyclolignans. This work addresses to modify the lactone moiety and prepare synthetic analogues with heteroatoms at different positions of the podophyllotoxin and further screening for a successful candidate drug in a computer-aided rational drug design.

Key words: ATPase domain, DNA Topoisomerase II, free energy of binding (FEB), FlexX, glide, GOLD and podophyllotoxin

INTRODUCTION

DNA Topoisomerase II (TP-II) is an enzyme that decatenates and disentangles DNA by passing one DNA helix through another (Berger and Wang, 1996). Due to the requirement for such a DNA strand passage activity in a number of critical nuclear processes, including replication, recombination, and chromosome segregation, TP-II is essential for the survival of proliferating eukaryotic cells (Goto *et al.*, 1985). As a prerequisite for its DNA passage reaction, TP-II generates transient double-stranded breaks in the nucleic acid backbone (Berger and Wang, 1996). In order to maintain the integrity of the cleaved genetic material during this process, the enzyme forms a proteinaceous bridge that spans the nucleic acid break. This bridge is anchored by covalent phosphotyrosyl bonds established between the active site residues of the homodimeric enzyme and the newly created 5'-DNA termini (Andersen *et al.*, 1989). Because the covalent TP-II-cleaved DNA complex (referred to as the *cleavage complex*) is normally a short-lived intermediate in the catalytic cycle of the enzyme, it is tolerated by the cell. However, when present in high concentrations, cleavage complexes become potentially toxic, promoting frameshift mutations, permanent double-stranded DNA breaks, illegitimate recombination, and apoptosis (Corbett and Osheroff, 1993; Froelich-Ammon and Osheroff, 1995). The cytotoxic potential of TP-II has been exploited clinically by the development of anticancer drugs that generate high levels of covalent enzyme-DNA cleavage complexes Froelich-Ammon and Osheroff, 1995).

Epipodophyllotoxin, teniposide and etoposide (synthetic analogues of podophyllotoxin) are the most widely prescribed chemotherapeutic agent currently used for the treatment of human cancers (Beck *et al.*, 1993), are targeted to TP- II (Osheroff, 1989; Chow *et al.*, 1988). These drugs increase TP-II-mediated DNA breakage primarily by inhibiting the ability of the enzyme to religate cleaved nucleic acid molecules (Osheroff, 1989). Efforts for improving their clinical efficacy further by overcoming the drug resistance, myelosuppresion and poor bioavailability problems (Aisner *et al.*, 1994) associated with them, were continued to be challenging.

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Consequently, the number of analogues of podophyllotoxin increased considerably. Most of these analogues prepared so far have the D-ring lactone intact. The importance of these compounds has once again gained momentum because of the discovery, showing that the D-ring modified podophyllotoxins were found to possess immunosuppressive activities (Brattain *et al.*, 1984). It was seen that changes in the configuration, size and chemical nature of substituents in the C ring of podophyllotoxin markedly affect the activity of the analogues. On the other hand, other podophyllotoxin derivatives, namely podophenazines, have also been reported to retain or even improve the cytotoxic activity, but these were weak inhibitors of TP-II in vitro (Bastow *et al.*, 1996). Over the past few years, large number of cyclolignan derivatives have prepared by modifications of different positions in the cyclolignan skeleton (Castro *et al.*, 2000). But their mechanism of action is not known. Recently, still other compounds have prepared by modifications to the A and E cyclolignan-rings and are tested on cultures of different tumoral cell lines (P-388 murine leukemia, A-549 human lung carcinoma, HT-29 human colon carcinoma and MEL-28 human melanoma) and some of them have shown an interesting and selective cytotoxicity (Castro *et al.*, 2000). The data revealed that such analogues exhibit a different, as yet unknown, mechanism of action. More studies are needed to understand such mechanisms. However, the main deficiency of these compounds is their cytotoxicity for normal cells and hence side effects derived from their lack of selectivity against tumoral cells. In this regard it is necessary to investigate and prepare new more potent and less toxic analogues, that is, with better therapeutic indices.

Modern approaches for finding new leads for therapeutic targets are increasingly based on 3-Dimensional information about receptors. An effective way to predict binding structure of a substrate in its receptor is docking simulation, which has been successfully used in many applications. Docking procedures basically aims to identify correct posses of ligands in the binding pocket of a protein and to predict the affinity between the ligand and the protein. In other words, it describes a process by which two molecules fit together in 3-Dimensional space. Combinations of the method with other methods, such as MD simulation, free binding energy calculation; comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) (Abraham *et al.*, 1994) enable to get a lot of insights on biological systems and to help rational drug design.

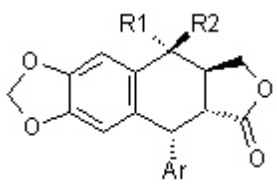
Unfortunately, the crystal structures of most the podophyllotoxin analogues complexed with TPP-II have not yet been reported. The need to determine their binding structures in the active site of TP-II and explore the interactions, as well as their binding energy for these new TP-II inhibitors is essential in order to improve the design of second generation inhibitors. Hence, in this study we have set out to dock a library of 126 podophyllotoxin analogues into TP-II (ATPase domain) and determine their binding pattern. We try to use a comparative approach based on the results obtained from docking softwares like Glide, Gold and FlexX, which implements different scoring functions and algorithms. These softwares use flexible docking approach to predict the "preferable" binding structure of the podophyllotoxin analogues in TP-II. To study the association of the ligands with the receptor further, we use the automated mechanism of Multi-Ligand Bimolecular Association with Energetics (eMBrAcE). The approach is simple, fast and straightforward. It benefits the calculation of relative binding affinity needed to evaluate the activity of large set of molecules in rational drug design.

MATERIALS AND METHODS

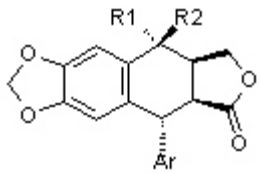
Preparation of compound libraries: A compound library of 126 podophyllotoxin analogues were built using the parental structure of podophyllotoxin (Fig. 1) as a template. These analogues were generated by structural modification of the ring structure with stearically and conformatically allowed substituents using reagent database and combinatorial design module (Schrödinger). For a better understanding of the influence of structural changes on the binding energy of these analogues and their suitability as anticancer drugs, they have been grouped into three subclasses; namely *trans* and *cis*-tetralinelactones (Table 1a-d), non-lactonic tetralines (Table 2a-g) and non-lactonic cyclolignans (Table 3a-f). Structural modifications were done as reported earlier (Castro *et al.*, 2000). Each structure was assigned an appropriate bond order using ligprep script shipped by Schrödinger. The analogues were converted to mae format (for Glide docking) and mol2 format (for use in GOLD and FlexX) using maestro (Schrödinger Inc.) and optimized by means of the MMFF94 force field using a default setting (Hayes *et al.*, 2004).

Preparation of protein target structure: The starting coordinates of the human Topoisomerase-II ATPase (TP-II)-AMP-PNP complex [PDB:1ZXM] was taken from the Protein Data Bank (www.rcsb.org) and further modified for docking calculations. For Glide (Schrödinger) calculations, TP-II complex was imported to Maestro (Schrödinger), the co-crystallized ligands were identified and removed from the structure and the protein was minimized using the protein preparation wizard (shipped by Schrödinger) by applying OPLS-AA forcefield (Jorgensen *et al.*, 1996) by application of autoref.pl script. Progressively weaker restraints (tethering force constants 3, 1, 0.3, 0.1) were applied to nonhydrogen atoms only. This refinement procedure is recommended by Schrödinger (technical notes for version 1.8), because Glide uses the full OPLS-AA force field at an intermediate docking stage and is claimed to be more sensitive to geometrical details than other docking tools.

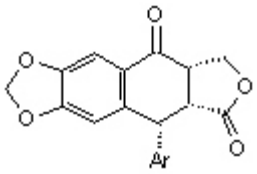
Table 1: *Trans*-tetralinelactones and *Cis*-tetralinelactones (a-d). Their docking score and calculated energies with topoisomerase II



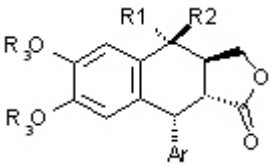
(a)(1-22)



(b)(23-42)



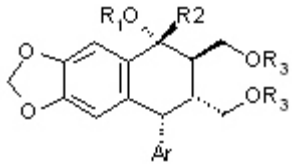
(c)(43-44)



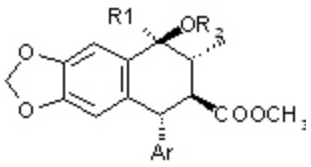
(d)(45-52)

S#	R1	R2	Calculated Energies (kcal/mol) Gvdw	Gele	Gsolv	Docked Energy FlexX (kcal/mol)	GOLD (Fitness Score)	Glide (Score)	Free Energy Binding FEB (KJ/mol)
1	OH	H	1.9	8.42	-6.97	-7.57	-5.84	-1.73	3.35
2	H	H	1.16	4.82	-4.84	-9.84	-6.72	-1.68	1.14
3	H	H (2-OMe)	-0.33	-1.15	2.93	-7.57	-6.04	-1.59	1.45
4	OH	H (2-OMe)	-0.42	-0.82	3.22	-2.44	-8.42	-1.24	1.98
5	OH	H (4'-OH)	0.21	0.74	-1.62	-12.69	44.48	-7.01	-0.67
6	OAc	H	1.26	4.90	-4.87	-5.15	23.23	-5.18	1.29
7	OAc	H (2-OMe)	2.89	4.57	-6.97	1.583	0.56	-6.04	0.49
8	OMe	H	-0.52	0.87	-1.4	-7.43	28.86	-5.64	-1.05
9	H	OH	1.66	6.96	-7.35	-1.56	24.83	-5.26	1.27
10	H	OAc	4.00	10.34	-9.7	-3.60	22.60	-3.82	4.64
11	H	OMe	-0.34	0.86	-1.24	-5.13	31.04	-5.62	-0.72
12	H	Cl	-0.12	0.45	-0.73	-6.43	22.61	-5.19	-0.4
13	Cl	H	-0.58	0.90	-1.5	-6.23	27.70	-5.52	-1.18
14	=O		-0.78	1.16	-1.24	-6.38	33.63	-5.48	-0.86
15	H	Br	3.47	6.62	-4.99	-7.08	11.94	-5.25	5.1
16	Br	H	-0.11	1.25	-8.68	-7.17	39.90	-5.39	2.47
17	H	H (4'-OH)	2.96	9.34	-8.6	-10.52	28.54	-5.56	3.7
18	H	H (4'-OAc)	-	-	-	-1.39	-	-	-
19	H	OAc (4'-OAc)	-	-	-	-1.39	-	-	-
20	=N-OH		-	-	-	-	-	-	-
21	=N-OAc		2.05	6.48	-6.07	-5.72	32.24	-5.86	2.46
22	=N-OMe		-	-	-	-	-	-	-
23	H	H	5.37	4.36	-5.04	-9.89	20.98	-5.47	4.69
24	H	H (2-OMe)	1.93	2.64	-4.36	-6.98	21.44	-5.72	0.21
25	OH	H	1.59	5.51	-5.23	-1.11	26.94	-5.43	1.87
26	OH	H (2-OMe)	-0.2	0.8	-1.42	-2.72	23.72	-5.93	-0.82
27	OAc	H	0.26	0.80	-2.03	-6.02	54.38	-7.04	-0.97
28	OAc	H (2-OMe)	1.39	5.48	-7.98	-5.77	40.29	-6.81	-1.11
29	OMe	H	-	-	-	-	-	-	-
30	H	OH	1.57	-0.12	-0.43	-4.42	18.14	-5.48	1.02
31	H	OH(2-OMe)	3.15	11.57	-9.89	-3.70	16.48	-5.77	4.83
32	H	OAc	-	-	-	-2.75	-	-	-
33	H	OAc	2.13	2.70	-4.62	-2.41	43.83	-6.04	0.15
34	H	OMe	-0.39	-0.35	-0.52	-0.13	49.98	-5.56	-1.26
35	H	Cl	2.68	7.95	-7.57	-5.21	39.37	-5.74	3.06
36	Cl	H	-0.4	1.0	-2.03	-3.27	51.87	-4.54	-1.43
37	H	H $\Delta^{8(8')}$	-0.23	0.69	-0.86	-10.24	58.42	-5.86	-0.4
38	H Δ^{7}	-	-0.91	-1.97	1.89	-7.46	43.96	-5.24	-0.99
39	=O		-	-	-	-	-	-	-
40	=N-OH		-	-	-	-	-	-	-
41	=N-OAc		-0.89	0.35	-1.93	-7.38	73.94	-8.32	-2.47
42	=N-OAc		-	-	-	-	-	-	-
	R								
43	-		-0.16	1.2	-2.32	-6.17	46.14	-5.56	-1.28
44	(4'-OH)		0.93	2.67	-3.07	-6.06	44.86	-5.09	0.53
	R1	R2 R3							
45	H	H H	-2.63	-0.66	1.98	-10.71	48.74	-8.24	-1.31
46	H	H Ac	-0.27	2.79	-1.19	-11.91	48.98	-4.48	1.33
47	OH	H H	3.19	8.84	-7.57	-4.75	48.16	-5.64	4.46
48	OAc	H Ac	2.49	3.59	-4.93	0.58	31.64	-5.04	1.15
49	H	OH H	2.54	5.34	-5.1	-8.44	36.83	-5.43	2.78
50	H	OAc Ac	1.59	3.98	-6.14	-0.59	41.95	-6.24	-0.57
51	H	H H $\Delta^{8(8')}$	-0.43	-0.33	-0.68	-11.26	54.94	-8.68	-1.44
52	H	H Ac $\Delta^{8(8')}$	0.28	6.05	-6.43	-11.91	45.94	-5.36	-0.1

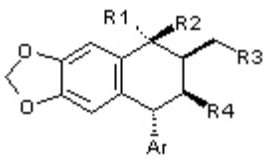
Table 2: Non-lactonic tetralines (a-g). Their docking score and calculated energies with topoisomerase II



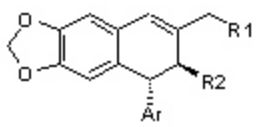
(a) (53-59)



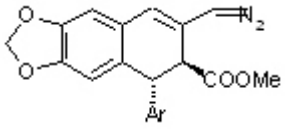
(b) (60-61)



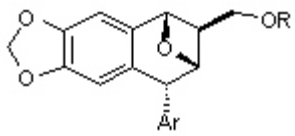
(c) (62-81)



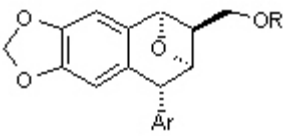
(d) (82-92)



(e) (93)



(f) (94-95)



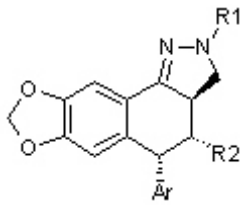
(g) (96-97)

S#	R1	R2	R3	R4	Calculated Energies (kcal/mol) Gvdw	Gele	Gsolv	Docked Energy FlexX (kcal/mol)	GOLD (Fitness Score)	Glide (Score)	Free Energy Binding FEB (KJ/mol)
53	OH	H	H		1.37	5.8	-6.82	0.02	25.07	-5.65	0.35
54	H	OH	H		2.84	7.74	-6.52	-2.48	49.84	-5.83	4.06
55	H	OAc	Ac		1.18	4.56	-5.94	5.53	43.47	-6.24	-0.2
56	H	OMe	H		1.6	1.65	-2.5	2.85	18.14	-5.29	0.75
57	H	OMe	Ac		-1.75	-0.71	1.81	1.83	55.55	-7.43	-0.65
58	OMe	H	H		2.8	6.39	-6.57	0.15	8.01	-5.72	2.62
59	OMe	H	Ac		-0.11	2.91	-1.23	-3.92	22.34	-4.32	1.57
60	H	OH			3.24	8.74	-9.71	-8.15	-56.11	-1.45	2.27
61	=O				-	-	-	-	-	-	-
62	H	H	OH	COOMe	4.57	5.21	-7.90	-9.27	-67.99	-2.34	1.88
63	H	H	OAc	COOMe	2.5	-2.31	-3.31	-4.25	49.12	-8.63	-3.12
64	H	H	OAc	CH_2OAc	3.94	6.73	-8.54	2.22	-157.49	-1.39	2.57
65	OH	H	OH	CH_2OH	2.39	6.36	-6.52	-1.43	-91.44	-2.67	2.03
66	OH	H	OH	COOMe	1.1	-2.21	-3.38	-6.71	81.87	-9.72	-4.59
67	OAc	H	OAc	CH_2OAc	-2.37	26.66	-22.04	2.14	-219.42	-1.21	2.25
68	OAc	H	OAc	COOMe	-1.28	25.86	-21.61	3.99	-224.79	-1.09	2.97
69	OMe	H	OH	CH_2OH	0.28	2.57	-0.6	-2.48	-91.06	-2.38	2.25
70	OMe	H	OAc	CH_2OAc	2.77	8.74	-7.66	0.26	-245.06	-0.31	3.85
71	H	OMe	OH	CH_2OH	-2.02	21.34	-19.03	0.74	28.94	-7.64	0.29
72	H	OMe	OAc	CH_2OAc	-1.4	-0.16	3.91	2.19	-225.45	-1.56	2.35
73	H	OH	OH	CH_2OH	-1.15	27	-23.93	-1.02	30.65	-7.88	1.92
74	H	OH	OH	COOMe	2.76	11.22	-10.2	-1.39	-82.15	-2.40	3.78
75	=O		OH	COOMe	-2.22	24.91	-21.99	-3.48	-105.48	-1.22	2.02
76	=O		OAc	COOMe	1.64	6.8	-5.87	-4.83	-154.21	-1.79	2.57
77	=N-OH		OAc	COOMe	1.26	17.71	-16.94	0.79	-203.32	-1.59	2.03
78	H	H	CHO	COOMe	-1.98	18.54	-14.93	-3.75	-119.14	-1.39	1.63
79	H	H	=N-OH	COOMe	-0.31	-0.77	0.43	-4.98	-121.14	-1.08	-0.65
80	H	H	=N-OMe	COOMe	2.27	6.92	-6.87	-3.46	-119.28	-1.77	2.32
81	H	H	=N-allyl	COOMe	3.60	8.58	-9.47	-2.13	-162.77	-1.62	2.71
	R1		R2								
82	CH_2OH		COOMe		-2.15	18.63	-14.67	-3.67	-100.02	-1.42	1.81
83	CHO		CH_2OH		-1.43	3.39	-1.98	4.66	-69.94	-2.10	1.05
84	CHO		COOMe		-4.29	32.87	-27.85	-3.53	-103.16	-1.34	0.73
85	CH=N=-NH_2		COOMe		-4.41	8.75	-12.32	-4.63	-79.29	-2.56	2.03
86	CH=N=-NH-CH_2CF_3		COOMe		-2.82	1.15	-16.4	-1.81	-100.72	-1.20	1.35
87	CH=N=-NH-Ph		COOMe		-3.33	23.46	-18.12	-5.60	-123.75	-1.93	2.01
88	CH=N=-NH-Ph		CH_2OH		-2.45	24.58	-22.51	-8.75	-88.85	-2.51	-0.38

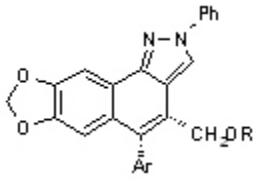
Table 2: (Continued)

89	CH=N-OH	COOMe	-2.98	25.21	-22.67	-1.71	-83.00	-2.91	-0.44
90	CH=N-OMe	COOMe	6.09	47.60	-50.06	-1.53	-113.88	-1.23	3.63
91		COOMe	-4.42	1.09	-19.32	-4.35	2.59	-3.54	-2.63
92		CH_2OH	1.13	5.7	-9.28	-1.18	3.99	-6.69	-1.55
93			-2.89	25.47	-23.97	-7.56	-102.48	-3.02	-1.39
94	H		1.67	60.1	-63.92	-5.71	17.36	-7.32	-2.15
95	Ac		-3.67	13.23	-11.06	-5.31	-20.66	-3.78	-1.1
	R								
96	H		-2.39	17	-16.29	-11.52	38.52	-7.53	-1.68
97	Ac		-6.62	24.98	-20.75	-8.03	24.64	-8.42	-2.39

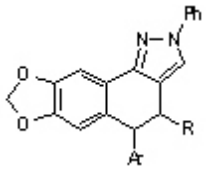
Table 3: Non-lactonic cyclolignans: Pyrazolignans and isoxagolignans (a-d) and Naphthalene lactones and non-lactonic naphthalenes (e-f). Their docking score and calculated energies with topoisomerase II



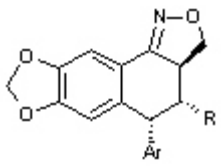
(a) (98-109)



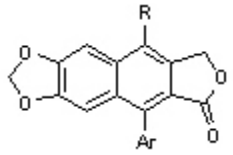
(b) (110-111)



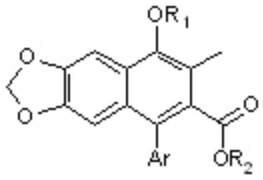
(c) (112-114)



(d) (115-120)



(e) (121-122)



(f) (123-126)

S#	R	R1	R2	Calculated Energies (kcal/mol) Gvdw	Gele	Gsolv (kcal/mol)	Docked Energy FlexX (Fitness Score)	GOLD (Score)	Glide (KJ/mol)	Free Energy Binding FEB
98		Ph	COOH	1.27	2.70	-3.29	-2.03	26.86	-8.42	-2.68
99		Ph	COOMe	2.31	-0.85	1.13	-5.96	-199.28	-1.61	2.59
100		Ph	CH_2OH	2.65	14.69	-13.45	-9.61	-186.57	-2.47	3.89
101		Ph	CH_2OAc	-0.53	3.91	-1.66	-5.86	-439.24	-1.02	1.72
102		m-NO_2Ph	COOH	-3.96	33.98	-28.15	-5.34	-273.69	-1.24	1.87
103		m-NO_2Ph	COOMe	-0.53	3.3	-1.38	-10.63	-290.82	-1.91	1.39
104		p-BrPh	COOH	-1.78	29.24	-25.42	-6.71	-494.41	-1.63	2.04
105		p-MePh	COOMe	-1.37	6.67	-2.78	-8.44	-405.21	-1.17	2.52
106		Me	COOH	-0.82	3.07	-0.73	-7.82	-104.26	-2.35	1.52
107		Me	COOMe	2.75	5.99	-7.92	-7.34	-169.02	-1.42	0.82
108		$CONH_2COOH$	COOH	-3.98	30.67	-25.11	-0.98	-149.68	-2.79	1.58
109		$COCH_3COOMe$	COOMe	-2.9	6.71	-1.67	-2.78	-248.00	-1.72	2.14
110	H			-1.5	1.04	-1.90	-7.68	-259.90	-1.34	2.16
111	Ac			-4.24	28.46	-24.63	-7.42	-126.32	-2.04	-0.41
112	CHO			-5.62	4.64	-16.45	-7.91	-187.70	-1.52	2.59
113	CH_2OH			-5.98	23.91	-15.94	-3.27	-150.51	-1.89	1.99
114	CH_2Ac			-3.82	25.92	-20.76	-6.54	-200.66	-1.45	1.44
115	COOH			1.9	8.54	-8.81	-12.05	-82.99	-1.92	1.63

Table 3: (Continued)

116	COOMe		-2.86	19.14	-15.23	-2.59	-96.21	-2.47	1.05
117	COOMe								
	(4'-OH)		3.03	-36.89	36.01	-3.14	-78.64	-1.99	2.05
118	CH_2OH			-2.18	27.81	-24.3	-4.68	-54.24	-2.341.33
119	CH_3			-2.9	26.09	-22.12	-3.36	-86.46	-1.411.07
120	CHO			-2.86	19.64	-15.23	-4.59	-61.99	-2.461.55
121	H		2.6	-22.15	22.16	-8.75	-32.30	-2.74	1.61
122	OAc			-0.94	28.02	-26.21	-5.25	-40.41	-2.400.89
123	H	H	1.98	-37.02	36.87	-4.29	-40.82	-2.56	1.83
124	H	Me	-0.53	1.61	-1.66	-3.89	-27.82	-3.68	-0.58
125	Ac	H	-1.5	5.73	-2.88	-4.70	-92.03	-2.45	1.35
126	Ac	Me	-1.37	5.67	-2.78	-3.42	-81.10	-2.68	1.52

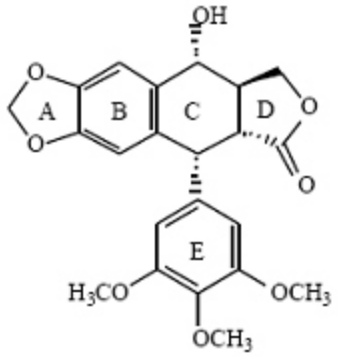


Fig.1: Parental structure of Podophyllotoxin

Water molecules were removed and H atoms were added to the structure. The most likely positions of hydroxyl and thiol hydrogen atoms, protonation states and tautomers of His residues, and Chi 'flip' assignments for Asn, Gln and His residues were selected by the protein assignment script shipped by Schrödinger. Minimizations were performed until the average root mean square deviation of the nonhydrogen atoms reached 0.3 A^o.

Glide Docking & Scoring function: Glide calculations were performed with Impact version v18007 (Schrödinger, Inc.) (Krovat *et al.*, 2005). It performs grid-based ligand docking with energetics and searches for favorable interactions between one or more typically small ligand molecules and a typically larger receptor molecule, usually a protein (Banks *et al.*, 2004). Schrödinger recommends the performance of test calculations with different scaling factors for the van der waal radii of the receptor and ligand atom, because steric repulsive interactions might otherwise be overemphasized, leading to rejection of overall correct binding modes of active compounds (Krovat *et al.*, 2005). After ensuring that the protein and ligands are in correct form for docking, the receptor-grid files were generated using a grid-receptor generation program. To soften the potential for nonpolar parts of the receptor, we scaled van der waal radii of receptor atoms by 1.00 with partial atomic charge of 0.25. A grid box of size 56 x 56 x 56 Å with coordinates X = 3.4860, Y = -36.84 and Z = 22.3853 was generated by centroid of the binding site of ATPase domain consisting of residues Asn91, Asn95, Asn 120, Lys123, Gly124, Pro126, Ile141, Ser149, Glu155 and Arg184 (Frei *et al.*, 2001) and the size of ligands to be docked was selected form the workspace. The ligands were docked with the binding site using the 'xtra precision' Glide algorithm. Glide generates conformations internally and passes these through a series of filters. The first places the ligand center at various grid positions of a 1Å grid and rotates it around the three Euler angles. At this stage, crude score values and geometric filters weed out unlikely binding modes. The next filter stage involves a grid-based force field evaluation and refinement of docking solutions including torsional and rigid body movements of the ligand. The OPLS-AA force field is used for this purpose. A small number of surviving docking solutions can then be subjected to a Monte Carlo procedure to try and minimize the energy score. The final energy evaluation is done with GlideScore and a single best pose is generated as the output for a particular ligand.

$$GScore = a * vdW + b * Coul + Lipo + Hbond + Metal + BuryP + RotB + Site$$

Where, vdW => van der Waals energy; Coul => Coulomb energy; Lipo => Lipophilic contact term; HBond => Hydrogen-bonding term; Metal => Metal-binding term; BuryP => Penalty for buried polar groups; RotB => Penalty for freezing rotatable bonds; Site => Polar interactions in the active site; and the coefficients of vdW and Coul are: a = 0.065, b = 0.130.

FlexX Docking: FlexX docking calculations were performed with conformational flexibility of ligands modeled by a discrete set of preferred torsion angles at acyclic single bonds and multiple conformations for ring systems (Kramer *et al.*, 1996). The torsional angles were taken from a database containing about 900 molecular fragments with a central single bond which has been derived from the Cambridge Structure Database (CSD) (Allen *et al.*, 1979; Klebe and Mietzner, 1994). Up to 12 low-energy torsion angles were assigned to each single bond. Multiple conformations for rings were computed with the program CORINA (Gasteigerr and Sadowski, 1993). The number of ring atoms was limited to seven. Larger rings were generally considered as rigid. FlexX modeled molecular interactions between receptor-ligand by defining the position of center and shape of interaction

surfaces which is usually part of a sphere. Different types of interactions are arranged on three levels, from level 3 for highly directional bond such as H-bonds down to level 1 for directionally unspecific bonds such as hydrophobic interactions. In placing the base fragment, FlexX first tries to direct itself by interactions of high-level types. If there are not enough such interactions, the algorithm descends to lower-level interaction types (Kramer *et al.*, 1999). FlexX uses a fragment based approach for docking a ligand to the active site of receptor. Ranking of the docking results is done with a modification of the scoring function developed by Bohm (Bohm, 1994).

$$\Delta G = \Delta Go + \Delta Grot * Nrot \quad (1)$$

$$+ \Delta ghb \sum_{\text{Neutral H-bonds}} f(\Delta R, \Delta\alpha) \quad (2)$$

$$+ \Delta gio \sum_{\text{Ionic int.}} f(\Delta R, \Delta\alpha) \quad (3)$$

$$+ \Delta garo \sum_{\text{Aro int.}} f(\Delta R, \Delta\alpha) \quad (4)$$

$$+ \Delta glipo \sum_{\text{lipo. cont}} f*(\Delta R) \quad (5)$$

The first two terms (1) of the function are a fixed ground term ($\Delta G0$ = 5.4 KJ/mol) and a term taking into account the loss of entropy during ligand binding due to the hindrance of rotatable bonds ($\Delta Grot$ = 1.4 KJ/mol). The following terms (2–4) are sums over all pairwise interactions. The last part (5) of the scoring function rates the atom–atom contacts between protein and ligand, which are hydrophobic contacts and forbiddingly close contacts (clashes).

GOLD Docking: GOLD (Glen *et al.*, 1997) uses genetic algorithm (GA) and performs automated docking with full acyclic ligand flexibility, partial cyclic ligand flexibility and partial protein flexibility in the neighborhood of the protein active site. In this study, the binding pocket of the receptor was defined from the reported article (Frei *et al.*, 2001). Dockings were performed under 'Standard default settings' mode-number of islands was 5, population size was 100, number of operations was 100,000, a niche size of 2 and a selection pressure of 1.1. The fitness score of the ligands with the receptor was determined by a sum of all the energy components as follows.

Fitness score = –(H Bond Energy) – (Internal Energy + Complex Energy).

MM and Free Energy of Binding: For the calculation of free energy of binding (FEB) of the ligands with TP-II we have used only the glide-XP docking results and only the best scoring pose for each ligand was taken into consideration. Bimolecular Association with Energetics (eMBrAcE) developed by Schrödinger was used for the physics-based rescoring procedure (Guvench *et al.*, 2002). For each ligand, the protein-ligand complex ($E_{lig\text{-}prot}$), the free protein (E_{prot}), and the free ligand (E_{lig}) were all subjected to energy minimization using OPLS-AA all-atom force field with the surface generalized Born implicit solvent model (water) with a constant dielectric electrostatic treatment of 1.0 (Todorov et.al., 2003). It uses traditional MM methods to calculate ligand-receptor interaction energies (*Gele, GvdW, Gsolv*) by a GB/SA method (Reynold's, 1995). A conjugate gradient minimization protocol with default values was used in all minimization. eMBrAcE minimization calculations were performed using an energy difference mode, in which the calculation is performed first on the receptor, then on the ligand and finally on the complex, taking as input the complexes obtained after docking analysis (Glide outputs). The energy difference is then calculated using the equation:

$$\Delta E = E_{complex} - E_{ligand} - E_{protein}$$

RESULTS

Molecular docking of podophyllotoxin and its analogues: To study the molecular basis of interaction and affinity of binding of the podophyllotoxin and its analogues, all the ligands were docked into the ATPase domain of TP-II. ATPase domain is the probable binding site for etoposide (an analogue of podophyllotoxin) as reported earlier (Frei *et al.*, 2001). The docking result of these ligands is given in Table-1, 2 & 3. The ranking of ligands was done based on the glide score for glide docking, fitness score for GOLD docking and docked energy for FlexX docking. In this study the three different docking approaches were used to cross-validated the predicted result, this is because all the three methods used different algorithms. It was seen that in few cases the ranking of the analogues was different for all the three docking approach. However, for ligands considered as good binder the ranking was consistent among GOLD and Glide docking with exception of FlexX in few cases. The Glide result also demonstrated that the docking simulations docked all the analogues into the same binding site as well (Fig. 2a, b).

Out of 52 podophyllotoxin analogues belonging to lactones tetralines (Table 1a-d) groups, only 4 analogues (serial number 1, 2, 3 & 4) were found not to be good binder with ATPase domain (low docking score). The docking score using FlexX varies from 1.58 Kcal/mol to a minimum of -12.69 Kcal/mol, fitness score using GOLD varies in between -5. 84 to 58.42 and Glide score varies from -3.82 to -8.68. The calculated free energy of binding (FEB) among lactones tetralines analogues varies in between -2.47 to 4.83 KJ/mol and the overall difference was also very small ($\pm$2.44 KJ/mol). Finally the analogues (S#: 5, 14, 16, 27, 28, 33-38, 41, 43, 44, 45-47 and 49-52) with FelxX docked energy < -5.77 Kcal/mol, Gold fitness score >32 and Glide score < -6.24 revealed that these

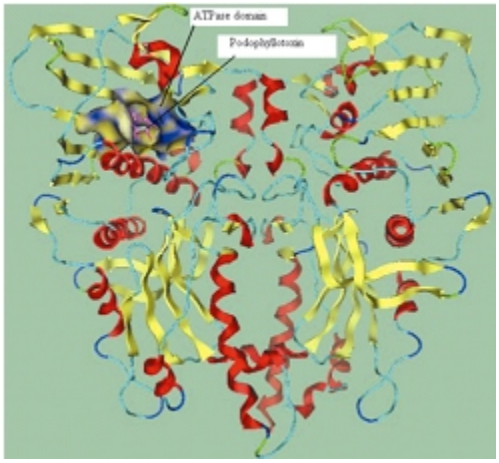


Fig. 2a: Podophyllotoxin analogue docked into ATPase domain of Topoisomerase II (tunnel view);

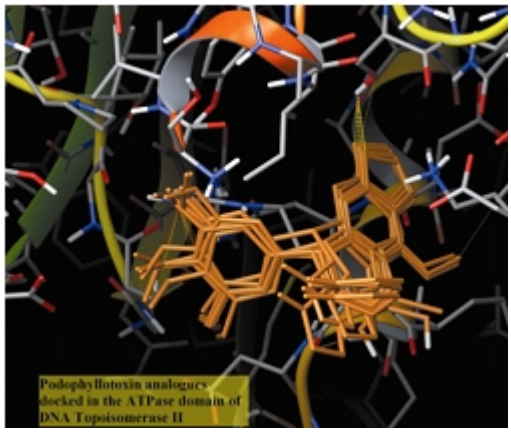


Fig. 2b: Docked podophyllotoxin analogues in ATPase domain of Topoisomerase II showing similar position and orientation in binding.

molecules bind in ATPase domain with high affinity and with free energy of binding (FEB) in between -2.47 to 3.06 KJ/mol.

The docking result of another group of podophyllotoxin analogues (nonlactonic tetralines) with ATPase domain of TP-II is given in Table 2a-g. Out of 45 analogues of this group only 7 analogues (S#: 54, 55, 57, 63, 66, 96 and 97) were considered as best binder with ATPase domain based on their docking score (FlexX docking score: -11.52 to1.83 Kcal/mol; Gold fitness score: 81.87 to 24.64; Glide score: -9.72 to -5.83) and the free energy of binding -4.59 to 4.06 KJ/mol. The energy difference (FEB) among these analogues was also very small (±1.94 KJ/mol).

For non-lactonic cyclolignans analogues (total29) the docking result is given in Table 3(a-f). Only one analogue (S#: 98) of this group was considered as best binder with ATPase domain based on its docking score (FlexX docking score -2.03 Kcal/mol; Gold fitness score 26.86; Glide score -8.42) and the free energy of binding is 8.63

Fig. 3: Superimposition of podophyllotoxin analogues displaying common tetra ring.

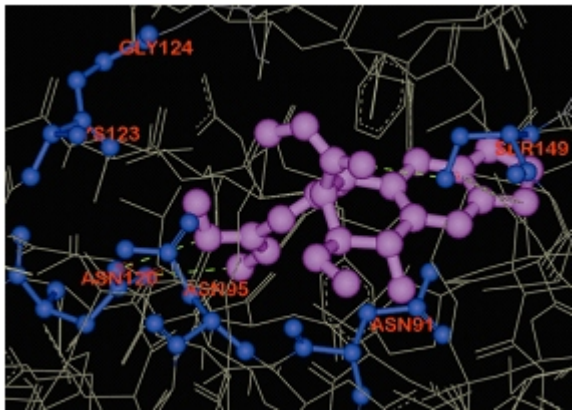


Fig. 4: Docked podophyllotoxin analogue displaying H-bond interaction (green dotted lines) in ATPase domain of Topoisomerase II.

KJ/mol. The energy difference (FEB) among them is found to be very large (± 2.97 KJ/mol).

By superposing the scaffold structure of the best binding analogues (based on the lowest glide score) (RMSD varies from 0.04 to1.32 Å), it is seen that these analogues bind in the same orientation and similar position in terms of the common structure (tetra-ring part) (Fig. 3). As these molecules have the same backbone structure of the tetra-ring, it is obvious that they bind in similar pattern in the ATPase domain of TP-II. The amino acids putatively involved in the binding of podophyllotoxin analogues are Asn91, Asn95, Asn120, Lys123, Gly124 and Ser149 (Fig. 4), which could collectively contribute three stabilizing hydrogen bonds to the ligands. The polycyclic central domain of podophyllotoxin and its analogues fits in the trough which

is define by Lys123, Pro126, Ile141, Ser149 and Glu155 residues. The binding of the podophyllotoxin and its analogues thus predicted to impede but not eliminate ATP binding as the ATP binding pocket of TP-II consisting of amino acid residues Asn46, Asp73, Ile78, Arg136 and Thr165 (Frei *et al.*, 2001) just above the binding site of podophyllotoxin analogues.

Calculated free energy of binding versus activity: For all the 126 podophyllotoxin analogues used in this studied the biological activities (IC_{50}) with respect to inhibiting the proliferation rate with different cell lines were obtained from Castro *et al.* (2000) but their mode of action at cellular level is not known. One docking structure with best pose (lowest glide score) for each ligand was taken into consideration for calculation of free energy of binding (FEB) using eMBrAcE (Schrödinger). The interaction energy was calculated after a minimization was performed on a docked ligand in which atoms within 7.5Å from the ligand were free to move (other atoms were fixed). The interaction energy includes an implicit solvation (H_2O) term. A vdW, solvation and electrostatic energy were calculated for each minimized complex. A similar scheme to linear response was used to develop a free energy of binding (FEB) relationship based on these energies of podophyllotoxin analogues (Table 1-3). The plot of the FEB and log ($1/IC_{50}$) is shown in Fig. 5. The linear trend in the plot indicated that the docking calculation produces reasonable binding modes. Based on a correlation study, it is seen that vdW energy (*GvdW*) has most significant correlation (R^2= 44.14%) followed by electrostatic energy (*Gele*) (R^2 = 3.8%) and solvation energy (*Gsolv*) (R^2= 1.5%) to the FEB. It indicates that in the binding of podophyllotoxin analogues, vdW energy terms estimated by GB/SA may be a major driving force to their binding and contributes to their activity.

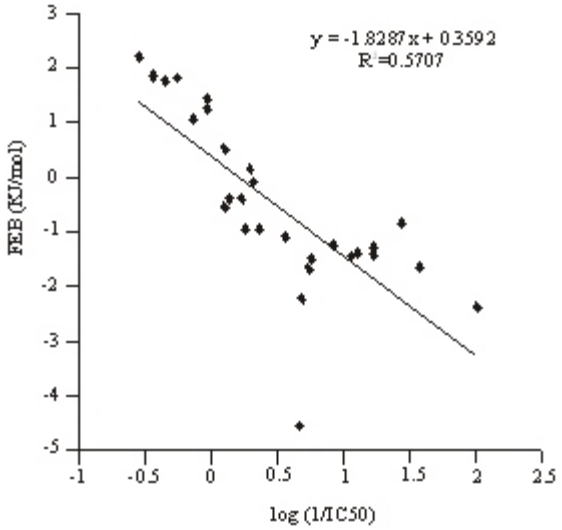


Fig. 5: Regression plot of calculated FEB (KJ\mol) vs. experimentally observed activity (IC_{50}) of podophyllotoxin analogues.

DISCUSSION

In Table 1-3, we have seen that the docking energies are different for the various algorithms (FlexX, GOLD & Glide). We know that the docking energy is a criterion used to score the docked configurations during a docking simulation and it is not the actual interaction energy. We realize that because all the three docking softwares used in the study uses different algorithms, which yield different results.

In the docking calculation, the calculated FEB of the 126 podophyllotoxin analogues in TP-II ATPase domain-based docked structures demonstrates a linear correlation (R^2 = 0.5707) with their experimental log ($1/IC_{50}$) value. This concludes that the structural modification implemented in this study is significantly related to their biologically activity and possibly these analogues interact with the ATPase domain of TP-II. Also this proved the reasonability and reliability of the docking results. Theoretically, FEB can be partitioned into several components: vdW, electrostatic, solvation and entropy energy term. However, to relative rigid molecules, the entropy is relatively small and normally is ignored or cancelled in relative free energy calculation. Further, in the rational drug design, the calculation of relative FEB rather than absolute FEB is important. Several works have been reported, in which reasonable correlation between calculated FEB and activity for a small set of molecules.

The prepared analogues were evaluated using docking approach and their free binding energy with TP-II. The docking results show that the structurally homologous inhibitors bind in a very similar position and orientation in TP-II, which suggest that the homologous inhibitors have similar binding patterns and interaction modes in ATPase domain, and further have similar inhibitory mechanism. Furthermore, the most potent inhibitor should have the best interaction with TP-II and were docked deepest into the pocket (lactonic analogues; S#:1 to 52) in general than that of molecules without lactonic moiety (S#: 53 to 97). Non-lactonic cyclolignans, the least potent inhibitor were docked shallowest. This suggests that a good inhibitor docked most deeply into the active site of TP-II would have the best interaction energy (FEB) with TP-II. Another important group of podophyllotoxin analogues are those having heterocycles fused to the cyclolignan's skeleton: pyrazoline and isoxazoline rings (S#: 98 to 120). All these derivatives found to be having more positive free binding energy in general comparing to other groups and thus were proved to be less binder than podophyllotoxin lignans with TP-II. Therefore, it is confirming that the presence of the lactone moiety is a prominent requirement for better binding and activity to be achieved by the podophyllotoxin analogues.

Despite the above, not much is known about the mode of interaction of podophyllotoxin analogues and it is still not clear whether the interaction between these

analogues and topoisomerase II is able to account for all the pharmacological effects observed for these types of substance. Instead, it is believed that a tertiary complex is formed by the lignan, topoisomerase II and the actual DNA itself. Another unknown aspect is whether these substances act as such or whether metabolites generated in the organism are responsible for the observed cytostatic effect. However, it is known that in the organism podophyllotoxin-like substances are transformed into their epimers or are demethylated and the demethylated derivatives seem to be involved in the action and mechanism of these compounds. Moreover, it is possible that lignan derivatives could act through a different mechanism, such that further studies are required to elucidate this mechanism of action.

CONCLUSION

A FEB calculation on the binding affinity of 126 podophyllotoxin analogues with the ATPase domain of human DNA Topoisomerase II is described. The binding structures of these ligands in TP-II were predicted by flexible docking simulations. Superposition of the binding structure of whole set of ligands from docking shows that these structurally similar ligand bind in a very similar pattern in TP-II. They bind in a similar position inside the ATPase domain (a tunnel) of TP-II to fit the binding pocket well. The calculated FEB for these ligands reasonably correlated with the experimental activity (IC_{50}) of same set of ligands and suggested that these analogues presumably interact with ATPase domain of TP-II. The result shows that the linear combination of three energy terms: vdW, electrostatic, and solvation (electrostatic part) has power to express the binding affinity of large set of ligands in receptor. The Docking-MM-GB/SA combination demonstrates a good ability on the binding structure prediction and binding energy determination.

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