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Design, synthesis, biological evaluation and QSAR studies of novel bisepipodophyllotoxins as cytotoxic agents

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Abstract—Two moieties of epipodophyllotoxin have been linked at C4-position to provide novel bisepipodophyllotoxin analogues. These have been evaluated for their anticancer potential and DNA–topoisomerase II poisoning activity. Most of these analogues have exhibited promising in vitro anticancer activity against different human tumour cell lines and interestingly 4′-*O*-methylated analogues have shown increased cytotoxic activity. Similarly, the DNA–topo II poisoning activity tested for these compounds has not only exhibited the DNA cleavage potential comparable to etoposide, but for some compounds this cleavage potential is superior to etoposide. Further, an interesting structure–activity relationship of these epipodophyllotoxin dimers have been generated on the basis of GI_{50} values. The equations indicated that GI_{50} activity is strongly dependent on structural and thermodynamic properties. These QSAR results are discussed in conjunction with conformational analysis from molecular modelling studies. QSAR models developed in these studies will be helpful in the future to design novel potent bispodophyllotoxin analogues by minor structural modifications.

1. Introduction

Podophyllotoxins belonging to the aryltetralin lactone class of compounds have been used as medications by folklore for more than 1000 years. In 1946, the antimitotic property of podophyllotoxin (**1**) was defined but its high toxicity prevented its use in clinic. Podophyllotoxin has a wide spectrum of effects on biological systems including inhibition of viral replication. Moreover, it has been used for the treatment of venereal warts by its topical application. Its powerful cytotoxic properties have been attributed by its binding to tubulin during mitosis and thus inhibiting microtubule assembly. **1** upon 4′-*O*-demethylation and the change of stereochemistry from α to β at the C4 position is referred to as epipodophyllotoxin (**2**). The programme on identifying antineoplastic agents based on podophyllotoxin ring system led to the discovery of etoposide (**3**) and teniposide (**4**) as shown in Figure 1.

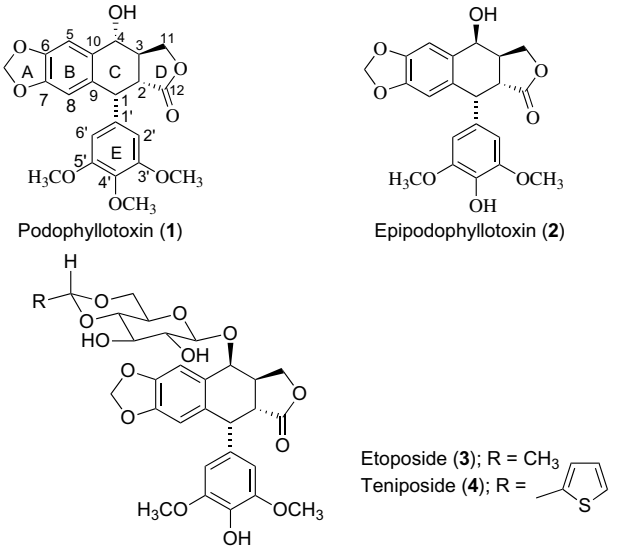

Figure 1. Structures of podophyllotoxin (**1**), epipodophyllotoxin (**2**), etoposide (**3**) and teniposide (**4**).

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Etoposide, a semisynthetic glucoconjugate of **2** has found widespread clinical application as an anticancer agent for more than two decades.[1] Its clinical success, as well as its partially understood mechanism of action[2] (DNA–topoisomerase II inhibition) has stimulated interest in further structural modifications of podophyllotoxin and epipodophyllotoxin. Recently a two-drug model for the action of etoposide against a human topoisomerase IIα has been suggested.[3] Synthesis and biological evaluation of epipodophyllotoxin analogues with di- and tri-substituted anilines at the 4β-position have been investigated extensively. It has also been demonstrated that *O*- and *S*-aryl substitutions at 4β-position on the epipodophyllotoxin ring system are comparatively less active than *N*-aryl substituted congeners. These efforts have led to the development of several congeners that have emerged as promising drug candidates, that includes: etopophos (**5**),[4] NPF (**6**),[5] GL-331 (**7**)[6] as shown in Figure 2. Recently, **5**, which is a disodium phosphate salt at the 4′-phenolic oxygen[7] of **3** is being used in the clinic as a prodrug of etoposide. Interestingly some of the C4-β-*N*-aminoaryl analogues such as **6** and **7** that contain *p*-fluoro and *p*-nitro anilino moieties respectively at 4β-position instead of a glycoside have demonstrated many fold increased potency in inhibiting human DNA–topo II and more activity in cellular protein–DNA complex formation when compared to **3**. Further, **7** is undergoing phase II clinical trials and has shown positive results against etoposide resistant malignancies.[8]

A comparative molecular field analysis (CoMFA) and novel CoMFA/q^2-GRS technique were developed[9] to identify the essential structural requirements for the podophyllotoxin derivatives to form topo II–DNA complexes. This comparative study revealed that the CoMFA steric and electrostatic fields are compatible with stereochemical properties of the DNA backbone. This investigation suggested that topo II inhibitory activity was related to three structurally distinct pharmacophoric domains,[10] the DNA intercalating moiety, the minor groove binding site and molecular region that can accommodate a number of structurally diverse substituents, which could also interact with minor groove (Fig. 3). This hypothesis has considerable strength in rationalizing and in predicting the future avenues in drug development by topo II inhibition.

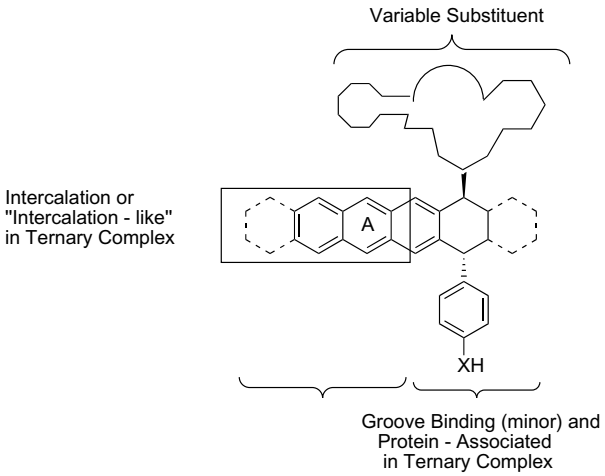


Figure 3. A composite pharmacophore model for etoposide-like analogues expressing the topoisomerase II activity.

There is current interest in dimeric analogues of lipholic, neutral, DNA mono-intercalating agents as potential anticancer drugs. The bis(naphthalimide) LU 79953 (**8**) is a DNA bis-intercalator and very potent cytotoxin, with broad-spectrum activity against a variety of human solid tumour cell lines and is in phase I clinical trials.[11] Similarly, a series of tricyclic aromatic carboxamide, the bis(acridine-4-carboxamide) and the related bis-compounds (**9**) have exhibited large increase in potency over their monomeric counterparts.[12] It is also reported in the literature that several benzimidazole derivatives act as topoisomerase I poisons and more recently bibenzimidazoles and 5-phenylterbenzimidazoles (**10**) have been identified as poisons for topoisomerase[13] (Fig. 4).

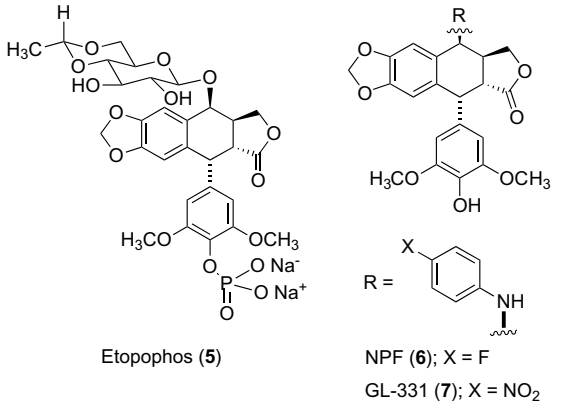


Figure 2. Epipodophyllotoxin congeners.

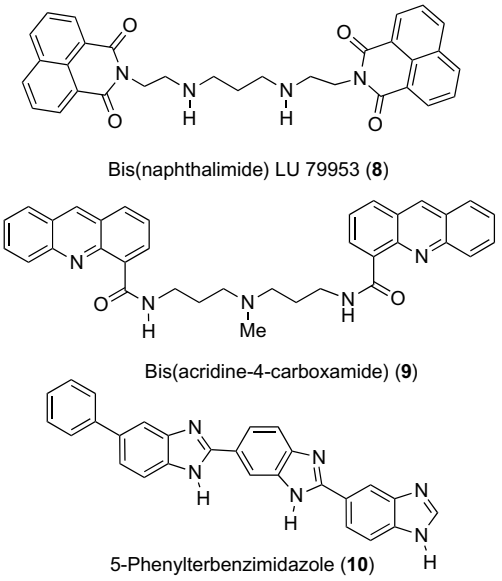


Figure 4. Dimeric analogues of DNA intercalators.

We have been interested in the design of new synthetic strategies[14] for the podophyllotoxin based derivatives and also the development of new congeners of podophyllotoxin as potential anticancer agents.[15] In continuation of these efforts it was considered of interest to synthesize bisepipodophyllotoxin congeners by linking two 4β-amino podophyllotoxin moieties with suitable aryl spacers.[16] It has been assumed that in such podophyllotoxin dimers the intercalation component is likely to be enhanced significantly along with the groove binding sites. There have been reports on the synthesis of various podophyllotoxin conjugates such as linking comptothecin,[17] lexitropsin[18] and pyrrolecarboxamidino components[19] at C-4β-position of podophyllotoxin moiety. Such structural modifications have displayed a combined spectrum of cytotoxic activity against various drug resistant KB-cell lines. This unique cytotoxic spectrum and interesting biochemical properties of such podophyllotoxin conjugates suggested the antitumour potential of these, and similar type of compounds. In this context, linking two podophyllotoxin moieties with different aryl spacers could display useful biochemical properties.

Therefore, the aim of the present work was to investigate the cytotoxicity, topoisomerase inhibition and SAR studies of such novel bisepipodophyllotoxins and the effect of different aryl spacer linkers at C-4β-position.

2. Results and discussion

2.1. Chemistry

The dimers of epipodophyllotoxin (**14**) and 4′-*O*-demethylepipodophyllotoxin (**15**) have been prepared from podophyllotoxin via their 4β-bromo intermediates (**12/13**). These 4β-bromo intermediates have been prepared by bromination and selective demethylation employing modified Kuhn's method.[20] The bromo intermediates: 4β-bromopodophyllotoxin or 4β-bromo-4′-*O*-demethylepipodophyllotoxin have been coupled with substituted diaminoaryl compounds to afford the desired bisepipodophyllotoxins (**14/15**) in good yields as illustrated in Scheme 1. The key step for the synthesis of 4β-anilino dimers is stereoselective nucleophilic displacement of bromine in 4β-bromoepipodophyllotoxin presumably via SN^1 mechanism, occurring on the C-4 benzylic carbonium ion. The bulky C-1 pendant aromatic E ring together with $Bu_4N^+I^-$ directs the substitution in an stereoselective manner, resulting in the formation of C-4β-orientation as the main product in improved yields. Recently, displacement reaction in the presence of $Bu_4N^+I^-$ indicated the feasibility of the dynamic kinetic resolution process.[21]

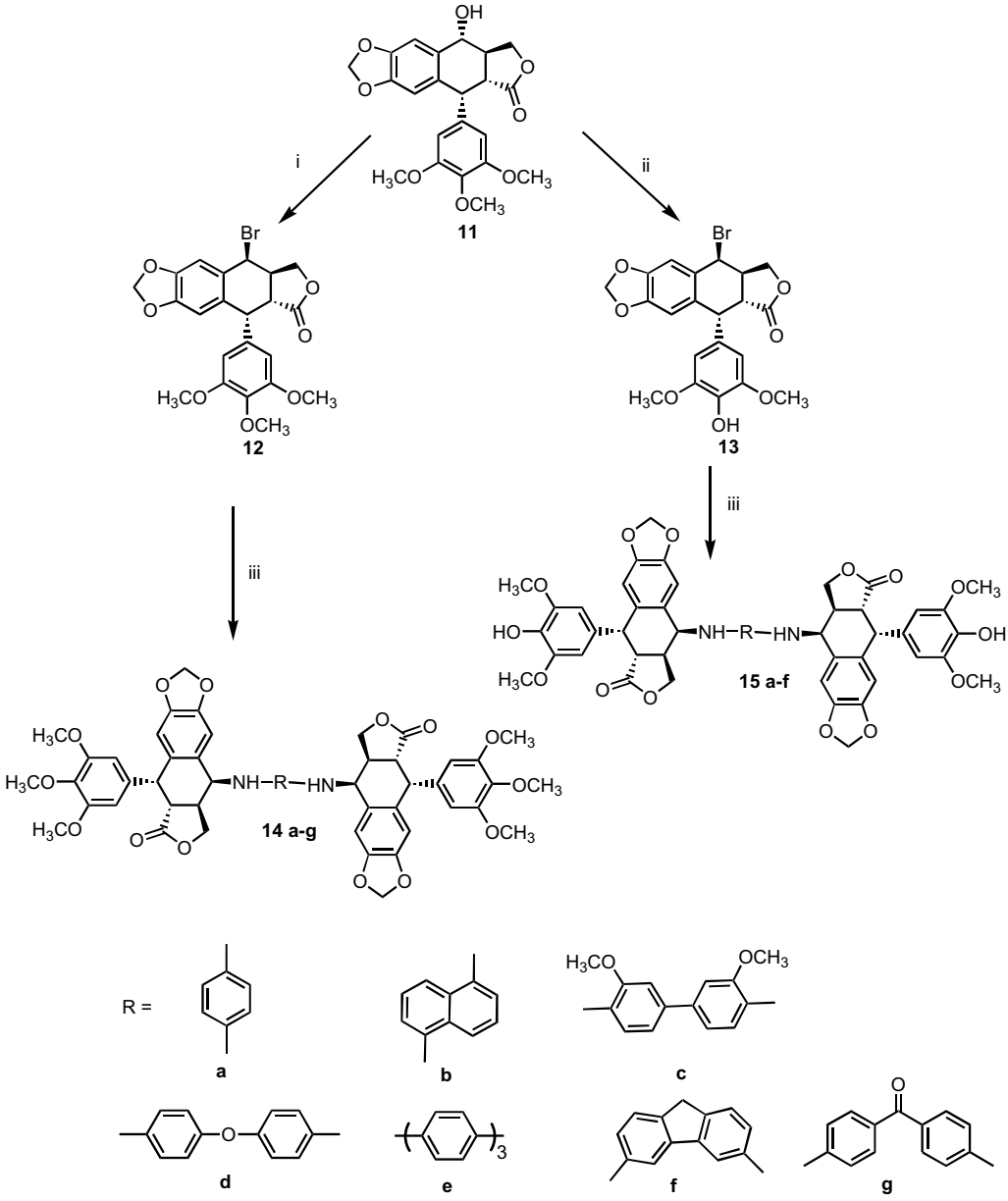
In order to further enhance the yields of these dimers particularly 4′-*O*-demethylbisepipodophyllotoxins, an alternate synthetic strategy has been attempted based on the application of iodotrimethylsilane (TMSI). The earlier literature studies[22] have demonstrated the effectiveness of TMSI attacking hindered methoxy group in a regiospecific fashion. Moreover, it is an excellent reagent for iodination of alcohols and in our earlier studies we have been interested in exploring the application of TMSI for different organic transformations such as azido reductive processes.[23] Therefore, TMSI has been used for both 4′-*O*-demethylation and C-4 epimerization for the podophyllotoxin ring system to provide the required key iodointermediate (**16**). Interestingly, the simultaneous iodination–demethylation sequence of **11** occurs to give **16** without the detection of γ-lactone opening.[24] This 4′-*O*-demethyliodointermediate (**16**) upon coupling with diamino substituted aryls in presence of a base like $BaCO_3$ yields 4′-*O*-demethylbisepipodophyllotoxins (**15**) in high yields as shown in Scheme 2.

2.2. In vitro cytotoxicity

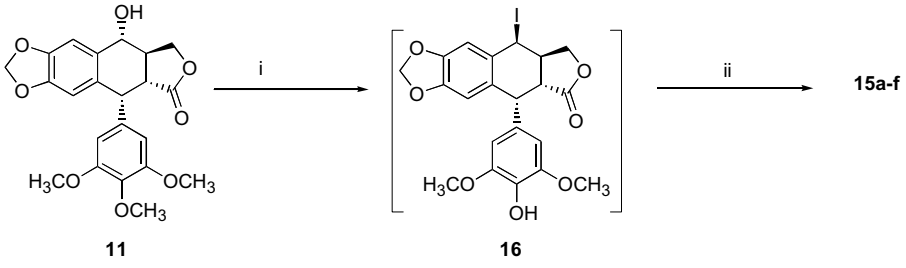
On the basis of the in vitro testing results, most of these new podophyllotoxin dimers linked through different substituted aryl rings exhibited significant anticancer activity against different human tumour cell lines as depicted in Table 1. It is interesting to observe that there is a decrease in activity for 4′-*O*-demethyl dimers of epipodophyllotoxin compared to their 4′-*O*-methylated analogues. However, 4′-*O*-demethylated epipodophyllotoxin dimers linked through phenyl and substituted biphenyl spacers, and also most of the 4′-*O*-methylated compounds show enhanced or comparable cytotoxic activity in comparison to etoposide. Further, these 4′-*O*-demethyl dimers linked through naphthyl (at 1, 5 position, **15b**) and fluorene (at 2, 7 position, **15f**) are almost inactive in most of the cell lines. Therefore, it appears that there is some correlation between anticancer activity and some type of steric crowding, which takes place between the two moieties of podophyllotoxin that are linked through such aryl groups. Some of these compounds have also been evaluated in vitro against 60 human tumour cell lines derived from nine cancer types (leukaemia, nonsmall cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast) and these results have been obtained from National Cancer Institute USA (Fig. 5). The compound **15e** exhibits cytotoxic potency in CNS cancer panel, in which the growth of SF-539, SNB-75 cell lines are inhibited, with GI_{50} value of $<0.01\,\mu M$. In the ovarian and CNS cancer panel growth of IGROVI, SF-539 cell lines are inhibited by compound **15f** with GI_{50} value as 1.56 and $0.978\,\mu M$, respectively.

2.3. DNA–topoisomerase II poisons

Topo II poisoning activity of these dimers has been investigated by the analysis of 'cleavable complex' formation by the enzyme. In the cleavage complex the compound has been covalently linked with topo II and broken DNA in the form of ternary complex, treatment of this complex with SDS leads to formation of linear DNA. Hence, analysis of linear DNA formation by the drug in presence of topo II reflects the ability of the



Scheme 1. Reagents and conditions: (i) HBr, CH_2Cl_2, 0 °C, 45 min; (ii) HBr, CH_2Cl_2, 48 h; (iii) NH_2–R–NH_2, $Bu_4N^+I^-$, Et_3N, THF, rt, 6 h.



Scheme 2. Reagents and conditions: (i) TMSCl/NaI, CH_3CN, rt; (ii) NH_2–R–NH_2, $BaCO_3$, rt, 2–10 h.

test compound in induction of cleavage complex as a transient intermediate thus exhibiting the topo II poisoning activity. Preliminary results of the experiments conducted for these bisepipodophyllotoxins show that most of them induce the formation of cleavable complex with topo II and this is illustrated by the formation of linear DNA in Figure 6 (lane #4–15). Among the compounds tested, **14e** (lane #4 and #5) has shown strong cleavage of DNA and thus suggesting its anticancer potential.

Table 1. In vitro cytotoxicity of various bisepipodophyllotoxin derivatives against human tumour cell lines (GI_{50} values)

Compound no	DU 145 (Prostate)	HT29 (Colon)	MCF7 (Breast)	MCF7ADR (Adr. Res. breast)	SKNMC (CNS Neuro blastoma)	U251 (CNS Glioma)	LOX (Melanoma)
14a	0.3	0.3	3.3				0.4
14b	1.9	0.2	2.8	0.7	0.3	1.2	
14c	0.2	0.2	0.6				
14d	0.1	0.1	0.2	0.2	0.01	0.1	
14e	0.1	0.01	0.5	0.02	0.01	0.1	
14f	0.1	0.04	0.2	0.1	0.03	0.04	
14g	0.2	0.2	0.1				0.1
15a	0.02	0.5	0.02	0.9			0.02
15b	11.0	7.1	22.2	6.8	4.1	4.8	
15c	0.3	0.3	0.5	0.01			0.65
15d	7.4	0.8	2.2	2.8	1.7	4.2	
15e	2.7	1.4	2.9	3.3	1.4	1.5	
15f	19.1	13.9	28.5	12.7	9.4	10.3	
Etoposide[a]	0.8	59	4.3	116		6.4	5.3

[a] Values from NCI database.

2.4. Quantitative structure–activity relationships

Structure–activity relationships of these dimers of epipodophyllotoxin have been investigated quantitatively by use of genetic function analysis. In this study relationships between the biological activities and various physicochemical descriptors have been obtained by the conversion of GI_{50} values to their log(1/molar activities) as depicted in Table 2. The preliminary survey of r^2 values have indicated that only structural and thermodynamic properties gave higher correlations and are considered for final regression.

The equations derived from some of the cell lines, for example, prostrate, colon and breast cancer are described in Table 4. These are the best equations (highest r^2 values) derived by deleting two of the compounds **14d** and **14g**. The degree of fit ranges from strong trends in colon cancer to potentially predictive level in prostrate and breast cancer. However, the best correlation coefficient (0.81) is obtained when the mean of all cell lines is considered. The high value of F (33.67) and a low PRESS value (1.3) support the findings. In order to confirm these findings, $-\log GI_{50}$ have been estimated from the above equation and compared with the observed values (Table 2) and such correlations are graphically presented in Figure 7. It has been observed from the cytotoxicity values (Table 1) that the most potent compounds are those with 4′-*O*-methyl substitution. The dimer (**14c**) linked through the biphenyl spacer is the most active compound as shown in Table 2. The dimers linked through biphenyls or substituted biphenyl spacers (**b**, **c**, **d** and **f**) are well predicted and this may be due to their enhanced hydrophobicity.

The correlation matrix (Table 3) shows that there is reasonable orthogonality between the variables of the equation except for dipole and MR, which have some colinearity (i.e., both have +ve values in the equation and almost same numbers). However, it is to be noted that the area contribution to the activity (GI_{50}) is minimal because this term has a small coefficient. Since MR is primarily a measure of substituted bulk, the positive MR term is presumed to model positive steric and/or dispersion effects, thus increasing the value of log(1/GI_{50}). Nevertheless steric effects are involved in this process, the hydrophobic and electronic factors also have their role.

Another important aspect, which has to be addressed is the distance between the two podophyllotoxin moieties. Molecular modelling studies show that in an extended conformation the biphenyl group which links the two moieties allows proper spacing between the two subunits that correspond to a distance of 9.91 Å. From the GI_{50} data (especially as expressed in the geometric mean), only the **14c**, **15c** and **14g** analogues are clearly superior to other aryl spacers. Therefore, in case of larger linkers than a biphenyl group such as triphenyl spacer (**15e**) and also a smaller spacer than the former such as **14a** and **14b** and **15a** and **15b** the GI_{50} increases.

However, QSAR equation without the molecular graphic models that are based on X-ray co-ordinates of the ternary complex cannot be convincing. The good agreement between the mathematical model and the graphics model[25] confirm our assumption in this approach towards the understanding of DNA–topo II–ligand interactions. In fact, in most of the examples described in the literature on SAR studies based on the aforesaid approach have shown convincing results.

3. Conclusions

In conclusion new epipodophyllotoxin dimers have been synthesized and evaluated for their in vitro anticancer potential. The cytotoxic assay for most of these analogues demonstrated many fold increase of activity in comparison to etoposide. These bisepipodophyllotoxins have shown moderate to strong DNA–topoisomerase II poisoning activity. Although earlier studies on etoposide or epipodophyllotoxin derivatives have shown that 4′-*O*-demethyl is crucial for activity whereas the present study has revealed that 4′-*O*-methyl contributes to the activity in these bisepipodophyllotoxins. Further studies are

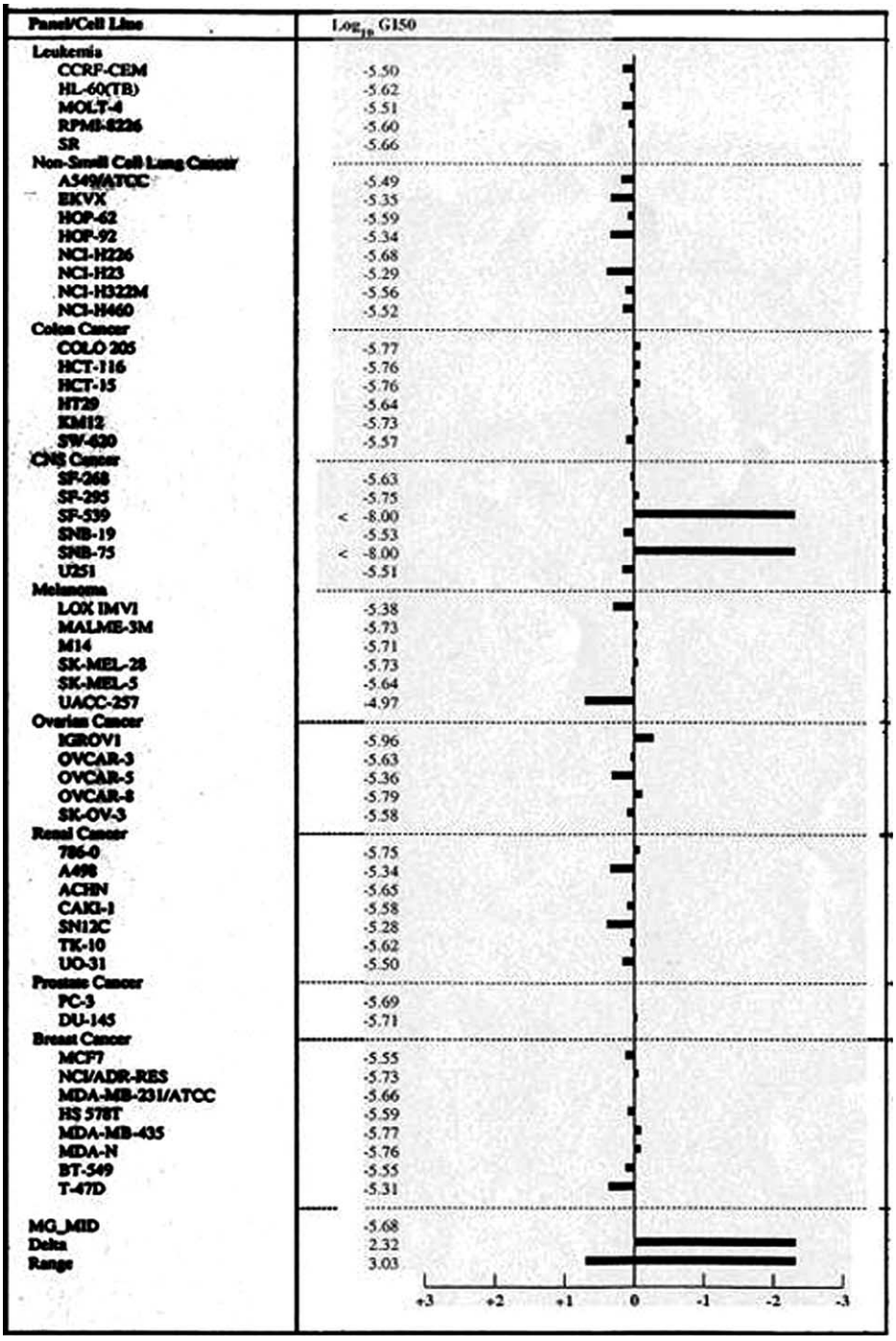


Figure 5.

underway to determine this aspect in other podophyllotoxin based derivatives. Moreover, some interesting QSAR equations have been developed for cytotoxic activity and the correlation between the actual versus predicted activities are in good agreement particularly by taking into account the molecular graphic models. Therefore, these models are expected to facilitate the rational design of these novel molecules based on the epipodophyllotoxin skeleton. Investigations are also underway to search the potential activity of these novel compounds against the DNA as minor groove binders in order to explain the mechanism of action.

4. Experimental section

4.1. Synthetic chemistry

Reaction progress was monitored by thin-layer chromatography (TLC) using GF_{254} silica gel with fluorescent indicator on glass plates. Visualization was achieved with UV light and iodine vapor unless otherwise stated. Chromatography was performed using Acme silica gel (100–200 mesh). The majority of reaction solvents were purified by distillation under nitrogen from the indicated drying agent and used

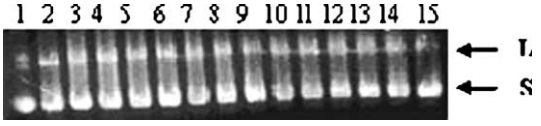


Figure 6. Topo II catalyzed cleavage of pRYG DNA in presence of bisepipodophyllotoxin analogues: Supercoiled DNA was incubated with 50 and 100 μM compound and cleavage assay was conducted. The samples were resolved on 1% agarose gel, ethidium bromide stained and photographed. L refers to linear DNA and S refers to supercoiled DNA. Lanes, #1: DNA alone, #2: Topo II and #3: in presence of 100 μM etoposide. #4 and #5: 50 and 100 μM **14e**, #6 and #7: 50 and 100 μM **15e**, #8 and #9: 50 and 100 μM **15b**, #10 and #11: 50 and 100 μM **14b**, #12 and #13: 50 and 100 μM **15f**, #14 and #15: 50 and 100 μM **14a**.

fresh: dichloromethane (calcium hydride), tetrahydrofuran (sodium benzophenone ketyl), acetone (potassium permanganate), acetonitrile (phosphorus pentoxide).

^{1}H NMR spectra were recorded on Varian Gemini 200 MHz spectrometer using tetramethyl silane (TMS) as an internal standard. Chemical shifts are reported in parts per million (ppm) down field from tetramethyl silane. Spin multiplicities are described as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Coupling constants are reported in Hertz (Hz). Low resolution mass spectra were recorded on VG-7070H Micromass mass spectrometer at 200 °C, 70 eV with trap current of 200 μA and 4 kV acceleration voltage. HRMS were recorded on VG Autospec N mass spectrometer at 200 °C, 70 eV with a trap current of 200 μA and 7 kV acceleration voltage.

4.2. 4β-Bromopodophyllotoxin (12)

To a solution of podophyllotoxin (**1**) (1.1 g, 2.65 mmol) in 10 mL of dichloromethane was added molecular sieves (4 Å) powder and dry HBr gas was bubbled through the solution for 45 min. Later nitrogen gas was bubbled to remove excess HBr and then filtered to remove the molecular sieves. The filtrate was evaporated under reduced pressure to leave a residue, which was used for next reaction with diamino aryl compounds without further purification.

4.3. 4′-*O*-Demethyl-4β-bromo-4-desoxypodophyllotoxin (13)

To a solution of podophyllotoxin (**1**) (1.1 g, 2.65 mmol) in 10 mL of dichloromethane was added molecular sieves (4 Å) powder and dry HBr gas was bubbled through the solution for 45 min. This reaction mixture was allowed to stir for 48 h at room temperature and filtered to remove the molecular sieves. The filtrate was evaporated under reduced pressure to leave a residue, which was used for next reaction with diamino aryl compounds without further purification.

Table 2. Parameters for regression analysis

Compound	Mean GI_{50} values	Activity (−log)	GFA Predictor	GFA Residual	Apol	Dipole-ma	Rad of gyra	Area	Density	Rotl bonds	H-bond acc	H-bond don	*A* log *P*	Mol Ref	Dist.[a] between Monomers
14a	0.58	0.236	0.244	−0.008	3.43*E* + 04	7.087	7.602	902.479	1.17	12	14	2	5.24	237.7	5.63
14b	1.80	−0.255	−0.285	0.030	3.68*E* + 04	4.284	7.654	974.341	1.16	12	14	2	6.24	254.2	6.26
14c	0.035	1.456	1.354	0.102	3.98*E* + 04	8.501	7.197	1083.142	1.15	15	16	2	8.82	280.5	9.91
14d	0.30	0.522	0.856	−0.334	3.83*E* + 04	6.268	8.638	1033.997	1.16	14	15	2	6.67	264.0	9.90
14e	0.15	0.809	0.666	0.143	4.17*E* + 04	4.682	9.896	1102.362	1.14	12	14	2	8.82	280.5	14.17
14f	0.12	0.903	0.838	0.065	3.89*E* + 04	5.89	7.303	1016.43	1.16	12	14	2	6.96	266.7	9. 68
14g	0.045	0.346	0.530	−0.184	3.90*E* + 04	2.392	8.247	1046.941	1.15	13	15	2	6.56	264.5	9.91
15a	0.46	0.337	0.139	0.198	3.33*E* + 04	7.111	7.364	868.473	1.18	12	14	4	5.18	228.2	5.63
15b	6.55	−0.816	−0.513	−0.303	3.57*E* + 04	8.815	6.248	907.632	1.18	12	14	4	6.18	244.6	6.25
15c	0.26	0.585	0.505	0.080	3.88*E* + 04	7.204	8.436	1028.917	1.16	15	16	4	6.36	266.3	9.88
15d	3.35	−0.525	−0.452	0.127	3.73*E* + 04	7.890	8.400	977.200	1.17	14	15	4	6.61	254.4	9.88
15e	10.5	−1.021	−1.230	0.209	4.07*E* + 04	3.547	9.625	1048.944	1.16	14	14	4	8.55	278.5	14.18
15f	18.05	−1.256	−1.075	−0.181	3.74*E* + 04	4.912	8.757	961.127	1.17	12	14	4	6.89	257.0	9.65

[a] This parameter is not included in the regression analysis.

Table 3. Correlation matrix

	Apol	Dipole-mag	Rad of gyrati	Area	Rotl bonds	H-bond acceptor	H-bond donor	*A* log *P*	Mol Ref
Apol	1.000								
Dipole-mag	−0.416	1.000							
Rad of gyrati	0.692	−0.664	1.000						
Area	0.966	−0.372	0.620	1.000					
Rotl bonds	0.515	0.165	0.148	0.596	1.000				
H-bond acceptor	0.361	0.278	−0.085	0.510	0.867	1.000			
H-bond donor	−0.227	0.197	−0.059	−0.494	0.000	−0.170	1.000		
A log *P*	0.869	−0.516	0.804	0.737	0.153	−0.116	−0.013	1.000	
Mol Ref	0.992	−0.374	0.656	0.969	0.581	0.413	−0.243	0.831	1.00
Activity_1	0.206	0.285	−0.055	0.416	0.429	0.649	−0.597	−0.142	0.27

Table 4. The development of QSAR for different cell lines

Cell Line	Equation	Eq. no
Mean of all cell lines	$-\log GI_{50} = 0.186 + 0.0370$ area $+ 0.186$ Dipole $+ 0.147$ Mol Ref., $n = 13$, $r^2 = 0.93$, $Xr^2 = 0.811$, $F = 33.67$, PRESS = 1.31	1
Prostrate cancer	$-\log GI_{50} = 92.35 - 0.49$ Dipole $- 81.5 + 0.73$ Dipole mag, $n = 11$, $r^2 = 0.88$, $Xr^2 = 0.63$, $F = 17.9$, PRESS = 1.68	2
Colon Cancer	$-\log GI_{50} = 72.41 + 0.21$ Dipole $z - 62.46$ density, $n = 13$, $r^2 = 0.85$, $Xr^2 = 0.78$, $F = 29.0$, PRESS = 2.3	3
Breast cancer	$-\log GI_{50} = 53.53 + 0.32$ Dipole $z - 0.20$ Dipole mag $- 45.8$ density, $n = 11$, $r^2 = 0.87$, $Xr^2 = 0.68$, $F = 16.1$, PRESS = 2.4	4

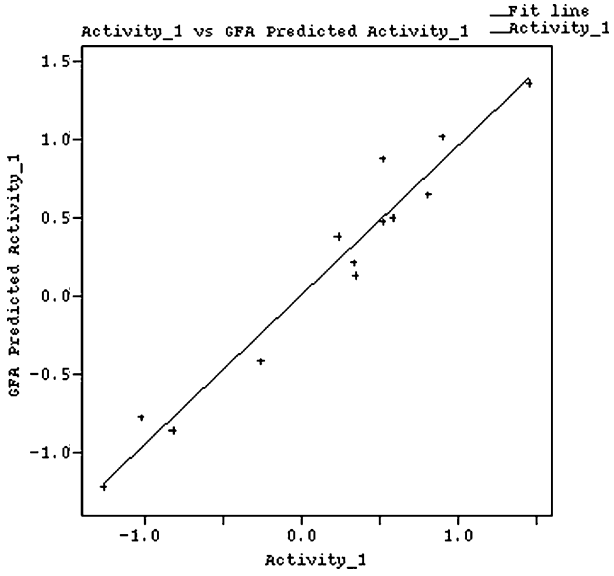


Figure 7. Graph showing actual versus predicted activities.

4.4. 1″,4″-[Bis-(4β-*N*-podophyllotoxin)]phenylenediamine (14a)

To a stirred solution of 1,4-phenylenediamine (54 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and $Bu_4N^+I^-$ (185 mg, 0.5 mmol) in dry THF (10 mL), was added a solution of 4β-bromopodophyllotoxin (476 mg, 1.0 mmol) in THF (10 mL) slowly at room temperature under nitrogen atmosphere. The stirring was continued for 6 h after the completion of the reaction (TLC), the THF was evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ and washed with water, it was then purified by column chromatography employing $CHCl_3$–CH_3COCH_3–EtOAc (9.0:0.5:0.5) as eluent to afford the pure dimer in 80% yield; mp 197–199 °C; $[\alpha]_D^{25}$ −175.10 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3378, 2906, 2360, 1772, 1588, 1507, 1482, 1418, 1387, 1320, 1230, 1160, 1125, 1037, 1000, 930, 863 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.78 (s, 2H), 6.50 (s, 2H), 6.32–6.45 (m, 4H), 6.26 (s, 4H), 5.95 (s. 4H), 4.50–4.61 (m, 4H), 4.28–4.40 (m, 2H), 3.98–4.10 (t, 2H, $J = 9.30$ Hz), 3.80 (s, 6H), 3.70 (s, 12H), 3.07–3.22 (dd, 2H, $J = 4.61$, 13.90 Hz), 2.82–3.05 (m, 2H); MS (FAB): *m*/*z* 900 $[M]^+$; HRMS $[M]^+$ calcd for $C_{50}H_{48}N_2O_{14}$ *m*/*z* 900.3105. Found (FAB) *m*/*z* 900.3104.

4.5. 1″,5″-[Bis-(4β-*N*-podophyllotoxin)]naphthalenediamine (14b)

This compound **14b** was prepared according to the method described for earlier compound **14a**, employing the 1,5-diaminonaphthalene (80 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4β-bromopodophyllotoxin (476 mg, 1.0 mmol) to afford the compound **14b** in 80 % yield; mp 227–230 °C; $[\alpha]_D^{25}$ −78.06 (*c* 1.04, $CHCl_3$); IR ($CHCl_3$) 2906, 2360, 1772, 1588, 1532, 1482, 1428, 1329, 1231, 1125, 1037, 1001, 962, 863 cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.39 (d, 2H, $J = 9.3$ Hz), 7.22 (t, 2H, $J = 6.97$ Hz), 6.78 (s, 2H), 6.60 (d, 2H, $J = 6.97$ Hz), 6.53 (s, 2H), 6.35 (s, 4H), 5.97

(s, 4H), 5.55 (m, 2H), 4.90–5.05 (m, 2H), 4.60 (d, 2H, $J = 4.65$ Hz), 4.38 (t, 2H, $J = 9.30$ Hz), 3.80–3.95 (m, 2H), 3.75 (br s, 18H), 3.55 (dd, 2H, $J = 4.65$, 13.95 Hz), 2.90–3.00 (m, 2H); MS (FAB): m/z 951 $[M+H]^+$; HRMS $[M+H]^+$ calcd for $C_{54}H_{50}N_2O_{14}$ m/z 950.3262. Found (FAB) m/z 950.3234.

4.6. 3″,3‴-[Bis-(4β-*N*-podophyllotoxin)]dimethoxybenzidine (14c)

This compound **14c** was prepared according to the method described for earlier compound **14a**, using the 3,3′-dimethoxybenzidine (122 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4β-bromopodophyllotoxin (476 mg, 1.0 mmol) to afford the dimer **14c** in 78% yield; mp 204–207 °C; $[\alpha]_D^{25}$ −131.60 (*c* 1.05, $CHCl_3$); IR ($CHCl_3$) 2936, 2240, 1776, 1725, 1586, 1482, 1331, 1229, 1126, 1035, 1001, 927 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.01 (d, 2H, $J = 7.12$ Hz), 6 94 (s, 2H), 6.78 (s, 2H), 6.55 (s, 2H), 6.49 (m, 4H), 6.32 (s, 4H), 5.99 (s, 4H), 4.70 (m, 2H), 4.63 (d, 2H, $J = 4.57$ Hz), 4.41 (d, 2H, $J = 6.89$ Hz), 4.00 (m, 2H), 3.93 (s, 6H), 3.80 (s, 6H), 3.75 (s, 12H), 3.20 (dd, 2H, $J = 4.59$, 16.09 Hz), 2.90–3.12 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 174.74, 152.58, 148.16, 147.55, 146.55, 137.38, 136.24, 135.20, 131.77, 131.24, 130.85, 119.14, 109.76, 109.29, 109.07, 108.50, 101.43, 68.93, 60.66, 56.28, 55.49, 52.26, 43.62, 41.86, 38.91; MS (FAB): m/z 1036 $[M]^+$, calcd for $C_{58}H_{56}N_2O_{16}$: C 67.17, H 5.44, N 2.70. Found: C 67.11, H 5.04, N 2.62.

4.7. 4″,4‴-[Bis-(4β-*N*-podophyllotoxin)]diaminodiphenylether (14d)

This compound **14d** was synthesized by the method described for earlier compound **14a**, employing 4,4′-diaminodiphenyl ether (100 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4β-bromopodophyllotoxin (476 mg, 1.0 mmol) to afford the compound **14d** in 80% yield; mp 201–204 °C; $[\alpha]_D^{25}$ −133.81 (*c* 1.01, $CHCl_3$); IR ($CHCl_3$) 3392, 2906, 2836, 2361, 1774, 1588, 1498, 1330, 1228, 1159, 1125, 1037, 1002, 942 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.85 (d, 4H, $J = 7.05$ Hz), 6.79 (s, 2H), 6.53 (s, 2H), 6.50 (d, 4H, $J = 7.05$ Hz), 6.29 (s, 4H), 5.97 (d, 4H, $J = 3.5$ Hz), 4.59 (d, 4H, $J = 4.70$ Hz), 4.38 (t, 2H, $J = 7.05$ Hz), 4.03 (t, 2H, $J = 9.41$ Hz), 3.80 (s, 6H), 3.75 (s, 12H), 3.70 (d, 2H, $J = 4.70$ Hz), 3.16 (dd, 2H, $J = 4.70$, 14.11 Hz), 2.90–3.07 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 174.68, 152.53, 150.13, 148.07, 147.49, 143.28, 137.35, 135.07, 131.60, 130.78, 119.72, 113.5, 109.78, 109.06, 108.42, 101.39, 77.64, 68.86, 60.59, 56.21, 53.06, 43.51, 41.67, 38.72; MS (FAB): m/z 993 $[M+H]^+$, calcd for $C_{56}H_{52}N_2O_{15}$: C 67.73, H 5.28, N 2.82. Found: C 67.79, H 5.21, N 2.80.

4.8. 4″,4‴-[Bis-(4β-*N*-podophyllotoxin)]-*p*-terphenyldiamine (14e)

This compound **14e** was synthesized by the method described for earlier compound **14a**, employing 4,4″-diamino-*p*-terphenyl (130 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4β-bromopodophyllotoxin (476 mg, 1.0 mmol) to afford the compound **14e** in 75% yield; mp 203–206 °C; $[\alpha]_D^{25}$ −126.00 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3378, 2905, 2361, 1774, 1610, 1587, 1482, 1417, 1329, 1231, 1189, 1125, 1037, 1001, 813 cm^{-1} ^1H NMR ($CDCl_3$) δ 7.63 (s, 4H), 7.54 (d, 4H, $J = 8.23$ Hz), 6.88 (s, 2H), 6.66 (d, 4H, $J = 8.20$ Hz), 6.60 (s, 2H), 6.37 (s, 4H), 6.04 (d, 4H, $J = 2.85$ Hz), 4.75–4.80 (m, 2H), 4.65 (d, 2H, $J = 3.76$ Hz), 4.47 (t, 2H, $J = 7.05$ Hz), 4.10 (t, 2H, $J = 14.11$ Hz), 3.95 (d, 2H, $J = 4.70$ Hz), 3.85 (s, 6H), 3.80 (s, 12H), 3.22 (dd, 2H, $J = 4.70$, 14.11 Hz), 2.97–3.13 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 174.65, 152.54, 148.14, 147.53, 146.69, 138.69, 135.06, 131.69, 130.78, 130.52, 127.91, 126.47, 112.57, 109.79, 109.16, 108.40, 101.43, 77.63, 68.87, 60.62, 56.22, 52.44, 43.53, 41.71; 38.71; MS (FAB): m/z 1052 $[M]^+$, calcd for $C_{62}H_{56}N_2O_{14}$: C 70.71, H 5.36, N 2.66. Found: C 70.82, H 5.41, N 2.72.

4.9. 2″,7″-[Bis-(4β-*N*-podophyllotoxin)]diaminofluorene (14f)

This compound **14f** was prepared according to the method described for the earlier compound **14a**, employing 2,7-diaminofluorene (135 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4β-bromopodophyllotoxin (476 mg, 1.0 mmol) to afford the compound **14f** in 75% yield; mp 215–218 °C; $[\alpha]_D^{25}$ −157.02 (*c* 0.5, $CHCl_3$); IR ($CHCl_3$) 3378, 2905, 2835, 1775, 1614, 1586, 1503, 1481, 1417, 1329, 1230, 1125, 1037, 999, 931, 864, 800 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.44 (d, 2H, $J = 7.59$ Hz), 6.82 (s, 2H),6.68 (s, 2H), 6.45–6.55 (m, 2H), 6.31 (s, 4H), 5.96 (d, 4H, $J = 2.53$ Hz), 4.65–4.80 (m, 2H), 4.60 (d, 2H, $J = 3.79$ Hz), 4.43 (t, 2H, $J = 7.59$ Hz), 4.06 (t, 2H, $J = 10.12$ Hz), 3.80 (s, 6H), 3.75 (s, 12H), 3.50–3.70 (m, 2H), 3.11 (dd, 2H, $J = 3.79$, 15.18 Hz), 2.90–3.10 (m, 2H); MS (FAB): m/z 988 $[M]^+$, calcd for $C_{57}H_{52}N_2O_{14}$: C 69.22, H 5.30, N 2.83. Found: C 69.28, H 5.15, N 2.79.

4.10. 4″,4‴-[Bis-(4β-*N*-podophyllotoxin)]diaminobenzophenone (14g)

This compound **14g** was prepared according to the method described for the earlier compound **14a**, employing 4,4′-diaminobenzophenone (106 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4β-bromopodophyllotoxin (476 mg, 1.0 mmol) to afford the compound **14g** in 75% yield.

4.11. 1″,4″-[Bis-(4′-*O*-demethyl-4β-*N*-4-desoxypodophyllotoxin)]phenylenediamine (15a)

4.11.1. Method A. To a stirred solution of 1,4-phenylenediamine (54 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and $Bu_4N^+I^-$ (185 mg, 0.5 mmol) in dry THF (10 mL) was added a solution of 4′-*O*-demethyl-4β-bromo-4-desoxypodophyllotoxin (462 mg, 1.0 mmol) in THF

(10 mL) slowly at room temperature under nitrogen atmosphere. The stirring was continued for 6 h after the completion of the reaction (TLC), the THF was evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ and washed with water, it was then purified by column chromatography employing $CHCl_3$–CH_3COCH_3–EtOAc (9.2:0.4:0.4) as eluent to afford the product **15a** in 76% yield.

4.11.2. Method B. To a solution of podophyllotoxin (414 mg, 1 mmol) and sodium iodide (600 mg, 4 mmol), in acetonitrile (10 mL), was added a solution of chlorotrimethylsilane (432 mg, 4 mmol) in acetonitrile (10 mL). This reaction mixture was stirred continuously for 60 min, after the formation of the iodo-intermediate as indicated by TLC, $BaCO_3$ (590 mg, 3 mmol) and 1,4-phenylenediamine (54 mg, 0.5 mmol) was added subsequently to the reaction mixture. After completion of the reaction as indicated by TLC, the reaction mixture was diluted with dichloromethane (30 mL) then quenched with saturated sodium thiosulfate solution (20 mL). The organic layer was separated and dried over anhydrous Na_2SO_4 and evaporated under vacuum to give the crude compound. This upon purification by column chromatography (dichloromethane–ethylacetate–acetone (9.2:0.4:0.4), yielded pure dimer in 75% yield; mp 216–219 °C; $[\alpha]_D^{25}$ −169.16 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3503, 2905, 2360, 2343, 1772, 1750, 1616, 1508, 1482, 1457, 1328, 1228, 1113, 1037, 999, 930 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.78 (s, 4H), 6.46 (s, 2H), 6.36–6.40 (m, 4H), 6.26 (s, 4H), 5.98 (s. 4H), 5.34 (s, 2H), 4.52 (m, 2H), 4.20–4.40 (m, 2H), 3.90–4.10 (m, 2H), 3.75 (s, 6H), 3.16–3.35 (m, 4H); ^{13}C NMR ($CDCl_3$) 174.89, 148.06, 147.47, 146.48, 134.22, 131.78, 131.38, 130.71, 113.93, 109.84, 109.03, 108.40, 108.16, 101.38, 96.10, 68.98, 56.44, 43.43, 41.83; MS (FAB): *m/z* 872 $[M]^+$; HRMS $[M]^+$ calcd for $C_{48}H_{44}N_2O_{14}$ *m/z* 872.2792. Found (FAB) *m/z* 872.2791.

4.12. 1″,5″-[Bis-(4′-*O*-demethyl-4β-*N*-4-desoxypodophyllotoxin)]-naphthalenediamine (15b)

4.12.1. Method A. This compound was synthesized by the procedure described for earlier compound **15a**, employing, 1,5-diaminonaphthalene (80 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4′-*O*-demethyl-4β-bromo-4-desoxypodophyllotoxin (462 mg, 1.0 mmol) to afford the product **15b** in 76% yield.

4.12.2. Method B. This compound was synthesized according to the procedure described for earlier compound **15a**, employing, podophyllotoxin (414 mg, 1 mmol), sodium iodide (4 mmol, 600 mg), chlorotrimethylsilane (432 mg, 4 mmol), $BaCO_3$ (590 mg, 3 mmol) and 1,5-diaminonaphthalene (80 mg, 0.5 mmol); mp 238–240 °C; IR ($CHCl_3$) 3426, 2905, 1776, 1530, 1482, 1427, 1328, 1227, 1113, 1037, 994, 931, 764 cm^{-1}; ^{1}HNMR ($CDCl_3$) δ 32 (d, 2H, $J = 9.0$ Hz)), 7.10–7.21 (m, 2H), 6.73 (s, 2H), 6.58 (s, 2H), 6.52 (d, 2H, $J = 7.52$ Hz), 6.33 (s, 4H), 5.98 (s, 4H), 5.34 (s, 2H), 4.87–4.90 (m, 2H), 4.66 (d, 2H, $J = 4.70$ Hz), 4.52–4.60 (m, 2H), 4.42 (t, 2H, $J = 9.41$ Hz), 3.85–3.95 (m, 2H), 3.82 (s, 12H), 3.25 (dd, 2H, $J = 4.70$, 14.11 Hz), 2.98–3.16 (m, 2H); MS (FAB): *m/z* 922 $[M]^+$, calcd for $C_{52}H_{46}N_2O_{14}$: C 67.67, H 5.02, N 3.04. Found: C 67.59, H 5.01, N 3.01.

4.13. 3″,3‴-[Bis-(4′-*O*-demethyl-4β-*N*-4-desoxypodophyllotoxin)]dimethoxy benzidine (15c)

4.13.1. Method A. This compound was prepared by the procedure described for earlier compound **15a**, employing 3,3′-dimethoxybenzidine (122 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4′-*O*-demethyl-4β-bromo-4-desoxy podophyllotoxin (462 mg, 1.0 mmol) to afford the product **15c** in 70% yield.

4.13.2. Method B. This compound was synthesized according to the procedure described for earlier compound **15a**, employing podophyllotoxin (414 mg, 1 mmol) in acetonitrile (5 mL), sodium iodide (4 mmol, 600 mg), chlorotrimethylsilane (432 mg, 4 mmol), $BaCO_3$ (590 mg, 3 mmol) and 3,3′-dimethoxybenzidine (122 mg, 0.5 mmol) to afford the product **15c** in 75% yield; mp 215–218 °C; $[\alpha]_D^{25}$ −135.23 (*c* 1.05, $CHCl_3$); IR ($CHCl_3$) 3637, 3413, 2906, 2241, 1777, 1611, 1502, 1482, 1462, 1329, 1227, 1113, 1036, 1000, 930, 800, 765 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.04 (d, 2H, $J = 7.12$ Hz), 6.97 (s, 2H), 6.81 (s, 2H), 6.58 (s, 2H), 6.52 (d, 2H, $J = 7.12$ Hz), 6.36 (s, 4H), 6.01 (s, 4H), 5.40 (s, 2H), 4.70–4.78 (m, 2H), 4.65 (d, 2H, $J = 4.59$ Hz), 4.37–4.50 (m, 4H), 4.00–4.10 (m, 2H), 3.95 (s, 6H), 3.84 (s, 12H), 3.24 (dd, 2H, $J = 4.59$, 14.97 Hz), 2.98–3.15 (m, 2H); MS (FAB) *m/z* 1008 $[M]^+$; HRMS $[M]^+$ calcd for $C_{56}H_{52}N_2O_{16}$ *m/z* 1008.331. Found (FAB) *m/z* 1008.334.

4.14. 4″,4‴-[Bis-(4′-*O*-demethyl-4β-*N*-4-desoxypodophyllotoxin)]diaminodiphenyl ether (15d)

4.14.1. Method A. This compound was prepared by the procedure described for earlier compound **15a**, employing 4,4′-diaminodiphenyl ether (100 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4′-*O*-demethyl-4β-bromo-4-desoxypodophyllotoxin (462 mg, 1.0 mmol) to afford the product **15d** in 75% yield.

4.14.2. Method B. This compound was synthesized according to the procedure described for earlier compound **15a**, employing podophyllotoxin (414 mg, 1 mmol) in acetonitrile (5 mL, sodium iodide (600 mg, 4 mmol), chlorotrimethylsilane (432 mg, 4 mmol), $BaCO_3$ (590 mg, 3 mmol) and 4,4′-diaminodiphenyl ether (100 mg, 0.5 mmol) to afford the product **15d** in 78% yield; mp 217–219 °C; $[\alpha]_D^{25}$ −135.09 (*c* 1.04, $CHCl_3$); IR ($CHCl_3$) 3400, 2906, 1775, 1611, 1499, 1328, 1226, 1113, 1037, 1001, 931, 839; ^1H NMR ($CDCl_3$) δ

6.90 (d, 4H, $J = 6.90$ Hz), 6.81 (s, 2H), 6.55 (s, 2H), 6.51 (d, 4H, $J = 6.94$ Hz), 6.34 (s, 4H), 6.01 (d, 4H,, $J = 3.65$ Hz), 5.39 (s, 2H), 4.59–4.69 (m, 4H), 4.40 (t, 2H, $J = 9.19$ Hz), 4.06 (t, 2H, $J = 9.19$ Hz), 3.86 (s, 12H), 3.72 (d, 2H, $J = 4.59$ Hz), 3.17 (dd, 2H, $J = 4.19$, 13.79 Hz), 2.94–3.10 (m, 2H); MS (FAB) m/z 964 $[M]^+$, calcd for $C_{54}H_{48}N_2O_{15}$: C 67.21, H 5.01, N 2.90. Found: C 67.26, H 5.21, N 2.80.

4.15. 4″,4‴-[Bis-(4′-*O*-demethyl-4β-*N*-4-desoxypodophyllotoxin)]-*p*-terphenyl diamine (15e)

4.15.1. Method A. This compound was prepared by the procedure described for earlier compound **15a**, employing 4,4″-diamino-*p*-terphenyl (130 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4′-*O*-demethyl-4β-bromo-4-desoxypodophyllotoxin (462 mg, 1.0 mmol) to afford the product **15e** in 72% yield.

4.15.2. Method B. This compound was synthesized according to the procedure described for earlier compound **15a**, employing podophyllotoxin (414 mg, 1 mmol) in acetonitrile (5 mL), sodium iodide (4 mmol, 600 mg), chlorotrimethylsilane (432 mg, 4 mmol), $BaCO_3$ (590 mg, 3 mmol) and 3,3′-dimethoxy benzadine (122 mg, 0.5 mmol) to afford the product **15e** in 70% yield; mp 226–229 °C; $[\alpha]_D^{25}$ −134.62 (*c* 1.03, $CHCl_3$); IR ($CHCl_3$) 3386, 2905, 1776, 1610, 1512, 1482, 1384, 1327, 1112, 1037, 1000, 930, 813, 765; ^{1}H NMR (DMSO-d_6) δ 7.57 (s, 4H), 7.43 (d, 4H, $J = 7.23$ Hz), 6.81 (s, 2H), 6.65 (d, 4H, $J = 7.23$ Hz), 6.55 (s, 2H), 6.32 (s, 4H), 5.98 (d, 4H, $J = 3.85$ Hz), 5.35 (s, 2H), 4.70–4.78 (m, 2H), 4.60 (d, 2H, $J = 4.81$ Hz), 4.42 (t, 2H, $J = 7.22$ Hz), 4.03 (t, 2H, $J = 9.63$ Hz), 3.90–4.00 (m, 2H) 3.82 (s, 12H), 3.15 (dd, 2H, $J = 4.81$, 15.66 Hz), 2.94–3.08 (m, 2H); MS (FAB) m/z 1024 $[M]^+$, calcd for $C_{60}H_{52}N_2O_{14}$: C 70.30, H 5.11, N 2.73. Found: C 70.19, H 4.99, N 2.70.

4.16. 2″,7″-[Bis-(4′-*O*-demethyl-4β-*N*-4-desoxypodophyllotoxin)]diaminofluorene (15f)

4.16.1. Method A. This compound was prepared by the procedure described for earlier compound **15a**, employing 2,7-diaminofluorene (135 mg, 0.5 mmol), $Bu_4N^+I^-$ (185 mg, 0.5 mmol), Et_3N (150 mg, 1.5 mmol) and 4′-*O*-demethyl-4β-bromo-4-desoxypodophyllotoxin (462 mg, 1.0 mmol) to afford the product **15f** in 70% yield.

4.16.2. Method B. This compound was synthesized according to the procedure described for earlier compound **15a**, employing podophyllotoxin (414 mg, 1 mmol) in acetonitrile (5 mL), sodium iodide (4 mmol, 600 mg), chlorotrimethylsilane (432 mg, 4 mmol), $BaCO_3$ (590 mg, 3 mmol) and 2,7-diaminofluorene (135 mg, 0.5 mmol) to afford the product **15f** in 65% yield; mp 242–243 °C; $[\alpha]_D^{25}$ −157.56 (*c* 1.08, $CHCl_3$); IR ($CHCl_3$) 3378, 2905, 1765, 1612, 1481, 1327, 1227, 1112, 1036, 998, 930, 800, 765; ^{1}H NMR ($CDCl_3$) δ 7.42 (d, 2H, $J = 7.14$ Hz), 6.78 (s, 2H), 6.66 (s, 2H), 6.50 (s, 2H), 6.43–6.48 (m, 2H), 6.30 (s, 4H), 5.96 (d, 4H, $J = 4.05$ Hz), 5.33 (s, 2H), 4.65–4.70 (m, 2H), 4.58 (d, 2H, $J = 4.76$ Hz), 4.39 (t, 2H, $J = 8.33$ Hz), 4.05 (t, 2H, $J = 9.52$ Hz), 3.65–3.9 (m, 14 H), 3.14 (dd, 2H, $J = 4.76$, 14.28 Hz), 2.90–3.08 (m, 2H); ^{13}C NMR ($CDCl_3$) 174.87, 148.13, 147.50, 146.47, 145.87, 144.22, 134.17, 131.84, 130.85, 130.67, 119.51, 111.25, 109.84, 109.14, 108.34, 108.10, 101.44, 68.98, 56.48, 52.93, 43.43, 41.90, 38.74; MS (FAB) m/z 960 $[M]^+$, calcd for $C_{55}H_{48}N_2O_{14}$: C 68.74, H 5.03, N 2.92. Found: C 68.20, H 4.99, N 2.90.

4.17. In vitro evaluation of cytotoxic activity

In routine screening, each agent is tested over a broad concentration range (10-fold dilutions starting from >100 μM to ~10 nM) against six human cancer cell lines comprised of different tumour types. Standard compound doxorubicin is tested in each assay as a positive control. The cells are maintained in growing condition in RPMI 1640 medium containing 10% foetal calf serum and incubated at 37 °C under 5% CO_2 atmosphere. All cell lines are inoculated onto a series of standard 96-well microtitre plate on day zero, followed by 24 h incubation in the absence of test compound. The inoculation densities used in this screen are as per the procedure of Monks et al.[26] All NCEs are dissolved in DMSO and diluted further in culture medium. An aliquot of each dilution is added to the growing cells in 96-well plates and incubated for 48 h. After incubation the assay is terminated by adding 50 μL of trichloroacetic acid and incubating at 4 °C for 30 min. The precipitated cells are washed and stained with sulforhodamine B dye for 30 min and the excess dye is washed off with acetic acid. Adsorbed dye is solubilized in Tris base (alkaline pH) and quantitated by measuring the OD at 490 nm in an ELISA reader. GI_{50} (concentration that inhibits the cell growth by 50%) is calculated according to the method of Boyd and Paull.[27]

4.18. In vitro activity in the NCI screen

For each compound, dose response curves for each cell line were measured at a minimum of five concentrations at 10-fold dilutions. A protocol of 48 h continuous drug exposure was used, and a sulforhodamine B (SRB) protein assay was used to estimate cell viability or growth. The concentration causing 50% cell growth inhibition (GI_{50}), total cell growth inhibition (TGI, 0% growth) and 50% cell death (LC_{50}, −50% growth) compared with the control was calculated.

4.19. Cleavage assay

The formation of cleavage complex was assayed following the procedure of Zechiedrich et al.[28] The 20 μL reaction mixture contained relaxation buffer (minus ATP), 0.6 μg of pBR322 supercoiled DNA and

increasing concentrations of the ruthenium complexes. The reaction was initiated by adding 10 units (40 nmol) of rat liver topo II and incubated at 30 °C for 15 min. The reaction was stopped with 2 μL of 0.5 M EDTA and 2 μL of 10% SDS. The DNA bound protein was degraded by incubating the reaction mixture with 2 μL of 1 mg/mL Proteinase K at 45 °C for 1 h. The products were separated on 1% agarose gel for 8 h at 50 V in 1 × TAE buffer (40 mM tris-acetate, 1 mM EDTA), stained and photographed. The linear DNA band was quantified as percentage of total DNA in a UVP gel documentation system.

4.20. Data processing and computational analysis

QSAR analysis were run on Octane Silicon Graphics workstation using cerius 4 package. Genetic function analysis (GFA) is used to obtain regression equations for modelling $-\log GI_{50}$ of the podophyllotoxins. A large pool of descriptors of cerius 4 were used which include molecular weight (MW), number of rotatable bonds (Rotl bonds), number of hydrogen bond acceptors (H-bond acceptor), number of hydrogen donors (H-bond donors), area (*A*), radius of gyration (Rad of gyra), density, molecular volume (Vm), $A \log P$, molar refractivity (Mol Ref) and dipole moment. This is somewhat large for 13-membered compound set. Therefore limitations were applied to reduce the risk of finding spurious relationships. Perhaps the most important MR and π were not considered simultaneously because they are highly correlated. Combinations with squared terms were not allowed. No combinations containing more than four terms were considered.

For each equation, *n* is the number of compounds, Xr^2 is the cross validated r^2, *F* is a significance test, PRESS is the sum of squared deviations between the predicted and the actual GI_{50} values.

Modelling of dimers was performed by using INSIGHT II program package. Models were constructed with ring systems and substituents available in this package and from the X-ray data.[25] Conformations were initially screened and the selected conformations were then optimized using the molecular mechanics by the discovery subroutine. These models were then verified by using the molecular orbital AM_1 program with geometry optimization for appropriate valence, torsional angles and distance between two podophyllotoxin moieties.

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