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Podophyllotoxin esters with alicyclic residues: an insight into the origin of microtubule-curling effect in cancer cells

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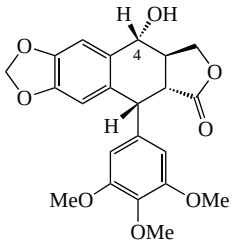
Immunofluorescent microscopy of cancer cells A549 treated with novel alicyclic (mostly bridged) podophyllotoxin C^4-esters at different concentrations gave evidence that the 'curling' of microtubules occurred at one of the first steps of their depolymerization. Molecular dynamics study revealed the differences in curved conformations of tubulin dimer in a complex with adamantane-comprising ester and in a complex with podophyllotoxin.

Keywords: molecular dynamics, podophyllotoxin, alicyclic compounds, esters, bridged moieties, tubulin, microtubules, human lung carcinoma A549, immunofluorescence microscopy.

Natural lignan podophyllotoxin (PPT) **1** possesses pronounced anticancer activity due to its ability to inhibit polymerization of cell protein α,β-tubulin to microtubules (MTs), which leads to a disruption of cell division.[1–4] However, this compound is not applied in chemotherapy because of its high general toxicity. Therefore, numerous PPT analogues were synthesized in a search of more safe drugs (see refs. 5–11 for reviews and recent examples). The majority of these analogues were obtained by modification at C^4 of PPT structure.



1, podophyllotoxin

In the course of these works a series of bridged esters were obtained (Figure 1) which showed cytotoxicity in a wide range from subnanomolar to micromolar.[12–15] Tested at a single concentration of 10 μM the compounds were found either to induce MTs shortening or depolymerization, typical for podophyllotoxin and its active derivatives, or to alter the dynamics of microtubule cytoskeleton in unusual manner by stimulating the formation of involuted structures defined as 'curled MTs'.[12,13] To the best of our knowledge, this action was described only for C^4-PPT esters with bridged residues. Therefore, in the present work we additionally synthesized five novel C^4-PPT esters with alicyclic (mostly bridged) moieties, tested them by immunofluorescence microscopy at different concentrations and performed molecular dynamics study with the purpose to hypothesize about the origin of MTs curling effect in cancer cells.

Compounds **2d**,**e**,**g**,**k**,**l** (see Figure 1) were synthesized by the Steglich esterification of podophyllotoxin **1** with commercially available alicyclic acids in acceptable yields (52–58%). Esters **2g**,**k**,**l** could not be separated to individual isomers by column or thin layer chromatography on silica gel and were isolated as diastereomeric mixtures (for the synthetic details and

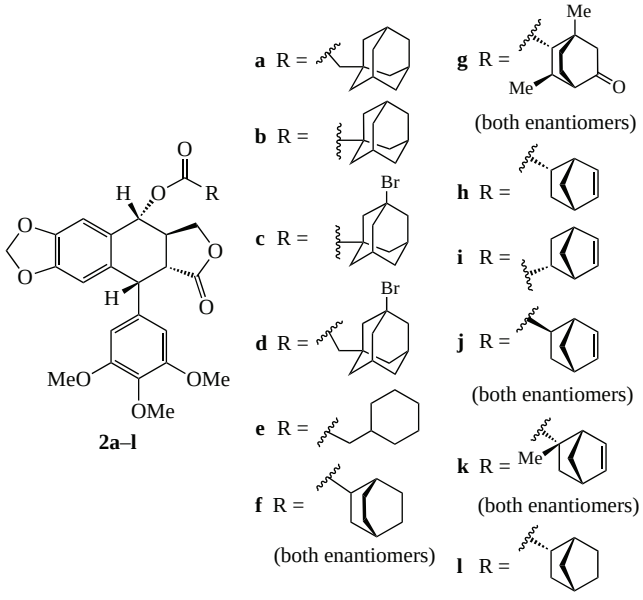


Figure 1 Examples of podophyllotoxin esters with alicyclic residues. Compounds **2d**,**e**,**g**,**k**,**l** are synthesized in the present work, the rest of compounds were described earlier (**2a**,[13] **2b**,**c**,**f**,**j**,[12] **2h**,**i**[14]). Esters **2f**,**g**,**j**,**k** were obtained as diastereomeric mixtures.

characteristics of compounds, see Online Supplementary Materials). Biotesting *in vitro* was performed with MTT test[16,17] and cell growth inhibition assay using human epithelial lung carcinoma cell line A549 (for the specific procedures, see refs. 18–20). The effect on microtubule dynamics[21] and ability to induce apoptosis[22] was investigated using immunofluorescence microscopy as described.[23–25]

As can be seen from Table 1, the highest antimitotic activity (with IC_{50} ~ 100 nM and EC_{50} = 290 nM) was observed for compound **2e** with cyclohexyl moiety. Esters with bridged residues inhibited cell proliferation at the concentrations in high nanomolar–submicromolar range and the extent of inhibition correlated with MTT data. Calculation of the cells undergoing the nuclear fragmentation revealed pronounced (**2e**,**k**,**l**) or weak (**2d**,**g**) pro-apoptotic activity of the podophyllotoxin derivatives at a concentration of 2 μM (see Table 1). Interesting data were evaluated by immunofluorescence microscopy (see Table 1 and Figure 2). At a very high concentration of 100 μM all tested compounds caused full or partial MTs depolymerization. However, at much lower concentrations of 2 or 10 μM the same action was observed only for ester **2e** [see Figure 2(*e*)], while all compounds with bridged residues stimulated slight or pronounced curling at any of these concentrations [see Table 1 and Figure 2(*b*),(*c*)].

The observed changes in effect on MTs with increasing concentration of esters **2d**,**g**–**l** (see Table 1) give evidence for the proposition that the 'curling' precedes full MTs depolymerization and passes before or in parallel with MTs shortening. If so then the division of bridged podophyllotoxin esters (studied in this work and described in refs. 12–15), into 'only depolymerizing' and 'only curling' is invalid. Indeed, the curling action correlates in part with EC_{50} values. For example, for compound **2l** with EC_{50} = 0.8 the curling was detected at 2 μM, while for **2g** with EC_{50} = 1.8 it was detected at 10 μM. For highly active compounds like **2e** the effect was not observed, because for this it should be tested at nanomolar concentrations. However, the results of compound test at the concentrations much lower than 2 μM which can provide the immunofluorescence microscopy are difficult to interpret due to the cell concentration of tubulin being 2–4 μM. Therefore, additional research is needed to verify if the active podophyllotoxin esters can or cannot induce MTs curling. It should be noted that kinetic studies carried out in the early works (see ref. 26 and citation therein) revealed the ability of substoichiometric concentrations of podophyllotoxin to suppress the dynamic instability of MTs due to the weak association of free tubulin–PPT complex with shortened MTs. This allows us to hypothesize that free tubulin complexes with bridged podophyllotoxin esters may have noticeable structural differences

Table 1 Results of biotests for compounds **2d**,**e**,**g**,**k**,**l**.

Compound	Microtubules[a]			Cell growth inhibition IC_{50}/μM[b]	Cytotoxicity, EC_{50}/μM[b]	Induction of apoptosis (%) (2 μM, 48 h)
	2 μM	10 μM	100 μM			
2d	+++	++	+	1.6 ± 0.01	2.2 ± 0.4	3
2e	–	–	–	0.109 ± 0.007	0.29 ± 0.08	51
2g	++++	++	–	1.8 ± 0.35	2.5 ± 0.1	3
2k	++/+	+	–	0.7 ± 0.1	0.7 ± 0.2	53
2l	++/+	–	–	0.55 ± 0.2	0.8 ± 0.1	58
1	–	n.d.	n.d.	0.02	0.02 ± 0.005	48
DMSO	++++	++++	++++			

[a] Effect on MTs of lung carcinoma A549 cells at the marked concentration after cell treatment for 24 h; the symbols indicate: '–' no MTs; '+' shorted MTs, '++' curled MTs, '+++' slightly curled MTs, '++++' intact MTs, 'n.d.' is not determined. [b] Results of minimum three independent experiments.

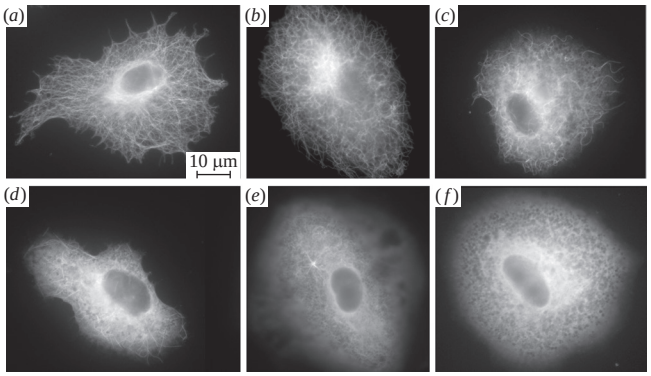


Figure 2 Immunofluorescence microscopy image of the microtubules in carcinoma A549 cells treated with 2 μM of tested compounds and DMSO as negative control: (*a*) 0.5% DMSO (intact MTs); (*b*) ester **2d** (slightly curled MTs); (*c*) ester **2d** (curled MTs); (*d*) ester **2l** (shorted MTs); (*e*) ester **2e** (full depolymerization of MTs, the rests of MTs near the centrosomes are seen as star-like structures); (*f*) podophyllotoxin **1** (full depolymerization of MTs).

that enable them to copolymerize with shortened MTs leading to 'MTs' with different dynamics and 'curled' morphology. To check if this was possible in principle, we carried out molecular modeling study.

Podophyllotoxin is known to interact with colchicine-binding site (PDB ID: 1SA1[27]) located in β-subunit of tubulin at the interface with α-subunit, where it is directed C^4-hydroxyl of the parent molecule.[28–30] The bulk residues of podophyllotoxin esters should be located in the voluminous gap between the two subunits and this is confirmed by the data of molecular docking performed for expanded series of compounds, namely **2a**–**e**,**h**,**i** and both diastereomers of esters **2f**,**g**,**j**–**l** (Figure 3). As it is seen from Figure 3, the positions of all alicyclic moieties of the studied esters (including diastereomers of the diastereomeric pairs) are close, except that of cyclohexane ring of ester **2e**.[†] Earlier we hypothesized that MTs curling may be a consequence of proximity the bridged moieties to GTP molecule, tightly bound to α-subunit of tubulin dimer.[15] Here we performed molecular dynamics simulations of the behavior of tubulin and two complexes ligand–tubulin in the CHARMM36/CGenFF 4.4 force field using GROMACS 2020.3 program[33,34] (for details, see Online Supplementary Materials).

First a reference model of α,β-tubulin dimer was obtained using a molecular dynamic procedure (Figure 4, shown in pink). Then two complexes of the protein with podophyllotoxin **1** and ester **2a** (IC_{50} 6.2 μM, very strong curling at 10 μM)[13] were modeled. The position of podophyllotoxin in the former was

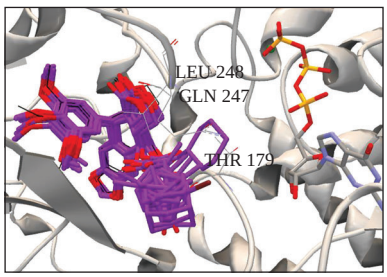


Figure 3 Complexes of esters depicted in Figure 1 (including diastereomers of diastereomeric pairs) with colchicine binding site in α,β-tubulin (PDB ID: 1SA1). β-Subunit is presented on the left, α-subunit with GTP molecule on the right, hydrogen atoms are omitted (docking procedure was performed using USCF Chimera 1.13.1 program[31] and AutoDock Vina 1.1.2 software[32]).

[†] The probability of close location of diastereomers in the binding site has an indirect confirmation, namely the identical cytotoxicity values for diastereomers **2h** and **2i** to A549 cells.[14]

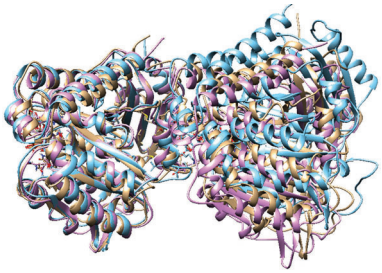


Figure 4 Final view of the α,β-tubulin dimer (in pink) and its complexes with podophyllotoxin **1** (in beige) or ester **2a** (in blue) predicted by molecular dynamics in the CHARMM36/CGenFF 4.4 force field; β-subunit is presented on the left and α-subunit – on the right.

identical to the obtained by X-ray analysis (PDB ID: 1SA1). Both models were superimposed on the reference model in such a way to achieve the best match of their β-subunits (see Figure 4, complexes with **1** and **2a** are shown in beige and in blue, respectively). It is seen that β-subunits of all three models coincide almost completely.

As it was expected, the arrangement of α-subunit in the complex tubulin–podophyllotoxin is slightly different from that of the reference model, and tubulin dimer takes on a curved conformation, which is known to be important for the MTs depolymerization caused by the ligands of colchicine binding site (the angle between α- and β-subunits is 11.7° for colchicine).[28–30] In the complex tubulin–ester **2a** the helixes of α-subunit are shifted from those in the control model much more significantly than in the complex with podophyllotoxin (see Figure 4), the difference in angle between tubulin–**1** and tubulin–ester **2a** models being 8.5°. Thus, molecular dynamics study reveals notable differences in the curved conformations of tubulin dimer in a complex with podophyllotoxin and its ester with 1-adamantaneacetic acid, having much less cytotoxicity, than the parent molecule, and a very pronounced MTs-curling effect.

In general, the data of this work allowed us to hypothesize that the 'curling' of microtubules caused by bridged podophyllotoxin C^4-esters takes place at one of the first steps of their depolymerization and occurs due to a change in the curvature of curved conformation of tubulin dimer. Additional studies of the effect are in progress and will be published in due course.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2022.03.006.

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