Cytotoxic activity of a podophyllotoxin-like lignan from *Linum tauricum* Willd

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The present study describes the preliminary evaluation of the cytotoxic activity of a podophyllotoxin-like compound 4'-demethyl-6-methoxypodophyllotoxin (4'-DM-6-Mptox), isolated as one of the main lignans of *Linum tauricum* Willd. ssp. *tauricum*. The cytotoxic effects 4'-DM-6-Mptox were assessed by the MTT-dye reduction assay against the human leukemic cell lines HL-60, BV-173 and LAMA-84. DNA-fragmentation analysis and NF-kB inhibition assay were performed in order to elucidate some of the mechanistic aspects of the cytotoxic action of the investigated compound. 4'-DM-6-Mptox was found to exert prominent cytotoxicity, with IC_{50} values being several-fold lower than those of the referent antineoplastic agent etoposide. The DNA-fragmentation analysis revealed that 4'-DM-6-Mptox treatment triggered apoptosis in BV-173 and HL-60 cells. In our hands 4'-DM-6-Mptox was found to induce concentration-dependent NF-kB inhibition in HeLa cells as assessed by the IL-6 luciferase gene reporter assay, which though not quite prominent, at least partly contributes to the cytotoxic potential of the tested lignan. On the basis of the results obtained it could be concluded that 4'-DM-6-Mptox necessitates further pharmacological and toxicological evaluation as a possible chemotherapeutic agent. Furthermore due to its relatively high concentrations in the described plant source the possibility for its use as a precursor for the semisynthetic production of lignan-based drugs, could be considered.

Key words: 4'-demethyl-6-methoxypodophyllotoxin, lignans, Linum tauricum Willd. ssp. tauricum, cytotoxicity, apoptosis, NF-k-B inhibition

Lignans are a large group of phenolic compounds defined as ββ'-dimers of phenylpropane (C_6C_3) units [3]. This widely spread group of natural products has drawn the attention since the discovery of the 2,7'-cyclolignan podophyllotoxin (Ptox) and the establishment of its potent cytotoxic effects by virtue of mitotic spindle inhibition [4]. Although the extreme gastrointestinal toxicity of Ptox precluded its use as potential antineoplastic agent, it is still widely utilized for the topical treatment of genital warts [3]. Furthermore Ptox is used as a starting material for production of the semi-synthetic anticancer drugs etoposide, teniposide and etopophos, which despite of the great structural resemblance to the prototype act as topoisomerase II inhibitors [1, 3, 8, 15]. Nowadays these agents are important antineoplastic agents used for the treatment of Hodgkin's disease, small cell anaplastic lung cancer, testicular cancer and other malignancies [1, 4, 14].

Ptox is a naturally occurring lignan that is extracted from the rhizomes of *Podophyllum peltatum* and *Podophyllum hexandrum* (Berberidaceae) [3, 13]. Both sources however are becoming increasingly limited because of extensive over-collection and lack of cultivation [3]. *P. hexahendrum* is even listed in the appendix II of CITES (Convention for International Trade in Endangered Species) [17]. Due to this serious supply problem, much effort has been focused upon the search for alternative sources of podophyllotoxin, including utilization of different plant species, chemical synthesis and biotechnological production [7].

The clinical efficacy of the marketed podophylotoxin-like agents attracted considerable attention to the synthesis and isolation of new active analogues of Ptox [9]. One important

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factor for the antitumor activity is the presence of 4'-phenolic hydroxyl group [8, 15]. In addition to the 4'-demethyl-3',5'-dimethoxy substitution pattern in the pendant ring, methylated derivatives at C-6 of Ptox glycosidic variants showed superior cytotoxic activity [9]. Therefore the lignan 4'-demethyl-6-methoxypodophyllotoxin (4'-DM-6-Mptox) (Fig. 1) might be an appropriate starting material for further structural modifications.

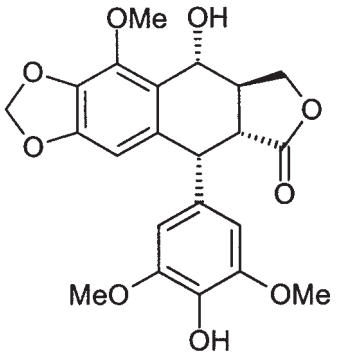

Figure 1. Chemical structure of the investigated compound 4'-demethyl-6-methoxypodophyllotoxin (4'-DM-6-Mptox).

To our knowledge there is only one report about the isolation of 4'-DM-6-Mptox from plant source [14]. However, the described *Linum flavum* cell cultures contained trace amounts of 4'-DM-6-Mptox; below 0.001% of the cell dry mass.

The present study describes the preliminary pharmacological evaluation of the cytotoxic activity of 4'-DM-6-Mptox, isolated as one of the main lignans of *L. tauricum* Willd. ssp. *tauricum*. In addition to the cytotoxicity screening program, DNA-fragmentation analysis and NF-kB inhibition assay were performed in order to elucidate some of the mechanistic aspects conditioning the cytotoxic action of 4'-DM-6-Mptox.

Material and methods

Plant material, extraction, isolation and identification of 4'-demethyl-6-methoxypodophyllotoxin (4'-DM-6-Mptox). Samples of *L. tauricum* Willd. *ssp. tauricum* were randomly collected from an area in the vicinity of Kavarna, Bulgaria. A fine powder (0.2 g) of the dried material, obtained from the aerial parts of the plant, was extracted with methanol (2 ml). The mixture was further homogenized in an ultrasonic bath (2x30 s) with intermediate cooling on ice. Distilled water (6 ml) was added, and the pH was adjusted to 5.0 by o-phosphoric acid. After adding of β-glucosidase (1 mg), the sample was incubated at 35 °C for 1 h. Methanol (12 ml) was added and the mixture incubated for another 10 min at 70 °C in an ultrasonic bath. After centrifugation, the supernatant was used for HPLC analysis. An Waters/Millipore HPLC system equipped with a photodiode array detector and a fraction collector for the collection of the unknown compounds was used. After the lignan extraction the methanolic part of the supernatant was evaporated. The aqueous remnant was extracted twice with equal volumes of ethylacetate p.a. After the ethylacetate phases were combined and evaporated, the remains were dissolved in methanol p.a. The peak collection was carried out by using a semipreparative GROM-SIL 120 ODS-5ST column with guard column (250 mm long, 8 mm i.d. and 50 mm long, 8 mm i.d., respectively; 5 μM particle; Grom Company, Herrenberg, Germany) and a gradient system with water (A) and acetonitrile (B) as eluents as follows: 0 to 17 min from 40 to 67% B, from 17 to 18 min to 40% B, and until 24 min back to 40% B. The flow rate increased from 1.6 ml/min at 0 min to 2.0 ml/min at 17.0 min and decreased again to 1.6 ml/min between 18 and 24 min. The tested compound 4'-demethyl-6-methoxypodophyllotoxin (4'-DM-6-Mptox) was identified by ^{1}HNMR.

Cell lines and culture conditions. The cytotoxic activity of 4'-DM-6-Mptox was evaluated in a panel of three leukemic cell lines of human origin, namely the pre-B cell leukemia BV-173, the chronic myeloid leukemia LAMA-84 and the chronic lymphoid leukemia SKW-3. LAMA-84 cells are distinguished by lower responsiveness to cytotoxic drugs because of the strong expression of the fusion oncoprotein BCR-ABL, a constitutive non-receptor tyrosin-kinase, which renders the cells less responsive to pro-apoptotic stimuli [2]. BV-173 cells have the same protein expression but at significantly lower level. The human tumor cell lines exploited herein were supplied from the German Collection of Microorganisms and Cell Cultures. They were maintained as suspension – type cultures in a controlled environment (RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum and 2mM L-glutamine, at 37 °C in a 'Heraeus' incubator with 5% CO_2 humidified atmosphere). In order to maintain the cells in log phase cellular suspension aliquots were re-fed with fresh RPMI-1640 medium two or three times/week. The stock solution of the tested compound was prepared in ethanol and consequently diluted in RPMI-1640. At the final dilutions obtained the concentration of the solvent never exceeded 0.5%.

The human cervical carcinoma-derived HeLa cells, exploited for the NF-kB inhibition assay were cultured at 37 °C, 95% humidity, in Dulbecco's Modified Eagle Medium with additives of 10% heat deactivated foetal bovine serum and 1% penicillin/streptomycin antibiotic. The cells were maintained as an IL-6/luciferase stably transfected culture (antibiotic resistant) with the addition to the media of 100 μg/ml of hygromycin B.

Cytotoxicity determination (MTT dye reduction assay). The MTT-dye reduction assay was carried out as described by MOSSMANN [5] with some modifications [2]. The clinically applied epipodophyllotoxin derivative etoposide was used as referent cytotoxic drug. Briefly, 100 μl aliquots of cell suspension ($1x10^5$ cells/ml) were seeded in 96-well microplates. Following 24 h incubation at 37 °C the cells

were exposed eighter to the newly isolated lignan or to etoposide for 72 h. After the incubation period MTT solution (10 mg/ml in PBS) was added (10 µl/well) and the plates were further incubated for 4 h at 37 °C. Thereafter the formazan crystals formed were dissolved through addition of 100 µl/well 5% formic acid in 2-propanol (Merck) and the absorption of the samples was measured with an ELISA reader (Uniscan Titertec) at 580 nm. 100 µl RPMI 1640 medium (Sigma), 10 µl MTT stock and 100 µl 5% formic acid in 2-propanol served as a blank solution. The results were expressed as survival fraction (% of untreated control).

DNA isolation and gel electrophoresis. The DNA extraction and horizontal gel electrophoresis procedures were carried out as previously described [2]. About $5x10^6$ BV-173 cells – treated with 4'-DM-6-Mptox (at concentrations of 0.0125, 0.025, 0.05, 0.1 and 0.2 µM) and untreated controls, were washed in PBS and spun at 2000 rpm for 5 min. The cell pellets were re-suspended in 0.25 ml PBS and lysed through addition of 0.5 ml buffer containing 0.5% Triton X-100, 20 mM Tris-HCL and 1mM EDTA (pH=7.4). Samples were incubated at 0 °C (on ice) for 5 min and thereafter spun at 13,000 rpm for 20 min. The supernatants were transferred into 2 ml 'safe lock' test tubes and then 0.937 ml 2-propanol as well as 0.187 ml 6M solution of NaCl were added to each sample. The tubes were gently agitated and incubated at –20 °C for 12 h in order to allow precipitation of the hydrophillic DNA. The samples were centrifuged for 20 min at 13 000 rpm, the supernatants were decanted and DNA was washed in 1 ml ice cold 70% ethanol and then air dried. The isolated DNA was re-dissolved in 20 µl distilled water and analyzed by gel electrophoresis in 0.8% agarose gel. Finally DNA was stained with ethidium bromide and visualized using an UV transilluminator and photographed with a fixed digital camera (Bio Doc IT™ system).

NFκB inhibition assay. The potential effects of the identified inhibitor were tested on the transcription of the inflammatory cytokine IL-6, which is mainly regulated by NFκB. The HeLa cells were transfected with a *luciferase* reporter gene controlled by the IL-6 promoter as described elsewhere [12]. Following 24 h of the transfection, the cells were preincubated with different concentrations of the compound in DMSO. Cells were exposed to the tested compound for 1 hr, at which point the stimulant PMA was added (50 ng/ml final concentration). The cells were incubated (37C) for more further 7 h before cell harvesting. Then, the cells were lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. 100 µl of this luciferase lysis reagent was added to each well and left for 15 mins to complete the lysing of the cells. 15 µl from each well was then added to a 96-well plate in preparation for the automated reading of the luciferase reaction. Luciferase activity was measured using an Autolumat LB 9501 (EG&G Berthold, USA) following the instructions of the luciferase assay kit (Promega, Madison, WI). At each well, 50 µl of luciferin was automatically added and the resulting luminometric reading recorded following a reaction time of 10 s. The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation expressed as total RLU induction. All the experiments were repeated at least three times. Positive (stimulated cells without a sample) and negative (resting cells without stimulation) controls were included to monitor assay consistency.

Statistics. The data processing included the Student`s t-test with $p \leq 0.05$ taken as significance level, using Microsoft EXCEL and OriginPlot software for PC.

Results

The dose-response curves obtained for the tested compound as well as for the referent cytotoxic agent etoposide are shown in Figures 2, 3 and 4 for SKW-3, BV-173, and LAMA-84 cells, respectively. The corresponding IC_{50} values are summarized in Table 1. As evident from the presented results both compounds caused concentration-dependent cytotoxic effects in the panel investigated. The newly isolated compound, however, proved to be profoundly more active in respect to relative potency with IC_{50} values being several-fold lower than those encountered for the referent drug.

The electrophoregrams from the DNA fragmentation assay in BV-173 and HL-60 cells is depicted on Figure 5. The DNA extracted from the cytosolic fraction of BV-173 and HL-60 cells treated with varying concentrations of 4'-DM-6-Mptox for 24 h demonstrated a typical laddering phenomenon that was more prominent at the higher concentration evaluated (Fig. 5).

The results from the NFκB inhibition examination (IL-6 luciferase gene reporter assay) are summarised in Table 2. As evident from the data presented at the lower concentrations of 0.0125 µM and 0.025 µM 4'-DM-6-Mptox caused only marginal inhibition by ca. 23%. At the highest concentration evaluated herein it exerted by ca. 10% more pronounced effect.

Discussion

The results from the preliminary screening program retrieved revealed that the newly isolated lignan 4'-DM-6-Mptox is a potent cytotoxic agent whose potency was 2 to 3.5 times higher in the different cell lines as compared to that of the referent antineoplastic drug etoposide. Thus in addition to the possible application of the plant *Linum tauricum* as source for lignan core structure, due to the high 4'-DM-6-Mptox content, the compound itself is of great interest for further evaluation. Indeed 4'-DM-6-Mptox is closely related to podophyllotoxin-like lignans, but has the distinction of having two substituents in the pendant ring, unlike the monosubstituted podophyllotoxin, peltatins and the non-pendant ring substituted deoxypodophyllotoxin, polygamain etc that possibly could attribute to its profound cytotoxic effects.

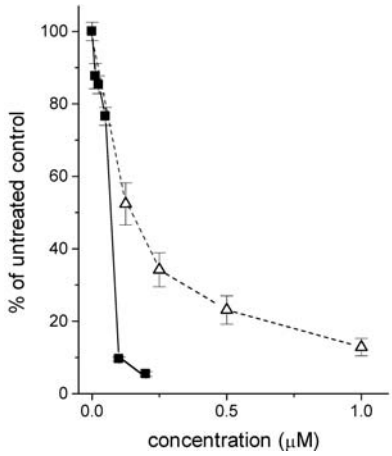

Figure 2. Cytotoxic effects of 4'-DM-6-Mptox (■) and etoposide (△) on the chronic lymphoid leukemia-derived SKW-3 cells after 72 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments. The error bars represent the corresponding standard deviations.

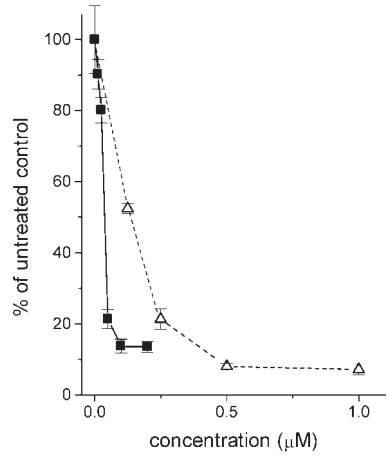

Figure 3. Cytotoxic effects of 4'-DM-6-Mptox (■) and etoposide (△) on the pre-B-cell lymphoma-derived BV-173 cells after 72 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments. The error bars represent the corresponding standard deviations.

Preliminary data indicate that 4'-DM-6-Mptox acts as an apoptosis inductor. The observed DNA laddering is indicative for oligonucleosomal DNA fragmentation, that is a consequence of the action of specific nucleases, which degrade the higher order chromatin-bound genomic DNA during the apoptotic process [6]. Now it is firmly established that despite of the mode of action of the specific classes of antineoplastic drugs it appears that the primary effect is currently followed by the activation of the cell death signalling pathways [6, 11]. Hereby the results obtained indicate that the induction of apoptosis at least partly mediates the cytotoxicity of 4'-DM-6-Mptox.

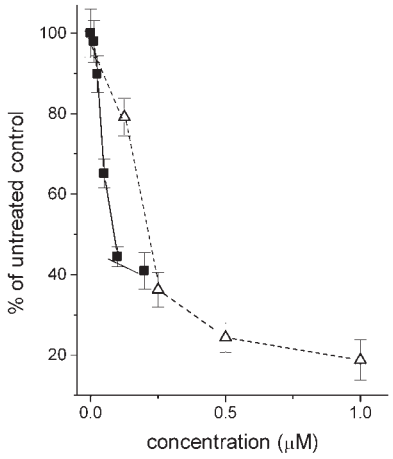

Figure 4. Cytotoxic effects of 4'-DM-6-Mptox (■) and etoposide (△) on the chronic myeloid leukemia-derived LAMA-84 cells after 72 h treatment, as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments. The error bars represent the corresponding standard deviations.

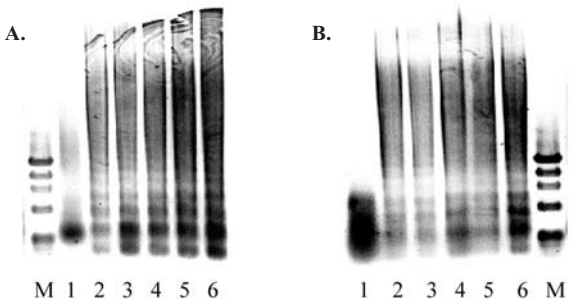

Figure 5. DNA-fragmentation analysis following 24 h treatment with 4'-DM-6-Mptox of BV-173 (A) and HL-60 cells (B). Untreated control (lane 1), 4'-DM-6-Mptox treated cells at concentrations of 0.2 μM (lane 2), 0.1 μM (lane 3), 0.05 μM (lane 4), 0.025 μM (lane 5), 0.0125 μM (lane 6), size marker (lane M).

Table 1. Relative potency of 4'-DM-6-Mptox and etoposide

Cell line	Cell type	IC_{50} value (μM)	
		4'-DM-6-Mptox	etoposide
LAMA-84	chronic myeloid leukemia	0.087	0.210
BV-173	pre-B-cell lymphoma	0.038	0.135
SKW-3	chronic lymphoid leukemia	0.071	0.140

Table 2. 4'-DM-6-Mptox induced NFκB inhibition (as assessed by the IL-6 luciferase gene reporter assay)

4'-DM-6-Mptox concentration (μM)	IL-6 luciferase gene reporter assay (Compared to 100% PMA stimulation
0.0125	76.90 %
0.025	77.17 %
0.05	66.79 %

The observed concentration-dependent inhibition of NFκB activity, as assessed by the IL-6 luciferase gene reporter assay, is an important feature of the cellular effects induced by 4'-DM-6-Mptox. NFκB is a transcription factor that regulates the expression of various cytokines, enzymes and cytokine receptors and intervenes in numerous signal transduction pathways, implicated in cellular proliferation, apoptosis inhibition and thence in the immune response, malignant disease progression and inflammation [10,16]. It is the primary trigger for the autocrine secretion of interleukin 6, which is established in various tumor species and is proven to stimulate their growth and survival [16]. Hence the observed NFκB inhibition, although not quite prominent even at the highest concentration evaluated could be expected to at least partially contribute to the cytotoxic effect of 4'-DM-6-Mptox.

Finally, on the basis of the results obtained it could be concluded that 4'-DM-6-Mptox deserves further pharmacological and toxicological evaluation as a possible chemotherapeutic agent. Furthermore, due to its structural resemblance to podophyllotoxin the possibility for its use as precursor for semisynthetic production of etoposide and analogues could be considered, since its concentrations in the dryed *Linum tauricum* aerial parts is higher than the corresponding concentrations of podophyllotoxin-like lignans in both *Podophyllum* species. Current studies devoted to the optimisation of the extraction and purification of 4'-DM-6-Mptox as well as for its biotechnological production from *Linum* spp. cell cultures are in progress.

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