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A Dual Topoisomerase Inhibitor of Intense Pro-Apoptotic and Antileukemic Nature for Cancer Treatment

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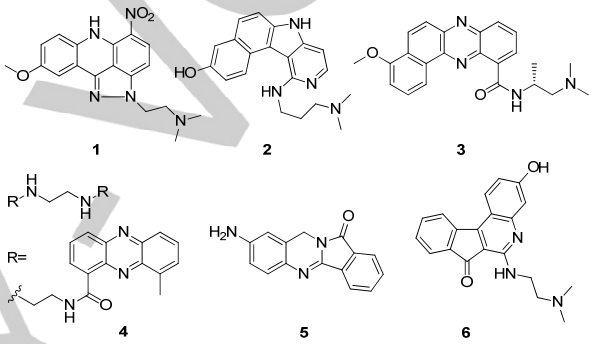
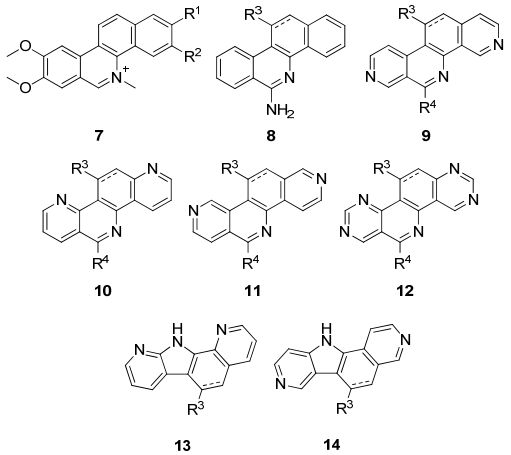
Abstract: *Classic cytotoxic drugs constantly remain an indispensable instrument in antitumor therapy due to their effectiveness and a more prevalent insensibility against tumor resistance mechanisms. We describe the favorable properties of P8-D6, a powerful inductor of apoptosis caused by an equipotent inhibition of human topoisomerase I and II activities. A broad spectrum effect against human tumor cell lines in nanomolar concentrations as well as strong antileukemic effects were shown and proven to be superior to marketed topo-targeting drugs and dual topoisomerase inhibitors in clinical trials. The facile four-step synthesis, advantageous drugability proper-ties, and first in vivo data encourage the application of P8-D6 in appropriate animal tumor models and further drug development.*

The human topoisomerase (topo) enzyme family plays a crucial role in a plenty of cellular actions involving movement and the solution of torsional strains of the genetic material. Its detailed intra-nuclear functions have been studied extensively and are well described in literature.[1-5] As these operations are required for vital processes such as DNA-replication, transcription, chromatin assembly and DNA-methylation, topoisomerase types I and II emerged as fundamental targets for the chemotherapeutic treatment of malignant tumor diseases.[6-10] Approved topo-targeting drugs like camptothecin derivatives, acting as topo I poisons, and primarily epipodophyllotoxins and anthracyclins that interfere with topo II are highly effective front-line cytostatics for the treatment of systemic cancers and solid tumor types.[5,8] The efficacy and experience in clinical use on the one hand, but also dose- and/or therapy-limiting side effects and upcoming resistance mechanisms against these agents on the other demonstrate a high medical need of novel topo-targeting molecules.[1,5,6,11-13] Many efforts were made designing dual topo I/II inhibitors that exhibit a high cytotoxicity and a strong affinity to both enzyme classes. In this regard, the complete abolishment of topological processes in the tumor cell was shown to be extremely effective in vitro and in vivo in a broad spectrum of tumor types.[1,14-20] Consequently, dual topo I/II inhibitors were evaluated in phase I/II clinical trials, pyrazoloacridine (PZA) **1**, the benzopyridoindole intoplicine **2**, the phenazine derivatives XR11576 **3** and XR5944 **4**, batracylin **5** and the indenoquinolinone TAS-103 **6** (Scheme 1), as well as the etoposide derivative tafluposide and the homocamptothecin elomotecan.[1,14,21-28] Although pre-clinical studies were very promising for these derivatives, their effectiveness could not be transferred to the clinic yet.



Scheme 1. Dual topoisomerase I/II inhibitors in clinical development.[1,14,21-27]

Naturally occurring benzo[*c*]phenanthridine alkaloids offer a broad variety of pharmacological activities.[29-33] Among them the quarternary ammonium salts fagaronine and nitidine **7** were found to possess moderate dual topo I/II poisoning activity.[34-36] In the mid-90's our laboratories evaluated a facile and straightforward synthetic route to 11-substituted 6-aminobenzo[*c*]-phenanthridines.[37] Antitumoral activity of derivatives **8** was observed to be high in vitro and in vivo, but unfortunately the dissatisfactory physicochemical properties (solubility: lower µM range, $logD_{(7.4)}$ ~5) limited further development of this substance class (Scheme 2).[38,39]



R^1= OH, R^2= OCH_3: fagaronine; R^1-R^2= OCH_2O: nitidine; R^3= versatilely substituted aryl; alkyl; R^4= H, OH, NH_2, various hydrophilic sidechains.

Scheme 2. Natural product-inspired development of aza-analogous benzo[*c*]-phenanthridines.[37-42]

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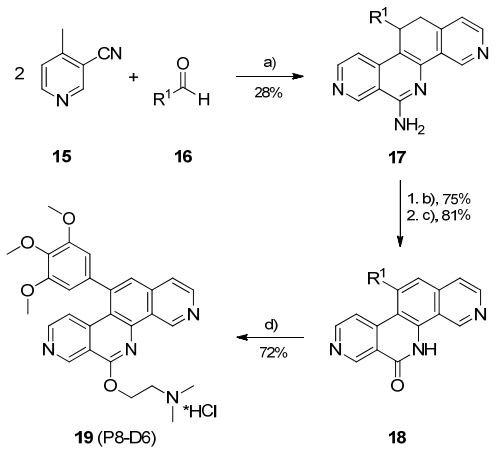
To overcome these disadvantages we synthesized aza-analogs of benzo[*c*]phenanthridines (**9**-**14**) using different *o*-methyl-hetarenecarbonitriles and the core system was systematically adapted and optimized regarding both physicochemical properties and cytotoxicity (Scheme 2).[39-42] Applying the concept of an amino-alkyl side chain linked to a planar core system to achieve DNA-intercalation as well as anti-topoisomerase activity,[18] the pyrido-[3,4-*c*][1,9]phenanthroline P8-D6 **19** was found to be the most potent derivative of our compound library by far. **19** was accessible via a simple four-step synthetic protocol commencing with commercially available starting materials to form the 6-amino-11,12-dihydro derivative **17** in a two-fold ring closure including one-pot synthesis (Scheme 3).[39]



R^1= 3,4,5-Trimethoxyphenyl; a) KO^{t}Bu, DMPU, 3h, rt; b) Pd/C, DMPU, 10 min, reflux; c) $NaNO_2$, H_3PO_2, H_2SO_4, AcOH, 1 h, 0°C; d) Dimethylaminoethanol, TPP, DIAD, THF, 72 h, rt; HCl(g), CH_2Cl_2.

Scheme 3. Four-step synthesis of P8-D6 **19** starting from commercially available building blocks.[39,40,43,44]

Dehydratation with Pd/C followed by diazotation with $NaNO_2$ in acidic aqueous medium yielded **18**.[40,43] The final step gave the *O*-alkylated derivative P8-D6 **19** via Mitsunobu reaction in an overall yield of 12% (Scheme 3).[44]

Cytotoxicity was evaluated by incubation of 60 human tumor cell lines of nine different tumor types with P8-D6 (NCI-60 DTP human tumor cell line screening, NCI, Bethesda, Maryland).

The substance showed an outstanding overall activity (expressed as average growth inhibition 50% over all 60 cell lines) of 49 nM in the range of the natural alkaloid camptothecin. Its growth-inhibitory effect was 4-10 fold higher compared with other dual topo I/II inhibitors recently elaborated in clinical trials (Table 1). Besides the good response of all tumor types, a sensitivity of leukemia cells and NSCLC cells (non-small cell lung cancer) towards P8-D6 was noticed. [45]

P8-D6 is able to inhibit the catalytic activities of both type I and II topoisomerases in low micromolar concentrations in vitro (Figure 1, A+B). In this connection there is no significant discrimination between topo IIα, which reaches maximum protein levels in G2/M-phase in human cells, and topo IIβ that is expressed independently of proliferative status and cell cycle.[1] A topo-independent DNA-attraction as it has been reported for structurally related substances before is postulated likewise.[18, 46]

Table 1. Cytotoxicity (μM) of topoisomerase targeting agents in comparison.

Compound	$\frac{\sum GI_{50}}{60}$ [a]	Leukemia[b]	NSCLC[b]	Colon cancer[b]
Topoisomerase I inhibitors				
CPT[c]	0.04	0.02	0.02	0.08
Irinotecan	0.84	0.20	0.07	1.70
Topotecan	0.04	0.01	0.02	0.09
Topoisomerase II inhibitors				
Etoposide	1.32	0.21	0.79	2.51
Teniposide	0.40	0.05	0.27	0.71
Doxorubicin	0.14	0.07	0.07	0.26
Dual topoisomerase I/II inhibitors				
P8-D6	0.05	0.02	0.03	0.06
PZA[d]	0.20	0.17	0.17	0.12
Intoplicine	0.53	0.47	0.23	0.50

Data taken from DTP-NCI databank, NCI-60 screening data set from 09/2014.[45] [a] Mean growth inhibition 50% over all 60 cell lines. [b] Average GI_{50} for all comparable cell lines of corresponding tumor type. [c] Camptothecin. [d] Pyrazoloacridine.

Using flow cytometric analysis of Annexin V-PE and 7-AAD stained HeLa cells as well as the detection of caspase-mediated PARP-cleavage it could be demonstrated that P8-D6 is a strong and rapid inductor of apoptosis (Figure 1, C-G).[47,48] Low micromolar concentrations of P8-D6 showed pro-apoptotic effects already after 8 h of incubation whilst no PARP cleavage was observed for etoposide (ETO) or the dual topo inhibitor PZA before reaching a concentration of 100 μM (Figure 1, D+E). After 24 h incubation time, 100-1000 fold lower amounts of P8-D6 were sufficient to induce apoptosis in cells compared to the cultivation in the presence of etoposide or PZA (Figure 1, D+E). PZA treatment also led to partial necrosis of the HeLa cells at these concentrations (Figure 1, F).

P8-D6 was furthermore tested for antileukemic effects on Jurkat ALL cells. With 30% of all childhood cancers, leukemia is the most common cancer in children with acute lymphoid leukemia (ALL) being most prevalent.[49] The Jurkat ALL cell line was shown to be sensitive towards P8-D6 treatment in accordance to the NCI-60 results. Using proliferation assays, flow cytometric analysis (referred to Nicoletti et al.) and Western Blotting, strong growth-inhibitory and pro-apoptotic effects at low nanomolar concentrations could be confirmed (Figure 2, A-C).[50,51]

Propidium iodide staining and FACS analysis was also used to evaluate the effects of P8-D6 on the cell cycle. As shown in Figure 2, D the presence of the substance led to an arrest in the G2-phase as expected for a topo inhibitor.[52]

Additionally, dose-dependent phosphorylation of histone H2A.X (γH2A.X) in Jurkat ALL cells clearly indicates DNA double-strand breaks as main apoptotic trigger of P8-D6 (Figure 2, E).[53]
In order to examine selectivity towards tumor cells, the cytotoxic effect of P8-D6 was also investigated on healthy human lymphocytes isolated from whole blood. Results indicate no response of lymphocytes towards P8-D6 concentrations that are already intensively cytotoxic and pro-apoptotic to the leukemia cell line (Figure 2, F).

Recently, the selective protein disulfide isomerase inhibitor PS89 was found to sensitize cancer cells towards etoposide treatment (structure shown in Figure 2, G).[54] In this regard, we analyzed the effects of P8-D6 in combination with the chemo-sensitizing compound PS89 pointing out a clearly enhanced growth inhibition and triggering of apoptosis of per se sub/less toxic P8-D6 concentrations towards Jurkat ALL cells while incubated with PS89 (Figure 2, H+I).

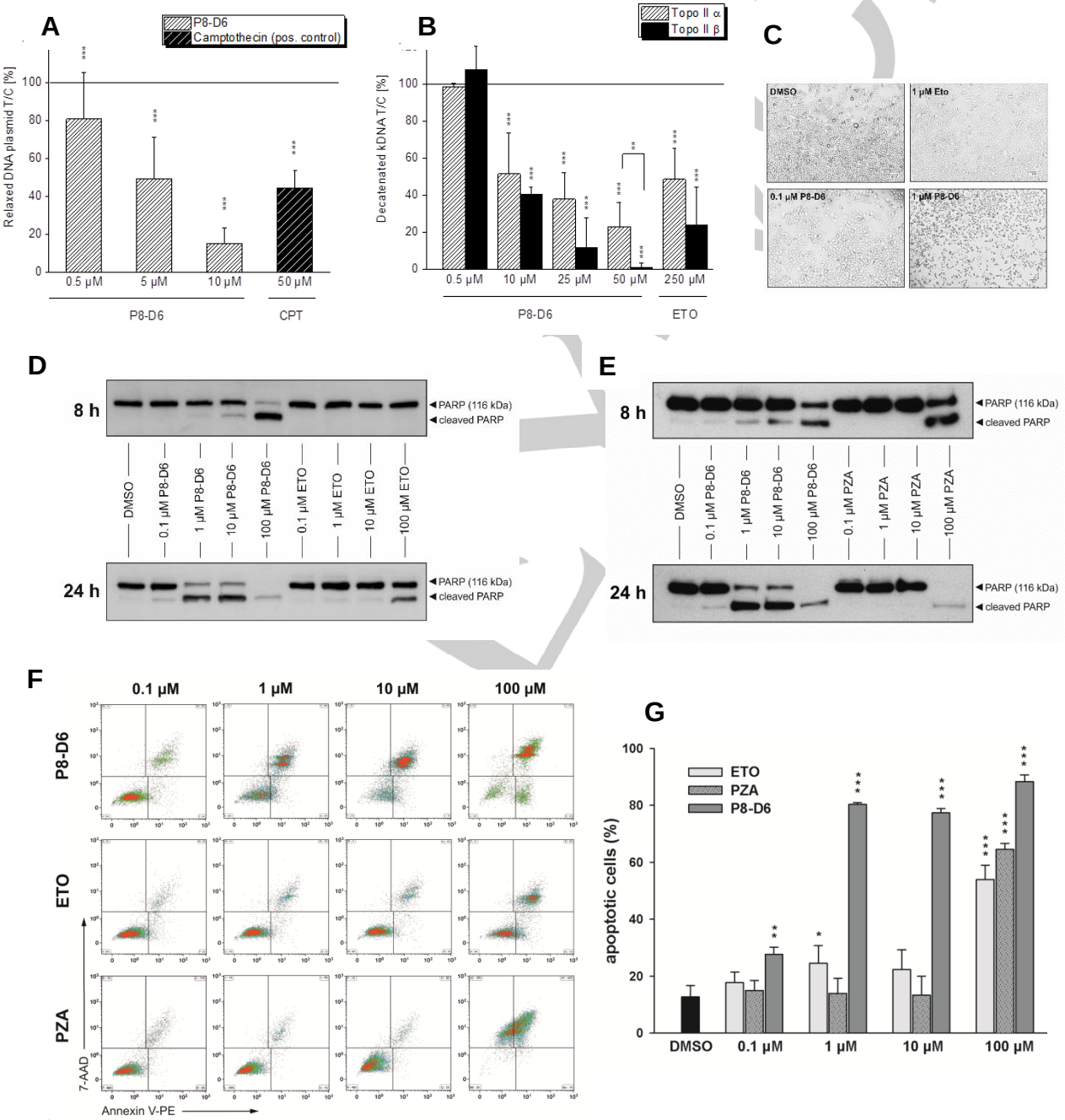


Figure 1. P8-D6 is a highly pro-apoptotic dual topoisomerase inhibitor. A) Inhibition of topoisomerase I activity (relaxation assay). B) Inhibition of topoisomerase IIα and IIβ (decatenation assay). C) HeLa cells after incubation (24 h) with P8-D6 or etoposide. D+E) PARP apoptosis assay in HeLa cells (ETO/PZA reference). F) Flow cytometric analysis of pro-apoptotic effects on HeLa cells (after 24 h). G) Quantitative determination of apoptotic cells using flow cytometry (after 24 h). *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ significant difference towards DMSO control.

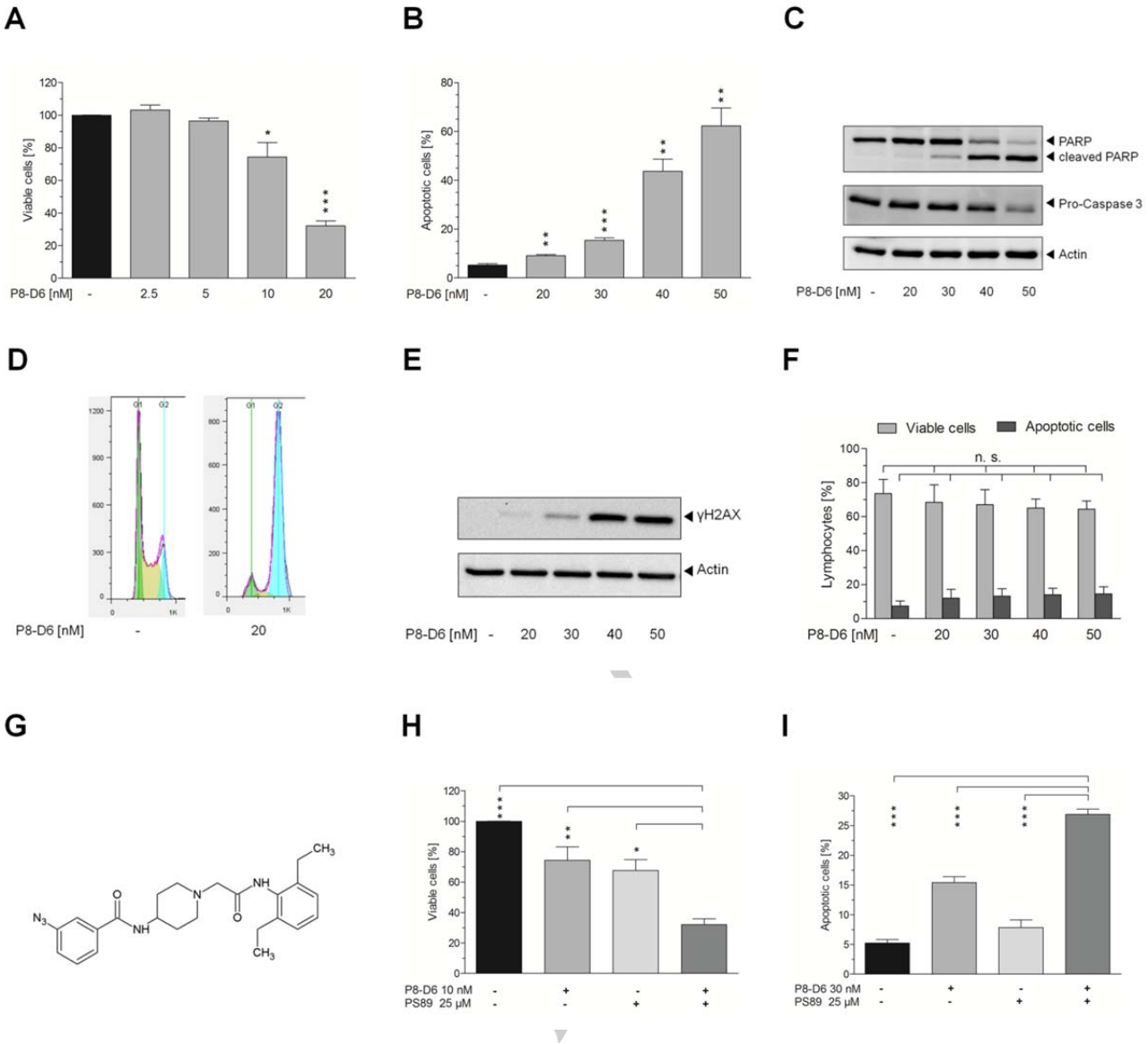


Figure 2. P8-D6's strong antileukemic effects and activity on healthy human lymphocytes. A) Dose-dependent inhibition of Jurkat ALL cell proliferation by P8-D6 treatment (Cell TiterBlue® assay, 72 h). B) Dose-dependent induction of apoptosis in Jurkat ALL cells by P8-D6 treatment (Nicoletti assay, 24 h). C) Decrease of Pro-Caspase-3 level and PARP-cleavage in Jurkat ALL cells as hallmarks of apoptosis (P8-D6 treatment, 24 h); Actin = loading control. D) Cell cycle arrest in the G2-phase by P8-D6 treatment (Propidium iodide staining, 24 h). E) Phosphorylation of Histone H2A.X as sensitive proof of DNA double-strand breaks in Jurkat ALL cells (P8-D6 treatment, 24 h). F) No pro-apoptotic effects of P8-D6 on healthy human lymphocytes (FACS analysis, 24 h). G) The chemosensitizing protein disulfide isomerase inhibitor PS89. H) Growth inhibition of Jurkat ALL cells by P8-D6 in combination with PS89 (Cell TiterBlue® assay, 72 h). I) Induction of apoptosis in Jurkat ALL cells by P8-D6 in combination with PS89 (Nicoletti assay, 24 h).

In addition to its pharmacological advantages, P8-D6 exhibits a physicochemical profile that encourages its development as promising drug candidate in antineoplastic chemotherapy. A solubility of approximately 1 mM in phosphate buffer pH 7.4 as well as the accordance of Lipinski Ro5-parameters for the prediction of oral bioavailability including a logD of 3.7 would allow even a peroral administration (parameters determined using HPLC analytics).[55]

To evaluate first in vivo tolerability of P8-D6, female athymic nude mice (n = 9 vs. 9 mice receiving PBS-control) were treated intravenously with different unique doses of P8-D6 in PBS. This MTD (maximum tolerated dose) study revealed a good tolerability of 1 mg/kg per body weight (bw) of P8-D6 with regard to further in vivo efficacy studies (Figure 3). In higher concentrations the substance showed dose-dependent toxicity (parameters: body weight, clinical signs), while loss of body weight and apathy of study animals in the 5 mg/kg group were reversible over the period of study (Figure 3).

Contrary to macroscopic alterations that had been reported for i.v. etoposide low and high dose treated mice in comparable studies,[56] no significant changes in organs could be observed for study animals that received P8-D6 injection up to 5 mg/kg bw (necropsy findings).

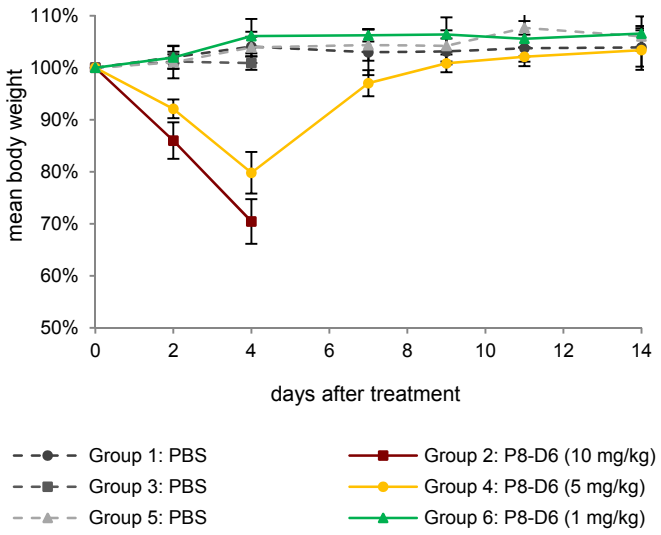


Figure 3. I.v. application of single dose P8-D6 vs. PBS-control (phosphate buffered saline) in athymic nude mice.

Since tumor cells require an enormous machinery of components involved in DNA-related growth and replication processes, the human topoisomerase family continuatively represents a pivotal target in antitumor therapy. In summary, our drug candidate P8-D6 introduced herein exhibits a plenty of advantages. The strong and equipotent inhibition of topo I, IIα and IIβ with a for a dual topoisomerase inhibitor hitherto undescribed excellent cytotoxicity, a fast and effective tumor cell-specific pro-apoptotic and particularly antileukemic activity and not least the easy synthetic access in combination with its favorable physicochemical properties underline the importance of further in vivo evaluation of P8-D6 in adequate animal tumor models. Due to the improved hydrophilicity of P8-D6 it is not necessary to provide solubilizing agents for i.v./i.m. or i.p. injections or infusions as it was already demonstrated in an in vivo tolerability study which also gave first i.v. dosage information.

Herein described studies clearly mark out P8-D6 as highly superior than the clinically evaluated dual topo inhibitor PZA regarding apoptosis-inducing effects. Therefore it can be considered as a substantial enrichment in the development of novel agents targeting both topoisomerases I and II raising hope for clinical efficacy, contrary to previously developed drug candidates of this substance class.

Besides the administration of single P8-D6 or in combination with other cytostatics or chemosensitizing agents the compound could be an excellent component in antibody-drug-conjugates (ADC's) or related modern techniques, most likely applicable against a broad variety of tumor types.[57]

Keywords: antitumor agents • apoptosis • heterocycles • ring closure • topoisomerase inhibitors

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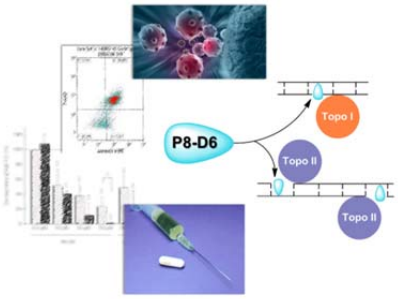
Entry for the Table of Contents

Layout 1:

COMMUNICATION

P8-D6 is a novel designed drug candidate for antineoplastic chemotherapy which acts as highly pro-apoptotic dual topoisomerase I and II inhibitor. A broad spectrum effect against human tumor cell lines, its strong anti-leukemic activity, the facile synthetic access, advantageous drugability properties as well as first in vivo data encourage the application of P8-D6 in appropriate in vivo efficacy studies and further drug development.



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A Dual Topoisomerase Inhibitor of Intense Pro-Apopotic and Anti-leukemic Nature for Cancer Treatment