Newly developed dual topoisomerase inhibitor P8-D6 is highly active in ovarian cancer

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Abstract
Background: Ovarian cancer (OvCa) constitutes a rare and highly aggressive malignancy and is one of the most lethal of all gynaecologic neoplasms. Due to chemotherapy resistance and treatment limitations because of side effects, OvCa is still not sufficiently treatable. Hence, new drugs for OvCa therapy such as P8-D6 with promising antitumour properties have a high clinical need. The benzo[c]phenanthridine P8-D6 is an effective inductor of apoptosis by acting as a dual topoisomerase I/II inhibitor.

Methods: In the present study, the effectiveness of P8-D6 on OvCa was investigated in vitro. In various OvCa cell lines and ex vivo primary cells, the apoptosis induction compared with standard therapeutic agents was determined in two-dimensional monolayers. Expanded by three-dimensional and co-culture, the P8-D6 treated cells were examined for changes in cytotoxicity, apoptosis rate and membrane integrity via scanning electron microscopy (SEM). Likewise, the effects of P8-D6 on non-cancer human ovarian surface epithelial cells and primary human hepatocytes were determined.

Results: This study shows a significant P8-D6-induced increase in apoptosis and cytotoxicity in OvCa cells which surpasses the efficacy of well-established drugs like cisplatin or the topoisomerase inhibitors etoposide and topotecan. Non-cancer cells were affected only slightly by P8-D6. Moreover, no hepatotoxic effect in in vitro studies was detected.

Conclusion: P8-D6 is a strong and rapid inductor of apoptosis and might be a novel treatment option for OvCa therapy.

Keywords: apoptosis, chemotherapy, drug development, dual topoisomerase inhibitor, hepatotoxicity, OvCa
causes single strand breaks while Topo II with its isoforms α and β is responsible for the double-strand break (Figure 1).5,6 Such topoisomerase poisoning leads to cell death by inducing apoptosis. The aza-analogous benzo[c]phenanthridine P8-D6 was synthesized in an optimized four-step process with advantageous physicochemical and cytotoxic properties.7 In the National Cancer Institute (NCI)-60 DTP Human Tumor Cell Line screening 49 nM of P8-D6 results in an average growth inhibition of 50% (GI50).7,8 The result for OvCa cell lines were 0.12 µM compared with cisplatin with 15.25 µM or topotecan with 0.23 µM, respectively.

Cell-based assays are an important pillar in drug development. In addition to traditional two-dimensional (2D) monolayer, co-culture and three-dimensional (3D) cell culture have recently gained importance because of greater comparability with in vivo set ups.9 Therefore, efficacy studies of P8-D6 were performed on different OvCa cell lines and patient-derived primary cells, compared with single drugs and combinational drug therapy.10–12

Preclinical cancer drug development addressed several topics, including target achievement, induction of apoptosis in cancer cells and toxicity in normal cells.

Methods

Materials

P8-D6 was synthesized as recently described7 and solved in phosphate-buffered saline (PBS). Topotecan, etoposide, cisplatin and doxorubicin were obtained from the UKSH dispensary.

In vitro experiments

Cell preparation and culture. Human OvCa cell lines A2780, BG-1, Igrov-1, OvCar8, SKOV-3 and fibroblasts Detroit 551 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum.
(FBS), 60 IU(µg)/ml penicillin–streptomycin. SKOV-3luc (firefly luciferase gene) were grown in DMEM (Dulbecco’s Modified Eagle Medium), supplemented with 10% FBS, 800 µg/ml neomycin. Primary OvCa cells were isolated from advanced stage OvCa patients during surgery at first diagnosis (UKSH, Campus Kiel). The tumour cells were extracted from tumour tissue and ascites as described previously.13,14 Human ovarian surface epithelial cells (HOSE) (Innoport) were cultivated in OSE medium containing 1% OEpiCGS, 100 IU(µg)/ml penicillin–streptomycin. Primary human hepatocytes were isolated from liver tissue under surgery (Uppsala University Hospital), isolated and cultured as described previously.15 Cells were grown at 37°C, 5% CO2 and short tandem repeat profiling and mycoplasma contamination by MycoAlert™ (Lonza) were checked.

Informed consent was obtained from all donors, in agreement with the approval from the Institutional Review Board of the UKSH, Campus Kiel (AZ: D578/20) and the Uppsala Regional Ethical Review Board (Ethical Approval 2009/028).

Western blot. Cells were harvested, protein contents determined and SDS-PAGE and western blot analysis was carried out as described previously.17 Membranes were incubated with primary antibodies (anti-TopoI 1:500 (Santa Cruz#sc-271285), anti-TopoIIα/β 1:10000 (Abcam#ab109524), anti-HSP 90 1:10000 (Santa Cruz#sc-13119)) and HRP-labelled anti-mouse IgG 1:3000 (Elabscience#E-AB-1003). Chemiluminescence was visualized using ECL Plus Western Blotting Detection System and ChemoStar ECL and Fluorescence Imager (Intas).

Fluorescence imaging. Due to its chemical structure, P8-D6 has fluorescent properties (462Ex/530Em). In all, 10,000 cells/well were seeded in a 96-well plate (Corning #3903) and treated for 48h. The measurement using ApoLive-Glo™ Multiplex Assay (Promega #G6410) was performed as described in the instruction (TM325) with a microplate reader (Infinite 200, Tecan). Relative caspase activity calculation: caspase activity divided by the viability (normalized to control). With viability data, dose–response curves were plotted and inhibitory concentration 50%(IC50) values were calculated (GraphPad).

Flow cytometric analysis. Cells were seeded in 6-well plates and treated for 48h. Cells were harvested and stained as described previously.17

The 2D viability and apoptosis assay. In all, 10,000 cells/well were seeded in a 96-well plate (Corning #3903) and treated for 48h. The measurement using ApoLive-Glo™ Multiplex Assay (Promega #G6410) was performed as described in the instruction (TM325) with a microplate reader (Infinite 200, Tecan). Relative caspase activity calculation: caspase activity divided by the viability (normalized to control). With viability data, dose–response curves were plotted and inhibitory concentration 50%(IC50) values were calculated (GraphPad).

The 3D cytotoxicity, viability and apoptosis assay: A2780 (200/well), SKOV-3 (8000/well), OvCar8 (1000/well) and BG-1 (450/well) cells were seeded into a 96-well Ultra-Low Attachment (ULA) plate (Corning #4520) and grown for 96h. Then, spheroids were treated for 48h. Simultaneously, CellTox™ Green assay (Promega #G8731) was added and detected (485Ex/520Em) 24 and 48h after treatment using NYONE® (SYNENTEC). Filters: BF Ex/GreenEm (530/43 nm); Blue Ex (475/28 nm)/GreenEm (530/43 nm). Subsequently, viability and apoptosis were determined by RealTime-Glo™ (460Em) (Promega #G9711) and Caspase-Glo 3/7 (565Em) (Promega #G8090) using microplate reader (Infinite 200, Tecan). The measurement was performed according to the instructions. Relative caspase activity: caspase activity divided by the viability (normalized to control). For live–dead staining, cells were grown and treated as described above. Then, 80% of the medium was removed and replaced with propidium iodide (PI) (10 µg/ml), calcein-AM (1 mM) and hoechst 33342 (0.001%) in medium for 3 h and imaged by NYONE® (SYNENTEC). Filters: BF Ex/GreenEm (530/43 nm); hoechst 33342: UV Ex (377/50 nm)/BlueEm (452/45 nm); calcein-AM: Blue Ex (475/28 nm)/GreenEm (530/43 nm); PI: LimEx (562/40 nm)/RedEm (628/32 nm).

Scanning electron microscopy (SEM). Spheroids were grown as described above, treated with 1 µM P8-D6 and PBS for 48h, and fixed with 2.5% glutaraldehyde (1 h room temperature) and then 1% osmium tetroxide (1.5 h room temperature). Spheroids were dehydrated with ethanol (25%, 50%, 75%, 96%, 100%) and air dried using hexamethyldisilazane on charcoal stubs overnight. Then, spheroids were coated with gold and measured with SEM (Phenom XL).
Co-culture. For co-culture, 40,000 Detroit 551 fibroblasts were seeded into 24-well plates, and 40,000 A2780 were cultured onto inserts (ThinCert™ translucent 0.4 µm). Cells were treated for 48 h. Cells were harvested and centrifuged (10 min, 250 g). Cell pellets were resuspended in 25 µl medium. Viability and apoptosis were measured using ApoLive-Glo™ Multiplex Assay.

Hepatotoxicity. Oxidative stress of hepatocytes caused by 48 h treatment was analysed via dihydroethidium fluorescence. The cell culture protocol was previously described. Apoptosis was analysed using ApoLive-Glo™ Multiplex Assay.

Statistical analysis. Statistical tests were performed using GraphPad Prism9 (GraphPad). Gaussian distribution was tested by Shapiro–Wilk normality test. Data of multiple groups were checked with one-way analysis of variance (ANOVA) for statistical significance. Statistically significant differences were assumed at p values < 0.05 (*) according to Tukey’s multiple comparison and Dunn’s method.

Results
Previous studies showed that P8-D6 functions as dual topoisomerase inhibitor. However, the effectiveness is highly dependent on reaching its nuclear target structure – topoisomerase I/II. In addition to colon cancer cells, we checked the location of P8-D6 and Topo I and II expression in OvCa. Using fluorescent microscopy, P8-D6 was identified in the nucleus (Additional file 1A and B in the Supplemental material). In addition, western blot provided evidence that all cells also express sufficient Topo I and Topo II (Additional file 1C in the Supplemental material).

P8-D6 is highly effective in OvCa 2D monolayers
The main aim of this study was to prove efficacy of P8-D6 in OvCa. Therefore, OvCa cell lines (A2780, Igrov-1, BG-1, OvCar8, SKOV-3, SKOV-3luc) were treated with P8-D6, and compared with topotecan, etoposide and cisplatin. Initially, the viability of OvCa cells after 48 h treatment was measured using an enzymatic assay and IC50-values were determined. P8-D6 exhibits a four times lower IC50-value and is, therefore, significantly more effective than the standard chemotherapeutics (Figure 2(a) and (h); Additional file 2A in the Supplemental material). A significantly higher increase of apoptosis after 48 h treatment with P8-D6 compared with its standard therapeutic drugs could be observed in all tested OvCa cell lines by using ApoLive-Glo™ Multiplex Assay (Figure 2(b), Additional file 2B–F in the Supplemental material) and flow cytometric analyses (Figure 2(d) and (e); Additional file 3B in the Supplemental material).

Since P8-D6 is a dual topoisomerase inhibitor, but the reference substances primarily inhibit one of the enzymes only, a combination of a Topo I (topotecan) and a Topo II (etoposide) inhibitor was analysed. Compared with this combination, P8-D6 shows a significantly higher rate of apoptosis (Figure 2(c); Additional file 3A in the Supplemental material). To validate the pronounced induction of apoptosis and anti-proliferative effect of P8-D6 in primary cells, we used ex vivo patient-derived cells from tumour tissue and ascites in a translational aspect. A significantly higher rate of anti-proliferative and apoptotic effect was observed in primary cells by P8-D6 compared with comparative substances (Figure 2(f) and (g); Additional file 2G and H in the Supplemental material). Altogether, we generated results of a plurality of different cells (established and primary cells) to emphasize the durable and robust effect of P8-D6 (Figure 2(h)).

P8-D6 induce strong effects in 3D target tumour and co-culture model
The 3D cell cultures are regarded to bridge the gap from 2D to in vivo models, since cell–cell interaction is considerable for the efficacy of a substance. The 3D spheroids mimic the physiological behaviour of solid tumours more closely. Spheroids were generated in ULA plates for 96 h and subsequently treated with P8-D6 or topotecan. The OvCa spheroids (A2780, SKOV-3, BG-1, OvCar8) showed a decrease in growth behaviour and stability after P8-D6 treatment (Figure 3(a); Additional file 4A–C in the Supplemental material). Cell toxicity was increased in all spheroids by P8-D6 compared with topotecan and PBS (Figure 3(b) and (c); Additional file 4D–F in the Supplemental material). Furthermore, P8-D6 exerted a significantly increased pro-apoptotic effect in all spheroids compared with control (Figure 3(d); Additional file 5A–C in the Supplemental material). To visualize the potency of P8-D6 in spheroids, a triple live–dead staining consisting of calcein-AM, PI and hoechst 33342 was used.
Figure 2. Antitumour responses in OvCa 2D monolayers. A2780 (cell line) and UF-168T (primary cells) were treated with different concentrations of P8-D6, topotecan, etoposide, cisplatin and negative control (PBS) for 48h. Subsequently, the viability and caspase activity were determined. (a) The IC_{50} values of each cytostatic drug were calculated by using the viability data. (b) The apoptosis is represented as relative caspase activity. (c) To compare the combinatorial apoptotic effect of topotecan (Topo I Inhibitor) and etoposide (Topo II Inhibitor) with P8-D6, a dual topoisomerase inhibitor was performed in A2780. (d) and (e) Flow cytometric analysis of pro-apoptotic effects with Annexin V-PE (An V) and 7AAD staining (n = 6). Representative flow cytometry dot plots of treated and stained A2780 cells were done. The mean distribution of viable (An V/7AAD-negative), early apoptotic (An V-positive, 7AAD-negative), late apoptotic/necrotic (An V/7AAD-positive) or necrotic (An V-negative, 7AAD-positive) tumour cells after treatment were calculated (d). (f) For primary OvCa cells (UF-168T) viability and apoptosis were measured. (g) In addition, the anti-proliferative effect after 24-h treatment was evaluated by microscopy. Scale bars, 50 µm. (h) Heat map of the IC_{50} values using the viability of all tested OvCa cells. Data are means + SD (standard deviation) one-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 3. Antitumour properties in 3D spheroids and 2D co-culture. For 3D culture, A2780, SKOV-3, OvCar8 and BG-1 cells were maintained in ULA plates for 96 h and subsequently treated with P8-D6 [0.1, 0.5, 1 and 10 µM], topotecan [1 and 10 µM] and PBS for 48 h. (a) Every 24 h images were generated by microscopy. Scale bars, 500 µm. (b) and (c) During treatment, the cell toxicity was measured by fluorescence microscope using CellTox™ Green [24 and 48 h]. Scale bars, 500 µm. The fluorescence signals for 24 and 48 h after treatment were quantified (relative fluorescence units RFU) and shown in a heat map (c). (d) After 48-h treatment, the viability and caspase activity were measured in A2780 spheroids. (e) A2780 spheroids were stained after the growth and treatment phase with PI [red], calcein-AM [green] and hoechst 33342 [blue], and measured by microscopy. Scale bars, 500 µm. (f) SEM images of A2780 and OvCar8 spheroids, which were treated with P8-D6 [1 µM] or PBS for 48 h were taken. Scale bars, 20 µm. (g) For co-culture experiments, A2780 cells were seeded in 2D monolayers on transwell inserts and fibroblasts on well bottoms. For comparison, mono-cultures were cultured and treated with P8-D6 [10 µM], etoposide [10 µM], topotecan [10 µM] and PBS in the same way. The apoptosis represented as relative caspase activity was measured in A2780 and fibroblasts. Data are means + SD (n=3) one-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
The decreased staining of calcein-AM and the increase of cells stained with PI proved the strong cytotoxic effect of P8-D6. In addition, P8-D6 treated cells showed disintegration of the spheroid and a considerably higher number of dead cells than topotecan. A 10-fold lower dose of P8-D6 showed similar results as 1 µM topotecan. Moreover, SEM identified surface changes of the spheroids like loss of membrane integrity due to treatment (Figure 3(f), Additional file 4G in the Supplemental material).

In addition, it was investigated whether fibroblast-A2780 co-culture could mediate changes in cancer cell responses to anti-cancer drugs by affecting cell–cell interaction (Figure 3(g)). Co-culture promotes the apoptosis induction significantly for P8-D6 treated cancer cells compared with mono-culture, while the co-culture treated cancer cells with etoposide and topotecan only exhibited minimal activity differences.

**P8-D6 only slightly affects non-cancer cells**

Since side effects often occur as a result of tumour therapy, it is important to determine the toxicity of P8-D6 in preclinical setting on non-cancerous cells such as HOSE. HOSE were treated for 48 h and the anti-proliferative effect of P8-D6 is only slightly increased compared with the reference (Figure 4(a)). While 1 µM P8-D6 is highly effective on cancer cells, no significant increase of apoptosis was measured compared with PBS control in HOSE cells. The application of 10 µM P8-D6 shows a similar value as positive controls (Figure 4(b)).

Hepatotoxicity is a common and serious side effect in chemotherapy. By measuring oxidative stress and induction of apoptosis after 48-h
treatment in primary human hepatocytes, P8-D6 showed no significant difference compared with PBS, while doxorubicin induces significant cell damage effects (Figure 4(c)). P8-D6 proved to be cytotoxic against OvCa cells without inducing cell death in hepatocytes.

Discussion

Since OvCa is one of the world’s deadliest gynaecological malignancies, there is a high clinical need for the development of new, effective and well-tolerated therapy options with suitable physicochemical properties. P8-D6 was investigated as a novel dual topoisomerase inhibitor in OvCa cell line and ex vivo patient-derived primary cells in 2D, 3D and co-culture, and was proven to be overall significantly more effective than standard therapeutics and negative control.

Side effects due to chemotherapy have significant impacts on therapy. However, P8-D6 shows only limited toxic effects on normal cells measured by oxidative stress and induces apoptosis. The significantly reduced influences on the normal cells could be explained by the reduced ability of these cells to divide, but these causes have to be investigated furthermore. If efficacy and toxicity studies are related to clinical therapy, P8-D6 induces significantly higher (>five-fold) apoptosis rates in OvCa than current standards. Thus, it could potentially be used in lower doses than the standard therapeutic agents to achieve the same effect.

Due to the dual topoisomerase inhibition of P8-D6, the question arises whether a combination therapy of two established mono-Topo I/II inhibitors reach the antitumour potency of P8-D6 in OvCa. Previous studies compared the combination of Topo I with Topo II inhibitor versus monotherapy and showed heterogeneous results. This study showed significantly higher induction of apoptosis by P8-D6 when compared with the additive effect of Topo I and Topo II inhibitor combination. So far, the combination of topotecan and etoposide shows no clinical benefit and is not considered as a clinical standard, whereas the monotherapy of topotecan is proposed to treat in second line therapy.

A combination of P8-D6 with a tyrosyl-DNA phosphodiesterase inhibitor or a Poly (ADP-ribose) polymerase (PARP) inhibitor could merit additional consideration. These enzymes are involved in the repair mechanisms of the Topo-DNA complex and would possibly have a further positive effect in apoptosis induction. Liposomal formulations could also be possible further approaches for P8-D6. This special drug formulation would significantly increase the selectivity of the active ingredient and thus contribute to better tolerability of the active ingredient, since topoisomerase are ubiquitous in cells and can thus trigger slight side effects there, as known with other cytostatics.

Conclusion

In summary, P8-D6 has promising antitumour properties in 2D, 3D and co-culture in OvCa. It has fewer effects on normal ovarian cells and hepatocytes than its references. To sum up, P8-D6 is a strong and rapid inductor of apoptosis.
and warrants further development. Further in vivo experiments for P8-D6 are needed to verify anti-tumour effects also for complex multiorgan systems. In addition, further studies on other side effects that could lead to dose-limitation should be performed.

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Supplemental material
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