Newly Developed Dual Topoisomerase Inhibitor P8-D6 is Highly Active in Ovarian Cancer

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Research

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RESEARCH

1 Newly developed dual topoisomerase inhibitor P8-

2 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 to standard therapeutic agents was determined in 2D monolayers. Expanded by 3D 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 standard therapeutic drugs. Non-cancer cells were affected only slightly by P8-D6. Moreover, no hepatotoxic effect in *in vitro* studies was detected.

Conclusions

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

Keywords: OvCa, chemotherapy; drug development; dual topoisomerase inhibitor; apoptosis, 2D, 3D, co-culture, primary cells, hepatotoxicity,
Background

Ovarian cancer (OvCa) is the fifth leading cause of cancer deaths among women and the most lethal gynaecological malignancy in the developed world (1). The frequent diagnosis in advanced stages and insufficient treatment options due to chemotherapy resistance and side effects lead to a poor prognosis. First line therapy usually consists of surgical cytoreduction followed by platinum/taxane-based combination chemotherapy, but most patients relapse with drug-resistance in the course of the disease (2). Previous studies demonstrated a benefit in second line therapy with topoisomerase inhibitors like liposomal doxorubicin or topotecan. However, response duration was commonly short (3). Consequently, an important aim is to reduce mortality by improved new therapeutic options.

P8-D6 acts as a dual topoisomerase poison by stabilizing the covalent Topo-DNA-intermediate of both topoisomerase (Topo) enzymes I and II (4). Topoisomerases regulate torsional stress in DNA to enable essential genome functions (e.g. transcription, replication, or recombination). Topo I causes single strand breaks while Topo II with its isoforms α and β is responsible for the double-strand break (5, 6) (Fig. 1). 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 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 to cisplatin with 15.25 µM or topotecan with 0.23 µM, respectively.
Fig. 1. Chemical structure and mechanism of P8-D6 action. P8-D6 acts as a dual topoisomerase inhibitor by stabilising the cleavable Topo–DNA complex, thereby inducing apoptosis. The effectiveness and the broad activity spectrum of P8-D6 were examined for the first time in a 60-tumour cell line panel by the NCI. In the evaluation, P8-D6 reached an average $\Sigma G_{I50}$ value of 49 nM in multiple tumour cell lines (7). For the ovarian carcinoma cells tested, this $G_{I50}$ average was 0.12 µM. For comparison, other active drugs are listed. $\Sigma G_{I50}$ (60): average growth inhibition 50% in 60 cancer cell lines (different cancer types), $\Sigma G_{I50}$ (OvCa): average growth inhibition 50% in OvCa cell lines.

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 to 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 described (7) and solved in PBS. Topotecan, etoposide, cisplatin and doxorubicin were obtained from the UKSH dispensary.

In vitro experiments

Cell Preparation and Culture: Human OvCa cell lines A2780, HEY, Igrov-1, OvCar8, SKOV-3 and fibroblasts Detroit 551 were maintained in RPMI 1640 supplemented with 10 % FBS, 60 IU(µg)/ml penicillin–streptomycin. SKOV-3lac (firefly luciferase gene) were grow in DMEM, 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 (16) 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 1:2000 (Santa Cruz#sc-516102) or HRP-labelled goat anti-rabbit IgG 1:3000 (Elabscience#E-AB-1003)
Chemiluminescence was visualized using ECL Plus Western Blotting Detection System and ChemoStar ECL & Fluorescence Imager (Intas).

Fluorescence Imaging: Due to its chemical structure, P8-D6 has fluorescent properties (462 Ex/530 Em).

10,000 cells/well were seeded in glass-bottomed 4-well chamber slides and treated with 10 µM P8-D6 or PBS for 30 min, washed with PBS and fixed with acetone (10 min at RT). Samples were stained by CellTracker™ Deep Red Dye (5 µM at 37 °C for 15 min) and DAPI (0.5 µg/ml) (Vectashield). Fluorescence imaging was performed using DAPI (350 Ex/470 Em), FITC (490 Ex/525 Em) and Cy5 (649 Ex/670 Em) filters. Microscope Axioplan 2 (Carl Zeiss Microscopy), Isis Version 5.8.8 (MetaSystems).

2D Viability & Apoptosis Assay: 10,000 cells/well were seeded in a 96-well plate (Corning #3903) and treated for 48 h. 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: caspase activity divided by the viability (normalized to control)).

With viability data dose-response curves were plotted and IC50 values were calculated (GraphPad).

Flow Cytometric Analysis: Cells were seeded in 6-well plates and treated for 48 h. Cells were harvested and stained as described previously (17).

3D Cytotoxicity, Viability & Apoptosis Assay: A2780 (200/well), SKOV-3 (8000/well), OvCar8 (1000/well) and HEY (450/well) cells were seeded into a 96-well ULA plate (Corning #4520) and grown for 96 h. Then, spheroids were treated for 48 h. Simultaneously, CellTox™ Green assay (Promega #G8731) was added and detected (485 Ex/520 Em) 24 h and 48 h after treatment using NYONE® (SYNENTEC). Filters: BFl/E/GreenEm(530/43 nm); BlueFl(475/28 nm)/GreenEm(530/43 nm).

Subsequently, viability and apoptosis were determined by RealTime-Glo™ (460 Em) (Promega #G9711) and Caspase-Glo 3/7 (565 Em) (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<sub>Ex</sub>/Green<sub>Em</sub>(530/43nm); hoechst3342: UV<sub>Ex</sub>(377/50nm)/Blue<sub>Em</sub>(452/45nm); calcein-AM: Blue<sub>Ex</sub>(475/28nm)/Green<sub>Em</sub> (530/43nm); PI: Lime<sub>Ex</sub>(562/40nm)/Red<sub>Em</sub>(628/32nm).

Scanning Electron Microscopy (SEM): Spheroids were grown as described above, treated with 1 µM P8-D6 and PBS for 48 h and fixed with 2.5% glutaraldehyde (1 h RT) and then 1% osmium tetroxide (1.5 h RT). 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 0.4µm). 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 (15). 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 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 (4, 7). However, the effectiveness is highly dependent on reaching its nuclear target structure -topoisomerase I/II. In
addition to colon cancer cells (4), 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-B). Additionally, western blot provided evidence that all cells also express sufficient Topo I and Topo II (Additional file 1C).

**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, HEY, OvCar8, SKOV-3, SKOV-3luc) were treated with P8-D6, and compared to 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 (Fig. 2A, 2H; Additional file 2A). A significantly higher increase of apoptosis after 48 h treatment with P8-D6 compared to its standard therapeutic drugs could be observed in all tested OvCa cell lines by using ApoLive-Glo™ Multiplex Assay (Fig. 2B, Additional file 2B-F) and flow cytometric analyses (Fig. 2D-E; Additional file 3B).

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 to this combination, P8-D6 shows a significantly higher rate of apoptosis (Fig. 2C; Additional file 3A). 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 to comparative substances (Fig. 2F; 2G; Additional file 2G-H). Altogether, we generated results of a plurality of different cells (established and primary cells) to emphasize the durable and robust effect of P8-D6 (Fig. 2H).
Fig. 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 48 h. Subsequently, the viability and caspase activity were determined. (A) The IC50 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) to P8-D6, a dual topoisomerase inhibitor was performed in A2780. (D) (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) Additionally, the anti-proliferative effect after 24 h treatment was evaluated by microscopy. Scale bars, 50 µm. (H) Heat map of the IC50 values using the viability of all tested OvCa cells. Data are means ± SD one-way ANOVA, * (p <0.05), ** (p <0.01), *** (p <0.001), **** (p <0.001).

**P8-D6 induce strong effects in 3D target tumour and co-culture model**

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. 3D spheroids mimic the physiological behaviour of solid tumours more closely (10, 11). Spheroids were generated in Ultra-Low Attachment (ULA) plates for 96 h and subsequently treated with P8-D6 or topotecan. The OvCa spheroids (A2780, SKOV-3, HEY, OvCar8) showed a decrease in growth behaviour and stability after P8-D6 treatment (Fig. 3A; Additional file 4A-C). Cell toxicity was increased in all spheroids by P8-D6 compared to topotecan and PBS (Fig. 3B-C; Additional file 4D-F). Furthermore, P8-D6 exerted a significantly increased proapoptotic effect in all spheroids compared to control (Fig. 3D; Additional file 5A-C). To visualize the potency of P8-D6 in spheroids, a triple live-dead staining consisting of calcein-AM, PI, and hoechst 33342 was used (Fig. 3E, Additional file 5D-F). The decreased staining of calcein-AM and the increase of cells stained with PI, proved the strong cytotoxic effect of P8-D6. Additionally, P8-D6 treated cells showed disintegration of the spheroid and a considerably higher number of dead cells than topotecan. 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 (Fig. 3F, Additional file 4G).

Additionally, it was investigated whether fibroblast-A2780 co-culture could mediate changes in cancer cell responses to anti-cancer drugs by affecting cell-cell interaction (Fig. 3G). Co-culture promotes the apoptosis induction significantly for P8-D6 treated cancer cells compared to mono-culture, while the co-culture treated cancer cells with etoposide and topotecan only exhibited minimal activity differences.
Figure 3. Antitumour properties in 3D spheroids and 2D co-culture. For 3D culture, A2780, SKOV-3, OvCar8 and HEY cells were maintained in ULA plates for 96 h and subsequently treated with P8-D6 [0.1 µM, 0.5 µM, 1 µM, 10 µM], topotecan [1 µM, 10 µM] and PBS for 48 h. (A) Every 24 h images were generated by microscopy. Scale bars, 500 µm. (B) (C) During treatment the cell toxicity was measured by fluorescence microscope using CellTox™ Green (24 h, 48 h). Scale bars, 500 µm. The fluorescence signals for 24 h and 48 h after treatment were quantified (fluorescence intensity 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), hoechst 33342 (blue) and measured by microscopy. Scale bars, 500 µm. (F) SEM images of A2780 and OvCa8 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 <0.001).

**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 human ovarian surface epithelium cells (HOSE). HOSE were treated for 48 h and the anti-proliferative effect of P8-D6 is only slightly increased compared to the reference (Fig. 4A). While 1 µM P8-D6 is highly effective on cancer cells no significant increase of apoptosis was measured compared to PBS control in HOSE cells. The application of 10 µM P8-D6 shows a similar value as positive controls (Fig. 4B).

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 to PBS, while doxorubicin induces significant cell damage effects (Figure 5C). P8-D6 proved to be cytotoxic against OvCa cells without inducing cell death in hepatocytes.
**Fig. 4.** Effects on non-cancer cells. (A) To examine toxic effects of therapy, human ovarian surface epithelial cells (HOSE) were treated with P8-D6, cisplatin, etoposide, topotecan and PBS for 48 h. The anti-proliferative effect after 48 h treatment was evaluated by microscopy. Scale bars, 100 µm. (B) The viability and apoptosis were measured after 48 h treatment in HOSE cells. Data are means ± SD (n=6) one-way ANOVA, * (p <0.05), ** (p <0.01), *** (p <0.001), **** (p <0.001). (C) To investigate the hepatotoxicity, primary human hepatocytes were treated with P8-D6, doxorubicin and PBS for 48 h. Afterwards, microscopy images were taken, and apoptosis and oxidative stress measurements were performed. Data are means ± SD (n=3) one-way ANOVA, * (p <0.05), ** (p <0.01), *** (p <0.001), **** (p <0.001).

**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 physiochemical properties (1). 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. 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 (18–21). This study showed significantly higher induction of apoptosis by P8-D6 when compared to the additive effect of Topo I and Topo II inhibitor combination. So far, the combination of topotecan and etoposide shows no clinical benefit (22) and is not considered as a clinical standard, whereas mono-therapy is (23).

A major advantage of a dual topoisomerase inhibitor is the reduced development of resistance through inhibition of both topoisomerases. The inhibition of only one enzyme causes a compensatory upregulation of the other (21, 24). The effectiveness of dual topo-inhibitors on solid tumours has been shown previously (25, 26). In clinical trials, however, these drugs showed intolerable side effects. (e.g. intoplicine, TA-103, batracylin), such as hepatotoxicity and severe neutropenia (26–32). Because of this increased hepatotoxicity with dual topoisomerase inhibitors (33), the hepatotoxicity of P8-D6 was determined. Importantly, this study shows that P8-D6 has no relevant effect concerning oxidative stress and apoptosis in human hepatocytes. The mechanism of action is the key benefit and novel about these drugs by affecting both topoisomerase inhibitors. However, it is a validated target since single Topo inhibitors are already standards in cancer therapy (23).

The current therapy for platinum-resistant/refractory OvCa consists mainly of a monotherapy with topotecan or PLD, but cancer control rate is limited (34–36). Hence, some novel drugs are in clinical trials like the tyrosine kinase inhibitor rivoceranib (37). The antibody drug conjugate (ADC) Mirvetuximab soravtansine (MIRV) comprise a FRα-binding antibody, cleavable linker, and the...
tubulin-targeting toxin DM4. A Phase III study evaluated the safety and efficacy of MIRV compared to chemotherapy in patients with platinum-resistant OvCa (38).

A combination of P8-D6 with a tyrosyl-DNA phosphodiesterase inhibitor or a 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 (39, 40). Liposomal formulations could also be possible further approaches for P8-D6.

Conclusions:

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 antitumour effects also for complex multiorgan systems. Additionally, further studies on other side effects that could lead to dose-limitation should be performed.

List of abbreviations

2D, two-dimensional; 3D, three-dimensional; 5-FU, fluorouracil; ADC, antibody drug conjugate; DAPI, 4′,6-Diamidin-2-phenylindol; FRα, Folate receptor alpha; GI50, Growth inhibitory of 50 %; HOSE, human ovarian surface epithelial cells; IC50, inhibitory concentration 50 %; MIRV, Mirvetuximab soravtansine; NCI, National Cancer Institute; OvCa, OvCa; PARP, Poly (ADP-ribose) polymerase; PI, propidium iodide; PLD, pegylated liposomal doxorubicin; RFU, relative fluorescence units; RLU, relative luminescence units; SD, standard deviation; SEM, scanning electron microscopy; Topo, topoisomerase; UKSH, university Hospital Schleswig-Holstein; ULA, Ultra-Low Attachment

Declarations

Ethics approval and consent to participate: Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the University Medical Centre Schleswig–Holstein, Campus Kiel (AZ: D578/20) and the Uppsala Regional Ethical Review Board (Ethical Approval no. 2009/028). Informed consent was obtained from all subjects involved in the study.

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Appendices

Additional file 1: Target control. (A) (B) A2780 (A) and OvCar8 (B) were treated with 10 µM P8-D6 (fluorophore: 462Ex/530Em) or negative control (PBS) for 30 min. Then cells were fixed, and membrane and nucleus were stained by CellTracker™ Deep Red Dye and DAPI. Fluorescence images show the fluorophore (green) on the left, membrane staining (red) in the middle and finally the combination of drug (green), membrane (red) and nucleus (blue) staining on the right. Scale bars, 20 µm. (C) Investigated cells were lysed and protein expression was analysed by using western blot. All tested cells except for the non-cancer HOSE cells are OvCa cells. HSP 90 was used as loading control.
Additional file 2: Cytotoxicity and apoptosis induction in OvCa 2D monolayers. Ovarian cancer cells were treated with different concentrations of P8-D6, topotecan, etoposide, cisplatin and negative control [PBS] for 48 h. Afterwards the viability and caspase activity were determined. A) The IC\textsubscript{50} value of each...
cytostatic drug in HEY cells was calculated by using the viability data. (B-H) The apoptosis is represented as relative caspase activity in HEY (B), Igrov-1 (C), OvCar8 (D), SKOV-3 (E), SKOV-3luc (F) and primary ovarian cancer cells UF-169T (G), UF-160AS (H). Data are means ± SD one-way ANOVA, *(p < 0.05), **(p < 0.01), *** (p < 0.001), **** (p < 0.001)).

Additional file 3: Apoptosis induction in OvCa 2D monolayers. Ovarian cancer cells were treated with different concentrations of P8-D6, topotecan, etoposide, cisplatin and negative control [PBS] for 48 h. Afterwards, the apoptosis rate was determined. (A) Comparison of the combinatorial apoptotic effect of topotecan (Topo I Inhibitor) and etoposide (Topo II Inhibitor) to P8-D6, a dual topoisomerase inhibitor was performed in Igrov-1 after 48 h. (B) (C) Flow cytometric analysis of pro-apoptotic effects on SKOV-3luc (B) and UF-168T. (C) cells after treatment and staining with Annexin V-PE and 7AAD (n=3) 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). Data are means ± SD one-way ANOVA, *(p < 0.05), **(p < 0.01), *** (p < 0.001), **** (p < 0.001)).
Additional file 4: Morphological changes and cell toxicity in OvCa spheroids. For 3D culture SKOV-3, OvCar8 and HEY cells were maintained in ULA plates for 96 h and subsequently treated with P8-D6 [0.1 µM, 0.5 µM, 1 µM, 10 µM], topotecan [1 µM, 10 µM] and PBS for 48 h. (A-C) Every 24 h, images were generated by microscopy (NYONE® Scientific (SYNENTEC)). Scale bars, 500 µm. HEY (A), OvCar8 (B), SKOV-3 (C). (D-F) During treatment, the cell toxicity was measured by fluorescence microscopy using CellTox™ Green. The fluorescence intensities for 24 h and 48 h after treatment were quantified and shown in heat maps. HEY (D), OvCar8 (E), SKOV-3 (F). (n=3) (G) SEM images of SKOV-3 and HEY spheroids, which were treated with P8-D6 [1 µM] or PBS for 48 h were taken. Scale bars, 20 µm.
Additional file 5: Apoptosis induction and live-dead staining in OvCa spheroids. For 3D culture SKOV-3, OvCar8 and HEY cells were maintained in ULA plates for 96 h and subsequently treated with P8-D6 [0.1 µM, 0.5 µM, 1 µM, 10 µM], topotecan [1 µM, 10 µM] and PBS for 48 h. (A-C) After 48 h treatment, the viability and caspase activity were measured in HEY (A), OvCar8 (B) and SKOV-3 (C) spheroids. Data are means (n=3) + SD one-way ANOVA, * (p <0.05), ** (p <0.01), *** (p <0.001), **** (p <0.001)). (D-F) HEY (D), OvCar8 (E) and SKOV-3 (F) spheroids were stained after growth and treatment phase with PI (red), calcein-AM (green), hoechst 33342 (blue) and measured by microscopy (NYONE® Scientific (SYNENTEC)). Scale bars, 500 µm.