Abstract. Background: D-116883 (Aeterna Zentaris GmbH, Frankfurt, Germany) is an orally effective drug that acts via inhibition of phosphatidylinositol 3-kinase (PI3K). The PI3K/AKT signal transduction pathway is involved in ovarian cancer tumorigenesis. Phosphatase and Tensin homolog (PTEN) loss and other activating mutations frequently contribute to the activation of this pathway. We tested whether D-116883 exerts cytostatic effects in in vitro models of ovarian cancer and analyzed the induced programmed cell death. Materials and Methods: We evaluated the potency of D-116883 in four ovarian carcinoma cell lines with different cellular assays. The effects of D-116883 on cell proliferation was analysed by crystal-violet staining and tetrazolium salt [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT] assay. The capacity for anchorage-independent growth was analyzed in two ovarian carcinoma cell lines without and with D-116883 addition by using the soft agar assay. Fluorescence activated cell sorting (FACS) cell cycle analyses were performed. Cells were incubated with multicaspase inhibitor benzoyloxycarbonyl-val-ala-asp(OMe)-fluoromethylketone (zVAD) and inhibitor of necroptosis necrostatin. Results: Growth inhibition occurred in all ovarian carcinoma cell lines studied (A2780, A2780cis, OAW42 and SKOV3) in a micromolar range (IC50<1 μM). By using soft agar assay, a reduced capacity for anchorage-independent growth, a hallmark of tumor cells, caused by D-116883 was demonstrated. Cell cycle analyses showed that D-116883 dose-dependently increased apoptotic cells. Multicaspase inhibitor zVAD and inhibitor of necroptosis necrostatin did not abrogate the growth-inhibiting effect of the compound. Conclusion: PI3K inhibitor D-116883 showed substantial cytotoxic effects in various in vitro models of ovarian cancer. Our results make D-116883 a good candidate for further ovarian cancer research including in vivo experiments.

Ovarian cancer is the major cause of gynecological cancer deaths worldwide (1). Present therapies fail mainly because of the advanced stage of the disease at the time of diagnosis. Advanced epithelial ovarian cancer is currently treated by cytoreductive surgery and platinum-based chemotherapy. Yet after an initial response (2), most patients eventually experience disease recurrence and progression due to chemoresistance, which unfortunately occurs in most cases (3). This underlines the importance of establishing new strategies for the treatment of ovarian cancer.

Phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/v-akt murine thymoma viral oncogene homolog (AKT)/mammalian target of rapamycin (mTOR) pathway is central in the transmission of growth regulatory signals (4). PTEN loss and other activating mutations frequently contribute to the activation of this PI3K/AKT signal transduction pathway. Furthermore, amplification of multiple components of the PI3K pathway is a hallmark of serious epithelial ovarian cancer. The resultant activation of the PI3K pathway in ovarian cancer contributes to cell-cycle progression, reduced apoptosis, and increased metastatic capability (5). Inhibition of PI3K and thereby shutting down this pathway seems to be a promising target for cancer therapy, as it is a main nodal point for transducing extracellular and intracellular oncogenic signals. Alterations of the PI3K/AKT pathway have been detected in several human malignancies and also in ovarian cancer (6). PI3K has a broad range of downstream effectors that regulate cell processes, such as cell growth, cell-cycle progression, survival, migration, and angiogenesis (7). Inhibition of the PI3K/AKT pathway signalling results in apoptosis and growth inhibition of tumor cells with elevated AKT. AKT was found to be activated in 68% of human ovarian cancer specimens (6) and PI3K was found to be mutated in 12% of the cases (8).
**PI3KCA** gene, encoding a catalytic subunit of PI3K, is mutated and/or amplified in various neoplasms, including ovarian cancer (9). PIK3CA amplification is associated with resistance to platin-containing chemotherapy in ovarian cancer patients (9). Targeting the PI3K/AKT pathway therefore seems to be a logical therapeutic strategy in ovarian cancer.

Huang *et al.* analyzed copy number alterations, using array comparative genomic hybridization (aCGH) on 93 primary ovarian tumors and identified PI3K/AKT pathway as the most frequently altered cancer-related pathway (10). Furthermore, these authors demonstrated survival analyses to correlate gene copy number and mutation data with patient outcome and showed that copy number gains of PIK3CA, PIK3CB, and PIK3R4 in these tumors were associated with reduced survival.

siRNAs targeting altered PI3K/AKT pathway genes inhibited proliferation and induced apoptosis in ovarian cancer cell lines (10). Huang *et al.* concluded that their results would strongly support the utilization of PI3K pathway inhibitors in ovarian cancer.

D-116883 is an orally effective drug, that acts via inhibition of PI3K. Ovarian cancer is believed to be selectively sensitive to pharmacological and genetic manipulation of the PI3K pathway, making molecular therapeutics targeting this pathway particularly attractive approaches for this type of cancer (5). We tested whether D-116883 exerts cytostatic effects in *in vitro* models of ovarian cancer and analyzed the induced programmed cell death with a panel of assays.

**Materials and Methods**

**Reagents and cell lines.** PI3K inhibitor D-116883 was kindly provided by Aeterna Zentaris GmbH (Frankfurt, Germany). Human ovarian cancer cell lines SKOV3, A2780 and A2780cis were obtained from the American Type Culture Collection (Manassas, VA, USA). OAW42 were obtained from Cell Line Services, Heidelberg, Germany. All cells were grown in RPMI-1640 medium (PAA, Cölbe, Germany) containing 10% fetal calf serum, 2 mM glutamine, and 1% penicillin/streptomycin, 0.5% sodium pyruvate solution. Primary human peripheral blood lymphocytes (PBL) were obtained by venipuncture from healthy individuals and purified by density gradient centrifugation (Biocoll, Biochrom, Berlin, Germany). Cells were maintained in X-Vivo 15 (Lonza, Bioscience, Vervier, Belgium). Propidium iodide (PI) and all other reagents, unless indicated otherwise, were purchased from Sigma (Taufkirchen, Germany). The chemiluminescent HRP substrate solution (Milipore, Schwalbach, Germany) was used for detection.

**Flow cytometry.** For cell cycle analysis, cells were treated with D-116883 as indicated, harvested, fixed and permeabilized overnight in ice-cold 70% ethanol (Merck, Darmstadt, Germany). The cells were washed twice with PBS. RNA was digested with RNase A (Gibco Life Technologies, Paisley, UK). The DNA was stained with PI (50 μg/ml). Fluorescence was recorded in a FACSCalibur instrument (Becton Dickinson, Heidelberg, Germany). Instrument settings were adjusted to move the G0/G1 peak to 200 relative fluorescence units. Cells to the left of this peak appeared to have a DNA content below 2n, indicative of cell death. Aggregated cells were gated out. A total of 20,000 events per condition were recorded.

**Soft agar assay to measure anchorage-independent growth of ovarian cancer cells.** Anchorage-independent growth of cells in soft agar is one of the characteristics of cellular transformation and uncontrolled cell growth, with normal cells typically not growing in semisolid matrices. We tested the effect of D-116883 in a soft agar assay, which has a 3-dimensional format. Parental A2780 and cisplatin-resistant A2780cis human ovarian cancer cells (105 cells in RPMI, 20% FCS) were mixed with an equal volume of 0.8% agarose (Roth, Karlsruhe, Germany) and poured onto a bed of 1.4% agarose in 6-well plates. Transformed tumor cells usually form multicellular colonies within a couple of days. Addition of PI3K inhibitor D-116883 after cell seeding allowed for the analyses of
antitumorigenic effects. The analysis was carried out as a screening looking at the effect of a concentration of 0.2 μM of D-116883. Medium was changed every three days. Evaluation of colony-forming ability was performed on day 18.

Results

D-116883 inhibits the growth of human ovarian cancer cells. A2780, A2780cis, OAW42 and SKOV3 human ovarian cancer cell lines were treated with increasing concentrations of PI3K inhibitor D-116883 or with solvent only for 24, 48 and 72 h. All experiments were repeated at least in triplicates. While the solvent DMSO did not affect cell growth, D-116883 displayed strong growth-inhibitory effects that became more pronounced with increasing treatment time. Half maximal inhibitory concentration (IC50) values at 24 h were approximately 0.4 μM for OAW42 and 1 μM for SKOV3 cells; at 48 h approximately 0.25 μM for OAW42 and 0.25 for SKOV3 cells; and at 72 h were approximately 0.2 μM for OAW42 and 0.15 μM for SKOV3 cells. With respect to proliferation of platin-sensitive cell line A2780 and platin-refractory cell line A2780cis, IC50 values were also determined. IC50 values at 24 h were approximately 0.4 μM for A2780 and 1 μM for A2780cis cells; at 48 h were approximately 0.25 μM for A2780 and 0.25 μM for A2780cis cells; and at 72 h were approximately 0.2 μM for A2780 and 0.15 μM for SKOV3 cells. With respect to proliferation of platin-sensitive cell line A2780 and platin-refractory cell line A2780cis, IC50 values were also determined. IC50 values at 24 h were approximately 0.4 μM for A2780 and 1 μM for A2780cis cells; at 48 h were approximately 0.25 μM for A2780 and 0.25 μM for A2780cis cells; and at 72 h were approximately 0.2 μM for A2780 and 0.15 μM for SKOV3 cells (Figure 1).

In order to analyze if D-116883 displays antiproliferative effect in metabolically inactive cells, we treated these four cell lines with the compound while cultivating them in serum-free medium, i.e. medium devoid of growth factors. Increasing concentrations of D-116883 which ranged between 0.05 and 25 μM were used, however, no or only minimal reduction of the cytotoxicity of the drug was observed (data not shown).

D-116883 inhibits anchorage-independent growth of ovarian cancer cells. Anchorage-independent growth is a hallmark of malignant tumor cells. We aimed to elucidate whether D-116883 has the capacity to inhibit anchorage-independent three-dimensional growth using the soft agar assay. D-116883 did inhibit anchorage-independent growth of platin-sensitive cell line A2780 and platin-refractory cell line A2780cis. D-116883 also inhibited growth of cell lines OAW42 and SKOV3 (data not shown).

D-116883 inhibits AKT phosphorylation in A2780 and A2780cis human ovarian cancer cells. The presence of phosphorylated AKT (pAKT) was assessed by western blotting in A2780 and A2780cis cells that had been treated with 0.25 μM D-116883. Specific antibody reactivity was detected at an apparent molecular weight of 60 kDa for pAKT. pAKT was dose-dependently reduced by treatment with D-116883 (Figure 3). In A2780cis cells (Figure 3 B), pAKT expression is stronger than in the parental A2780 cells (Figure 3 A).

DNA cell cycle analysis by FACS. In order to assess whether D-116883 arrested cell growth in a specific phase of the cell cycle or rather caused cell death, we performed a flow cytometric DNA cell cycle analysis on D-116883-treated A2780, A2780cis and SKOV3 cells. This revealed a dose-dependent decrease of cells in the G0/G1, S and G2 phases of the cell cycle, whereas the fraction of hypodiploid cells appearing to the left of the G0 cell population was concomitantly increased (Figure 4). Since this subG0-population is indicative of cell death, it can be concluded that D-116883 not only arrests the growth of ovarian cancer cells, but also actually kills tumor cells irrespective of their current state in the cell cycle. In contrast, D-116883 did not affect the cell-cycle distribution of primary human peripheral blood lymphocytes (data not shown).

Co-treatment of human ovarian cancer cells with D-116883 and multicaspase inhibitor z-VAD-FMK as well as with necrostatin, an inhibitor of necroptosis. We incubated A2780 and A2780cis ovarian cancer cells with increasing concentrations of D-116883 and the multicaspase inhibitor z-VAD-FMK (30 μM) for 48 h. In addition A2780 and A2780cis ovarian cancer cells were incubated with increasing concentrations of D-116883 and necrostatin, an inhibitor of necroptosis (50 μM), for 48 h (Figure 2). There was no difference in the D-116883-induced cytotoxicity regardless of caspase inhibition by z-VAD-FMK or necroptosis inhibition by necrostatin in A2780 and A2780cis ovarian cancer cells (Figure 2).

Discussion

Ovarian cancer patients can be divided into two different prognostic groups. The worse prognostic group has platin-refractory disease and for these patients disease recurrence occurs within 6-12 months after initial cytoreductive and chemotherapeutic treatment. The second group has a distinctly better outcome but even in this group, therapy eventually fails. The most commonly used therapeutic regimen combines carboplatin and paclitaxel. Systemic treatment of recurring ovarian cancer remains a major problem, mostly due to secondary resistance to platinum-derived substances, which are still the most active therapeutic agents in the treatment of this disease (2). Accordingly, it is important to identify new drugs which effectively inhibit ovarian cancer cells. As the PI3K/AKT signal transduction pathway is frequently activated in ovarian cancer (7, 8), we tested the PI3K inhibitor D-116883 in four human ovarian cancer cell lines.

D-116883 inhibited proliferation in all cell lines investigated with IC50<1 μM. The growth-inhibiting impact
Figure 1. Growth inhibition mediated by D-116883 in OAW42, SKOV3, and A2780, A2780cis ovarian cancer cells. These human ovarian cancer cells were incubated for 24 h (A, B), 48 h (C, D) and 72 h (E, F) with increasing concentrations of D-116883.
of the drug was not dependent on whether cells were platin-refractory (A2780cis) or platin-sensitive (A2780, OAW42, SKOV3).

We also tested the effect of D-116883 on cell growth using soft agar assay. Some investigators consider this assay superior to using monolayer cultures because cell growth in 3D might be more similar to the *in vivo* cellular environment. The growth-inhibiting effect yielded with D-116883 using platin-refractory and platin-sensitive human ovarian cancer cells resembled the results obtained with monolayer culture experiments.

We demonstrated by western blotting (Figure 3) that inhibition of PI3K leads to a dramatic decrease of phosphorylated AKT in the investigated ovarian cancer cells. The PI3K/AKT pathway is strongly involved in cell survival and inhibition of the pathway is known to induce programmed cell death in various experimental tumor systems (12). Cell cycle analysis revealed that PI3K inhibition induced cell death rather than a mere growth arrest of human ovarian carcinoma cells. This was evidenced by an increase in the number of hypodiploid, *i.e.* non-viable cells, while the cell numbers in all phases of the cell cycle showed a similar dose-
dependent decline upon treatment with D-116883 (Figure 4). Accordingly, D-116883 appears to kill ovarian cancer cells irrespective of their current phase in the cell cycle.

In order to elucidate if D-116883 induces classical apoptosis in the cell lines, we evaluated the effect of co-administration of the multicaspase inhibitor zVAD-FMK. Furthermore, we investigated if necroptosis is involved in the cell death caused by D-116883. Necroptosis has been described as an alternative nonapoptotic cell death mechanism (13). This cell death pathway can be inhibited by necrostatin, an inhibitor of necroptosis. We also investigated whether the effects of D-116883 on cell growth could be abrogated by necrostatin (Figure 2). Interestingly, cell death induced by D-116883 was inhibited neither by zVAD-FMK nor by necrostatin. It has been suggested that clonogenic survival of cancer cells may rely on the simultaneous blockade of both apoptotic and nonapoptotic cell death mechanisms (13). Our results suggest that D-116883 certainly induces cell death. As neither zVAD-FMK nor necrostatin prevented this cell death, classic apoptosis and necroptosis did not take place in the cell lines we tested. One can only speculate that an alternative death mechanism such as apoptosis-like programmed cell death took place, as has been described for other inhibitors of the PI3K/AKT pathway (14).

In conclusion, we demonstrated substantial antitumor activity of PI3K inhibitor D-116883 in platinum-sensitive and -resistant human ovarian cancer cells in vitro. Therefore, D-116883 should be considered for clinical studies in patients with platinum-resistant ovarian cancer. In animal experiments in other tumor entities, PI3K inhibitor D-116883 did not show any surprising toxicity. Therefore the compound seems an appropriate candidate for use in treatment combinations with classical chemotherapeutic agents.

References


