Sunitinib (SU11248) Inhibits Growth of Human Ovarian Cancer in Xenografted Mice

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Abstract. *Background: Treatment of ovarian cancer is still challenging especially in recurrent platinum refractory cases. Sunitinib is a multi tyrosine kinase inhibitor targeting receptors for vascular endothelial growth factor and platelet-derived growth factor which play a role in tumor angiogenesis. It has been approved for the treatment of recurrent gastro intestinal stroma tumors and metastatic renal cancer. Materials and Methods: In this study, sunitinib was tested for its effectiveness as a single agent in an ovarian cancer xenograft mouse model. Skov3 cells stably expressing firefly luciferase were injected into SCID beige mice. Mice received either 40 mg/kg bodyweight sunitinib or vehicle control. Tumor growth was monitored longitudinally by luciferase signal. Results: Sunitinib significantly reduced tumor growth ($p=0.0052$) and peritoneal metastases, and was associated with a significantly reduced microvessel density count ($p<0.001$). Conclusion: These results suggest that clinical trials are warranted for the evaluation of sunitinib for treatment of patients with recurrent or advanced ovarian cancer.*

Ovarian cancer is the fifth most common malignancy in women. In the US approximately 22,000 new cases occur each year (1) and approximately 16,000 patients die from this disease each year. The vast majority (>75%) of patients are diagnosed at an advanced stage of disease progression (FIGO III/IV) with an unfavorable prognosis. Once ovarian tumor has spread beyond the pelvis the 5-year survival rate decreases dramatically (2). Standard treatment is cytoreductive surgery followed by a chemotherapy combination of carboplatin and paclitaxel. However, a high recurrence rate, even in optimally treated patients, is a major problem and new therapeutic strategies are urgently needed.

Vascular endothelial growth factor (VEGF) plays a major role in ovarian cancer progression and metastases (3). Many patients present with an increase in abdominal girth due to ascites accumulation. The genesis of this intra-abdominal fluid is not fully understood, but capillary leakage due to increased VEGF levels may play a pivotal role in creating irregular vascular permeability (4, 5). VEGF is also required for ovarian cancer spread across the peritoneal layer of the abdominal cavity. Tumor nodules require a blood supply, which is mediated through the induction of neo-angiogenesis. Therefore, targeting the VEGF signaling pathway is a promising strategy to improve clinical outcome of ovarian cancer patients.

Sunitinib is a multi-tyrosine kinase inhibitor targeting vascular endothelial growth factor receptor (VEGF-R) –1 to –3, platelet-derived growth factor receptor alpha and beta, stem cell factor (KIT) and tyrosine protein kinase receptor (Flt3). Signaling blockade of these pathways is associated with antitumor and anti-angiogenesis activity. It has been approved for treating advanced stage or recurrent renal cell carcinoma (6) as well as imatinib-resistant gastrointestinal stromal tumors (GIST) (7). In preclinical settings sunitinib has been shown to be effective against breast and small cell lung cancer (8, 9). In breast cancer xenografts models sunitinib alone and in combination with standard chemotherapeutics reduced xenograft tumor growth significantly (9).

In this study, a xenograft mouse model was used to evaluate the efficacy of sunitinib in inhibiting ovarian cancer growth and angiogenesis.

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Key Words: Ovarian cancer, tyrosine kinase inhibitor, mouse model, xenograft, luciferase.

Materials and Methods

Cells. SKOV3 cells stably transfected with the firefly luciferase reporter gene (SKOV-3 luc) under the regulation of a constitutively active CMV promoter were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Munich, Germany) cell culture medium in the presence of heat inactivated 10% fetal bovine serum (Sigma-Aldrich, Munich, Germany). To maintain stable expression, cells were maintained under selection with neomycin (Sigma-Aldrich) at a concentration of 800 μg/ml. Cells were incubated in standard 5% CO_2 humidified incubators at 37˚C. Cells were split when confluence reached 80%.

SKOV3 luc xenografts and treatments. Skid beige mice (bodyweight on average 17g, n=15) were purchased form Charles River (Sulzfeld, Germany) after: institutional approval was obtained (Ministerium für Landwirtschaft, Umwelt und Ländliche Räume des Landes Schleswig-Holstein, V 321-72241.121-10). Mice were maintained under pathogen-free conditions. Mice received irradiated rodent chow and water *ad libidum*.

Xenograft model. SKOV3 luc cells (5×10^6 cells) suspended in PBS were injected into the left lower peritoneal cavity (i.p.). Twenty-eight days after tumor cell implantation one group of mice (n=8) were treated daily with sunitinib as a single agent therapy by oral gavage at a previously established efficacious and clinically meaningful dose of 40 mg/kg/day (9-11). The mice of the control group (n=7) were fed accordingly with phosphate-buffered saline (PBS) as vehicle control. Mice were anesthetized with fentanyl/ midazolam/medetomidin (dosage 0.05 mg/kg; 5 mg/kg; 0.5 mg/kg). For detection of tumor growth, mice received luciferin (Sigma-Aldrich) 150 mg/kg body weight *i.p*. in a volume of 100 μl isotonic saline solution. After 15min, bioluminescence imaging was performed by measuring luciferase activity *in vivo* using a Peltier cooled charged-coupled device camera (NightOWL LB 983; Berthold, Bad Wildbach, Germany) with exposure time for 2 minutes.

Immunohistochemical staining. Mice were sacrificed in accordance with our institutional guidelines when the control group reached critical conditions (tumor burden and ascites) around week 6 to 7. Tumor was dissected out, snap frozen and kept at –80˚C until further analyses. Frozen sections were taken and stained using a mouse antibody to CD31 (BD Pharmingen 553370, New Jersey, USA; dilution 1:500) as described elsewhere. In brief, sections were mounted on slides and dried at room temperature overnight. Slides were archived at –20˚C until staining was performed. Sections were fixed in acetone and the endogenous peroxidase was blocked with Dako S 2001, for 20 min (DAKO North Amerika, Inc., CA, USA). Primary anti-CD31 antibody was applied for 45 min at room temperature. After a washing step, sections were incubated with biotinylated secondary rabbit anti Rat IgG (H+L) antibody (Cat. No. BA-4001; Vector Lab, USA) for 30 min. Development was carried out using the ABC kit and for nuclear counterstaining, Meyer's hemalaun (Sigma-Aldrich) staining was applied. Microvessel density (MVD) was evaluated by calculating the surface (μm^2) of the tumor using the software Axiovision Rel 4 (Carl Zeiss, Jena, Germany). This software allows the tissue area on the slide to be delineated so as to measure the surface in μm^2. Stained vessels were counted and MVD was calculated as counts of vessels/μm^2 tumor surface.

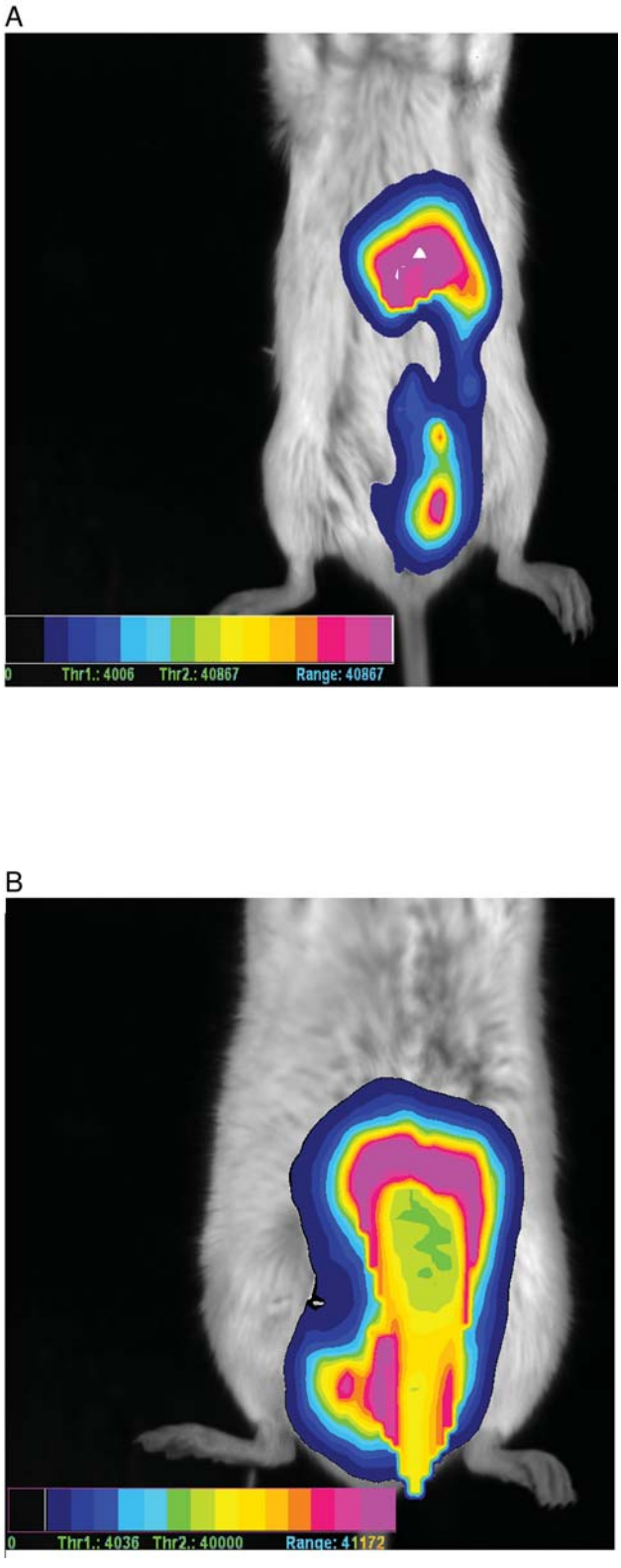

Figure 1. *Measurement of luciferase activity (day 60) imaged as a false color image showing that measurements were take with the same threshold as indicated by the color bar. A: Mouse treated with sunitinib 40 mg/kg bodyweight; B: Mouse treated with PBS control.*

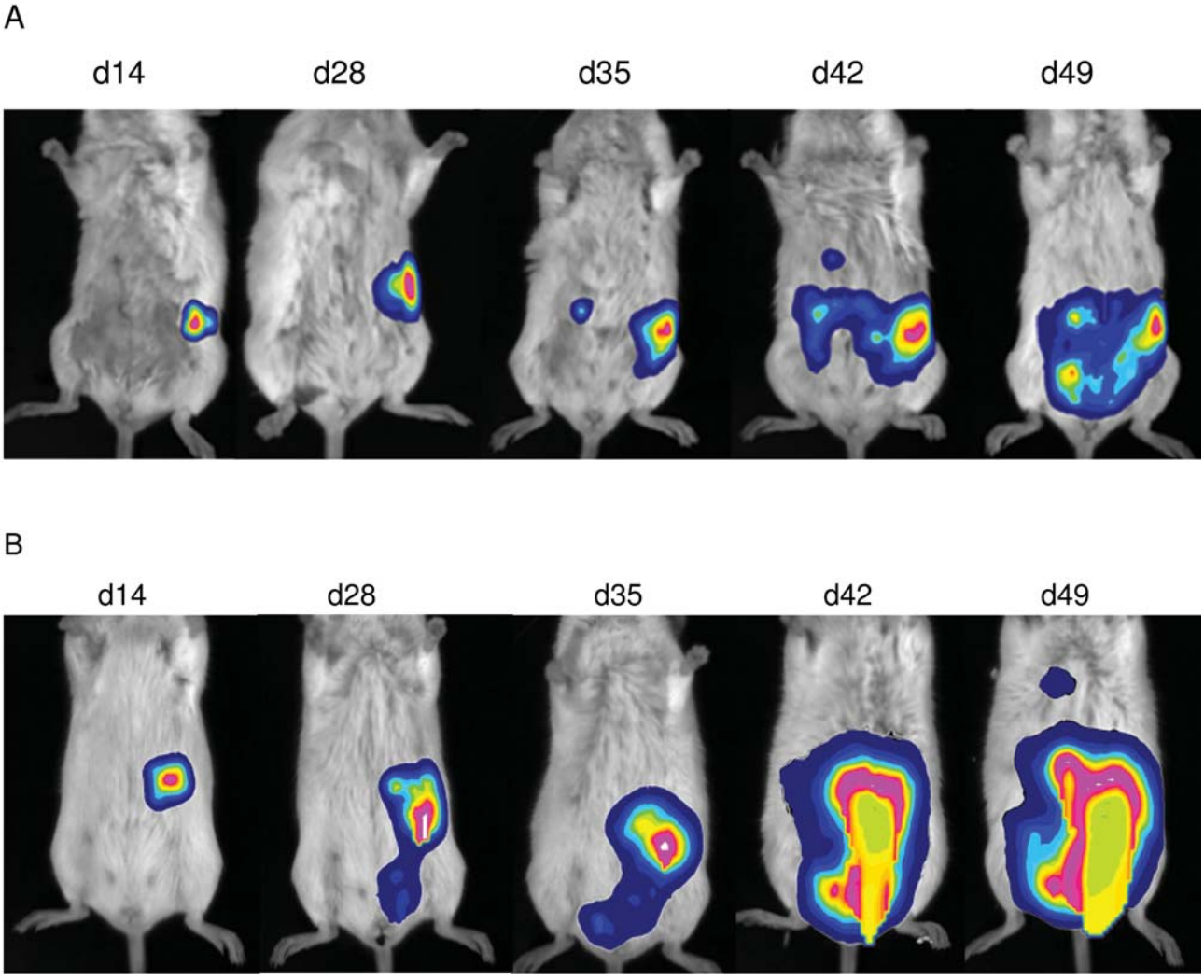

Figure 2. *Longitudinal bioluminescent measurements of treated mice. Mice of the treatment group (A) received 40 mg/kg bodyweight sunitinib daily by oral gavage and showed a significant reduction in peritoneal metastasis compared to the control mice receiving vehicle control (B). Day 1 corresponds to the start of drug treatment.*

Statistical analysis. Statistical analyses were performed with the SPSS program version XVI (IL, USA). *P*-values were calculated using a mixed linear model to assess differences in tumor luciferase signals between treatment and control groups (statistical significance was assumed with a p-value of <0.05). For the assessment of the MVD the t-test was applied; statistical significance was assumed with a p-value of <0.05.

Results

In this study, luciferase activity detection was performed with the same camera setup and with the same threshold for all mice, exemplarily shown at day 60 (Figure 1A, B).

We observed a steady increase in the tumor size until 7 weeks (post initiation of drug treatment) after which point there was widespread tumor dissemination throughout the peritoneal cavity (Figure 2A, B). These features of tumor growth are very similar to those of human ovarian cancer. In addition, SKOV3 cells are considered to be cisplatin-resistant and secrete high amounts of VEGF as is common amongst patients with advanced ovarian cancer.

Since SKOV3 tumors are initially slow growing, tumor growth was allowed to be established for 4 weeks before drug administration. The treatment group received 40 mg/kg bodyweight sunitinib daily by oral gavage. This dosage has been previously established and shown to be efficacious. The control group received vehicle control only.

Single-agent treatment with sunitinib led to a significantly slower increase in luciferase signal (p=0.0052) over time as compared to the control group. The effects of sunitinib were particularly pronounced at the latter stages of tumor

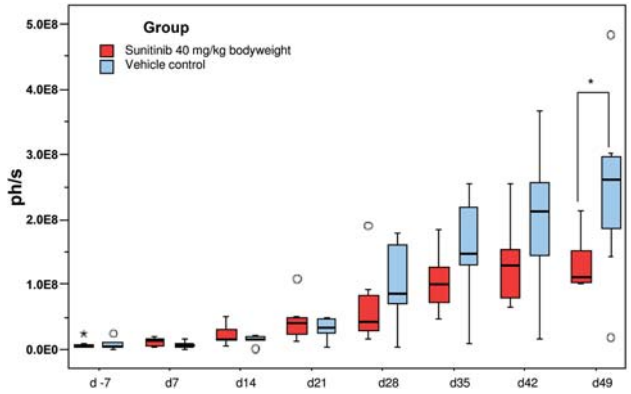

Figure 3. *Changes in luminescence signal photons per second (ph/s) of SKOV3 tumor xenografts in SCID mice in treated (red; n=8) and untreated (blue; n=7) groups. Mice were given sunitinib at 40 mg/kg body weight starting 4 weeks after tumor cell inoculation. The rate of peritoneal metastatic spread was significantly less in the treated group. At day 49, the tumor burden in the treated group was significantly less than that in the control group (* significant difference at p<0.05; o outliers).*

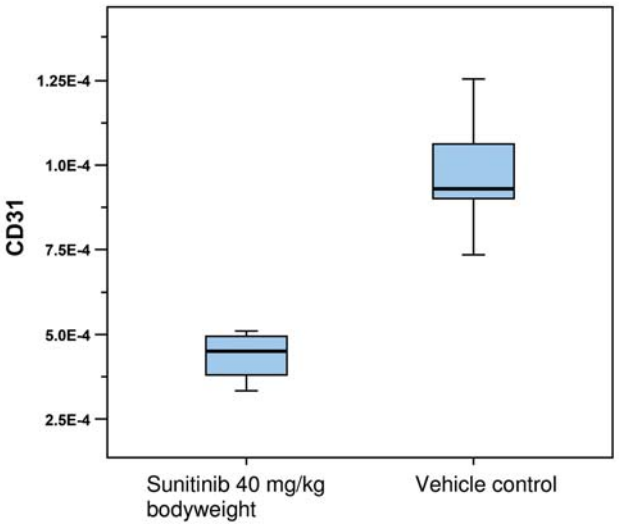

Figure 4. *Tumors of mice treated with sunitinib show a significant decrease in microvessel density (Box plot depiction). Microvessel density (microvessel count/μm^2) was assessed by staining with anti-mouse CD31 antibody. A: Treatment group; B: Control group.*

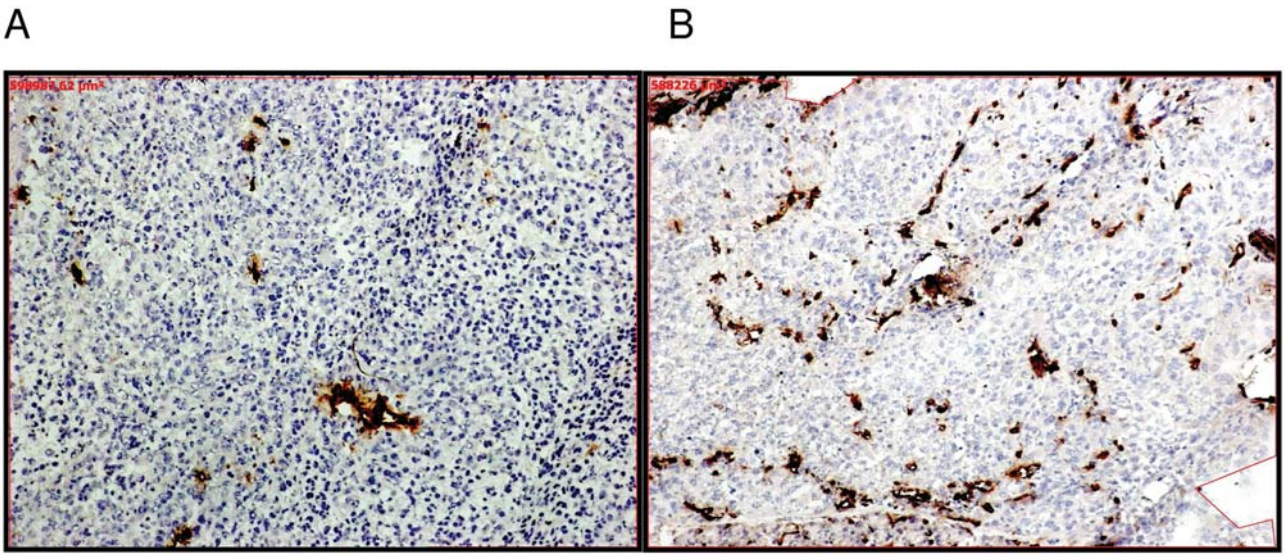

Figure 5. *Anti-CD31 antibody imunohistochemical staining on frozen sections of tumor nodules. A: Treatment group; B: Control group. Magnification 100-fold. The red border line shown in the pictures was used to delineate the area of tissue under the microscope. Microvessels show a brown staining reaction.*

progression when the tumor disseminated throughout the peritoneum in the control group. This is evidenced by the increasing difference in the bioluminescent values between the treated group and the control group. At the point of sacrifice of mice, there was a 2.3-fold difference in the median luciferase signal compared to the control group (Figure 3). Changes in girth were not observed in the treated group of mice and *ex vivo* examination revealed no ascites build up in these mice. In contrast ascites was detected in control mice.

MVD. To determine whether the reduction in tumor burden with sunitinib treatment is mediated through inhibition of angiogenesis, we investigated the MVD in tumor nodules

obtained from both the treatment and the control group. Staining was performed using an anti-mouse CD31 antibody which was applied to frozen sections.

The MVD in the treatment group was significantly lower when compared to the control group ($p<0.001$) (Figure 4). In sections analyzed from control groups, a moderately dense network of blood vessels was observed in the tumor mass. In sunitinib-treated mice there was sparse density of blood vessels in tumors (Figure 5).

Discussion

In this study, we investigated the potency of sunitinib as a single-agent therapy in platinum-refractory ovarian cancer. We have shown that the treatment using this multi tyrosine kinase inhibitor in a preclinical ovarian cancer mouse model significantly reduced tumor peritoneal metastasis and tumor angiogenesis. These results suggest that sunitinib can potentially be utilized to target primary tumors with intrinsic platinum-resistance and recurrent tumors with acquired platinum-resistance. SKOV3 cells are considered to be cisplatin resistant and secret high amounts of VEGF (12) as is common amongst patients with advanced ovarian cancer. Therefore this cell line seems to be a good model to investigate *in vivo* the single drug approach.

VEGF plays a critical role in mediating angiogenesis in ovarian cancer. High expression of VEGF correlates with poor disease-free and overall survival in early stage ovarian cancer (13). High serum levels of VEGF are also associated with ascites (3, 14, 15). Enhanced angiogenesis is indicated by high levels of VEGF and high MVD and is correlated with the presence of metastases and survival (16). Yamamoto *et al*. (17) found that VEGF contributes to tumor progression in the vast majority of ovarian tumors. Using the monoclonal antibody bevacizumab in ovarian cancer, *e.g*. as single agent in recurrent disease or in combination with chemotherapy, the proof of principle that inhibiting the VEGF pathway in ovarian cancer is a successful approach has been established (18-20). Therefore, the effectiveness of sunitinib in this study is likely due to the blockade of VEGF-mediated pathways leading to suppression in tumor growth, angiogenesis and ascites build up in ovarian cancer patients.

This is the first investigation on treating ovarian cancer xenografts with sunitinib. Sunitinib, targeting VEGF-R-1 to -3, PDGF-R alpha and beta, Kit and Flt3, has been approved for treatment of recurrent GIST (7) and renal cell carcinoma (RCC) (6). In RRC, sunitinib was established as one option for standard of care in first-line treatment.

In other tumor models, such as in breast cancer xenograft models, sunitinib alone and in combination with standard chemotherapeutics reduced xenograft tumor growth significantly (9, 21). Cumashi *et al*. (22) showed that sunitinib is effective as a single-agent therapy in mice xenograft prostate cancer model. They showed in prostate xenograft models that treatment with sunitinib alone or in combination with docetaxel gave rise to an anti-tumorigenic effect. The combination of sunitinib and low-dose (10 mg/kg/week) docetaxel was as effective as high-dose (30 mg/kg/week) docetaxel treatment but tolerability was better. In ovarian cancer, SU6668 as precursor of SU11248 was tested successful pre clinically (23). Besides the reduction of tumor burden, a significant reduction in VEGF and PDGF concentration was observed. SU6668 was not further pursued because of toxicity problems. In contrast, SU11248 was well tolerated, with mice showing only a yellow discoloration of the fur.

We tested sunitinib as a single-agent treatment in a platinum-resistant ovarian cancer xenograft mouse model, where luciferase-expressing SKOV3 cells were injected *i.p*. The single-agent regimen did reduce tumor burden significantly ($p<0.05$). Our analysis did show a significant reduction of tumor growth and MVD displaying the importance of tumor-induced neoangiogenesis for ovarian cancer growth. Histological staining using the antibody CD31 revealed a significant reduction of MVD ($p<0.001$) in tumors of treated mice. Preventing the tumor from inducing neoangiogenesis leads to a limited tumor growth potential in ovarian cancer.

Acknowledgements

We would like to thank Mrs. S. Hamann for her excellent work staining the frozen sections. We would also like to thank Mrs. U. Schulz from Medistat GmbH for doing the statistical analyses. This study was supported by an unrestricted research grant form Pfizer Pharma Germany (NRA6180007). Sanjay Tiwari is supported by the BMBF 'TOMCAT; 01EZ0825 grant and by MOIN-SH.

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Received August 28, 2010
Revised June 16, 2010
Accepted June 28, 2010