Chapter 9

Low-dose angiostatic tyrosine kinase inhibitors improve photodynamic therapy for cancer: lack of vascular normalization

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Low-dose angiostatic TKIs improve PDT for cancer: lack of vascular normalization
Abstract

Photodynamic therapy (PDT) is an effective clinical treatment for a number of different cancers. PDT can induce hypoxia and inflammation, pro-angiogenic side effects, which may counteract its angio-occlusive mechanism. The combination of PDT with anti-angiogenic drugs offers a possibility for improved anti-tumor outcome. We used two tumor models to test the effects of the clinically approved angiostatic tyrosine kinase inhibitors sunitinib, sorafenib and axitinib in combination with PDT, and compared these results with the effects of bevacizumab, the anti-VEGF antibody, for the improvement of PDT. Best results were obtained from the combination of PDT and low-dose axitinib or sorafenib. Molecular analysis by PCR revealed that PDT in combination with axitinib suppressed VEGFR-2 expression in tumor vasculature. Treatment with bevacizumab, although effective as monotherapy, did not improve PDT outcome. In order to test for tumor vessel normalization effects, axitinib was also applied prior to PDT. The absence of improved PDT outcome in these experiments, as well as the lack of increased oxygenation in axitinib treated tumors, suggests that vascular normalization did not occur. The current data imply that there is a future for certain anti-angiogenic agents to further improve the efficacy of photodynamic anti-cancer therapy.
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Introduction

Photodynamic therapy (PDT) is a minimally invasive therapy in which visible or near infrared light irradiation is combined with light sensitive molecules (photosensitizers) to produce reactive oxygen species (ROS). These ROS can damage blood vessels in such a way that vascular occlusion occurs. Several photosensitizers have been approved by the FDA to treat a number of oncological applications by PDT (see supplemental Table 1). PDT is also used in ophthalmology and for many years PDT was the mainstay for treating exudative age-related macular degeneration, the main cause of blindness in the aged western population. Angio-occlusive PDT can cause tissue responses, such as hypoxia and inflammation, both of which play a role in inducing angiogenesis. This angiogenic tissue response following PDT can in principle counteract the angio-occlusive effect of PDT, thus leading to a reduced tumoricidal outcome. Therefore, PDT results may be improved by co-treatment with an angiogenesis inhibitor. We previously showed in a tumor-free model that vessel regrowth after angio-occlusive PDT can effectively be inhibited by anti-angiogenic agents. In the present study, we tested the effect of combining PDT with an anti-angiogenic drug, by monitoring tumor vasculature and tumor growth. This was done in two different tumor models on the chorioallantoic membrane (CAM) of the chicken embryo.

Therapeutic anti-angiogenesis strategies have been established in the clinical management of cancer, both as monotherapies and in combination with other anti-tumor modalities. Among these are bevacizumab (Avastin®, an antibody-based drug that neutralizes VEGF), and the broad-spectrum (tyrosine) kinase inhibitors (TKIs) that inhibit signaling of growth factor receptors. Examples of the latter are sunitinib (Sutent®), clinically approved for the treatment of metastatic renal cell carcinoma and imatinib-resistant gastrointestinal stromal- and pancreatic neuroendocrine tumors. We also tested sorafenib (Nexavar®), approved for metastatic renal cell cancer and unresectable hepatocellular carcinoma. While sunitinib inhibits VEGF receptors 1, 2 and 3 (VEGFR-1, -2 and -3), platelet-derived growth factor receptor beta (PDGFR-β) and mast/stem cell growth factor receptor (c-KIT) with medium affinity, and FGFR-1 with low affinity, sorafenib inhibits the RAF/MEK/ERK pathways, as well as VEGFR-1, -2 and -3, c-KIT and PDGFR-β with relatively low affinity. A second-generation TKI with improved affinity to VEGFR-2 and a better toxicity profile, is axitinib (Inlyta®). Axitinib has fewer targets and has a higher affinity for VEGF receptors. It should be noted that combination of PDT with the antibody-based agents bevacizumab and ranibizumab has been tested clinically for the treatment
of wet age-related macular degeneration\textsuperscript{14}. For cancer only preclinical studies are available. Very limited research has been focused so far on the combination of PDT with TKIs\textsuperscript{15}.

It has previously been shown that angiogenesis inhibition can normalize cancer vessels\textsuperscript{16}. As the efficacy of PDT depends on tissue oxygenation, we tested sequencing of the combination therapy. We found that PDT treatment can be significantly improved by angiostatic compounds. The tested TKIs were more effective enhancers of PDT effects than bevacizumab. In addition, antiangiogenic drugs were found to be best applied after PDT. These results, as well as tissue oxygenation measurements, suggested that the observed improvements were not dependent on vascular normalization.

Results

\textit{Clinically used angiostatic tyrosine kinase inhibitors prolong the vaso-occlusive effect of PDT}

Visudyne\textsuperscript{®}-PDT (PDT) was performed on the CAM at embryo development day (EDD) 10 (Fig. 1A), leading to blood flow stasis in the smaller blood vessels and in the capillary bed. Vessels with a diameter \textgreater 70\,\mu m stayed perfused (Fig. 1B). New capillaries were first seen in the most peripheral zone of the treated area (Fig. 1B) and a completely regrown vasculature was observed after 48 hours (Fig. 1C). Quantification of the data was performed by digital image analysis in four concentric areas (Fig. 1C, most right image).

To prolong the effect of PDT, treatment with anti-angiogenic compounds, axitinib, sorafenib, sunitinib or bevacizumab, was performed. Angiostatic compounds were first tested alone by administering i.v. injection on EDD 10 and 11, followed by imaging and quantification was performed on EDD 12. Representative fluorescence images of the CAM treated with 0.9% NaCl (control) or axitinib (13\,\mu g/kg) are presented in Fig. 1D and 1E, respectively. Low concentrations of all four drugs were identified where a statistically significant inhibitory effect was observed (**p \leq 0.01, Fig. 1F). These doses were tested in combination with PDT. All drugs were administered twice, immediately after PDT and 24 hours later. Interestingly, all three tested tyrosine kinase inhibitors markedly suppressed the regrowth of blood vessels, as determined by a significant reduction in the number of branching points. This activity was not observed for bevacizumab. Axitinib and sunitinib were the most effective drugs (Fig. 1G and 1H). An approximate 90% reduction in the number of branching points per mm\textsuperscript{2} was observed in treatment area 1, while bevacizumab was completely ineffective.
Figure 1. Clinically used angiostatic tyrosine kinase inhibitors prolong the vaso-occlusive effect of PDT. (A) Fluorescence angiograms of the CAM before PDT. The circle represents the diaphragm, which limits CAM exposed with light. (B) 24 h and (C) 48 h after PDT showing the start of micro-vascular regrowth and complete revascularization of the treated area, respectively. (C) Right panel shows the skeletonization and area numbers used for the image processing. (D and E) Natural growth of CAM vasculature and inhibition of angiogenesis by axitinib and skeleton images of EDD 12. White arrows indicate the avascular zones induces by axitinib. (F) Quantification of the number of branching points per mm² after treatment with an ineffective and an effective dose of each compound. Effective doses: axitinib (13 µg/kg; n = 7), sorafenib (85 µg/kg; n = 7), sunitinib (71 µg/kg; n = 5), and bevacizumab (497 µg/kg; n = 5). (G) Fluorescence angiogram of the CAM treated with PDT+axitinib at its effective dose taken 48 h post PDT. (H) Quantification of the results for all four tested compounds. Data are shown as means (± SEM, **p < 0.01 as compared to the control in each respective area of vascular regrowth (1-4), n = 3-6 per condition). The white bars in (A, D and G) represent 200µm.
Angiostatic tyrosine kinase inhibitors, but not bevacizumab, improve the anti-tumor effect of PDT

A2780 ovarian carcinoma cells were inoculated at EDD 7 and monitored for 11 days. Established and vascularized tumors were detected 3 days post implantation (EDD 10). Tumors grew to an average size of approximately 140 mm$^3$ by EDD 17 when left untreated (supplemental Fig. 1A). The chicken vasculature in these tumors was efficiently perfused, as demonstrated by the prompt distribution of India ink throughout the tumor vasculature within 5 seconds after intravenous injection (Supplementary Fig. S1B). As expected, the tumor vessels were leaky, as of the ink was present in the extracellular space of the tumor already after 20 seconds (Supplementary Fig. S1C).

Figure 2. Defining suboptimal drug concentrations and PDT conditions for tumor treatment on the CAM. Treatment regimens for CAM tumors tested for PDT alone (A) or drug alone (C). Tumor growth curves for PDT (B) and angiostatic drug (D) are shown. Arrows indicate treatment days. Data are shown as means (±SEM). n = 3-10 per condition, **p < 0.01.
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Sub-optimal treatment strategies were defined, both for PDT (Fig. 2A) and angiostatic compounds (Fig. 2C) in A2780 tumors. The PDT conditions were selected such that tumor growth was inhibited by approximately 60% (Fig. 2B). Dose selection for axitinib is shown in Fig. 2D. For sorafenib, sunitinib, and bevacizumab the sub-optimal doses in A2780 model were defined at 85 μg/kg, 71 μg/kg and 497 μg/kg, respectively. The same doses were applied in the HCT-116 model.

**Figure 3.** PDT and anti-angiogenesis combination therapy. (A) Treatment regimens for CAM tumros treated with schedule 1. (B) Representative images A2780 human ovarian tumors for control and various treated groups resected on treatment day 8. (C) Tumor growth curves of tumors treated by each anti-angiogenic drug, PDT and the combination of both therapies (**p = 0.0033 for PDT+axitinib and **p = 0.0025 for PDT+sorafenib as compared to PDT alone, Figure 3C, n = 6-10 per condition). S indicates synergy (CI<1).
Combination of PDT and i.v. drug administration immediately after and 24 hours later was performed in the A2780 xenographs (Fig. 3A). The representative images of tumors resected on treatment day 8 from different treatment groups are presented in Fig. 3B. PDT in combination with axitinib and sorafenib significantly improved PDT outcome (**p = 0.0033 and **p = 0.0025, respectively, Fig. 3C, n = 6-10). Surprisingly, sunitinib and bevacizumab did not or only marginally improve the effect of PDT. Synergy, as defined by the Chou-Talalay equation as combinations with a ‘combination index’ (CI) less than 1, was calculated for the combination of PDT with axitinib (CI = 0.36) and PDT with sorafenib (CI = 0.59). Neither sunitinib nor bevacizumab gave a statistically significant difference in tumor size together with PDT as compared to PDT alone. Similar experiments with axitinib and sorafenib were performed on human HCT-116 colorectal carcinoma tumors (Fig. 3C). In this model, comparable results were observed for PDT+axitinib (n = 6, **P=0.0008 as compared to control) and PDT+sorafenib (n = 6; *p = 0.02), as shown in Fig. 6D (schedule 1) and 6G, respectively, as a percentage of the control on the experiment day.

**Combination therapy reduces vessel density and modulates vascular morphology and angiogenesis-related gene expression**

Immunohistochemical staining for CD31 was performed 3 and 8 days after treatment (Fig. 4) in both tumor models. It was found that the combination of PDT and TKIs (both axitinib and sorafentib) suppressed microvessel density as shown at the last (8th) experiment day (Fig. 4A, **p = 0.0009, *p = 0.022, respectively and n = 6-14). Angiogenesis inhibitors alone did not significantly suppress microvessel density (supplemental Fig. 2). Microvessel density in the bevacizumab combination group was not different from the PDT monotherapy group, while sunitinib combination group was increased as compared to the control. Another interesting difference was observed in the morphology of the tumor vessels. While control tumors had a large numbers of small vessels with compressed lumens, the combination of PDT with axitinib and sorafenib resulted in larger vessels with an open lumen (***p < 0.001, *p = 0.051, respectively, Fig. 4A). PDT initially, (treatment day 3, Fig. 4B) suppressed microvessel density significantly, but after a longer period (day 8) this effect had largely disappeared presumably due to the PDT-induced angiogenesis. Combination treatment of PDT+axitinib in the HCT-116 tumors revealed a statistically significant decrease in vessel density (**p = 0.0006, n = 10) as compared to control tumors resected at the last (8th) experiment day (Fig. 4C).
Based on the above-described results, we performed real-time quantitative PCR studies for tumors exposed to axitinib and its combination with PDT (Fig. 5A-C). We investigated the expression of angiogenic growth factor receptors in vasculature (chicken specific primers, 5A and B) and growth factors secreted by tumor cells (human specific primers, Fig. 5C). It was observed that early after treatment (day 3), i.v. administered axitinib, but not PDT, suppressed VEGFR-2 in the vasculature. VEGFR-2 was still downregulated eight days after treatment, at which time this effect was also seen for the expression of PDGFR-β. Assessment of growth factor expression in the tumor cells (Fig. 5C) did not reveal a strong angiogenic response.

The change in the Ct values of the control and treatment group tumors was examined between tumors excised on treatment days 3 and 8. A detectable, but not significant, change in gene levels was noticed between days 3 and 8 in control tumors (not shown). The only

![Figure 4](image_url)  
**Figure 4.** Histology of resected tumors showing CD31 positive staining used to assess microvessel density/mm². (A) CD31 stained sections of the A2780 tumors excised at day 8 for control, PDT and combination treatment groups. Graphs of microvessel density/mm² and the percent of vessels with open lumens (right of images) for control, PDT and combination treatment groups showing a statistically significantly decrease in microvessel density and increase in the number of vessels with an open lumen for PDT+axitinib treated tumors as compared to the control tumors. (B) CD31 stained sections of the A2780 tumors excised at day 3 for the most effective treatment group (PDT+axitinib, 13 µg/kg) and quantification of microvessel density/mm² (right). (C) CD31 stained sections of HCT-116 tumors excised on day 8 for the most effective treatment group and quantification of microvessel density/mm² (right) showing significant inhibition of vessel density in the combination PDT+axitinib treatment group. **p < 0.01. n = 5-22 per condition.
significant increase in gene levels was noted for VEGFA, whose expression was upregulated by 10.7% in the host cells on day 3 vs. day 8 (*p = 0.044, n = 5-7) and by 11.7% in the tumor cells between days 3 and 8 (*p = 0.052, n = 2-3).

**Figure 5.** Real-time RT-PCR molecular profiling of the tumors treated with PDT, axitinib (13 μg /kg), or their combination. The expression of some of the angiogenesis-related genes determined by quantitative real-time PCR performed at day 3 (A) and day 8 (B) post PDT using chicken (gg)-specific primers for: VEGFA, VEGFR2, PDGFR-β. (C) Quantification of human genes in tumors excised on day 3 using human (hs)-specific primers for VEGFA, bFGF and PLGF. Mean relative expressions are shown with the standard error of the mean. Between 5 and 7 eggs were used per condition. N = 5-6 per condition.

**Scheduling of PDT and angiostasis: lack of vascular normalization**

Next to the above used schedule (Fig.6A, now called schedule 1), also a treatment schedule starting with angiostatic compounds axitinib (Fig. 6B-D) or sorafenib (Fig. 6E-G) 24 hours prior to PDT (Fig. 6B, schedule 2) was also tested in the two tumor models. Interestingly, for none of the angiostatic compounds application prior to PDT (schedule 2) resulted in better anti-tumor photodynamic activity than for schedule 1 at the conditions applied. While for sorafenib similar results for schedule 1 and 2 were observed (Fig. 6E and 6G), for axitinib treatment schedule 2 resulted in a worse outcome (Fig. 6B and D), as compared to schedule 1 in both tumor models. In the HCT-116 model, all tumors treated with combination therapy using either schedule were inhibited significantly as compared to the control tumors (control: N=6-12; axitinib schedule 1: **p = 0.0008, axitinib schedule 2: *p = 0.01; sorafenib schedule 1: *p = 0.022; and sorafenib schedule 2: *p = 9.924, as compared to the control).
The most unexpected result was that bevacizumab pretreatment even resulted in a loss of the anti-tumor activity resulting from the PDT treatment (Fig. 6H). To further investigate the origin of differences in tumor growth after treatment with the two treatment schedules, intra-tumoral oxygenation was measured at 24 hours (when PDT was performed in schedule 2) after the first...
bolus injections of axitinib (13 µg/kg), sorafenib (85 µg/kg), and bevacizumab (497 µg/kg), see Fig. 6C, F and I, respectively. The pO\textsubscript{2} measurements performed 24 h after the first injection with the inhibitors showed a small, but not significant, increase in oxygenation (e.g. 6.7% for bevacizumab, as compared to control tumors, p = 0.27, n = 10). Moreover, there was no difference between the latter groups and the PDT group.

**Discussion**

A major limitation in the use of PDT against cancer is the PDT-induced angiogenic tissue response. As there are now many clinically approved effective angiogenesis inhibitors, it is proposed that these compounds can significantly prolong the beneficial angi-oclusive effect of PDT\textsuperscript{4}. The results of the present study show that angiostatic small molecule tyrosine kinase inhibitors (TKI) can synergistically improve the anti-tumor effect of PDT, in both an ovarian and a colorectal tumor model. A major observation of this study is that this improvement of PDT outcome was due to the inhibition of PDT-induced angiogenesis, and not to the vascular normalization processes, as TKI-induced enhancement of tumor oxygenation was not observed. Synergy between PDT and anti-angiogenic TKIs for tumor growth suppression was best observed for axitinib when applied at a sub-optimal dose and combined with a sub-optimal PDT regimen. Sorafenib also showed a synergistic activity, but these results were not observed for sunitinib and bevacizumab. The results suggest that a combination of PDT and axitinib might be a promising strategy for translation into the clinic.

Photodynamic therapy has been most successfully used in the treatment of ophthalmological neovascularization-based disorders. These were in the past mainly wet age-related macular degeneration patients\textsuperscript{18} and at present mainly patients with polypoidal choroidal vasculopathy\textsuperscript{19}. The treatment of solid tumors with PDT is currently receiving renewed interest because it is being realized that its combination with anti-angiogenesis therapy has promising applications\textsuperscript{4}. Several studies have been reported on such combinations for the treatment cancer. These include preclinical studies assessing the activity of cetuximab and/or bevacizumab with hypericin-PDT in a human bladder carcinoma model\textsuperscript{20}, SU5416 and SU6668 with hypericin-PDT in a human nasopharyngeal carcinoma model\textsuperscript{21} and PD166285 and PD173074 with hexylether pyropheophorbide-a-PDT in a murine mammary carcinoma model\textsuperscript{22}. In all these studies, the anti-angiogenic drugs were applied after PDT, and the combination treatment was shown to be more potent than the monotherapies. A comparative study in which the PDT was combined in varying treatment schedules, with clinically approved TKIs has not yet been performed. In our study, the
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best results, i.e. a synergistic improvement of PDT, were observed in combination with axitinib, making a clinical translation of this treatment a promising option. This would most likely be best developed for tumor types that have been shown to be successfully treated with PDT, such as basal cell carcinoma (BCC) or non-metastatic base of the tongue squamous cell carcinoma.

In BCC diagnosed patients, the average recurrence was shown to be 10% at 12 months after topical Metvix® (methyl aminolevulinate)-mediated PDT. Unfortunately, the follow-up of these patients is not continued longer than 1 year post PDT, whereas it was shown in many studies that the recurrence peak post Metvix®-PDT is at 36 months. Moreover, patients treated with such PDT strategies had a better cosmetic outcome and the treatment outcome was typically superior to that achieved with existing standard therapies. Recurrent base of the tongue malignancies develop usually loco-regionally at previously irradiated fields. Also, interstitial PDT (with mTHPC) of recurrent non-metastatic carcinoma of the tongue base showed promising results.

It is most interesting to see that when PDT was followed by angiogenesis inhibition at the applied conditions, synergisms were observed for axitinib and sorafenib, but not for sunitinib and bevacizumab. For the latter, there even seemed to be a lack of additive effect (Fig. 3). It should be noted that part of the VEGF signaling in this model may be derived from chicken VEGF, and bevacizumab probably binds chicken VEGF with a lower affinity than human VEGF. However, a number of studies have shown the efficacy of anti-VEGF antibodies (bevacizumab or ranibizumab) against chicken VEGF, so this argument may not be very significant. The question remains why neutralizing VEGF does not work so well, while inhibition of VEGFR signaling does. This could mean that neutralization of VEGF by a large molecule - an antibody - is much less efficient inside the microenvironment of a tumor in situ than inhibiting the VEGFR by a small molecule. Another explanation could be the broader activity spectrum of axitinib. This then raises the question why axitinib works better than sunitinib. However, the most likely explanation for this is that the affinity of axitinib for VEGFR-2 is some 40 times higher.

A similar discussion is valid for the situation of angiogenesis inhibition prior to PDT. Here, bevacizumab not only lacks improvement of PDT, but also seems to counteract the efficacy of PDT. Apparently, the presence of VEGF is necessary for an effective PDT outcome. It can be assumed that VEGF induced active cell metabolism is necessary for effective PDT. This also suggests that the major effect of PDT, at the applied conditions, is through its effect on the vasculature. The fact that the results from the axitinib treatment groups do not seem to support this option may be explained by the broad activity spectrum of TKIs. Relatedly, this may also
explain the overt difference between axitinib and sunitinib, being the two drugs mainly inhibiting the VEGFRs. Although VEGFRs and other growth factor receptors are considered the primary targets of these compounds, it has been shown before that more than one hundred kinases are affected by sunitinib\(^\text{29}\), and it would thus be quite difficult to pin-point the exact mechanism of action of these drugs\(^\text{30}\). Moreover, it cannot be ruled out that part of the success of axitinib is through a direct activity on the tumor cells.

Another aim of this study was to study the consequences of the treatment sequence. Previous studies on such combination therapies for cancer were all performed by timing the angiostatic therapy starting either at the same time as PDT, or after\(^\text{22,31,32}\) PDT. As suggested by Jain\(^\text{33}\) angiogenesis inhibition can normalize the tumor vasculature, as well as the blood flow, interstitial pressure, vessel wall permeability and oxygenation. We and others have shown that this effect of angiogenesis inhibitors can improve the combination with e.g. chemo- and radiotherapy\(^\text{34-36}\). For example, Dings et al. found a time-window of increased tumor oxygenation over the first 4 days of treatment with either bevacizumab (10 mg/kg i.v. in a single injection) or anginex (10 or 20 mg/kg/d i.p.). Elevated oxygenation was also accompanied by reduced vessel density and increased pericyte coverage. When radiotherapy was initiated within this window, tumor growth delay was significantly enhanced in relation to alternative treatment schedules\(^\text{34}\). Hiber et al.\(^\text{37}\) showed that SU11657 (a multi-target small molecule inhibitor of VEGFRs and PDGFR) was more effective when administrated one day prior to radiotherapy as compared to one day after radiotherapy. Since PDT, like radiotherapy, is dependent on oxygenation of the tissue, we put forward the hypothesis that anti-angiogenesis, at least in some cases, could effectively be given prior to PDT\(^\text{38}\). In the present study, we observed that the latter treatment schedule does not improve the anti-tumor activity, or even, it can make the overall outcome worse. This suggests that vascular normalization does not take place to a significant extent at the applied conditions. Indeed, in our experimental conditions, we did not observe significantly increased oxygenation after treatment with axitinib (13 µg/kg), sorafenib (85 µg/kg), or bevacizumab (497 µg/kg) over a period of 2 days. It should, however, be noted that in these studies we used very low drug doses, i.e. 0.497 mg/kg of bevacizumab, as compared to a dose of 10 mg/kg reported to induce vascular normalization by Dings et al.\(^\text{34}\).

To summarize the data from the current study, it can be concluded that PDT and anti-angiogenic therapy can synergistically inhibit tumor growth. Through the indirect neutralization of VEGF, and the direct inhibition of growth factor receptors, the anti-tumor effect of PDT can be improved.
Acknowledgments

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Materials

Cell culture, preparation and implantation on the CAM model.
A2780 human ovarian carcinoma cells (ECACC, Salisbury, UK) were maintained in RPMI-1640 cell culture medium supplemented with GlutaMAX™ (Gibco, Carlsbad, USA), 10% bovine calf serum (Sigma-Aldrich, St. Louis, USA) and 1% antibiotics (Sigma-Aldrich). Human colorectal carcinoma (HCT-116, ECACC) cells were maintained in DMEM medium (Gibco) supplemented as above. Fertilized chicken eggs were incubated in a hatching incubator (relative humidity 65%, 37°C), as previously described. On EDD 7, 10⁶ HCT-116 cells were mixed with ice-cold matrigel medium (BD Biosciences, Franklin Lakes, USA) and transplanted on the surface of the CAM as a 30 μl drop. 10⁶ A2780 cells were prepared as a spheroid in a 25 μl hanging drop and 3 hours later were transplanted on the surface of the CAM.

Image acquisition and quantification
Visualization of the CAM vasculature and irradiation with light during PDT was performed under an epi-fluorescence microscope (Nikon AG, Eclipse E 600 FN, Japan) with objectives (Plan Apo 4x/0.2, working distance: 20 mm or Plan Fluor 10x/0.3, working distance: 16 mm; Nikon AG, Japan), as previously described. Shortly, PDT was performed (λex = 420 ± 20 nm, λem ≥ 470 nm, Nikon, Japan) using Visudyne® (Novartis Pharma Inc., Hettlingen, Switzerland). Visualization of blood vessels was achieved through fluorescence angiography after intravenous (i.v.) injection of fluorescein isothiocyanate dextran (FITC-dextran, 20 kDa, 20 μl, 25 mg/ml, Sigma-Aldrich). 20 μl of India ink from Pelikan (Witzikon, Switzerland) was administered to enhance vascular contrast. Fluorescence images were taken using an F-view II 12-bit monochrome Peltier-cooled digital CCD camera run by ‘analySIS DOCU’ software (Soft Imaging System GmbH, Munster, Germany). Image-processing and quantification of the
fluorescence angiographies was achieved using a macro written in ImageJ (version 1.40 a), as previously described\textsuperscript{42}. The four concentric circles with “1” being the central area, and “4” being the most peripheral area, create four zones of revascularization, each of which is analyzed separately by the software.

**Combination therapy on the CAM**

Bevacizumab was purchased from Genentech (San Francisco, USA), sunitinib from Pfizer Inc. (New York, USA), axitinib and sorafenib from LC Laboratories (Woburn, MA, USA). Drugs were administered intravenously (20 μl) on EDD 10 and 11 at two concentrations: axitinib (6.5 or 13 or μg/kg), sorafenib (21 or 85 μg/kg), sunitinib (35.5 or 71 μg/kg), and bevacizumab (99 or 497 μg/kg). Concentrations were calculated for an estimated embryo weight of 3 g\textsuperscript{43}. Angiograms of the CAM were taken on EDD 12.

Visudyne®-PDT (subsequently referred to as PDT) was performed at a low-fluence rate (5 J/cm\textsuperscript{2}, with irradiance of 35 mW/cm\textsuperscript{2} at 420 ± 20 nm). The irradiation area was limited to a circular spot of 0.02 cm\textsuperscript{2} using an optical diaphragm. Directly after PDT, 20 μl of the angiogenesis inhibitors was administered intravenously in the CAM at the following effective doses: axitinib (13 μg/kg), sorafenib (85 μg/kg), sunitinib (71 μg/kg), and bevacizumab (497 μg/kg). Treatment was repeated 24 hours after PDT.

**Tumor treatment**

Vascularized tumors appeared approximately 3 days after inoculation on the surface of the CAM and the average tumor volume was 1.66±0.09 mm\textsuperscript{3}. Visudyne®-PDT, as described above, was performed at this moment, while adjusting the diaphragm to the tumor size. Aniostatic therapy was performed by administering 20 μl of axitinib (13 μg/kg), sorafenib (85 μg/kg), sunitinib (71 μg/kg), and bevacizumab (497 μg/kg) intravenously on EDD 10 and 11.

**Combination therapy**

Tumors receiving combination treatment were injected twice intravenously with 20 μl of each angiogenesis inhibitor (at doses as above) according to two different schedules: (1) right after PDT and 24h after PDT, or (2) 24 hours before PDT and right after PDT.
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(Fig. 6A). PDT with 5 J/cm² and 35 mW/cm² at 420±20 nm was applied. Tumors were measured daily, volume = (the smaller diameter)² x (perpendicular diameter) x 0.5.

**Immunohistochemistry**

Tumors were resected at treatment day 8, fixed overnight in zinc fixative solution and stained as previously described. In short, 4 µm sections were treated with 0.3% H₂O₂ in methanol for 30 minutes, a citrate buffer (20 min at 95°C) antigen retrieval step was applied, blocking with 10% goat serum and 1% BSA was performed. Primary antibody (Dianova, DIA-310, Hamburg, Germany) incubations were performed overnight.

**RNA isolation, cDNA synthesis, and quantitative real-time RT-PCR**

Total RNA isolation, cDNA synthesis and quantitative real-time RT-PCR (qRT-PCR) were performed as previously described. Each target gene was quantified relative to the expression of the reference genes (β-Actin and Cyclophilin-A). Chicken (gg) and human (hs) primers were synthesized by Eurogentec (Liege, Belgium).

**pO₂ measurements**

Intra-tumoral oxygenation was measured 24 hours after the first treatment intervention (corresponding to treatment day 2). Measurements of the partial pressure of oxygen (pO₂) within the treated tumors were obtained using an OxyLab pO₂ meter (Oxford Optronix Ltd., Oxford, UK) coupled to a calibrated fiber optic probe (NP/O/E) placed in a 23G surgical steel needle. Each measurement was taken over 60 s after the intra-tumoral probe insertion.

**Statistical analysis**

Values are given as mean values ± standard error of the mean. Data are represented as averages of independent experiments. Statistical analysis was done using the ANOVA test and t-test. *p indicating p-values lower than 0.05 and **p indicating p-values lower than 0.01 were considered statistically significant. Synergy was calculated using the CompuSyn application.
References

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Supplementary Material

Low-dose angiostatic tyrosine kinase inhibitors improve photodynamic therapy for cancer: lack of vascular normalization.

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**Supplementary Figure S1.** Functional vasculature of a tumour growing on the CAM. After injection of 30 μl of India ink into the CAM vessels (A–C), the India ink is immediately distributed within the bloodstream and perfused the tumour vessels within 5 sec. and tumour interstitial space shortly after 20 sec.

**Supplementary Figure S2.** Effect of anti-angiogenic inhibitors on microvessel density/mm² in A2780 tumours. Quantification of microvessel density/mm² at day 8 of experiments for axitinib (13 μg/kg), sorafenib (85 μg/kg), sunitinib (71 μg/kg) and bevacizumab (497 μg/kg) administrated at days 1 and 2 of the experiments. Each group represents the mean with the SEM.
Supplemental Table S1:

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<td>Photofrin</td>
<td>48</td>
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<tr>
<td>relieve airway obstruction</td>
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<tr>
<td>Inoperable, early centrally located lung cancer</td>
<td>Photofrin</td>
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<tr>
<td>Pleural malignancies</td>
<td>mTHPC</td>
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</tr>
<tr>
<td><strong>Upper aerodigestive track</strong></td>
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<tr>
<td>Oral, laryngeal, head and neck cancers</td>
<td>various</td>
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<tr>
<td>Upper aerodigestive track</td>
<td>mTHPC</td>
<td>52</td>
</tr>
<tr>
<td>Head and neck tumors</td>
<td>mTHPC</td>
<td>53</td>
</tr>
<tr>
<td>Non-metastatic, base of the tongue tumors</td>
<td>mTHPC</td>
<td>26</td>
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<tr>
<td><strong>Esophageal cancers</strong></td>
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<tr>
<td>Precancerous lesions of Barrett esophagus</td>
<td>Photofrin</td>
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<tr>
<td>Esophageal cancer</td>
<td>Photofrin</td>
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<td>Early SCC of the esophagus</td>
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<td><strong>Skin cancers</strong></td>
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<tr>
<td>BCC, actinic keratoses, Bowen's disease</td>
<td>mALA</td>
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<td>Bowen's disease</td>
<td>ALA</td>
<td>57,58</td>
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<td>Skin lymphomas</td>
<td>ALA</td>
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<td>ALA</td>
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<tr>
<td><strong>Urological</strong></td>
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<td>Bladder cancer</td>
<td>Motexafin lutetium</td>
<td>62</td>
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<tr>
<td>Recurrent prostate cancer after failure of radiotherapy</td>
<td>Padoporfin/WST09</td>
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<tr>
<td>Bladder cancer</td>
<td>HPD</td>
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</table>

ALA: 5-aminolevulinic acid; HPD: Hematoporphyrin derivative; mALA: methyl ester of 5-aminolevulinic acid; mTHPC: m-tetrahydroxyphenylchlorin.