Chapter 5

Optimal treatment scheduling of ionizing radiation and sunitinib improves the antitumor activity and allows dose reduction


Chapter 5

Abstract

The combination of radiotherapy with sunitinib is clinically hampered by rare but severe side effects and varying results with respect to clinical benefit. We studied different scheduling regimes and dose reduction of sunitinib and radiotherapy in pre-clinical tumor models to improve potential outcome of this combination treatment strategy.

The chicken chorioallantoic membrane (CAM) was used as an angiogenesis in vivo model and as a xenograft model with human tumor cells (HT29 colon carcinoma, OE19 esophageal adenocarcinoma). Treatment consisted of ionizing radiation (IR) and sunitinib as single therapy or in combination, using different dose-scheduling regimes. Sunitinib potentiated the inhibitory effect of IR (4 Gy) on angiogenesis. In addition, IR (4 Gy) and sunitinib (4 days of 32.5 mg/kg/day) inhibited tumor growth, both with approximately. Ionizing radiation induced tumor cell apoptosis and reduced proliferation while sunitinib decreased tumor angiogenesis and reduced tumor cell proliferation. When IR was applied before sunitinib, this almost completely inhibited tumor growth while concurrent IR was less effective and IR after sunitinib had no additional effect on tumor growth. Moreover, optimal scheduling allowed a 50% dose reduction of sunitinib while maintaining comparable anti-tumor effects. The current study shows that the therapeutic efficacy of combination therapy improves when proper dose-scheduling is applied. More importantly, optimal treatment regimes permit dose reductions of the angiogenesis inhibitor, which will likely reduce the side effects of combination therapy in the clinical setting. Our study provides important leads to optimize combination treatment in the clinical setting.
Introduction

With approximately 50% of all cancer patients receiving radiotherapy, this strategy is among the most commonly applied anti-cancer treatments worldwide (1,2). Apart from technical advances that continue to improve the accurate dose delivery to the malignant tissue, efforts are being made to develop drugs that increase the sensitivity of tumor cells to ionizing radiation (IR) (3-5). These radiosensitizers usually target cellular pathways that mediate radioresistance in tumor cells, e.g. DNA repair mechanisms, cell-cycle checkpoints, and cell survival signaling pathways (6,7). In recent years it has been suggested that drugs that inhibit tumor angiogenesis, i.e. the growth of tumor blood vessels, can also potentiate the anti-tumor effect of IR. Indeed, this combination has demonstrated promising results in animal tumor models in vivo (8-10). However, how both treatment modalities should be scheduled to obtain the maximum anti-vascular and anti-tumor effect is still poorly understood (11).

Sunitinib (Sutent, SU11248) is a receptor tyrosine kinase (RTK) inhibitor that targets multiple receptors involved in angiogenesis, including vascular endothelial growth factor receptor (VEGFR)-1, -2 and -3 and platelet-derived growth factor receptor (PDGFR) (12). Treatment with sunitinib is currently approved by the FDA for different cancer types, including metastatic renal cell carcinoma and certain gastro-intestinal stromal tumors. Several preclinical in vivo studies that combined sunitinib with IR have demonstrated promising antitumor effects (13-15). In addition, a number of phase I/II clinical trials have shown that this combination is a generally well tolerated combination therapy with promising tumor response rates (16-19). However, there is a major concern about rare but severe side effects, such as hemorrhages or gastro-intestinal perforations (18;20). While some pre-clinical studies have demonstrated that precise scheduling of the two treatment modalities is essential for the anti-tumor effect, it is still poorly understood if optimal scheduling also permits dose reductions of either treatment modality (13;15). This is an important issue to address, since dose reductions could lead to lower toxicity. This is supported by clinical trials in which decreased sunitinib doses resulted in lower toxicity rates (16;19) and by case reports that observed no change in response upon dose reduction (21;22). This warrants more pre-clinical research to resolve the optimal dose-scheduling of radiotherapy and sunitinib.

In this study, we transplanted human tumors on the chorioallantoic membrane (CAM) of the chicken embryo to study the effects of IR and sunitinib on angiogenesis and tumor growth in vivo. In addition, we evaluated whether proper scheduling would allow dose reductions of either sunitinib or IR. Our data show that optimization of dose-scheduling enhances the effects of IR and sunitinib on angiogenesis and tumor growth. More importantly, optimal dose-scheduling allows dose reduction of sunitinib by at least 50% while maintaining the same anti-tumor effect. In addition, by adding sunitinib to IR, the dose of IR can be reduced while maintaining the same anti-tumor effect as IR alone. Altogether, our results demonstrate that the combination of IR and angiogenesis inhibition can be improved by optimizing the dose and scheduling of both treatment modalities.
Materials and Methods

Cell culture
Cancer cell line HT29 (colon carcinoma) was cultured in DMEM (Lonza) and OE19 (esophageal adenocarcinoma) in RPMI (Lonza), both supplemented with 10% fetal calf serum (FCS) and 1% Penicillin-Streptomycin (Lonza) and for OE19 (kindly provided by Dr. H van Laarhoven, Amsterdam Medical Centre, The Netherlands) with 1% L-glutamine (Invitrogen) in a 37°C humified incubator, with 5% CO2. Both cell lines were authenticated before start of the experiments and with were repeatedly found negative for mycoplasm infection as checked by PCR.

Proliferation assay
Six hours after plating 2x10^3 HT29 cells/ well in a 96-well plate, 4 Gy IR and sunitinib was applied. Non-irradiated cells served as a control and for each condition a minimum of 4 wells were plated. On indicated time points, the amount of viable cells was determined, adding 100ul CellTiter-Glo (Promega) reagent to the cells. After the suspension was incubated for 20 min at room temperature, luminescence was measured. Experiments were performed in duplicate, with a minimal of four replicates in each experiment.

Analysis of cell cycle and apoptosis
In a 6-well plate, 2x10^5 HT29 cells/ well were plated and 6 hours later 4 Gy IR was applied. Non-irradiated cells served as a control. On indicated time points, cells were collected with trypsin EDTA (Lonza), resuspended in 70% ethanol and stored in -20 ºC for at least 2h for fixation. After spinning cells at 400g for 5 min, ethanol was discarded and cells were then incubated in DNA extraction buffer (90 parts 0.05M Na_2HPO_4.2H_2O, 10 parts 0.025M citric acid, 1 part 10% Triton X-100) for 20 min at 37 ºC. Propidium iodide was then added (20µg/ml) and cell cycle distribution was measured using flow cytometry (23). Experiments were performed in duplicate.

Chorioallantoic membrane assay
Fertilized white leghorn chicken eggs were incubated at 38°C in a fan-assisted humidified egg incubator. From embryonic development day (EDD) 0 to EDD3 the eggs were placed horizontally, rotating 90° each hour. On EDD3 eggs were put in an upright position and with fine tweezers a hole was made in the narrow end of the shell. On EDD6 a treatment window of ±1 cm² was created which was sealed with adhesive tape.

For drug treatment experiments without tumor xenografts, a non-latex dental elastic ring (Ø 9.5 mm) was carefully applied onto the CAM on EDD6. Anti-angiogenesis treatment consisted of daily application of the indicated concentrations in 50 µL within the ring. Sunitinib (20 mM in DMSO, Pfizer) was diluted in 0.9% saline, as a control 0.9% saline with the required concentration of DMSO was used.

At the end of each experiment, the chicken embryos were first euthanized by hypothermia (30 minutes at 4ºC). After injection of ±1 mL contrast agent (zinc oxide in pure vegetable oil)
under the CAM, pictures were taken with a Leica DFC425 camera mounted on a Leica M125 microscope. Multiple vascular parameters, including vessel length, vessel area, number of branching points and number of endpoints were quantified in each picture using CAM analysis software (HetCAM, DCILabs). All experiments were performed on a minimal number of 8 eggs in at least two independent experiments.

**CAM tumor grafts**

For tumor growth experiments on the CAM, $5 \times 10^6$ HT29 cells or $7.5 \times 10^6$ OE19 cells were resuspended in 50 µL cold growth factor reduced Matrigel (Becton Dickinson) and kept on ice until grafting. After slightly lacerating a small area of the CAM with a soft tissue, the cell-matrigel suspension was applied to the CAM on EDD6. The eggs were incubated under standard conditions and any subsequent treatment of the tumor started on EDD10. The sunitinib in saline was applied topically on the CAM close to but not directly onto the tumor at the indicated dose. Tumor size was measured each day and the volume was calculated as follows: $(\text{length})^2 \times \text{width} \times 0.5$, with length and width in mm. At the end of each experiment the tumors were harvested and weighed. Tumors were stored in ZincFix (0.5 gr calcium acetate, 5.0 gr zinc acetate, 5.0 gr Zinc Chloride in 1 L of 0.1M Tris Buffer, pH 7.4). Subsequently, the tissues were paraffin embedded according to standard procedures. Each experiment was performed with a minimum of 9 tumors per group.

**Ionizing radiation**

The cells and eggs were irradiated on the indicated day at room temperature by γ-radiation using a $^{60}$Co source (Gamma Cell 200; Atomic Energy of Canada). Following IR, the eggs were routinely checked on a daily basis.

**Immunohistochemistry**

Immunohistochemical (IHC) stainings were performed on 4 µm thick paraffin sections of CAM tumors. Following deparaffinization in xylene and rehydration through a graded series of alcohol, endogenous peroxidase activity was blocked by 20 minutes incubation in 0.3% $\text{H}_2\text{O}_2$/PBS. Next, antigen retrieval was performed in sodium citrate solution (pH 6.0) using a pressure cooker. After a blocking step of 30 minutes in 5% BSA/PBS at RT, the samples were incubated for 1 hour at room temperature (RT) with the primary antibody diluted in 0.5% BSA/PBS. The following primary antibodies were used: CD31 (SZ31, Dianova), cleaved caspase-3 (5A1E, Cell Signal Technology), and Ki-67 (M7240; Dako). Control slides were incubated with 0.5% BSA/PBS. Next, the slides were incubated for 30 minutes at RT with the appropriate secondary biotinylated antibody, followed by incubation with strep-ABC-HRP for 30 minutes at RT (1 uL avadin and 1 uL biotin in 500 uL PBS). Finally, staining was visualized with 3,3-diaminobenzidine-tetra hydrochloride (DAB, 0.3 mg/mL) in 1 mL PBS with 0.3% $\text{H}_2\text{O}_2$. All slides were counterstained with hematoxylin and mounted in Entellan (Merck) for microscopy. A minimum of 4 pictures of each slide were taken at 100x magnification and quantification of positive DAB staining was done using Image J with color deconvolution (24).
Measuring intratumoral and circulating sunitinib concentrations
Sunitinib concentration in tumors dissected from the CAM and in peripheral blood, withdrawn from a CAM vein, was determined by liquid chromatography tandem mass spectrometry (LCMS) as described previously (25).

Statistical analysis
All data are shown as mean +/- standard error of mean (SEM), unless indicated otherwise. For statistical analysis of the CAM angiogenesis experiments the Mann-Whitney rank sum test was used. The two tailed Students t-test for was applied for IHC quantification and the two-way ANOVA for tumor growth experiments with the post-hoc multiple comparison Bonferroni test. \( P \)-values < 0.05 were considered statistically significant.

Results
Ionizing radiation has a fast but transient effect on angiogenesis.
We set out to study the effects of scheduling the combination of ionizing radiation (IR) with angiogenesis inhibitors in vivo. For this, we used the chicken chorioallantoic membrane (CAM) assay, which is commonly used in angiogenesis research to evaluate the efficacy of angioregulatory drugs (26-29). The CAM is a highly vascularized membrane that facilitates gas exchange between the growing chicken embryo and the environment. It develops unidirectional from embryonic day of development (EDD) 3 to EDD10 (Supporting information Figure 1A). Subsequently, endothelial cell proliferation decreases and vessel maturation occurs (30). This was confirmed by analysis of different vascular parameters within the established macro- and microvascular bed throughout CAM development (Supporting information Figure 1B and 1C). To determine whether the CAM could be used to study the effects of IR, we first performed a dose escalation study (0-10 Gy) at EDD6. This resulted in a dose-dependent increase in embryonic lethality with 4 Gy as the maximal tolerable dose (Figure 1A). Analysis of the vascular parameters 24 hours after 4 Gy showed an almost 50% reduction in the vascular parameters, including vessel length, number of branchpoints and number of endpoints in the capillary bed (Figure 1B). No changes were observed in the macrovascular bed, i.e. the established vessels (data not shown). Furthermore, when 4 Gy was applied at EDD12 there was only a 7-10% reduction of the vascular parameters (Figure 1C). All this confirms previous observations that growing and immature blood vessels are more sensitive to IR as compared to mature blood vessels (31). Finally, we determined whether the effects of irradiation sustained. Time series experiments showed that all vascular parameters normalize to the level of the non-irradiated CAM within 3 days following 4 Gy (Figure 1D). Altogether, these data identify the CAM as a suitable model to study the fast and transient effects of IR on growing vessels.
Sunitinib potentiates the effect of IR on angiogenesis.

Next, we set out to combine IR with angiostatic therapy. For the latter, the FDA-approved drug sunitinib was used, a tyrosine kinase inhibitor mainly targeting VEGF- and PDGF receptors. First, the effect of sunitinib as a monotherapy was evaluated. Dose response experiments identified 5.3 µg/mL sunitinib (50 uL, daily application) to effectively inhibit vascular development in the CAM (Supporting information Figure 2A). Similar to IR, sunitinib only affected the microvasculature and the effects normalized to the level of the non-treated CAM within 4 days after treatment (Supporting information Figure 2B and 2C). Of note, 2 days of sunitinib treatment (EDD6+7) resulted in similar but smaller effects (Supporting information Figure 2D).

Next, we tested the effect of IR preceding sunitinib, with 4 Gy administered on EDD6 and sunitinib from EDD6-9. Measuring vascular parameters on EDD10 did not reveal any effects of IR alone while the combination was as effective as sunitinib alone (Figure 2A). To further address the importance of scheduling, next we applied IR (EDD 9) after sunitinib treatment for four days (EDD6-9). This schedule reduced the microvasculature more than either treatment alone (Figure 2B). These data illustrate the importance of scheduling both treatment modalities in order to obtain maximal efficacy.
Ionizing radiation reduces the tumor growth of human tumors cells on the CAM and transiently affects the tumor vasculature.

To investigate the effects of dose-scheduling on tumor angiogenesis and tumor growth, we next grafted HT29 human colon carcinoma cells on the CAM at EDD6. On EDD10 tumors had reached a volume of ±18 mm³. The tumors predominantly grew just below the CAM surface and hematoxylin/eosin (H/E) staining of resected tumors revealed an organized structure of clear tumor cells nests surrounded by stromal compartments (Figure 3A). The tumors also showed the presence of red blood cells, indicative of tumor vascularization which was confirmed by CD31 staining (Figure 3B). Furthermore, the tumors showed a clear response to IR as reflected by a decrease in tumor volume and weight four days after 4 Gy (Figure 3C). A comparable response was observed in tumors of a human esophageal adenocarcinoma cell line OE19 (Supporting information Figure 3). Ionizing radiation appeared to induced a decrease in proliferating cells in the HT29 tumors (Figure 3D). While did this not reach significance when scoring the number of Ki-67⁺ cells by IHC, in vitro data confirmed significantly lower proliferation rates 4 days after IR (Supporting information Figure 4A). Furthermore, in vitro analyses also showed a G2/M and S phase arrest respectively after 4 Gy (Supporting information Figure 4B). This suggests an overestimation of proliferating cells with Ki-67 IHC in the tumors as described previously (32;33). The apoptotic fraction was not enhanced 24h after 4 Gy IR, but an increase was observed 4 days after IR (EDD 14) (Figure 3E). This was in line with in vitro analyses (Supporting information Figure 4C). Together, these mechanisms likely underlie the decrease in tumor volume by IR.

Of note, we detected a decrease in endothelial cells 24h after 4 Gy (EDD11). Interestingly, the reduction in the vessel density in non-irradiated tumors, which is caused by vessel growth lagging behind tumor expansion, was reversed in IR tumors (Figure 3F). Thus, while IR impairs tumor growth, the initial inhibition of vessel growth rapidly recovers, similar as observed in our previous experiments.
Figure 3. Effect of ionizing radiation on HT29 tumor growth in vivo. A) Representative images of HT29 tumor grafts on the CAM. The two panels on the left show the same tumor on EDD10 and EDD14. The ‘cap’ on top of the CAM consists of Matrigel and cell debris. The viable tumor is growing just beneath the CAM (dotted lines). The two panels on the right show a resected tumor on EDD17 and H/E staining on a resected tumor with clear tumor cell nests surrounded by stromal tissue. B) H/E staining showing the presence of red blood cells (arrows) in the tumor tissue (upper panel). IHC showing CD31+ (brown) vessels within the tumor tissue (lower panel). C) Tumor growth curves showing growth inhibition following single dose IR (4 Gy) on EDD10. The bar graph shows the average weight of tumors resected either on EDD11 or EDD14 (average + SD, n ≥ 9). D) IHC stainings (left) for Ki67 (proliferation marker). The bar graph shows the quantification of Ki67+ cells in control and irradiated tumors that were resected either on EDD11 or EDD14. Quantification was performed using ImageJ with color deconvolution. E) Similar as in (D), but now staining was performed for cleaved caspase 3 (CC3, apoptosis marker). F) Similar as in (D), but now staining was performed for CD31 (endothelial cell marker).
Sunitinib reduces the tumor growth on the CAM by reducing proliferation and inhibiting angiogenesis.

To determine the effect of monotherapy with sunitinib on tumor growth, first a dose-safety study was performed. Determining the intra-tumor sunitinib concentration with LCMS demonstrated that topical administration of sunitinib on the CAM close to the tumor resulted in an almost 4 times higher sunitinib concentration as compared to direct intravenous administration (82 nmol vs 21.8 nmol) 24 hours after 1 dose of 160 µg sunitinib. Thus, while sunitinib was applied topically on the CAM vasculature, it acted systemically. The systemic effect was also confirmed by observed toxicities in the chicken embryos (black necrotic claws, recognized as the hand-foot syndrome) 48 hours after sunitinib application. Due to the toxicities, the dose had to be reduced to 50% divided over 4 days (EDD10-13), i.e. 20 µg sunitinib per day, corresponding to 32.5 mg/kg/day, as the maximum tolerable dose. This treatment schedule significantly reduced tumor growth and weight in HT29 tumors (Figure 4A and 4B) as well as in OE19 tumors (Supporting information Figure 5). In line with the described angiostatic activity, sunitinib treatment significantly reduced the percentage of CD31+ cells (Figure 4C). In contrast to IR, sunitinib treatment did not increase the apoptotic fraction but decreased the percentage of proliferating tumor cells (Figure 4D), which was confirmed in vitro (Supporting information Figure 4D). These data not only confirm that sunitinib monotherapy inhibits tumor growth via angiogenesis inhibition and tumor cell growth but also indicate that sunitinib treatment might be complementary to IR.

Figure 4. Effect of sunitinib on HT29 tumor growth in vivo. A) Growth curves of HT29 tumors grafted on the CAM on EDD6. Treatment with sunitinib (20 µg/day in 50µL) was applied from EDD10-13. B) Weight of tumors resected on EDD17 (average + SEM, n ≥ 9). C) IHC stainings for CD31 (endothelial cell marker). The bar graph shows the quantification of CD31+ cells in control and sunitinib treated tumors that were resected on EDD17. Quantification was performed using ImageJ with color deconvolution. D) Similar as in (C) but staining was performed for cleaved caspase 3 (CC3, apoptosis marker) or Ki67 (proliferation marker).
Ionizing radiation given before sunitinib effectively inhibits tumor growth.

To study the possible complementarity of both treatment modalities, we next combined 4 Gy IR with 20 µg sunitinib using three different treatment schedules. The tumors in the control group received sunitinib from EDD10-13. In the additional three groups the tumors also received 4 Gy IR, either on EDD10 (neo-adjuvant), EDD12 (concurrent) or EDD14 (adjuvant). The combination of sunitinib with pre-adjuvant IR resulted in an almost complete inhibition of tumor growth and significant reduction in tumor weight (Figure 5A and 5B). The concurrent IR inhibited tumor growth to a lesser extent while adjuvant IR did not further affect tumor growth or weight (Figure 5A and 5B). Again, we observed an induction of apoptosis after 4 Gy IR, while Ki-67 IHC did not demonstrate a difference in proliferating fraction (Figure 5C). These experiments illustrate that proper scheduling of combination therapy with sunitinib and IR can almost completely block tumor growth.

Figure 5. Effect of scheduling combination therapy on HT29 tumor growth in vivo. A) Growth curves of HT29 tumors grafted on the CAM on EDD6. Treatment with sunitinib (20 µg/day in 50µL) was applied from EDD10-13. Ionizing radiation (4 Gy) was applied on either EDD10, EDD12 or EDD14. B) Weight of control and treated tumors resected on EDD17 (average + SEM, n ≥ 10). C) IHC stainings for CC3 (apoptosis marker) and Ki67 (proliferation marker). The bar graph shows the quantification in tumors resected on EDD17. Quantification was performed using ImageJ with color deconvolution.
Optimal scheduling allows dose reduction of sunitinib without affecting therapeutic efficacy.

Finally, we determined whether optimal scheduling would allow dose reduction of sunitinib. We reduced the dose of sunitinib with 50% to 10 µg/day for 4 days. As expected, monotherapy with 10 µg sunitinib had less effect on tumor growth than 20 µg sunitinib (Figure 6A). This was accompanied by less inhibition of the CD31+ fraction and tumor cell proliferation (Figure 6B). However, when 10 µg sunitinib was combined with 4 Gy IR, this also resulted in a complete inhibition of the tumor growth, comparable to 20 µg sunitinib with 4 Gy IR (Figure 6A). While the combination treatment was significantly more effective than 10 µg sunitinib alone, no statistically significant difference to 4 Gy alone was observed. This is most likely due to the considerable effect of 4 Gy alone and the limited follow up time due to hatching of the eggs. Therefore, we also reduced the dose of IR with 50% to 2 Gy. Though the timing of 2 Gy IR (EDD10, 12 or 14) had no significant influence on reducing the tumor growth (Supporting information Figure 6A), 2 Gy induced a significant inhibitory effect on tumor growth, albeit less as compared to 4 Gy (Figure 6C and Supporting information Figure 6B). This was also reflected by the percentage of necrotic tissue and apoptotic cells in these tumors (Figure 6D). When 2 Gy IR in the pre-adjuvant schedule was combined with 20 µg sunitinib an additional reduction in tumor growth was achieved (Figure 6C). Next, we reduced the dose of sunitinib by 50% to 10 µg/day for 4 days. The monotherapy of 10 µg sunitinib was less effective than 2 Gy IR, while the combination was similarly effective as 20 µg sunitinib + 2 Gy IR. In addition, 10 µg sunitinib + 2 Gy IR was significantly more effective then sunitinib alone and reached borderline significance with 2 Gy IR (Figure 6C). Of note, both combinations had comparable effects as 4 Gy IR alone (Supporting information Figure 6C). We also applied the combination of this sub-optimal treatment on grafted OE19 cells. While monotherapy with either 10 µg sunitinib (EDD10-13) or 2 Gy IR (EDD10) resulted in a tumor growth reduction of already approximately 50% compared to the non-treated tumors, combining the two treatment modalities resulted in a tumor growth reduction of nearly 80%. This was also reflected in tumor weight (Supporting information Figure 7).

These data demonstrate that optimization of dose-scheduling allows halving the dosage of sunitinib without loss of therapeutic efficacy.
Discussion

Despite encouraging pre-clinical studies that combine radiotherapy with angiostatic drugs, varying results and increased side effects in the clinical setting demonstrate that more research is warranted to optimize this combination treatment. We therefore studied the effects of dose-scheduling of IR and the clinically available angiostatic drug sunitinib on both angiogenesis and tumor growth. Our observations show the importance of proper scheduling of both treatment modalities in order to obtain maximum treatment effects. Moreover, our data demonstrate that optimal scheduling allows dose reductions of sunitinib while maintaining the same anti-tumor effect. The latter is clinically relevant as it could reduce side effects in patients without affecting...
treatment efficacy. This is in line with two case reports in which sunitinib dose reductions did not affect the clinical benefit of the treatment (21;22).

In the current study we demonstrate that the CAM assay is a feasible method to study the effect of combination therapy on both angiogenesis and tumor growth. Both IR and sunitinib had a significant inhibitory effect on angiogenesis. In addition, both treatments significantly reduced tumor growth. As expected, IR had a dose dependent effect on tumor cell apoptosis and proliferation while sunitinib reduced the microvessel density (MVD) in the tumors as well as the tumor cell proliferation. This is in line with previous observations in different mouse tumor models, where it was shown that sunitinib not only has a direct effect on the tumor vasculature, but also on the tumor cells (34-36). All these observations identify the CAM tumor model as a representative model to study (tumor) angiogenesis and tumor growth in vivo. A potential limitation of the CAM tumor model is the relatively short time frame during which therapy can be applied. Nevertheless, our current data show that already within this time frame comparable observations can be made as in e.g. mouse tumor models. Moreover, since the model is reliable and affordable it provides a good alternative for rapid drug screening or monitoring the efficacy of (combination) therapy. The latter is illustrated in the current study.

While the effect of a limited number of drugs in combination with IR has been studied on the CAM (37;38), we now demonstrate that the efficacy of combining IR with sunitinib is dependent on the schedule that is used. In agreement with others, we show that IR alone reduces angiogenesis and that this recovers within 96 hours (37-39). Interestingly, our data suggest that the effect of IR on the vasculature is also transient when IR is followed by sunitinib treatment, since this combination treatment schedule was as effective as sunitinib alone. On the other hand, we observed that IR applied after sunitinib treatment was more effective than either treatment alone. While this already shows the importance of proper scheduling, the opposite effect of scheduling was observed in the tumor model. Here, IR given after sunitinib had no additional effect on tumor growth compared to sunitinib alone, while IR given before sunitinib stopped tumor growth completely. This not only further exemplifies the importance of scheduling, it also shows the importance of the environmental context, e.g. normoxic CAM vs. hypoxic tumor tissue, when studying combination therapies. Our observation in the CAM tumor model is in agreement with most xenograft mouse models which show that the combination therapy has beneficial effects on tumor growth reduction (40-42). However, in the mouse studies, mostly only a concomitant treatment schedule was used. We now show that IR applied before sunitinib treatment can lead to better tumor growth reduction as compared to concomitant treatment. This is in agreement with two previous pre-clinical studies in mice (13;15) as well as with a clinical trial where it was suggested that sunitinib after radiotherapy is the main factor contributing to tumor response rates (43). All this further confirms the applicability of the CAM tumor model and the clinical relevance of addressing the scheduling of combination therapy.

The precise mechanism by which adjuvant sunitinib improves outcome compared to neo-adjuvant or concomitant treatment is subject for further investigations. Our data show that sunitinib decreases tumor cell proliferation and tumor vascularization. The latter was also
observed in a breast cancer xenograft model and in patient tumor samples (44;45). The reduced vascularization could lead to increased hypoxia, as described in a melanoma xenograft model (46). Consequently, neo-adjuvant sunitinib might reduce therapeutic efficacy of IR due to increased hypoxia and reduced cell proliferation. In addition, IR might sensitize tumor cells to sunitinib or lead to an enhanced angiogenic response which is then counteracted by sunitinib. On the other hand, it has also been suggested that sunitinib could improve efficacy of IR by transiently inducing vessel normalization leading to better oxygenation of the tumor tissue (47;48). While we did not evaluate vessel normalization or tumor oxygenation in this study, our current results do not suggest that this mechanism occurred in this particular tumor model. Further research is required to unravel the exact underlying mechanism and to establish whether the current observations are a commonality when combining IR with angiostatic therapies.

An important finding of the current study is that optimal scheduling of IR and sunitinib allows dose reduction without affecting the therapeutic benefit. To our knowledge we are the first reporting on the effects of reducing the dose of sunitinib in combination with IR. This dose reduction is relevant for the clinical setting since there is a concern of increased side effects when both treatment modalities are combined, in particular with regard to bowel perforations and haemorrhagic events (17;43;49). The results of our study suggest that dose reduction of sunitinib in patients will not affect tumor response while it might result in a better toxicity profile, provided that optimal scheduling is applied. In addition, when high dose radiotherapy is not possible for a patient, due to the risk of normal tissue toxicities, sunitinib could be added to the radiotherapy. This might allow reduction of the IR dose, without compromising the anti-tumor effect. To elucidate whether these dose-reductions in the combination treatment have similar benefits in patients as we observed in our research, clinical trials are warranted.

Taken together, our results demonstrate that the CAM assay provides a feasible model to study the combination of different treatment modalities on angiogenesis and tumor growth, resulting in similar results as observed in other in vivo xenograft models. The major clinically relevant findings of our study are that precise scheduling of sunitinib and radiotherapy improves the therapeutic outcome and that this allows dose reduction of sunitinib without hampering the therapeutic efficacy. Further research should focus on the extension of different schedules and different doses of sunitinib and IR in order to improve the anti-tumor outcome with minimal toxicity. Especially since our current results suggest that the maximum effective dose of sunitinib in combination with IR is significantly lower than the maximum tolerated dose. Furthermore, the most optimal and clinically relevant schedule should be tested in a clinical trial, with a focus on dosing and scheduling of both sunitinib and radiotherapy. This will lead to a better, faster, and more rational translation of this promising combination therapy to the clinic.
References


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Supporting information

**Figure 1. The chicken chorioallantoic membrane model.**

A) Images of ex ovo CAM development from EDD3 to EDD10 showing unidirectional growth of the vasculature with the embryo growing in the center of the CAM (arrow). B) Representative image of CAM microvasculature analysis showing the different parameters that are acquired by the HetCAM software. C) Vascular parameters obtained using HetCAM software during CAM development.
Supporting Information Figure 2. Dose-response analysis of sunitinib. A) Vascular parameters obtained using HetCAM software on EDD10 following 4 days of treatment with sunitinib at the indicated dose. B) Vascular parameters obtained using HetCAM software on EDD10 of the macrovasculature following 4 days of 50 µl of 5.3 µg/mL sunitinib. C) Vascular parameters obtained using HetCAM software on EDD10 of the microvasculature following 2 days of 5.3 µg/mL sunitinib.

Supporting Information Figure 3. Effect of irradiation on OE19 tumor growth in vivo. Tumor growth curves showing growth inhibition following single dose irradiation (4 Gy) on EDD10. The bar graph shows the average weight of resected tumors (average ± SD, n ≥ 6)
Supporting Information Figure 4. Proliferation, cell cycle and apoptosis analysis for HT29 in vitro. A) Proliferation assay, measuring luminescence of HT29 cells with CellTiterGlo. Cells either received 4 Gy or no irradiation. * p < 0.05 B) Cell cycle analysis of HT29 cells in vitro following 4 Gy IR, using PI FACS. C) Apoptosis quantification of HT29 cells in vitro following 4 Gy IR, using PI FACS. D) Cell cycle analysis of HT29 cells in vitro following sunitinib treatment (1 µM), using PI FACS. * p < 0.05
Supporting Information Figure 5. Effect of sunitinib on OE19 tumor growth in vivo. Tumor growth curves showing growth inhibition following treatment with sunitinib (20 µg/day in 50uL) applied from EDD10-13. The bar graph shows the average weight of resected tumors (average ± SD, n ≥ 6).

Supporting Information Figure 6. Tumor growth curves of HT29 following 2 Gy IR alone or in combination with sunitinib. A) Tumor growth curves of HT29 showing growth inhibition following single dose IR (2 Gy) on EDD10, 12 or 14. B) Tumor growth curves of HT29 showing difference in growth inhibition following 2 Gy or 4 Gy single dose IR on EDD10. C) Tumor growth curves of HT29 showing growth inhibition following 2 Gy IR (EDD10) or 2 Gy IR (EDD10) in combination with sunitinib (10 or 20 µg/day in 50 µL EDD10-13).

Supporting Information Figure 7. Combination of radiation and sunitinib reduces the growth rate of OE19 tumor on the CAM more than either treatment alone. A) Growth curves of OE19 tumors on CAM subjected to different treatment regimes. Treatment with sunitinib (10 µg/day in 50 µL) was applied from EDD10-13. Irradiation (2 Gy) was applied on EDD10. B) Weight of OE19 tumors after resection on EDD17.