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TITLE: Potentiation of Prostate Cancer Radiotherapy using Combined Antiangiogenic and Antitumor Therapies

PRINCIPAL INVESTIGATOR: Bruce M. Fenton, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, NY 14642

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14. ABSTRACT The focus of this grant period was to delineate the effects of monotherapies or combinations of radiotherapy and AG-013736 (a multiple angiogenic receptor inhibitor) on vascular maturity and function, based on perfusion and hypoxia indices as well as pericyte coverage. In 3 major experiments, ~250 tumors were frozen for immunohistochemical staining and image analysis. Almost complete growth inhibition was observed for the combined therapy. Total and perfused vessel counts were significantly decreased, but overall hypoxia was also increased in comparison to volume-matched controls. Although pericyte coverage increased with therapy, dissociated PDGFR+ cells also increased. These findings could be explained by either selective vessel ablation or pericyte recruitment, but clearly indicate that a "normalization" of vessels into a more efficiently functioning network is not occurring in response to these specific combined treatments. Ongoing studies are expanding these studies into PC-3 tumor models, and specifically evaluating the effects of alternative scheduling.					
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Introduction:

Although antiangiogenic strategies have been shown highly promising in preclinical studies, only combinations of antiangiogenic and cytotoxic therapies have been shown effective in recent Phase III clinical trials. Unfortunately, almost no guidelines exist for the optimal scheduling of such combinations. Previous work has been contradictory, with some studies showing decreased tumor oxygenation and/or blood flow following antiangiogenic strategies and others demonstrating the opposite. The current work concentrated on evaluating the pathophysiological consequences of both single and combined treatments with fractionated radiation plus AG-013736, a potent receptor tyrosine kinase inhibitor of VEGFRs that also inhibits PDGFRs. Our first question was whether vascular density decreases following single or combination treatment, and if so, whether tumor hypoxia is thereby increased. Given the well known dependence of radiosensitivity on tumor oxygen levels, such an increase in hypoxia could compromise further treatments. Previous studies have also reported alterations in vessel maturity, as measured by the incorporation of periendothelial support cells, or pericytes, following antiangiogenic therapies. The consensus in the literature appears to be that the presumably more mature pericyte coated vessels are more resistant to treatment. Our second objective was to compare alterations in the tightness and coverage of three specific pericyte markers. Our hypothesis was that AG-013736, a specific small molecule inhibitor of both VEGFRs and PDGFRs, would inhibit tumor progression through an increase in endothelial and tumor cell apoptosis, and that this effect would be further enhanced through the addition of fractionated radiotherapy (RT). Our results provide evidence that although combination treatment reduces functional vascular density and increases tumor hypoxia in relation to volume-matched controls, combination therapy remains effective and tumor progression continues to be significantly inhibited over three weeks of therapy. Effects of treatment on pericyte coverage are more complex and vary strikingly with specific marker, tumor model, and treatment.

Body:

In the first year of this project, the focus was primarily on tumor pathophysiological response to two antibodies from ImClone Systems: A12 (an antibody to insulin growth factor receptor-1) and DC101 (an antibody to VEGFR-2). Towards the end of Year 1, we were able to obtain two developmental drugs from Pfizer Global Research: AG-028262 (a small molecule tyrosine kinase inhibitor of VEGFR-1,2,3) and AG-013736 (an inhibitor of both VEGFRs and PDGFRs), and we modified our research focus from injected antibodies to orally administered small molecule inhibitors. The primary advantage of comparing the latter drugs is that the AG-028262 targets only the VEGF receptors, and was thus expected to be primarily antiangiogenic, while the AG-013736 targets both VEGF and PDGF receptors, and was expected to act as both an antiangiogenic and an antivasular agent due to the previously described interdependence between PDGFRs and vascular pericyte coverage. Somewhat contradictory reports in the literature have shown that VEGFR2 inhibition can

either preferentially target and destroy non-pericyte coated blood vessels, or instead tighten pericyte-endothelial coverage, thus conferring a more mature phenotype on the vessels. A primary goal was therefore to specifically address this question.

Three major animal experiments were performed during Year 2. The first, initiated late in Year 1, included ~100 DU145 tumors treated with combined or monotherapies, with tumor groups frozen at each of 3 weekly timepoints. During Year 2, a major amount of effort went into cutting and processing multiple frozen sections of these tumors, further optimizing the immunohistochemistry, and defining the image processing algorithms for the newest pericyte stains. In addition to imaging for the functional vessels, endothelial coverage, and hypoxia, additional sections were cut for tumor cell and endothelial apoptosis, proliferation, and four different pericyte markers: PDGFR, desmin, NG2, and α -sma.

The second experiment (~70 tumors) utilized PC-3 tumors and repeated many of the same therapeutic regimens as before, but with treatment initiated at relatively large tumor volumes. These experiments took somewhat longer to initiate than expected due to difficulties in growing the PC-3 tumors from our collaborator's existing stock of frozen PC-3 cells. These cells and the implanted tumors grew much more slowly than anticipated, and we ultimately switched to a new PC-3 line obtained from ATCC. The final experiment (~110 tumors), now in progress compares monotherapies to combined therapies, with the emphasis on evaluating alternative treatment scheduling: AG-013736 delivered either 1 hr pre- or post-RT and following temporal changes in both single and multiple agents.

Recently, we also acquired additional funding for a second laboratory technician, and over the past two months she has been trained in most of the immunohistochemistry, animal handling, and cell culture techniques. This should substantially improve our productivity over the next year.

Image analysis – Vascular spacing, apoptosis, and proliferation. As described previously¹, tumor blood vessel spacing was determined using a combination of image segmentation and distance map filtering to obtain a spatial sampling of distance filter intensities, which are directly proportional to the distribution of distances to the nearest vessel. These distances (related to tumor blood vessel spacing) are reflective of the median distances over which oxygen and nutrients must diffuse to reach all cells of the tumor. Colocalized and thresholded images of panendothelial cell antigen staining, DiOC₇ (for perfusion), and TUNEL staining (for apoptosis) were obtained, and population densities were determined for each as well as for the overlap between TUNEL and perfused vessel staining (using custom ImagePro macros that were developed over this period to obtain population densities within defined areas of interest). Percentage apoptotic vessels was calculated by dividing the overlap population density (which represents apoptotic endothelial cells) by vessel population density. Ki-67 stained images (proliferation marker) were quantified by determining % positively stained area (again using ImagePro thresholding and counting operations).

Image analysis – Pericyte coverage and tightness. We acquired colocalized and thresholded stained images of panendothelial cell antigen and three pericyte markers: 1) PDGFR β ⁺ cells, which are generally believed to correspond to perivascular progenitors or less mature pericytes, 2) NG2⁺ cells, which have variously been reported to correspond to both immature and mature pericytes, and 3) α -sma⁺ cells, generally thought to mark more mature pericytes and vascular smooth muscle cells. Percent areas of each as well as the % area of the overlap between endothelial and pericyte markers were determined (using ImagePro measurement operations within defined areas of interest). Percent coverage was defined as the % area overlap divided by the % area endothelial cells, and % dissociated was defined as 100 - (% area overlap divided by the % area pericytes).

Effects of AG-013736 and/or fractionated radiotherapy on tumor growth. In Experiment 1, male nude mice bearing DU145 tumors were treated for a period of from 1-3 weeks with either fractionated radiation (5 x 2 Gy / wk) or AG-013736 (25 mg/kg/day, i.g.) or the combination (groups of ~8 tumors were frozen for each treatment at each of the three timepoints for a total of ~97 tumors). Tumor volumes were measured 3 times/wk, mice were sorted into groups having roughly equal mean volumes, and treatments were initiated at volumes between 200-400 mm³. From previous studies, it was determined that tumor inhibition in response to doses of 10 and 25 mg/kg was roughly equivalent, but 50 mg/kg resulted in almost total cessation of tumor growth over the three weeks of treatment. A dose of 25 mg/kg was therefore selected for the combination experiments, in order to produce a limited reduction in tumor growth and allow synergistic combination effects to be seen.

Based on preliminary studies (not shown), a schedule of 5 x 2 Gy / wk of radiation was found to slow tumor growth to a rate similar to that following 25 mg/kg AG-013736. The combination studies compared the two single therapies with the daily combination of RT followed 4-5 hrs later by AG-013736. Tumor volume at the end of two weeks was significantly reduced for either single or combination treatments. Percent increase in tumor volume was very similar between RT (40% $\pm$ 9.8) and AG-013736 (48% $\pm$ 9.2), but was reduced following the combination treatment (12% $\pm$ 5.7) in respect to both single therapies (p = 0.06 and 0.004), and significantly reduced versus controls (77% $\pm$ 11, p < 0.001).

Total and perfused blood vessel spacing increases following either single or combination treatments. As we have reported previously, automated image analysis techniques are able to quantify tumor vessel spacing much more precisely than vascular density. Vessel spacing is also of more direct pathophysiological significance, since it reflects the median distance that oxygen and nutrients must diffuse to reach the surrounding tumor cells. Measurements of vascular density, in contrast, could conceivably remain constant despite a wide variance in vessel distributions among different tumors, i.e., ranging from optimally spaced vessels to highly clustered distributions.

We first examined the effects of treatment on total and perfused vascular spacing, to gauge whether RT, AG, or the combination compromised the tumor vascular distributions. As shown in Fig. 1A for the two week timepoint, either AG, RT, or the combination significantly increased both total and perfused blood

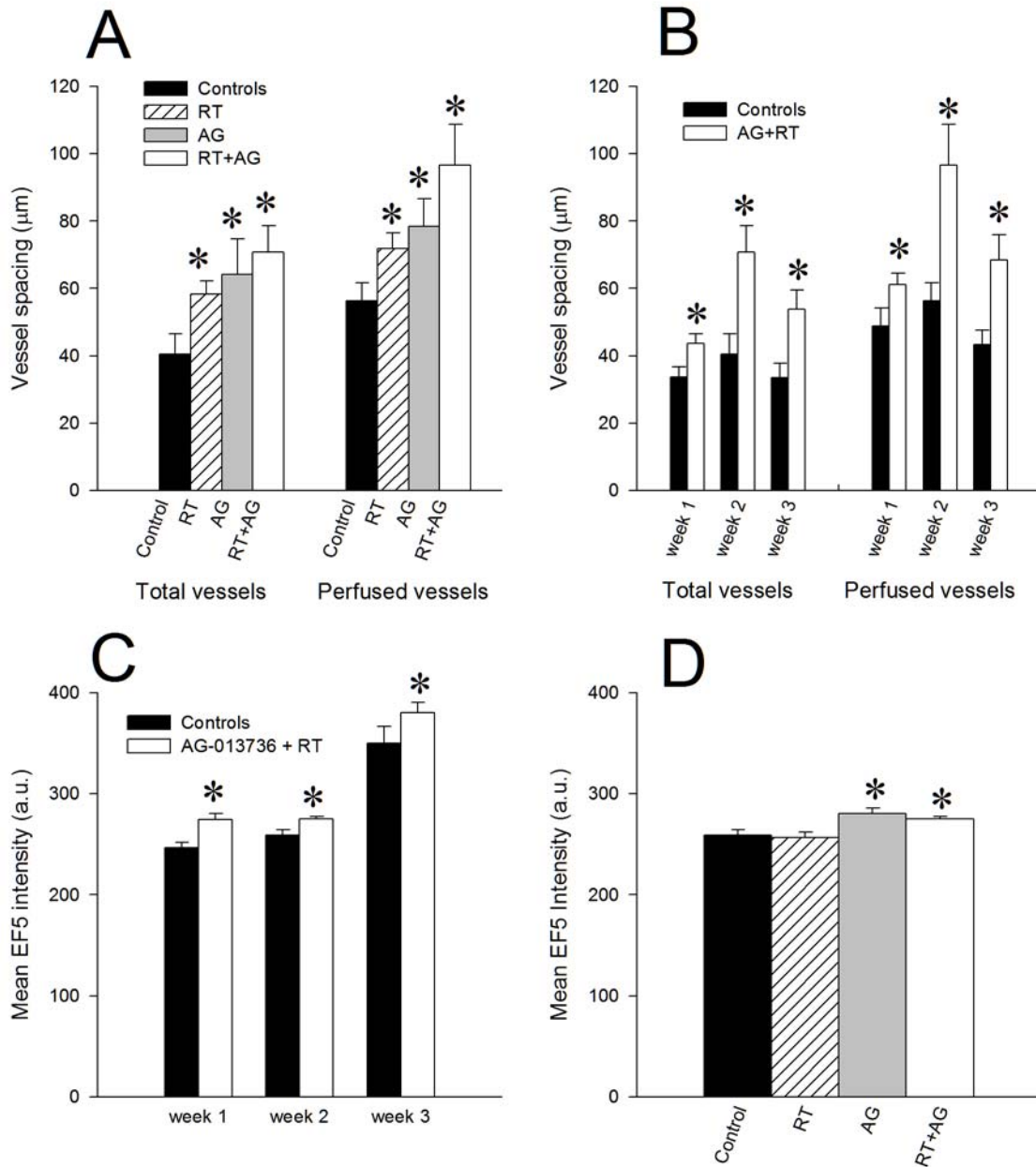


Figure 1 Tumor pathophysiological changes following single and combined treatments. A) Effect of single and combined treatments on total (left columns) and perfused (right columns) vessel spacing at week 2. B) Effect of combination treatment on total and perfused vessel spacing at weeks 1, 2, and 3. C) Effect of combination treatment on mean overall tumor hypoxia at weeks 1, 2, and 3. D) Effect of single and combined treatments on mean EF5 intensity at week 2. 5-8 tumors / group; In panel C, asterisks denote significant differences of treated tumors in relation to week 1 controls ($p \leq 0.05$).

vessel spacing, although none of the treatments were significantly different from each other. Fig. 1B compares plots of combination versus control groups at 1, 2, or 3 weeks of treatment. Although combination treatment produced significant increases in total and perfused vessel spacing at each timepoint, the changes were minimal at 1 week, most pronounced at 2 weeks, and again reduced by 3 weeks of treatment.

Tumor hypoxia increases following combination therapy. Overall tumor hypoxia was next determined by EF5 hypoxia marker binding, which was quantified in frozen sections based on the overall intensity of the fluorescently conjugated antibody to EF5. Combination treated groups are shown paired with day-matched control tumors at each week, although a more relevant comparison is to pair treated groups with volume-matched controls in order to better differentiate the pathophysiological effects of treatment from those of tumor growth alone. Although it might be expected that alterations in hypoxia should mimic changes in perfused vessel spacing, this was not necessarily the case. Thus hypoxia increased significantly for controls over the 3 weeks of treatment ($p = 0.003$, Fig. 1C), while perfused spacing changed minimally over this same period (Fig. 1B). This suggests that although the vessel spacing was essentially unaltered, the blood flow within the perfused vessels may have steadily decreased in parallel with tumor growth, resulting in the observed increase in hypoxia. Since combination treated tumors grew extremely slowly, mean tumor volumes over the entire course of therapy were similar ($350\text{--}550\text{ mm}^3$), and roughly equal to control tumors at week 1 (480 mm^3). Comparing the control tumors at week 1 to the volume-matched combination treated tumors of week 3 reveals a significant increase in overall hypoxia in the treated tumors after 3 weeks of treatment ($p < 0.001$), in concert with a parallel increase in perfused vessel spacing ($p = 0.05$). This suggests that although the combination tumors are minimally progressing in terms of volume, vascular functionality is steadily decreasing, with a resultant increase in tumor hypoxia. Finally, Fig. 1D compares single and combined treatments at week 2. Both AG-013736 ($p = 0.011$) and the combination ($p = .033$) significantly increased mean tumor hypoxia at this timepoint.

Apoptosis but not proliferation is altered transiently. The effects of therapy on tumor cell apoptosis and proliferation were evaluated both in relation to single treatments and following 1, 2, and 3 weeks of combination therapy. AG-013736 alone had no significant effect on tumor cell apoptotic cell density ($p = 0.38$, Fig. 2A), as identified using TUNEL staining, while RT significantly increased apoptosis ($p = 0.038$). When AG-013736 was added to RT, apoptosis increased significantly compared to controls ($p = 0.011$), but was only marginally higher than RT alone ($p = 0.52$), arguing against any AG-013736 associated tumor cell radiosensitization. Previous work has shown that endothelial and tumor cell apoptosis increases immediately following RT in murine melanomas, but reverts to baseline at 3, 7, and 14 days post-RT². In our studies (Fig. 2B), total apoptosis was instead somewhat higher for combination treated tumors by week 1 ($p = 0.09$), significantly higher at week 2 ($p = 0.01$), and returned to controls levels by week 3 ($p = 0.67$). The percentages of apoptotic blood vessels were also

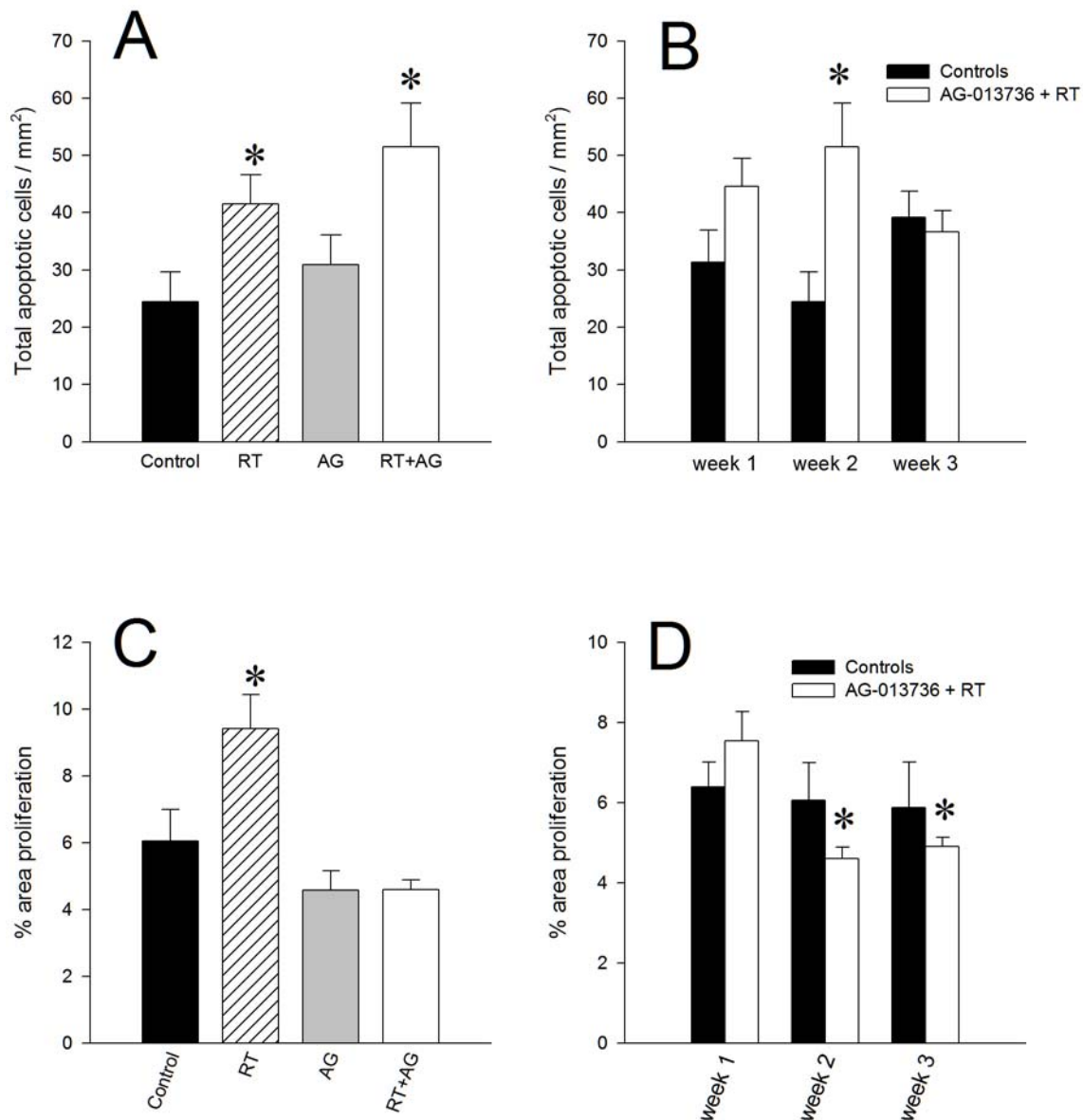


Figure 2 Tumor pathophysiological changes following single and combined treatments. A) Effect of single and combined treatments on total apoptotic cell density at week 2. B) Effect of combination treatment on total apoptotic cell density at weeks 1, 2, and 3. C) Effect of single and combined treatments on % area proliferation at week 2. D) Effect of combination treatment on % area proliferation at weeks 1, 2, and 3. 5-8 tumors / group. In Panel D, asterisks denote significant differences from volume matched controls (week 1).

automatically counted by combining the TUNEL and perfused vessel images, but in comparison to controls, combination treatment had no significant effect at any of the three timepoints (data not shown).

Tumor cell proliferation was quantitated based on % positive Ki-67 staining and was only significantly increased by RT at 2 weeks post-therapy (Fig. 2C, $p < 0.001$). As with tumor cell apoptosis, the % area of proliferating cells remained fairly constant for controls from weeks 1 to week 3 (Fig. 2D), despite the accompanying increase in tumor volume. For the combination treated tumors, in contrast, % proliferation decreased at both weeks 2 ($p = 0.014$) and 3 ($p = 0.033$). In combination with the increased apoptosis at week 2 (Fig. 2B), this produces a net decrease in tumor cell burden.

Pericyte coverage is altered by both tumor growth and combination

treatment. To determine the effect of combination treatment on pericyte coverage, three different pericyte markers were evaluated (one of which, NG2, is being recut to improve the auto-segmentation process). The expectation was that the proportion of blood vessels having a more mature phenotype would increase following treatment, as the less mature vessels were preferentially targeted and eliminated. Previous work has shown that pericyte coverage does not increase following RT alone, and can either increase or decrease following antiangiogenic strategies. Fig. 3 summarizes our results for PDGFR- β and α -sma in terms of % pericyte coverage at week 2 of treatment (defined as the endothelial / pericyte overlap area divided by the total endothelial area) and % dissociated (defined as the endothelial / pericyte overlap area divided by the total pericyte area).

Although only AG-013736 resulted in a significant increase in % PDGFR- β coverage (Fig. 3A), the levels were fairly similar among the different treatments. This was also the case for the % α -sma coverage (Fig. 3B), although coverage was markedly reduced for this pericyte marker in relation to the PDGFR- β . The % dissociated PDGFR- β , however, increased substantially with all treatments, and the combination treatment was significantly higher than either of the monotherapies (Fig. 3C). for the % dissociated α -sma, no significant changes were seen with any of the treatments.

Fig. 4 further delineates the effect of treatment time on pericyte coverage and dissociation for the PDBFR- β and α -sma markers. In this figure, alterations in pericyte coverage and dissociation from week to week for the control tumors are a reflection of growth-related changes as these tumors doubled in volume. Since vascular spacing was fairly constant over this time period for controls (Fig. 1B), the increase in % coverage corresponds to a maturation of the vessels. For the combination treated tumors, however, tumor growth was essentially dormant over the 3 wks of therapy, while at the same time vessel spacing was increased (Fig. 1B). Thus the tumors at weeks 2 and 3 are roughly equivalent to the volume of wk 1 controls. This means that the increase in PDGFR- β coverage was occurring at the same time that vessel counts were decreasing in the treated tumors, and suggests that non-PDGFR- β coated vessels are being preferentially ablated. Alternatively, it is possible that vessels are non-selectively ablated, in combination with pericyte recruitment, in the combination treated tumors, but this seems less likely based on the dissociation results of Fig. 4C. Here, PDGFR- β dissociation also increases substantially in wks 2 and 3 for the combination treated tumors, while controls are unchanged. This suggests a loosening of these

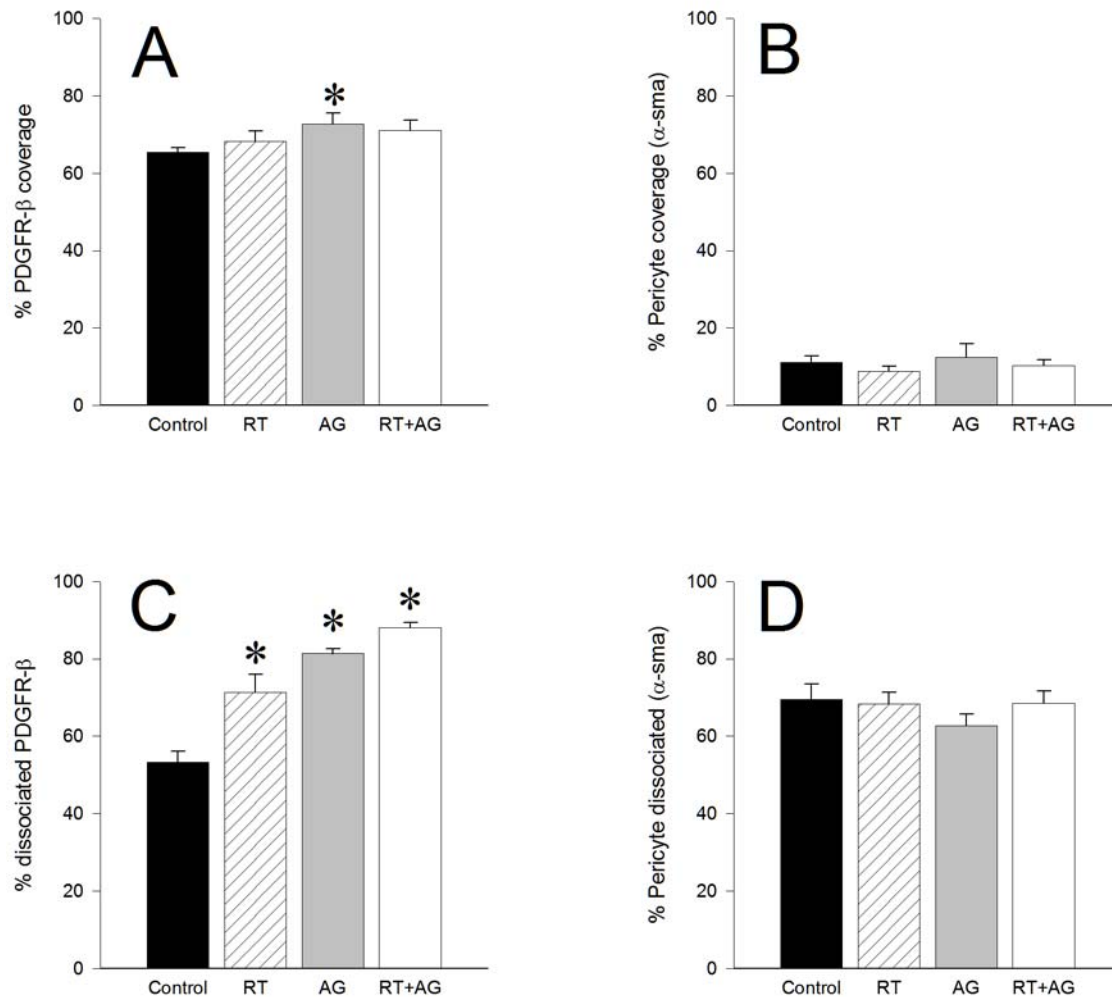


Figure 3 % coverage of PDGFR- β + (A) and α -sma+ (B) pericytes as a percentage of blood vessel area, and % dissociated PDGFR- β + (C) and α -sma+ (D) pericytes as a percentage of total pericyte area. Data from week 2 for controls, 5x2 Gy radiation / wk (RT), AG-013736 (AG), and the combination (RT+AG).

pericytes. Based on these data, we cannot rule out the possibility that the increased coverage shown in Fig. 4A is also a reflection of these less tightly bound pericytes. This could relate to the fact that the AG-013736 specifically inhibits the PDGFR- β . For the α -sma+ pericytes, combination treatment also leads to a net increase in coverage (Fig. 4B) with an eventual increase in % dissociation at wk 3. In this case, since dissociation was less strikingly increased with treatment, the increase in coverage may more likely reflect a resistance to vessel disruption in the α -sma coated vasculature.

Combination treatment significantly delays tumor progression in late stage PC-3 tumors. The second major experiment was the first designed to evaluate the effect of single and combined treatments on PC-3 tumors. Although treatment were intended to begin at tumor volumes of $\sim 300 \text{ mm}^3$, due to logistical

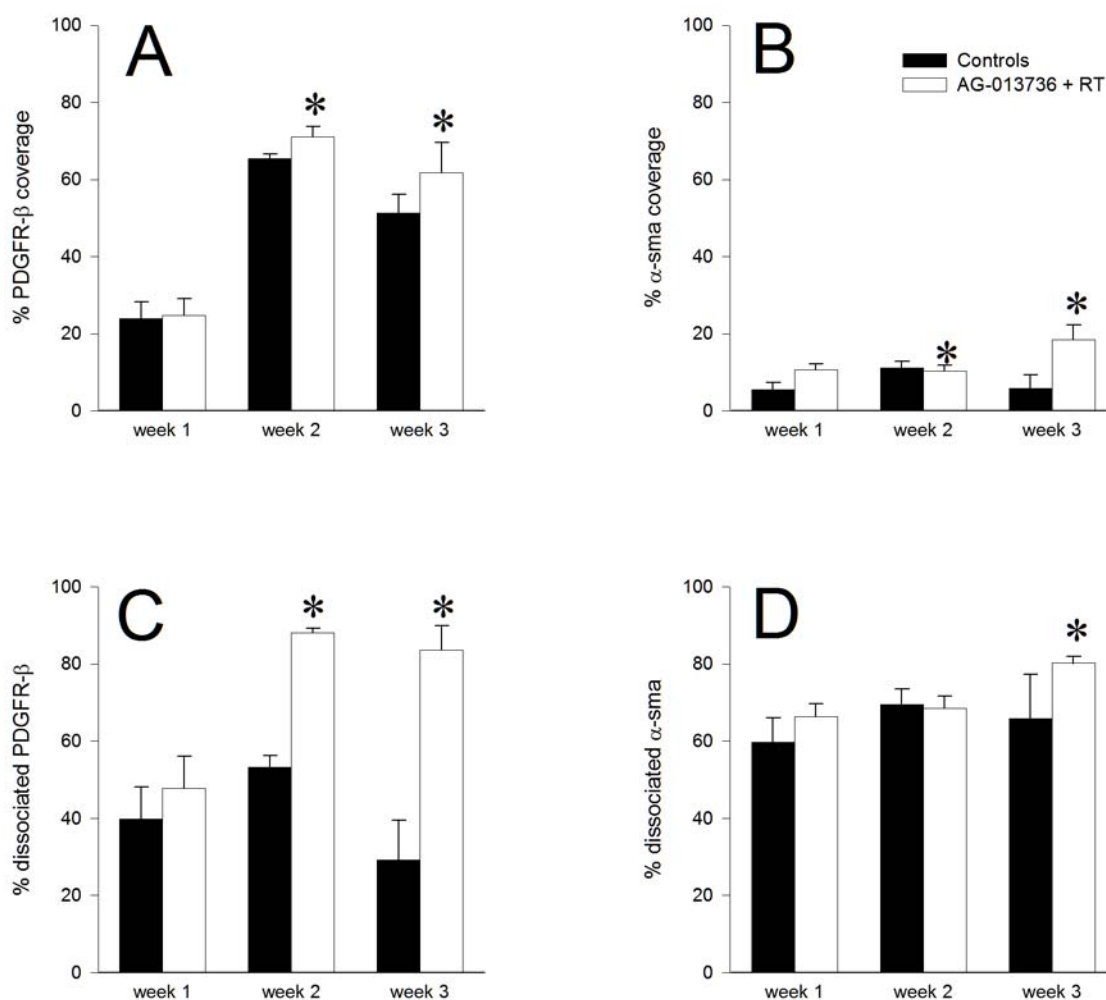


Figure 4 % coverage of PDGFR- β + (A) and α -sma+ (B) pericytes as a percentage of blood vessel area, and % dissociated PDGFR- β + (C) and α -sma+ (D) pericytes as a percentage of total pericyte area. Asterisks denote significant differences between treated tumors and volume-matched controls at week 1.

problems, treatments could not be initiated until the tumors had progressed to volumes of ~ 500 - 600 mm^3 . Based on previous experience with other tumor models at this volume, it was not expected that any of the treatments would have a significant effect on tumor growth inhibition. Surprisingly, both RT and AG-013736 significantly inhibited tumor growth at week 2, and the combination even resulted in a regression in tumor volume (Fig. 5). In light of more accurate information regarding the expected pharmacodynamics of AG-013736 treatment, and in view of the expected potentiation of RT effects by AG-013736, our combination treatment scheduling for the PC-3 tumors was modified somewhat in comparison to the previous DU145 studies. Rather than administering the AG-013736 immediately following each fraction of radiation, the drug was

administered 1 hr pre-irradiation, which may account for the striking response to the combination treatments.

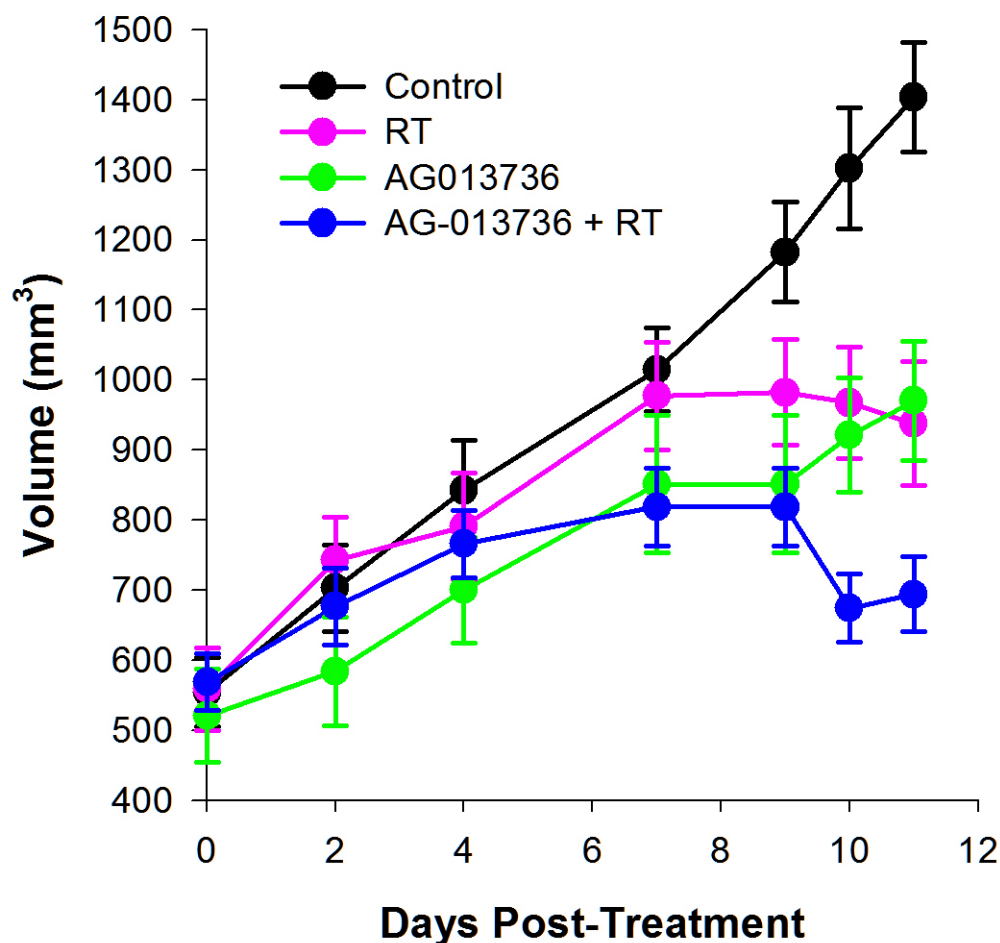


Figure 5 Increase in tumor volume as a function of days following initiation of treatment for vehicle treated controls, 5 x 2 Gy irradiation / wk, AG-013736, or the combination of AG-013736 + fractionated radiation (~8 tumors / group). Radiation was delivered 1 hr post-drug treatment.

A third experiment (110 tumors) is currently in progress that directly addresses this issue by comparing single therapies with two alternative combination schedules: AG-013736 is administered either 1 hr pre- or 1 hr post-RT each day. When treatment is completed for this experiment, 11 groups of tumors will be frozen based on tumor volume-matching, rather than day-matching: a) 250, 500, and 750 mm³ for controls, and b) 250 and 500 mm³ for RT, AG-013736, and each of the different scheduled combinations. We believe this is a more physiologically relevant comparison than the day-matched controls commonly

reported in the literature, and serves to remove the growth-related dependencies from the treatment effects.

Key Research Accomplishments:

- 1) The PC-3 tumor xenograft model was established and the radiosensitivity of this model was quantified in order to define a fractionated RT schedule that produced tumor growth inhibition without regression.
- 2) Immunohistochemistry was optimized for four pericyte markers (PDGFR- β , NG2, desmin, and α -sma) in order to enable the use of automated image processing algorithms rather than relying on manual thresholding of positive staining. Automated ImagePro macros were developed to acquire composite images of pericyte and apoptosis overlap with endothelial cells, process the images, and output the data to Excel. Excel Visual Basic macros were written to reformat the raw data and perform the statistical analyses.
- 3) Approximately 250 tumors (DU145 and PC-3 prostate carcinomas) were treated or are being treated with single and combined therapies: controls, AG-013736, fractionated 2 Gy RT, and alternative scheduling of AG-013736 combined with RT.
- 4) DU145 tumors were frozen at 3 weekly timepoints, cryosectioned, immunostained, and image processed to quantitate total and perfused vessel spacing, hypoxia, endothelial and tumor cell apoptosis, tumor cell proliferation, and pericyte coverage. Results varied substantially with specific pericyte marker, each of which is believed to reflect a different stage of pericyte maturity.
- 5) It has been widely suggested that antiangiogenic agents tend to preferentially target non-pericyte coated blood vessels, leading to a more efficient, or “normalized” tumor vasculature. Results from this year’s studies clearly demonstrate that, in the case of small molecule inhibitors of VEGFRs and PDGFRs at least, this phenomenon does not predominate.
- 6) Despite the fact that combination therapies with RT and AG-013736 reduce vascular densities and increase tumor hypoxia, which would be expected to compromise RT, tumor growth is substantially inhibited.
- 7) PC-3 tumors were frozen following late initiation single and combined therapies following a modified combination schedule. This schedule of AG-013736 1 hr prior to RT resulted in an unanticipated tumor regression, even in these large volume tumors.

Reportable Outcomes:

Abstracts and Presentations:

- 1) 2005 University of Rochester 10th Annual Cancer Center Symposium, Rochester, NY. Pathophysiological effects of VEGF/PDGF receptor kinase inhibitors plus fractionated radiation in DU145 prostate carcinoma xenografts. BM Fenton and SF Paoni.

- 2) 2005 International Society of Magnetic Resonance in Medicine Meeting, Miami, FL. Estimation of vascular functionality in two separate mammary carcinoma tumors during growth and exposure to carbogen using an interleaved DGE/DSE acquisition. PR Connelly, S Kennedy B Fenton, S Paoni, J Zhong.
- 3) 2006 American Association for Cancer Research Meeting, Washington, DC. Combined effects of VEGF/PDGF receptor tyrosine kinase inhibitors plus fractionated radiation on DU145 prostate carcinoma vasculature and oxygenation. BM Fenton and SF Paoni.
- 4) 2006 Tumor Microenvironment Meeting, Boston, MA. Antiangiogenic treatment enhances the tumor inhibitory effects of radiation. BM Fenton.

Funding applied for based on this work:

- 1) NIH NCI R01 – Tumor oxygenation, vascularization, and radiation response
- 2) NIH NCI R01 – Monitoring tumor response using ultrasound and pathophysiological microimaging

Degrees obtained partially supported by this grant:

- 1) Patrick Connelly, PhD in Biomedical Engineering, University of Rochester, 2006

Conclusions:

Combinations of RT with antiangiogenic strategies have almost universally demonstrated enhanced tumor growth delay in comparison to RT alone, despite the belief that antiangiogenics alone generally tend to decrease tumor oxygenation. Since neither unirradiated nor irradiated tumor cells are usually affected by antiangiogenic compounds in vitro, this suggests a radiosensitizing effect that is most likely restricted to the endothelial cells.

This has been further validated with alternate drugs by Schueneman et al.³, who administered RT fractions of 3 Gy at 30 min after each daily dose of SU11248 and found that the combination resulted in reduced tumor blood flow, increased endothelial-specific apoptosis, and increased vascular destruction. Huber et al.⁴ showed that SU11657, a similar multi-targeted small molecule inhibitor of VEGF and PDGF receptor tyrosine kinases, was most effective when a single dose of RT was delivered one day after SU11657 rather than one day before. In response to RT alone, Tsai, et al.² showed that vessel counts were unchanged at 7 days, but significantly lower by 14 days post-irradiation. In addition, vessel apoptosis increased at day one following RT, but recovered by day three. Ki-67+ vessels were unchanged at day 7, but increased significantly by day 14.

The literature has also been conflicting in selecting optimal antibodies for identification of pericytes and in defining their relationship to pericyte maturity, and the interpretation of previous studies is complicated by the inclusion of numerous alternative markers. Perhaps the major obstacle to pericyte identification has been that expression of different markers can vary substantially

among tissue, vessel, and tumor types. Studies by Song et al.⁵ have convincingly demonstrated that PDGFR β ⁺ cells are a progenitor of both NG2 and α -sma positive perivascular cells in pancreatic tumors. In addition, studies of vascular regression in the retina following hyperoxia, and in tumors following VEGF withdrawal, have shown that vessels covered by α -sma positive pericytes are protected^{6,7}. However, retinal vessels covered by NG2 positive pericytes can also undergo regression during normal development⁸. Together these studies suggest that NG2⁺ pericytes could be intermediate in maturity compared to PDGFR β ⁺ and α -sma⁺ cells. Different tumor types have been reported to have variable coverage of α -sma positive pericytes, with some expressing NG2 in the absence of α -sma. A common observation has been that tumor vessel pericytes are generally more loosely associated with endothelial cells than in normal vessels.

Reports as to the effects of drug administration or RT on pericyte coverage have also been varied. AG-013736 administration has been shown to produce a tightening of α -sma-positive pericyte coverage in spontaneous pancreatic tumors, in conjunction with a loss of both pericyte-coated and pericyte-free vessels⁹. Although vascular area decreased by 79%, α -sma-immunoreactive pericyte area was only reduced 33%. In contrast, PDGFR- β -positive pericytes were unchanged following administration or withdrawal of AG-013736. Also, mismatch between lectin perfused vessels and CD31 increased to 57% at 2 days post-AG-013736, but minimal at 7 days. Thus vessels that survived treatment were perfused. Following RT alone, however, pericyte coverage has been shown to remain constant². Alternate studies have shown that tyrosine kinase inhibitors of VEGFRs can be used to target early-stage tumors by disrupting endothelial cells, while inhibitors of PDGFRs can block growth of end-stage tumors by eliciting detachment of pericytes¹⁰. Thus although pericytes can confer resistance to a variety of VEGFR targeting agents, the inclusion of specific PDFGR inhibitors can overcome this resistance, leading to vessel destabilization and regression.

The current studies are the first to combine AG-013736 with fractionated RT and perhaps the only studies to attempt to quantitatively monitor alterations in tumor vasculature and function in combination with changes in pericyte coverage. Although the results for the final pericyte marker (NG2) are still in progress, several conclusions are evident. First, previous studies utilizing VEGF or VEGFR blockers have generally shown a preferential vulnerability of non-pericyte coated blood vessels to treatment, although other studies have suggested instead that pericytes are recruited and tightened. In either case, the net result is an increased "maturation index", or % of pericyte coverage. In conjunction with this increase in vessel maturity, Dr. Jain's group has proposed that such agents tend to actually increase vascular function and tumor oxygenation through a selective ablation of nonfunctional blood vessels that increases blood flow efficiency (vessel "normalization")¹¹. Also, it has been shown that VEGFR blockers have no effect on pericytes¹². In contrast to VEGF or VEGFR blockers, multiple receptor inhibitors such as AG-013736 or combinations of separate inhibitors of VEGFR2 (SU5416) and PDGFR (SU6668) can cause pronounced vascular regression and

pericyte loosening without a substantial change in vessel coverage¹³. This is similar to our results when combining RT and AG-013736. In our studies, PDGFR- β pericytes were not only dissociated, but in addition, vessels were ablated and tumor hypoxia increased. Although this was clearly not a vessel “normalization” phenomenon, the combination treatment resulted in an extreme slowdown in tumor progression. Further studies in Year 3 will incorporate more detailed timepoints for both single and combined treatments and will also include alternate scheduling of the combination treatments, in an attempt to better understand the complex pathophysiological changes. Finally, Year 3 will incorporate molecular assays to our preexisting bank of frozen tumors from the combined modality experiments. Using gene arrays, we will characterize each of the tumor models in terms of initial endogenous angiogenic-antiangiogenic cytokine balance and determine how this balance is altered temporally with single and combined modality therapies.

Since antiangiogenic strategies have only been shown effective when combined with conventional modalities such as RT or chemotherapy, increasing our understanding of the underlying mechanisms is a vital first step to optimizing treatment scheduling. Based on our work to date, we propose that studies seeking to quantify pathophysiological changes following combined modality treatments are most meaningful under two conditions: 1) a panel of pericyte markers is used to define treatment-induced changes in pericyte function, and 2) volume-matched controls rather than day-matched controls (or temporal changes during treatment) are used, which provide a more physiologically relevant comparison when evaluating treatment effects on tumor vascular function.

References:

1. Fenton, B. M., Paoni, S. F., and Ding, I. Effect of VEGF receptor-2 antibody on vascular function and oxygenation in spontaneous and transplanted tumors. *Radiother.Oncol.*, 72: 221-230, 2004.
2. Tsai, J. H., Makonnen, S., Feldman, M., Sehgal, C. M., Maity, A., and Lee, W. M. Ionizing Radiation Inhibits Tumor Neovascularization by Inducing Ineffective Angiogenesis. *Cancer Biol.Ther.*, 4: 1395-1400, 2005.
3. Schueneman, A. J., Himmelfarb, E., Geng, L., Tan, J., Donnelly, E., Mendel, D., McMahon, G., and Hallahan, D. E. SU11248 maintenance therapy prevents tumor regrowth after fractionated irradiation of murine tumor models. *Cancer Res.*, 63: 4009-4016, 2003.
4. Huber, P. E., Bischof, M., Jenne, J., Heiland, S., Peschke, P., Saffrich, R., Grone, H. J., Debus, J., Lipson, K. E., and Abdollahi, A. Trimodal cancer treatment: beneficial effects of combined antiangiogenesis, radiation, and chemotherapy. *Cancer Res.*, 65: 3643-3655, 2005.
5. Song, S., Ewald, A. J., Stallcup, W., Werb, Z., and Bergers, G. PDGFR β + perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nat.Cell Biol.*, 7: 870-879, 2005.

6. Benjamin, L. E., Golijanin, D., Itin, A., Pode, D., and Keshet, E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal [see comments]. *Journal of Clinical Investigation*, **103**: 159-165, 1999.
7. Benjamin, L. E., Hemo, I., and Keshet, E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development*, **125**: 1591-1598, 1998.
8. von Tell, D., Armulik, A., and Betsholtz, C. Pericytes and vascular stability. *Exp. Cell Res.*, **312**: 623-629, 2006.
9. Inai, T., Mancuso, M., Hashizume, H., Baffert, F., Haskell, A., Baluk, P., Hu-Lowe, D. D., Shalinsky, D. R., Thurston, G., Yancopoulos, G. D., and McDonald, D. M. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am. J. Pathol.*, **165**: 35-52, 2004.
10. Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E., and Hanahan, D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest*, **111**: 1287-1295, 2003.
11. Jain, R. K. Tumor angiogenesis and accessibility: Role of vascular endothelial growth factor. *Semin. Oncol.*, **29**: 3-9, 2002.
12. Yokoi, K., Sasaki, T., Bucana, C. D., Fan, D., Baker, C. H., Kitadai, Y., Kuwai, T., Abbruzzese, J. L., and Fidler, I. J. Simultaneous Inhibition of EGFR, VEGFR, and Platelet-Derived Growth Factor Receptor Signaling Combined with Gemcitabine Produces Therapy of Human Pancreatic Carcinoma and Prolongs Survival in an Orthotopic Nude Mouse Model. *Cancer Res.*, **65**: 10371-10380, 2005.
13. Erber, R., Thurnher, A., Katsen, A. D., Groth, G., Kerger, H., Hammes, H. P., Menger, M. D., Ullrich, A., and Vajkoczy, P. Combined inhibition of VEGF- and PDGF-signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *FASEB J*, **18**: 338-340, 2004.

Appendices:

Copies of four abstracts: one presented at the 2005 Cancer Center Symposium in Rochester, NY, one presented at the 2006 AACR Meeting in Washington, D.C., one presented at the 2006 Tumor Microenvironment Meeting in Boston, and one presented at the 2005 International Society of Magnetic Resonance in Medicine Meeting, Miami, FL.

PATHOPHYSIOLOGICAL EFFECTS OF VEGF/PDGF RECEPTOR KINASE
INHIBITORS PLUS FRACTIONATED RADIATION IN DU145 PROSTATE
CARCINOMA XENOGRAFTS

Bruce M Fenton and Scott F Paoni, Department of Radiation Oncology
University of Rochester Medical Center, Rochester, New York 14642

Introduction: Several recent reports have demonstrated that the specific scheduling of antiangiogenic strategies with conventional therapies (chemotherapy or radiotherapy) can be critical to ultimate therapeutic response. Our previous studies have shown that alternative antiangiogenic strategies can produce quite opposite effects on tumor oxygenation and blood flow in murine mammary tumors: monoclonal antibodies against VEGF receptor-2, resulted in substantial tumor hypoxia, while endostatin instead improved tumor oxygenation. The current experiments were designed to determine the pathophysiological effects of both single and combined treatments with VEGF/PDGF receptor kinase inhibitors plus fractionated radiotherapy (RT).

Methods: DU145 human prostate xenograft tumors in mice were treated with either: a) vehicle, b) AG-013736 (a potent receptor tyrosine kinase inhibitor of VEGFRs and PDGFRs provided by Pfizer, La Jolla, CA), c) 5×2 Gy /wk RT fractions, or d) the combination of AG-013736 and RT. Tumor volumes were measured 3 times weekly for 3 wks. Using automated image processing techniques combined with immunohistochemical staining, total and perfused blood vessels were quantified, and tumor hypoxia was estimated by EF5 hypoxia marker uptake.

Results: In comparison to controls, all treatment regimens produced substantial growth delay, although radioresponse was quite variable. Maximal growth inhibition was observed for the combination of AG-013736 plus RT. Although total and perfused vessel counts decreased significantly following both AG-013736 alone and the combination with RT, overall tumor hypoxia was not substantially increased in either case.

Conclusions: Since oxygen delivery was not markedly affected by the decrease in perfused vessel numbers following AG-013736, radiotherapy would not be predicted to be compromised when administered concurrently with this antiangiogenic therapy. Ongoing studies are comparing the effects of alternative scheduling (antiangiogenics given pre-, post-, or concurrently with fractionated radiation) to gauge the importance of the accompanying pathophysiological alterations in potentiating combined treatment response.

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Combined effects of VEGF/PDGF receptor tyrosine kinase inhibitors plus fractionated radiation on DU145 prostate carcinoma vasculature and oxygenation

Bruce M Fenton and Scott F Paoni, Department of Radiation Oncology
University of Rochester Medical Center, Rochester, New York 14642

Introduction: Although antiangiogenic strategies have been shown highly promising in preclinical studies, only combinations of antiangiogenic and cytotoxic therapies have been shown effective in recent Phase III clinical trials. Unfortunately, almost no guidelines exist for the optimal scheduling of such combinations. Furthermore, an ongoing concern has been whether antiangiogenic treatments in themselves may compromise conventional radiation and chemotherapy. The present studies were designed to determine the pathophysiological consequences of both single and combined treatments, using AG-013736, a potent receptor tyrosine kinase inhibitor of VEGFRs and PDGFRs (provided by Pfizer, La Jolla, CA) plus fractionated radiotherapy (RT).

Methods: DU145 human prostate xenograft tumors were implanted in the hind legs of nu/nu mice and beginning at tumor volumes of $\sim 200\text{-}400\text{ mm}^3$ were treated with either: a) vehicle alone, b) AG-013736, c) $5 \times 2\text{ Gy}$ /wk RT fractions, or d) the combination of AG-013736 and RT. Tumor volumes were measured 3 times weekly for 3 wks. Using automated image processing techniques combined with immunohistochemical staining, total and perfused blood vessels were quantified, tumor hypoxia was estimated by EF5 hypoxia marker uptake, and % pericyte coverage was determined with antibodies to $\alpha\text{-sma}$, PDGFR- β , and NG2.

Results: In comparison to controls, all treatment regimens produced substantial growth delay, although radioresponse was variable. Maximal growth inhibition was observed for the combination of AG-013736 plus RT. Although total and perfused vessel counts decreased significantly by the completion of 3 wk therapy for both AG-013736 alone and the combination with RT, overall tumor hypoxia was not substantially increased in either case.

Conclusions: The combination of AG-013736 plus RT produced a striking and prolonged inhibition of DU145 tumor progression compared with either RT or AG-013736 alone. Since oxygen delivery was not markedly affected by the decrease in perfused vessel numbers following AG-013736, radiotherapy would not be predicted to be compromised when administered concurrently with the antiangiogenic therapy.

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ANTIANGIOGENIC TREATMENT ENHANCES THE TUMOR INHIBITORY EFFECTS OF RADIATION

Bruce M Fenton, Department of Radiation Oncology
University of Rochester Medical Center, Rochester, New York 14642

An ongoing concern regarding combined therapies of antiangiogenic and cytotoxic therapies has been whether antiangiogenic treatments in themselves may compromise conventional radiation or chemotherapy. The present studies were designed to determine the pathophysiological consequences of both single and combined treatments, using AG-013736, a potent receptor tyrosine kinase inhibitor of VEGFRs and PDGFRs (kindly provided by Drs. Dana Hu-Lowe and David Shalinsky of Pfizer, La Jolla, CA) plus fractionated radiotherapy (RT).

DU145 human prostate xenograft tumors were implanted in the hind legs of nu/nu mice and beginning at tumor volumes of ~ 200-400 mm³ were treated with: a) vehicle alone, b) AG-013736, c) 5 x 2 Gy /wk RT fractions, or d) the combination of AG-013736 and RT. Tumor volumes were measured 3 times weekly for 3 wks. Using automated image processing techniques combined with immunohistochemical staining, total and perfused blood vessels were quantified, tumor hypoxia was estimated by EF5 hypoxia marker uptake, and % pericyte coverage and apoptosis was determined in relation to functional blood vessels.

In comparison to controls, all treatment regimens produced substantial growth delay, with maximal growth inhibition observed for the combination of AG-013736 plus RT. Total and perfused vessel counts were significantly decreased at 1, 2, and 3 week post-therapy for both single therapies and the combination. Although overall hypoxia in combination treated tumors was not increased in comparison to day-matched controls at week 3, treated tumors were significantly more hypoxic than volume-matched controls. Although PDGFR+ pericyte coverage increased with therapy, % dissociated PDGFR+ also increased. These combined findings could be explained by either selective vessel ablation or pericyte recruitment, but clearly indicate that a “normalization” of vessels into a more efficiently functioning network is not occurring in response to these specific combined treatments. Furthermore, day-matched controls are clearly not the most relevant comparison when evaluating treatment-induced changes in pathophysiological parameters in the absence of tumor growth.

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Estimation of vascular functionality in two separate mammary carcinoma tumors during growth and exposure to carbogen using an interleaved DGE/DSE acquisition

P. R. Connelly¹, S. Kennedy², B. Fenton³, S. Paoni³, J. Zhong⁴

¹Biomedical Engineering, ²Biochemistry and Biophysics, ³Radiation Oncology,

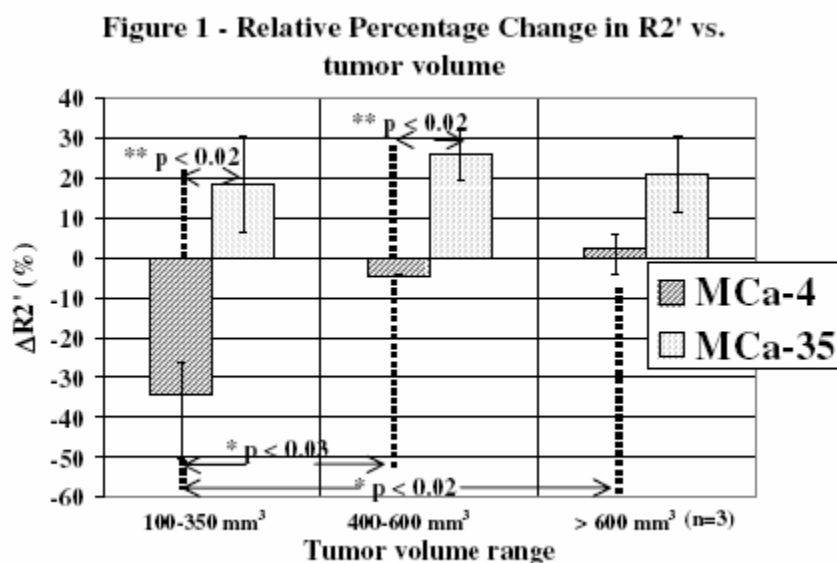
⁴Radiology and Biomedical Engineering, University of Rochester, Rochester, NY

Introduction: Analysis of tumor vascular function and maturation has been proven to be difficult using a variety of MRI techniques. We present the use of an interleaved Double Gradient echo (DGE) and Double spin echo (DSE) acquisition for the subsequent estimation of $R2^*$, $R2$, and $R2'$ as a basis for this analysis. $R2^*$ is the total transverse relaxation rate, $R2$ the irreversible relaxation rate and $R2'$ the rate due only to susceptibility where $R2' = R2^* - R2$. This analysis was performed on data collected from mammary carcinoma tumors of different volumes in mice exposed to the gas carbogen (95%O₂/5%CO₂). There have been similar investigations performed using blood oxygen level dependent (BOLD) contrast to analyze different treatment paradigms and growth [1,2].

Methods: MCa-4 and MCa-35 murine mammary carcinoma cells were injected into the hind limbs of C3H mice (8 mice total). Mice were exposed to carbogen (2L/min) through a mask with scavenger. Individual experiments were performed over a 10-day period of tumor growth. Control acquisitions were performed with room air at 2L/min. DGE/DSE imaging was performed on a 9.4 T scanner (GE Omega) using a 1.5 cm surface coil. The imaging parameters for DGE images were TE1/TE2/TR = 3.3ms/12ms/200ms, flip angle = 15°, NEX 4, and for DSE images TE1/TE2/TR = 6.5ms/18ms/1s, NEX 2. For both imaging protocols, matrix size 128x64, slice thickness = 0.75 mm, 8 slices, and FOV = 25mm. Region of interest (ROIs) were selected from a DW image ($b = 660 \text{ sec/mm}^2$) of the same slice orientation and thickness as with the DGE/DSE. A single diffusion gradient was applied parallel to the muscle fiber direction in the leg. Image analysis was performed using MATLAB (Mathworks, Natick, Ma.). Data sets with substantial motion artifacts were removed. Statistical comparisons were performed using Student's t-test.

Results: To verify consistency of signal acquisition, a series of 4 DGE/DSE images were taken of each tumor for air- baseline, carbogen, and return to air. The acquisition time for each set of 4 image pairs was 12 minutes (i.e., 4 baseline-4 carbogen-4 return). Mice were given 2 minutes between gas changes to reach equilibrium. Percent changes in $R2^*$, $R2$ and $R2'$, following carbogen exposure were calculated for each slice for individual tumors. The bar graph in Figure 1 shows the relative changes in $R2'$ when gas is switched from air to carbogen for three different ranges of tumor volumes for MCa-4 and MCa-35 tumors. A total of 3 separate tumors were analyzed for each volume group. Consistent decreases were observed in $R2'$ following carbogen exposure in the small (100-350mm³) and medium (400-600 mm³) MCa-4 tumor volume groups. Also, significant differences were noted in the relative change of $R2'$ during carbogen exposure between the MCa-4 small tumor group and both the medium group and the large group ($> 600 \text{ mm}^3$, $p < 0.03$) The opposite effect was seen in MCa-35 tumors where $R2'$ increased in all three size groups. This opposing difference is significantly different in the small and medium tumor groups ($p < 0.02$).

Discussion: Alterations in $R2'$ should correspond to changes in blood oxygen levels assuming that no changes in tumor blood volume. The low flip angle of 15° reduces any potential effect due to in-flow. We present the changes in relaxation rates in two different mammary carcinoma cell lines of varying vasculature in mice exposed to carbogen. As expected, these results show that there is a relative decrease in $R2'$ following carbogen exposure in MCa-4 tumors. Unexpectedly the results show a relative increase in $R2'$ in MCa-35 tumors following carbogen exposure. The vasculature in the MCa-35 tumors has a different structure than that seen in MCa-4 tumors, where the MCa-4 vessels are much larger and contain a greater number of sinusoids. It is hypothesized that the increase in $R2'$ is due to the vasodilatory effect of the CO_2 in carbogen resulting in an increased tumor blood volume of MCa-35 tumors. This increase in blood volume results in an increased relative deoxyhemoglobin concentration within the tumor. Additionally, the difference between the $R2'$ of the two groups was shown to be insignificant when tumor volume is large, suggesting that as tumors pass a specific growth stage, the relative amount of functional vasculature is reduced. These results will be further explored by performing steady state contrast enhanced imaging in these two tumor types during carbogen exposure. The DGE/DSE acquisition during a vasomodulatory stimulus may present a non-invasive, non contrast-enhanced method to evaluate the functionality of the microcirculation in tumors.



References

- [1] Abramovitch et al., 1999. *Cancer Res*, **59**:5012-5016
- [2] Thomas et al., 2003. *MRM*, **50**, 522-530