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14. ABSTRACT Vascular targeting agents (VTA) are able to disrupt tumor vasculature, leading to extensive tumor necrosis. Interesting findings have shown that VTA kills cells predominantly in the more hypoxic tumor center, while the better perfused peripheral rim is less affected. This apparently limits the effectiveness of such agents and rapid regrowth of tumor residues occurs. However, these findings suggest a potential of a combination of VTA with treatments specifically targeting the viable tumor rim. Radiation can certainly be expected to be most effective against the well-perfused and oxygenated cell populations at the peripheries of the tumors. One major goal of this project is to fully understand and precisely assess the dynamic changes in blood perfusion and oxygenation after VTA, so that we may predict response and optimize the therapy. I propose to use in vivo MRI to measure and assess physiological changes, e.g. tumor blood perfusion and dynamic tissue oxygenation, in the tumors before and after treatment. I believe non-invasive MRI approaches may provide a valuable prognostic tool to predict the response of specific breast tumors to VTA.					
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Introduction:

Targeting tumor vasculature promises new effective therapy for prostate cancer (1, 2). It avoids issues of drug delivery and is potentiated by massive downstream effects where one blood vessel may supply the nutrients for thousands of tumor cells. Thus, disrupting the vascular supply should generate magnified tumor cell kill. Thus, inhibiting the growth of new blood vessels, i.e., antiangiogenesis, should prevent growth and metastasis of the primary tumor (1, 2). In addition to the focus on the antiangiogenic approaches, vascular targeting, directly attacking the existing neovasculature, is an alternative strategy against the tumor blood vessel network. Tubulin binding agents, *e.g.*, combretastatin A-4-phosphate (CA4P) represent one kind of vascular targeting agent (VTA) (3, 4). Promising preclinical studies have shown that such agents selectively cause tumor vascular shutdown and subsequently trigger a cascade of tumor cell death in experimental tumors (4, 5). However, survived tissues in a thin viable rim of tumor usually re-grow in spite of induction of massive necrosis. Thus, a combination of VTAs with additional conventional therapeutic approaches will be required (6, 7). To better understand the mode of action, and hence, optimize such combinations, we plan to apply *in vivo* MR imaging approaches to monitoring physiological changes in response to VTA administration. Dynamic contrast enhanced (DCE) MRI based on the transport properties of gadolinium-DTPA (Gd-DTPA) is the most commonly used imaging approach to study tumor vascular perfusion and permeability (8, 9). For combination with radiotherapy, measurement of tumor oxygen dynamics will be especially important since hypoxia affects radiation response. By applying ^{19}F FREDOM (Fluorocarbon Relaxometry using Echo planar imaging for Dynamic Oxygen Mapping) MRI (10), dynamic tumor oxygenation can be monitored following the treatment with CA4P. Based on pathophysiological changes monitored by MRI, optimum scheme of the combined radiation and CA4P will be designed and experimental treatment will be performed on the syngeneic rat breast tumors.

Successful completion of this project will confirm the potential of this new therapeutic approach to breast cancer. It will lay the foundation for future clinical application for treating breast cancer patients.

Body:

The Statement of Work in this project had two major tasks:

Task 1. To assess vascular and oxygen dynamics in response to VTA, Months 1-18.

- a. *In vivo* MRI assessment of vascular and oxygen dynamics in response to VTA, Months 1-18
- b. To study morphological and biological changes of tumor vasculature and hypoxia at cellular and molecular levels in response to VTA, Months 1-18.

Task 1 was completed during the Years 1 and 2.

Task 2. Experimental tumor therapy, Month 19-36

Task 2 is in process.

Due to replacement of the irradiation instrument at our institute, the proposed experimental radiotherapy was not able to be completed on time. We requested and were granted no additional cost extension to end May 31, 2008. Fortunately, the new machine has been recently installed and this project has resumed. I believe remaining tasks will be completed successfully.

Progress in Task 2:

- a. Learn and become proficient in operating state of the art irradiation system (AccuRay).
Completed.
- b. Design therapeutic protocol based on the MR and histological findings: compare the order and timing of the combined therapy (CA4-P 100mg/kg, i.p., irradiation 30 Gy single dose).
Completed.

Doses for CA4P and Radiation:

CA4P dose: Based on the MRI studies, a dose of 30 mg/kg induced significant reduction in tumor vascular perfusion/permeability and tissue pO_2 (11). Therefore, we decided to use this dose instead of proposed 100 mg/kg to investigate experimental treatment.

Radiation dose: While a 30 Gy single dose was proposed originally, we found this dose was well over the TCD_{50} for the proposed 13762NF breast tumor. A 10 Gy single dose was then investigated. Results showed that this dose also significantly inhibited tumor growth. To study potential effects by adding CA4P, we decided to further lower the radiation dose to 5 Gy.

Order and timing of the combination: Our MRI results from Years 1 and 2 have shown that tumor blood perfusion/permeability decreased significantly to ~30% of baseline pretreatment level at 2 h after CA4P (30 mg/kg, i.p.) infusion, which recovered fully after 24 h in a thin peripheral region, but not the tumor center. More importantly, dynamic tumor regional pO_2 , which is well recognized to correlate closely with radiation outcome, was evaluated by ^{19}F MRI. Tumor pO_2 was found to decline within 60 min, become significantly lower at 90 min, and decrease further at 2 h after CA4P infusion. Some regional recovery was seen 24 h later but the pO_2 was still significantly lower than the pretreatment level. However, oxygen breathing at this point modified tumor pO_2 significantly, which resulted in essential elimination of tumor hypoxia. All the MRI findings have been confirmed by histological and immunohistological studies. These results have recently been published (11).

c and d. Evaluate tumor growth delay after the vascular targeting treatment and/or irradiation.

In process

Based on these observations, we proposed to administer CA4P (30 mg/kg) on Day 1, and 5 Gy radiation on Day 2, while having animals breathe 100% O_2 from 20 min before to the end of radiation. Previous studies by others have demonstrated that administration of VTA 1 h post radiation produced better improvements in tumor response than other combination schemes. Here, we plan to test and compare our combination approach with other possible combinations on the 13762NF tumors. The preliminary treatment data has been presented at an international conference (Fig. 1) (12).

Animals bearing pedicle 13762NF tumors were grouped as:

1. Control without treatment (n = 6);
2. CA4P (30 mg/kg, i.p. single dose) alone (n = 6)
3. Radiation alone (5 Gy single dose, n = 6)
4. Radiation (5 Gy) + O_2 (n = 5), The animals started to breathe oxygen (100% O_2 + 1% isofluorane) 20 min before receiving a 5 Gy radiation delivered by Accuray system.
5. Radiation (5 Gy) + CA4P (1 h post Rx, 30 mg/kg, i.p. n = 6)

6. Radiation with O₂ (5 Gy) + CA4P (1 h post Rx, 30 mg/kg; n = 5); The animals started to breathe oxygen (100% O₂ + 1% isoflurane) 20 min before radiation
7. CA4P (30 mg/kg) + radiation (24 h later, 5 Gy; n = 5)
8. CA4P (30 mg/kg) + radiation with O₂ (24 h later, 5 Gy; n = 6). The animals started to breathe oxygen (100% O₂ + 1% isoflurane) 20 min before receiving a 5 Gy radiation delivered by Accuray system.

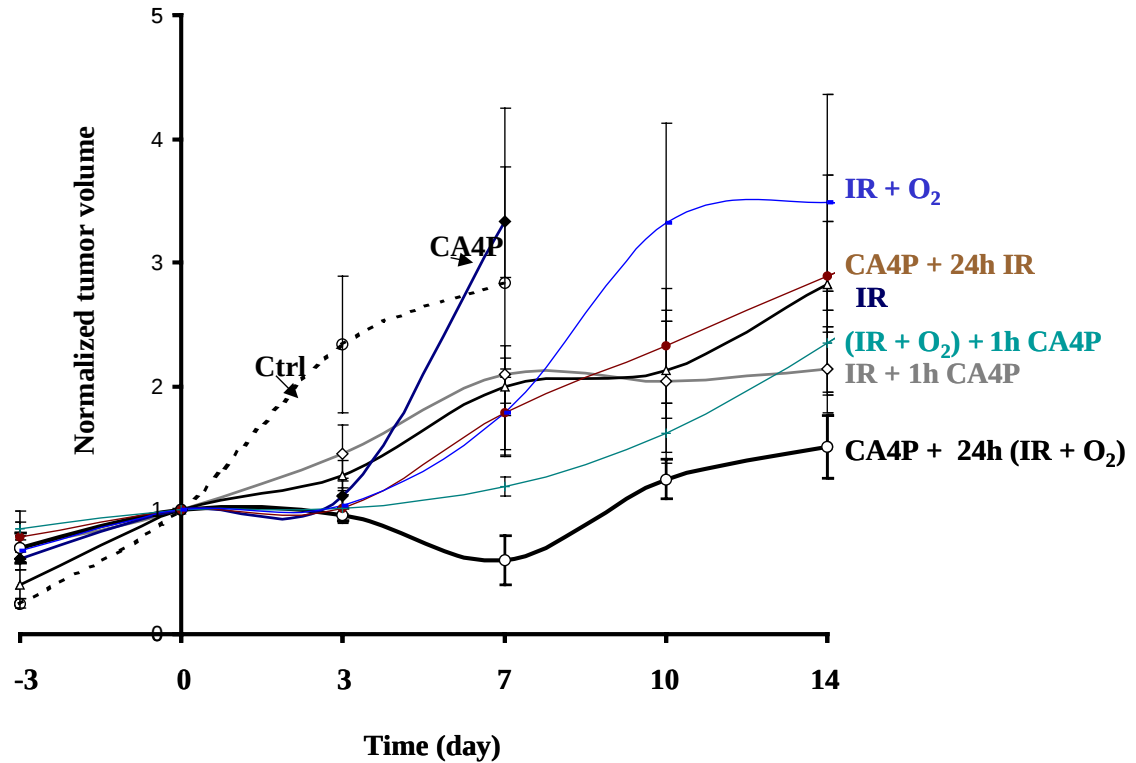


Figure 1. Growth delay versus time curve for the 13762NF tumors. Significant growth inhibition was observed in the CA4P + 24h (IR + O₂) treated group.

Results and Discussion:

Tumor volume change (normalized mean $\pm$ s.e.) versus time curve was plotted, as shown in Fig. 1. Most tumors in radiation alone or the combination groups became severely ulcerated, which led to termination of this study on Day 17. The results showed that a single dose (5 Gy; **Group 3**) radiation alone inhibited tumor growth significantly ($p < 0.05$). The relatively higher radiosensitivity of this tumor line is in a good agreement with the lower hypoxia fractions observed by MRI. While significant growth delay was achieved during the first 3 days after CA4P (30 mg/kg, i.p.; **Group 2**), tumors started to re-grow and caught up with the Control group rapidly on Day 7. This observation is in common with other studies (6, 7). Oxygen breathing didn't improve radiosensitivity in tumors of **Group 4**. This result again supported our MRI observation that this tumor line is relatively better oxygenated, which typically has a hypoxia fractions (< 5 torr) less than 20% (11). Similar observations has been reported in our previous studies in the Dunning prostate HI tumors that have a hypoxia range similar to the breast 13762NF tumors in this study (13). The approach with CA4P 1 h post radiation (IR + 1h CA4P; **Group 5**) showed no beneficial effects over the IR alone by Day 10. However, tumors in this group seemed to stop growing after

Day 10 while the IR alone tumors started to grow rapidly on Day 10. Unfortunately, longer term of growth delay in these tumors could not be achieved because of tumor ulceration. Again, tumors in **Group 6** didn't benefit from addition of oxygen breathing. Our MRI data showed that significantly increased hypoxic fractions induced by CA4P was still observed 24 h later (11). Thus, delivery of radiation at this time point will not induced significant tumor growth delay. This has been proved in the tumors of **Group 7** when compared to tumors in **Group 5** or **6**. However, oxygen breathing 24 h after CA4P administration was found to essentially eliminate tumor hypoxia in the survived peripheral rim (11), which will improve radiosensitivity. Indeed, the tumors in **Group 8** showed significantly slower growth rate from Day 3 than any other group ($p < 0.05$).

Key Research Accomplishments

- **Experimental therapy**

Based on *in vivo* study of tumor physiological dynamics evaluated by MRI, we designed a treatment scheme to administer CA4P 24 h before a single dose radiation plus oxygen inhalation. The results showed significantly slower tumor growth in this treatment group than those in other groups.

Reportable Outcomes

Years 1-3: Two peer-reviewed papers and six published conference proceedings.

Year 4:

Abstracts (Published Conference Proceedings): Oral presentation

Zhao, D., Jiang, L., and Mason, R.P. MRI evaluation of tumor physiological response to combretastatin A4 phosphate: correlation with a combined radiation response. *Joint ISMRM-ESMRMB*, Berlin, Germany, May 2007.

Manuscripts in preparation:

1. **Zhao, D.**, Jiang, L., Hahn, E.W., and Mason, R.P. Evaluation of breast tumor microcirculation and oxygenation using a combination of BOLD, DCE and ^{19}F MRI. *Magn. Reson. Med.*
2. **Zhao, D.**, Harper, A., Richer, E., Liu, Li., Slavine, N., Shay, J. W., Antich, P. P., Mason, R. P. Novel application of bioluminescent imaging: Interrogating acute effects of the vascular targeting agent combretastatin. *Cancer Res.*
3. **Zhao, D.**, Chang, K., Jiang, L., Mason, R.P. *In vivo* monitoring of tumor physiological response to combretastatin A4 phosphate: correlation with a combined radiation response. *Radiat. Res.*

Conclusion:

Based on the data of *in vivo* tumor perfusion and oxygenation dynamics in response to the vascular targeting agent, combretastatin A-4-phosphate (CA4P) evaluated by MRI, we

successfully designed a scheme to combine the radiation treatment and CA4P to treat breast tumors. This is the major goal of the proposed project. Moreover, the pathophysiological information will be especially useful for designing a complicated scheme, which usually involves combination of fractionated radiation and multiple dose of systemic chemotherapy at clinical settings. I am confident that the proposed project will be fulfilled by the next term.

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Appendices

Publication enclosed:

Zhao, D., Jiang, L., and Mason, R.P. MRI evaluation of tumor physiological response to combretastatin A4 phosphate: correlation with a combined radiation response. Joint ISMRM-ESMRMB, Berlin, Germany, May 2007.

MRI evaluation of tumor physiological response to combretastatin A4 phosphate: correlation with a combined radiation response

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Introduction: The vascular targeting agent, combretastatin A-4-phosphate (CA4P) causes tumor vascular shutdown inducing massive cell death. Although massive necrosis can be induced, tumors usually regrow from a thin viable rim. Thus, a combination of VTAs with additional conventional therapeutic approaches, *e.g.*, radiation, will be required (1). For combination with radiotherapy, measurement of tumor oxygen dynamics will be especially important, since reduced perfusion can induce hypoxia, potentially modulating radiation response. Thus, we have assessed dynamic changes in tumor oxygenation as compared with vascular perfusion/permeability after CA4P treatment by combining ¹H and ¹⁹F MRI (2). Based on pathophysiological changes monitored by MRI, optimum scheme of the combined radiation and CA4P treatment was designed and experimental treatment was initiated on rat breast tumors.

Materials and Methods: Rat mammary carcinoma 13762NF was implanted syngeneically in a skin pedicle surgically created on the foreback of Fisher 344 adult female rats and allowed to grow to ~ 1 cm diameter.

MRI study: MRI studies were performed using a 4.7 T Varian Inova imaging system. Each rat was maintained under general anesthesia (air and 1% isoflurane). A tunable (¹H/¹⁹F) volume RF coil was placed around the tumor-bearing pedicle. ¹H MRI R₂^{*} maps were obtained before and 2 h after CA4P (30 mg/kg, i.p., OXIGENE, Inc.) by gradient echo sequence (GEMS) with 8 echoes (TR = 195 ms, TE = 7 ms and spacing = 6 ms). Dynamic contrast enhanced (DCE) MRI using a T₁-weighted spin echo sequence (TR = 70 ms, TE = 12 ms) based on i.v. bolus injection of Gd-DTPA-BMA through a tail vein catheter was also acquired before, 2 h and 24 h after CA4P. For ¹⁹F NMR oximetry, hexafluorobenzene (50 µl) was injected directly into the tumor along two or three tracks in a single central plane of the tumor using a fine sharp needle (32G), as described in detail previously (25). **FREDOM** (Fluorocarbon Relaxometry using Echo planar imaging for Dynamic Oxygen Mapping) MRI was performed to acquire a series of pO₂ maps under air or oxygen breathing before and at different time points after CA4P. Data analysis was carried out on a voxel-by-voxel basis with IDL based house made software.

Experimental treatment: Animals bearing 13762NF tumors (n = 30) were grouped as: 1) control without treatment; 2) CA4P (30 mg/kg, i.p.) alone; 3) Radiation (IR) alone (5 Gy single dose); 4) IR (5 Gy) + CA4P (1 h post IR, 30 mg/kg, i.p.); 5) CA4P (30 mg/kg) + IR plus O₂ (24 h post CA4P, 5 Gy). The animals started to breathe oxygen (100% O₂ + 1% isoflurane) 20 min before receiving a 5 Gy IR delivered by Accuray system.

Immunohistochemistry: Immunostaining for Hoechst 33342 (perfusion marker) and CD31 (vascular endothelium) was performed to correlate with imaging findings.

Results: ¹H MRI showed that tumor blood perfusion/permeability by DCE MRI decreased significantly to ~30% of baseline pretreatment level at 2 h after CA4P (30 mg/kg, i.p.) infusion, which recovered fully after 24 h in a thin peripheral region, but not the tumor center. Analysis of R₂^{*} maps revealed significantly increased values after 2 h, compared to pretreatment (88.6 v.s. 85.1 s⁻¹, p < 0.05). Tumor pO₂ by ¹⁹F MRI was found to decline within 60 min, become significantly lower at 90 min, and decrease further at 2 h after CA4P infusion. At this time there was no response to breathing O₂. Some regional recovery was seen 24 h later, but the pO₂ was still significantly lower than the pretreatment level. However, oxygen breathing at 24 h point modulated tumor pO₂ significantly, which resulted in essential elimination of tumor hypoxia (Fig. 1). Correlating well with MRI observations, the tumors of Group 5 with radiation plus oxygen 24 h post CA4P showed a significantly prolonged growth delay (*p < 0.05, Fig. 2). Histological data using the perfusion marker, Hoechst 33342, confirmed a significant decrease in perfused vessels 2 h after CA4P, while there was recovery evident at 24 h.

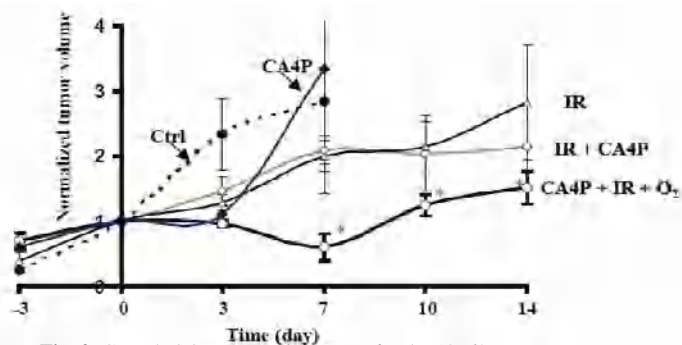
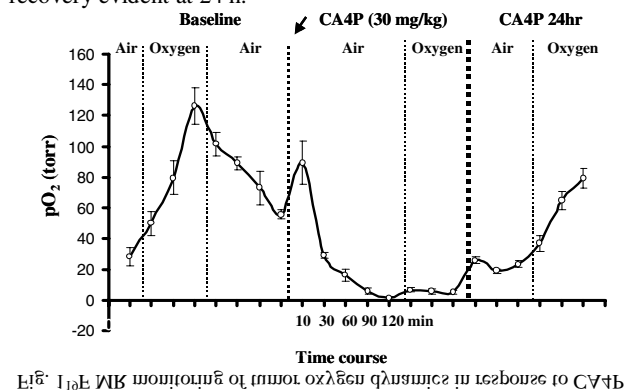


Fig. 2. Growth delay versus time curve for the 13762NF tumors.

Discussion: There is a distinct similarity between the results of the pO₂ measurements and the more traditional DCE, but the quantitative pO₂ values provide the potential for exploiting synergy with other oxygen dependent therapies. The observations further demonstrate the value of **FREDOM** in assessing dynamic changes in regional tumor pO₂ *in vivo* in response to intervention. We believe that dynamic measurements are particularly valuable for understanding the mode of action of therapeutic response to VTAs. Most significantly, these measurements lay a foundation to optimize the timing of combination therapy involving fractionated radiotherapy and multiple doses of VTAs.

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1. Stevenson, J. P., Rosen, M., Sun, W., Gallagher, M., et al. *J Clin Oncol*, 21: 4428-4438, 2003.
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