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Award Number: DAMD17-03-1-0363

TITLE: Therapeutic Vascular Targeting and Irradiation: Correlation of MRI Tissue Changes at Cellular and Molecular Levels to Optimizing Outcome

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REPORT DATE: June 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-06-2006	Annual	1 Jun 2005 - 31 May 2006

4. TITLE AND SUBTITLE
Therapeutic Vascular Targeting and Irradiation: Correlation of MRI Tissue Changes at Cellular and Molecular Levels to Optimizing Outcome

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0363

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas Southwestern Medical Center
Dallas, TX 75390-9058

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
Vascular targeting agents (VTA) are new types of anticancer drugs that act on existing tumor vasculature, causing vascular disruption, which ultimately leads to extensive ischemic tumor cell death. Interesting findings showed that VTA killed cells predominantly in the more hypoxic tumor center, as a consequence of hemorrhagic necrosis after vascular collapse, whereas the better perfused peripheral rim was less affected. This apparently limits the effectiveness of such agents and rapid regrowth of tumor residues occurs. However, these findings suggest the possibility and promise 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.

15. SUBJECT TERMS
Vascular targeting, Magnetic resonance imaging (MRI), pO2, Perfusion, irradiation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	13	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction:

Tumor growth, survival and metastasis depend critically on the development of new blood vessels (1). 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 tumors in a thin viable rim usually regrow 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.

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 ongoing.

While we have not completed all tasks within the original three year time table, we requested and were granted a 1 year no additional cost extension. 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.**

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 (Fig. 1). To study potential effects by adding CA4P, we decided to further lower the radiation dose to 5 Gy.

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.

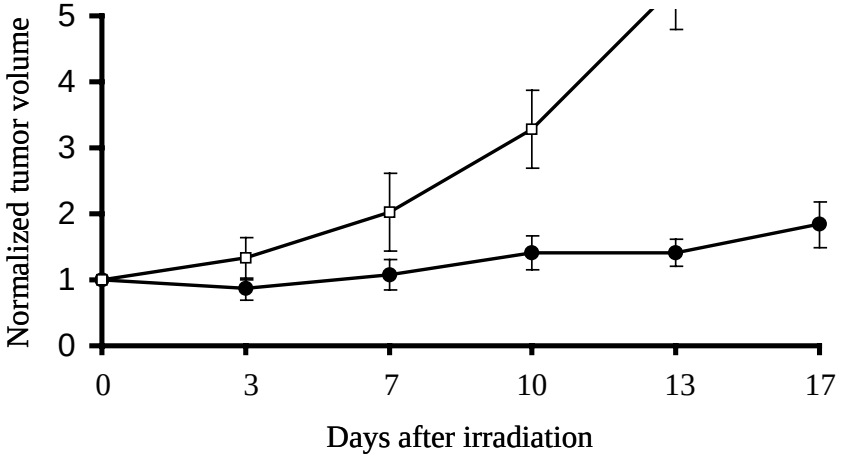

Figure 1. Tumor growth was significantly delayed by a single dose of 10 Gy radiation (●) compared to control tumors (□).

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 (11). 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 (Fig. 2) (11). Based on these observations, we decided 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. All the MRI findings have been confirmed by histological and immunohistological studies (11). 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 our combination approach on the 13762NF tumors.

c and d. Evaluate tumor growth delay after the vascular targeting treatmnet and/or irradiation. **Ongoing**

Animals bearing subcutaneous 13762NF tumors were grouped as: 1) control without treatment (n = 6); 2) CA4P (30 mg/kg, i.p.) alone (n = 6); 3) Radiation alone (5 Gy single dose, n = 6); 4) Radiation (5 Gy) + CA4P (1 h post Rx, 30 mg/kg, i.p.); 5) CA4P (30 mg/kg) + radiation with O_2 (next day, 5 Gy). The animals started to breathe oxygen (100% O_2 + 1% isofluorane) 20 min before receiving a 5 Gy radiation delivered by Accuray system.

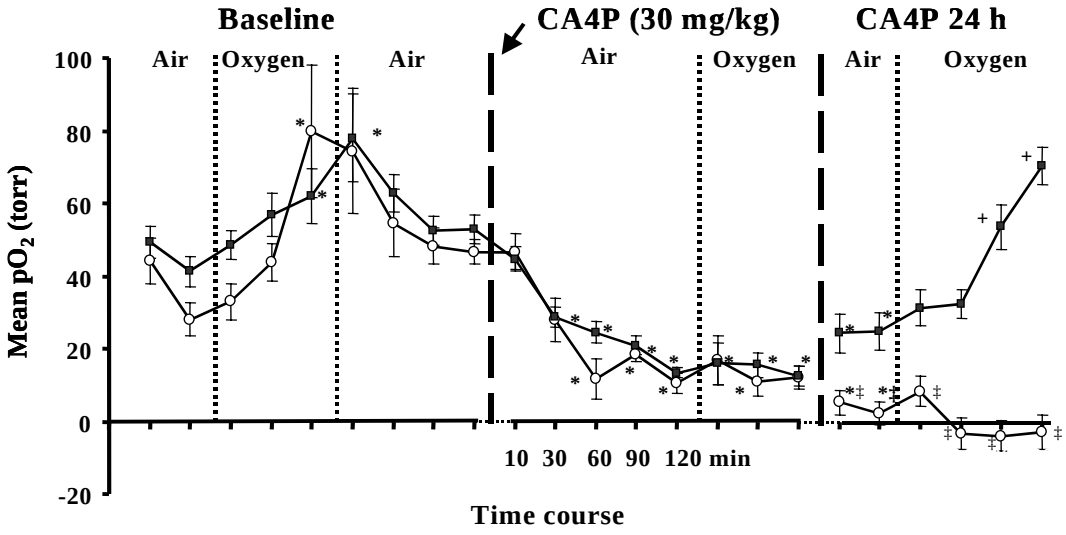

Figure 2 ^{19}F MRI evaluation of tumor pO_2 dynamics in response to CA4P. Mean pO_2 curves are shown for the peripheral (■) and central (○) voxels of the tumor shown in Fig. 2. Significant decrease in pO_2 was found as early as 30 min after CA4P (30 mg/kg) for both peripheral and central tumor. * $p < 0.05$ from baseline air, $^{+}$ $p < 0.05$ from 24 h air, ‡ $p < 0.05$ from periphery.

Tumor volume change (normalized mean ± s.e.) versus time curve was plotted, as shown in Fig. 3. The results showed that a single dose (5 Gy) radiation alone inhibited tumor growth significantly ($p < 0.05$). While significant growth delay was achieved during the first 3 days after CA4P (30 mg/kg, i.p.), tumors started to regrow from Day 3 and caught up with the Control group rapidly on Day 7. The approach with CA4P 1 h post radiation (IR + CA4P) 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. Our approach (CA4P + IP + O_2), which is designed based on the results of tumor pO_2 dynamics monitored by MRI, showed significantly slower tumor growth rate from Day 3 than other groups ($p < 0.05$).

In addition to the 13762NF subline, we proposed to perform studies on two other sublines of 13762: PAM-CTX and MTLn2. However, we could not obtain these two sublines. Instead, we decided to use human breast MDA-MB-231 tumor. The MDA-MB-231 cells were transfected stably with firefly luciferase. Extensive MRI and bioluminescent imaging (BLI) studies were performed to monitor physiological change in response to CA4P. In good agreement with the 13762NF line, DCE MRI revealed significant reduction in tumor vascular perfusion/permeability (Fig. 4). Comparable results were obtained by cheaper and high throughput BLI approach (Fig. 5).

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Figure 3. Growth delay versus time curve for the 13762NF tumors. Significant growth inhibition was observed in the CA4P + IR + O_2 treated group, compared to other treatment approaches (* $p < 0.05$).

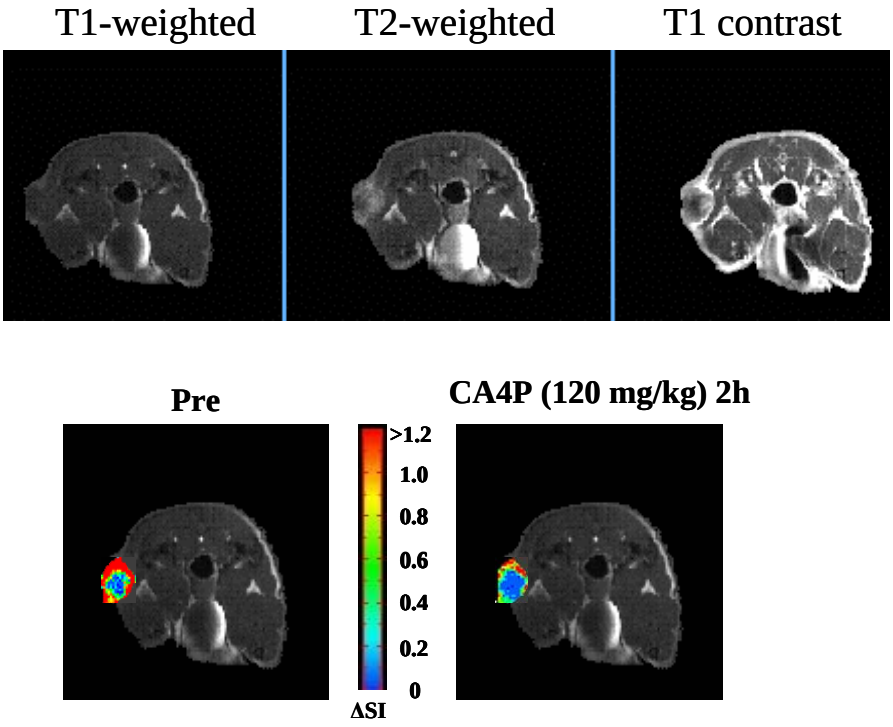

Figure 4. DCE MRI monitoring of tumor response to CA4P. **Top row**: Conventional T1- and T2-weighted, and T1- weighted contrast enhanced MR images of a nude mouse with MDA-MB-231-luc mammary tumor. **Bottom row**: Dynamic contrast enhance MRI was performed in the mouse

before (left) and 2 h after (right) CA4P (120 mg/kg) i.p. injection. A normalized contrast enhanced image at 30 s after a bolus injection of Gd-DTPA-BMA acquired before and 2 h after treatment, respectively is superimposed on the T1-weighted image. Significantly decreased signal enhancement, compared to pretreatment, was observed 2 h after i.p. injection of CA4P.

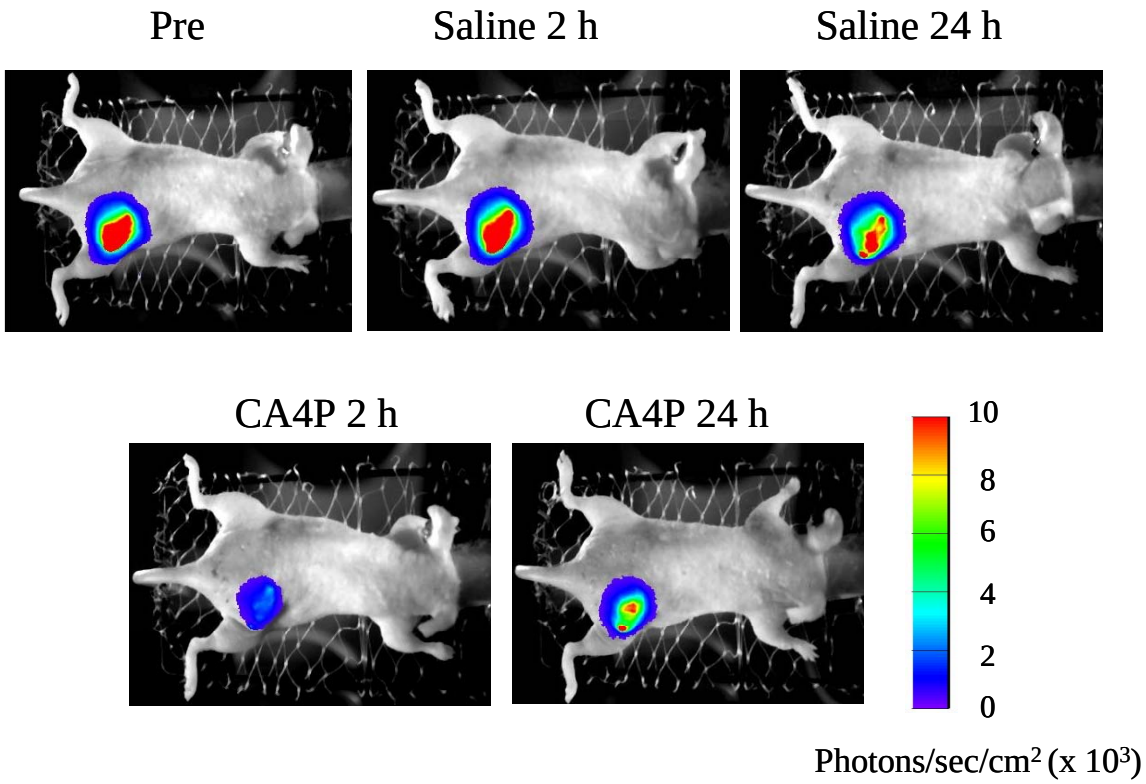

Figure 5. BLI monitoring of tumor response to CA4P. A representative MDA-MB231-luc tumor was monitored sequentially following saline (0.15 ml) or CA4P (120 mg/kg) i.p. infusion. Each image was acquired 4 min after s.c. luciferin injection. In contrast to saline treatment, CA4P caused a significant decrease in BLI signal intensity 2 h after treatment, which remained lower 24 h later.

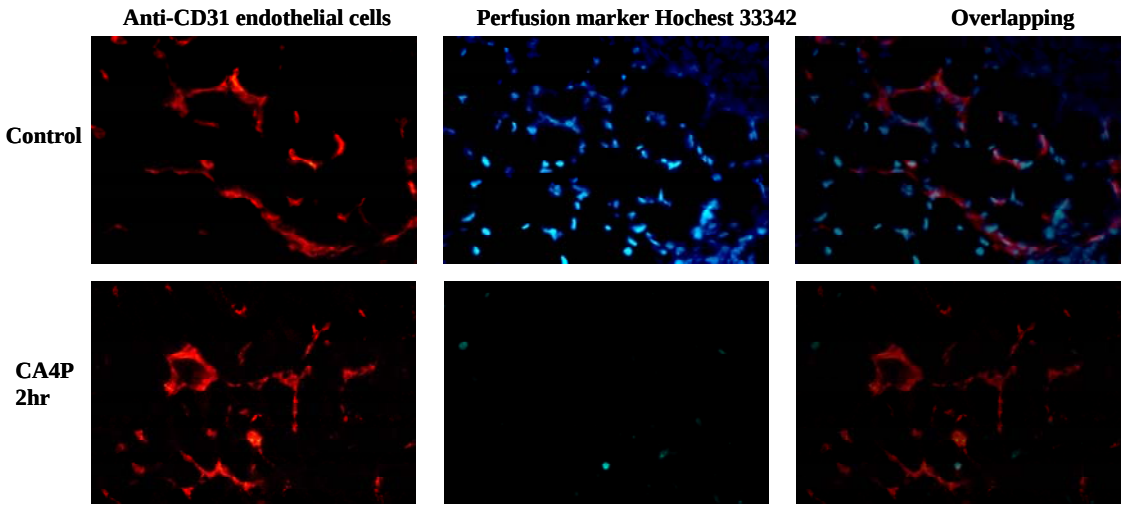

Figure 6 Immunohistochemical study of MDA-MB-231 tumor vascular response to CA4P. Perfusion marker Hoechst staining pre and 2 h post CA4P (120mg/kg). Vascular endothelium of the same field was immunostained by anti-CD31 (red). A good match between Hoechst and anti-CD31 stained vascular endothelium was found in the pretreated tumor. Two hours after treatment, significant reduction in perfused vessels was detected, in line with the significant reduction in BLI signals.

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.

- **Assessment of tumor perfusion dynamics in the human breast tumors in response to the vascular targeting agent, Combretastatin A4 phosphate, by *in vivo* MR approaches**

Similar to our previous data of rat 13762NF tumor, DCE MRI showed significant reduction in perfusion and permeability of human MDA-MB-231 breast tumors 2 h after administration of CA4P.

- **High throughput bioluminescent imaging evaluation of tumor perfusion in response to CA4P**

Significant reduction in signal intensity after infusion of CA4P correlates well with decrease in tumor perfusion monitored by MRI.

- **Correlation of MR findings with histological studies**

Consistent with MRI and BLI findings, histological study of tumor perfusion using Hoechst dye 33342 showed a significant reduction in perfused vessels at 2hr after CA4P, which recovered 24 h later.

Reportable Outcomes

Years 1 and 2: Two peer-reviewed papers and five published conference proceedings.

Year 3:

Abstracts (Published Conference Proceedings):

Zhao, D., Richer, E., Liu, Li., Ya Ren, Slavine, N., Shay, J. W., Antich, P. P., Mason, R. P. *In vivo* monitoring of antivascular effects of combretastatin A4 phosphate in a breast cancer xenograft model. *97th AACR*, Washington, DC, Apr 2006.

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.*

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., Richer, E., Liu, Li., Ya Ren, Slavine, N., Shay, J. W., Antich, P. P., Mason, R. P. *In vivo* monitoring of antivascular effects of combretastatin A4 phosphate in a breast cancer xenograft model. *97th AACR*, Washington, DC, Apr 2006.

1005 *In vivo* monitoring of antivascular effects of combretastatin A4 phosphate in a breast cancer xenograft model

Dawen Zhao, Edmond Richer, Li Liu, Ya Ren, Nikolai Slavine, Jerry W. Shay, Peter P. Antich, Ralph P. Mason.
UT Southwestern Medical Center, Dallas, TX.

The vascular targeting agent, combretastatin A-4-phosphate (CA4P) causes tumor vascular shutdown inducing massive cell death. We have recently shown acute hypoxiation within 90 mins following CA4P administration to rats bearing syngeneic breast 13762NF tumors using MRI. We have now applied MRI and bioluminescent imaging (BLI) to probe the acute effects of CA4P on human breast MDA-MB-231 tumors.
231 cells were infected with a lentivirus expressing a luciferase reporter and highly expressing clones isolated. 10^6 cells were implanted in the flank of nude mice and allowed to grow to ~ 6 mm diameter. For BLI studies, mice were anesthetized (isoflurane/O_2) and a solution of D-luciferin (450 mg/kg) was administered s.c. in the neck region and light images acquired immediately using one camera of our Light Emission Tomography System (LETS). Serial images (30 s each) were acquired over a period of 20 - 30 mins and the light intensity-time curves evaluated. Saline or CA4P in saline (120 mg/kg; OXiGENE, Inc. Waltham, MA) were injected i.p. immediately after baseline BLI and then 2 h and 24 h later the BLI time course was repeated. We also undertook 3D imaging by acquiring multiple images simultaneously using 3 cameras arranged in a circular gantry around the mouse. MRI studies were performed using a 4.7 T Varian Inova imaging system. The transverse relaxation rate R_2^* was measured using multigradient echo sequence before and 2 h after i.p. CA4P (120 mg/kg). Dynamic contrast enhanced (DCE) MRI based on i.v. bolus injection of Gd-DTPA-BMA through a tail vein catheter was also acquired before and 2 h after CA4P, respectively.
Control tumors showed intense light emission peaking within 8 mins and generally decreasing to about 50-70% after 20 mins. By contrast, CA4P led to a significantly lower light emission (peak ~ 2 to 10 times lower) and delayed peak emission when animals were imaged after 2 h. 24 h later signal remained considerably decreased. Traditional BLI provides a planar image only, but tumors are 3D and known to exhibit heterogeneity, particularly, after vascular targeting agents. LETS successfully provides a 3D representation of the tumors. In good agreement with the BLI data, DCE MRI revealed a ~70% decrease in perfusion/permeability of tumors 2 h after CA4P ($p < 0.001$), while little change was observed in perfusion of the femoral artery. R_2^* measurement showed a significant increase in R_2^* values 2 h after CA4P treatment (mean of 2 h = 131 s^{-1} vs pre = 113 s^{-1}, $p < 0.01$), which may suggest an elevated deoxyhemoglobin from hemorrhagic thrombosis.
Both BLI and MRI enabled accurate imaging of tumor vascular shutdown after CA4P treatment. However, BLI is much cheaper and offers a high throughput method for evaluating novel drugs and drug combinations and scheduling.