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**13. ABSTRACT** *(Maximum 200 Words)*

The goal of the studies is to determine optimal strategies for combining radiation and drug therapies that destroy tumor vasculature using murine tumors and to elucidate microenvironmental (blood flow, oxygen, etc.) changes in the tumor to guide the planning of combined treatment. The specific aims are:

Aim 1: To measure the response of murine tumors to combined radiation and drug therapies that target tumor vasculature under variations of order and timing of the two modalities.

Aim 2: To measure microenvironmental changes such as blood flow and tumor oxygen following the combined therapies.

The results to date have resulted in two published papers, one paper in preparation, three scientific presentations and one grant submission. The significant findings to date include:

1. Blood flow changes are predictive of tumor response following drug therapy that is designed to target tumor vasculature.
2. Radiation therapy sensitizes drug therapy with respect to tumor response in either a single dose regimen or a fractionated schema.
3. Timing of radiation and drug therapy is critical to the magnitude of tumor response. If radiation follows drug administration, the effect of the therapies on tumor response is additive. If radiation proceeds drug delivery by 1 hour, the effect of the therapies on tumor response is more than additive.

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NMR with Combined Antiangiogenic and Radiation Therapies - Breast Cancer

Introduction

New therapies that target tumor vasculature are receiving intense attention because they exhibit their effect preferentially on tumor vasculature compared with that of normal tissue. New therapies are combined with conventional treatment; radiation therapy is a standard therapy for breast cancer. There are reasons to believe timing of drug and radiation would be important. The proposal is designed to investigate the effect of sequence and timing on tumor response following the combined administration of drug and radiation. Furthermore, non-invasive tools for measuring blood flow and oxygen are used which have a potential direct clinical utility.

NMR with Combined Antiangiogenic and Radiation Therapies - Breast Cancer

Body – Final Report

This document is a revision of a Final Report dated June 2002. The revision was necessary to address comments by DOD Research Data Management. There are four changes from the original version.

1. A discussion of the tumor oxygen studies (Task 2.1) is included.
2. All cited papers listed as Reportable Outcome are specified as either "Attached" or "Previously submitted in Annual Report Year 1, or Year 2".
3. Further discussion of a "new quantitative, non-invasive measurement of vascular permeability using MRI" as well as new preliminary data suggesting the importance of the new tool for monitoring the efficacy of antivascular agents are provided.
4. Further discussion of the results that resulted from Task 2 that will be reported in manuscripts being prepared for publication is provided.

Changes from the original version are in *italics*.

The research accomplishments have been significant. We have studied tumor response to two drug therapies that target tumor vasculature alone and in combination with radiation therapy. The two drug therapies used were combretastatin (kindly provided by OxiGene, Sweden) and arsenic trioxide (purchased from Sigma Scientific). Our results indicate that in response to drug alone, there is a small but significant tumor response. In combination with radiation, antivascular therapies are much more effective especially in a fractionated regimen (Lew et al. 2002 for arsenic trioxide; Brown et al. 2001 for combretastatin). This represents a completion of Task 1 (using two separate antivascular therapies).

We have also measured changes in tumor physiology (specifically, blood flow, oxygen and vascular permeability) in response to the drug therapies with and without radiation. We have completed the tumor blood flow measurements (using $^{86}Rb$ uptake and $^{14}C$-iodoantipyrine quantitative autoradiography, QAR). Of note is the completion of the QAR blood flow measurements using spontaneous breast carcinoma, since it was a major technical accomplishment to perform QAR in mice (the challenge results from a small mouse blood volume necessarily sampled over time to obtain a quantitative blood flow determination). We have completed our measurements of tumor oxygen in response to arsenic trioxide (using Eppendorf glass electrodes in collaboration with Dr. C.W. Song, University of Minnesota). These studies are encompassed by Task 2. Finally, we have further extended tumor physiologic measurements to include a new quantitative, non-invasive measurement of vascular permeability using MRI.

Some significant findings have resulted from our invasive measurements of tumor physiology. We found a positive correlation between blood flow decrease in response to drug therapies that target tumor vasculature and tumor response. Furthermore, under

optimal conditions of sequence and timing, radiation therapy combined with drug therapy enhances both the tumor blood flow reduction and eventual tumor response (as measured by growth delay; Lew et al. 2002). Our interpretation is that radiotherapy acts to sensitize cancer to drug therapies that target tumor vasculature. Our measurements of tumor oxygen suggest that antivascular agents actually improve tumor oxygen (Lew et al. 2000), contrary to conventional wisdom. One hypothesis is that arsenic trioxide causes cellular death within the tumor leading to decreased oxygen consumption and increased oxygen availability. Our observation of tumor response to fractionated radiation plus drug regimen is consistent with this theory (Lew et al. 2002). Finally, our results indicate that blood flow changes in response to the combined treatment of radiation and drug therapies can be used to predict tumor response to the combined treatment.

The tasks of the project are as follows.

Task 1. To measure the response of spontaneous murine mammary carcinoma to combined radiation and antiangiogenic/antivascular therapies under variations of order and timing of the two modalities. (months 1 to 36)

- measure tumor response to 20 Gy (120 mice; months 1 to 9). Completed task. Presented at Chemical Modifier/Tumor Physiology Meeting in Banff, Oct. 2000 (arsenic studies), Radiation Research Meeting in Puerto Rico, April 2001 (combretastatin studies), and recently accepted for publication in Cancer Research (see Appendix).
  - radiotherapy before antiangiogenic/antivascular therapy
    Standard Therapy and Group A (40 mice; months 1 to 3)
  - antiangiogenic/antivascular therapy before radiation
    Group B (40 mice; months 3 to 6)
  - control experiments
    Group C (40 mice, months 7 to 9)

- measure tumor response to 10 or 30 Gy (100 mice; months 9 to 18). This task is completed and is being prepared for publication. Presented at Chemical Modifier/Tumor Physiology Meeting in Banff, Oct. 2000 (arsenic studies), Radiation Research Meeting in Puerto Rico, April 2001 (combretastatin studies), and recently accepted for publication in Cancer Research (see Appendix).
  - radiotherapy before antiangiogenic/antivascular therapy
    Standard Therapy and Group A (40 mice; months 9 to 12)
  - antiangiogenic/antivascular therapy before radiation
    Group B (40 mice; months 13 to 15)
  - control experiments
    Group C (20 mice, controls not repeated, months 16 to 18)

Task 2: To measure changes in tumor oxygen and blood flow following radiotherapy or antiangiogenic therapy (150 to 176 mice depending on statistics; months 1 to 36). This task is completed and is being prepared for publication.

- Imaging Group I: effect of antiangiogenic/antivascular therapy on oxygen (48 to 64 mice; months 1 to 12). This task is completed. In addition we have developed a non-invasive, quantitative measurement of blood vessel permeability using MRI and used the tool to measure changes in tumors following vascular targeting agents.
- Imaging Group II: effect of radiation on tumor blood flow measured using NMR and Iodo-AntiPyrine quantitative autoradiography (72 mice total split between two radiation doses; months 18 to 36). This task is completed.
- Imaging Group III: effect of antiangiogenic/antivascular therapy on tumor physiology in the same tumor over time (30 to 40 mice; months 13 to 18). This task is completed. Manuscript in preparation.

*Details of Results of Task 2.*

*Task 2.1: Effect of antiangiogenic/antivascular therapy on tumor oxygen.*

*Changes in oxygen were assessed in three ways: Polarographic measurements, $^{19}F$-based method, pimonidazole staining. Polarographic measurements were found to be the most useful even though invasive electrodes are used that only measure oxygen at a single location at a time. The results of oxygen changes following arsenic trioxide are shown in figure 1.*

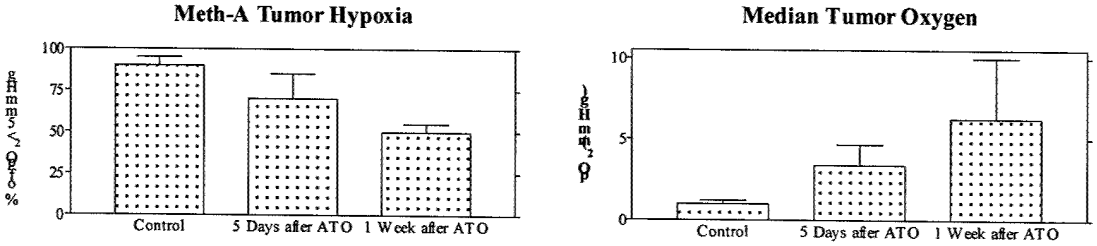



*Figure 1. Oxygen polarographic measurements following ATO administration (8mg/kg). Tumor oxygen was measured in over 100 locations in 8 to 10 tumors. The values presented represent average values and standard error of the means.*

*The second oxygen measurement used was a $^{19}F$-based method. The advantage of the $^{19}F$-based method is that the same animal can be measured sequentially. However despite our efforts, no meaningful $^{19}F$-based measurements of tissue oxygen were obtained. Specifically, tissue oxygen was measured to be zero before and after the administration of arsenic trioxide. We have not fully digested this data, but are considering that it may be artifactual since the contrast agent, hexafluorobenzene may be cleared from well-perfused tissue regions better than from regions far from blood vessels such as severely hypoxic and anoxic regions. The third method of oxygen measurement used was*

*pimonidazole binding (Hypoxyprobe, Chemicon International, Inc.). An example is shown in figure 2. The results although qualitative, were consistent with the increase in tissue oxygen one week after ATO.*

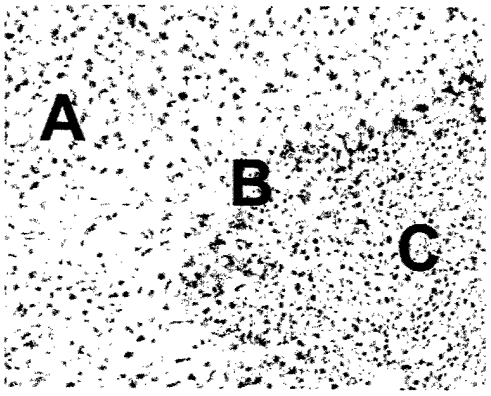



*Figure 2. Immunostaining for pimonidazole binding in an intra-dermal Meth-A tumor treated with 10 mg/kg i.p. arsenic trioxide (Lew et al. 1999). Three distinct tissue regions are identified: A) viable well-oxygenated cells, B) hypoxic cells and C) apoptotic cells. Tissue was paraffin embedded. Magnification: 400 times.*

*Task 2.2: Effect of radiation on tumor blood flow.*

*Changes in tumor blood flow following irradiation were measured using $^{86}Rb$ uptake, the gold-standard technique, and NMR, the non-invasive technique that allowed for multiple measurements in the same animal (referred to as Arterial Spin Tagging, AST, and Variable Tip Angle Gradient Refocused Echo). Rubidium uptake measurements demonstrated a decrease in tumor blood flow to approximately 75% of pre-irradiation levels one day after radiation followed by a gradual return to pre-irradiation levels by two and a half days after irradiation (Lew et al. Cancer Research 62: 4202-4205, 2002, see Appendix). In contrast to $^{86}Rb$ uptake results, no changes in blood flow after irradiation were observed using AST. However, the results are not inconsistent since the technique sensitivity (AST can not discern changes less than 20%) is approximately equal to the expected blood flow changes. Quantitative autoradiography was not used for this task since the single value measurements of $^{86}Rb$ uptake were adequate ($^{86}Rb$ uptake was used to measure blood flow changes in different regions of the tumor. Radiation changes that occurred were consistent throughout the tumor unlike that observed with the antivascular agents that affect the tumor core much more than the tumor periphery.*

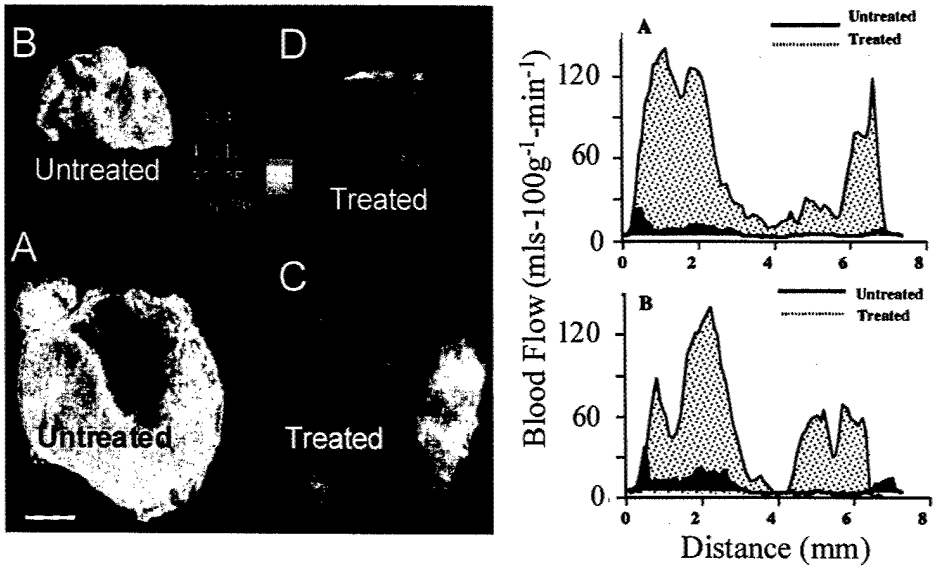
*Task 2.3: Effect of antiangiogenic/antivascular therapy on tumor physiology*

*Changes in tumor blood flow following antiangiogenic/antivascular agents were measured using four techniques. To obtain local or regional blood flow measurements, $^{86}Rb$ uptake and $Na^{99m}TcO_4$ clearance were used (Lew et al. 2002 in Appendix, Lew et al. 1999). To obtain blood flow measurements with submillimeter resolution, quantitative autoradiography was used (see Figure 3). To obtain blood flow in the same animal over a period of time, AST was used (see Figure 4).*

*Blood flow changes were measured in response to radiation (Lew et al. 2002), antiangiogenic/antivascular agents (Lew et al. 1999, 2002) and the combined treatments (Brown et al. 2001, Lew et al. 2002). Also blood vessel permeability was measured in response to antiangiogenic/antivascular agents and combined drug plus radiation regimens (see Figure 5).*

*Figure 3. Quantitative autoradiography ($^{14}$C-Iodo-AntiPyrine) in Murine Spontaneous Mammary Carcinoma*

**Examples of Quantitative Autoradiography Results with Murine Spontaneous Mammary Carcinoma**




*These studies represent the first time quantitative autoradiographs have been performed using a spontaneous breast cancer. These studies are technically demanding because of the small blood volume in mice and difficulty in sampling blood repeatedly. The results of these studies are being prepared for publication.*

*METHODS AND MATERIALS*

*Combretastatin-A4-P (provided by OxiGene Inc., Sweden) was administered i.p. at 100 mg/kg 1 hour before sampling of blood for flow measurements. Female Oncomice™ were purchased from Charles River Laboratories when they were approximately 42 days old. Spontaneous tumors grew when the mice reached puberty. Tumors had a volume doubling time of 6 days. Between 1 and 7 tumors grew per animal. Mice were used for blood flow studies when at least one tumor was 9mm in diameter. At the time of the experiment, mice were between 23 and 25g in weight. Tumor blood flow was measured using uptake of radiolabeled [$^{14}$C]IAP (New England Nuclear). Mice were anesthetized using sodium pentobarbital at 60 mg/kg, 10 to 15 minutes prior to surgery. Left leg femoral artery and right leg femoral vein were catheterized with PE-10 tubing. Arterial blood pressure was monitored up to the point of blood flow measurement. At the appropriate time, blood flow was measured by infusing 5μCi of [14C]IAP dissolved in 0.1 mls of buffered saline into the femoral vein for 20 s. During the 20 s period, free-flowing blood from the femoral artery was collected at 1 s intervals on pre-weighed filter paper. At the end of the 20 s, the mice were killed by decapitation. Tumors were removed as quickly as possible. In some experiments, brain and other tissues were also removed to assess blood flow in normal tissues. Tumors and tissues were frozen in 2-methylbutane cooled to 45°C with dry ice. Blood and tissue samples were counted using a scintillation well-counter. Radioactive counts per ml of blood as a function of time were determined from the ratio of radioactivity on filter paper divided by the difference between the weight of the blood soaked filter paper and the pre-weighed filter paper. Tumors and normal tissues were cryostat sectioned at 20μm thickness at intervals of 400 to 600μm, rapidly dried, mounted on cardboard and placed next to autoradiographic film along with appropriate standards for 10 days. The standards were used to construct a curve of nC as a function of optical density. Regions of darkened film were converted to nC from measurements of optical density using the standard curve. Blood flow rates to tumor and normal tissues were calculated from tissue counts, the equilibrium partition coefficient for IAP in the different tissues, and the arterial input function derived from the arterial blood counts. Autoradiographic images were digitized and converted to blood flow images pixel-by-pixel (AIS, Hamilton Ontario).*

*Tumor Segmentation Based on Blood Flow Response to Vasoactive Agents being prepared for publication*

*Concept: The different blood flow responses in tumor and adjacent normal tissue and the ability to measure changes non-invasively using AST suggested to us a new avenue of investigation, whether sequences of images obtained prior to and following administration of a vasoactive challenge could be used to produce a single image of high contrast incorporating the different responses of tumor and normal tissue vasculature.*

*Proof of principle was carried out using rat brain tumors and combretastatin A-4, the tubulin-binding agent that is thought to exhibit a differential tumor/normal vasculature effect deriving from the conversion to its active form by an enzyme, phosphatase, believed to be more abundant in cells lining tumor vasculature than normal tissue vasculature.*

*Image Processing: Our image processing technique consisted of 1) generating a pharmacokinetic signal in the core of tumor, periphery or normal tissue, 2) producing one or two images having optimal contrast(s) reflecting different vascular responses in the tumor core, tumor periphery, or normal brain, and 3) segmenting tumor and normal tissue. Pharmacokinetic signals in the tumor and normal tissue were obtained by manually sampling a few pixels in respective tumor and contralateral normal brain. A non-linear transformation was applied to images in a dynamic series by matching filter pixel-by-pixel with the pharmacokinetic signal as a reference function. This produced a single image from one reference function. Thus, a series of dynamic images was reduced to one or two images in which contrasts represent different tissue responses to the vasoactive agent.*

*In the case that more than one image was generated, a principal component analysis was applied to find the first principal image. The purpose of this step is two-fold: 1) contrasts will be optimized if the original ones are not. 2) The procedure further reduces dimensionality for segmentation. In general, the first principal image contains close to the maximum amount of information for segmentation. A simple segmentation algorithm was adopted, in which a histogram, as well as structural analysis, was done in a large ROI in the contralateral normal tissue. Two segmented images were generated by thresholding the pixel intensity in the first principal image 2.3 times the standard deviation above and below the mean of the normal tissue. Tumor delineation method based on blood flow dynamics is clearly beyond the scope of the present proposal, however the new analysis builds directly on the studies funded in the present grant. An example is shown in the schematics in Figure 4.*

**Figure 4**

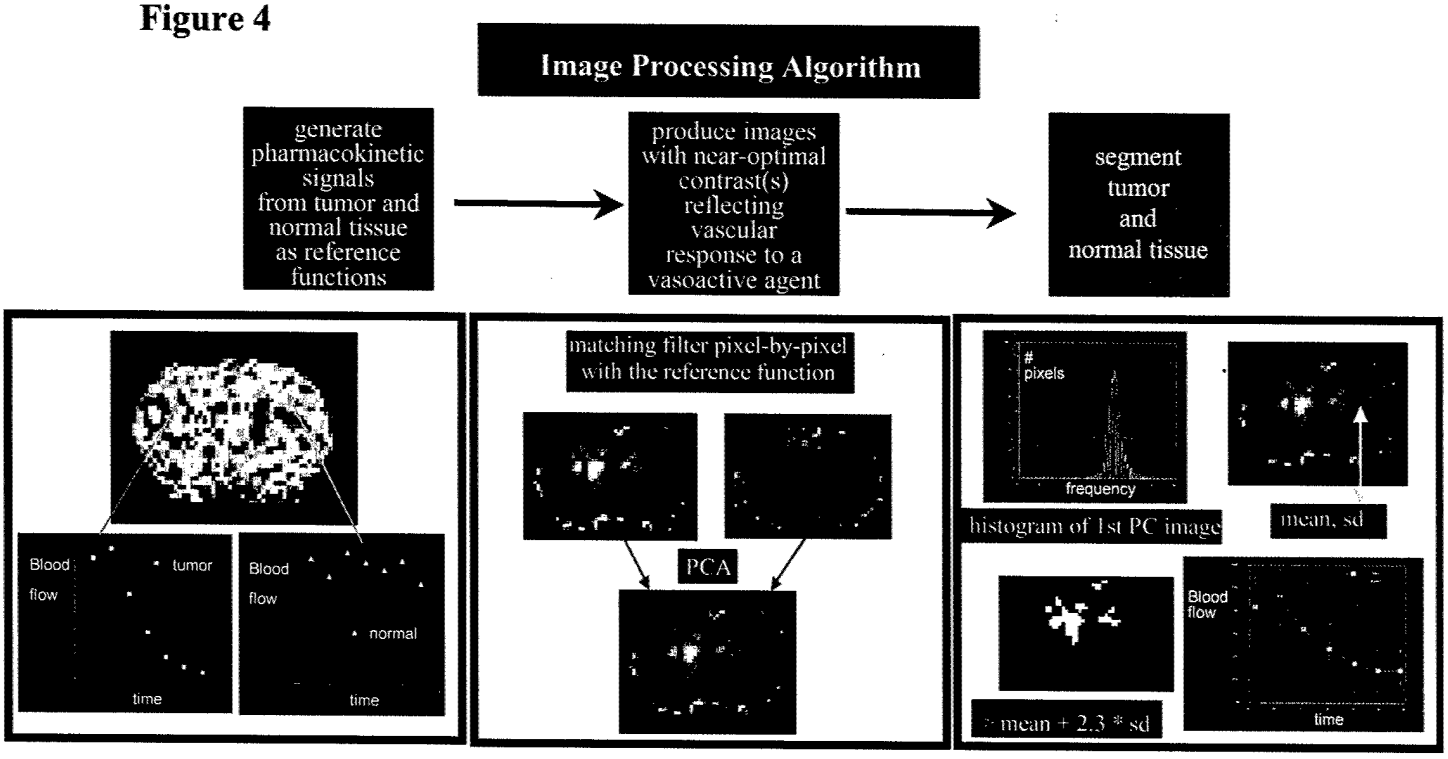
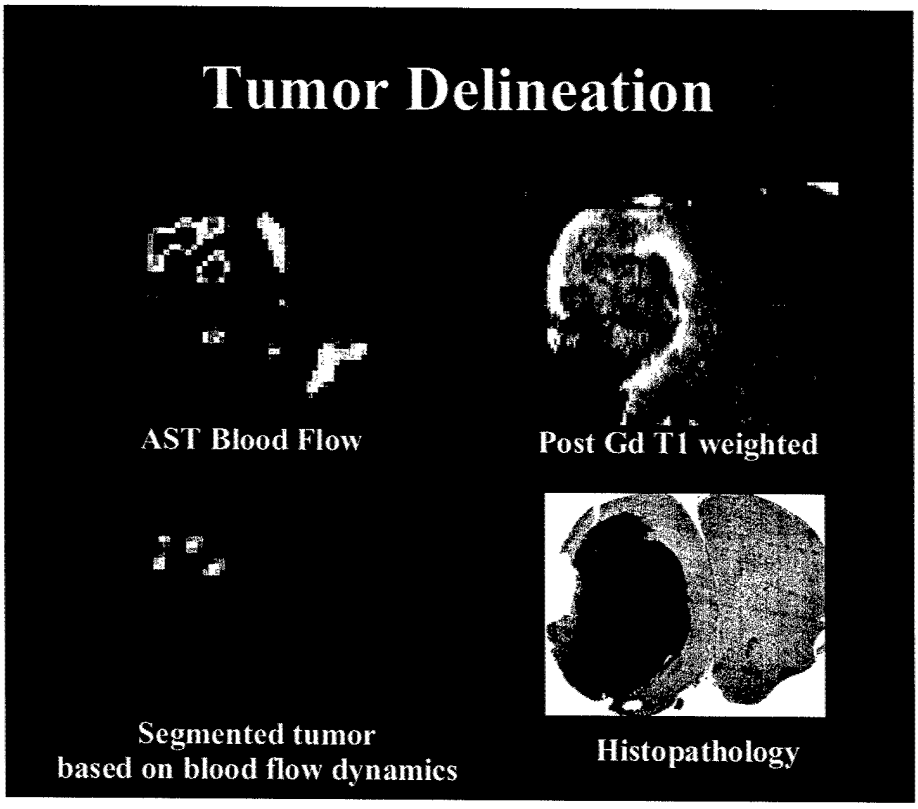


Image Processing Algorithm
generate pharmacokinetic signals from tumor and normal tissue as reference functions
produce images with near-optimal contrast(s) reflecting vascular response to a vasoactive agent
segment tumor and normal tissue
Blood flow
tumor
time
Blood flow
normal
time
matching filter pixel-by-pixel with the reference function
PCA
# pixels
frequency
histogram of 1st PC image
mean, sd
Blood flow
time
> mean + 2.3 * sd


Tumor Delineation
AST Blood Flow
Post Gd T1 weighted
Segmented tumor based on blood flow dynamics
Histopathology

*Blood Vessel Permeability*

*We have made the new observation that the antivascular agent, arsenic trioxide, causes an increase in tumor vessel permeability. Tumor vessel permeability has been measured using the leakage of a large fluorescent molecule observed using a laser scanning confocal microscope. Figure 5 illustrates the result of arsenic trioxide alone or in combination with radiation on FITC labeled dextran (1 million Dalton molecular weight) leakage from the vessels of 9L tumors. Our data indicates that early changes in vessel permeability correlate with eventual tumor response and suggest that a measurement of vessel permeability may predict tumor response.*

*In parallel to the vessel permeability studies described, Dr. James R. Ewing (a co-investigator on the grant) developed a novel MRI measurement of vessel permeability. The new technique uses progressively smaller molecular weight MRI contrast agents delivered as an intravenous bolus to determine the size of vessel pores. MRI signal intensity is plotted as a function of "stretch time" following the theory of Patlak such that the initial slope of the line yields a quantitative measurement of vessel leakiness.*

*The new technique forms a logical extension of the present grant because tumor permeability changes can be expected following delivery of either antiangiogenic agents (vessel permeability changes are the hallmark of angiogenesis, the evaluation of therapies designed to stop new vessel growth may benefit from the development of a non-invasive, quantitative measurement of vessel permeability) or antivascular agents (See figure 5). The evaluation of therapies designed to stop new vessel growth may benefit from the development of a non-invasive, quantitative measurement of vessel permeability.*

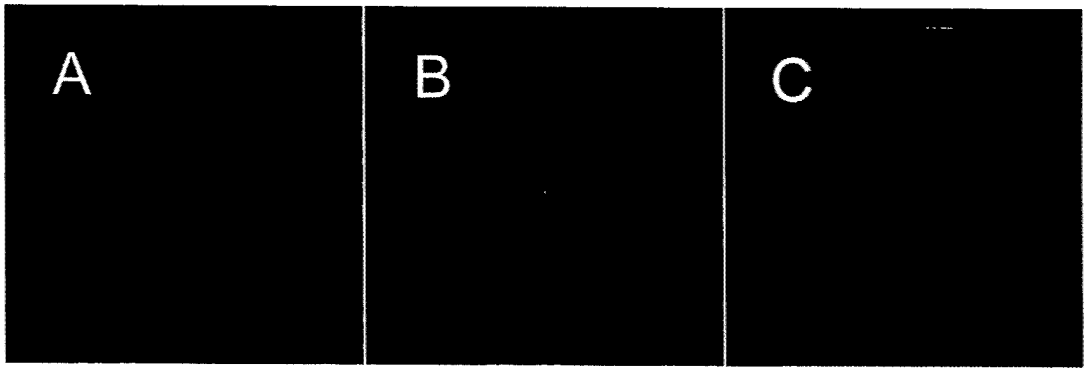



*Figure 5.*
*FITC labeled dextran administered intravenously to A) rats bearing 9L tumors untreated, B) treated 48 hours previously with 6mg/kg arsenic trioxide, C) treated 48 hours previously with 6mg/kg arsenic trioxide and radiation (25Gy). Images represent the sum of approximately fifty 1μm thick scans.*

Key Research Accomplishments

- Blood flow changes are predictive of tumor response following drug therapy that is designed to target tumor vasculature.

- Radiation therapy sensitizes drug therapy with respect to tumor response in either a single dose regimen or a fractionated schema.

- Timing of radiation and drug therapy is critical to the magnitude of tumor response. If radiation follows drug administration, the effect of the therapies on tumor response is additive. If radiation proceeds drug delivery by 1 hour, the effect of the therapies on tumor response is more than additive.

- The magnitude of blood flow decrease 60 hours following the combined treatment of radiation and drug is consistent with and predicts increased tumor response.

Reportable Outcomes

Manuscripts

1. Lew YS, Brown SL, Griffin RJ, Song CW and Kim JH. Arsenic trioxide causes selective necrosis in solid murine tumors by vascular shutdown. Cancer Research 59: 6033-6037, 1999. *(Previously submitted in Annual Report Year 1, dated May 2000).*

2. Lew YS, Kolozsvary A, Brown SL, Kim JH. Synergistic interaction with arsenic trioxide and fractionated radiation in locally advanced murine tumor. Cancer Research *62: 4202-4205, 2002. Attached.*

Presentations

1. Lew YS, Kolozsvary A, Brown SL, Song CW and Kim JH. Arsenic trioxide: anti-vascular effect and radiosensitization. Proceedings of the American Association for Cancer Research. 41: 294, March 2000. *(Previously submitted in Annual Report Year 1, dated May 2000).*

2. Lew YS, Kolozsvary A, Brown SL, Song CW, Kim JH. Arsenic trioxide: a novel vascular targeting agent and radiosensitizer. Presented at the 11th Meeting of Chemical Modifiers of Cancer Treatment – Tumor Physiology, Banff, Alberta, Canada, October 4-8, 2000. *(Previously submitted in Annual Report Year 2, dated May 2001).*

3. Brown SL, Wei L, Tavarekere TN, Marshall M, Kolozsvary A, Fenstermacher JD, Kim JH. Magnitude of radiosensitization by combretastatin depends on initial tumor blood flow. Presented at the 48th Annual Meeting of Radiation Research, San Juan, Puerto Rico, April 21-25, 2001. *(Previously submitted in Annual Report Year 2, dated May 2001).*

Other Reportable Outcomes

1. Grant Submissions *Partially* Resulting from the Present Funded Studies: NIH-NHLBI RO1 HL70023 (PI: J.R. Ewing). Title: MRI Measure of Blood Brain Barrier Permeability. *Re*-submitted *October* 1, 2002.

2. Clinical Trial *Partially* Resulting from the Present Funded Studies: NABITT Sponsored to Assess Toxicity (PI: S. Ryu). Title: Phase I Study of Combined Radiotherapy and Arsenic Trioxide for the Treatment of Malignant Glioma.

## Conclusions

The results to date provide critical data on the use of drugs that have an effect on tumor blood flow particularly in combination with radiation therapy. The data indicates that antivascular therapy should follow radiation therapy by one hour for maximum effect. The combined effects of drug and radiation are more than additive with tumor response as the endpoint. Blood flow changes are predictive of tumor response both following drug alone and combined with radiation. The non-invasive MRI physiological measurements are of paramount interest since the techniques are directly applicable to humans.

## References

1. Lew YS, Kolozsvary A, Brown SL, Kim JH. Synergistic interaction with arsenic trioxide and fractionated radiation in locally advanced murine tumor. Cancer Research *62: 4202-4205, 2002. Attached.*

2. Brown SL, Wei L, Tavarekere TN, Marshall M, Kolozsvary A, Fenstermacher JD, Kim JH. Magnitude of radiosensitization by combretastatin depends on initial tumor blood flow. Presented at the 48th Annual Meeting of Radiation Research, San Juan, Puerto Rico, April 21-25, 2001. *(Previously submitted in Annual Report Year 2, dated May 2001).*

3. Lew YS, Kolozsvary A, Brown SL, Song CW, Kim JH. Arsenic trioxide: a novel vascular targeting agent and radiosensitizer. Presented at the 11th Meeting of Chemical Modifiers of Cancer Treatment – Tumor Physiology, Banff, Alberta, Canada, October 4-8, 2000. *(Previously submitted in Annual Report Year 2, dated May 2001).*

## Appendix

1. Lew YS, Kolozsvary A, Brown SL, Kim JH. Synergistic interaction with arsenic trioxide and fractionated radiation in locally advanced murine tumor. Cancer Research *62: 4202-4205, 2002. Attached.*

[CANCER RESEARCH 62, 4202–4205, August 1, 2002]

*Advances in Brief*

# Synergistic Interaction with Arsenic Trioxide and Fractionated Radiation in Locally Advanced Murine Tumor[1]

**Young S. Lew, Andrew Kolozsvary, Stephen L. Brown, and Jae Ho Kim[2]**

*Department of Radiation Oncology, Henry Ford Health System, Detroit, Michigan 48202*

## Abstract

**We have shown previously that arsenic trioxide (ATO) preferentially shuts down tumor blood flow, leading to pronounced cell death in the central part of the solid tumor with a minimal effect on the surrounding normal tissues. On the basis of the histopathological and tumor perfusion studies, we hypothesized that the tumor control rate of locally advanced solid tumors would increase after the combined treatment of ATO and radiation relative to either radiation or ATO alone. The antitumor action and quantitative tumor perfusion studies were carried out with locally advanced methylcholanthrene-induced fibrosarcoma grown in BALB/c mice. The s.c. methylcholanthrene-induced fibrosarcoma leg tumors were treated with ATO alone (10 mg/kg), radiation alone (30 Gy), or drug plus radiation together. Radiation alone and drug alone delayed the growth of the tumor by a few days compared with untreated tumors. In contrast, when radiation and drug were given together, the tumor growth delay was longer than 1 month, resulting local tumor cure of 55%. The fractionated radiation combined with ATO showed a similar pronounced tumor growth delay relative to the drug alone or radiation alone. Sustained reduction in tumor blood flow after the combined treatment measured using a rubidium uptake method paralleled enhanced tumor response. There was an immediate 10-fold increase in the production of tumor necrosis factor-$\alpha$ in the tumor tissue after the drug treatment, concomitant with the onset of prompt reduction of the tumor blood flow. The present results indicate that tumor response was better with combined treatment than with either treatment alone, suggesting that ATO has potential as an adjuvant to radiotherapy.**

## Introduction

ATO[3] has a therapeutic benefit in the treatment of some hematologic malignancies. Used as an Asian medicinal for more than 1000 years, ATO recently has been accepted by Western medicine as a part of the arsenal in the treatment of recurrent acute promyelocytic leukemia. A significant percentage of patients with recurrences after conventional chemotherapy subsequently achieve a complete remission with ATO alone (1, 2).

We have demonstrated previously that a single systemic administration of ATO induced a prompt and pronounced vascular shutdown in large murine solid tumors and produced extensive central necrosis in the poorly perfused compartment of the tumor (3). The large tumors were more vulnerable to the action of the antivascular effect of ATO, whereas the peripheral zone of the tumor was minimally affected and became a source of eventual recurrence. Hence, the tumor response to ATO alone produced varying degrees of tumor growth delays in rapidly growing tumors, but the cure was seldom obtained.

The fact that ATO preferentially targets the poorly perfused fraction of the tumor, which may be radiation resistant, whereas radiation therapy is effective against well perfused tissues of the tumor periphery, led us to hypothesize that the combined treatment of drug and radiation would enhance the tumor response relative to that of radiation or drug alone. We report the first *in vivo* results demonstrating a pronounced enhancement of the radiation tumor response in well established large murine tumors after the combined treatment of drug and radiation.

## Materials and Methods

**Mice, Tumors, and Compounds.** BALB/c male mice, 6–8 weeks of age and weighing 20–25 g, that were obtained from Charles River Laboratories (Portage, MI) were used. ATO was purchased from Sigma-Aldrich (St. Louis, MO). The murine tumor line used was Meth-A grown in mice. For s.c. tumor, which was used for tumor response, approximately $1 \times 10^6$ viable Meth-A cells in 50 $\mu$l of MEM were inoculated into the hind leg s.c. For intradermal tumor, which was used for the study of blood perfusion and TNF-$\alpha$ assay, approximately $1 \times 10^5$ viable Meth-A cells in 100 $\mu$l of MEM were inoculated into the abdominal skin of mice. Maintenance of tumor cells, preparation of cells, and making ATO solution were performed as described previously (3).

**Effect of ATO and Radiation on Tumor Growth.** s.c. hind leg tumors were used for tumor growth studies. When tumors were palpable, mice were randomly divided into four groups, untreated, ATO alone, radiation alone, and radiation combined with the drug. When mean tumor volume reached 0.3~0.5 $cm^3$, radiation was delivered to the leg with tumors using a cobalt-60 unit (Theratron 780; AECL, Kanata, Ontario, Canada) with a secondary collimator of 2-inch-thick cerroband. A 2-cm-thick tissue-equivalent bolus was used to bring the maximal radiation dose on the surface of the target tissue. ATO (10 mg/kg) was administered i.p. 1 h after the beginning of each radiation exposure. Tumor growth delay was defined as the additional time necessary for a tumor to grow to 1 $cm^3$ in average volume compared with untreated tumors. Cured tumors were defined as no evidence of palpable tumors 60 days after the treatment.

**Blood Perfusion Measurement.** Changes in the blood perfusion were assessed by means of $^{86}Rb$ uptake as described elsewhere (4). BALB/c mice with intradermal Meth-A tumors, 1.2 cm in mean diameter, were used. Briefly stated, 5 $\mu$Ci of $^{86}RbCl$ in a 0.1-ml volume were injected through a tail vein after anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg), and mice were sacrificed by cervical dislocation 60 s after the injection. Whole tumors were excised and counted using a well counter (1282 Comugamma Cs; Amersham Biosciences Inc., Piscataway, NJ). The ratio of radioactive counts from the tissue of interest (counts per gram) to the counts in the standard $^{86}Rb$ solution equivalent to the total $^{86}Rb$ activity injected multiplied by 100 gave the percentage of cardiac output to the tissue of interest. Relative blood perfusion values, *i.e.*, a percentage of the untreated tissue, were calculated. $^{86}Rb$ uptake was measured before, at 24 h, and 60 h after treatment. At least five tumor-bearing mice were used at each time point.

**Measurement of Tissue TNF-$\alpha$ Levels.** The extractable TNF-$\alpha$ levels in tumor, liver, spleen, and serum were measured at 0 min, 30 min, 2 h, and 4 h after i.p. injection of ATO (10 mg/kg). BALB/c mice with intradermal Meth-A tumors, 1.2 cm in mean diameter, were used. Excised tissues were weighed and homogenized in minimal essential medium using a tissue grinder. Homogenates were centrifuged at 2000 $\times$ *g* for 30 min at 4°C, and collected supernatant was kept in a freezer. Tissue TNF-$\alpha$ was assayed in batches on the same day using the same conditions. Samples were thawed and centrifuged a second

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[2] To whom requests for reprints should be addressed. Phone: (313) 916-7271; Fax: (313) 916-3235; E-mail: jkim1@hfhs.org.

[3] The abbreviations used are: ATO, arsenic trioxide; TNF, tumor necrosis factor; VTA, vascular targeting agent; Meth-A, methylcholanthrene-induced fibrosarcoma.

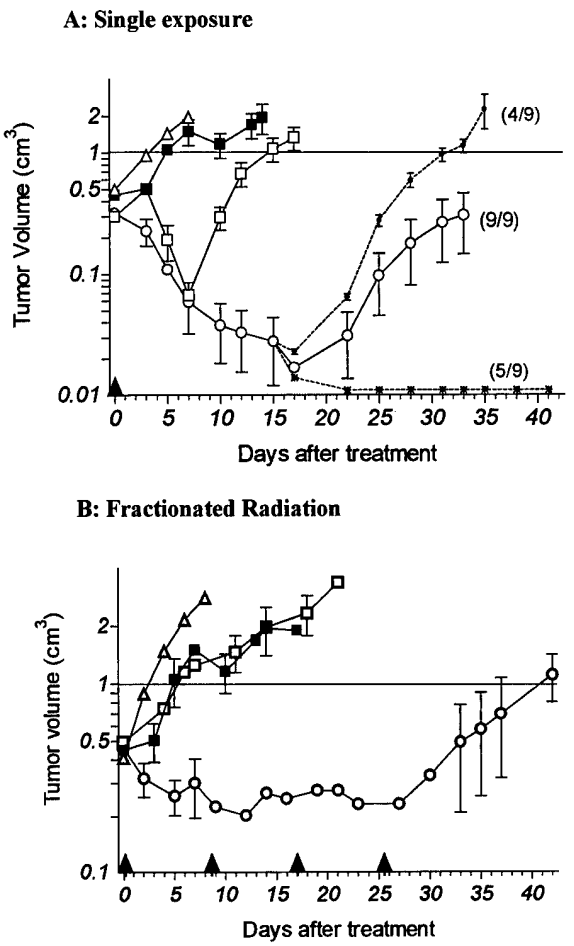


Fig. 1. The effect of combined treatment with radiation and ATO on s.c. Meth-A tumor grown in the hind leg of mice. Radiation was given at 30 Gy in a single dose (*A*) or in four weekly fractions of 12 Gy (*B*). ATO was given at 10 mg/kg, i.p., 1 h after each radiation exposure in both experiments. △, untreated control; ■, ATO alone; □, radiation alone: ○, combined treatment (·, not cured; *, cured). *Arrowheads*, treatment time. Data are the means of 9–10 mice per group. *Bars*, SE.

Table 1 *Tumor growth delays by combined treatment with fractionated radiation and ATO*

Subcutaneous Meth-A tumors grown in the hind leg of BALB/c mice were used. The combined fractionated schedule consists of once a week radiation and ATO. ATO (10 mg/kg) was given i.p., 1 h after each radiation dose.

| Dose/fraction × no. of fraction (total dose) | ATO alone (10 mg/kg, i.p.) | RT alone | RT + ATO |
|---|---|---|---|
| 12 Gy × 2, once a wk (24 Gy) | 2.3 ± 0.9 days | 0.8 ± 0.4 days | 5.4 ± 2.6 days |
| 12 Gy × 4, once a wk (48 Gy) | 2.4 ± 0.6 days | 0.8 ± 0.4 days | 27.3 ± 4.8 days |
| 15 Gy × 4, once a wk (60 Gy) | 2.4 ± 0.6 days | 1.6 ± 1.4 days | 27.0 ± 1.0 days |

time on the day of TNF measurement. TNF levels were measured with the ELISA method using a mouse TNF-α immunoassay kit (Quantikine M, R&D Systems, Minneapolis, MN).

## Results

**Tumor Response to the Combined Treatment of ATO and Radiation.** Before proceeding to a determination of the combined effect of ATO and radiation, we carried out two preliminary studies. (*a*) We wanted to determine which specific treatment sequence and time of ATO with respect to radiation would produce a maximal local tumor control or tumor growth delay. The maximal synergistic effect was observed only when the drug was administered shortly after irradiation (1-h interval). No synergism was obtained when the drug was given before radiation or 24 h after radiation (data not shown). (*b*) The next study was to determine whether the initial tumor size would influence the tumor growth delays after the combined treatment, because the initial tumor size limits the number of radiation fractions, and in our previous study, the drug exerted its maximal antivascular effect on a large tumor (3, 5). In contrast to the results with drug alone, both large and small tumors (between 0.3 $cm^3$ and 1.0 $cm^3$ in volume) showed similar enhanced tumor response to the combined treatment. On the basis of these preliminary findings, all subsequent studies with the combined treatment were carried out with Meth-A tumors of moderate size (average of 0.5 $cm^3$), using the following timing and sequence: i.p. administration of ATO 1 h after the beginning of radiation exposure. Fig. 1*A* shows the combined effect of radiation and ATO after single-dose radiation (30 Gy) or fractionated radiation (12 Gy, four times). The tumor growth delay from the combined treatment was estimated to be about 42 days (29 days if we exclude cured mice), significantly greater than the tumor growth delay after either radiation alone or ATO alone, approximately 12 days and 2 days, respectively. Significantly, more than 50% of tumors were cured after the combined treatment. Fig. 1B shows the effect of fractionated radiation and ATO. As with the foregoing result of single-dose radiation and ATO, the tumor growth delay was significantly increased (34 days) relative to that of radiation alone (3 days) or ATO alone (3 days). Table 1 shows the tumor growth delays obtained with different fractionation schemes.

**Relationship Between Tumor Response and Blood Flow Changes in Tumor.** Changes in the tumor blood flow were measured using $^{86}Rb$ uptake to evaluate whether the synergistic effect of the combined treatment was related to blood flow. Fig. 2 illustrates the blood flow changes of whole tumor at 24 h and 60 h after the treatment. The percentage of blood perfusion relative to untreated tumor at 24 h after ATO alone, radiation alone, or the combined treatment was 64.3 ± 13.3%, 69.9 ± 13.5%, and 45.9 ± 21.1%, respectively, all showing a significant decrease from pretreatment values. At 60 h, the percentage of blood perfusion after ATO alone or radiation alone rebounded to 108.3 ± 22.5% and 121.3 ± 34.2%, respectively. In contrast, the percentage of blood perfusion at 60 h after the combined treatment remained reduced at 61.6 ± 11.9%. The sustained reduction of blood flow in the tumor after the combined treatment paralleled the enhanced tumor response to the treatment.

**Intratumoral TNF-α After ATO Treatment.** Fig. 3 shows TNF-α levels in the tumor tissue before and after systemic ATO treatment. The TNF-α level in untreated tumor was 0.54 ± 0.12 μg/g. After the drug treatment, there was more than a 10-fold increase in intratumoral TNF-α (6.65 ± 2.98 μg/g) at 30 min. TNF-α gradually decreased by 4 h to 5-fold the initial level. In contrast, the changes of TNF-α in other tissues showed different kinetics, *i.e.*, 2-fold

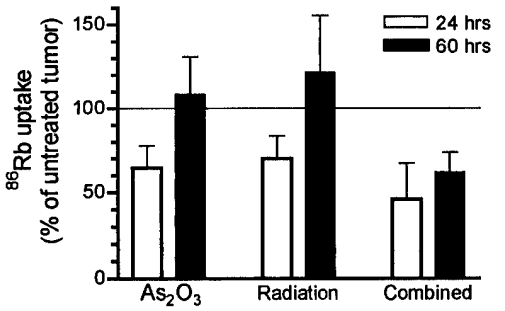


Fig. 2. Changes in the tumor blood flow after ATO (10 mg/kg) alone, radiation alone (30 Gy), or combined treatment. Blood flow was measured by $^{86}Rb$ uptake in intradermal Meth-A tumors grown in BALB/c mice. Plotted data are the means of calculated blood perfusion rates relative to untreated tumors; five mice per group. *Bars*, SE.

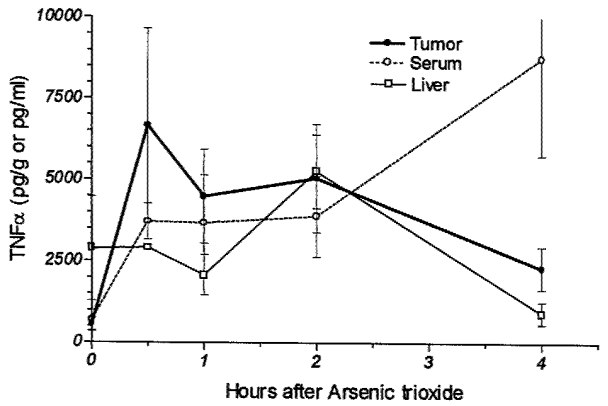


Fig. 3. Changes in TNF-$\alpha$ as a function of time after ATO (10 mg/kg, i.p.) treatment from intradermal Meth-A tumor, liver, and serum from BALB/c mice. TNF-$\alpha$ was measured by ELISA. Values are the means of at least five mice per time point. *Bars*, SE.

(5.25 ± 1.13 $\mu$g/g) increase at 2 h in the liver. TNF-$\alpha$ levels in serum gradually increased over 4 h.

## Discussion

The data herein presented demonstrate that the combination of radiation and ATO significantly increased tumor growth delay and local tumor control, apparently without a comparable increase in the reaction of the normal tissues. Although the experiments were not specifically designed to study normal tissue response, we found that the combined treatment did not increase the extent of hair loss in the irradiated limbs despite enormous tumor damage. Because hair loss is a simple, useful indicator of normal tissue toxicity by radiation, normal tissue damage appears not to be enhanced by the combined administration of ATO and radiation. The drug did not need to be present at the time of irradiation to produce the maximal effect, indicating that its synergistic effects are perhaps not at the cellular level but at the tissue level. Indeed, our cell culture studies with the combined radiation and drug showed no radiation enhancement, using similar time sequence schedules.[4] The most remarkable finding of the combined treatment was the radiation-enhancing effect by ATO retained under a fractionated schedule (Fig. 1*B;* Table 1). This effect differentiates ATO from the other hypoxic radiosensitizers wherein the drug-induced enhancement decreases with reoxygenation during fractionated irradiation (6, 7). Others using VTAs such as 5,6-dimethylxanthenone-4-acetic acid or combretastatin-A4P observed various degrees of enhanced tumor response with single doses of radiation (8, 9). However, quantitative comparisons of the degree of radiation enhancement between other VTAs and ATO are problematic because of differences in tumor models (type, size, locations), endpoints, and fractionation schedule used.

Because one of the main actions of ATO on the solid tumors has been its capacity to destroy tumor vasculature, we investigated changes in the tumor blood flow after the combined treatment. As shown in Fig. 2, enhanced tumor response paralleled a sustained reduction of blood flow in the tumor after the combined treatment. Blood flow recovered at 60 h after ATO alone or radiation therapy alone, even though a large portion of the tumor became necrotic. The recovery of blood flow 60 h after ATO or radiation may be explained by vasodilation and increased blood flow of normal tissue vessels immediately adjacent to the tumor, which feed the peripheral rim of the tumor. Inflammatory processes associated with ischemia and necrosis are known to induce vasoactive mediators and subsequent vasodilation (10). Furthermore, changes in blood flow of the peripheral rim of the tumor, not the central core of the tumor, predicted the recurrence of the tumor after radiation plus ATO.

We sought to determine the kinetic changes of the cytokine, TNF-$\alpha$, for two reasons, although the mechanism of interaction between ATO and radiation remains unknown. (*a*) TNF-$\alpha$ increases vascular permeability of the tumor vessels followed by extensive hemorrhagic necrosis of tumor, which was described by Carswell *et al.* (11) using Meth-A tumors. The gross observation after ATO treatment is similar to that seen with endotoxin-induced necrosis (3). (*b*) The elevation of intratumoral TNF-$\alpha$ occurs after treatment with other VTAs including flavone acetic acid and 5,6-dimethylxanthenone-4-acetic acid (12, 13). Fig. 3 shows an immediate and pronounced increase in production of TNF-$\alpha$ in the tumor tissue within a half-hour after the drug administration, whereas the TNF-$\alpha$ levels in other organs including the liver showed a delayed elevation at 2 h or later. The implication is that the antivascular effect of ATO is mediated by an increased production of TNF-$\alpha$. TNF-$\alpha$ has been shown to enhance the antitumor effect of radiation (14, 15). Also, there is evidence that pre-irradiated cell lines including endothelial cells are more sensitive to TNF-$\alpha$ (16, 17). This suggests that the increased production of TNF-$\alpha$ by ATO might be responsible for the synergistic effect of radiation and ATO.

Although VTAs are not curative when delivered alone, they do exhibit major tumor necrosis and, as such, are distinct from antiangiogenic agents that target newly formed tumor vasculature. VTAs have been categorized as one of two types (18). Drugs in one group, flavone acetic acid (19) and related compounds (12), exert their effect through the production of TNF-$\alpha$. The other group is tubulin-binding agents such as colchicine, the *Vinca* alkaloids, and the combretastatins, which are toxic to endothelial cells (20). Drugs in both groups enhance the antitumor activity of ionizing radiation. At present, ATO does not clearly belong to one group or the other.

Although the radiation-enhancing effect of ATO is clear, both after single doses and fractionated irradiation, it has been assumed that the drug is augmenting tumor response to irradiation. It is also conceivable that irradiation could affect cellular metabolism in a manner that would sensitize cells to the effects of the drug, because the synergy is obtained with the sequence of radiation followed by the drug administration. Additional studies are needed to examine the interaction and timing of various cytokine production and radiation schedules.

In summary, the results presented demonstrate that ATO retains its synergistic effect with radiation under a fractionated schedule. Furthermore, the data support the use of combining ATO with radiation as a strategy for the treatment of large tumors resistant to conventional therapies.

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## References

1. Soignet, S. L., Frankel, S. R., Douer, D., Tallman, M. S., Kantarjian, H., Calleja, E., Stone, R. M., Kalaycio, M., Scheinberg, D. A., Steinherz, P., Sievers, E. L., Coutre, S., Dahlberg, S., Ellison, R., and Warrell, R. P., Jr. United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia. J. Clin. Oncol. *19:* 3852–3860, 2001.
2. Murgo, A. J. Clinical trials of arsenic trioxide in hematologic and solid tumors: overview of the National Cancer Institute Cooperative Research and Development Studies. Oncologist, *2:* 22–28, 2001.
3. Lew, Y. S., Brown, S. L., Griffin, R. J., Song, C. W., and Kim, J. H. Arsenic trioxide causes selective necrosis in solid murine tumors by vascular shutdown. Cancer Res., *59:* 6033–6037, 1999.
4. Sapirstein, L. A. Regional blood flow by fractional distribution of indicators. Am. J. Physiol., *193:* 161–168, 1958.
5. Griffin, R. J., Lee, S. H., Rood, K. L., Stewart, M. J., Lyons, J. C., Lew, Y. S., and Song, C. W. Use of arsenic trioxide as an anti-vascular and thermosensitizing agent in solid tumors. Neoplasia, *2:* 555–560, 2000.

[4] J. H. Kim and Y. S. Lew, unpublished data.

6. Fowler, J. F. Eighth annual Juan del Regato lecture. Chemical modifiers of radiosensitivity—theory and reality: a review. Int. J. Radiat. Oncol. Biol. Phys., *11:* 665–674, 1985.
7. Lindenberger, J., Hermeking, H., Kummermehr, J., and Denekamp, J. Response of human tumour xenografts to fractionated X-irradiation. Radiother. Oncol., *6:* 15–27, 1986.
8. Wilson, W. R., Li, A. E., Cowan, D. S., and Siim, B. G. Enhancement of tumor radiation response by the antivascular agent 5,6-dimethylxanthenone-4-acetic acid. Int. J. Radiat. Oncol. Biol. Phys., *42:* 905–908, 1998.
9. Murata, R., Siemann, D. W., Overgaard, J., and Horsman, M. R. Interaction between combretastatin A-4 disodium phosphate and radiation in murine tumors. Radiother. Oncol., *60:* 155–161, 2001.
10. Male, D. Cell migration and inflammation. *In:* I. Roitt, J. Brostoff, and D. Male (eds.), Immunology, pp. 13.2–13.8. London, Mosby, 1993.
11. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA, *72:* 3666–3670, 1975.
12. Ching, L-M., Goldsmith, D., Joseph, W. R., Korner, H., Sedgwick, J. D., and Baguley, B. C. Induction of intratumoral tumor necrosis factor (TNF) synthesis and hemorrhagic necrosis by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in TNF knockout mice. Cancer Res., *59:* 3304–3307, 1999.
13. Mahadevan, V., Malik, S. T., Meager, A., Fiers, W., Lewis, G. P., and Hart, I. R. Role of tumor necrosis factor in flavone acetic acid-induced tumor vasculature shutdown. Cancer Res., *50:* 5537–5542, 1990.
14. Gridley, D. S., Archambeau, J. O., Andres, M. A., Mao, X. W., Wright, K., and Slater, J. M. Tumor necrosis factor-alpha enhances antitumor effects of radiation against glioma xenografts. Oncol. Res., *9:* 217–227, 1997.
15. Hallahan, D. E., Spriggs, D. R., Beckett, M. A., Kufe, D. W., and Weichselbaum, R. R. Increased tumor necrosis factor alpha mRNA after cellular exposure to ionizing radiation. Proc. Natl. Acad. Sci. USA, *86:* 10104–10107, 1989.
16. Herzog, T. J., Nelson, P. K., Mutch, D. G., Wright, W. D., Kao, M. S., and Collins, J. L. Effect of radiation on TNF alpha-mediated cytolysis of cell lines derived from cervical carcinomas. Gynecol. Oncol., *47:* 196–202, 1992.
17. Eussner, G., Kohlhuber, F., Grell, M., Ueffing, M., Scheurich, P., Hieke, A., Multhoff, G., Bornkamm, G. W., and Holler, E. Critical involvement of transmembrane tumor necrosis factor-alpha in endothelial programmed cell death mediated by ionizing radiation and bacterial endotoxin. Blood, *86:* 4184–4193, 1995.
18. Siemann, D. W., Warrington, K. H., Jr., and Horsman, M. R. Targeting tumor blood vessels: an adjuvant strategy for radiation therapy. Radiother. Oncol., *57:* 5–12, 2000.
19. Hill, S. A., Sampson, L. E., and Chaplin, D. J. Anti-vascular approaches to solid tumor therapy: evaluation of vinblastine and flavone acetic acid. Int. J. Cancer, *63:* 119–123, 1995.
20. Chaplin, D. J., Pettit, G. R., Parkins, C. S., and Hill, S. A. Antivascular approaches to solid tumor therapy: evaluation of tubulin binding agents. Br. J. Cancer, *74:* S86–S88, 1996.