

AD _____

Award Number: DAMD17-99-1-9215

TITLE: NMR with Combined Antiangiogenic and Radiation Therapies -
Breast Cancer

PRINCIPAL INVESTIGATOR: Stephen Brown, Ph.D.

CONTRACTING ORGANIZATION: Henry Ford Health System
Detroit, Michigan 48202

REPORT DATE: May 2000

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20000829 041

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE May 2000	3. REPORT TYPE AND DATES COVERED Annual (1 May 99 - 30 Apr 00)	
4. TITLE AND SUBTITLE NMR with Combined Antiangiogenic and Radiation Therapies - Breast Cancer		5. FUNDING NUMBERS DAMD17-99-1-9215	
6. AUTHOR(S) Stephen Brown, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Henry Ford Health System Detroit, Michigan 48202 E-MAIL: sbrown1@hfhs.org		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. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES This report contains colored photographs			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The original goal of the present study was to determine optimal strategies for combining radiation and antiangiogenic therapies in spontaneous murine tumors and to evaluate the potential of Nuclear Magnetic Resonance (NMR) blood flow, arterial spin tagging, and oxygen measurements, ¹⁹ F oximetry, to guide the planning of combined treatment. Antiangiogenic therapy was found to have only minimal effect in the tumor model. In contrast, we have discovered that antivascular therapies have a significant effect on tumor blood flow (Lew et al. Cancer Res. 59: 6033-7, 1999) and when combined with radiation have a profound effect on tumor response (manuscript in preparation, results presented at AACR in San Francisco in 1999, Abstract #1869). The specific aims are modified slightly: <u>Aim 1:</u> 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. <u>Aim 2:</u> To measure changes in tumor oxygen and blood flow following radiotherapy or antiangiogenic/antivascular therapy. We have completed the tumor response studies using radiation plus drug and we have completed the autoradiography blood flow studies (manuscript in preparation). The NMR blood flow and ¹⁹ F oximetry studies are in progress. The project is on schedule.			
14. SUBJECT TERMS Breast cancer, antiangiogenic, antivascular, radiation, blood flow, oxygen			15. NUMBER OF PAGES 15
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

JS X Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

JS X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

JS X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Stephen J. Ben

PI - Signature *May 30, 2000*
Date

Table of Contents

Cover.....	1
SF 298.....	2
Foreword.....	3
Introduction.....	5
Body.....	6-7
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	8
Appendices.....	8

NMR with Combined Antiangiogenic and Radiation Therapies - Breast Cancer

Introduction

New therapies that target tumor vasculature are receiving intense attention. Vascular targeting agents either act on the growth of new vessels, termed antiangiogenic therapy, or existing ones, termed antivascular therapies. All the compounds are of interest because they exhibit their effect preferentially on tumor vasculature compared with that of normal tissue. We reasoned that the new therapies would be combined with conventional treatment, particularly radiation. Furthermore, there are reasons to believe timing of drug and radiation would be important. The proposal was 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 – Annual Progress Report

The original proposal had two tasks. The most significant change from the original proposal is the addition of antivascular agents to the experimental design. We have not abandoned the use of antiangiogenic agents, but to date have seen little anti-tumor activity using VEGF antibody particularly in large tumors, and therefore thought it prudent to incorporate other potential anti-tumor agents into the protocol.

We have incorporated arsenic trioxide into the studies due in large part to the efforts of Dr. Young S. Lew. Dr. Lew came to the Radiation Oncology Research Laboratories as a visiting professor from Korea. In his clinical practice in Korea, Dr. Lew witnessed remarkable solid tumor regression in patients that received an elixir. Dr. Lew believes the elixir contained arsenic trioxide. We investigated the effect of arsenic trioxide (available from Sigma) using an experimental animal model and found it to have a preferential effect on tumor blood flow. The results were published as *Advances in Brief in Cancer Research* late last year (Lew et al. Arsenic trioxide causes selective necrosis in solid murine tumors by vascular shutdown Cancer Research **59**: 6033-7, 1999, please see Appendix). A most important part of this work is that we know the compound is well tolerated in humans with minimal side effect.

We have investigated the anti-tumor effect of the arsenic trioxide in combination with radiation. There is a profound sequence and timing dependence. The results were presented in San Francisco at the American Association for Cancer Research this year and are being prepared for publication. In summary the results indicate that arsenic trioxide administered 1 hour after radiation therapy is superior to other sequences and timings of administration.

NMR blood flow and ^{19}F oxygen studies are progressing slower than expected due to technical difficulties. The FAIR blood flow technique which allows for three-dimensional blood flow measurements has not yet been implemented on our 7T NMR machine. The program is written. It is at the stage of being debugged. We currently have available a quantitative three-dimensional measurement that provides relative blood flow but not absolute blood flow based on Gadolinium uptake. However, because we want to obtain absolute blood flow values, we plan to implement the FAIR blood flow technique instead of the Gd uptake measurement.

On the other hand, blood flow studies with the “gold-standard” IAP-autoradiography are a year ahead of schedule. We have completed the effect of drug on tumor blood flow using IAP-autoradiography. A manuscript summarizing the results is in preparation. The result is that blood flow in the tumor core and to a lesser extent the tumor periphery was substantially decreased following the administration of antivascular therapy.

The following is the original task outline with modifications indicated by *italics*.

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). *This task is completed as scheduled was presented at the AACR and is being prepared for publication.*
 - 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 ahead of schedule and is being prepared for publication.*
 - 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 on schedule overall. See details in each sub-task.*

- Imaging Group I: effect of antiangiogenic/*antivascular* therapy on oxygen ^{19}F and electrode pO_2 (48 to 64 mice; months 1 to 12). *This task is behind schedule by at least 12 months.*
- Imaging Group II: effect of radiation on tumor blood flow NMR and Iodo-AntiPyrene quantitative autoradiography (72 mice total split between two radiation doses; months 18 to 36). *This task is ahead of schedule regarding autoradiography and on schedule with regards to NMR. A manuscript describing the effect of *antivascular* therapy on a spontaneous tumor model is being prepared for publication.*
- 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 on schedule.*

Key Research Accomplishments

- Arsenic trioxide causes preferential vascular shutdown in tumor compared to normal tissue measured using rubidium uptake and pertechnetate clearance techniques.
- The effect of arsenic trioxide and radiation appear to be more than additive in their anti-tumor activity.
- Antivascular agent administered 1 hour after radiation is superior to other sequences and timings of administration with regards to tumor response.
- Antivascular agent causes a rapid decrease in blood flow as measured by the “gold-standard” IAP-autoradiography technique in spontaneous mouse tumors.

Reportable Outcomes

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.
2. 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.

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 preliminary data indicates that antivascular therapy should follow radiation therapy by one hour for maximum effect. The combined effects of drug and radiation appear to be more than additive with tumor response as the endpoint.

References

None

Appendix

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.
2. 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.

*Advances in Brief***Arsenic Trioxide Causes Selective Necrosis in Solid Murine Tumors by Vascular Shutdown¹**Young S. Lew,² Stephen L. Brown, Robert J. Griffin, Chang W. Song, and Jae Ho Kim

Department of Radiation Oncology, Henry Ford Health System, Detroit, Michigan 48202 [Y. S. L., S. L. B., J. H. K.] and Department of Therapeutic Radiology-Radiation Oncology, University of Minnesota Medical School, Minneapolis, Minnesota 55455 [R. J. G., C. W. S.]

Abstract

To investigate the antitumor action of arsenic trioxide in solid tumors, we carried out quantitative tumor perfusion studies, using locally advanced methylcholanthrene-induced fibrosarcoma grown in BALB/c mice. The tumor perfusion studies were assessed by two separate methods: ^{99m}Tc clearance and ⁸⁶Rb uptake. A single administration of arsenic trioxide (10 mg/kg i.p.) produced a preferential vascular shutdown in the tumor tissue at 2 and 6 h, leading to massive necrosis in the central part of the tumor. The phenomenon was repeatable at intervals of weekly administration of the drug in the same tumor. Normal skin, muscle, and kidney were relatively unaffected by arsenic trioxide. These results suggest that the drug may be investigated as an adjunct to the standard cancer therapeutic modalities.

Introduction

Arsenic compounds have been used in traditional medicine in many countries for >1000 years. Approximately 100 years ago, Western medicine adopted some of these compounds, used them widely (1), and subsequently abandoned them because of their toxic and oncogenic effects after chronic exposure. Arsenic compounds have received renewed attention in recent years following clinical observations of their effect on APL.³ In 1996, a group at Shanghai Institute of Hematology in China reported that As₂O₃ was an effective agent against APL even in patients who relapsed after retinoic acid-induced remission (2). The rate of clinically complete remission with As₂O₃ treatment was >80% without obvious acute toxicity, including bone marrow suppression. These spectacular clinical observations, confirmed by others (3-5), initiated investigations into the molecular mechanisms of As₂O₃-induced cell death.

It has been documented that arsenic can induce apoptosis in leukemic cells by activating apoptotic pathways (5, 6). However, the primary target of arsenic-induced cytotoxicity remains unknown. Trivalent arsenicals are regarded primarily as sulfhydryl reagents at the biochemical level. As₂O₃ inhibits many enzymes by reacting with biological ligands containing available sulfhydryl groups. Among them, arsenic targets vicinal thiol groups in tubulin with a high specificity (7). We postulated that As₂O₃ may have an antivascular effect on solid tumors because tubulin has been shown to be a primary target for other antitumor and antivascular agents, such as colchicine (8), flavone acetic acid (9), and combretastatin-A4 (10). We report the first *in vivo* observation demonstrating the antivascular effect of As₂O₃ in well-established murine solid tumors.

Received 9/13/99; accepted 10/29/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported in part by NIH Grants NIH-CA59542, NIH-CA44114, and DAMD17-99-9215.

² To whom requests for reprints should be addressed, at Department of Radiation Oncology, Henry Ford Health System, Detroit, MI 48202 Fax: (313) 916-3235; E-mail: younglew@yaho.com.

³ The abbreviations used are: APL, acute promyelocytic leukemia; As₂O₃, arsenic trioxide; Meth-A, methylcholanthrene-induced fibrosarcoma.

Materials and Methods*Mice, Tumors, and Compounds*

The murine tumor line used was Meth-A in BALB/c mice (11). Male mice, 6-8 weeks of age weighing 20-25 g were obtained from the Charles River Laboratories (Portage, MI). Meth-A cells were maintained by serial i.p. passage in syngeneic mice. For tumor implantation, Meth-A cells from ascitic fluid were collected between days 7 and 10 after cell implantation and washed twice with MEM medium. Approximately 1×10^5 viable Meth-A cells were inoculated into the skin of the right upper abdominal quadrant of the mouse. All experiments were carried out with intradermal tumors except for rubidium-uptake studies, which used s.c. tumors grown in the right rear leg of the mice.

As₂O₃ purchased from Sigma Chemical (St. Louis, MO) was dissolved in H₂O by continuous stirring. As₂O₃ was kept at 4°C as a stock solution. Dilution was made with 0.9% NaCl to a final concentration of 0.5 mg/ml As₂O₃. Dextrose was added to a final concentration of 5% to minimize acute arsenic toxicity (12).

Gross Morphology and Histopathology

Gross morphological changes were followed up to 48 h after a single injection of As₂O₃ (10 mg/kg i.p.). At selected time points, tumors were excised and histopathological examinations were performed. Tumor tissues from different groups were fixed in 10% neutral formalin and processed in paraffin wax. Sections (4 μm thick) were stained with H&E and assessed microscopically to investigate the temporal effect of As₂O₃.

Inhibition of Tumor Growth

Mice, typically five per group, with mean tumor diameters of 12.0 ± 1.0 mm were used in treatment or control studies. As₂O₃ was administered i.p. in doses of 10 mg/kg once a week for the duration of the experiment, starting 13 days after tumor inoculation. The same volume of normal saline with 5% dextrose was given to the control group. Tumor volume was assessed three times a week. Tumors were measured with a caliper, and tumor volumes were calculated assuming cylindrical growth using the formula $a/2 \times b/2 \times h \times \pi$, where *a*, *b*, and *h* are the minor and major dimensions and height, respectively. No attempt was made to correct the tumor volume measurement for the area of central necrosis.

Blood Perfusion Measurement

Two methods, technetium clearance and rubidium uptake, were used to assess the antivascular effect of a single injection of As₂O₃ (10 mg/kg i.p.). Both methods have been described in detail elsewhere (13, 14).

^{99m}Tc Clearance. Clearance measurements were performed as described previously (13). Briefly, mice were anesthetized with a 60 mg/kg i.p. injection of sodium pentobarbital and were immobilized using a jig that did not restrict blood flow to the tissue of interest. Technetium bound as sodium pertechnetate, Na^{99m}TcO₄ (typical activity, 6 μCi/ml), was injected directly into the central area of the intradermal tumor 10 min after the pentobarbital injection. A total volume of 10 μl was deposited, using a 29-gauge needle. Radioactivity was measured and recorded every 10 s, 200 times, using a multichannel scintillation counter (AccuSpec; Canberra Industries, Meriden, CT) interfaced with a personal computer. Raw data were corrected for the physical decay of tech-

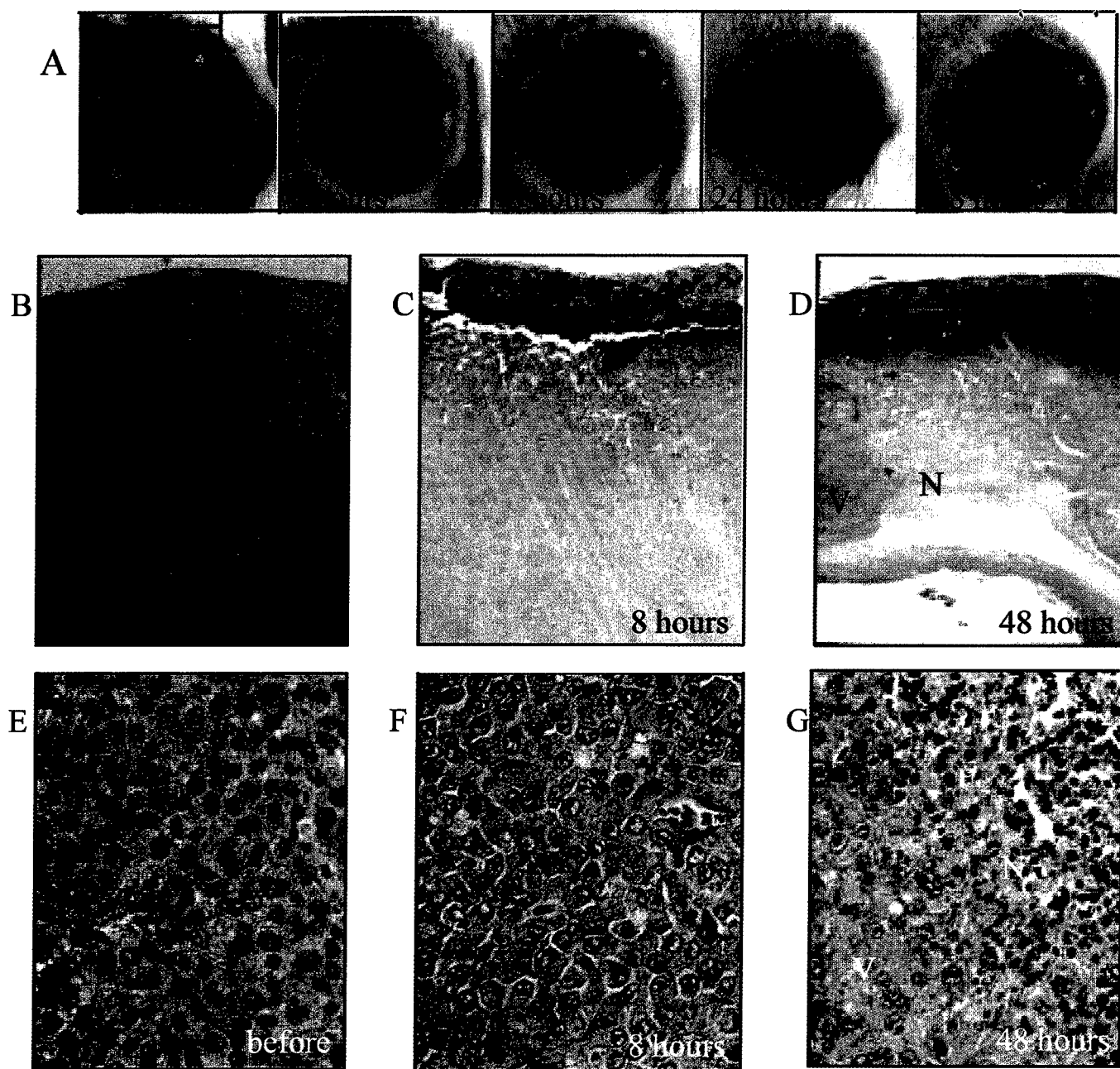


Fig. 1. Morphological features of Meth-A tumors in BALB/c mice. A, sequential gross morphological changes before and after the administration of As₂O₃ (10 mg/kg i.p.). B-G, histological features of the tumors stained with H&E. B-D, $\times 25$ magnification; E-G, $\times 400$ magnification. V, nonnecrotic zone; N, necrotic zone.

netium (6 h) and fit to a single exponential decay constant. No attempt to measure the partition coefficient was made, and the results are reported as clearance rate, not blood perfusion. The clearance rate was measured in each mouse before and 2, 6, and 24 h after arsenic treatment. Six tumor bearing mice with 12.0 ± 1.0 mm mean diameter tumors were used to study the effect of As₂O₃ on tumor clearance rate at each time point. Control experiments included clearance rate measurements in normal skin after As₂O₃ and also in untreated control tumors.

⁸⁶Rb Uptake. Changes in blood perfusion were measured in mouse tissue, using the rubidium uptake method (14). The rubidium studies were performed using s.c. Meth-A tumors. The tumor site was not critical to the results because we obtained similar results with rubidium-uptake studies using intradermal tumors at selected time point. In all experiments, 5 μ Ci of ⁸⁶RbCl in a 0.1-ml volume were injected through a tail vein after anesthesia with 100 mg/kg ketamine and 10 mg/kg xylazine, and mice were sacrificed by cervical dislocation 60 s after injection. The tumors, kidney, skin, and muscle were excised and counted in a well-counter (1282 Comugamma Cs; Pharmacia LKB Nu-

clear Inc., Gaithersburg, MD). The ratio of radioactive counts from the tissue of interest to the counts in the standard ⁸⁶Rb solution equivalent to the total ⁸⁶Rb activity injected multiplied by 100 gives the percentage of cardiac output to the tissue of interest. In this study, we did not attempt to measure absolute blood flow; relative blood perfusion values are presented. ⁸⁶Rb uptake was measured before and 2, 6, and 24 h after arsenic treatment. At least 10 tumor-bearing mice were used for each time point. The results are reported as the percentage of ⁸⁶Rb uptake per gram of tissue and plotted as a percentage of the control tissue.

Results

Tolerance and Acute Effect of As₂O₃. The toxicity of As₂O₃ was studied using the BALB/c mouse strain prior to initiating the tumor response studies. The approximate LD₅₀ dose of an i.p. administration of As₂O₃ was 17 mg/kg. We used the maximally tolerated dose in this strain, which was 10 mg/kg. This dose induced reliable antitumor

effects on locally advanced tumors and was well tolerated even in repeated administrations. Within 30 min after injection, mice showed signs and symptoms of acute toxicity, consisting of slurred motion, lethargy, and piloerection, but recovered within 6 h. No weight loss or other subacute toxicity was observed during the course of experiment.

Gross Morphological and Histopathological Changes. Fig. 1A illustrates gross tumor morphology before and after a single injection of the drug. As₂O₃ induced a pronounced central necrosis in the tumor, which was observed within 4 h and steadily progressed until at least 48 h. More than 70% of the tumor tissue underwent central necrosis, and the necrotic area formed a crust that became stony hard by 48 h. The tumor tissue under the crust was malleable. The peripheral zone of tumor tissue appeared unaffected. Tumor regrowth appeared to be initiated from the peripheral zone.

The sequential histopathological changes after drug treatment are shown in Fig. 1, B–G. Untreated control tumors consist of undifferentiated round cells with evidence of focal necrosis and a thin layer of amorphous debris on the surface. Beginning at 8 h after the treatment, there were early signs of cell death. The majority of cells had separated from one another and rounded. Amorphous debris on the surface of the tumor became thicker. As early as 4 h, there were foci of vascular congestion and a minimal amount of interstitial hemorrhage continuing up to 8 h, but there was no evidence of intravascular coagulation or thrombosis. By 24 h, the central portion of the tumor became uniformly necrotic. Most of necrotic cells showed faint cytoplasmic staining and condensed, pyknotic nuclei. The peripheral area of the tumor was unaffected, with a well-demarcated interface between the viable and necrotic tissues. This phenomenon became more pronounced by 48 h.

Tumor Growth Delay. Fig. 2 shows changes in the tumor volume as a function of time after a weekly administration of the drug. Immediately after the drug was administered, the tumor ceased to grow for ~2 days and then resumed growing. The regrowth was always seen from the periphery of the tumor. After administration of a second dose of the drug, tumor growth stopped for ~2 days, after which the tumor continued growing. A similar growth inhibitory pattern was observed after a third dose of the drug.

Effect of As₂O₃ on Regional Blood Perfusion in Tumor Tissue and Various Organs. Fig. 3 shows the ^{99m}Tc clearance rate and ⁸⁶Rb uptake as a function of time after a single administration of As₂O₃.

Clearance. In the absence of As₂O₃, technetium cleared from the tumor site rapidly. The initial half-time of clearance was ~8–9 min,

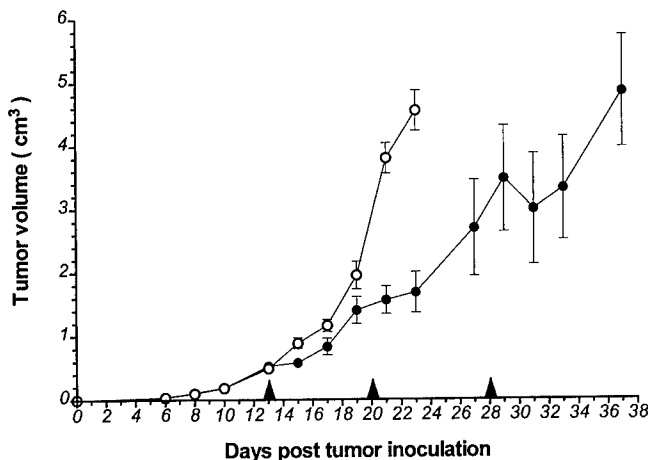


Fig. 2. Effect of As₂O₃ on the growth of Meth-A tumors in BALB/c mice. ○, untreated control group (vehicle only); ●, As₂O₃ group, 10 mg/kg i.p. The arrowheads indicate the times of As₂O₃ administration. Data are the means of five animals per group. Bars, SE.

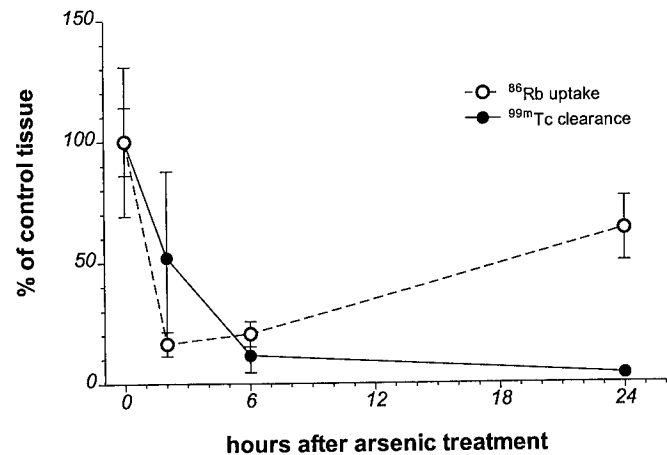


Fig. 3. Effect of As₂O₃ (10 mg/kg) on the blood perfusion in Meth-A tumors in the BALB/c mice measured by ⁸⁶Rb uptake and ^{99m}Tc clearance. Each point represents the mean of >10 mice for the rubidium uptake and 6 mice for ^{99m}Tc clearance. Bars, SE.

and this clearance rate did not change with subsequent injections even with seven repeat injections in the same area (data not shown). In contrast, the rate of technetium clearance was markedly reduced by the administration of As₂O₃ and remained low at 24 h, consistent with morphological observations.

Technetium clearance was $4.94 \pm 1.53 \text{ h}^{-1}$ before treatment and 2.57 ± 1.70 , 0.58 ± 0.35 , and $0.18 \pm 0.09 \text{ h}^{-1}$ at 2, 6, and 24 h after treatment, respectively. The calculated percentages relative to the control tumor were 52.1 ± 35.5 , 11.6 ± 7.1 , and $3.9 \pm 1.8\%$ of the control at 2, 6, and 24 h, respectively.

Uptake. Rubidium uptake in Meth-A tumors showed that a significant decrease in tumor blood perfusion occurred at 2 and 6 h after As₂O₃ injection and that tumor blood perfusion had recovered by 24 h. Rubidium uptake was $1.50 \pm 0.21\%/g$ tissue before treatment, and it decreased to 0.20 ± 0.07 , 0.30 ± 0.08 , and $1.0 \pm 0.20\%/g$ tissue at 2, 6, and 24 h after treatment, respectively. The calculated percentage relative to control tumor blood perfusion was 16.4 ± 5.0 , 20.5 ± 5.2 , and $64.3 \pm 13.3\%$ of the control at 2, 6, and 24 h respectively. The data of 2 and 6 h after the drug treatment were similar to the trends for the clearance rate. The uptake data at 24 h were inconsistent with both technetium clearance and morphological observation (see "Discussion"). Similar trends in uptake were observed in two other tumor lines studied (FSaII in C3H mice and SCK in A/J mice; data not shown).

Fig. 4 illustrates the effect of As₂O₃ (10 mg/kg) on rubidium uptake in the kidney, skin, and leg muscle measured at the same time as the measurement in the tumor. Blood perfusion in the skin and muscle was not changed significantly compared with control values. The maximum reduction of blood perfusion in normal tissues was that of the kidney at 2 h after As₂O₃ ($60.0 \pm 5.5\%$ of control). The reduction in blood perfusion reduction to the tumor was much greater than that in the normal tissues studied.

Discussion

The data clearly demonstrate that As₂O₃ induces a prompt selective vascular shut down leading to massive central necrosis in Meth-A sarcoma grown in BALB/c mice. The profound tumor response occurred at a dosage of As₂O₃ that was well tolerated even in repeated administrations. The phenomenon was repeatable on subsequent administration on the same tumor.

Two different methods of blood perfusion measurements gave complementary information. Technetium clearance, which allows

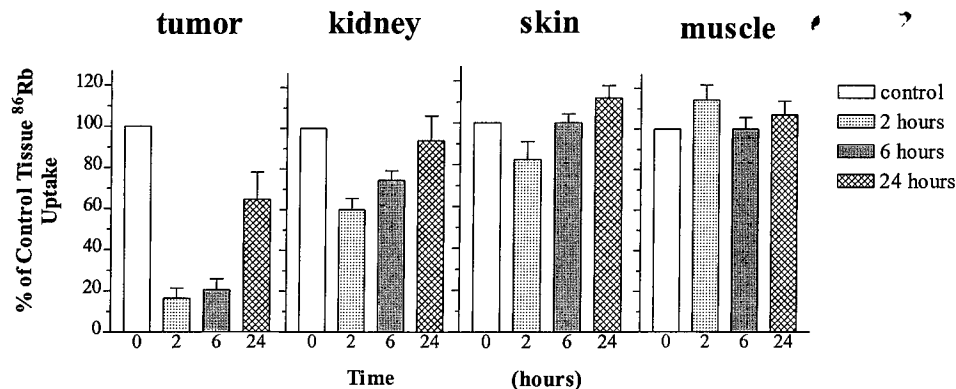


Fig. 4. Changes in blood perfusion as a function of time in normal tissues and Meth-A tumor measured by ⁸⁶Rb uptake. Values are the means of >10 mice at each time point.

multiple measurements in the same tumor, demonstrated a decreased clearance rate with increasing time after As₂O₃ treatment. This finding corresponded well with the morphological findings. Rubidium uptake, a single measurement in different animals, yielded decreased blood perfusion relative to controls up to 6 h after As₂O₃ treatment, with some recovery at 24 h. Further analysis of the perfusion data at the 24-h time point showed that perfusion in half of the group of tumors ($n = 6$ of 14) remained low (mean \pm SE, 24.4 \pm 10.9% of the control tumor) and that perfusion in the other tumors ($n = 8$) recovered to near control values (94.2 \pm 46.1%). Separate uptake studies with intradermal Meth-A tumors showed results similar to the results for the s.c. tumors. When rubidium activity was assessed from the central and peripheral parts of the tumor separately, the central part of the tumors had a lower uptake than the tumor periphery (37.6 \pm 25.1% versus 82.2 \pm 53.3%). These data indicate that the discrepancy between technetium clearance and rubidium uptake could be explained by the rubidium activity in the peripheral zone of the tumor. Regardless of the 24-h data, As₂O₃ treatment causes a rapid, selective shut down of tumor blood perfusion and induces massive necrosis in the central part of the tumor.

Several other antitumor agents, including combretastatin A-4 (10), colchicine (8), flavone acetic acid (9) and hydralazine (15), have been shown to have their primary effects on tumor vasculature. Their effects are characterized by massive vascular shut down in the central region of the tumor. Flavone acetic acid, hydralazine, and colchicine are either ineffective or toxic in species other than mice. Combretastatin A-4 and new flavone acetic acid derivatives have shown promise in overcoming these past problems. Similarly, As₂O₃ has been used in humans for many years, and preliminary studies on rats in this laboratory indicate that its antitumor effects may not be species specific.⁴

The underlying mechanisms of vascular shut down and central necrosis are not easily explained by our current knowledge of the known biological effects of As₂O₃. However, several possible scenarios are being entertained to illustrate this phenomenon. One possible mechanism is that As₂O₃ can induce selective endothelial cell injury, resulting in vascular shut down to tumor tissue. This mechanism is based on the observation that As₂O₃ is a noncompetitive inhibitor of GTP binding to tubulin (7). Combretastatin A-4 affects normal microtubule function and has been shown to have a greater toxicity toward endothelial cells compared with tumor cells *in vitro* and induces selective vascular shut down in P22 rat carcinosarcoma *in vivo* (10). Another tubulin-binding agent, colchicine, produced hemorrhagic necrosis in experimental tumors (8).

Another possible scenario is tumor necrosis factor-mediated

vascular shut down, because the gross observation after As₂O₃ treatment is similar to that seen with endotoxin-induced necrosis. The evidence is strengthened by the observation that cultured human keratinocytes overexpress tumor necrosis factor mRNA after arsenic exposure (16).

Apoptosis or/and necrosis represents a third possible scenario. As₂O₃ triggers apoptosis in APL cells by degrading promyelocytic leukemia/retinoic acid receptor- α fusion protein (6, 17), but it also induces apoptosis in other hematological malignancies and several solid tumor cells *in vitro* (7, 18, 19) at a lower concentration (0.5–5 μ M). At higher concentrations (>5 μ M), As₂O₃ causes acute necrosis in various cell lines (7). In this scenario, we can speculate massive apoptosis or/and necrosis in the central area of the tumor after As₂O₃ exposure.

The decrease in blood perfusion in the kidney and tumors observed with rubidium uptake suggests a systemic effect of As₂O₃. Changes in blood pressure measured using plethysmography on the tail revealed that the systolic pressure dropped to 50% of the original pressure 1 h after As₂O₃ injection (data not shown), remained low for ~1 h, and recovered gradually to near normal levels by 6 h. As₂O₃-induced systemic hypotension in the presence of high tumor interstitial fluid pressure, especially in the central area of the tumor (20), may be considered a contributing factor in inducing a vascular shut down.

We currently are investigating these possible scenarios, but most of our preliminary data do not unequivocally confirm any of the possible scenarios. Nonetheless, the present findings that As₂O₃ induces a rapid tumor mass reduction with a viable, presumably well-oxygenated peripheral zone of the tumor may provide an opportunity to combine this approach with standard cancer therapy that includes radiation and cytotoxic agents.

References

- Sears, D. A. History of the treatment of chronic myelocytic leukemia. *Am. J. Med. Sci.*, 296: 85–86, 1988.
- Shen, Z. X., Chen, G. Q., Ni, J. H., Li, X. S., Xong, S. M., Qiu, Q. Y., Zhu, J., Tang, W., Sun, G. L., Yang, K. Q., Chen, Y., Zhou, L., Fang, Z. W., Wang, Y. T., Ma, J., Zhang, P., Zhang, T. D., Chen, S. J., Chen, Z., and Wang, Z. Y. Use of arsenic trioxide (As₂O₃) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood*, 89: 3354–3360, 1997.
- Westervelt, P., Pollock, J., Haug, J., Ley, T. J., and DiPersio, J. F. Response and toxicity associated with dose escalation of arsenic trioxide in the treatment of resistant acute promyelocytic leukemia. *Blood*, 90(Suppl. 1): 249b, 1997.
- Matthews, J. H., Tompkins, K., Hasegawa, W., Brander, M. J., Rapson, D. A., and Galbraith, P. R. Successful arsenic therapy in relapsed acute promyelocytic leukemia resistant to all-trans retinoic acid. *Proc. Am. Soc. Clin. Oncol.*, 17: 122, 1998.
- Soignet, S. L., Maslak, P., Wang, Z. G., Jhanwar, S., Calleja, E., Dardashti, L. J., Corso, D., DeBlasio, A., Gabrielove, J., Scheinberg, D. A., Pandolfi, P. P., and Warrell, R. P., Jr. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. *N. Engl. J. Med.*, 339: 1341–1348, 1998.
- Chen, G. Q., Zhu, J., Shi, X. G., Ni, J. H., Zhong, H. J., Si, G. Y., Jin, X. L., Tang, W., Li, X. S., Xong, S. M., Shen, Z. X., Sun, G. L., Ma, J., Zhang, P., Zhang, T. D.,

⁴ Y. S. Lew *et al.*, unpublished results.

AS₂O₃ INDUCES CENTRAL NECROSIS IN MURINE TUMORS

- Gazin, C., Naoe, T., Chen, S. J., Wang, Z. Y., and Chen, Z. In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As₂O₃) in the treatment of acute promyelocytic leukemia: As₂O₃ induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR α /PML proteins. *Blood*, 88: 1052-1061, 1996.
7. Li, Y. M., and Broome, J. D. Arsenic targets tubulins to induce apoptosis in myeloid leukemia cells. *Cancer Res.*, 59: 776-780, 1999.
 8. Ludford, R. J. Colchicine in the experimental chemotherapy of cancer. *J. Natl. Cancer Inst.*, 6: 89-101, 1945.
 9. Zwi, L. J., Baguley, B. C., Gavin, J. B., and Wilson, W. R. Blood flow failure as a major determinant in the antitumor action of flavone acetic acid. *J. Natl. Cancer Inst.*, 81: 1005-1013, 1989.
 10. Tozer, G. M., Prise, V. E., Wilson, J., Locke, R. J., Vojnovic, B., Stratford, M. R., Dennis, M. F., and Chaplin, D. J. Combretastatin A-4 phosphate as a tumor vascular-targeting agent: early effects in tumors and normal tissues. *Cancer Res.*, 59: 1626-1634, 1999.
 11. Ryu, S., Brown, S. L., Kolozsvary, A., and Kim, J. H. Increased tumour response of a murine fibrosarcoma to low temperature hyperthermia and low dose rate brachytherapy. *Int. J. Hyperthermia*, 12: 635-643, 1996.
 12. Reichl, F. X., Kreppel, H., Szinicz, L., Fichtl, B., and Forth, W. Reduction of arsenic trioxide toxicity in mice by repeated treatment with glucose. *Arch. Toxicol. Suppl.*, 14: 225-227, 1991.
 13. Brown, S. L., Hunt, J. W., and Hill, R. P. A comparison of the rate of clearance of xenon (¹³³Xe) and pertechnetate ion (^{99m}TcO₄⁻) in murine tumors and normal leg muscles. *Nucl. Med. Biol.*, 15: 381-390, 1988.
 14. Song, C. W., Patten, M. S., Chelstrom, L. M., Rhee, J. G., and Levitt, S. H. Effect of multiple heatings on the blood flow in RIF-1 tumours, skin and muscle of C3H mice. *Int. J. Hyperthermia*, 3: 535-545, 1987.
 15. Lin, J. C., and Song, C. W. Effects of hydralazine on the blood flow in RIF-1 tumors and normal tissues of mice. *Radiat. Res.*, 124: 171-177, 1990.
 16. Germolec, D. R., Yoshida, T., Gaido, K., Wilmer, J. L., Simeonova, P. P., Kayama, F., Burleson, F., Dong, W., Lange, R. W., and Luster, M. I. Arsenic induces overexpression of growth factors in human keratinocytes. *Toxicol. Appl. Pharmacol.*, 141: 308-318, 1996.
 17. Shao, W., Fanelli, M., Ferrara, F. F., Riccioni, R., Rosenauer, A., Davison, K., Lamph, W. W., Waxman, S., Pelicci, P. G., Lo Coco, F., Avvisati, G., Testa, U., Peschle, C., Gambacorti-Passerini, C., Nervi, C., and Miller, W. H., Jr. Arsenic trioxide as an inducer of apoptosis and loss of PML/RAR α protein in acute promyelocytic leukemia cells. *J. Natl. Cancer Inst.*, 90: 124-133, 1998.
 18. Zhu, X. H., Shen, Y. L., Jing, Y. K., Cai, X., Jia, P. M., Huang, Y., Tang, W., Shi, G. Y., Sun, Y. P., Dai, J., Wang, Z. Y., Chen, S. J., Zhang, T. D., Waxman, S., Chen, Z., and Chen, G. Q. Apoptosis and growth inhibition in malignant lymphocytes after treatment with arsenic trioxide at clinically achievable concentrations. *J. Natl. Cancer Inst.*, 91: 772-778, 1999.
 19. Rousselot, P., Labaume, S., Marolleau, J. P., Larghero, J., Noguera, M. H., Brouet, J. C., and Femand, J. P. Arsenic trioxide and melarsoprol induce apoptosis in plasma cell lines and in plasma cells from myeloma patients. *Cancer Res.*, 59: 1041-1048, 1999.
 20. Boucher, Y., Baxter, L. T., and Jain, R. K. Interstitial pressure gradients in tissue-isolated and subcutaneous tumors: implications for therapy. *Cancer Res.*, 50: 4478-4484, 1990.

RADIOBIOLOGY/RADIATION ONCOLOGY 2

#1868 PRECLINICAL STUDIES TO EVALUATE TOXICITY AND EFFICACY OF IPDR AS A RADIOSENSITIZING PRODRUG. Timothy J Kinsella, Jane E Schupp, Thomas W Davis, Katherine Vielhuber, Keith Kunugi, Suzanne E Berry, Hwa-shin Hwang, Frank Balis, C. Warren, John Barnett, and Howard Sands, *Case Western Reserve Univ, Cleveland, OH*

Many previous studies have shown the thymidine analog iododeoxyuridine (IUdR) to be an effective radiosensitizing agent, achieving differential radiosensitization of rapidly proliferating tumor cells when given as a prolonged infusion prior to and during irradiation. However, systemic toxicities to rapidly proliferating normal tissues limit the dose rate and duration of the infusion. The studies reported here evaluate the effectiveness of an IUdR prodrug, 5-iodo-2-pyrimidinone-2 deoxyribose (IPdR), which is converted to the active drug IUdR by a liver enzyme aldehyde oxidase, resulting in high IUdR plasma levels for up to one hour following oral administration IPdR. Our studies have shown that p.o. IPdR is well tolerated, with doses of up to 1500 mg/kg/d x 14 days producing no side effects in athymic mice. Similar dosings in ferrets resulted in some moderate systemic toxicities, mild liver enlargement, and decreased motor activity. In athymic mice with two different human tumor xenograft tissues, there was a > 2 fold increase in % IUdR-DNA incorporation in tumor tissue with IPdR and < 2 fold decrease in normal tissue compared to IUdR. This increase of analog incorporation into tumor cell DNA translates to a sensitizer enhancement ratio (SER) of 1.31 to 1.5 with the p.o. IPdR plus irradiation treatment versus the SER of >1.1 for IUdR continuous infusion followed by irradiation. We have also determined that this drug crosses the blood/brain barrier in Rhesus monkeys in a recent pharmacokinetic study in which concentrations of the prodrug IPdR and active drug IUdR of up to 2 µM were found in CSF levels at 30 minutes following a single i.v. bolus dose of IPdR at 50 mg/kg.

#1869 ARSENIC TRIOXIDE: ANTI-VASCULAR EFFECT AND RADIOSENSITIZATION. Young S Lew, Andrew Kozlosvary, Stephen L Brown, Chang W Song, and Jae H Kim, *Henry Ford Health Sci Ctr, Detroit, MI, and Univ of Minnesota Med Sch, Minneapolis, MN*

The purpose of the study was to investigate the interaction of arsenic trioxide and radiation. Our data indicates that arsenic trioxide causes a preferential decrease in tumor blood flow, particularly in the central region of the tumor. Furthermore, arsenic trioxide administered after radiation enhances the effect of radiation on tumor growth. Tumor blood flow was assessed using clearance and uptake techniques. A single administration of arsenic trioxide (10mg/kg, i.p.) produced a preferential vascular shut down in the tumor tissue at 2 and 6 hours leading to extensive necrosis in the tumor core. Normal skin, muscle and kidney were relatively unaffected by arsenic trioxide. Based on the temporal effect of arsenic trioxide on tumor blood flow, radiation was delivered 1 hour before drug. Tumor growth delay, defined as the additional time necessary for a tumor to grow to 1cm in average diameter compared to untreated tumors, was 2.5, 10.5, and 26.1 days for groups of tumors administered radiation alone, drug alone and the combined therapies, respectively. The data is consistent with the hypothesis that enhancement of drug and radiation result from arsenic trioxide killing cells at low oxygen, which are resistant to radiation cytotoxicity and vulnerable to a reduction in blood flow.

#1870 EFFECTOR GENES RESPONSIBLE FOR IONIZING RADIATION INDUCED TUMOR RESISTANCE. Zhongkui Li, Jeffrey Y C Wong, Jianyi Wang, Alexander Khaletskiy, Leo M Lee, and Jian-Jian Li, *City of Hope National Med Ctr, Duarte, CA, and National Cancer Inst, Frederick, MD*

One of the challenges to the control of tumor growth by ionizing radiation is the prevention of resistance. Although several signal pathways have been described in the acute response to ionizing radiation, genes responsible for the development of radioresistance in tumor cells following fractionated radiation have not been well addressed. In the present study, we investigated the gene expression profiles in human breast cancer MCF-7 cells receiving fractionated gamma irradiation (2Gy per fraction, total dose 40 Gy). The resulted cells demonstrated a moderate radioresistance (1.5 ~ 1.7 fold). Using cDNA microarray, we found 19 genes were up-regulated in the irradiated cells. Among them, cyclin B1 and *CDKN1A* (*WAF-1*) were preferably expressed. Since both and the other up-regulated genes are related to TP53 transactivation, TP53 activity was measured in the radioresistant cells using luciferase reporters. Unexpectedly, TP53 transcriptional activity was decreased in the irradiated cells compared to the other two transcription factors AP-1 and NFκB. To confirm the radioresistant effects contributed by cyclin B1 and *CDKN1A*, clonogenic survival was measured in the radioresistant MCF-7 cells transfected with antisense oligonucleotides of cyclin B1 and/or *CDKN1A*. Antisense cyclin B1 inhibited 42% clonogenic survival at 5 Gy single dose irradiation. No effect in antisense *CDKN1A*, nor synergistic effect of both was observed. In summary, these results indicate that multiple effector genes are involved in signaling radioresistance formation in tumor cells receiving fractionated radiation. Cyclin B1 and/or other cell cycle elements may play a key role in this pathway which may be TP53 independent.

#1871 CHROMOSOMAL DAMAGE IN MELANOCYTES INDICATES THE GENOTOXIC ACTION OF ULTRAVIOLET(UV)-A AND UVB. Piet van Erp, Ria Kolb, Stan Pavel, Miklos Wenczl, and Albert Schothorst, *Leiden Univ Med Ctr, Leiden, Netherlands, Markusovski Hosp, Szombathely, Hungary, and Univ Hosp Nijmegen, Nijmegen, Netherlands*

Chromosomal defects are frequently present in malign and premalign pigmented skin disorders. It is not known, however, in what extent the UVA and the UVB part of sunlight play a role in their induction and how melanin acts. UVA induces DNA strand breaks in cultured human melanocytes and melanin acts as a photosensitizer for DNA damage (1). These lesions are rapidly repaired thus complicating an assessment of the genotoxic action of UV light. As micronuclei (MN) represent an irreversible chromosomal damage, MN induction in UV-irradiated asynchronously growing melanocytes were examined by flow cytometry. Melanocytes from an individual with white skin and red hair were cultured parallel, at basic (B) or at high tyrosine (HT) concentration to stimulate melanogenesis. Melanin content was monitored, cells were exposed to UVA or UVB irradiation and induction of MN and cell cycle changes were measured by flow cytometry. Dose dependent MN induction and G₂/M delay was observed after UVB or UVA irradiation. Melanocytes became less sensitive to UVB after increasing their pigment content than the same cells grown in the B medium and MN induction showed a positive correlation with G₂/M delay. However, after UVA irradiation more MN were found in the HT medium cultured cells than in those grown in the B medium but there was no difference in G₂/M delay. In conclusion, these results demonstrate the applicability of the MN assay for the assessment of the genotoxic effect of both UVA and UVB in melanocytes and that melanin acts as a 'two edged sword'. (1) E. Wenczl et al. *J. Invest. Dermatol.* 111:678-682, 1998

MOLECULAR CARCINOGENESIS 9: Site-specific Carcinogenesis

#1872 HRAS1 MUTATIONS FREQUENTLY OCCUR IN SMOKELESS TOBACCO-INDUCED ORAL CAVITY TUMORS BUT ARE RARE IN TUMORS OCCURRING IN CIGARETTE SMOKERS. Ernest M Cardwell, Shawn Glisson, Michael Gaugler, Alice Shum-Siu, Lan Su, Jeffery M Bumpow, and Fred J Hendler, *Univ of Louisville, Louisville, KY*

More than 30% of quid-induced oral cavity tumors (OCT) in India have *HRAS1* mutations whereas a recent report from the US suggests that *HRAS1* mutations are rare in American smokeless tobacco induced OCT (OCT_{OT}). To determine the incidence of *HRAS1* mutations in OCT_{OT}, exons 1 and II DNA was isolated from 26 OCT_{OT} developing in individuals who had never smoked, and from 32 tumors developing in cigarette smokers (OCT_{CS}). *HRAS1* mutations that affected the coding sequence were detected 8 of 26 OCT_{OT}. Only 2 of 33 OCT_{CS} had a *HRAS1* mutation and only 1 affected the coding sequence (P < .01). Among the OCT_{OT} 4 mutations occurred at codon 12. Mutations were also detected at codons 36, 56, 57, 60, 66, and 81. 2 tumors had multiple mutations involving codons 57 & 81 and 60 & 66. Loss of heterozygosity at *HRAS1* was detected in 4 OCT_{OT} and only in an OCT_{CS} codon 12 mutant which did not affect the *HRAS1* coding sequence (codon 12 C to T). G to A mutations occurred at 9 of 10 bases in OCT_{OT} and the OCT_{CS} where the mutation affected the coding sequence. An OCT_{CS} had an A to G transition. 50% of OCT analyzed have an apparent polymorphism in codon 27 (T to C) that does not affect the coding sequence. These data indicate that *HRAS1* mutations in OCT_{OT} have a similar incidence as observed in quid users. The preponderance of G to A mutations strongly suggests that OCT_{OT} have been induced by nitrosamines in the smokeless tobacco. Studies are underway to determine if G to A mutations are more prevalent in OCT_{CS}. Since 50% of mutations were observed at sites other than codons 12, 60, 61, *HRAS1* mutations apparently have been under reported in studies in which tumor DNA has not been sequenced.

#1873 CIGARETTE SMOKING IS STRONGLY ASSOCIATED WITH MUTATION OF THE K-RAS GENE IN PRIMARY ADENOCARCINOMA OF THE LUNG. E A Alawi, P A Decker, K M Doffek, M J Demeure, S C Yang, J J Sidransky, and Steven A Ahrendt, *Med Coll of Wisconsin, Milwaukee, WI, and Johns Hopkins Univ, Baltimore, MD*

The majority of lung cancer cases occur in current or former smokers, though little data exist directly linking tobacco carcinogens with common gene mutations in lung cancer. We hypothesized that smoking would be associated with K-ras gene mutations, which are common in primary lung adenocarcinoma. Smoking histories were obtained from 97 patients with adenocarcinoma of the lung; mutations were detected in the primary tumor using an allele-specific PCR assay. Eighty-seven of the 97 patients (90%) with lung adenocarcinoma were smokers. The nonsmokers were significantly older than 95 smokers (72 ± 64 ± 1 yrs, p=0.01). Nonsmokers also had smaller tumors (2.6 ± 0.2 cm vs 3.6 ± 0.2 cm) and less commonly had lymph node metastases (10% versus 44%). K-ras mutations were detected in 38 of 97 tumors and were significantly more common in smokers than in nonsmokers (44% versus 0%, p<0.01). Smokers with adenocarcinoma of the lung were younger, more likely to have K-ras mutations, and tended to have larger, more advanced tumors than nonsmokers. This link between cigarette smoking and K-ras mutations in adenocarcinoma of the lung supports the role of specific carcinogens in tobacco in the etiology of lung malignancy.

T
F
C
K-
ne
ing
ty;
for
ca
fac
lat-
no-
ER
E1
ele
cor
inh.
alth
form
turr.

#18
LUN
Blar
eng:
Dep
In
silic-
sque
12m
chio
stud
and
VEG
and/r
carci
high
obse-
carci
tumo
serve
Pro-a
adenc
plasti
distrib
concl
mode
towar
contrc

#187
BY O
CLAR
TX
The
respira
charac
can lea
model
the Clar
promot-
visible
approx
istent
the deve
with alte
The rate
Bromod-
cyclins.
Biotin N
pathway
E in the
lower lev
lungs th
generate
igation
understa
of the Ci
lung can
Proceeo