[CANCER RESEARCH 57, 2922-2928, July 15, 1997]

Tirapazamine-induced Cytotoxicity and DNA Damage in Transplanted Tumors: Relationship to Tumor Hypoxia[1]

Bronwyn G. Siim, Douglas R. Menke, Mary Jo Dorie, and J. Martin Brown[2]

Department of Radiation Oncology, Stanford University School of Medicine, Stanford, California 94305-5468 [B. G. S., D. R. M., M. J. D., J. M. B.], and Section of Oncology, Department of Pathology, The University of Auckland, Private Bag 92019, Auckland, New Zealand [B. G. S.]

ABSTRACT

Tirapazamine (TPZ) is a hypoxia-selective bioreductive drug currently in Phases II and III clinical trials with both radiotherapy and chemotherapy. The response of tumors to TPZ is expected to depend both on the levels of reductive enzymes that activate the drug to a DNA-damaging and toxic species and on tumor oxygenation. Both of these parameters are likely to vary between individual tumors. In this study, we examined whether the enhancement of radiation damage to tumors by TPZ can be predicted from TPZ-induced DNA damage measured using the comet assay. DNA damage provides a functional end point that is directly related to cell killing and should be dependent on both reductive enzyme activity and hypoxia. We demonstrate that TPZ potentiates tumor cell kill by fractionated radiation in three murine tumors (SCCVII, RIF-1, and EMT6) and two human tumor xenografts (A549 and HT29), with no potentiation observed in a third xenograft (HT1080). Overall, there was no correlation of radiation potentiation and TPZ-induced DNA damage in the tumors, except that the nonresponsive tumor xenograft had significantly lower levels of DNA damage than the other five tumor types. However, there was a large tumor-to-tumor variability in DNA damage within each tumor type. This variability appeared not to result from differences in activity of the reductive enzymes but largely from differences in oxygenation between individual tumors, measured using fluorescent detection of the hypoxia marker EF5. The results, therefore, suggest that the sensitivity of individual tumors to TPZ, although not necessarily the response to TPZ plus radiation, might be assessed from measurements of DNA damage using the comet assay.

INTRODUCTION

TPZ[3] (3-amino-1,2,4-benzotriazine 1,4-dioxide; SR 4233; WIN 59075; Tirazone) is the lead compound in a series of benzotriazine di-*N*-oxides that exhibit selective toxicity under hypoxic conditions. TPZ is currently undergoing Phase II clinical trials in combination with radiotherapy and Phase III trials in combination with the anticancer drug cisplatin. The hypoxic cytotoxicity of TPZ results from activation by reductive enzymes that add an electron to the parent drug to produce a radical species that causes DNA ssb, dsb, and chromosome aberrations (1, 2). The mechanism of aerobic drug toxicity, which generally requires drug concentrations 50–200-fold higher than concentrations required for equivalent cell killing under hypoxia, has not been unequivocally defined,but appears to result largely from the production of active oxygen species by redox cycling of the TPZ radical in the presence of oxygen (3–5).

The rationale for the use of hypoxia selective bioreductive drugs, such as TPZ, in cancer therapy is that: (*a*) the presence of hypoxic regions in solid tumors should allow reductive metabolism to a cytotoxic species to occur preferentially within the tumors rather than in normal tissues; and (*b*) it is the hypoxic cells in solid tumors that are most resistant to cancer therapy, including radiation and chemotherapeutic drugs (6–8). The presence of hypoxic cells has been demonstrated both in transplanted rodent tumors and in human tumor xenografts (9, 10) and in many different types of human malignancies, including glioblastomas, carcinomas of the breast, head and neck, cervix, and rectum, and melanomas (11). Strong evidence that hypoxic cells adversely affect response to radiotherapy has been provided from studies with head and neck carcinomas (12, 13) and cervical cancers (14–16). Because of this tumor-specific hypoxia, the addition of TPZ to a fractionated radiation protocol would be expected to be beneficial because TPZ would kill the radioresistant hypoxic tumor cells, thereby complementing the radiation killing of the aerobic tumor cells (8, 17). This has been demonstrated in preclinical studies in which TPZ produced a large potentiation of radiation killing in transplanted murine tumors (18).

In the clinic, it would be an advantage to be able to predict which patients were going to respond to treatment. Mechanistic studies (1) suggest that the response of tumors to TPZ will be largely determined by two parameters; (*a*) the activity of the reductive enzyme(s) that activate TPZ to a cytotoxic species; and (*b*) the extent of hypoxia. Thus, although it is possible in theory to predict the response of individual tumors to TPZ by measuring both their oxygen levels (19) and reductive enzyme activity, this approach is currently limited by the uncertain identity of the key enzyme(s) involved in the bioactivation of TPZ (20). In addition, because of the difficulty of knowing how to combine measurements of enzyme activity with oxygen levels, it would be preferable if a single measurement could be made that would predict tumor response.

We and others have previously suggested that it might be possible to use the alkaline comet assay, which measures DNA damage (ssb) to predict the response of individual tumors to TPZ (20, 21). DNA damage measured using this assay should provide a functional end point that is dependent on both the oxygen tension and the activity of reductase(s) that convert TPZ to a DNA-damaging and cytotoxic species (Fig. 1). We previously demonstrated a highly significant correlation between TPZ-induced cell killing under hypoxia and DNA damage measured using the alkaline comet assay for a number of murine (SCCVII, RIF-1, and EMT6) and human (A549, HT1080, and HT29) tumor cell lines *in vitro* (20). In the present study, we have grown these same cell lines as transplanted tumors in mice and investigated whether TPZ-induced DNA damage is predictive of the enhancement by TPZ of cell killing by fractionated radiation. Also, because the level of hypoxia is likely to be a key determinant of tumor response, we have assessed the oxygen dependence of DNA damage both *in vitro* and *in vivo* using the fluorinated etanidazole derivative EF5 as a hypoxia marker.

MATERIALS AND METHODS

Animals and Tumors. Human fibrosarcoma HT1080 and lung carcinoma A549 cells were obtained from the American Type Culture Collection, and colon adenocarcinoma HT29 cells were obtained from Dr. R. M. Sutherland (SRI International, Menlo Park, CA). Derivation of the SCCVII squamous carcinoma (22), EMT6 mammary carcinoma (23), and RIF-1 fibrosarcoma (24) cell lines has been described previously. All cell lines were maintained in

Received 1/8/97; accepted 5/13/97.

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 by Grant CA 15201 from the National Cancer Institute.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: TPZ, tirapazamine; ssb, single strand break(s); dsb, double strand break(s); FNA, fine needle aspirate; EF5, 2-(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide; CV, coefficient of variation.

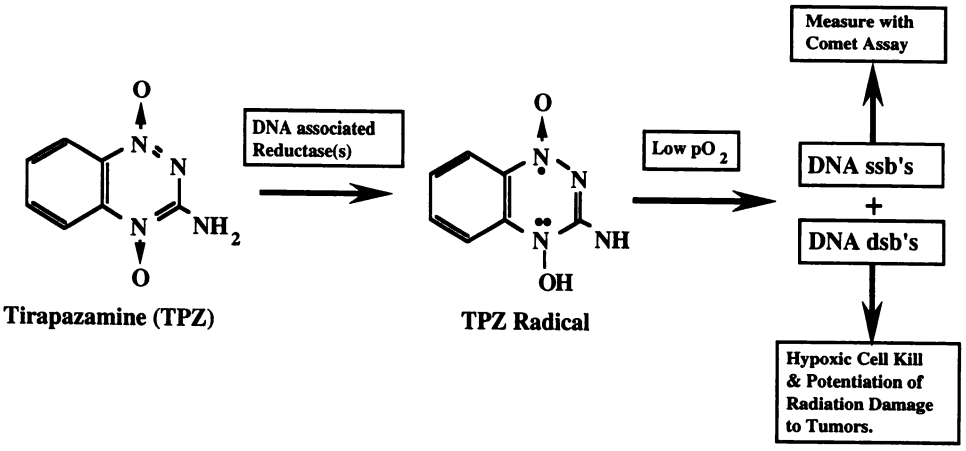

Fig. 1. Diagrammatical representation of the requirements for both reductive enzymes and hypoxia (low pO_2) for TPZ to produce DNA dsb, which lead to cell killing, and DNA ssb, which can be assayed with the alkaline comet assay.

culture as detailed previously (20). Tumors were grown from cells (2×10^5 in 0.05 ml medium) implanted intradermally in the lower back of C3H/Km (SCCVII and RIF-1), BALB/c (EMT6), or scid (HT1080, A549, and HT29) mice. Experiments were started when the tumors reached a mean diameter of 8–10 mm.

Drugs. TPZ was kindly supplied by Sanofi-Winthrop, Inc. (Great Valley, PA). The drug was dissolved in physiological saline at a concentration of 1 mg/ml and injected i.p. on a mmol/kg basis. EF5, a pentafluorinated derivative of etanidazole, and the monoclonal antibody ELK3-51, conjugated with the fluorescent dye cy3 for detection of covalent EF5 adducts (25), were generously supplied by Drs. Cameron Koch (Department of Radiation Oncology, University of Pennsylvania, Philadelphia, PA) and Edith Lord (University of Rochester Cancer Center, Rochester, NY). The EF5 was dissolved in saline at a concentration of 2 mM and injected i.p. at 0.1 mmol/kg body weight. Hoechst 33342 (Sigma Chemical Co., St. Louis, MO) was dissolved in saline at a concentration of 8 mg/ml, and 0.1 ml was injected i.v. into the tail vein. Nicotinamide (Sigma), dissolved in saline at 100 mg/ml, was injected i.p. at a dose of 1000 mg/kg. Fresh solutions of Hoechst 33342 and nicotinamide were prepared for each experiment. For modulation of tumor oxygenation, nicotinamide was administered 30 min before injection of EF5 and the start of carbogen (95% O_2 + 5% CO_2) breathing. TPZ was administered 30 min later, and tumors were excised after another 30 min. A 1-h interval between nicotinamide and radiation has been shown to be optimal for achieving radiosensitization in the SCCVII (26), RIF-1 (27), and KHT (28) tumors. In experiments in which tumor oxygenation was lowered, mice were injected with EF5 and placed in an atmosphere of 10% oxygen. TPZ was injected 30 min later, and tumors were sampled by FNA and/or excised and dissociated with enzyme cocktail after an additional 30 min.

Radiation Studies. Mice were treated with eight doses of 2.5 Gy radiation (two times daily) with 0.12 mmol/kg TPZ or saline administered 30 min prior to each radiation dose. Irradiation of unanesthetized mice was performed using individual lead boxes with the tumors protruding through a cut-out portion at the rear of the box and a 250-kVp X-ray machine that delivered a dose rate of 1.36 Gy/min.

Tumor response was evaluated using the *in vivo/in vitro* excision assay. Mice were killed 18 h after the final 2.5 Gy irradiation, and tumors were excised, minced, and dissociated using an enzyme cocktail [DNase (bovine pancreas, 3000 units/tumor), collagenase (type 1A, 280 units/tumor), and Pronase (*Streptomyces griseus*, 225 units/tumor) in HBSS] with incubation at 37°C for 30 min, followed by plating of cells for clonogenic survival. Colonies were stained and counted after 13 days incubation at 37°C, except for HT1080, which was stained after 14 days.

Measurement of DNA Damage by the Comet Assay. Thirty min after administration of a single dose of TPZ, single-cell suspensions were obtained from tumors either by enzymatic disaggregation of excised tumors as described above or by FNA biopsy from mice anesthetized with 0.016 ml/g Nembutal administered 5 min before biopsy (Abbott Laboratories, Chicago, IL). On average, 10^5 cells were obtained from each FNA. Cells were suspended in ice-cold calcium- and magnesium-free PBS at a concentration of approximately 2×10^4 cells/ml. The comet assay was performed according to the method of Olive *et al.* (29, 30). Briefly, 0.5 ml of cell suspension was mixed with 1.5 ml of a 1% solution of low gelling temperature agarose (Sigma type VII), pipetted onto a microscope slide, and allowed to gel on a cold block. The cells were lysed by immersing the slides in a alkaline solution (30 mM NaOH, 1 M NaCl, and 0.1% *N*-lauroylsarcosine) for 60 min. This was followed by $3 \times$ 20-min rinses in 30 mM NaOH, 2 mM EDTA, and then electrophoresis at 0.6 V/cm for 22 min in a fresh solution of 30 mM NaOH, 2 mM EDTA. Slides were rinsed with distilled water for 15 min, then stained with propidium iodide (2.5 μg/ml) for 15 min, and analyzed within 48 h. The DNA from individual cells was visualized using a ×20 objective with a Nikon Optiphot microscope attached to an Ikegami 4612 CCD camera and fluorescence image analysis system described by Olive *et al.* (31). DNA damage was quantitated as the tail moment, the product of percentage of DNA in the tail, and the mean tail length. At least 200 comets were analyzed per sample.

***In Vitro* Drug Exposure.** TPZ-induced DNA damage was compared in SCCVII and RIF-1 cells grown in tissue culture or freshly prepared from tumors disaggregated as above. The hypoxic exposure of single-cell suspensions was carried out as described previously (20), with DNA damage measured using the comet assay after 5 or 30 min drug exposure.

For experiments at varying oxygen concentrations, SCCVII cells (3×10^4/dish) were seeded onto glass Petri dishes (50-mm diameter). Two days later, when the cell density was $1–2 \times 10^5$ cells/dish, the dishes were rinsed with 2 ml of 10 μM TPZ in α-MEM supplemented with 10% fetal bovine serum plus penicillin (100 IU/ml) and streptomycin (100 μg/ml); then 2 ml of drug solution were added to the dishes for the drug exposure. The dishes were placed in aluminum chambers, attached to a manifold, and shaken while the gas phase was exchanged for the desired oxygen concentration by serial evacuation to 0.1 atmosphere, followed by gassing with N_2/5% CO_2 (<10 ppm O_2). The chambers were sealed and transferred to a 37°C incubator for 30 min, during which time they were gently shaken. Cells were trypsinized, centrifuged, and resuspended in cold calcium-/magnesium-free PBS to a density of $\sim 2 \times 10^4$ cells/ml; then DNA damage was assessed using the comet assay as detailed above.

EF5 Binding. Tumors were excised from anesthetized mice 60 min after administration of EF5 and were rapidly cooled in ice-cold PBS; then either: (*a*) placed on a piece of premoistened filter paper and quickly frozen in liquid nitrogen, coated in OCT embedding medium (Miles, Elkhart, IN), and stored at −80°C; or (*b*) single-cell suspensions were obtained by mincing and enzymatic disaggregation. In some cases, tumors were bisected; one-half was frozen for subsequent sectioning, and one-half was dissociated. Cells were fixed and stained with the ELK3-51 antibody conjugated with the fluorescent dye cy3 as described by Koch *et al.* (25) and analyzed by flow cytometry using a FACStar plus instrument, with excitation at 514 nm. At least 20,000 cells were analyzed per sample. Sections (15 μm) were cut from the frozen tumors using an American Optical Corporation Cryo-Cut Microtome cryostat and collected on poly-L-lysine (Sigma)-coated microscope slides. Tumor sections were fixed and stained as described previously (32). Sections were photographed using a Nikon Optiphot epifluorescence microscope with a ×10 Fluor objective, a bandpass filter (510–560 nm), a Nikon FX-35DX camera, and Kodak Gold 400 ASA film. Some mice bearing RIF-1 tumors were injected

Table 1 *Potentiation by TPZ of tumor cell killing by fractionated irradiation and DNA damage induced by TPZ in six different tumors*

Tumor	Logs of cell killing after 8 × 2.5 Gy[a]				
	Radiation only	Radiation + TPZ (0.12 mmol/kg)	Enhancement ratio	Median tail moment[b] (± SE)	CV (*n*)[d]
SCCVII	3.05 ± 0.18	5.13 ± 0.28	1.7 ± 0.14	12.7 ± 1.1	19 (5)
RIF-1	3.01 ± 0.21	5.01 ± 0.08	1.7 ± 0.12	12.1 ± 2.2	44 (6)
EMT6	2.54 ± 0.27	3.48 ± 0.13	1.4 ± 0.16	10.3 ± 2.5	55 (5)
A549	1.45 ± 0.06	1.93 ± 0.09	1.3 ± 0.08	13.9 ± 2.1	33 (5)
HT1080	3.31 ± 0.31	3.14 ± 0.21	0.95 ± 0.11	5.7 ± 1.5[c]	58 (5)
HT29	1.99 ± 0.31	3.07 ± 0.07	1.5 ± 0.24	13.4 ± 1.1	16 (4)

[a] Mean data from two independent experiments for each tumor type. Each experiment had at least three mice in each treatment group.
[b] SE refers to variability for individual tumors.
[c] Significantly different from all other tumors ($P < 0.05$, two-tailed Student's t test) except for EMT6 tumor ($0.05 < P < 0.10$).
[d] CV, as a percentage, for DNA damage (median tail moment) in individual mice. n, the number of mice.

with Hoechst 33342 i.v. 10 min prior to tumor excision. Hoechst 33342 binding was visualized in RIF-1 tumor sections using the system described above with an excitation bandpass filter of 340–380 nm.

RESULTS

Fractionated TPZ plus Radiation Experiments. The response of six different transplanted tumor types to a standard protocol of 8 × 2.5 Gy over 4 days with or without 0.12 mmol/kg TPZ administered 30 min before each radiation dose was compared using clonogenic assay as the end point for tumor cell killing. With the exception of human HT1080 xenografts, the addition of TPZ to each dose of the fractionated radiation protocol produced an enhancement of cell kill relative to radiation alone (Table 1). The potentiation of radiation cell killing by TPZ was quantitated as an enhancement ratio, based on previous data showing exponential cell killing as a function of number of radiation doses (18). Enhancement ratios ranged from 1.7 for SCCVII and RIF-1 tumors to 0.95 for HT1080 tumors (Table 1). Although the effect of adding TPZ was greatest in the mouse tumors, there was substantial potentiation of the radiation effect in human A549 and HT29 tumors, with enhancement ratios of 1.3 and 1.5, respectively.

DNA Damage from TPZ Exposure. TPZ-induced DNA damage was measured in cells obtained from SCCVII tumors either by FNA or following enzymatic disaggregation of tumors (Fig. 2*a*). DNA damage increased with TPZ dose, with a higher level of damage observed in the cells obtained by FNA (Fig. 2, *a–c*). The differential in damage between the two methods of preparing single-cell suspensions is expected because DNA damage will be repaired during the enzymatic dissociation of the tumors at 37°C. A large number of normal mouse cells could be detected, on the basis of DNA content, in the SCCVII tumor cell preparations (Fig. 2*d*). SCCVII cells are predominantly tetraploid, and comparison of the DNA content of cells grown *in vitro* and cells from freshly excised tumors clearly indicates the presence of a population of cells at 1N and 2N DNA content in the tumor cell suspensions (Fig. 2, *d* and *e*). However, the distribution of DNA damage following TPZ administration was similar in the normal mouse cells and in the tumor cells (Fig. 2*f*), implying a similar oxygenation distribution and reductase activity for host cells and tumor cells. This result is similar to that of Olive *et al.* (33), who found comparable misonidazole binding in tumor cells and tumor associated macrophages. The normal mouse cells were, therefore, not excluded from the analysis of DNA damage.

TPZ-induced DNA damage was measured in each of the six different tumor types 30 min after drug administration (*i.e.*, the time after TPZ injection at which tumors were irradiated in the fractionated irradiation studies). After tumor excision and disaggregation of individual tumors, DNA damage was assessed using the comet assay. The lowest median level of damage was observed in the HT1080 tumors, in which TPZ produced no enhancement of radiation cell killing (Table 1). In the other five tumor types, similar levels of DNA damage were observed with no correlation between damage and the enhancement ratios (Table 1). However, there was a large variability in DNA damage within each tumor type with coefficients of variation up to 58%, indicating that within a single tumor transplant, there were large variations in response to TPZ, suggesting intertumor variations in the extent of hypoxia and/or activity of reductive enzymes.

To investigate the possibility that the large intertumor variability in DNA damage arises from differences in enzyme activity between individual tumors, damage was measured in RIF-1 cell suspensions,

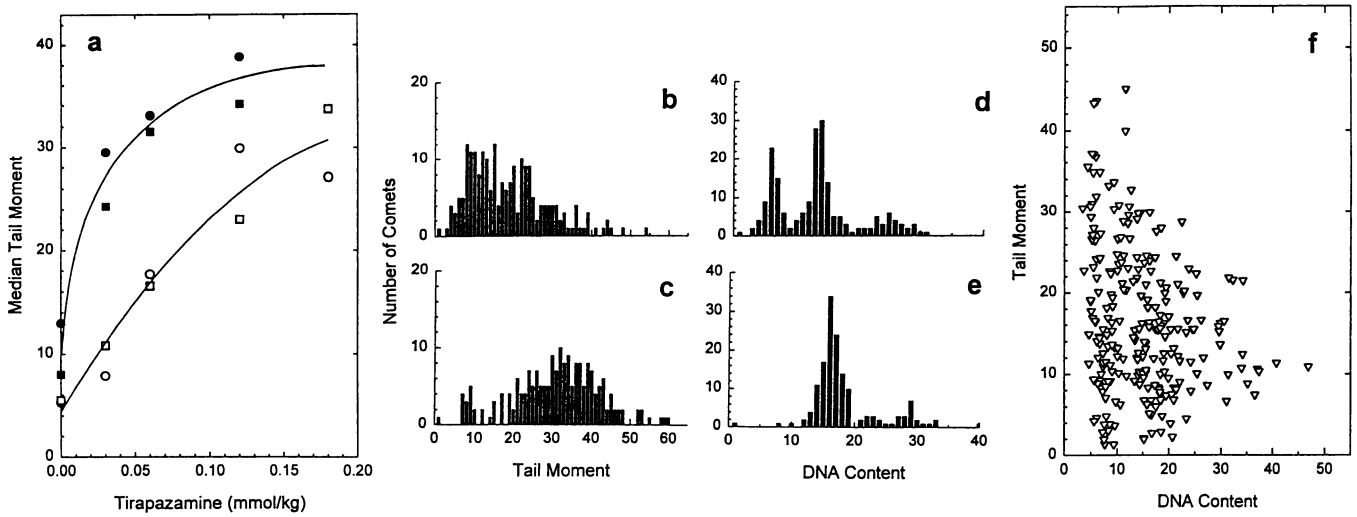

Fig. 2. *a*, DNA damage as a function of TPZ dose in SCCVII tumors determined 30 min after drug administration. Cells were obtained from individual tumors by FNA (*filled symbols*), after which the same tumor was excised, and a single-cell suspension was prepared by enzymatic dissociation (*open symbols*). The different symbols represent independent experiments. *b* and *c*, representative histograms of DNA damage in SCCVII tumors 30 min after injection of 0.06 mmol/kg TPZ, with cells obtained by enzymatic dissociation (*b*) or by FNA (*c*). *d* and *e*, representative histograms of DNA content for cells obtained by FNA biopsy from a transplanted SCCVII tumor (*d*) or SCCVII cells grown in culture (*e*). *f*, bivariate plot of DNA damage and DNA content for cells obtained by enzymatic disaggregation of an SCCVII tumor 30 min after injection of 0.12 mmol/kg TPZ.

prepared from freshly excised individual tumors, and treated with TPZ under hypoxic conditions *in vitro* as described previously (20). DNA damage measured after 5 or 30 min exposure to 10 μM TPZ showed little variation between individual tumor cell preparations (median tail moment, 13.8 ± 0.5, CV = 8.5%, and 19.3 ± 0.9, CV = 11.2%, respectively; $n = 6$) in contrast to the large variability (CV = 44%; $n = 6$) in DNA damage observed in cells from RIF-1 tumors treated with TPZ *in situ*. We also compared TPZ-induced DNA damage in hypoxic suspensions of SCCVII and RIF-1 cells prepared from cells grown either in culture or as tumors. In SCCVII cell suspensions from freshly excised tumors, exposure to 15 μM TPZ under hypoxic conditions for 5 min resulted in a median tail moment of 11.2 ± 1.4 (mean ± SE; $n = 10$). A comparable level of DNA damage (median tail moment, 10.6 ± 0.6; $n = 9$) was observed in SCCVII cells grown in culture and exposed to TPZ under the same conditions. Similar results were obtained with RIF-1 cells from freshly excised tumors or grown in culture, with median tail moments of 13.8 ± 0.5 ($n = 6$) and 10.2 ± 1.9 ($n = 3$), respectively, observed following exposure to 10 μM TPZ under hypoxic conditions.

Oxygen Dependence of DNA Damage. To determine whether differences in the level of hypoxia between individual tumors contribute to the observed intertumor variability in DNA damage, the oxygen dependence of TPZ-induced DNA damage was assessed both in cell cultures and in tumors. DNA damage in SCCVII cells treated with TPZ under various defined oxygen concentrations decreased with increasing oxygen concentration (Fig. 3). Damage was half-maximal at an oxygen concentration of 2 μM (0.2%).

To investigate the oxygen dependence of TPZ-induced damage *in vivo*, we modified the oxygenation of SCCVII tumors by treating the mice with carbogen and nicotinamide or placing the mice in an atmosphere of 10% oxygen. These treatments altered the level of TPZ-induced DNA damage measured in cells obtained by FNA biopsy 30 min after a single dose of TPZ (Fig. 4). Breathing an atmosphere of 10% oxygen resulted in greater DNA damage than in air-breathing mice, whereas TPZ produced less damage in the tumors of mice treated with carbogen and nicotinamide (Fig. 4). Each of the mice was also administered the hypoxia marker EF5 30 min prior to injection of TPZ. EF5 alone did not produce any DNA damage, as shown by the similar tail moments observed in the control mice (saline only) and the mice treated with EF5 only (Fig. 4).

Assessment of Tumor Hypoxia by EF5 Binding. Binding of EF5 with immunohistochemical detection was used to assess the level of hypoxia in transplanted tumors. In sections from RIF-1 tumors (Fig. 5*a*) and SCCVII tumors (Fig. 5*e*), the distribution of fluorescence shows large variation in intensity over distances of 100–200 μm, consistent with hypoxia occurring at the periphery of rims of tumor cords surrounding blood vessels. To check this, in addition to EF5,

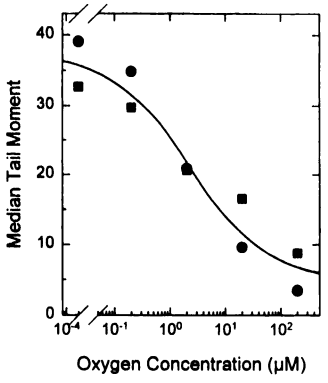

Fig. 3. Relationship between DNA damage and gas phase oxygen concentration for SCCVII cells treated with 10 μM TPZ for 30 min. Different symbols represent independent experiments.

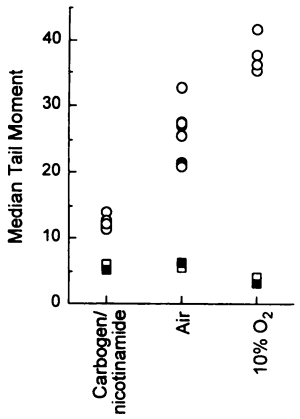

Fig. 4. DNA damage in SCCVII tumor cells obtained by FNA biopsy after injection of: saline (□); 0.1 mmol/kg EF5 (■); and 0.1 mmol/kg EF5 with 0.06 mmol/kg TPZ injected 30 min prior to sampling (○). Mice were air-breathing, breathing 10% oxygen, or treated with carbogen and nicotinamide as described in "Materials and Methods." Each point represents an individual tumor.

some mice were administered the fluorescent dye Hoechst 33342 10 min prior to tumor excision. Following i.v. injection, Hoechst 33342 distributes rapidly and binds to cells surrounding capillaries, resulting in a drug gradient away from functional capillaries (Fig. 5*b*). A double exposure (Fig. 5*c*) of a RIF-1 tumor section from a mouse treated with both EF5 and Hoechst 33342 shows largely complementary staining by EF5 and Hoechst 33342, confirming that EF5 is binding to the regions in tumors most distant from blood vessels.

Treatment with carbogen/nicotinamide decreased EF5 binding in SCCVII tumors (Fig. 5*d*) relative to binding in tumors in air-breathing mice (Fig. 5*e*), whereas placing the mice in an atmosphere of 10% oxygen resulted in increased binding (Fig. 5*f*). Quantitation of the different levels of EF5 binding was obtained from flow cytometric analysis of cells from EF5-treated tumors (Fig. 6). Representative flow cytometry traces demonstrating increased fluorescence in tumor cells from mice breathing 10% oxygen and decreased fluorescence in cells from mice treated with carbogen and nicotinamide relative to air-breathing mice are shown in Fig. 6. In each experiment, a low level of fluorescence was observed in the controls for nonspecific binding of the antibody (cells from untreated tumors stained with antibody) and autofluorescence (cells from tumors treated with EF5 and not stained with antibody; data not shown).

Relationship between DNA Damage and Hypoxia in Tumors. Because both TPZ-induced DNA damage (measured by the comet assay) and EF5 binding were determined in individual SCCVII tumors, we examined the relationship between DNA damage and hypoxia, quantitated as relative EF5 fluorescence intensity (fluorescence intensity relative to controls for nonspecific binding in the same experiment). Although there was considerable scatter in the data, there was a significant correlation ($r = 0.732$, $P < 0.001$) between DNA damage and tumor hypoxia assessed by mean fluorescence intensity of EF5 binding (Fig. 7).

DISCUSSION

TPZ is a bioreductive drug with selective toxicity toward hypoxic cells both *in vitro* and in tumors. The combination of TPZ with fractionated radiation is currently under evaluation in Phase II clinical trials, and the combination of TPZ with cisplatin is in Phase III trials. Because not all patients would be expected to respond equally to TPZ, it would be extremely useful to have a simple method of predicting the response of individual tumors to the drug. To this end we have been investigating the utility of using DNA ssb measured by the comet assay to assess the sensitivity of individual tumors to TPZ. As a first

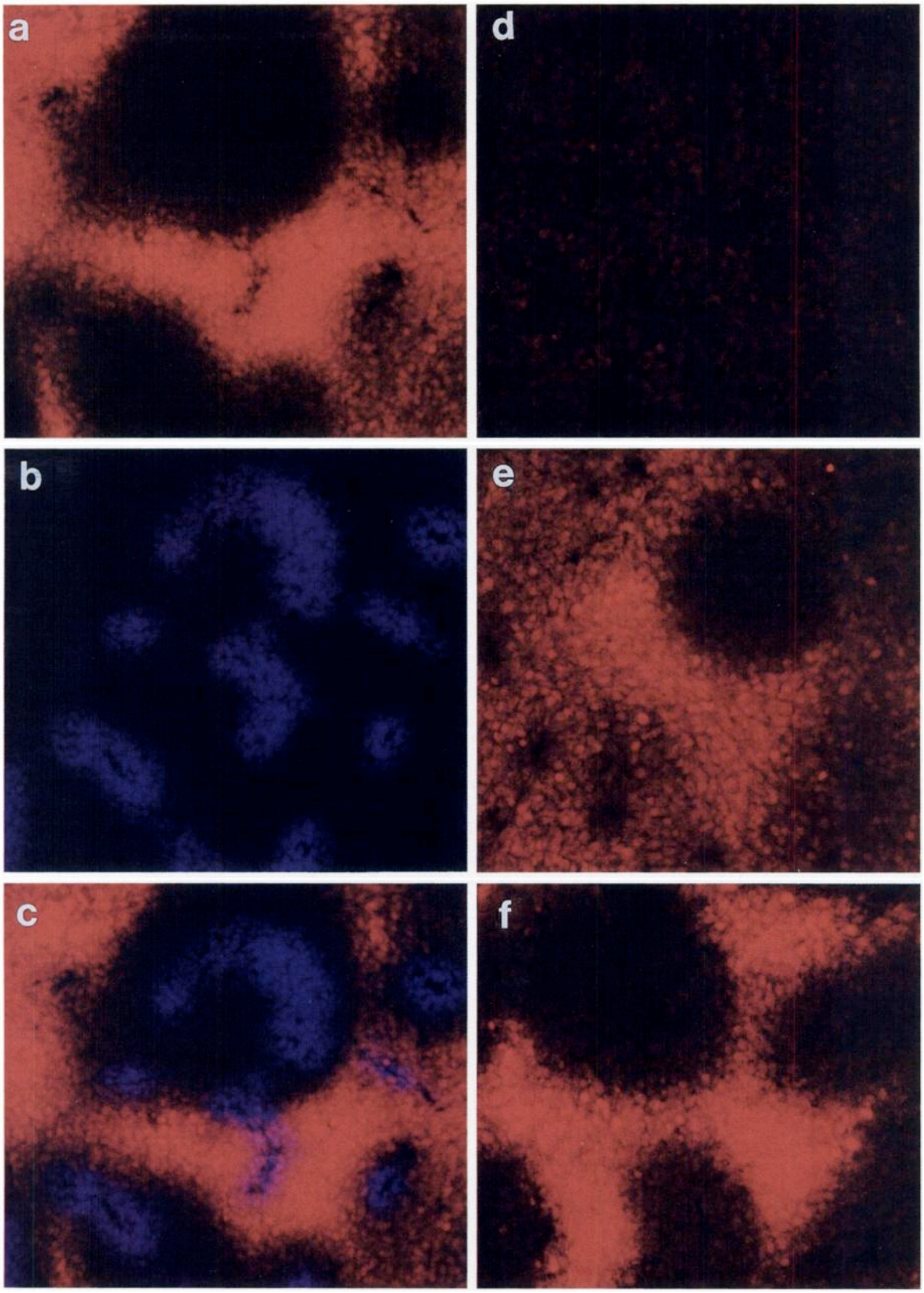

Fig. 5. Representative fields from sections of RIF-1 (*a–c*) or SCCVII tumors (*d–f*) excised 1 h after EF5 administration with immunohistochemical staining using a monoclonal anti-EF5 antibody (ELK3-51). Ten min before the RIF-1 tumor was excised, Hoechst 33342 was injected i.v. Tumor sections were photographed as described in "Materials and Methods." *a*, RIF-1 tumor section showing binding of EF-5. *b*, the same RIF-1 tumor section, photographed using a UV filter, showing distribution of Hoechst 33342. *c*, double exposure of the RIF-1 tumor section showing both EF5 binding (*red*) and Hoechst 33342 binding (*blue*). *d*, SCCVII tumor section from a mouse treated with carbogen and nicotinamide. *e*, SCCVII section from an air-breathing mouse. *f*, SCCVII tumor section from a mouse breathing 10% oxygen. *d–f*, processed and photographed under identical conditions.

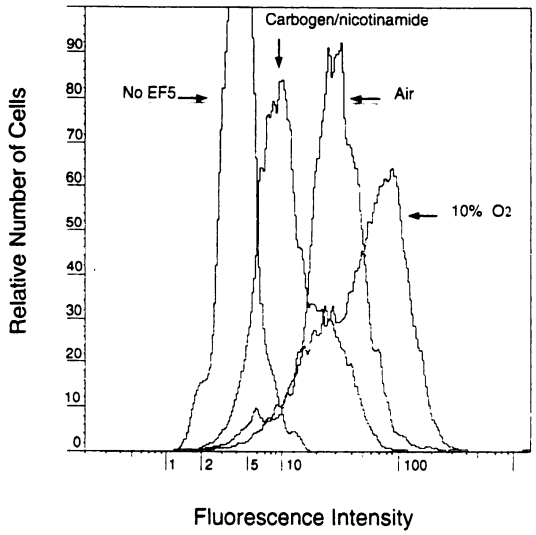

Fig. 6. Flow cytometric analysis of cells from SCCVII tumors dissociated 1 h after EF5 administration. The tumors were removed from air-breathing mice, from mice treated with carbogen and nicotinamide, from mice breathing 10% oxygen, or were from controls for nonspecific binding of the ELK3-51 antibody (no EF5).

step, we have previously demonstrated a strong correlation between TPZ-induced DNA ssb and hypoxic cytotoxicity for a number of human (A549, HT1080, and HT29) and murine tumor cell lines (EMT6, RIF-1, and SCCVII) *in vitro* (20), indicating that ssb can be used as a surrogate measure of cytotoxicity in hypoxic cells.

In the present study, the potentiation produced by the addition of TPZ to a fractionated radiation protocol was evaluated in transplanted tumors grown from the same six mouse and human tumor cell lines. Our data confirm and extend previous findings with mouse tumors (18) that the combination of TPZ and fractionated radiation produces enhancement of the tumor cell killing achieved by radiation alone. However, we also demonstrate that the extent of this potentiation varies considerably between different tumor types in a manner inconsistent with cellular sensitivities to TPZ under hypoxic conditions. For example, we have reported previously that each of the three human tumor cell lines (A549, HT1080, and HT29) had similar sensitivity to killing by TPZ under defined hypoxic conditions *in vitro* (20). This suggested that the activity of the reductive enzymes that activate TPZ are similar in each of these lines. Because these three cell lines growing as tumors *in vivo* showed considerable variation in potentiation of fractionated irradiation by TPZ (enhancement ratios of 0.95 to 1.5, Table 1), the present result, therefore, suggests that in tumors there are additional factors that determine response to TPZ. These could include differences in vascularization affecting both drug delivery and tumor oxygenation or differences in the kinetics of reoxygenation and rehypoxiation following each fraction of TPZ plus radiation (8, 17).

As a step closer to measuring the sensitivity of the tumor cells to TPZ *in vivo*, we measured TPZ-induced DNA damage in cell suspensions obtained from tumors treated *in situ*. We found that the tumor cell line (HT 1080) showing no enhancement of radiation by TPZ had less TPZ-induced damage than the other five tumor types (Table 1). However, within these five other tumor types, there was no correlation between the extent of potentiation of radiation cell kill and DNA damage induced by TPZ. A possible reason for this is that any such relationship could be masked by the extremely large intertumor variation in TPZ-induced DNA damage, shown by the CV for DNA damage in Table 1. Because such variation could be important in the clinical situation, we investigated the two likely causes of these variations, *i.e.*, intertumor differences in reductive enzymes and/or oxygen levels.

In terms of intertumor differences in reductive enzymes, our finding of the low degree of variability in TPZ-induced DNA *in vitro* in SCCVII and RIF-1 cells prepared from freshly excised individual tumors indicates that the activity of the enzymes that activate TPZ to a DNA-damaging species do not vary substantially between individual tumors. However, because recent reports have suggested that reductive enzyme activity can change when cells are grown in culture or as transplanted tumors (34, 35), we examined TPZ metabolism in cells maintained in tissue culture and freshly excised from tumors. We found similar levels of TPZ-induced DNA damage following hypoxic exposure of RIF-1 or SCCVII cell suspensions prepared from cells grown in culture or from freshly excised tumors, indicating that the activity of the enzymes that activate TPZ to a DNA-damaging species are not altered when these cell lines are grown as transplanted tumors in mice.

To assess whether variations in the level of hypoxia between individual tumors could produce the observed variability in DNA damage, we determined the oxygen dependence of TPZ-induced DNA damage. Damage in SCCVII cell cultures *in vitro* decreased with increasing gas phase oxygen concentration (Fig. 3). The oxygen dependence of DNA damage in tumors treated with TPZ was investigated by modulating tumor oxygenation using either carbogen and nicotinamide or 10% oxygen breathing. Treatment with nicotinamide and carbogen breathing has been shown to oxygenate hypoxic tumor cells *in vivo* and improve tumor response to radiation (28, 36–38). As shown in Fig. 4, the level of TPZ-induced DNA damage in SCCVII tumors treated with carbogen and nicotinamide was lower than in air-breathing tumors, whereas breathing an atmosphere of 10% oxygen, which has been shown to increase tumor hypoxia (39), resulted in a higher level DNA damage by TPZ, consistent with decreased tumor oxygenation. These results clearly show the oxygen dependence of DNA damage by TPZ both *in vitro* and *in vivo*.

As a test of whether oxygen levels could be quantitated and correlated with DNA damage in individual tumors, we assessed tumor oxygenation by binding of the pentafluorinated etanidazole derivative EF5 with detection using a monoclonal antibody conjugated to the fluorescent dye cy3. Binding of EF5 is oxygen dependent, with a large dynamic range of at least 50-fold between aerobic and hypoxic cells (25). The fact that in tumors EF5 is activated to form macromolecular adducts in the cells at greatest distance from functional blood vessels was confirmed by the administration of Hoechst 33342 shortly before tumor excision, resulting in almost total complementary staining by the two fluorescent dyes (Fig. 5).

Treatment of SCCVII tumors with carbogen and nicotinamide or with 10% oxygen-breathing produced changes in the binding of EF5 that were

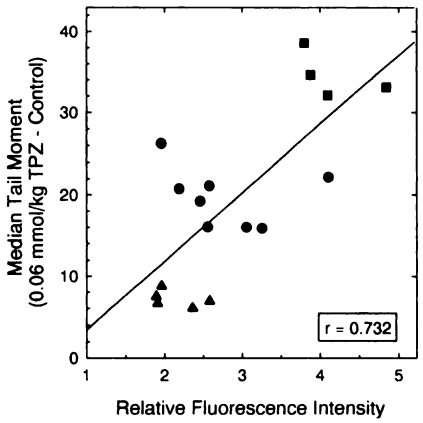

Fig. 7. Relationship between TPZ-induced DNA damage and hypoxia, measured as relative fluorescence intensity (mean fluorescence intensity relative to controls for nonspecific binding) in individual SCCVII tumors treated with 0.06 mmol/kg TPZ and 0.1 mmol/kg EF5. Cells are from tumors in mice breathing 10% oxygen (■), breathing air (●), or treated with carbogen and nicotinamide (▲).

clearly visible in tumor sections and could be quantitated by flow cytometric analysis of tumor cell suspensions. An interesting observation from these data was that in tumors in mice breathing 10% oxygen, there were cells with greater fluorescence than in air-breathing mice (Fig. 5, *e* and *f*, and Fig. 6). Thus, breathing 10% oxygen did not merely increase the number of hypoxic tumor cells, it appeared to decrease the oxygenation of the most "hypoxic" cells in the air-breathing mice, implying that many of the latter were at intermediate oxygen levels.

Analysis of the relationship between TPZ-induced DNA damage and hypoxia in individual SCCVII tumors, quantitated as mean fluorescence intensity relative to controls for nonspecific binding, showed a highly significant correlation ($r = 0.73$, $P < 0.001$) between DNA damage and tumor hypoxia as measured by these two assays. Least squares regression analysis indicates that 54% of the variation in DNA damage can be accounted for by variations in tumor hypoxia as measured by EF5 binding. The remainder could be due to experimental or sampling variations and/or to the reported different oxygen dependencies for the reductive activation of nitroimidazoles and TPZ (40).

In conclusion, this study has demonstrated that TPZ potentiates cell killing by fractionated radiation in all three murine tumors and two of three human tumor xenografts investigated. DNA damage measured after a single dose of TPZ identified the one nonresponsive tumor, but for the remaining five, it did not correlate with the enhancement of radiation cell killing for the tumors pooled by type. This could have been due to the fact that factors other than absolute levels of cell kill are expected to modify the potentiation by TPZ of fractionated radiation (17). However, the large variations in TPZ-induced DNA damage between individual tumors could have masked correlations between DNA damage and radiation potentiation. These variations in DNA damage did not result from variations in reductive enzyme activity within a tumor type but appeared to result primarily from differences in oxygen levels between individual tumors. This is consistent with the *in vitro* and *in vivo* experiments that the level of hypoxia is an important determinant of response to TPZ. These studies, therefore, encourage clinical testing of TPZ-induced DNA damage and/or tumor oxygen levels to assess individual tumor sensitivity to TPZ as a means of individualizing treatment.

ACKNOWLEDGMENTS

We thank Drs. Cameron Koch and Edith Lord for the generous supply of reagents and advice for measuring tumor hypoxia using the EF5 method; we also thank James Evans for skilled assistance with the flow cytometry measurements.

REFERENCES

1. Brown, J. M. SR 4233 (tirapazamine): a new anticancer drug exploiting hypoxia in solid tumours. Br. J. Cancer, *67:* 1163–1170, 1993.
2. Wang, J., Biedermann, K. A., and Brown, J. M. Repair of DNA and chromosome breaks in cells exposed to SR 4233 under hypoxia or to ionizing radiation. Cancer Res., *52:* 4473–4477, 1992.
3. Elwell, J. H., Siim, B. G., Evans, J. W., and Brown, J. M. Adaptation of human cells to tirapazamine under aerobic conditions: implications of increased antioxidant enzyme activity to mechanism of aerobic cytotoxicity., Biochem. Pharmacol., in press, 1997.
4. Herscher, L. L., Krishna, M. C., Cook, J. A., Coleman, C. N., Biaglow, J. E., Tuttle, S. W., Gonzalez, F. J., and Mitchell, J. B. Protection against SR 4233 (tirapazamine) aerobic cytotoxicity by the metal chelators desferrioxamine and tiron. Int. J. Radiat. Oncol. Biol. Phys., *30:* 879–885, 1994.
5. Silva, J. M., and O'Brien, P. J. Molecular mechanisms of SR 4233-induced hepatocyte toxicity under aerobic *versus* hypoxic conditions. Br. J. Cancer, *68:* 484–491, 1993.
6. Wilson, W. R. Tumour hypoxia: challenges for cancer chemotherapy. *In:* M. J. Waring and B. A. J. Ponder (eds.), The Search for New Anticancer Drugs, Cancer Biology and Medicine, Vol. 3, pp. 87–131. Lancaster: Kluwer Academic Publishers, 1992.
7. Sartorelli, A. C. Therapeutic attack of hypoxic cells of solid tumors. Cancer Res., *48:* 775–778, 1988.
8. Brown, J. M., and Siim, B. G. Hypoxia-specific cytotoxins in cancer therapy. Semin. Radiat. Oncol., *6:* 22–36, 1996.
9. Moulder, J. E., and Rockwell, S. Tumor hypoxia: its impact on cancer therapy. Cancer Metastasis Rev., *5:* 313–341, 1987.
10. Rockwell, S., and Moulder, J. E. Hypoxic fractions of human tumors xenografted into mice: a review. Int. J. Radiat. Oncol. Biol. Phys., *19:* 197–202, 1990.
11. Vaupel, P. W., and Hockel, M. Oxygenation status of human tumors: a reappraisal using computerized pO2 histography. *In:* P. W. Vaupel, D. K. Kelleher, and M. Gunderoth (eds.), Tumor Oxygenation, pp. 219–232. Stuttgart: Gustav Fischer Verlag, 1995.
12. Nordsmark, M., Overgaard, M., and Overgaard, J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother. Oncol., *41:* 31–40, 1996.
13. Gatenby, R. A., Kessler, H. B., Rosenblum, J. S., Coia, L. R., Moldofsky, P. J., and Hartz, W. H. Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. Int. J. Radiat. Oncol. Biol. Phys., *14:* 831–838, 1988.
14. Fyles, A., Milosevic, M., Kavanagh, M-C., Pintilie, M., Sun, A., Chapman, W., Levin, W., Manchul, L., Keane, T. J., and Hill, R. Hypoxia measured with a polarographic electrode correlates with radiation response in cervix cancer. Radiother. Oncol., in press, 1997.
15. Hockel, M., Knoop, C., Schlenger, K., Vorndran, B., Baussmann, E., Mitze, M., Knapstein, P. G., and Vaupel, P. Intratumoral pO_2 predicts survival in advanced cancer of the uterine cervix. Radiother. Oncol., *26:* 45–50, 1993.
16. Kolstad, P. Vascularization, oxygen tension, and radiocurability in cancer of the cervix. Copenhagen: Scandinavian University Books, 1963.
17. Brown, J. M., and Koong, A. Therapeutic advantage of hypoxic cells in tumors: a theoretical study. J. Natl. Cancer Inst., *83:* 178–185, 1991.
18. Brown, J. M., and Lemmon, M. J. Potentiation by the hypoxic cytotoxin SR 4233 of cell killing produced by fractionated irradiation of mouse tumors. Cancer Res., *50:* 7745–7749, 1990.
19. Stone, H. B., Brown, J. M., Phillips, T. L., and Sutherland, R. M. Oxygen in human tumors: correlations between methods of measurement and response to therapy. Radiat. Res., *136:* 422–434, 1993.
20. Siim, B. G., van Zijl, P. L., and Brown, J. M. Tirapazamine-induced DNA damage measured using the comet assay correlates with cytotoxicity toward hypoxic tumour cells *in vitro*. Br. J. Cancer, *73:* 952–960, 1996.
21. Olive, P. L., Vikse, C. M., and Banath, J. P. Use of the comet assay to identify cells sensitive to tirapazamine in multicell spheroids and tumors in mice. Cancer Res., *56:* 4460–4463, 1996.
22. Hirst, D. G., Brown, J. M., and Hazlehurst, J. L. Effect of partition coefficient on the ability of nitroimidazoles to enhance the cytotoxicity of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea. Cancer Res., *43:* 1961–1965, 1983.
23. Rockwell, S. C., Kallman, R. F., and Fajardo, L. F. Characteristics of a serially transplanted mouse mammary tumor and its tissue culture-adapted derivative. J. Natl. Cancer Inst., *49:* 735–749, 1972.
24. Twentyman, P. R., Brown, J. M., Gray, J. W., Franko, A. J., Scoles, M. A., and Kallman, R. F. A new mouse tumor model system (RIF-1) for comparison of end-point studies. J. Natl. Cancer Inst., *64:* 595–604, 1980.
25. Koch, C. J., Evans, S. M., and Lord, E. M. Oxygen dependence of cellular uptake of EF5 [2-(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl)acetamide]: analysis of drug adducts by fluorescent antibodies *vs.* bound radioactivity. Br. J. Cancer, *72:* 865–870, 1995.
26. Horsman, M. R., Brown, J. M., Hirst, V. K., Lemmon, M. J., Wood, P. J., Dunphy, E. P., and Overgaard, J. Mechanism of action of the selective tumor radiosensitizer nicotinamide. Int. J. Radiat. Oncol. Biol. Phys., *15:* 685–690, 1988.
27. Horsman, M. R., Chaplin, D. J., and Brown, J. M. Radiosensitization by nicotinamide *in vivo*: a greater enhancement of tumor damage compared to that of normal tissues. Radiat Res., *109:* 479–489, 1987.
28. Siemann, D. W., Horsman, M. R., and Chaplin, D. J. The radiation response of KHT sarcomas following nicotinamide treatment and carbogen breathing. Radiother. Oncol., *31:* 117–122, 1994.
29. Olive, P. L., Wlodek, D., Durand, R. E., and Banath, J. P. Factors influencing DNA migration from individual cells subjected to gel electrophoresis. Exp. Cell Res., *198:* 259–267, 1992.
30. Olive, P. L., and Durand, R. E. Detection of hypoxic cells in a murine tumor with the use of the Comet assay. J. Natl. Cancer Inst., *84:* 707–711, 1992.
31. Olive, P. L., Banath, J. P., and Durand, R. E. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. Radiat. Res., *122:* 86–94, 1990.
32. Evans, S. M., Joiner, B. J., Jenkins, W. T., Laughlin, K. M., Lord, E. M., and Koch, C. J. Identification of hypoxia in cells and tissues of epigastric 9L rat tumours using EF5. Br. J. Cancer, *72:* 875–882, 1995.
33. Olive, P. L. Distribution, oxygenation, and clonogenicity of macrophages in a murine tumor. Cancer Commun., *1:* 93–100, 1989.
34. Collard, J., Matthew, A. M., Double, J. A., and Bibby, M. C. EO9: relationship between DT-diaphorase levels and response *in vitro* and *in vivo*. Br. J. Cancer, *71:* 1199–1203, 1995.
35. Hejmadi, M. V., McKeown, S. R., Friery, O. P., McIntyre, I. A., Patterson, L. H., and Hirst, D. G. DNA damage following combination of radiation with the bioreductive drug AQ4N: possible selective toxicity to oxic and hypoxic tumour cells. Br. J. Cancer, *73:* 499–505, 1996.
36. Kjellen, E., Joiner, M. C., Collier, J. M., Johns, H., and Rojas, A. A therapeutic benefit from combining normobaric carbogen or oxygen with nicotinamide in fractionated X-ray treatments. Radiother. Oncol., *22:* 81–91, 1991.
37. Chaplin, D. J., Horsman, M. R., and Aoki, D. Nicotinamide, fluosol DA, and carbogen: a strategy to reoxygenate acutely and chronically hypoxic cells *in vivo*. Br. J. Cancer, *63:* 109–113, 1991.
38. Dorie, M. J., Menke, D., and Brown, J. M. Comparison of the enhancement of tumor responses to fractionated irradiation by SR 4233 (tirapazamine) and by nicotinamide with carbogen. Int. J. Radiat. Oncol. Biol. Phys., *28:* 145–150, 1994.
39. Sasai, K., and Brown, J. M. Discrepancies between measured changes of radiobiological hypoxic fraction and oxygen tension monitoring using two assay systems. Int. J. Radiat. Oncol. Biol. Phys., *30:* 355–361, 1994.
40. Koch, C. J. Unusual oxygen concentration dependence of toxicity of SR-4233, a hypoxic cell toxin. Cancer Res., *53:* 3992–3997, 1993.