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

Bronwyn G. Sum, Douglas R. Menke, Mary Jo Dorie, and J. Martin Brown

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 as 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 fractional radiation in three human tumors (SCCIVII, 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 these DNA damage within each tumor type. This variability appeared not to result from differences in enzyme activation, but rather 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

TPZ3 (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 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 (SCCIVII, 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 SCCIVII squamous carcinoma (22), EMT6 mammary carcinoma (23), and RIF-1 fibrosarcoma (24) cell lines has been described previously. All cell lines were maintained in...
culture as detailed previously (20). Tumors were grown from cells ($2 \times 10^5$ 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 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 0.6 V/cm for 22 mm 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 Ikeyemi 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 ($3 \times 10^4$ dish) were seeded onto glass Petri dishes (50-mm diameter). Two days later, when the cell density was 1–2 × 10^6 cells/dish, the dishes were rinsed with 2 ml of 10 μM TPZ in a-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 ~2 × 10^6 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 ×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...
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. 2a). 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 damage in 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. 2d). 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. 2f), 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, 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 SCC VII tumors, with enhancement ratios of 1.3 and 1.5, respectively.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Radiation only (0.12 mmol/kg)</th>
<th>Radiation + TPZ (0.06 mmol/kg)</th>
<th>Enhancement ratio</th>
<th>Median tail moment (± SE)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCVII</td>
<td>3.05 ± 0.18</td>
<td>5.13 ± 0.28</td>
<td>1.7 ± 0.14</td>
<td>12.7 ± 1.1</td>
<td>19 (5)</td>
</tr>
<tr>
<td>RIF-1</td>
<td>3.01 ± 0.21</td>
<td>5.01 ± 0.08</td>
<td>1.7 ± 0.12</td>
<td>12.1 ± 2.2</td>
<td>46 (6)</td>
</tr>
<tr>
<td>EMT6</td>
<td>2.54 ± 0.27</td>
<td>3.48 ± 0.13</td>
<td>1.4 ± 0.16</td>
<td>10.3 ± 2.5</td>
<td>55 (5)</td>
</tr>
<tr>
<td>A549</td>
<td>1.45 ± 0.06</td>
<td>1.93 ± 0.09</td>
<td>1.3 ± 0.08</td>
<td>13.9 ± 2.1</td>
<td>33 (5)</td>
</tr>
<tr>
<td>HT1080</td>
<td>3.31 ± 0.31</td>
<td>3.14 ± 0.21</td>
<td>0.95 ± 0.11</td>
<td>5.7 ± 1.5</td>
<td>58 (5)</td>
</tr>
<tr>
<td>HT29</td>
<td>1.99 ± 0.31</td>
<td>3.07 ± 0.07</td>
<td>1.5 ± 0.24</td>
<td>13.4 ± 1.1</td>
<td>16 (4)</td>
</tr>
</tbody>
</table>

- Mean data from two independent experiments for each tumor type. Each experiment had at least three mice in each treatment group.
- SE refers to variability for individual tumors.
- CV, as a percentage, for DNA damage (median tail moment) in individual mice. n, the number of mice.

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 disaggregation (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 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. 5a) and SCCVII tumors (Fig. 5e), 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, 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. 5b). A double exposure (Fig. 5c) 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. 5d) relative to binding in tumors in air-breathing mice (Fig. 5e), whereas placing the mice in an atmosphere of 10% oxygen resulted in increased binding (Fig. 5f). 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-I (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.
In the previous experiments, we found that the extent of potentiation of radiation cell kill and DNA damage induced by TPZ varied considerably between different tumor types in a manner inconsistent with cellular senescence. From these findings, we concluded 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 that 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 is 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 pentafuorinated 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. 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).

---

**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 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 potentiating effect of TPZ on 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

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


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/14/2922

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.