Biochemical Pharmacology, Vol. 54, pp. 249–257, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00171-8

Adaptation of Human Tumor Cells to Tirapazamine under Aerobic Conditions

IMPLICATIONS OF INCREASED ANTIOXIDANT ENZYME ACTIVITY TO MECHANISM OF AEROBIC CYTOTOXICITY

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ABSTRACT. Tirapazamine (TPZ, 3-amino-1,2,4-benzotriazine 1,4-di-*N*-oxide, SR 4233, WIN 59075) is a bioreductive antitumor agent with a high selective toxicity for hypoxic cells. The selective hypoxic toxicity of TPZ results from the rapid reoxidation of the one-electron reduction product, the TPZ radical, in the presence of molecular oxygen with the concomitant production of superoxide radical. Under hypoxia the TPZ radical kills cells by causing DNA double-strand breaks and chromosome aberrations. However, the mechanism of aerobic cytotoxicity is still a matter of debate. In this study, we investigated the mechanism of aerobic cytotoxicity by adapting human lung adenocarcinoma A549 cells to aerobic TPZ exposure and characterizing the changes associated with drug resistance. The adapted cells were resistant to aerobic TPZ exposures (with dose-modifying factors of up to 9.2), although hypoxic sensitivity was largely unchanged. Relative to the parental A549 cell line, adaptation to continuous aerobic TPZ exposure resulted in increased levels of manganese superoxide dismutase (up to 9.4-fold), moderate increases in glutathione reductase (up to 2.1-fold), and loss of both quinone oxidoreductase (DT-diaphorase) activity and NADPH cytochrome P450 reductase activity. There was essentially no change in the activity of the cytoplasmic form of superoxide dismutase (CuZnSOD), catalase, or glutathione peroxidase. The increased activity of antioxidant enzymes in the resistant cell lines (in particular MnSOD) strongly suggests that reactive oxygen species are, in large part, responsible for the toxicity of TPZ under aerobic conditions, and is consistent with aerobic and hypoxic drug cytotoxicity resulting from different mechanisms. BIOCHEM PHARMACOL **54**;2:249–257, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. tirapazamine; bioreductive drugs; oxidative stress; manganese superoxide dismutase; NADPH cytochrome P450 reductase

A problem often encountered in cancer therapy is the presence of tumor cell subpopulations that are resistant to treatment. Solid tumors frequently contain hypoxic cells [1, 2] that are resistant to killing both by ionizing radiation [3] and probably also by many chemotherapeutic agents [4, 5]. There is considerable clinical evidence that these hypoxic cells contribute to treatment failure, at least for radiotherapy [6–8]. Hypoxic cell cytotoxins were developed for use in combination with radiation or chemotherapy, both to combat this problem and to exploit these areas of low oxygenation for therapeutic advantage [9–11]. TPZ† (3-amino-1,2,4-benzotriazine 1,4-di-*N*-oxide, SR 4233, WIN 59075) is the lead compound in a series of potent hypoxic cell cytotoxins that kill hypoxic rodent and human tumor cells both *in vitro* and *in vivo* [12–14]. Unlike other bioreductive agents that require extremely low oxygen levels to produce cytotoxicity, TPZ toxicity extends over a broad range of oxygen concentrations [15]. It should, therefore, be active at oxygen concentrations expected in the hypoxic regions of human solid tumors. Preclinical studies have demonstrated the efficacy of using TPZ in combination with both fractionated radiotherapy [16, 17] and chemotherapeutic agents [18–20]. TPZ is currently in Phase II clinical trials for use in combination with fractionated radiotherapy, and in Phase III trials in combination with cisplatin chemotherapy.

TPZ exhibits high hypoxic selectivity with the ratio of drug concentrations required for the same level of killing in aerobic and hypoxic cells ranging between 25- and 200-fold in most cell lines investigated [12, 21]. The drug is reduced enzymatically by a one-electron process under both aerobic and hypoxic conditions to a free radical species [21, 22]. Several enzymes [cytochrome P450, NADPH:cytochrome P450 oxidoreductase (EC 1.6.2.4, P450 reductase), and xanthine oxidase] are capable of reducing TPZ to its radical species [22–26]. In the presence of molecular oxygen, the TPZ radical is reoxidized rapidly to the parent compound concomitant with the production of superoxide [22]. This

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† *Abbreviations*: DMF, dose-modifying factor; HCR, hypoxic cytotoxicity ratio; MnSOD, manganese superoxide dismutase; SOD, superoxide dismutase; TPZ, tirapazamine.

Received 21 August 1996; accepted 12 February 1997.

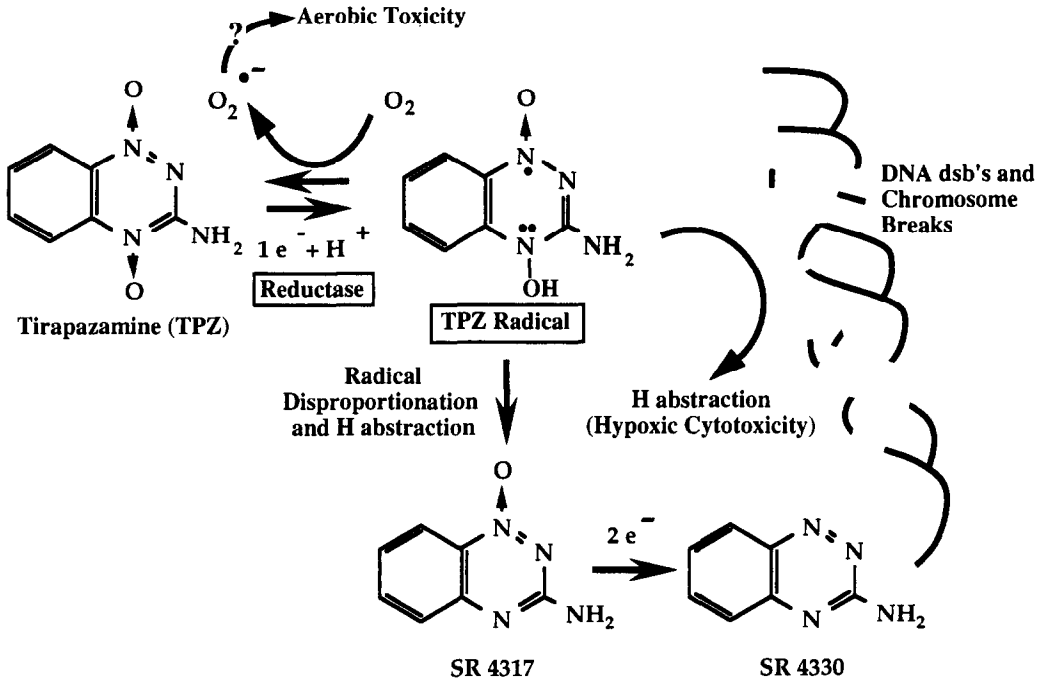

FIG. 1. Proposed model for TPZ metabolism and cytotoxicity under aerobic and hypoxic conditions.

reaction is thought to be the basis for the hypoxic selectivity of TPZ cell killing. Under hypoxic conditions the toxic radical species is longer lived and is free to diffuse and interact with cellular macromolecules (Fig. 1). The hypoxic toxicity of TPZ results from the formation of TPZ-induced DNA double-strand breaks and chromosomal breaks [27].

Although TPZ is preferentially toxic to hypoxic cells, it does kill aerobic cells at high drug concentrations. It is also likely that the side-effects of muscle cramping [28] and reversible ototoxicity [29] observed in clinical studies are a result of the effect of the drug on aerobic cells. The mechanism of aerobic cytotoxicity has not been elucidated and could result from at least three different mechanisms: (1) direct interaction of the low number of TPZ radicals that escape reoxidation by molecular oxygen with cellular macromolecules; (2) production of superoxide and other reactive oxygen species during the futile redox cycling of the TPZ molecule between its reduced and oxidized species (Fig. 1); or (3) direct cytotoxicity of the parent TPZ molecule by an unknown mechanism.

To further investigate the mechanism of aerobic TPZ toxicity, we have adapted the human lung tumor A549 cell line to continuous aerobic TPZ exposure and characterized the changes associated with drug resistance. We show that A549 cells selected for resistance to the aerobic cytotoxicity of TPZ largely retain their sensitivity to TPZ under hypoxic conditions. The acquisition of resistance of TPZ aerobic cytotoxicity is associated with elevation of the levels of antioxidant enzymes involved in superoxide detoxification, the magnitude of which correlates well with the observed degree of resistance. These results implicate the production of superoxide and other reactive oxygen species as responsible for the aerobic toxicity of TPZ.

MATERIALS AND METHODS

Reagents

TPZ was supplied by Sterling–Winthrop Inc. (now Sanofi–Winthrop Inc., Great Valley, PA). SR 4317 (3-amino-1,2,4-benzotriazine 1-*N*-oxide) and SR 4330 (3-amino-1,2,4-benzotriazine) were synthesized by Dr. Mike Tracy (SRI International, Menlo Park, CA). Bathocuproinedisulfonic acid and diethylpentaacetic acid (for the SOD assays) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). All other reagents were obtained from the Sigma Chemical Co. (St. Louis, MO).

Cell Lines and Culture Conditions

A549 human lung adenocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in complete medium (Alpha Minimal Essential Medium) containing 10% (v/v) heat-inactivated fetal bovine serum plus penicillin (100 U/mL) and streptomycin (0.1 mg/mL). TPZ-resistant cell lines were prepared in a step-wise fashion by growing the cells in increasing concentrations (10, 25, 50, 75, and 100 μM) of TPZ under aerobic conditions. Initially, parental A549 cells were plated in T-25 flasks at a density of 2×10^5 cells/flask containing 10 μM TPZ. Adapted cell lines were refed with fresh medium containing drug twice weekly, subcultured every 7–10 days using 0.05% trypsin/0.1% EDTA, and plated for clonogenic survival. When the surviving fraction determined from the weekly subculturing exceeded approximately 50% survival in adapted lines, the drug concentration was escalated to the next concentration step. Approximately 6 months were spent adapting the lines as described. All adapted lines were maintained under selec-

tive pressure until the 100 μM adapted lines exceeded 50% survival. All lines maintained resistance to TPZ in the absence of selective pressure.

Cell Survival Assays

A549 cells were seeded onto plastic (for aerobic exposures) or notched glass (for hypoxic exposures) 60-mm dishes in complete medium at a concentration of 10^5 cells/dish. Their doubling time was 22 h. Drug exposures were performed 3–4 days later when the cells had reached a concentration of approximately 5×10^5 cells/dish. Cells were exposed to graded concentrations of TPZ for 90 min (hypoxic exposures) or 6 hr (aerobic exposures) in complete medium at 37°. Hypoxia was achieved by five cycles of evacuation to 80 mm Hg with gassing with 95% N_2/5% CO_2 to 1850 mm Hg after each evacuation using a modification of the jig and gassing system described by Koch [30], which allows rapid gas equilibration. The purpose of the 95% N_2 gassing to greater than atmospheric pressure was to achieve a higher degree of oxygen exchange than would have been obtained at atmospheric pressure. Following the final evacuation, the jigs were returned to ambient pressure and placed in a 37° incubator. Aerobic exposure was carried out in a humidified incubator containing 5% CO_2. At the end of the drug treatment period the TPZ-containing medium was removed, and the cell monolayers were washed twice in PBS, trypsinized, and plated for clonogenic survival. The plating efficiency of the parental and resistant A549 cells was approximately 70%. Fourteen days later, the dishes were stained with crystal violet (0.25% in 95% ethanol) and colonies containing more than 50 cells were counted.

Preparation of Cell Homogenates

A549 parental and TPZ-adapted lines were seeded in 100-mm plastic culture dishes at 2×10^5 cells/dish and maintained in complete culture medium until they had reached late exponential phase. The exponentially growing monolayers were washed twice with ice-cold PBS, scraped off the dish into cold PBS, and pelleted by centrifugation (400g). The cells were resuspended in a volume of 50 mM phosphate buffer (pH 7.8) sufficient to yield a final protein concentration of 5–10 mg protein/mL. The cell suspension was then sonicated (2×15 sec) at 15% output from a Virsonic 300 Cell Disrupter (Virtis, Gardiner, NY). The suspension was frozen, thawed, and sonicated again (2×15 sec) prior to determination of protein content using a BCA Protein Assay Kit (Pierce Biochemical, Moline, IL) with bovine IgG as a standard. Cell lysates for determination of NADPH cytochrome P450 reductase activity were prepared as above with the following modifications: following centrifugation, the cells were resuspended in ice-cold isolation buffer (10 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.05 mM dithiothreitol), and held on ice for 10 min before sonication. The protein content of the cell lysates was determined using a Bio–Rad Bradford Kit (Bio-Rad, Hercules, CA).

Enzymatic Assays

SOD activity was measured by the method of Spitz and Oberley [31] with the following minor modification: when NaCN was added to the assay solution to inhibit CuZnSOD and allow measurement of MnSOD alone, the assay reagent mixture was readjusted to pH 7.8 by the addition of 0.05% phosphoric acid. Catalase activity was determined by the method of Brannan *et al.* [32]. Glutathione peroxidase activity was determined by the method of Lawrence and Burk [33] using hydrogen peroxide as a substrate and sodium azide to inhibit catalase activity. Glutathione reductase activity was determined by the method of Ray and Prescott [34]. NAD(P)H (quinone acceptor) oxidoreductase (EC 1.6.99.2) (DT-diaphorase) activity was measured using the method of Prochaska *et al.* [35]. P450 reductase activity was determined by spectrophotometric assay of the NADPH-dependent reduction of cytochrome c, using published methods [36]. Changes in enzyme activity were evaluated by ANOVA and subjected to Dunnett's post-hoc test to determine if the values in the adapted cell lines were significantly different from the values in the parental line.

TPZ Metabolism and Cellular Uptake

Confluent A549 cultures (of parental and drug-adapted cells) were harvested by trypsinization, and a 2-mL suspension of 5×10^5 cells/mL in complete medium was placed in a 60-mm glass dish immediately prior to the addition of TPZ to give a final concentration of 50 μM. Hypoxia was achieved as described above, and after the final evacuation the jigs were held at 760 mm Hg with continuous shaking at 37° for the duration of the drug exposure. After 0, 15, 30, or 45 min of hypoxic drug exposure, the jigs were reoxygenated, and aliquots of cell suspension were removed. The cells were pelleted by centrifugation, and the supernatant was saved for HPLC analysis of SR 4317 production. Aliquots were stored at −80° until analyzed. To determine whether cellular retention of SR 4317 would bias the results of sampling the supernatant derived from whole cell suspensions, representative samples were pelleted by centrifugation, and the cell pellet and supernatant were analyzed separately. Cell pellets were lysed with 70 μL water and precipitated using 2 vol. of methanol containing the internal standard (100 μM pimonidazole). Extracellular samples were precipitated with 2 vol. of methanol containing the internal standard. Supernatants of these samples were analyzed by HPLC on a Waters Nova Pak-phenyl column (3.9×150 mm) eluted with an isocratic mobile phase (22.5% methanol, balance water) at a flow rate of 1.2 mL/min. Concentrations were calculated from integrations performed using Waters Maxima 820 software using absorbance at 269 nm for TPZ (R_t = 3.9 min) and 238 nm for SR 4317 (R_t = 9.2 min). Under these conditions, the

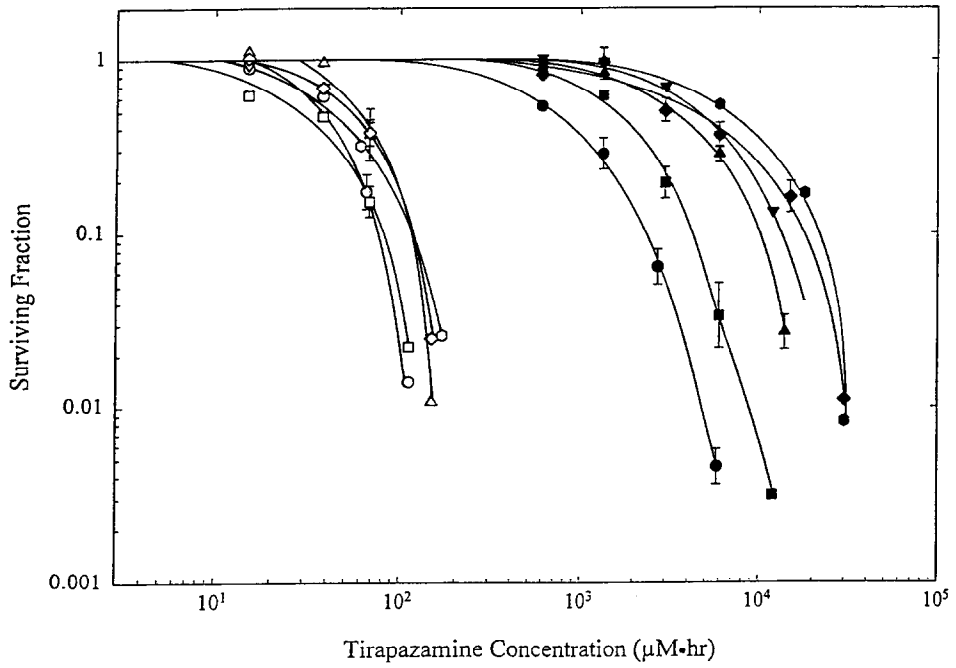

FIG. 2. TPZ cytotoxicity in parental and adapted cell lines under aerobic (filled symbols) and hypoxic conditions (open symbols). The cell lines are: parental A549 (○, ●), 10 μM TPZ adapted (□, ■), 25 μM TPZ adapted (△, ▲), 50 μM TPZ adapted (▽, ▼), 75 μM TPZ adapted (◇, ◆), and 100 μM TPZ adapted (○, ●). The results are pooled from three independent experiments; error bars are SEM. The unit "μM · hr" is used so as to equate the 90-min hypoxic exposures with the 6-hr aerobic exposures. We found that cytotoxicity was closely dependent on concentration × time over this time range (data not shown).

sensitivity of the assay was 0.1 fmol/cell/min. Cells were found to preferentially retain SR 4317 by a factor of 4 on a volume basis, but since the volume of extracellular medium was in over 1000-fold excess of the pellet volume, sampling of extracellular medium was judged to be a valid method for determining SR 4317 production.

RESULTS

Survival of TPZ-Adapted Lines under Aerobic and Hypoxic Conditions

Adaptation of A549 cells to continuous aerobic exposure to TPZ was performed as described in Materials and Methods. Following completion of the selection process, the parental A549 cell line and the five adapted cell lines were characterized with respect to their sensitivity to TPZ under both aerobic and hypoxic conditions. The TPZ-adapted cell lines were maintained free of selective pressure and exhibited similar survival results when examined at 21 and 180 days following the end of the selection protocol. Figure 2 shows the pooled results of three independent clonogenic survival assays performed on the parental and TPZ-adapted cell lines following TPZ exposure under aerobic or hypoxic conditions. The cellular response to TPZ was assessed using two endpoints; (1) the DMF calculated as the ratio of drug concentrations required to reduce survival to 10% of untreated controls in the parental and drug-adapted cell lines, and (2) the HCR, the ratio of drug concentrations required to reduce survival to 10% under aerobic and hypoxic conditions. The HCR is a measure of the selective toxicity of the drug under hypoxic exposure conditions.

The response of the parental and TPZ-adapted cell lines to hypoxic and aerobic TPZ exposure is summarized in Table 1. All adapted cell lines exhibited increased resistance to cell killing by TPZ under aerobic conditions with DMF values ranging from 1.8 to 9.2. The degree of aerobic resistance was proportional to the concentration of TPZ used for adaptation ($r = 0.989$, $F = 183$). The sensitivity of the aerobically adapted lines to killing by TPZ under

TABLE 1. Comparison of hypoxic and aerobic TPZ-mediated cytotoxicity in adapted lines

Cell line*	Hypoxic 10% survival (μM · hr)	Aerobic 10% survival (μM · hr)	DMF (10%) hypoxic exposure	DMF (10%) aerobic exposure	Hypoxic cytotoxicity ratio	SR 4317† production (fmol/cell/min)	Cytochrome P450 reductase activity† (mmol/mg/min)
A549	73	2,400	1.0	1.0	33	20.3 ± 8.2	33.0 ± 1.4
10 μM^R	81	4,400	1.1	1.8	54	21.6 ± 7.6	9.9 ± 0.4‡
25 μM^R	110	10,000	1.5	4.2	91	20.7 ± 5.4	0.88 ± 0.17‡
50 μM^R	110	14,000	1.5	5.8	127	18.3 ± 6.1	0.51 ± 0.09‡
75μM^R	110	19,000	1.5	7.9	173	8.4 ± 2.8‡	0.52 ± 0.11‡
100 μM^R	110	22,000	1.5	9.2	200	9.9 ± 2.3‡	0.42 ± 0.02‡

* The superscript "R" after the concentration (e.g. 50 μM^R) refers to the TPZ concentration to which the cell line was adapted to grow under aerobic conditions.
† Values are means ± SEM, N = 6.
‡ Significantly different from parental control values at $P < 0.05$.

TABLE 2. Antioxidant enzyme levels in TPZ-adapted A549 cell lines

Cell line*	MnSOD (U/mg)	CuZnSOD (U/mg)	Catalase (U/mg)	Glutathione peroxidase (mU/mg)	Glutathione reductase (mU/mg)	Quinone oxidoreductase (nmol/min/mg)
A549	19 ± 5	28 ± 4	13 ± 1	164 ± 12	21 ± 6	3.56 ± 0.25
10 μM^R	34 ± 4	32 ± 5	11 ± 1	155 ± 16	18 ± 4	1.51 ± 0.65†
25 μM^R	70 ± 12†	29 ± 6	13 ± 2	172 ± 18	26 ± 3	0.10 ± 0.03†
50 μM^R	93 ± 14†	24 ± 5	14 ± 2	179 ± 9	45 ± 8†	0.12 ± 0.05†
75 μM^R	110 ± 21†	22 ± 6	16 ± 2	181 ± 14	44 ± 4†	0.16 ± 0.01†
100 μM^R	179 ± 29†	19 ± 6	18 ± 2	168 ± 22	34 ± 4	0.15 ± 0.01†

Values are means ± SEM, N = 3–5.

* The superscript "R" after the concentration (e.g. 50 μM^R) refers to the TPZ concentration to which the cell line was adapted to grow under aerobic conditions.

† Significantly different from parental control values at $P < 0.05$.

hypoxic conditions was largely unchanged, with DMF values ranging from 1.1 to 1.5. The increased resistance to aerobic drug exposure coupled with minor changes in the hypoxic sensitivity resulted in an increased HCR value in each of the adapted cell lines. The HCR increased from 33 in the parental A549 line up to 200 in the 100 μM^R line. Survival experiments under aerobic or hypoxic conditions were also performed on parental A549 cells following exposure to 10 or 50 μM TPZ under aerobic conditions for 24 hr. The surviving fraction of the previously exposed A549 cells to TPZ was not different from that of cells not pretreated, when toxicity resulting from the aerobic pretreatment was corrected for.

Drug Metabolism in TPZ-Adapted Cell Lines

TPZ metabolism was measured in the adapted cell lines to determine if the aerobic resistance to TPZ was the result of reduced bioreductive capacity. TPZ metabolism was estimated by measuring the production of SR 4317, the two-electron reduction product of TPZ, under hypoxic conditions using HPLC analysis. SR 4317 can be formed by direct two-electron reduction of TPZ, by hydrogen abstraction (Fig. 1), or by disproportionation of two TPZ radicals. Table 1 shows the rate of SR 4317 production in parental and adapted cell lines. At lower levels of selection (10, 25, and 50 μM^R), the rate of SR 4317 production was not altered significantly from the level of metabolism in the parental line. However, in the cell lines adapted using higher TPZ concentrations (75 and 100 μM^R), there was a 2-fold decrease in the rate of SR 4317 production, indicating that cells adapted to higher drug concentrations had partially lost the ability to metabolize TPZ.

NADPH Cytochrome P450 Reductase Activity in TPZ-Adapted Cell Lines

The activity of NADPH cytochrome P450 reductase (P450 reductase) was measured in each of the cell lines in order to assess the role of this enzyme in the decreased rates of hypoxic metabolism of TPZ observed in the 75 and 100 μM^R cell lines. P450 reductase activity was significantly lower in each of the adapted cell lines (Table 1). In the 10 μM^R line, the activity of this enzyme was 30% of that in the parental line, while in the lines adapted to 25–100 μM P450 reductase activity was only 1–3% of the parental value.

Antioxidant Enzyme Levels in TPZ-Adapted Cell Lines

The activity of antioxidant enzymes was measured in the TPZ-adapted cell lines to determine whether changes in the activity of these enzymes were associated with the increased resistance to cell killing by TPZ under aerobic conditions. Table 2 shows the levels of SOD, catalase, glutathione peroxidase, glutathione reductase, and quinone oxidoreductase (DT-diaphorase) in parental and adapted cell lines. All adapted lines demonstrated increased levels of the mitochondrial enzyme MnSOD. MnSOD activity was between 1.8- and 9.4-fold higher than in the parental cell line with the increase being statistically significant in the 25, 50, 75, and 100 μM^R cell lines. Activity of the cytoplasmic form of superoxide dismutase (CuZnSOD) was not altered significantly in any of the lines examined. The adapted cell lines also did not demonstrate a change in the levels of hydrogen peroxide metabolizing enzymes (catalase and glutathione peroxidase), but two lines (50 and 75 μM^R) exhibited significantly increased levels of glutathione reductase.

In addition to measuring the activities of antioxidant enzymes, the activity of the drug detoxifying enzyme DT-diaphorase was also determined. In contrast to MnSOD and glutathione reductase activity, DT-diaphorase activity in the adapted cell lines was significantly lower than in the parental cell line, reaching a minimum value of 2.8% of parental levels in the 25 μM^R line. No further decrease in activity was observed in the lines adapted to higher concentrations of TPZ.

Role of DT-Diaphorase in Cell Killing by TPZ

The relevance of the large decrease in DT-diaphorase activity in the adapted cell lines to killing by TPZ was investigated by determining the cytotoxicity of TPZ in the parental A549 cell line with and without the addition of

dicoumarol, an inhibitor of DT-diaphorase. Survival data were averaged from at least three determinations in two independent experiments. The plating efficiency of the untreated controls was not affected by the addition of dicoumarol (2 mM). The surviving fraction following aerobic exposure to 900 μM TPZ for 18 hr was 5.44 ± 1.38×10^{-2} (N = 3, SEM) in the absence of dicoumarol, and 9.24 ± 1.98×10^{-2} (N = 4, SEM) when dicoumarol (2 mM) was present throughout the drug exposure period. Following a 90-min exposure to 40 μM TPZ under hypoxic conditions, the surviving fractions were 7.89 ± 6.96×10^{-2} (N = 3, SEM) and 7.99 ± 2.46×10^{-2} (N = 4, SEM) with or without dicoumarol, respectively. Thus, dicoumarol provided a small degree of protection against cell killing by TPZ under aerobic, but not under hypoxic conditions.

DISCUSSION

In this investigation we have demonstrated that adaptation of a human lung adenocarcinoma cell line to the aerobic toxicity of the bioreductive agent TPZ significantly increased the aerobic resistance of these cells to acute TPZ exposures with only small effects on the response to the drug under hypoxic conditions. The adaptation process increased the hypoxic selectivity of TPZ in the adapted lines by predominantly modifying the aerobic toxicity.

Adaptation of A549 cells to aerobic TPZ exposure resulted in two distinct cellular changes that may account for the increased drug resistance: (1) increased expression of enzymes that detoxify reactive oxygen species, and (2) decreased bioreductive activation of the drug at high levels of selection. This pattern is consistent with the adaptive response seen in other cells selected for resistance to agents, such as paraquat, that produce cytotoxicity via futile redox cycling. It has been shown that resistance to paraquat is associated with both increased expression of antioxidant enzymes, and the loss of reductive enzymes that allow the drug to redox cycle [37]. Since reactive oxygen species such as superoxide will only be produced under aerobic conditions, via redox cycling reactions of the TPZ radical, increased levels of antioxidant enzymes should only modify aerobic drug toxicity. In fact, the adaptation process had little effect on the hypoxic drug toxicity, with the adapted cell lines (25–100 μM^R) being only 1.5-fold less sensitive than the parental line. This supports the hypothesis proposed in a number of recent studies that different mechanisms are responsible for aerobic and hypoxic TPZ toxicity.

Different types of TPZ-induced DNA damage under aerobic and hypoxic conditions were suggested by the observation that radiation sensitive irs-1 cells, which are defective in the fidelity of DNA strand break repair, were 15-fold more sensitive than parental V79 cells to TPZ under aerobic conditions, but only 1.7-fold more sensitive under hypoxic conditions [38]. Studies with isolated rat hepatocytes have also provided evidence that the mechanism of TPZ toxicity differs under aerobic and hypoxic conditions [39]. Inactivation of catalase and glutathione reductase, two antioxidant enzymes, resulted in increased aerobic toxicity (as judged by trypan blue exclusion), with no effect on the hypoxic toxicity of TPZ. As mentioned earlier, Chinese hamster V79 cells were protected against aerobic, but not hypoxic TPZ toxicity by addition of the metal chelators desferrioxamine or Tiron® [40]. These observations are consistent with aerobic cell killing by TPZ, at least in part, resulting from the production of reactive oxygen species via redox cycling of the TPZ radical in the presence of molecular oxygen. One target of superoxide produced in the intracellular compartment may be the mitochondria. Aerobic TPZ exposure has been reported to cause uncoupling of mitochondrial respiration in primary rat hepatocytes and in the MCF-7 human tumor cell line [39, 41]. This mitochondrial uncoupling has been shown to result from one-electron redox cycling of TPZ [39].

In the present study, the increased resistance to TPZ under aerobic conditions correlates well with the increased activity of MnSOD ($r = 0.966$, $F = 55$), with the 100 μM^R line being 9.2-fold less sensitive to TPZ than the parental line, and having a 9.4-fold higher level of activity of MnSOD. This suggests that the acquired resistance to cell killing by TPZ under aerobic conditions can largely be accounted for by the increase in activity of MnSOD. The fact that both antioxidant enzymes which demonstrated increased activity in the TPZ-adapted lines (MnSOD and glutathione reductase) are mitochondrial in origin is consistent with mitochondria being a target for aerobic TPZ toxicity, and suggests that the adaptation protocol used here may have selected for cells that were able to maintain ATP production via coupled respiration under continuous TPZ exposure.

One unexpected observation in this investigation was the loss of DT-diaphorase activity in the TPZ-adapted cell lines. DT-diaphorase is an obligate two-electron reductase that is important in the bioreductive activation of a number of quinone bioreductive drugs such as EO9 [42], mitomycin C [43], and CB 1954 [44]. DT-diaphorase reduces TPZ directly, by the addition of two electrons, to SR 4317, bypassing the cytotoxic one-electron reduction product, the TPZ radical. Purified rodent Walker DT-diaphorase reduces TPZ to SR 4317 and the four-electron reduction product SR 4330 [45], both of which are non-toxic [46]. Thus, DT-diaphorase detoxifies TPZ and should protect against TPZ toxicity. Therefore, it might be expected that cells adapted to continuous aerobic exposure to TPZ would express increased levels of this enzyme. In fact, the opposite was observed in this study with the adapted cell lines possessing very low levels of this enzyme (activity down to 3% that of parental A549 cells).

Although there is evidence that DT-diaphorase contributes to the metabolism of TPZ under hypoxic conditions [25], there is no evidence that it has any effect on TPZ toxicity. The studies reported here show that the addition of dicoumarol (an inhibitor of DT-diaphorase) had little effect on aerobic or hypoxic TPZ toxicity towards the

parental A549 cell line. Other recent studies have also reported evidence that DT-diaphorase is not an important enzyme in the bioreductive activation of TPZ. Using a panel of human breast and lung cancer cell lines, Patterson *et al.* [47] found no correlation between DT-diaphorase expression and aerobic or hypoxic TPZ cytotoxicity. Further, the cytotoxicity of TPZ towards the two human colon carcinoma cell lines (HT29 cells, which express a high level of DT-diaphorase, and BE cells, which have a mutation in the *NQO1* gene and do not express this activity) was essentially the same [48]. It is thus unlikely that the changes in DT-diaphorase activity seen in the present study in the adapted cell lines contributed to their decreased aerobic toxicity.

While DT-diaphorase does not appear to contribute to TPZ toxicity, several studies have suggested cytochrome P450 and NADPH cytochrome P450 reductase (P450 reductase) to be important enzymes in drug reduction, although there is disagreement as to which is the more important. Some investigators have reported that the majority of reduction is carried out by cytochrome P450 [23–25], while others have identified P450 reductase as the most important enzyme [22, 26]. There is also disagreement as to the relationship between P450 reductase activity and sensitivity to TPZ under hypoxia. For example, Patterson and colleagues have shown that the toxicity of TPZ under hypoxic conditions correlates strongly with NADPH cytochrome P450 reductase activity in a panel of six human breast adenocarcinoma cell lines [36], but found no correlation in a similar series of experiments with lung cancer cell lines [49]. Also Plumb *et al.* [50] found no correlation between P450 reductase activity and sensitivity to TPZ using a large panel of human tumor cell lines.

The activity of P450 reductase was measured in the present study in order to assess whether the decreased capacity of the adapted cell lines to reduce TPZ was related to decreased activity of this enzyme. While adaptation to continuous TPZ exposure resulted in a 2-fold decrease in bioreductive capacity, determined as production of SR 4317 under hypoxic conditions, and a 1.5-fold decrease in sensitivity to cell killing by TPZ under hypoxic conditions, the adapted cell lines demonstrated a very large decrease (in the order of 50-fold) in P450 reductase activity relative to the parental cell line. Although a one-to-one correlation between P450 reductase activity and killing by TPZ is unlikely, given the possible reactions of the toxic TPZ radical including detoxifying bimolecular disproportionation reactions, the much greater decrease in enzyme activity than in hypoxic metabolism and cytotoxicity in the adapted cell lines questions the role of P450 reductase in the bioreductive activation of TPZ in the A549 human lung adenocarcinoma line.

Thus, both from our own data and from that in the literature, it is apparent that there is unlikely to be a clear correlation between P450 reductase and TPZ cytotoxicity for all cell lines. One likely reason for this is that the activating enzyme(s) responsible for drug toxicity may need to be close to DNA, thereby producing high local concentrations of the active TPZ radical [21]. If this were the case, it would mean that the metabolism responsible for cytotoxicity might represent only a small fraction of the overall drug metabolism by the cell, thereby making correlations between cytotoxicity and cellular enzyme (or metabolism) levels difficult to make. Indeed we have shown recently that there is a much better correlation between DNA damage induced by TPZ and hypoxic cytotoxicity in a panel of cell lines than between drug metabolism and cytotoxicity in the same panel [51].

In summary, we have shown that the aerobic toxicity of TPZ can be modified with little change in the cytotoxicity of the drug under hypoxic conditions. This suggests that different mechanisms of toxicity operate under aerobic and hypoxic conditions. While direct interaction of the TPZ radical with intracellular macromolecules and/or direct cytotoxicity of the parent TPZ molecule may still contribute to aerobic toxicity, it appears that other mechanisms, particularly the production of reactive oxygen species as a result of redox cycling of the TPZ radical, play the major role. The process of adaptation to aerobic TPZ resistance was associated with increases in the levels of antioxidant enzymes that are responsible for detoxifying reactive oxygen species. There was a strong correlation between the degree of aerobic resistance and activity of MnSOD. This suggests that increases in intracellular antioxidant enzymes or radical scavengers may provide protection against the aerobic toxicity of TPZ. Muscle cramping and reversible ototoxicity are side-effects observed in the clinical trials of TPZ [28, 29]. If these effects are the result of reactive oxygen species from the futile cycling of TPZ under aerobic conditions, then it is possible that administration of antioxidants, metal chelators, or radical scavengers could protect against this normal tissue toxicity of TPZ.

This work was supported by Grant CA 15201 awarded by the U.S. National Cancer Institute, DHHS.

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