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Metabolism of tirapazamine by multiple reductases in the nucleus

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Abstract

Tirapazamine (TPZ, 3-amino-1,2,4-benzotriazine 1,4-di-*N*-oxide, SR4233, Tirazone), a bioreductive drug currently in clinical trials, is selectively toxic to hypoxic cells commonly found in solid tumors. The toxicity results from the intracellular metabolism of TPZ to a highly toxic radical. When oxygen levels are low, the TPZ radical reacts with cellular molecules, producing DNA damage and cell death. The much lower toxicity towards aerobic cells results from the back-oxidation of the TPZ radical by oxygen. A major unresolved aspect of the mechanism of TPZ is the identity of the reductase(s) in the cell responsible for activating the drug to its toxic form. We have studied both the metabolism of the drug using HPLC and the formation of the TPZ radical with a fluorescence assay using dihydrorhodamine 123. We also measured DNA double- and single-strand breaks produced by TPZ, using the comet assay. We demonstrated that multiple reductases in the nucleus metabolize TPZ under hypoxia. Using the cofactor dependence of the reductases for metabolizing TPZ and of the DNA damage caused by TPZ, we show that DNA single-strand breaks after TPZ metabolism are probably caused by the most abundant source of reductase in the nucleus. DNA double-strand breaks, on the other hand, are formed by TPZ metabolism by an unknown nuclear reductase that requires only NADPH for its activity. This study is the first to characterize multiple nuclear reductases capable of activating TPZ.

Keywords: Tirapazamine; Bioreductive drug; Hypoxia; Tumor; NADPH; Cytochrome P450 reductase

1. Introduction

The microenvironment of human solid tumors is heterogeneous and contains regions that are poorly oxygenated [1]. As a result, most primary tumors have lower median pO_2 levels than does normal tissue at the site of growth and contain regions of cells that are severely hypoxic. However, the median pO_2 levels can vary from one tumor to another of the same histological type and are independent of such tumor and patient parameters as tumor size, extent of necrosis, and patient hemoglobin level [2].

The cells in these hypoxic regions may contribute to the failure of cancer therapy. Hypoxic cells are resistant to radiotherapy [3] because oxygen "fixes," or makes permanent, cellular damage caused by radiation. When cells are irradiated in low-oxygen conditions, the free radicals produced in DNA react with hydrogen-donating species, including glutathione, which reverses the free radical damage and protects the cell. Additionally, hypoxic cells are resistant to some drugs because they are non-proliferating and, therefore, do not respond to drugs active against only proliferating cells, and because they are distant from the blood vessels that carry the drug [4]. Clinically, low tumor oxygenation predicts for a poor treatment outcome following radiotherapy to head and neck and cervix cancers [5–7].

However, extensive hypoxia is unique to solid tumors as it is potentially an exploitable target. TPZ (3-amino-1,2,4-benzotriazine-1,4-di-*N*-oxide, WIN 59075, SR 4233, Tirazone) belongs to a class of cytotoxins [8] that selectively kill hypoxic cells in mammalian cell lines [9]. The hypoxic cytotoxic ratio (HCR) of TPZ, defined as the ratio of drug concentrations under aerobic to hypoxic conditions that give equal cell kill, is between 50 and 200 for most cell types [9]. This selectivity allows TPZ to be given in the clinic at doses that are toxic to hypoxic tumor cells and non-toxic to well-oxygenated cells [10]. To date, results of phase II and phase III clinical testing of TPZ in combination with radiation and

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Abbreviations: TPZ, tirapazamine; P450 reductase, NADPH:cytochrome P450 reductase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; DHR123, dihydrorhodamine 123; DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride; SN, supernatant from nuclei; LS, low salt; HS, high salt; and NM, nuclear matrix.

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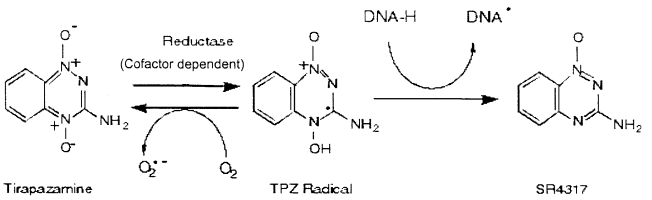

Fig. 1. TPZ activation under both hypoxic and aerobic conditions. TPZ undergoes an enzyme-mediated one-electron reduction to form a toxic radical intermediate. When oxygen is present, the TPZ radical reacts with oxygen to form superoxide and the parent TPZ molecule. Under low oxygen conditions, the active free radical can abstract hydrogen from DNA and other macromolecules.

with cisplatin show significant promise for both head and neck cancers and for non-small-cell lung cancer [11,12].

Current knowledge of the mechanism for TPZ activation is shown in Fig. 1. In hypoxic conditions, TPZ undergoes a one-electron reduction that is mediated by a cofactor-requiring reductase to form a free radical intermediate. The TPZ free radical has been detected by EPR using *in vitro* systems [13] and can produce DNA single-strand breaks, double-strand breaks, and chromosome aberrations [14] with the concomitant formation of the two-electron metabolite SR4317 (3-amino-1,4-benzotriazine-1-*N*-oxide). The lifetime of the TPZ radical has been measured under hypoxia as approximately 10 msec [15]. However, the lifetime of the radical in the cell is not known. Under aerobic conditions, the TPZ radical preferentially reacts with oxygen to form a superoxide radical and the parent compound. The reaction of the TPZ radical with oxygen is the source of its selective toxicity to hypoxic cells [16]. Both TPZ and the two-electron metabolite, SR4317, are stable and relatively non-toxic. This implicates the TPZ free radical intermediate as responsible for DNA damage and toxicity.

A major unresolved question is the identity of the enzyme(s) that activates TPZ to cause cell death. Numerous enzymes, including NADPH:cytochrome P450 reductase (EC 1.6.2.4), cytochrome P450, xanthine oxidase (EC 1.2.3.1), and DT-diaphorase (EC 1.6.99.2), are able to metabolize TPZ *in vitro* under hypoxic conditions [15,17–20]. One approach to identifying the relevant metabolizing enzyme has been to correlate enzyme activity with sensitivity to TPZ, and such a correlation between NADPH:cytochrome P450 reductase levels and sensitivity to TPZ has been reported for some cell lines [21], but not for others [22].

We recently found that only the metabolism of TPZ in the nucleus contributes to DNA damage [23]. Using isolated nuclei from A549 lung carcinoma cells, we found that although the nuclei accounted for only ~20% of the overall cellular metabolism of TPZ, this produced all of the TPZ-induced DNA single-strand breaks. We also demonstrated that TPZ radicals formed outside of the nucleus do not enter the nucleus and damage DNA. Taken together, these results indicate that only a fraction of the cellular metabolism of TPZ is responsible for its toxic lesions. Thus, measuring overall TPZ metabolism or total cellular TPZ reductase levels would most likely not be useful in identifying tumors responsive to TPZ.

These results also suggest that the cellular localization of the TPZ radical is important for its effect on the cell. Consistent with this idea is the fact that there are approximately 10,000 times more DNA double-strand breaks formed by TPZ in cells than would be predicted from random distribution of single-strand breaks in the DNA [24,25]. This also provides evidence for a non-random distribution of TPZ radicals in the cells.

In this study, we investigated the role of nuclear proteins in the activation of TPZ. Identification of the relevant reductase(s) not only would aid in further drug development, but also would be useful in identifying which tumors are sensitive to TPZ. We have shown that there are nuclear reductases that can metabolize TPZ to its toxic form and that TPZ metabolism is not uniformly distributed in the nucleus. Furthermore, we found that the dependence of TPZ metabolism on either of the cofactors NADH or NADPH differs for different nuclear fractions, providing evidence for multiple nuclear reductases capable of metabolizing TPZ. We also provide evidence that different enzymes cause the DNA single- and double-strand breaks induced by TPZ.

2. Materials and methods

2.1. Cell lines

Cell lines were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°. HeLa cells were obtained from the American Type Culture Collection and were maintained as monolayers in α-MEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 200 units/mL of penicillin–0.2 mg/mL of streptomycin. HeLa cells were also grown in spinner flasks in RPMI medium supplemented with 10% FBS and antibiotics. All experiments utilized cells in logarithmic-phase growth.

2.2. Drugs and chemicals

TPZ was obtained from Sanofi-Synthelabo. SR4317 was supplied by Dr. Michael Tracy (SRI International) and used as an internal control for HPLC. Methanol was from Mallinckrodt. Triton X-100 was from Boehringer Mannheim. DHR123 and rhodamine 123 were from Molecular Probes. Purified P450 reductase was obtained from Promega. DT-diaphorase was a gift from Dr. David Ross (University of Colorado Health Sciences Center). All other chemicals were obtained from the Sigma Chemical Co. and were of the highest analytical quality.

2.3. Clonogenic assay for measuring cell kill

Cells were seeded as monolayers onto 60 mm^2 glass dishes in 5 mL of complete medium 2 days before treatment such that each dish would have 1×10^6 cells at the time of drug exposure. A stock solution of TPZ (4 mM) was dissolved in complete medium and added at the appropriate concentration in medium in a final volume of 2 mL. The cells were then added to prewarmed, leak-proof aluminum jigs, placed on a shaking platform at room temperature, and treated with five rapid evacuations and fillings with either 95% nitrogen–5% CO_2 for hypoxia or with 95% air–5% CO_2 for aerobic controls. The oxygen concentration in the hypoxic jigs was less than 0.02%. The jigs were then placed in an incubator at 37° for 1 hr with shaking. After the 1 hr of drug exposure, the dishes were removed from the jigs. The cells were then washed with PBS, trypsinized, and plated in complete medium for cloning. After 10 days, the plates were stained with crystal violet, and colonies with more than 50 cells were counted as survivors.

2.4. Nuclear protein purification

HeLa cells were harvested from spinner flasks, washed twice with ice-cold PBS, and resuspended in cold hypotonic buffer A (20 mM HEPES-KOH, pH 7.5, at 4°, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM DTT). Cells were held on ice for 10 min and then homogenized gently at a density of 3×10^6 with a ground glass Dounce homogenizer. The nuclei content of the sample was monitored constantly with DAPI staining under a fluorescent microscope using a 580 nm UV filter. Nuclei content was greater than 95% with this method, and the nuclei themselves remained intact. The lysed cells were transferred to 15-mL tubes, held on ice for 30 min, and then spun at 1000 *g* for 10 min at 4°; the supernatant was kept as the cytoplasm fraction. The nuclei were fractionated by standard procedures with some modifications The nuclear pellet was resuspended in 20 vol. of buffer B (0.25 M sucrose, 10 mM Tris–HCl, pH 7.4, at 4°, 5 mM $MgCl_2$, 1 mM PMSF, 1 μg/mL of leupeptin, 0.1 mM DTT). DNase I (0.5 mg/mL) and RNase A (0.25 mg/mL) were added, and the nuclei were incubated at room temperature for 15 min. The nuclei were then quick-chilled on ice and centrifuged at 1000 *g* for 10 min at 4°. The supernatant was removed and labeled as the SN fraction. The pellet was resuspended in TMP1 (0.2 M $MgCl_2$, 10 mM Tris–HCl, pH 7.4, at 4°, 1 mM PMSF, 1 μg/mL of leupeptin, 0.1 mM DTT), held on ice for 10 min, and centrifuged at 1000 *g* for 15 min at 4°. The pellet was washed twice with TMP1, and the combined supernatant was labeled as LS. Then the pellet was resuspended in one-half volume of TMP1 and held on ice. The same volume of TMP2 (4 M NaCl, 0.2 M $MgCl_2$, 10 mM Tris–HCl, pH 7.4, at 4°, 1 mM PMSF, 0.1 mM DTT, 1 μg/mL of leupeptin) was added over 30 min in five aliquots with constant mixing to avoid areas of local HS concentration. The sample was then centrifuged at 1000 *g* for 15 min at 4°. The pellet was washed twice with TMP3 (2 M NaCl, 0.2 M $MgCl_2$, 10 mM Tris–HCl, pH 7.4, at 4°, 1 mM PMSF, 1 μg/mL of leupeptin, 0.1 mM DTT), and all supernatants were combined and labeled HS. The pellet was washed once with TMP1, and the supernatant was added to the HS fraction. Then the pellet was resuspended in TMP1, and one-tenth volume of 10% Triton X-100 (1% final concentration) was added slowly on ice. The sample was centrifuged at 1000 *g* for 15 min at 4°. The pellet was washed twice with TMP1, and all supernatants were combined and labeled NM. The remaining pellet was suspended in buffer C (50 mM KPO_4, pH 7.2, at 4°, 10% glycerol, 1 mM DTT, 1 mM EDTA) and labeled NMP (nuclear matrix pellet). All fractions were dialyzed overnight at 4° against buffer D (20 mM Tris–HCl, pH 7.0, at 4°, 5 mM $MgCl_2$, 25 mM NaCl, 5% glycerol, 1 mM PMSF, 0.1 mM DTT, 1 μg/mL of leupeptin) in dialysis tubes with a molecular weight cutoff of 2000 from Spectrum. The NM fraction was dialyzed against buffer D with 0.05% Triton X-100.

2.5. Protein determination

The amount of protein in the cellular extracts was determined by the BCA assay (Pierce) [26] using both bovine serum albumin and IgG as protein standards.

2.6. TPZ metabolism in vitro

A hypoxia chamber (Sheldon Manufacturing) was used for TPZ metabolism assays. The chamber was maintained at less than 0.05% O_2 by continuous gassing with 5% H_2, 5% CO_2, and 90% N_2. An oxygen electrode (Oxygen Systems Inc.) with a sensitivity of 0.01% O_2 was placed inside the chamber to monitor ambient oxygen levels. At least 90 min before an experiment, the following were placed inside the chamber: TPZ stock solution, 10× metabolism buffer (250 mM Tris–HCl, pH 7.0, 250 mM NaCl, 500 mM KCl, 100 mM EDTA), NADPH (10 mM), NADH (10 mM), water, and the protein sample. All solutions were in glass tubes to avoid contamination from the dissolved oxygen in plastic. The protein and cofactor solutions were placed in a container with dry ice to keep them cold without introducing water into the chamber. The solutions were bubbled with N_2 for 1 min before allowing them to equilibrate in the chamber for 1 hr prior to the experiment. The liquids were bubbled through with N_2 again immediately before the start of incubation. The final reaction mixture contained TPZ (5–100 μM), NADPH (500 μM) and/or NADH (500 μM), 1× metabolism buffer, and protein extract (5–50 μg) in a final volume of 100 μL.

2.7. HPLC

After incubation for 1 hr under hypoxia, the TPZ metabolism samples were removed from the chamber and placed on ice. An aliquot of 25 μL was loaded onto an HPLC

column to detect TPZ and its metabolite, SR4317, the two-electron reduction product of TPZ, as previously described [16]. The amount of activity in each extract was calculated as the micromolar amount of SR4317 produced per microgram of protein per unit of time.

2.8. TPZ radical detection with DHR123

TPZ radicals were detected by using DHR123 oxidation as an indicator of free radical formation [27]. DHR123 is a non-fluorescent compound that is oxidized by highly reactive species, including the TPZ radical, to form the highly fluorescent rhodamine 123 compound. DHR123 (25 μM) was added to the TPZ metabolism system described in a previous section in a final volume of 2 mL. The reaction was placed in 60 mm^2 glass dishes, and hypoxia was created in the aluminum jigs with alternate flushing and filling of 95% N_2, 5% CO_2. After a 1-hr incubation at 37°, the solution was transferred to a cuvette, and the fluorescence was measured using a Perkin Elmer LS-3 fluorescence spectrophotometer at 509 nm absorbance, 529 nm emission, with a slit-width of 10 nm. A blank containing 1× metabolism buffer and cofactors was used to zero the spectrophotometer.

2.9. Nuclei isolation

Intact nuclei were isolated from HeLa cells as previously described with minor modifications [28]. Briefly, cells were harvested in log phase, washed twice with cold PBS, and resuspended at 12.5 μL/10^6 cells in NI buffer (115 mM KCl, 5 mM NaCl, 1 mM KH_2PO_4, 20 mM HEPES, 0.3 mM $MgCl_2$, 0.5 mM PMSF, and 0.5 mM DTT at pH 7.4). An equivalent volume of NI buffer with 1% IGEPAL CA-630 was added slowly, and the reaction was held on ice for 15 min. The permeable cells were disrupted by pipetting 25–30 times with a 1-mL pipette tip. The isolated nuclei were diluted with a 10× volume of NI buffer, centrifuged at 200 *g* for 15 min at 4°, and washed with 10 mL of NI buffer.

2.10. Measurement of DNA breaks using the comet assay

Single-strand DNA breaks were measured using the alkaline comet assay [29]. Isolated nuclei and whole cells were treated with TPZ in glass dishes under hypoxic conditions for 1 hr in aluminum jigs as described earlier. Cells were treated with TPZ dissolved in α-MEM + 10% FBS. Isolated nuclei were treated with TPZ in nuclei treatment buffer (100 mM KCl, 1 mM KH_2PO_4, 25 mM $NaHCO_3$, 20 mM HEPES, 10 mM EDTA, 5 mM gluthathione, and 0.9 mM spermine at pH 7.4). After a 1-hr treatment, cells and nuclei were resuspended in cold PBS without Mg^{2+} and $CaCl_2$ at a concentration of 3×10^4 cells/mL, and slides were prepared following the protocol of Olive [29]. A 500-mL aliquot of cell or nuclei suspension was placed in a labeled 5-mL glass tube, and 1.5 mL of 1% low-gelling temperature agarose (Sigma type VII dissolved in deionized water and held in a 45° water bath) was added to the suspension. The agarose solution was pipetted onto a microscope slide and allowed to gel for 1 min at 4° on a cold metal block. The slides were then placed in an alkaline lysis solution containing 1 M sodium chloride, 0.03 M sodium hydroxide, and 0.1% *N*-lauroylsarcosine for 1 hr at room temperature. After lysis, slides were washed three times, for 20 min each, in 2 mM EDTA with 0.03 M sodium hydroxide. The slides were placed in a gel box and electrophoresed in rinse buffer at 0.6 V/cm for 20 min. The slides were then removed from the gel box and placed in water for 15 min, followed by staining in propidium iodide (2.5 μg/mL in water) for 15 min. Cells were scored as described by Olive [29], and the images were analyzed using a commercially available system and software (Loats Associates, Inc.). The tail moment is calculated as the product of the percentage of DNA in the comet tail multiplied by the length between the means of the head and the tail distributions. Mean tail moments were determined from 150 or more individual comets for each data point.

Double-strand breaks were measured in cells and isolated nuclei by the neutral comet assay as described by Olive [29]. Briefly, cells and nuclei were treated as described above, placed in agarose, pipetted on slides, and lysed in a neutral lysis buffer. The slides were rinsed in neutral buffer, and electrophoresed and visualized as described above.

3. Results

3.1. Detection of the TPZ radical

HPLC quantifies TPZ metabolism by measuring the amount of TPZ converted into the two-electron reduction product, SR4317. However, the production of this product does not always indicate that the enzyme formed the toxic one-electron intermediate. DT-diaphorase, for example, metabolizes TPZ in a single two-electron step, bypassing the free radical, and producing SR4317 in a detoxifying reaction [17]. Thus, a fluorescence-based assay was developed for measuring the production of the TPZ radical. DHR123 is a non-fluorescent compound that can be oxidized by high-energy molecules, including the TPZ radical, to become the highly fluorescent rhodamine 123 (Rh123). The assay gave a linear concentration response for TPZ radical formation with both purified P450 reductase (data not shown) and the NM protein fraction as the source of reductase (Fig. 2A). We also confirmed with this assay that DT-diaphorase metabolizes TPZ to SR4317 without producing the toxic free radical intermediate (Fig. 2B).

3.2. Nuclear protein extraction

Isolated nuclei were washed with solutions of increasing salt and detergent concentrations, with each protein fraction

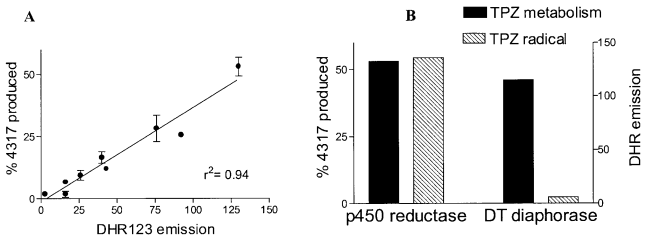

Fig. 2. Detection of the TPZ radical by the DHR123 assay. (A) TPZ metabolism was measured by HPLC detection of SR4317, and TPZ radical formation was measured by the DHR123 assay described in the text using the NM protein fraction. TPZ metabolism was calculated as the percentage of the initial TPZ (100 μM) converted to the two-electron metabolite, SR4317. The amount of SR4317 detected by HPLC measurements and the level of Rh123 fluorescence after the reaction was complete were linear, with a correlation coefficient of 0.94. The means ($\pm$ SD) for four independent experiments are shown. (B) Purified NADPH:cytochrome P450 reductase reduces TPZ by one-electron, producing both TPZ metabolism as measured by the amount of two-electron metabolite SR4317 and TPZ radical production as measured by the DHR123 assay. Purified DT-diaphorase reduced TPZ by a single two-electron reduction and bypassed the TPZ radical intermediate. Each experiment was performed three times, and the results from a representative experiment are shown. The standard deviation between the experiments was less than 5%.

being tested for the presence of proteins of known cellular localization to ensure quality control. Lamin B and topoisomerase IIα are both NM proteins, and both were associated with the NM-associated fractions (HS, NM) (data not shown). Xanthine oxidase and DT diaphorase were found exclusively in the cytoplasm. Cytochrome P450 reductase was associated with the cytoplasm and (to a minor extent) the nuclear wash fraction (SN) but not with proteins obtained with higher salt washes (data not shown). This pattern of extraction is consistent with published results [23].

3.3. Distribution of TPZ metabolism in the nucleus

We next investigated the distribution of TPZ metabolism throughout the nucleus (Table 1) by incubating the different fractions with NADH (500 μM) and NADPH (500 μM) and measuring the production of the TPZ metabolite SR4317. The cytoplasm was responsible for the majority of TPZ activation (71 $\pm$ 6.7%) in the cell, consistent with previous results with A549 human tumor cells [23]. In the nucleus, the majority of TPZ metabolism occurred in the LS fraction, which is comprised of nuclear proteins not associated with the NM. The fractions associated with the NM contributed approximately 30% of the nuclear TPZ metabolism (Table 1, HS, NM). This indicates that TPZ metabolism occurs throughout the nucleus and is not associated exclusively with any location in the nucleus.

3.4. Cofactor dependence of nuclear TPZ metabolism

TPZ activation *in vitro* is enzyme-mediated and needs the cofactors NADH or NADPH to supply reducing equivalents to the enzyme. To further characterize the various protein fractions, we analyzed the cofactor requirements of each protein fraction to metabolize TPZ. We found that the different fractions had different cofactor dependencies (Table 1). The cytoplasm proteins needed NADPH mainly, as did the LS nuclear proteins (Table 1, LS), although both fractions were capable of metabolizing TPZ with NADH. The HS nuclear fraction, on the other hand, was dependent more on NADH for TPZ metabolism (Table 1, HS). The NM-associated fraction could use either cofactor to metabolize TPZ. To distinguish between the possibilities of one enzyme in this fraction being dependent on either cofactor equally well versus one enzyme using NADH and another using NADPH, we tested whether adding both cofactors together doubled the rate of TPZ metabolism. The data (Table 1, NM) suggest that a single enzyme in this fraction is capable of using each cofactor equally well since having both cofactors at the same time gave no additional activity. This is reflected by the fact that each cofactor supported 92–95% of the maximum rate of TPZ metabolism obtained when both cofactors were present.

Table 1
TPZ-metabolizing activity in HeLa cells and nuclei

Protein source	Fraction name	% TPZ metabolism in cell	% TPZ metabolism in nuclei	% Metabolism supported by NADPH	% Metabolism supported by NADH
Cytoplasm		71 (6.7)		83 (2.2)	43.2 (1.8)
Nuclear		28.5 (4.9)			
	LS		67.2 (4.8)	99 (1.0)	68.7 (3.7)
	HS		7.7 (1.1)	57 (5.3)	98 (1.2)
	NM		22.3 (3.6)	95 (0.7)	92 (2.9)

Reactions were analyzed by both HPLC to quantify the amount of SR4317 produced, and DHR123 fluorescence to determine the amount of TPZ radical produced. The results obtained are normalized to the amount of protein per HeLa cell obtained in the protein isolation. The nuclear fraction contained approximately 42.3 $\pm$ 6.9% of the cellular protein compared with the cytoplasmic fraction. The percentage of TPZ metabolized in the nuclear fractions LS, HS, and NM is based on the percentage of TPZ metabolized in the nucleus normalized to 100%. The results represent the means (SD) for three independent experiments. The cofactor dependence of the nuclear fractions isolated from HeLa cells was determined by incubating 200 μM TPZ with the indicated protein fraction (100 μg) and cofactors (50 μM) for 1 hr under hypoxia. Each experiment was performed in triplicate, and the means (SD) for all experiments are shown. The results for the cofactor dependence are normalized to the amount of TPZ metabolism that occurs when both cofactors together are incubated with the protein reductase source and TPZ.

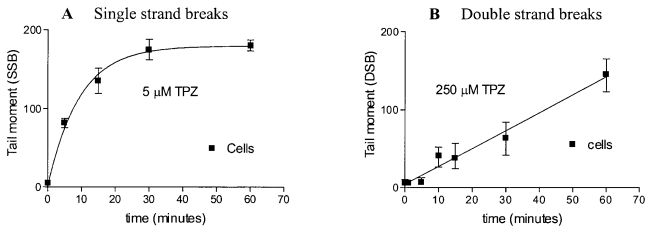

Fig. 3. Kinetics of induction of DNA damage by TPZ in HeLa cells. (A) Single-strand breaks (SSB) from tirapazamine, as measured by the alkaline comet assay described in the text. The means (± SD) for 300 comets are shown for selected time points. (B) Double-strand breaks (DSB) as measured using the neutral comet assay described in the text. The means (± SD) for 200 comets are shown.

3.5. Characterization of DNA damage by TPZ with the comet assay

The cofactor dependence of the nuclear proteins indicated that more than one enzyme was involved in the nuclear activation of TPZ. Therefore, we measured TPZ-induced DNA single- and double-strand breaks in isolated nuclei using the alkaline and neutral comet assay, respectively, in order to determine whether either type of damage had cofactor requirements that matched a particular protein fraction. To verify the linearity of measuring the comet 'tail moment' with strand breaks, we measured comet tail moment under both alkaline and neutral conditions as a function of radiation dose. Irradiated cells and nuclei showed a linear DNA tail moment under both alkaline and neutral conditions proportional to the dose of irradiation (data not shown).

3.6. Kinetics of single- and double-strand breaks from TPZ metabolism

We examined the kinetics of induction of DNA damage after TPZ in HeLa cells. Single-strand DNA breaks increased linearly after the initial time of drug exposure and reached a plateau after 15 min (Fig. 3A). TPZ was not depleted, and DNA breaks were produced and repaired continuously, possibly accounting for the plateau effect. On the other hand, double-strand breaks increased linearly throughout the entire 1-hr TPZ treatment (Fig. 3B). Thus, the two types of lesions differ in formation kinetics in whole cells. Due to the decreased sensitivity of the neutral comet assay as compared with the alkaline comet assay, higher levels of TPZ were needed to produce DNA double-strand breaks.

3.7. Comparison of levels of double- and single-strand breaks in whole cells and in nuclei

We next compared the level of DNA damage in cells with that in isolated nuclei given saturating levels (500 μM) of both cofactors. Cells and isolated nuclei had similar

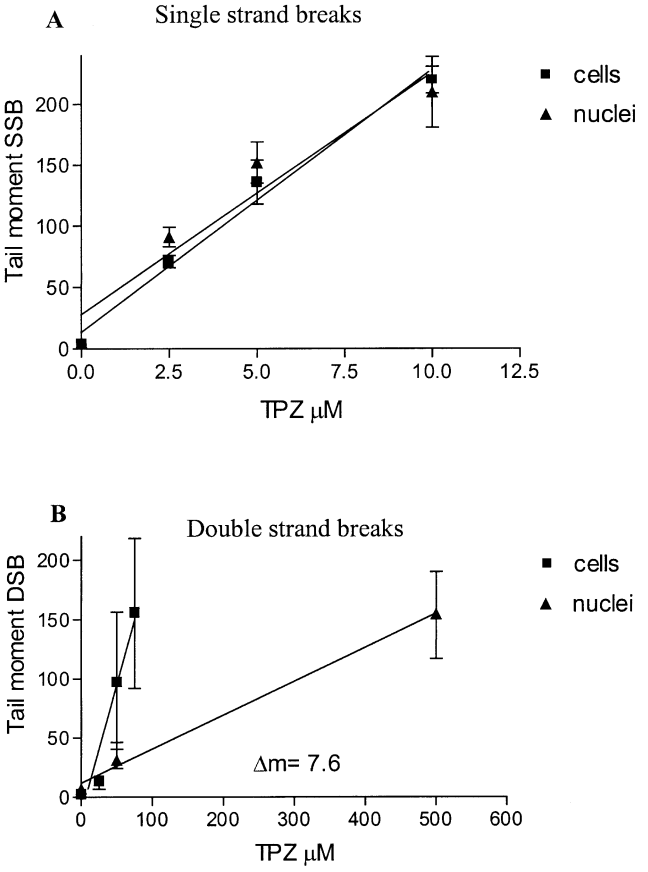

Fig. 4. Comparison of levels of double- and single-strand DNA breaks in whole cells and in isolated nuclei after incubation with TPZ. HeLa cells and nuclei were treated with increasing concentrations of TPZ for 1 hr as described in the text. (A) Single-strand DNA breaks (SSB) were measured using the alkaline comet assay. Data shown represent the means (± SD) of 200 comets. (B) Double-strand DNA breaks (DSB) by TPZ were quantified using the neutral comet assay. Data shown represent the means (± SD) of 200 comets.

levels of single-strand breaks after TPZ (Fig. 4A), even though compared with whole cells, nuclei metabolized only a fraction of TPZ. However, under identical conditions whole cells had approximately 7.6-fold higher levels of DNA double-strand breaks than isolated nuclei per dose of TPZ (Fig. 4B). This suggests either that a large portion of the DNA double-strand breaks are formed by TPZ metabolism outside of the nucleus, or that preparation of the nuclei inactivated most of the ability of the nuclei to form TPZ-induced double-strand breaks. The first of these possibilities is not consistent with the finding of equal amounts of single-strand breaks in cells and nuclei (Fig. 4A). To investigate this further, we added back various factors, including whole cell lysate and the cytoplasmic proteins, to the isolated nuclei before exposure to TPZ. However, this did not increase the amount of DNA double-strand breaks in the nuclei, indicating that the procedure to isolate nuclei may cause the loss of some of the ability of the nuclear proteins to metabolize TPZ and/or to form DNA double-strand breaks. We do have evidence that using less stringent

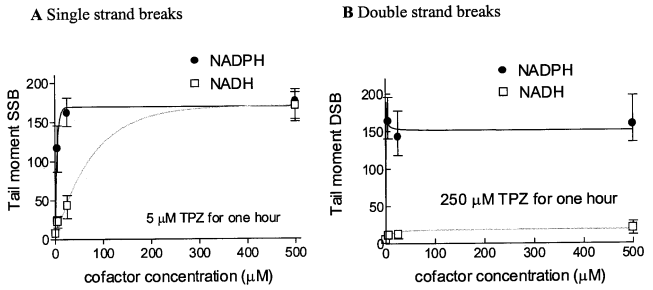

Fig. 5. Cofactor dependence of single- and double-strand DNA breaks induced by TPZ. (A) HeLa cells and nuclei were treated with increasing concentrations of cofactor and with a single concentration (5 μM) of TPZ for 1 hr as described in the text. Single-strand DNA breaks (SSB) were measured using the alkaline comet assay. Results are the means (± SD) of three independent experiments for selected cofactor concentrations. (B) TPZ-induced double-strand DNA breaks (DSB) were measured at 250 μM TPZ and increasing cofactor concentration. Results are the means (± SD) for 300 comets.

conditions to isolate the nuclei increases the amount of DNA double-strand breaks formed by TPZ activation (Evans JW, unpublished observation).

3.8. Cofactor dependence of nuclear reductases responsible for forming TPZ-induced DNA breaks

By analyzing the cofactor dependence of TPZ-induced DNA damage in the isolated nuclei, we found that single-strand breaks were produced primarily by reductases using NADPH at low cofactor concentrations (<100 μM), but they were also produced by reductases that used both NADPH and NADH at physiologic cofactor concentrations (500 μM) (Fig. 5A). This agrees with previous findings using alkaline elution to measure DNA single-strand breaks after TPZ with high levels of cofactors [23]. However, the cofactor dependence for the induction of DNA double-strand breaks was quite different: even at high levels of cofactor, TPZ could only be activated by reductases using NADPH to form these breaks (Fig. 5B).

4. Discussion

4.1. Metabolism of TPZ by multiple enzymes

Many cellular enzymes are able to metabolize TPZ, and their location within the cell is significant in governing the cellular response to the drug. For example, we recently found that TPZ is metabolized by enzymes in the mitochondria, and under aerobic conditions this leads to a loss of mitochondrial transmembrane potential following TPZ exposure [30]. Under hypoxic conditions, enzymes within the nucleus are responsible for the reduction of TPZ that causes DNA damage. The experiments reported here demonstrate that multiple enzymes in the nucleus reduce TPZ to its DNA damaging radical, and that the formation of single- and double-strand breaks may be caused by enzymatic reduction of TPZ by different reductases in distinct locations.

4.2. Cytochrome P450 reductase levels and TPZ toxicity

Many candidate enzymes are able to activate TPZ *in vitro.* DT diaphorase, xanthine oxidase, NADPH:cytochrome P450 reductase, and the cytochrome P450 family all metabolize TPZ *in vitro.* However, these enzymes are located in the cytoplasm, and we have found previously that only metabolism of TPZ within the nucleus contributes to the production of DNA single-strand breaks [23]. Nonetheless, Patterson *et al.* [21] have reported a positive correlation between NADPH:P450 reductase activity and TPZ toxicity in a panel of breast cancer cell lines, thereby implicating this enzyme in the cytotoxic activity of TPZ. However, they have shown recently that a similar correlation also occurs for many anticancer drugs of very diverse mechanisms, and have therefore concluded that it is the ability of cells to respond to DNA damage that may be responsible for the toxicity of TPZ [31]. Also, they found no correlation of NADPH:cytochrome P450 reductase activity and TPZ sensitivity in a panel of lung cancer lines [22].

To address the question of whether P450 reductase was involved in nuclear TPZ metabolism, we showed by immunoblot that there was no P450 reductase protein in the nuclear protein fractions [23]. We also used diphenyliodonium chloride (DPIC), an irreversible inhibitor of the enzyme [32], to inhibit the activity of P450 reductase in TPZ metabolism assays. DPIC completely abolished the ability of the cytoplasm and purified P450 reductase to metabolize TPZ but had no effect on TPZ activation by nuclear protein fractions (data not shown). Taken together, these data strongly argue that TPZ metabolism and DNA damage in nuclei are not mediated through P450 reductase.

4.3. Cofactor dependence of nuclear proteins to metabolize TPZ

In earlier work, we reported that the induction of DNA single-strand breaks in isolated nuclei by TPZ was dependent upon the presence of either NADPH or NADH as reducing equivalents for the reductases [23]. Each of the cofactors with TPZ produced similar levels of DNA damage, but adding the cofactors together gave no additional damage. This suggested that one enzyme that can use either cofactor equally well, rather than two or more enzymes, each capable of using a single cofactor, was responsible for activating TPZ to produce single-strand breaks. This unusual cofactor dependency also ruled out major involvement by cytochrome P450 reductase in causing DNA single-strand breaks, since it is dependent solely on NADPH. There are no known nuclear reductases that have a similar cofactor dependence. We therefore studied the cofactor dependence of TPZ metabolism to a free radical by the dif-

ferent nuclear protein fractions to determine if any had a similar cofactor dependence to the whole nuclei in producing DNA damage. We found that the NM protein fraction is capable of using both cofactors in an equal and non-additive manner. However, this makes up only a small fraction of the nuclear TPZ metabolism (~22%). The majority of TPZ metabolism in the nucleus (~67%) is associated with the LS fraction, which depends mainly on NADPH for its activity, although it can utilize NADH with lower efficacy. We also showed that there is another active fraction, the HS fraction, which needs primarily NADH. This provides strong evidence that there are multiple reductases in the nucleus that can activate TPZ.

4.4. Induction of single- and double-strand breaks by TPZ

The procedure to isolate nuclei for the comet assay is gentle but could cause loss of factors necessary to produce certain types of DNA damage after TPZ exposure. To test for this we compared the levels of DNA single- and double-strand breaks after TPZ treatment in whole cells and isolated nuclei. Levels of single-strand breaks were equivalent in cells and nuclei, indicating that the nuclei contain all of the single-strand break-producing reductase(s). Although double-strand breaks in nuclei were induced in nuclei by TPZ in a concentration-dependent manner, the nuclei had approximately 8-fold lower levels of double-strand breaks than did whole cells, suggesting that nuclei may have lost important enzymes or factors in the isolation procedure. Attempts to increase TPZ-induced double-strand breaks in the nuclei by adding back both cytoplasm and whole cell lysate to the nuclei before the drug exposure were unsuccessful. This suggests that double-strand breaks may be caused by cellular processes responding to damage produced by the TPZ radicals and not by the TPZ radicals directly. The data clearly do not support a model similar to the mechanism of ionizing radiation in which TPZ radicals induce DNA single- and double-strand breaks directly.

4.5. Cofactor dependence of TPZ-induced single-strand breaks and the nuclear proteins

By comparing the cofactor dependence of DNA strand breaks and TPZ metabolism of the various nuclear fractions, it is apparent that the cofactor dependence of single-strand breaks corresponds to that of the LS fraction, which uses NADPH slightly more efficiently than NADH. Reductases capable of using NADPH at low cofactor concentrations (<50 μM) and both NADH and NADPH at a high physiological concentration (~500 μM) metabolize TPZ to produce single-strand breaks. The LS fraction is responsible for the majority of TPZ metabolism in the nucleus. As one TPZ radical produced by the reductase would be predicted to yield one single-strand break, it is reasonable that the fraction with the most TPZ activation, the LS fraction, appears to dominate in the production of single-strand breaks in the nucleus.

4.6. NASPH requirement for DNA double-strand breaks by TPZ metabolism

The enzyme(s) responsible for producing DNA single-strand breaks does not appear to produce the DNA double-strand breaks after TPZ treatment as these are formed by at least one nuclear reductase that requires only NADPH. NADH alone with nuclei gave no TPZ-induced DNA double-strand breaks, whereas at physiological concentrations NADH is able to serve as a cofactor for the reductases producing single-strand breaks from TPZ. However, none of the nuclear protein fractions have a similar cofactor dependence to that of double-strand break formation. This is not surprising in view of the fact that the protein fractions contained several proteins. The double-strand break-producing reductase could be in any nuclear fraction with its activity and cofactor dependence obscured by other reductases.

Although the present investigation did not identify the nuclear reductase responsible for producing TPZ-induced DNA double-strand breaks, and by implication, TPZ cytotoxicity, knowledge of the unusual cofactor requirements for the reductase producing TPZ-induced double-strand breaks will guide further efforts to isolate the enzyme. Identification of this nuclear enzyme may help in the future profiling of tumors before drug treatment. The present data, as well as earlier studies [33], indicate that the overall level of reductases is unlikely to correlate with tumor sensitivity to the drug. However, tumors expressing high levels of the reductase involved in producing DNA double-strand breaks would be expected to be highly sensitive to TPZ under hypoxic conditions.

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