Tirapazamine Is Metabolized to Its DNA-damaging Radical by Intracellular Enzymes

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Abstract

Tirapazamine (TPZ), a new anticancer drug that is currently in Phase II and III clinical trials, has a unique mechanism of action. Its cytotoxicity is selective for hypoxic cells in solid tumors and results from DNA damage produced by a free radical, which is generated by enzymatic reduction of the parent molecule. However, there is no agreement as to which enzymes are involved. Here, we have measured both DNA damage and TPZ metabolism in A549 human lung cancer cells and in isolated nuclei derived from the cells. We show that, although the nuclei metabolize TPZ at a rate that is only 20% of that of whole cells, they have levels of DNA damage that are similar to those of the cells. We also show that TPZ radicals that are formed outside nuclei do not contribute to intranuclear DNA damage. Thus, essentially all of the DNA damage from TPZ results from radicals generated within the nucleus, and the 80% of the drug metabolism that occurs in the cytoplasm is probably irrelevant for the activity of this drug in killing hypoxic cells.

Introduction

TPZ\(^2\) (3-amino-1,2,4-benzotriazine-1,4-dioxide, SR 4233, WIN 59075, and Tirazone) is currently in Phase II and III clinical trials as an adjunct to radiotherapy and to cisplatin-based chemotherapy, respectively. It has a unique spectrum of activity in that its toxicity is toward those cells in solid tumors that are at low oxygen tension (\(T\)). Such hypoxic cells have been shown to be present in many different types of human malignancies, including glioblastomas, melanomas, and carcinomas of the breast, head and neck, cervix, and rectum (\(T\)). Furthermore, there is considerable preclinical and clinical evidence that these hypoxic cells can be detrimental to cure by both radiotherapy and chemotherapy (3–5). Because TPZ selectively kills these radiation- and chemotherapy-resistant cells, its combination with radiation and with some anticancer drugs would be expected to improve the therapeutic response. Preclinical studies combining TPZ with fractionated radiation (6) and with cisplatin (7) clearly demonstrate the potential for such combinations, and early reports of clinical results have shown the promise of this combination with radiotherapy (8) and with cisplatin-based chemotherapy (9).

The 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 SSBs, double-strand breaks, and chromosome aberrations (1). The much lower aerobic cytotoxicity results from "back-oxidation" of the radical by oxygen to the nontoxic parent molecule. Because knowledge of the enzyme(s) involved in drug reduction would provide a convenient means of identifying tumors that are likely to be responsive to TPZ, a considerable effort has been expended to identify the relevant enzyme(s). Such studies have involved metabolism of TPZ by isolated enzymes or by whole cells in the presence of specific enzyme inhibitors, as well as attempts to correlate cytotoxicity with enzyme activities. However, there is no agreement as to the relevant enzymes involved (10–12). We show here that, although only a small fraction of the cellular metabolism of TPZ occurs in the cell nucleus, essentially all of the DNA damage and, hence, cytotoxicity result from intranuclear metabolism. The data, therefore, imply that it is unlikely that measurement of overall cellular metabolism or of cellular enzymes, will be fruitful in identifying either the enzymes relevant to drug toxicity or individual tumors responsive to this new anticancer agent.

Materials and Methods

Reagents and Cell Culture. TPZ (3-amino-1,2,4-benzotriazine-1,4-dioxide) was obtained from Sanofi-Winthrop (Malvern, PA). SR 4317 (3-amino-1,2,4-benzotriazine-1-N-oxide) was generously provided by Dr. Michael Tracy (SR International, Menlo Park, CA). All other reagents (unless otherwise stated) were obtained from Sigma Chemical Co. (St. Louis, MO).

A549 human lung adenocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in eMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum and 200 units/ml penicillin-0.2 mg/ml streptomycin. Cells were maintained in logarithmic-phase growth for all experiments.

Preparation of Nuclei. Nuclei were prepared in isotonic buffer with non-ionic detergent by a modification of published techniques (13). Briefly, cells were harvested, washed twice in PBS, and resuspended at 12.5 \(\mu\)l per 10\(^6\) cells in nuclei isolation buffer (115 mM KCl, 5 mM NaCl, 1 mM KH\(_2\)PO\(_4\), 20 mM HEPES, 0.3 mM MgCl\(_2\), 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT (pH 7.4)) in a 13-ml round-bottomed tube. An equal volume of nuclei isolation buffer containing 2% IGEPAL CA-630 was gently added to the cells, mixed, and allowed to incubate for 15 min at 4°C. The resulting permeabilized cells were sheared by gently pipetting 20–30 times through a 1-ml plastic pipette tip. The isolated nuclei were diluted with a 10X volume of nuclei isolation buffer, centrifuged, and washed with 10 ml of nuclei isolation buffer. All samples were monitored for purity of the nuclei by phase-contrast microscopy.

Irradiation, Drug, and Hypoxia Treatments. Irradiations were performed on ice under aerobic conditions in a J. L. Shepherd Mark I 13\(^7\)Cs irradiator at a dose rate of 86 cGy/min.

For exposure to TPZ, cells and nuclei were treated in suspension in glass dishes in 2 ml at 1 \(\times\) 10\(^6\) cells or nuclei per ml. TPZ stocks (2 mM) were dissolved in PBS and added either to cells in complete medium or to nuclei in treatment buffer (100 mM KCl, 1 mM KH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), 20 mM HEPES, 10 mM EDTA, 5 mM glutathione, and 0.9 mM spermidine (pH 7.4)). When appropriate, nuclei were supplemented with cofactor at 1 mM NADH, 1 mM NADPH, or a combination of 0.5 mM NADH and 0.5 mM NADPH. In some reactions, the NADPH-generating system G6P/G6PDH [1 mM NADP, 10 mM G6P, and 2.5 units of G6PDH (EC 1.1.1.49) type XII from Torula yeast; Sigma] was added.

Hypoxia (~200 ppm O\(_2\)) was achieved by exposing the glass dishes in warmed, air-tight aluminum jigs to a series of five rapid evacuations and flushings with 95% nitrogen-5% CO\(_2\) on a shaking platform at room temperature (controls were flushed with 95% air-5% CO\(_2\)). The jigs were immediately
that effect on the ability of the cell to scavenge radicals close to DNA and
of the radiation-induced breaks during the 1-h incubation nor any
DNA degradation in the nuclei.
Alkaline Elution. Alkaline elution to measure DNA SSBs was performed
as outlined by Koch and Giandomenico (14). Briefly, cells radiolabeled with
[2-14C]thymidine (Abersham, Cleveland, OH) were either resuspended in
medium for treatment or used to prepare isolated nuclei, as outlined above.
Irradiated or drug-treated cells or nuclei suspended in ice-cold PBS were
loaded onto a 2-μm polycarbonate filter; lysed with 2 M NaCl, 0.2% sarkosyl,
and 25 mM EDTA (pH 10.0) containing 1 mg/ml proteinase K (Boehringer
Mannheim, Indianapolis, IN); and incubated for 1 h at room temperature. DNA
was eluted from the filter by alkaline buffer (0.1% SDS, 25 mM EDTA-free
acid, and sufficient tetrapropylammonium hydroxide to yield pH 12.2) at a
flow rate of 0.033 ml/min. Radioactivity from the eluted fractions or from the
DNA retained on the filter, was measured on a Beckman LS6000IC (Fullerton,
CA). Data were analyzed by plotting the logarithm of the fraction of labeled
DNA retained on the filter versus time. The slope of these linear profiles
equalled the elution rate.
HPLC. Analysis of TPZ and its two-electron reduction metabolite (SR
4317) was performed as described previously (15).
Plasmid Nicking Assay. The conversion of supercoiled plasmid DNA
(form I) to the nicked relaxed circular form (form II) was used to assess DNA
damage caused by the activation of TPZ in vitro by purified NADPH P450
reductase (recombinant human NADPH P450 oxidoreductase; Pan Vera Cor-
poration, Madison, WI). Briefly, 1 μg of supercoiled plasmid DNA (pBlue-
script; Stratagene, La Jolla CA) was preequilibrated in a hypoxic chamber at
37°C [10 mM EDTA, 25 mM Tris (pH 7.5), 50 mM KCl, 25 mM NaCl, 1 mM
DTT, and 0.5 mM phenylmethylsulfonyl fluoride] with 100 μM NADPH as
cofactor and was mixed with 100 μM TPZ and 0.1 μg of purified enzyme for
1 h. Products were separated on a 1% agarose gel, and the scanned image
was analyzed with NIH Image software.
Results and Discussion
To test the relative contribution to DNA damage produced by
intranuclear metabolism of TPZ, we prepared nuclei from A549
human lung tumor cells. Because the experiments required a quanti-
tative comparison of DNA breaks induced by TPZ metabolism in
nuclei and those induced in whole cells, it was necessary to eliminate
potential artifacts that could arise from the nuclear preparation.
First, possible contamination of the nuclei with endoplasmic retic-
umulum, which contains high levels of NADPH P450 reductase, which is
known to metabolize TPZ (12), was examined by immunoblot anal-
ysis of equal numbers of cells and nuclei. Quantitative scanning on the
blots (Fig. 1) showed that the nuclei contained only 3.8% of the P450
reductase levels in whole cells.
Second, to test whether the nuclear preparation had altered levels of
radical-scavenging species compared to whole cells, we irradiated
both cells and nuclei with a range of doses of 0–4 Gy on ice and
measured DNA SSBs from both samples using alkaline elution. The
dose-response curves were linear and were not significantly different
for cells and nuclei (slopes and SEs of 0.062 ± 0.007 and
0.053 ± 0.004 for nuclei and whole cells, respectively). As a further
test of the similarity of our nuclei to whole cells, we incubated nuclei
with TPZ (5 μM for 1 h), with or without 250 mM DMSO, a potent free
radical scavenger. DMSO protected against DNA damage in the
nuclei by a factor of 1.74, a value similar to the protection for cell
survival in whole cells of 1.7–1.8 under identical conditions (16).
Because the state of chromatin condensation can have a dramatic
effect on the ability of the cell to scavenge radicals close to DNA and
also on the sensitivity of cells to radiation-induced strand breakage
(17), we conclude from these data that the nuclei had a state of
chromatin condensation and radical-scavenging ability that was simi-
lar to that of whole cells.
Finally, because it was necessary in our experiments to incubate
both nuclei and cells for 1 h at 37°C with TPZ, we tested whether
incubation of the nuclei would affect the yield of DNA SSBs. We
found that nuclei irradiated on ice and then incubated for 1 h at 37°C
in the same buffer as that used for drug exposure showed no change
in the elution profile over time, indicating that there was neither repair
of the radiation-induced breaks during the 1-h incubation nor any
DNA degradation in the nuclei.
To examine the cofactor requirements for TPZ metabolism leading
to SSBs, we exposed isolated nuclei from A549 cells to 5 μM TPZ for
1 h, with or without the cofactors NADH and/or NADPH. The data
are shown in Fig. 2 and demonstrate that DNA damage induction by

Fig. 1. Immunoblots of whole-cell extracts or of stages in the preparation of the nuclei,
incubated with antibodies to NADPH P450 reductase (A) and lamin B (B).

![Immunoblots of whole-cell extracts or of stages in the preparation of the nuclei, incubated with antibodies to NADPH P450 reductase (A) and lamin B (B).](image-url)
TPZ has an absolute requirement for either NADPH or NADH in the nuclei. Each of the cofactors gave similar results, but adding both cofactors together did not increase the yield of SSBs over each cofactor given alone. This suggests that the enzyme(s) responsible for TPZ metabolism in the nucleus can use electrons from either NADH or NADPH for donation to TPZ. The fact that the combination of the two cofactors produces no more DNA damage than does either cofactor alone shows that the effect is not the result of two enzymes, one requiring NADH and the other NADPH, but rather, it is the result of an enzyme or enzymes that are capable of using both cofactors. Various controls (data not shown) were performed, including groups in which each cofactor was tested at 0.1 and 1 mM, with no differences being found. We also showed that elution rates much higher than 0.4 were possible with higher doses of TPZ (Fig. 3), thus eliminating the possibility that the similar response with both cofactors was the result of saturation of the assay.

To test the influence on DNA damage of intranuclear and whole-cell metabolism of TPZ, we prepared nuclei from A549 tumor cells and incubated these nuclei or whole cells under identical conditions, with varying concentrations of TPZ for 1 h under hypoxia (Fig. 3). The data show that in the initial region of the dose-response curve the nuclei are 1.5–2-fold more sensitive to DNA damage by TPZ than are whole cells. Higher DNA damage at the end of the incubation period in the nuclei would be expected if equal damage were induced in cells and nuclei because repair of the damage would occur in the cells but not in the nuclei during the 1-h treatment (there was no repair of radiation-induced SSBs in the nuclei at 37°C). Because the half-life for repair of TPZ-induced SSBs in cells is approximately 50 min (18), this difference (factor of 1.5–2) is close to that expected for a 1-h incubation at 37°C. We conclude from these data that, under identical hypoxic conditions, TPZ induces similar levels of DNA damage in cells and nuclei.

We next measured metabolism of TPZ under hypoxic conditions by both nuclei and whole cells. Drug metabolism was calculated by production of the two-electron metabolite, SR 4317, measured using HPLC (15). As expected, nuclei metabolized considerably less TPZ than does the same number of whole cells. The mean value of TPZ metabolism by nuclei from five experiments was 21 ± 4% of that of whole cells.

Finally, to determine whether the small (3.8%) contamination of the nuclei with NADPH P450 reductase might be sufficient to cause the observed DNA damage in the nuclei, we incubated either nuclei or supercoiled plasmids with TPZ, with or without purified NADPH P450 reductase. The data are shown in Fig. 4. We found that, although P450 reductase, together with TPZ and NADPH, caused extensive nicking of isolated plasmids, the addition of exogenous P450 reductase produced no increase in the DNA SSBs induced by TPZ in

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**Fig. 3.** Alkaline elution rates of DNA from A549 cells (□) or isolated nuclei (●), exposed to various concentrations of TPZ for 1 h under hypoxic conditions.

**Fig. 4.** Nicking of supercoiled plasmids (left) and alkaline elution rates of DNA from A549 cells (right) treated for 1 h at 37°C with 5 or 10 µM TPZ, with purified P450 reductase and NADPH cofactor, or with combinations of the enzyme and cofactor with TPZ, with or without the G6P/G6PDH NADPH-generating system.
isolated nuclei. This was the case when we used the same amount (1×) of P450 reductase equal to that in 2×10^6 cells (equal to the number of nuclei used), as well as 20-fold more (20×), although >50% of the TPZ was metabolized by the P450 reductase during the 1-h exposure with the higher level of enzymes used. To control for the possibility that 1 mM NADPH might have been limiting with the higher level of P450 reductase, we included groups in which the G6PDH NADPH-generating system was added to the reaction. This did not change the amount of DNA damage in the nuclei (Fig. 4, right hatched columns). We demonstrated the efficacy of the generating system by spectrophotometric assay of NADPH. These data, therefore, demonstrate not only that minor contamination of P450 reductase on the surface of the nuclei cannot account for the DNA damage but also that TPZ radicals formed outside the nucleus do not cause damage to intranuclear DNA.

These experiments, therefore, show that TPZ can be metabolized in the nuclei of these human tumor cells, and although this only represents 21% of total cellular metabolism, it produces essentially all of the DNA damage (and, presumably, cytotoxicity). This provides direct experimental evidence to support the model proposed previously (1), based on the finding that chromosome breaks produced by TPZ are less easily repaired than those produced by X-rays (19). We suggested that this was the result of metabolism by reductases located close to DNA, which would cause high local concentrations of the damaging radical molecules. In turn, this could lead to highly localized damage to DNA, similar to that produced by densely ionizing radiation, which also produces chromosome breaks that are more difficult to repair than those from sparsely ionizing radiation, such as X-rays (20).

The data also explain why there is disagreement as to which enzymes are important for TPZ-induced cytotoxicity under hypoxia. For example, Patterson and colleagues (12) have shown that the toxicity of TPZ under hypoxia correlates with NADPH cytochrome P450 reductase activity in a panel of six human breast adenocarcinoma cell lines, but they found no correlation in similar experiments with lung cancer cell lines (10). Also, we have recently shown that cell lines adapted to continuous TPZ exposure under aerobic conditions had only a small (1.5-fold) decrease in sensitivity to cell killing by TPZ under hypoxic conditions, but they had a very large decrease (by some 50-fold) in P450 reductase activity (11). Here, we can rule out NADPH cytochrome P450 reductase as the intranuclear enzyme responsible for TPZ metabolism because there was very little of this enzyme (<4%) in our nuclear preparations, as well as the fact that the intranuclear enzyme(s) could use both NADH and NADPH as cofactors.

In summary, we have shown that essentially all of the DNA damage produced by TPZ under hypoxic conditions can be accounted for by intranuclear metabolism of the drug, although this metabolism only represents some 20% of the total cellular metabolism. This is consistent with a model of the reductase(s) responsible for the metabolism of TPZ leading to cytotoxicity being located close to DNA. The data also show that measurements of overall cellular metabolism or of the activity of cytoplasmically located enzymes capable of metabolizing TPZ are unlikely to be useful as predictors of individual tumor sensitivity to TPZ. Identification and quantitation of the intranuclear enzyme(s) responsible for TPZ metabolism could, however, provide a means of selecting tumors that are sensitive to this drug.

References
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