

# Tirapazamine: A Hypoxia-activated Topoisomerase II Poison<sup>1</sup>

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## ABSTRACT

Tirapazamine (TPZ), a hypoxia-selective cytotoxin, has demonstrated activity in cancer clinical trials. Under hypoxic conditions, TPZ is reduced to a radical that leads to DNA double-strand breaks (DSBs), single-strand breaks, and base damage. A previous finding of an association of the DSBs with protein led us to investigate the involvement of topoisomerase II (topo II) in their formation. Nuclear extracts from human lung cancer cells treated with either the topo II poison etoposide or TPZ under hypoxic conditions had markedly reduced topo II activity as judged by an inability to convert kinetoplast DNA from the catenated to the decatenated form. Because topo II poisons, such as etoposide, cause DNA DSBs, we hypothesized that pretreatment of cells with merbarone or aclarubicin, known catalytic inhibitors of topo II, would abrogate DNA DSBs caused by topo II. Cells pretreated with these catalytic inhibitors abrogated both DNA DSBs and cell kill induced by etoposide or by TPZ. Etoposide- and TPZ-mediated DSBs were also greatly reduced in a small cell lung cancer cell line with low levels of nuclear topo II $\alpha$ . We also showed that topo II $\alpha$  becomes covalently bound to DNA after TPZ treatment under hypoxic conditions, and that the cleavable complexes formed by TPZ are more stable over time than those formed by etoposide. Taken together, these data suggest that TPZ exerts its cytotoxic effect at least in part through poisoning topo II. Because TPZ is activated only under hypoxic conditions, which are characteristic of solid tumors, these data implicate TPZ as a tumor-specific topo II poison.

## INTRODUCTION

A key goal in the treatment of solid tumors is the development of chemotherapeutic agents with tumor specificity to decrease drug side effects and/or increase antitumor efficacy. One possible way to achieve this is to exploit the lower oxygen tensions of tumors compared with normal tissues. Such hypoxic regions in tumors confer resistance to standard radiotherapy and chemotherapy (1, 2), but a drug selectively toxic to hypoxic cells would be both tumor specific and kill the cells resistant to conventional therapy (3, 4). The first such selective hypoxic cytotoxin to enter clinical trials is TPZ<sup>3</sup> [SR 4233, 3-amino-1,2,4-benzotriazine-1,4-dioxide; Refs. 4 and 5], a drug that has shown significant activity in Phase II and III clinical trials in combination with radiotherapy and cisplatin-based chemotherapy (6–8).

TPZ is converted by intracellular reductase(s) to a cytotoxic radical that under hypoxic conditions generates base damage and DNA SSBs and DSBs (9, 10). However, when oxygen is present, the TPZ radical is back-oxidized to the nontoxic parent compound, thereby decreasing the cytotoxicity. Although many intracellular enzymes can convert TPZ to its cytotoxic radical (9, 11), it is the nuclear reductase(s) that

are responsible for the DNA damage caused by TPZ (12). Like the cytoplasm, the nucleus is highly compartmentalized, and the nuclear matrix, a nonchromatin protein network, dictates this subnuclear organization (13). On the basis of recent studies showing that TPZ can be selectively reduced by nuclear matrix-associated reductases (14), we hypothesized that TPZ would affect metabolic activities associated with the nuclear matrix. Consistent with this, we have recently shown that TPZ, under hypoxic conditions, produces a marked inhibition of DNA replication, one of the activities that occur at the nuclear matrix (15).

The nuclear matrix, a protein scaffold highly enriched in lamins A, B, and C and topo II $\alpha$ , organizes 30-nm chromatin fibers into 5–200-kb chromatin loops and tethers these loops via matrix-attachment regions (16–18). Mammals have two isoforms of topo II ( $\alpha$  and  $\beta$ ) with molecular masses of 170 and 180 kDa, respectively. The  $\alpha$  isoform is enriched in the nuclear matrix and is increased during S phase and mitosis, whereas the  $\beta$  isoform is expressed independently of the cell cycle (19). Traditionally, it was believed that topo II $\beta$  resided exclusively at the nucleolus (20, 21), but more recent reports suggest that topo II $\beta$  localization is throughout the nucleus and does not sequester itself to the nucleolar compartment (22, 23). topo II usually exists as a homodimeric species ( $\alpha\alpha$  or  $\beta\beta$ ), but heterodimers of  $\alpha$  and  $\beta$  can also be present and are enzymatically active (24, 25). topo II is essential in mammalian cells because it resolves the unfavorable topological structures in DNA that are encountered during replication and transcription (26). To unwind DNA, topo II introduces transient DSBs that allow the passage of unbroken double-stranded DNA. The enzyme then religates the transient break and dissociates from the DNA.

Generally, the DNA DSBs caused by topo II are short-lived, but many common chemotherapeutic agents, including Adriamycin and etoposide, block the religation stage, thereby generating frank DSBs (27). These drugs that stabilize topo II with a DNA DSB (the so-called “cleavable complex”) are termed topo II poisons. This distinguishes them from other agents, such as aclarubicin, merbarone, and ICRF-187, which block topo II at specific sites in its catalytic cycle prior to or subsequent to the formation or religation of DNA DSB (so-called topo II catalytic inhibitors).

Because of the proposed location of the enzyme responsible for the reduction of TPZ and other evidence that TPZ DNA DSBs are protein associated (28, 29), we hypothesized that TPZ would affect topo II activity. Consistent with this, we show that nuclear extracts from LXFL 529 human lung carcinoma cells treated with TPZ under hypoxic conditions had reduced the activity of topo II. Furthermore, we demonstrate that inhibitors of the topo II catalytic cycle abrogate TPZ-generated DNA DSBs and cytotoxicity using the neutral comet and clonogenic survival assays, respectively. TPZ also stabilizes DNA topo II cleavable complexes, and these complexes remain bound to DNA longer than etoposide-induced cleavable complexes. Taken together, these data provide strong support for the conclusion that TPZ is a hypoxia-selective topo II poison.

## MATERIALS AND METHODS

**Reagents.** TPZ (3-amino-1,2,4-benzotriazine-1,4-dioxide) was obtained from Sanofi-Syhelabo (Malvern, PA). Merbarone (NSC 336628) was generously provided by the United States National Cancer Institute. All of the other

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<sup>3</sup> The abbreviations used are: TPZ, tirapazamine; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride hydrate; DSB, double-strand break; kDNA, kinetoplast DNA; SSB, single-strand break; topo II, topoisomerase II; TARDIS, trapped in agarose DNA immunostaining (assay).

reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

**Cell Culture.** HeLa cells, graciously provided by Dr. George Iliakis (Department of Radiation Oncology, Jefferson Medical College, Philadelphia, PA), were grown and maintained in monolayer culture in Joklik-modified MEM supplemented with 5% iron-supplemented calf serum. LXFL 529 non-small cell lung carcinoma cells were obtained from the United States National Cancer Institute and were grown and maintained in monolayer culture in RPMI modified medium supplemented with 10% fetal bovine serum. H69 and H69/VP small cell lung carcinoma cells were graciously provided by Dr. Maxwell Sehested (Laboratory Center, Copenhagen, Denmark) and were grown and maintained in RPMI modified medium supplemented with 10% fetal bovine serum. All of the cells were used in the logarithmic phase of growth.

**Drug and Aerobic/Anaerobic Treatments.** LXFL and HeLa cells were treated with etoposide or TPZ at the concentrations and times indicated in the relevant Figure captions. For hypoxia treatment, cells were grown in notched glass dishes, which were loaded into prewarmed aluminum jigs. The jigs were evacuated five times to 0.1 atmosphere with  $N_2 + 5\%$   $CO_2$  reintroduction and constant shaking to achieve hypoxia (less than 200 ppm  $O_2$ ) as previously described (30). For aerobic treatment, jigs were evacuated and refilled five times with 95% air + 5%  $CO_2$ . All of the etoposide and TPZ exposures were for 1 h at 37°C unless otherwise indicated. For the experiments with the topo II inhibitors, the cells were pretreated with either merbarone or aclarubicin at various concentrations for either 30 or 20 min before exposure to either TPZ or etoposide. Irradiations were performed with  $^{137}Cesium$   $\gamma$ -rays from a J. L. Shepherd and Associates Mark I Model 25 Irradiator (San Fernando, CA) operating at 369.1 cGy/min. Cells were placed on ice during treatment with irradiation.

**Preparation of Nuclear Lysates for topo II Activity.** Nuclear extracts were prepared as described previously with some modifications (31). Briefly, untreated and treated LXFL cells were pelleted and lysed in 1.0 ml of nuclear buffer A [1 mM potassium phosphate, 150 mM sodium chloride, 5 mM magnesium chloride, 1 mM disodium EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM DTT, and 10% glycerol (v/v)]. After initial lysis, the cells were rinsed with nuclear buffer A and spun at  $460 \times g$  for 10 min. Pelleted cells were resuspended in 1.0 ml of nuclear buffer A and 9.0 ml of nuclear buffer B (nuclear buffer A + 0.3% Triton X-100). Samples were gently rotated for 10 min and spun at  $460 \times g$  for 10 min. For salt extraction, cell pellet was resuspended in 1.0 ml of nuclear buffer C [1 mM potassium phosphate, 350 mM sodium chloride, 5 mM magnesium chloride, 1 mM disodium EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM DTT, and 10% glycerol (v/v)]. Samples were rotated for 30 to 60 min at 4°C and centrifuged at  $670 \times g$  for 20 min. After this centrifugation, the supernatants were removed and centrifuged again at  $12,000 \times g$  for 15 min. The supernatant was obtained and protein content was determined using BCA Protein Assay Kit (Pierce, Rockford, IL).

**topo II Activity Assay.** We used the topo II assay kit from TopoGEN, Inc. (Columbus, OH) according to the manufacturer's instructions to determine topo II activity in nuclear extracts from LXFL cells.

**Neutral Comet Assay for DNA DSBs.** We used the neutral comet assay to measure DNA DSBs caused by TPZ and etoposide as described previously (28). In brief, after TPZ or etoposide treatment, cells were trypsinized, washed in PBS, and resuspended at  $2 \times 10^4$  cells/ml. To embed cells onto glass slides, 1% low-melt type VII agarose was added to the cell suspension, which was then spread onto glass slides, and after attachment to the slides, the cells were lysed in 0.5% SDS, 30 mM EDTA (pH 8.3) for 4 h at 50°C. Proteinase K (0.5 mg/ml) was then added to the lysis buffer, and lysis was extended overnight at 37°C. The slides were then rinsed in 90 mM Tris, 90 mM boric acid, 2 mM EDTA (pH 8.5) for 6–16 h at room temperature, electrophoresed at 0.6 V/cm for 25 min, rinsed in double-deionized water for 15 min and stained with 2.4  $\mu$ g/ml propidium iodide for 15–60 min. After staining, DNA damage was analyzed using custom-designed software (Loats Associates, Inc., Westminster, MD). For every slide, 200 or more comets were analyzed. The yield of DNA DSBs was determined by averaging the comet "tail moments" 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. We established the validity of this endpoint in preliminary experiments by showing that it gave a linear dose-response curve with ionizing radiation.

**Clonogenicity Assays.** Colony-forming assays were performed as described earlier (32). For treatment with catalytic inhibitors, cells were pretreated for the indicated times and concentrations before exposure to TPZ or etoposide. Briefly, cells treated with various doses of TPZ under hypoxia for 1 h or with various doses of etoposide for 1 h were collected by trypsinization, resuspended in ice-cold media, and plated in 60-mm or 100-mm Petri dishes. After 2 weeks, the medium was removed from the dishes, and the cells were stained with crystal violet. Individual colonies of more than 50 cells were counted and the survival of treated groups obtained by comparing the number of colonies in the treated groups *versus* the number of colonies in the control group.

**TARDIS Assay.** The TARDIS assay was performed as described by Willmore *et al.* (33) with few minor modifications. Slides were stained with anti-topo II $\alpha$  rabbit polyclonal antibody (1:100; Neomarkers, Fremont, CA) in PBS containing 0.1% Tween 20 and 1% BSA for 1 h at room temperature. Slides were then stained with FITC-conjugated goat antirabbit IgG antibody (Zymed Laboratories, South San Francisco, CA) in PBS containing 0.1% Tween 20 and 1% BSA for 1 h at room temperature and with the DNA stain DAPI containing Vectashield (Vector, Burlingame, CA) and visualized using a Nikon Optiphot fluorescent microscope (Melville, NY).

**Cesium Chloride Isolation of DNA Protein Cross-Links.** After TPZ or etoposide treatment, HeLa cells in 100-mm dishes were lysed. The cesium chloride isolation was performed as described by TopoGEN, Inc. (Columbus, OH). After application of DNA to slot-blots, nitrocellulose membranes (Schleicher and Schuell GmbH, Dassel, Germany) were blocked with TBST [10 mM Tris (pH 7.5), 150 mM sodium chloride, and 0.1% Tween 20] with 5% milk and 0.5% BSA and blotted with anti-topo II $\alpha$  rabbit polyclonal antibody (1:300; Neomarkers, Fremont, CA). Blots were then stained with goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Zymed Laboratories, South San Francisco, CA) and visualized using the ECF Western Blotting kit (Amersham Biosciences, Piscataway, NJ). Data were analyzed using a STORM optical scanner (Molecular Dynamics, Sunnyvale, CA).

## RESULTS

**Inhibition of topo II Activity by TPZ.** We used decatenation of the DNA substrate kDNA to assess topo II activity. Catenated DNA minicircles, which comprise the kDNA from *Crithidia fasciculata*, requires catalytically active topo II to unwind to the decatenated form. We treated LXFL lung carcinoma cells with various concentrations of TPZ under aerobic or hypoxic conditions or with etoposide, and we prepared nuclear extracts to determine whether the extracts could support decatenation of the kDNA. Whereas extracts from untreated LXFL cells could efficiently convert kDNA from the catenated to the decatenated form (Fig. 1, Lane 1), extracts from LXFL cells exposed to 50 or 100  $\mu$ M TPZ under hypoxic conditions or etoposide for 1 h were unable to convert kDNA to the decatenated forms (Fig. 1, Lanes 2–5). TPZ's inhibition of topo II activity in LXFL cells occurs only

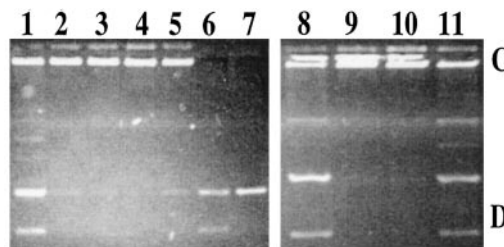
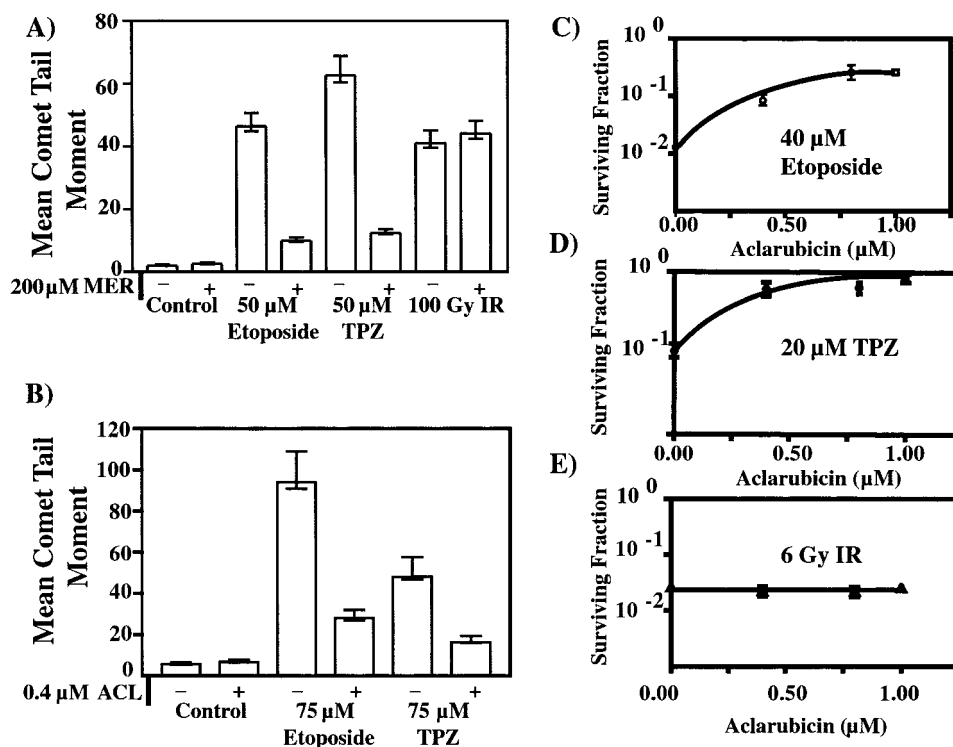


Fig. 1. Inhibition of topo II activity in LXFL non-small cell lung carcinoma cell nuclear extracts by TPZ. LXFL cells were treated with either TPZ or etoposide. Nuclear extracts were obtained and analyzed for topo II activity using the kDNA decatenation assay. To detect decatenation, 1% agarose gel stained with ethidium bromide separates catenated kDNA (C) and decatenated kDNA (D). Treatment groups: Lane 1, control; Lane 2, 50  $\mu$ M TPZ (hypoxic); Lane 3, 100  $\mu$ M TPZ (hypoxic); Lane 4, 50  $\mu$ M etoposide; Lane 5, 100  $\mu$ M etoposide; Lane 6, decatenated marker DNA; Lane 7, linear marker DNA; Lane 8, hypoxia only; Lane 9, 100  $\mu$ M etoposide; Lane 10, 100  $\mu$ M TPZ (hypoxic); Lane 11, 100  $\mu$ M TPZ (aerobic).

Fig. 2. Effect of topo II catalytic inhibitors on DNA DSBs and cytotoxicity produced by TPZ and etoposide. To assess the effects of merbarone and aclarubicin on DNA DSBs, LXFL cells were pretreated with either 200  $\mu\text{M}$  merbarone for 30 min (A) or 0.4  $\mu\text{M}$  aclarubicin for 20 min (B). After pretreatment, cells were exposed to TPZ under hypoxic conditions, etoposide, or ionizing radiation. DNA DSBs were measured using the neutral comet assay and quantitated using the mean comet tail moment. Survival of HeLa cells pretreated with aclarubicin (0–1.0  $\mu\text{M}$ ) for 20 min before exposure to 40  $\mu\text{M}$  etoposide (C), 20  $\mu\text{M}$  TPZ + hypoxia (D), or 6 Gy irradiation (E). Survival was assessed using the clonogenic assay.



under hypoxic conditions (Fig. 1, Lanes 2, 3, and 10) and does not take place when oxygen is present (Fig. 1, Lane 11). These data suggest that TPZ under hypoxic conditions acts similarly to etoposide in inhibiting the activity of topo II.

**TPZ-induced DNA DSBs and Cytotoxicity Are Mediated by topo II.** topo II poisons, such as etoposide and doxorubicin, are cytotoxic as a result of their ability to stabilize the cleavable complex, thereby decreasing the ability of the enzyme to religate the transient DNA DSBs. Other agents, such as ICRF-187, merbarone, and aclarubicin, block topo II in specific parts of its catalytic cycle: aclarubicin prevents topo II binding to DNA (34), and merbarone inhibits DNA cleavage (35). Because the cytotoxicity of topo II poisons can be abrogated if cells are pretreated with these catalytic inhibitors (36–38), we hypothesized that a similar effect should occur with TPZ if its cytotoxicity was mediated by poisoning topo II.

Pretreatment of cells with merbarone (Fig. 2A) or with aclarubicin (Fig. 2B) decreased etoposide-induced DNA DSBs as measured by the neutral comet assay. Consistent with the hypothesis that TPZ acts by poisoning topo II, pretreatment of cells with merbarone (Fig. 2A) or aclarubicin (Fig. 2B) also inhibited TPZ-induced DNA DSBs. To rule out the possibility of nonspecific abrogation of DNA DSBs by these catalytic inhibitors, we showed that merbarone pretreatment did not affect the induction of DNA DSBs by ionizing radiation (Fig. 2A).

Because DNA DSBs caused by TPZ are believed to be responsible for the cytotoxicity of TPZ (4), we investigated whether catalytic inhibitors of topo II also abrogated the cytotoxicity of TPZ. Using clonogenic survival, we show that increasing concentrations of aclarubicin increased the surviving fraction of cells treated with etoposide (Fig. 2C) and TPZ (Fig. 2D), thereby implicating topo II in the cytotoxicity of TPZ. Again, as a negative control, we showed that aclarubicin did not abrogate the cytotoxicity of ionizing radiation (Fig. 2E).

As a further test of the involvement of topo II in TPZ-mediated DNA damage, we used H69 and H69/VP small cell lung carcinoma

cell lines. H69/VP cells are resistant to topo II poisons such as etoposide primarily as a result of a mutation in the nuclear localization signal of topo II $\alpha$  that results in low nuclear levels of the enzyme (39). We show that in the H69 parental cell line, but not in the H69/VP mutant, etoposide is effective in producing DNA DSBs (Fig. 3A). A similar result was found with TPZ (Fig. 3B). Although studies have shown that H69/VP are also resistant to topo II poisons because of the overexpression of P-glycoprotein (40), it is unlikely that this is the explanation for the reduced number of TPZ-induced DNA DSBs

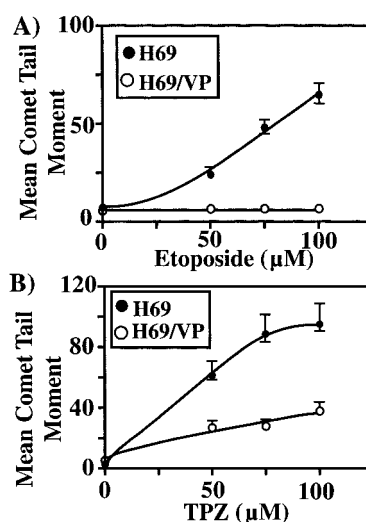


Fig. 3. Production of DNA DSBs in H69 Small cell lung carcinoma cells and H69/VP mutant cells by etoposide (A) and TPZ under hypoxic conditions (B). H69 small cell lung carcinoma cells and mutant H69/VP cells were treated with increasing doses of etoposide (0–100  $\mu\text{M}$ ) and TPZ (0–100  $\mu\text{M}$ ) under hypoxic conditions. Cells were analyzed for DNA DSBs using neutral comet assay to detect DNA DSBs. Mean comet tail moment, the measurement of DNA DSBs.



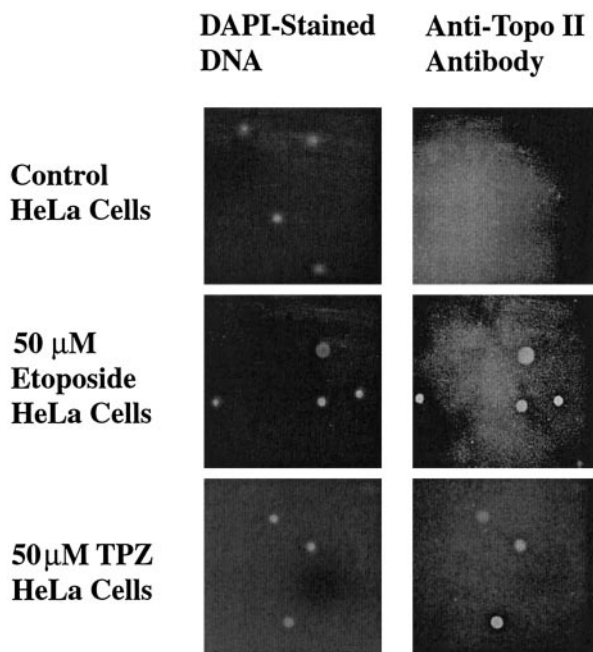


Fig. 4. Detection of DNA-topo II complexes in HeLa cells using the TARDIS assay. HeLa cells were treated with either 50  $\mu\text{M}$  etoposide for 1 h or 50  $\mu\text{M}$  TPZ under hypoxic conditions for 1 h. After treatment, cells were suspended in agarose gels on glass slides, lysed, and probed with anti-topo II $\alpha$  antibodies. Anti-topo II $\alpha$  binding was visualized with secondary antibody conjugated to FITC. *Left panels*, DAPI-stained DNA; *right panels*, fluorescein-stained topo II.

because TPZ is not a substrate for P-glycoprotein.<sup>4</sup> However, to check this, we pretreated the H69/VP cells for 24 h with 2  $\mu\text{M}$  cyclosporin PSC 833 (a concentration and time known to abrogate multidrug resistance; Ref. 41). We found no increase in DNA damage produced by a 1-h exposure to 100  $\mu\text{M}$  TPZ (data not shown). Thus, the reduction in TPZ-induced DSBs in H69/VP cells is most likely caused by a lack of nuclear topo II $\alpha$ , again implicating this enzyme in the action of TPZ.

**Production of Stable DNA-topo II Complexes by TPZ.** We chose two separate methods to determine whether TPZ produced DNA-topo II complexes: the TARDIS assay (33) and cesium chloride isolation of DNA-topo II complexes (42).

In the TARDIS assay, topo II, covalently attached to DNA, is detected by staining cells with anti-topo II $\alpha$  antibodies and secondary antibodies conjugated with FITC (33). In untreated HeLa cells, we found little or no staining present for anti-topo II $\alpha$  but significant staining for topo II $\alpha$  when the cells were treated with either etoposide or with TPZ under hypoxic conditions (Fig. 4). The doses used for both agents produced only moderate cytotoxicity ( $\sim 10^{-1}$  to  $10^{-2}$  survival) for these cells (data not shown).

We next used cesium chloride isolation of DNA-topo II complexes to quantify further TPZ-induced topo II binding to DNA. In this assay, DNA-topo II complexes are isolated using cesium chloride and detected by immunoblot analysis (35). HeLa cells were lysed rapidly using ionic detergent, and the lysates were centrifuged through 1.5 g/cm cesium chloride at 80,000 rpm, thereby separating free topo II from topo II covalently bound to DNA. We compared the stability of DNA-topo II complexes in cells treated with either etoposide or TPZ under hypoxic conditions. Immediately after exposure, cells treated with etoposide had more DNA-topo II complexes than the did the cells treated with TPZ (Fig. 5, A and B). Yet the amount of DNA-topo II complexes in HeLa cells treated with etoposide steadily decreased to one-half the initial amount by 1 h after the end of the exposure (Fig.

5A), whereas the amount of DNA-topo II complexes in cells treated with TPZ remained relatively constant for at least 3 h after the initial exposure (Fig. 5B). Fig. 5C shows a comparison of the amount of DNA-topo II complexes in HeLa cells treated with either 100  $\mu\text{M}$  etoposide or 100  $\mu\text{M}$  TPZ under hypoxic conditions immediately after treatment and 90 min after treatment. Again, these data show that the DNA-topo II complexes are more stable in cells treated with TPZ than those treated with etoposide. Also shown is the fact that DNA-topo II complexes are not produced in cells exposed to TPZ under aerobic conditions (Fig. 5C).

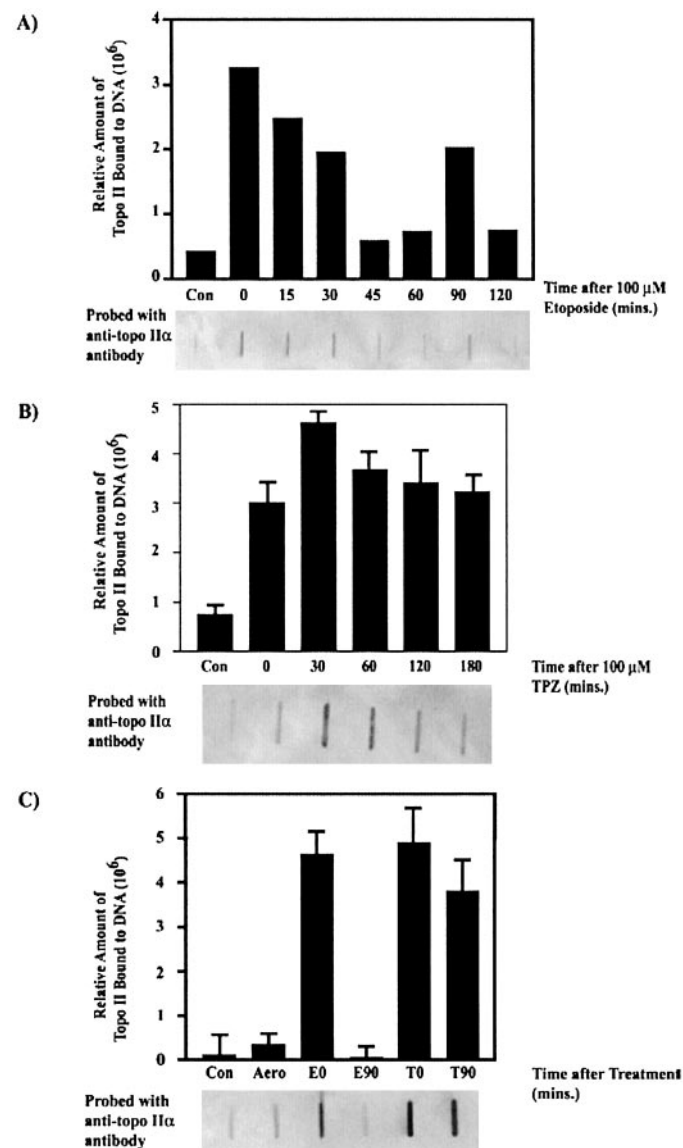


Fig. 5. Stability of DNA-topo II complexes in HeLa cells by etoposide and TPZ. HeLa cells were treated with either 100  $\mu\text{M}$  etoposide for 1 h or 100  $\mu\text{M}$  TPZ under hypoxic conditions for 1 h, and high-ionic lysates were analyzed for DNA-topo II complexes using cesium chloride isolation of DNA-topo II complexes. DNA-topo II complexes were isolated using cesium chloride gradients, and DNA fractions were identified and quantified. Samples were analyzed 0–120 min after 100  $\mu\text{M}$  etoposide (A, single experiment) and 0–180 min after 100  $\mu\text{M}$  TPZ under hypoxic conditions (B, pooled data from two experiments; error bars,  $\pm 1$  SE). For a direct comparison of the two agents, samples were analyzed 0 min and 90 min after 100  $\mu\text{M}$  etoposide and 0 min and 90 min after 100  $\mu\text{M}$  TPZ under hypoxic conditions (C, pooled data from three experiments). Samples that were untreated (Con) and samples that were treated with 100  $\mu\text{M}$  TPZ under aerobic conditions (Aero) were also analyzed for DNA-topo II complexes (C). DNA (2.0  $\mu\text{g}$ ) was blotted onto nitrocellulose using a slot-blot. Membranes were probed with anti-topo II $\alpha$  antibody and with a secondary antibody conjugated to alkaline phosphatase. The blots were visualized using the ECF Western Blotting kit and relative amounts of DNA-topo II complexes were calculated using a Storm optical scanner.

<sup>4</sup> Mary S. Kovacs and J. Martin Brown, unpublished observations.

## DISCUSSION

TPZ is an anticancer agent designed to exploit the unique hypoxic microenvironment of solid tumors (4). At low levels of oxygen, the TPZ radical, produced by intracellular reduction of the parent drug, leads to extensive DNA damage including base damage, DNA SSBs, and DNA DSBs, the latter of which can account for the cytotoxicity of the drug (29, 43). Recent studies have shown the nuclear metabolism of TPZ is not only responsible for all of the damage caused by TPZ (12) but is also compartmentalized with different reductases being responsible for the production of DNA SSBs and DSBs (14). We hypothesized, based on the finding that TPZ is metabolized by reductases associated with the nuclear matrix (14), that this selective localization of TPZ reduction could lead to a disruption of the components of the nuclear matrix or of the activities that take place there. This protein-rich scaffold maintains organization of the chromatin loops and aids in critical nuclear functions, such as DNA replication and transcription, and DNA repair (13). topo II $\alpha$  is highly enriched in the nuclear matrix and is responsible for resolving topological states that are encountered during replication and transcription. By virtue of its location and its role in the cytotoxicity of several other chemotherapeutic agents (27), in particular, the topo II poisons doxorubicin and etoposide, we investigated whether the cytotoxic effects of TPZ could be the result of its interaction with topo II.

Using the kDNA unwinding assay, we showed that exposure to TPZ under hypoxic conditions can inhibit topo II activity in nuclear extracts from LXFL non-small cell lung cancer cells (Fig. 1). Although this inhibition of activity could be the result either of direct disruption of the enzyme or of depletion of topo II from the available pool, our inability to affect the purified enzyme in solution by TPZ reduction (data not shown), as well as the data showing formation of DNA-topo II complexes (Figs. 4 and 5), suggests that inhibition of the activity of topo II is most likely the result of the depletion of free topo II.

In the topo II catalytic cycle, the enzyme binds to double-strand DNA, introduces a transient DNA DSB and passes the unbroken strand through the break. topo II then religates the transient break and dissociates from DNA. The topo II poison etoposide stabilizes the DNA-topo II complex and prevents religation, producing DNA DSBs that are associated with topo II. The fact that certain drugs, notably aclarubicin and merbarone, inhibit the topo II catalytic cycle before the formation of the transient DNA DSB, and thereby abrogate the cytotoxicity of etoposide, allowed us to test directly the involvement of topo II in the action of TPZ. We found that both catalytic inhibitors inhibited the cytotoxicity and DNA DSBs caused by TPZ under hypoxic conditions (Fig. 2). These results are consistent with published studies showing that DNA DSBs caused by TPZ are protein associated and that proteinase K must be used to detect these breaks (28, 29).

Because of the possibility of the effects of these inhibitors on targets other than topo II [merbarone, for example, in addition to acting on various signaling pathways (44), is a barbiturate derivative (45) and could, therefore, interfere with TPZ reduction], we also used a genetic approach to test the involvement of topo II in TPZ-induced DSBs. For this we used the H69 small cell lung carcinoma cells and its etoposide-resistant mutant H69/VP cells. H69/VP cells are resistant to etoposide because of an overexpression of P-glycoprotein (40), which is a large transmembrane protein that pumps epipodophyllotoxins and anthracyclines out of the cell, and because of a mutation in the topo II nuclear localization signal that localizes the enzyme in the cytoplasm rather than in the nucleus (39). As TPZ is not a substrate for P-glycoprotein,<sup>4</sup> we used the H69/VP cell line to investigate whether nuclear topo II $\alpha$  is required for the induction of DNA DSBs by TPZ. As expected, although etoposide-induced DNA DSBs are produced effectively in H69 cells, they are not produced in the mutant H69/VP

cells at similar doses (Fig. 3A). Similarly, DNA DSBs were induced much less efficiently by TPZ in the mutant than in the parental cells (Fig. 3B). The greater reduction in DNA DSBs, induced by etoposide than by TPZ, in the mutant H69/VP cells is consistent with the additional mechanism of overexpression of P-glycoprotein for etoposide resistance. These results again suggest that topo II $\alpha$  mediates, at least in part, the DNA DSBs caused by TPZ.

When topo II binds and transiently cleaves DNA, the enzyme is covalently bound to the DNA. Etoposide stabilizes this DNA-topo II complex, resulting in increased levels of covalently bound topo II to DNA. Because this increased covalent binding of topo II to DNA is characteristic of topo II poisons, we used two separate assays to determine whether TPZ leads to an increase in DNA-topo II complexes.

First developed by Frank *et al.* (46) to study melphalan adducts and modified by Willmore and colleagues to detect DNA-topo II complexes (33), the TARDIS assay detects topo II that is covalently bound to DNA in individual cells. Using this assay, we showed qualitatively that TPZ leads to the formation of DNA-topo II complexes (Fig. 4) at doses that produce only modest cytotoxicity.

We also measured topo II binding to DNA after cesium chloride isolation of the complexes, both immediately and as a function of time after etoposide or TPZ treatment. In HeLa cells treated with 100  $\mu$ M etoposide, the DNA-topo II complexes are transient and are removed effectively within 1–2 h after treatment (Fig. 5, A and C). In contrast, cells treated with 100  $\mu$ M TPZ removed the DNA-topo II complexes less efficiently (Fig. 5, B and C). These results suggest that TPZ-induced DNA-topo II complexes may be less able to be repaired than the complexes produced by etoposide, or that TPZ produced additional damage to the repair enzymes. Relevant to this is an earlier finding that TPZ exposure can inhibit the repair of DNA DSBs induced by ionizing radiation (43). It should be added that, although these experiments focused on the involvement of topo II $\alpha$  with TPZ, because of the fact that the  $\beta$ -isoform can exist both as a heterodimer with topo II $\alpha$  and as a homodimer, we cannot dismiss the possibility that the  $\beta$ -isoform may also be involved in the cytotoxicity of TPZ.

An issue as yet unresolved by the present data is the fact that quiescent cells are equally sensitive as proliferating cells to TPZ-induced cytotoxicity under hypoxia<sup>5</sup> although levels of topo II $\alpha$  (but not the  $\beta$ -isoform) are much reduced in quiescent cells (19, 47). This suggests either the involvement of topo II $\beta$ , as indicated above, or another mode of cytotoxicity in quiescent cells.

Although the present data suggest that the formation of DNA DSBs and cytotoxicity by TPZ under hypoxic conditions are mediated by topo II, they do not address the question of how this occurs. Recent work by Li *et al.* and Wang *et al.* (48, 49) has demonstrated that hydroxyl radicals and/or reactive thiol compounds can damage topo II, and this damage can stimulate topo II-mediated cleavage. Thus, it is possible that TPZ radicals damage topo II leading to topo II-mediated DNA cleavage. Alternatively, work by Kingma and Osheroff (50, 51) and Sabourin and Osheroff (52) has shown that DNA damage, in particular base damage, can poison topo II leading to DNA DSBs. Because TPZ has been shown to produce extensive base damage that is different from that produced by ionizing radiation (53), this could stimulate topo II to cleave DNA to form DNA-topo II complexes. Because they are less repairable (Fig. 5), the TPZ-induced complexes are likely to be more toxic than those produced by etoposide.

In summary, the present data indicate that at least some of the cytotoxicity of TPZ in hypoxic cells is mediated through topo II. At the doses of TPZ used in these studies, all of the effects seen depended on the TPZ exposure being under hypoxic conditions. There were no

<sup>5</sup> James E. Evans and J. Martin Brown, unpublished observations.

effects on cell viability nor on any of the topo II assays if the exposures to TPZ were under aerobic conditions, nor if the cells were exposed to hypoxia without TPZ. As the cells in solid tumors are at lower oxygen levels than those of normal tissues, the present results suggest that TPZ is a tumor-specific topo II poison.

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