

Inhibition of DNA Replication by Tirapazamine¹

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ABSTRACT

Tirapazamine (TPZ) is a hypoxia-selective cytotoxin that is currently being examined in Phase II and III clinical trials in combination with radiotherapy and cisplatin-based chemotherapy. Reductases convert TPZ to a cytotoxic radical that produces DNA damage under hypoxic conditions. Because one or more of the enzymes responsible for the bioactivation of TPZ is/are thought to be at or near the nuclear matrix, we hypothesized that TPZ may have a major effect on DNA replication, a process that is known to occur predominantly at the nuclear matrix. To assess the effect of TPZ on DNA replication, we measured the incorporation of radioactive thymidine into DNA of HCT116 human colon cancer cells and HeLa cells. We show that incorporation of radioactive thymidine is dramatically inhibited in cells that are pretreated with TPZ under hypoxic conditions. TPZ-induced inhibition of DNA synthesis was much greater than that produced by more toxic doses of ionizing radiation. We used the SV40-based *in vitro* DNA replication assay to study the mechanism of inhibition of DNA synthesis in cells treated with TPZ. Using this assay, we show that extracts prepared from cells treated with TPZ under hypoxic conditions had only 25–50% of the DNA replication activity measured in control cells. This reduction in DNA replication activity was associated with a reduction in levels of replication protein A (RPA) in cytoplasmic extracts used for the *in vitro* DNA replication assay and could be overcome by addition of recombinant human RPA. Furthermore, we show by indirect immunofluorescence that TPZ leads to a localization of the p34 subunit of RPA (RPA2) to small subnuclear foci. These results show that TPZ dramatically inhibits DNA replication and that the mechanism of inhibition, at least in part, involves changes in RPA that alter its cellular localization.

INTRODUCTION

TPZ³ (SR 4233; WIN 59075; 3-amino-1,2,4-benzotriazine-1,4-dioxide; Tirazone) is a chemotherapeutic agent that is activated at low oxygen levels to become a cytotoxic agent (1, 2). It is currently showing considerable promise in Phase II and III clinical trials in combination with radiation and cisplatin (3, 4). The basis for the tumor selectivity of TPZ is that most solid tumors have a much lower average oxygen tension than normal tissues. These hypoxic tumors are often refractory to radiotherapy and are probably also refractory to chemotherapy (5–8). However, because TPZ is selectively toxic to hypoxic cells, it can exploit low tumor oxygenation for therapeutic advantage (9).

Intracellular reductases convert TPZ to a cytotoxic radical that, under hypoxic conditions, produces base damage, DNA single-strand breaks, DNA double-strand breaks, and chromosome aberrations (2, 10–12). In aerobic cells, the cytotoxicity of the TPZ radical is greatly reduced because oxygen causes back-oxidation of the radical to the

nontoxic parent compound. Many enzymes (including cytochrome P450 and NADPH cytochrome P450 reductase) can reduce TPZ to its cytotoxic radical (11, 13–19). However, although a large majority of the TPZ is metabolized in the cytoplasm, nuclear metabolism of TPZ accounts for essentially all of the TPZ-induced DNA damage (20).

The efficient production of DNA double-strand breaks by TPZ, as well as their inefficient repair compared with those produced by ionizing radiation (10), suggested that TPZ may be producing radicals in a highly localized area (2). The nuclear matrix, a proteinaceous scaffold composed of lamins A, lamins B, and topoisomerase II α , provides a structure to organize DNA in the nucleus. Matrix attachment regions are the regions of DNA that attach to the nuclear matrix (21, 22). Whereas specific nuclear reductases associated with the nuclear matrix that metabolize TPZ have yet to be identified, we have proposed that these reductases could produce highly localized damage at the matrix attachment regions (23).

The possibility that the enzymes responsible for the cytotoxicity of TPZ associate with the nuclear matrix led us to examine whether the TPZ radical modifies or disrupts cellular functions that occur at or near the nuclear matrix. One critical cellular function that occurs at the nuclear matrix is DNA replication (24–28). Thus, we hypothesized that because TPZ radicals may be highly concentrated in this region, TPZ might have a disproportionately large effect on DNA replication compared with other DNA-damaging agents at comparable levels of cytotoxicity. In agreement with this hypothesis, we show that TPZ dramatically inhibits DNA synthesis in HCT116 colon cancer cells and HeLa cells. This inhibition of DNA synthesis by TPZ (75–80% reduction after 1 h at 20 μ M) is of greater magnitude and duration than that caused by more toxic doses of ionizing radiation, suggesting that the TPZ radicals may directly affect components involved in replication. In support of this model, we show using a SV40-based *in vitro* DNA replication assay that extracts from TPZ-treated cells have a reduced ability to support *in vitro* DNA replication (20–50% of control after 1 h at 20 μ M). The reduced DNA replication activity in these extracts could be traced to a reduction in the levels of RPA, a heterotrimeric protein essential for DNA replication but also involved in DNA repair and recombination (reviewed in Ref. 29). Addition of rRPA to the extracts of TPZ-treated cells restores replication activity to control levels. Using indirect immunofluorescence, we demonstrate that the p34 subunit of RPA (RPA2) forms small subnuclear foci after cells are treated with TPZ under hypoxic conditions. These results reveal that exposure of cells to TPZ under hypoxic conditions greatly inhibits DNA synthesis and suggest that relocation of RPA or damage to RPA may contribute to this effect.

MATERIALS AND METHODS

Reagents. TPZ was obtained from Sanofi-Winthrop (Malvern, PA). [¹⁴C]Thymidine and [³H]thymidine were obtained from Amersham (Cleveland, OH). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Cell Culture. HeLa cells were grown and maintained in monolayer culture in Joklik-modified MEM supplemented with 5% iron-supplemented calf serum. HCT116 (p21^{+/+}) human colon carcinoma cells and HCT116 (p21^{-/-}) cells were graciously provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD) and grown and maintained in monolayer culture in McCoy's 5A modified medium supplemented with 10% fetal bovine serum.

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³ The abbreviations used are: TPZ, tirapazamine; RPA, replication protein A; rRPA, recombinant RPA; TAG, SV40 T antigen.

For all experiments, both HeLa cells and HCT116 cells were used in the logarithmic phase of growth.

TPZ and Aerobic/Anaerobic Treatment. HCT116 cells and HeLa cells were treated with TPZ at the concentrations and times indicated. 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 described previously (30). For aerobic treatment, jigs were evacuated and refilled five times with 95% air + 5% CO_2 . After evacuation, the jigs were placed in a 37°C incubator for 1 h. All TPZ exposures were for 1 h unless otherwise indicated.

For shorter exposures to TPZ (15–45 min), spinner flasks were used to achieve hypoxia. The control spinner flask contained 10.0 ml of control media, the treatment spinner flask contained 10 ml of media supplemented with 26.0 μM TPZ, and the cell spinner flask contained 10.0 ml of cells at a concentration of 4.3×10^6 cells/ml. Control spinner and treatment spinner flasks were allowed to become hypoxic for 1.0 h by circulating $N_2 + 5\%$ CO_2 gas through the flasks. Cells from the cell spinner flask were then added to both the control spinner flask and the treatment spinner flask to bring the final concentration of TPZ to 20 μM . At the appropriate time, 1.0 ml of cells was removed from the control spinner flask and the treatment spinner flask. Cells were centrifuged and washed twice with PBS. After washing, the cells were immediately pulse-labeled with [3H]thymidine as indicated below.

Cell Irradiation. Irradiations were performed with $^{137}Cesium$ γ -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.

Radioactive Labeling for DNA. Before treatment with TPZ or ionizing radiation, cells were continuously labeled for 24 h with 1 $\mu Ci/ml$ [^{14}C]thymidine. Immediately before treatment, the [^{14}C]thymidine-containing media was removed, and the cells were rinsed with PBS. After treatment with TPZ or with irradiation, the cells were incubated at 37°C for 30 min. After recovery, the cells were pulse-labeled with 100 $\mu Ci/ml$ [3H]thymidine for 30 min. The radioactive media were removed after pulse-labeling, and the cells were washed twice with PBS.

Measurement of DNA Synthesis in Intact Cells. HCT116 or HeLa cells were lysed after pulse-labeling in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS] at 4°C. The lysate was precipitated on ice for 30 min with 10% trichloroacetic acid + 2% Na PP_i. The lysate was then resuspended to a final volume of 2.0 ml. Aliquots (400 μl) of each sample were applied in triplicate to prewetted 24.0 mm Whatman glass filters (Maidstone, Kent, United Kingdom) placed on a filtration system (Millipore, Bedford, MA). The samples were then washed three times with 5.0 ml of 10% trichloroacetic acid + 2% Na PP_i and twice with 5.0 ml of 95% ethanol. Filters were allowed to air dry and then transferred to scintillation tubes. A total of 10.0 ml of Ecolume were added to each sample. The samples were analyzed using a Beckman LS6000IC liquid scintillation counter (Fullerton, CA). DNA synthesis was calculated as the ratio of the [3H]thymidine counts:[^{14}C]thymidine counts. The percentage of replication activity was calculated by comparison with identically treated controls not exposed to TPZ or to ionizing radiation.

Clonogenic Assays. Colony-forming assays were performed as described previously (31). Briefly, HCT116 p21^{+/+} cells treated with varying doses of TPZ under hypoxia for 1 h or varying doses of irradiation were collected by trypsinization, resuspended in ice-cold media, and plated in 60- or 100-mm Petri dishes. After 2 weeks, the media were 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 was obtained by comparing the number of colonies in the treated groups *versus* the number of colonies in the control group.

In Vitro Assay of DNA Synthesis. HeLa cells were grown in 1-liter spinner flasks, filled to 250 ml, to a final concentration of $\sim 10^6$ cells/ml. Cells were made hypoxic by a 1-h exposure to a N_2/CO_2 mixture and treated for 1 h with TPZ. Hypoxia was monitored using an O_2 electrode (kindly provided by Dr. Cameron Koch, Department of Radiation Oncology, University of Pennsylvania School of Medicine, Philadelphia, PA). After treatment, cells were collected, an aliquot was taken to measure DNA replication *in vivo*, and the remaining cells were processed for cytoplasmic extract preparation using standard protocols (32–35). *In vitro* DNA replication activity was measured

using an SV40-based *in vitro* DNA replication assay. In this assay, replication of plasmids carrying SV40 origin of DNA (ori⁺ DNA) replication is accomplished *in vitro* with cytoplasmic extracts, with TAg as the only noncellular protein. Reaction mixtures (25 μl) contained the following: 40 mM HEPES; 8 mM $MgCl_2$; 0.5 mM DTT; 3 mM ATP; 200 μM each of CTP, GTP, and UTP; 100 μM each of dATP, dGTP, and dTTP; 25 μM [α - ^{32}P]dCTP (1000 cpm/pmol); 40 mM creatine phosphate; 2.5 mg of creatine phosphokinase; 0.3 μg of superhelical plasmid DNA; 100–150 μg of cytoplasmic extracts; and 0.5 μg of TAg. The reaction mixture was incubated at 37°C for 1 h. The extent of DNA synthesis was evaluated by measuring incorporation into acid-insoluble material. DNA replication products were analyzed by gel electrophoresis followed by radioautography.

Western Blots. Standard procedures were followed for Western blots using nuclear or cytoplasmic extracts. We used whole cell lysates to assess RPA2 levels and RPA2 phosphorylation. Whole cell lysates from HeLa cells were obtained by lysing cells with radioimmunoprecipitation assay buffer supplemented with protease inhibitor mixture for 30 min at 4°C. Lysates were analyzed with SDS-PAGE using the Novex NuPAGE Electrophoresis System (San Diego, CA). Membranes were blocked with 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 RPA2 mouse monoclonal antibody (1:400; Neomarkers, Fremont, CA). Blots were then stained with secondary antibody conjugated to alkaline phosphatase and visualized using the Enhanced Chemifluorescence (ECF) system (Amersham Life Science, Little Chalfont, United Kingdom). Data were analyzed using a STORM Optical Scanner (Molecular Dynamics, Sunnyvale, CA).

Indirect Immunofluorescence. To determine cellular localization of RPA, HeLa cells were plated onto glass chamber microscope slides at a density of 1×10^4 cells/slide. Slides were treated with media containing 0 or 50 μM TPZ and then exposed to hypoxia using a Bacton anaerobic/environmental chamber (Sheldon Manufacturing, Inc., Cornelius, OR). After 1 h of treatment with TPZ under hypoxic conditions, slides were removed from the chamber, and treatment media were removed. Slides were washed twice with PBS and then fixed with 2 ml of $-20^\circ C$ methanol. To insure fixation, slides were incubated in methanol for 15 min at $-20^\circ C$. After fixation, the methanol was removed, and slides were washed twice with PBS and then blocked with PBS + 3% BSA for 30 min at 25°C. Fixed HeLa cells were probed with RPA2 mouse monoclonal antibody (1:250) for 1 h at 25°C and washed with PBS + 3% BSA to remove unbound primary antibody. Bound antibodies were detected with mouse anti-human IgG conjugated to fluorescein. The slides were stained with the DNA stain 4',6-diamidino-2-phenylindole dihydrochloride hydrate containing Vectashield (Vector, Burlingame, CA) and visualized using a Nikon optiphot fluorescence microscope (Melville, NY).

RESULTS

Inhibition of DNA Synthesis by TPZ. We measured DNA synthesis as a function of dose and time after TPZ treatment of HCT116 (p21^{+/+}) and (p21^{-/-}) human colon carcinoma cells under both aerobic (Fig. 1A) and hypoxic (Fig. 1B) conditions. After a 1-h treatment under aerobic conditions and for concentrations of up to 50 μM (Fig. 1A), TPZ produced only a small inhibition of DNA synthesis. However, when cells were treated with TPZ under hypoxic conditions, TPZ inhibited DNA synthesis in a dose-dependent manner in both p21^{+/+} and p21^{-/-} cells (Fig. 1B). After a 1-h exposure to 20 μM TPZ under hypoxic conditions, the rate of DNA synthesis decreased by approximately 70% (Fig. 1B). HCT116 cells show no signs of toxicity to 20 μM TPZ for up to 24 h after treatment, as judged by trypan blue exclusion and cell detachment (data not shown). Therefore, the inhibition of DNA synthesis caused by TPZ is not the result of the cells being metabolically dead. We obtained very similar results with HeLa cells (data not shown).

We next examined the recovery of DNA synthesis inhibition in cells treated with TPZ under hypoxic conditions. HCT116 (p21^{-/-}) cells were treated with TPZ (20 μM , 1 h) and allowed to recover at 37°C. DNA synthesis remained at 15–30% of controls for up to 24 h (Fig. 1C). The lack of recovery of DNA synthesis may be due in part

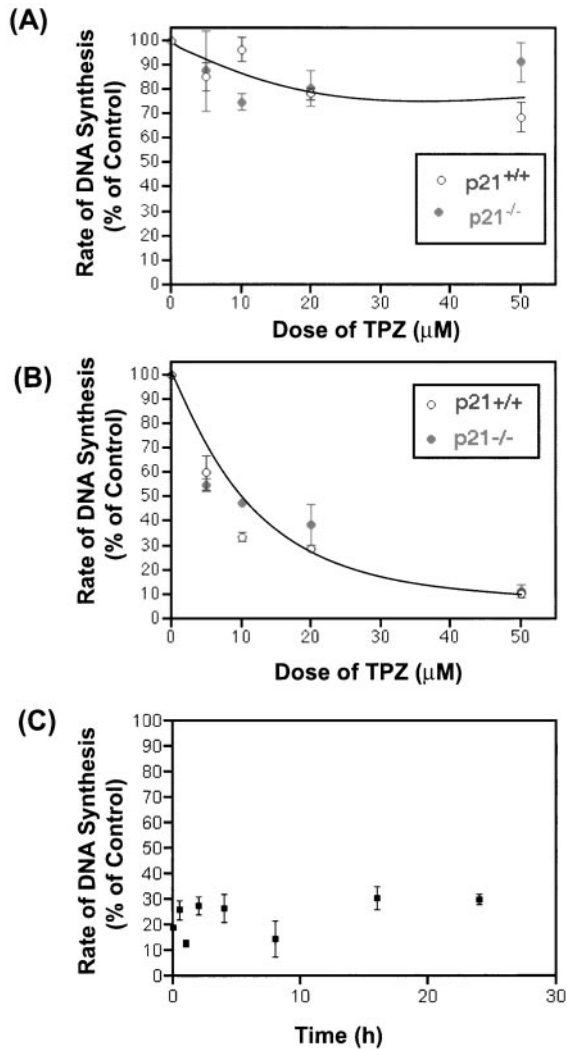


Fig. 1. Inhibition of DNA synthesis in $p21^{+/+}$ and $p21^{-/-}$ HCT116 colon carcinoma cells by TPZ under aerobic (A) or hypoxic conditions (B) and continued inhibition of DNA synthesis from 0 to 24 h in $p21^{-/-}$ HCT116 colon carcinoma cells treated with 20 μM TPZ for 1 h (C). HCT116 colon carcinoma cells [$p21^{+/+}$ (○) and $p21^{-/-}$ (●)] were treated with increasing doses of TPZ (0–50 μM) under aerobic or hypoxic conditions for 1 h. After treatment, cells were allowed to recover for 30 min and then pulse-labeled with [^3H]thymidine. The rate of DNA synthesis was calculated by comparison with identically treated controls not exposed to TPZ. To examine continued inhibition of DNA synthesis, HCT116 $p21^{-/-}$ cells were allowed to recover after a 1-h exposure to 20 μM TPZ under hypoxic conditions for time periods between 0 and 24 h (C). After the recovery time, the cells were pulse-labeled with [^3H]thymidine, and the rate of DNA synthesis was calculated by comparison with identically treated controls not exposed to TPZ.

to a reduction in the fraction of S-phase cells as a result of their accumulation in G_2 -M phase. To determine the kinetics of inhibition of DNA synthesis, we treated HeLa cells with 20 μM TPZ under hypoxic conditions for a period of 15–60 min. After only 15 min of treatment, the rate of DNA synthesis was approximately 50% of the control and continued to decrease with increasing time of exposure to TPZ (Fig. 2). The rapidity of the decrease seen argues against a G_1 -S checkpoint here with HeLa cells and in Fig. 1C with HCT116 cells, particularly because the HCT116 cells had both alleles of p21 deleted and would therefore not be expected to delay in G_1 (36–39).

Comparison of Inhibition of DNA Synthesis by TPZ and Irradiation. Both TPZ and ionizing irradiation introduce DNA single-strand breaks and DNA double-strand breaks and produce equal numbers of lethal chromosome breaks at equal levels of cell kill (10). Ionizing irradiation also produces a dose-dependent inhibition of DNA synthesis (reviewed in Ref. 40). To test our hypothesis that TPZ,

unlike ionizing radiation, produces nonrandom damage in DNA possibly concentrated at the nuclear matrix, we compared the inhibition of DNA synthesis caused by TPZ under hypoxic conditions with that caused by ionizing irradiation under hypoxic conditions. Doses of 15–60 Gy of irradiation had a modest effect on DNA synthesis in comparison with 20 μM TPZ under hypoxic conditions, which inhibited the rate of DNA synthesis by approximately 80% (Fig. 3A).

Fig. 3, B and C, shows the clonogenic survival of HCT116 $p21^{+/+}$ cells after exposure to varying doses of TPZ (under hypoxia) or ionizing radiation (under euoxia). Comparison of these results with those of Fig. 2 demonstrate that TPZ, under hypoxic conditions, inhibits DNA synthesis to a much greater extent than doses of ionizing radiation that produce greater cell kill (even allowing for the 3-fold reduction in equivalent dose for the hypoxic irradiation; Fig. 3, A–C). These observations suggest that in addition to introducing DNA damage, TPZ may also interfere directly with the replication machinery. We examined this possibility using an *in vitro* assay of DNA replication.

In Vitro Measurement of DNA Replication. To evaluate the effects of TPZ on DNA replication at the biochemical level, we treated HeLa cells under hypoxic conditions with varying concentrations of TPZ and then prepared cytoplasmic extracts. The ability of these extracts to support *in vitro* DNA replication was evaluated using plasmids containing the SV40 origin of DNA replication and recombinant Tag as the only noncellular protein (35). Although extracts prepared from untreated cells could actively support *in vitro* DNA replication, those of cells treated with 20 or 50 μM TPZ under hypoxic conditions showed a 50% reduction in this activity (Fig. 4). This observation suggests that extracts of TPZ-treated cells are deficient in a factor or activity that is critical for DNA replication. Because RPA is the rate-limiting factor in this type of reaction (29), we evaluated DNA replication after the addition of rRPA in reactions assembled with extracts prepared from TPZ-treated cells. The results shown in Fig. 4 indicate that rRPA restored the DNA replication activity of extracts from TPZ-treated cells to control levels (Fig. 4). Thus, exposure of cells to TPZ under hypoxic conditions leads to a partial loss of RPA activity from the cytoplasmic component of the extract.

Other DNA-damaging agents, such as camptothecin and high doses of ionizing irradiation, also decrease DNA replication as measured in extracts of treated cells by the SV40-based *in vitro* DNA replication assay. One component in this inhibition is a reduction of the available pool of RPA in the cytoplasmic fraction of the extract (32–34). We therefore tested this possibility in extracts prepared from TPZ-treated

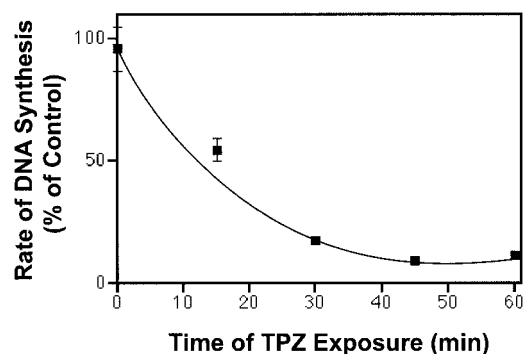


Fig. 2. Initial inhibition of DNA synthesis in HeLa cells by 20 μM TPZ under hypoxic conditions. HeLa cells were continuously labeled with [^{14}C]thymidine for 24 h. Subsequent to labeling, 1×10^6 cells were exposed to 20 μM TPZ for 15–60 min under hypoxic conditions. Cells were immediately pulsed with [^3H]thymidine-containing media for 30 min and then lysed, and DNA was precipitated from the lysates onto filters. The relative amounts of [^{14}C]thymidine and [^3H]thymidine were assessed using a scintillation counter. The rate of DNA synthesis was calculated by comparison with identically treated controls not exposed to TPZ.

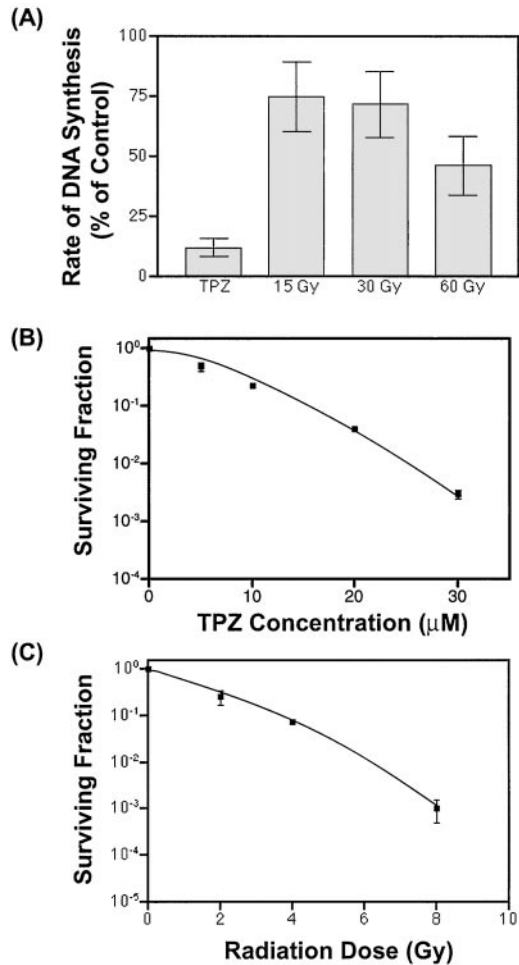


Fig. 3. Comparison of the inhibition of DNA synthesis by TPZ and γ -irradiation (A) and survival of HCT116 p21^{+/+} cells treated with either TPZ (B) or γ -irradiation (C). HCT116 p21^{+/+} colon carcinoma cells were treated with 20 μM TPZ under hypoxic conditions for 1 h or exposed to γ -irradiation (15–60 Gy) using a Mark I Model 25 Irradiator. After treatment, the cells were allowed to recover for 30 min and then pulse-labeled with [³H]thymidine. The rate of DNA synthesis was calculated by comparison with identically treated controls not exposed to TPZ or to γ -irradiation. Survival of HCT116 p21^{+/+} cells exposed to increasing doses of TPZ (B) or increasing doses of γ -irradiation (C) was assessed using the standard clonogenic assay.

cells. Western blots of cytoplasmic extracts of TPZ-treated HeLa cells show a decrease in both the p70 (RPA1) and p34 (RPA2) subunits of RPA when compared with untreated cells (Fig. 5A). This reduction is not directly obvious in nuclear extracts of TPZ-treated HeLa cells, suggesting that the overall levels of RPA in the cells probably remain unchanged.

After exposure of cells to UV irradiation or X-rays, the RPA2 subunit is found in a partly phosphorylated form (29). After treatment of HeLa cells with 50 μM TPZ, there is a shift in the electrophoretic mobility of a portion of RPA2 present in whole cell extracts (Fig. 5B) that suggests phosphorylation. Moreover, the phosphorylation of RPA2 persists for up to 28 h after a 1-h exposure to 50 μM TPZ (data not shown). We note that there is no reduction in the total levels of RPA2 after exposure to TPZ (Fig. 5B). Localization of RPA2 was further examined with indirect immunofluorescence. HeLa cells that were plated on glass chamber slides were exposed to hypoxia alone or 50 μM TPZ under hypoxic conditions. Under hypoxic conditions only, RPA2 localized uniformly throughout the nucleus (Fig. 5C). After treatment with 50 μM TPZ, RPA2 localizes to small subnuclear foci as detected with indirect immunofluorescence (Fig. 5C). In conclusion, these results suggest that TPZ under hypoxic conditions either traps

RPA in the nucleus or enhances the recruitment of RPA into the nucleus, possibly to sites of TPZ-induced damage.

DISCUSSION

TPZ, a promising chemotherapeutic agent, takes advantage of the hypoxic environment of solid tumors. At low oxygen levels, TPZ radicals formed by 1-electron reduction produce extensive DNA damage, which leads to cytotoxicity (10). We have proposed that although TPZ metabolism occurs throughout the cell, it is the nuclear metabolism that is responsible for the DNA damage and cytotoxicity (9, 20). Moreover there is evidence that this metabolism is associated, at least in part, with the nuclear matrix (23). Because DNA replication occurs at sites of DNA attachment to the nuclear matrix (24–28), we speculated that TPZ might disrupt DNA replication to a greater degree than other agents that produce DNA damage more randomly. The results presented here confirm that TPZ produces a profound inhibition of DNA replication in both HCT116 and HeLa cells. Other hypoxia-activated cytotoxic drugs such as metronidazole, misonidazole, and nitrofurazone have been tested for their effect on DNA synthesis (41, 42). Variable effects were seen, which, in some cases, were not hypoxia specific. It is possible that some of their similarities with TPZ to inhibit DNA synthesis could be the result of activation by the same nuclear matrix-activating enzymes that are responsible for the reduction of TPZ.

Ionizing irradiation also inhibits DNA replication, but the extent of inhibition at equitoxic or more toxic doses is much less than that produced by TPZ (Fig. 3, A–C). The extent to which ionizing radiation inhibits DNA replication is cell line dependent (40), and with the HCT116 cells used in our studies, there was only a small level of inhibition at doses <15 Gy under hypoxic conditions (Fig. 3A). Because DNA damage is believed to be directly or indirectly responsible for the bulk of the inhibition of DNA replication (40), it is relevant to ask why ionizing radiation and TPZ inhibit DNA replication to such different efficiencies. We hypothesize that this difference arises because damage induced by ionizing radiation is randomly

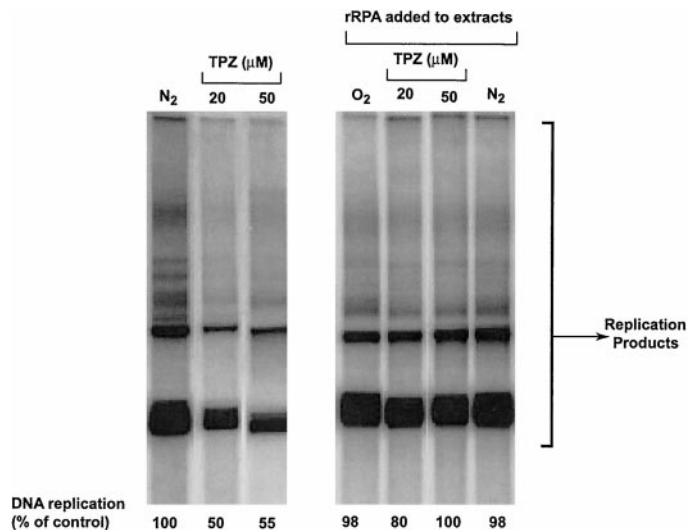


Fig. 4. *In vitro* measurement of DNA replication using cytoplasmic extracts from HeLa cells treated with TPZ under hypoxic conditions and the SV40-based assay for DNA replication. Extracts from either control-treated HeLa cells or TPZ-treated HeLa cells were prepared and used to assess the ability to replicate SV40 plasmid *in vitro* by the incorporation of ³²P-labeled nucleotides. The samples were electrophoresed and radioautographed to identify the replication products. Incorporation of radioactive nucleotides was restored by the addition of rRPA to TPZ-treated cellular extracts. The amount of replication products was quantified and used to calculate the DNA replication activity (percentage of control). These results are representative of several repeated experiments.

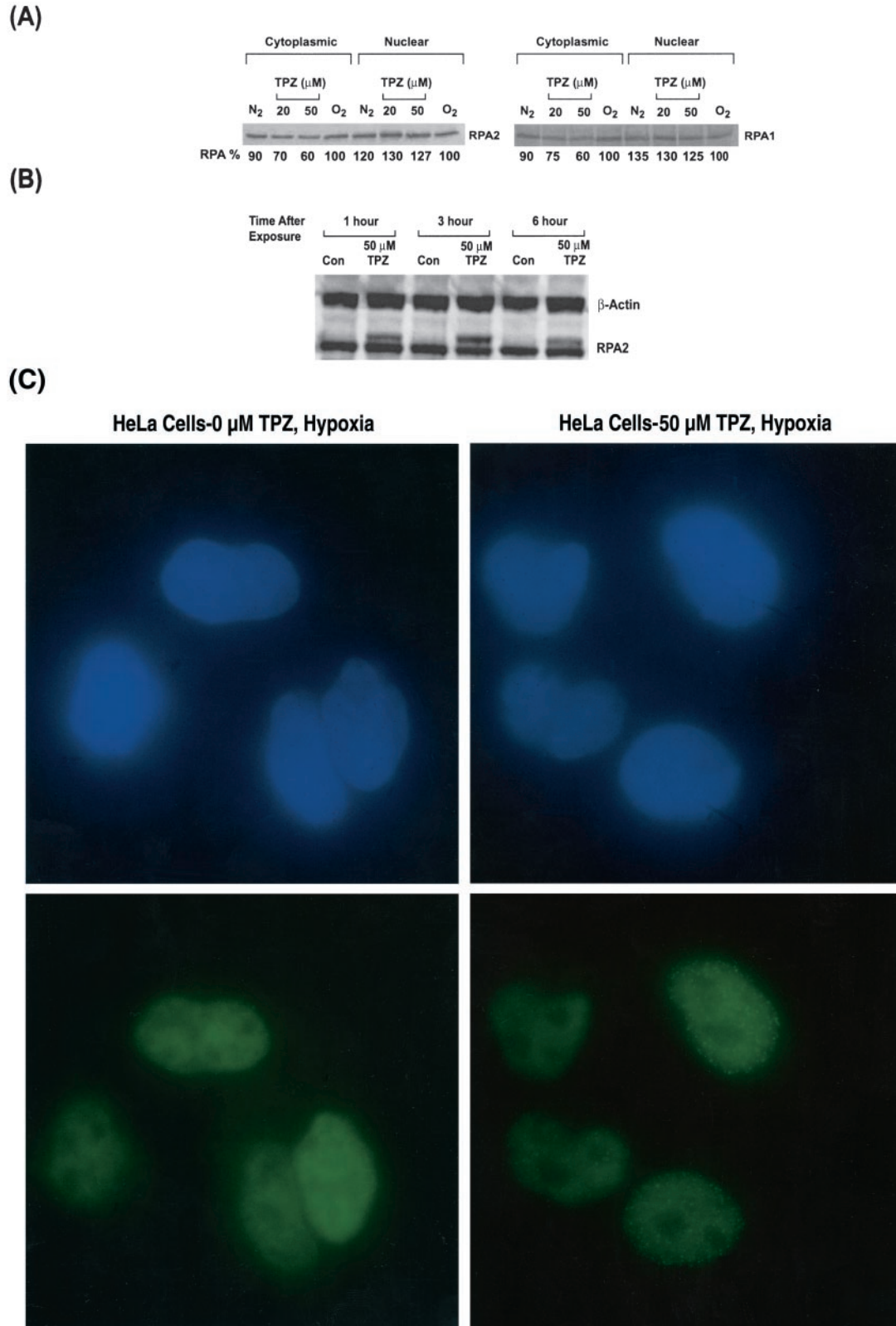


Fig. 5. Change in localization of RPA subunits and phosphorylation of RPA2 caused by TPZ as assessed by Western blot (A and B) and indirect immunofluorescence (C). The amount of RPA2 (left panel) and RPA1 (right panel) in cytoplasmic and nuclear HeLa cell extracts exposed to hypoxia (N₂), air, or TPZ (20 or 50 μM) under hypoxia was assessed by Western blot (A). The relative amounts of RPA are indicated as *RPA %*. Phosphorylation of the RPA2 subunit by TPZ was determined by comparing the Western blot of whole cell HeLa extracts that had been treated with 0 or 50 μM TPZ (B). An upward shift in the electrophoretic mobility of RPA2 is indicative of the phosphorylation of RPA2. Localization of RPA2 was examined further by indirect immunofluorescence using anti-RPA2 antibody and a secondary antibody conjugated to fluorescein (C). *Top panels of C* show the 4',6-diamidino-2-phenylindole dihydrochloride hydrate blue-stained nuclei, and the *bottom panels of C* show localization of secondary antibody conjugated to fluorescein (*green*). These results are representative of several repeated experiments.

distributed throughout the DNA, whereas TPZ-induced damage may be localized preferentially at the nuclear matrix. This could result in a strong inhibition of the DNA replication activity. Because the number of chromosome breaks produced by radiation and by TPZ are comparable at equitoxic doses (10), our data suggest that many more DNA replication-inhibiting lesions are produced per lethal event after exposure to TPZ than after exposure to ionizing radiation. A possible reason for this would be direct damage to the replication machinery.

As a direct test of this possibility, we investigated DNA replication *in vitro* using a SV40-based *in vitro* DNA replication assay. The results obtained using this system demonstrated a decrease in the ability of extracts prepared from TPZ-treated cells to support SV40 DNA replication (Fig. 4), suggesting that a component required for DNA replication was lost or damaged. However, we do not believe that such damage to the replication machinery can account for all of the inhibition because at doses of TPZ that gave 80% inhibition *in vivo*, we observed only 50% inhibition *in vitro*.

When rRPA was added to extracts of TPZ-treated cells, SV40 DNA synthesis was restored to control levels (Fig. 4). This implies that RPA may be affected by TPZ treatment. RPA, a component of the origin recognition complex, is critical for eukaryotic DNA replication and is present in cells as a heterotrimeric complex of 70, 34, and 14 kDa referred as RPA1, RPA2, and RPA3 (p14 subunit of RPA), respectively. Other cellular processes, including DNA repair and recombination, have also been shown to require RPA (29). Western blots of TPZ-treated cell extracts revealed that there was a reduction in RPA1 and RPA2 subunits of RPA in the cytoplasmic extracts (Fig. 5A). On the other hand, nuclear extracts of TPZ-treated cells contained RPA1 and RPA2 at levels similar to those of extracts from untreated cells (Fig. 5A). This is consistent with the hypothesis that the localized action of TPZ at the nuclear matrix traps or damages RPA and prevents it from leaching into the cytoplasmic fraction during preparation of the extract. However, similar effects may also be induced by either redirection of the activities of RPA or posttranslational modifications.

Whereas RPA is critical for DNA replication, it also participates in DNA repair (29). It is therefore possible that on DNA damage induction by TPZ, RPA redistributes to sites of DNA repair, thereby abandoning sites of DNA replication. We believe that this could be due to RPA's known association with hRad51, a protein critical for repair of DNA damage by homologous recombination that we have recently found to be the major pathway of repair of TPZ damage.⁴ In support of this, we show that small subnuclear foci of RPA2 are seen in TPZ-treated cells (Fig. 5C). Whatever the cause, this redistribution of RPA activity would likely reduce the cytoplasmic component of RPA or further prevent its leaching into the cytoplasmic fraction during extract preparation. Indeed, the Western blots in Fig. 5A indicate a reduction in both RPA1 and RPA2 in cytoplasmic extracts prepared from TPZ-treated cells. Moreover, on treatment with TPZ, HeLa cells phosphorylate RPA2 (Fig. 5B). Such phosphorylation of RPA2 after treatment with DNA-damaging agents has been linked to the role of RPA in DNA repair (29), and recently, it was also found that this phosphorylation of RPA2 led to a partial dissociation of the heterotrimeric complex (43). This posttranslational modification may therefore also contribute to the altered distribution of RPA in the cytoplasmic fraction in extracts of TPZ-treated cells.

In summary, our results show that TPZ, a hypoxia-selective cytotoxin, inhibits DNA replication to a greater extent than ionizing irradiation. This inhibition is independent of *p21^{Waf1/Cip1}* and may

result, at least in part, from direct damage to or intracellular redistribution of RPA, a protein critical for DNA replication.

REFERENCES

- Zeman, E. M., Brown, J. M., Lemmon, M. J., Hirst, V. K., and Lee, W. W. SR-4233: a new bioreductive agent with high selective toxicity for hypoxic mammalian cells. *Int. J. Radiat. Oncol. Biol. Phys.*, *12*: 1239–1242, 1986.
- Brown, J. M. SR 4233 (tirapazamine): a new anticancer drug exploiting hypoxia in solid tumours. *Br. J. Cancer*, *67*: 1163–1170, 1993.
- von Pawel, J., von Roemeling, R., Gatzemeier, U., Boyer, M., Elisson, L. O., Clark, P., Talbot, D., Rey, A., Butler, T. W., Hirsh, V., Olver, I., Bergman, B., Ayoub, J., Richardson, G., Dunlop, D., Arcenas, A., Vescio, R., Viallet, J., and Treat, J. Tirapazamine plus cisplatin versus cisplatin in advanced non-small-cell lung cancer: a report of the international CATAPULT I study group. Cisplatin and tirapazamine in subjects with advanced previously untreated non-small-cell lung tumors. *J. Clin. Oncol.*, *18*: 1351–1359, 2000.
- Rischin, D., Peters, L., Hicks, R., Hughes, P., Fisher, R., Hart, R., Sexton, M., D'Costa, I., and von Roemeling, R. Phase I trial of concurrent tirapazamine, cisplatin, and radiotherapy in patients with advanced head and neck cancer. *J. Clin. Oncol.*, *19*: 535–542, 2001.
- Sartorelli, A. C. Therapeutic attack of hypoxic cells of solid tumors: presidential address. *Cancer Res.*, *48*: 775–778, 1988.
- Nordmark, M., Overgaard, M., and Overgaard, J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. *Radiother. Oncol.*, *41*: 31–39, 1996.
- Brizel, D. M., Sibley, G. S., Prosnitz, L. R., Scher, R. L., and Dewhurst, M. W. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. *Int. J. Radiat. Oncol. Biol. Phys.*, *38*: 285–289, 1997.
- Fyles, A. W., Milosevic, M., Wong, R., Kavanagh, M. C., Pintilie, M., Sun, A., Chapman, W., Levin, W., Manchul, L., Keane, T. J., and Hill, R. P. Oxygenation predicts radiation response and survival in patients with cervix cancer. *Radiother. Oncol.*, *48*: 149–156, 1998.
- Brown, J. M. The hypoxic cell: a target for selective cancer therapy—Eighteenth Bruce F. Cain Memorial Award Lecture. *Cancer Res.*, *59*: 5863–5870, 1999.
- Wang, J., Biedermann, K. A., and Brown, J. M. Repair of DNA and chromosome breaks in cells exposed to SR 4233 under hypoxia or to ionizing radiation. *Cancer Res.*, *52*: 4473–4477, 1992.
- Wang, J., Biedermann, K. A., Wolf, C. R., and Brown, J. M. Metabolism of the bioreductive cytotoxin SR 4233 by tumour cells: enzymatic studies. *Br. J. Cancer*, *67*: 321–325, 1993.
- Daniels, J. S., Gates, K. S., Tronche, C., and Greenberg, M. M. Direct evidence for bimodal DNA damage induced by tirapazamine. *Chem. Res. Toxicol.*, *11*: 1254–1257, 1998.
- Cahill, A., and White, I. N. Reductive metabolism of 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233) and the induction of unscheduled DNA synthesis in rat and human derived cell lines. *Carcinogenesis (Lond.)*, *11*: 1407–1411, 1990.
- Lloyd, R. V., Duling, D. R., Rumyantseva, G. V., Mason, R. P., and Bridson, P. K. Microsomal reduction of 3-amino-1,2,4-benzotriazine 1,4-dioxide to a free radical. *Mol. Pharmacol.*, *40*: 440–445, 1991.
- Walton, M. I., Wolf, C. R., and Workman, P. The role of cytochrome P450 and cytochrome P450 reductase in the reductive bioactivation of the novel benzotriazine di-N-oxide hypoxic cytotoxin 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233, WIN 59075) by mouse liver. *Biochem. Pharmacol.*, *44*: 251–259, 1992.
- Riley, R. J., Hemingway, S. A., Graham, M. A., and Workman, P. Initial characterization of the major mouse cytochrome P450 enzymes involved in the reductive metabolism of the hypoxic cytotoxin 3-amino-1,2,4-benzotriazine-1,4-di-N-oxide (tirapazamine, SR 4233, WIN 59075). *Biochem. Pharmacol.*, *45*: 1065–1077, 1993.
- Patterson, A. V., Barham, H. M., Chinje, E. C., Adams, G. E., Harris, A. L., and Stratford, I. J. Importance of P450 reductase activity in determining sensitivity of breast tumour cells to the bioreductive drug, tirapazamine (SR 4233). *Br. J. Cancer*, *72*: 1144–1150, 1995.
- Patterson, A. V., Saunders, M. P., Chinje, E. C., Patterson, L. H., and Stratford, I. J. Enzymology of tirapazamine metabolism: a review. *Anticancer Drug Des.*, *13*: 541–573, 1998.
- Chinje, E. C., Patterson, A. V., Saunders, M. P., Lockyer, S. D., Harris, A. L., and Stratford, I. J. Does reductive metabolism predict response to tirapazamine (SR 4233) in human non-small-cell lung cancer cell lines? *Br. J. Cancer*, *81*: 1127–1133, 1999.
- Evans, J. W., Yudoh, K., Delahoussaye, Y. M., and Brown, J. M. Tirapazamine is metabolized to its DNA-damaging radical by intranuclear enzymes. *Cancer Res.*, *58*: 2098–2101, 1998.
- Mirkovitch, J., Mirault, M. E., and Laemmli, U. K. Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. *Cell*, *39*: 223–232, 1984.
- Cockerill, P. N., and Garrard, W. T. Chromosomal loop anchorage of the κ immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell*, *44*: 273–282, 1986.
- Delahoussaye, Y. M., Evans, J. M., and Brown, J. M. Tirapazamine is metabolized by multiple reductases in the nucleus. *Biochem. Pharmacol.*, in press, 2001.
- Van der Velden, H. M., Poot, M., and Wanka, F. *In vitro* DNA replication in association with the nuclear matrix of permeable mammalian cells. *Biochim. Biophys. Acta*, *782*: 429–436, 1984.
- Jackson, D. A., and Cook, P. R. Replication occurs at a nucleoskeleton. *EMBO J.*, *5*: 1403–1410, 1986.

⁴ J. Evans, G. Birrell, and J. M. Brown, unpublished observations.

26. Dijkwel, P. A., Wenink, P. W., and Poddighe, J. Permanent attachment of replication origins to the nuclear matrix in BHK-cells. *Nucleic Acids Res.*, *14*: 3241–3249, 1986.
27. Brylawski, B. P., Tsongalis, G. J., Cordeiro-Stone, M., May, W. T., Comeau, L. D., and Kaufman, D. G. Association of putative origins of replication with the nuclear matrix in normal human fibroblasts. *Cancer Res.*, *53*: 3865–3868, 1993.
28. Cook, P. R. The organization of replication and transcription. *Science (Wash. DC)*, *284*: 1790–1795, 1999.
29. Iftode, C., Daniely, Y., and Borowiec, J. A. Replication protein A (RPA): the eukaryotic SSB. *Crit. Rev. Biochem. Mol. Biol.*, *34*: 141–180, 1999.
30. Kovacs, M. S., Hocking, D. J., Evans, J. W., Siim, B. G., Wouters, B. G., and Brown, J. M. Cisplatin anti-tumour potentiation by tirapazamine results from a hypoxia-dependent cellular sensitization to cisplatin. *Br. J. Cancer*, *80*: 1245–1251, 1999.
31. Wouters, B. G., Giaccia, A. J., Denko, N. C., and Brown, J. M. Loss of p21^{Waf1/Cip1} sensitizes tumors to radiation by an apoptosis-independent mechanism. *Cancer Res.*, *57*: 4703–4706, 1997.
32. Wang, Y., Perrault, A. R., and Iliakis, G. Down-regulation of DNA replication in extracts of camptothecin-treated cells: activation of an S-phase checkpoint? *Cancer Res.*, *57*: 1654–1659, 1997.
33. Wang, Y., Perrault, A. R., and Iliakis, G. Replication protein A as a potential regulator of DNA replication in cells exposed to hyperthermia. *Radiat. Res.*, *149*: 284–293, 1998.
34. Wang, Y., Zhou, X. Y., Wang, H., Huq, M. S., and Iliakis, G. Roles of replication protein A and DNA-dependent protein kinase in the regulation of DNA replication following DNA damage. *J. Biol. Chem.*, *274*: 22060–22064, 1999.
35. Iliakis, G., Wang, Y., and Wang, H. Y. Analysis of inhibition of DNA replication in irradiated cells using the SV40-based *in vitro* assay of DNA replication. *Methods Mol. Biol.*, *113*: 543–553, 1999.
36. Waldman, T., Kinzler, K. W., and Vogelstein, B. p21 is necessary for p53-mediated G₁ arrest in human cancer cells. *Cancer Res.*, *55*: 5187–5190, 1995.
37. Waldman, T., Lengauer, C., Kinzler, K. W., and Vogelstein, B. Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature (Lond.)*, *381*: 713–716, 1996.
38. Brown, J. P., Wei, W., and Sedivy, J. M. Bypass of senescence after disruption of p21^{CIP1/WAF1} gene in normal diploid human fibroblasts. *Science (Wash. DC)*, *277*: 831–834, 1997.
39. Chang, B. D., Xuan, Y., Broude, E. V., Zhu, H., Schott, B., Fang, J., and Roninson, I. B. Role of p53 and p21^{Waf1/Cip1} in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. *Oncogene*, *18*: 4808–4818, 1999.
40. Rowley, R., Phillips, E. N., and Schroeder, A. L. The effects of ionizing radiation on DNA synthesis in eukaryotic cells. *Int. J. Radiat. Biol.*, *75*: 267–283, 1999.
41. Olive, P. L. Inhibition of DNA synthesis by nitroheterocycles. I. Correlation with half-wave reduction potential. *Br. J. Cancer*, *40*: 89–93, 1979.
42. Olive, P. L. Inhibition of DNA synthesis by nitroheterocycles. II. Mechanisms of cytotoxicity. *Br. J. Cancer*, *40*: 94–104, 1979.
43. Treuner, K., Findeisen, M., Strausfeld, U., and Knippers, R. Phosphorylation of replication protein A middle subunit (RPA32) leads to a disassembly of the RPA heterotrimer. *J. Biol. Chem.*, *274*: 15556–15561, 1999.