

# Homologous Recombination Is the Principal Pathway for the Repair of DNA Damage Induced by Tirapazamine in Mammalian Cells

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## Abstract

**Tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide) is a promising hypoxia-selective cytotoxin that has shown significant activity in advanced clinical trials in combination with radiotherapy and cisplatin. The current study aimed to advance our understanding of tirapazamine-induced lesions and the pathways involved in their repair. We show that homologous recombination plays a critical role in repair of tirapazamine-induced damage because cells defective in homologous recombination proteins XRCC2, XRCC3, Rad51D, BRCA1, or BRCA2 are particularly sensitive to tirapazamine. Consistent with the involvement of homologous recombination repair, we observed extensive sister chromatid exchanges after treatment with tirapazamine. We also show that the nonhomologous end-joining pathway, which predominantly deals with frank double-strand breaks (DSB), is not involved in the repair of tirapazamine-induced DSBs. In addition, we show that tirapazamine preferentially kills mutants both with defects in XPF/ERCC1 (but not in other nucleotide excision repair factors) and with defects in base excision repair. Tirapazamine also induces DNA-protein cross-links, which include stable DNA-topoisomerase I cleavable complexes. We further show that  $\gamma$ H2AX, an indicator of DNA DSBs, is induced preferentially in cells in the S phase of the cell cycle. These observations lead us to an overall model of tirapazamine damage in which DNA single-strand breaks, base damage, and DNA-protein cross-links (including topoisomerase I and II cleavable complexes) produce stalling and collapse of replication forks, the resolution of which results in DSB intermediates, requiring homologous recombination and XPF/ERCC1 for their repair. [Cancer Res 2008;68(1):257–65]**

## Introduction

Tirapazamine (SR4233; 3-amino-1,2,4-benzotriazine-1,4-dioxide) is the first of a new class of anticancer agents that specifically kill

**Note:** J.W. Evans, S.B. Chernikova, and L.A. Kachnic contributed equally to this work.

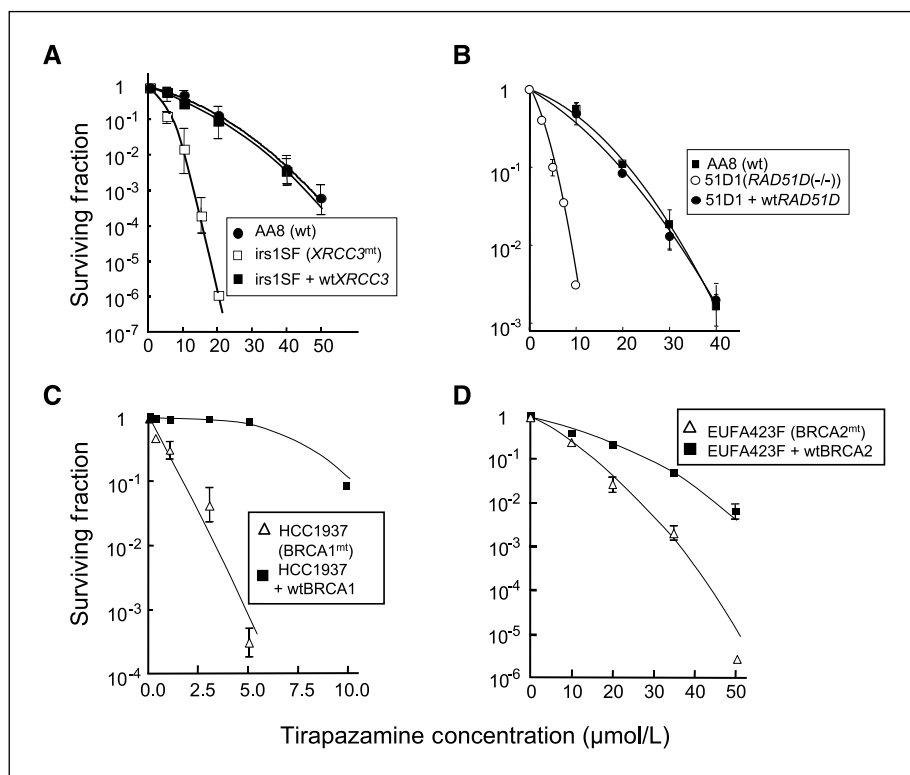
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the hypoxic cells of solid tumors to be tested clinically. Cells at very low oxygen concentrations are unique to solid tumors and confer resistance to standard radiotherapy and chemotherapy (1–3). Thus, a drug that selectively kills hypoxic cells would be expected to improve standard treatment of solid tumors by killing the most resistant cells in these tumors (4). Consistent with this, positive results have been reported in a multicenter, randomized phase III clinical trial combining tirapazamine with cisplatin (5), and promising results have been reported with tirapazamine in combination with chemoradiotherapy of head and neck cancer (6). Phase III clinical trials of tirapazamine in combination with chemotherapy and radiotherapy are ongoing.

The hypoxic selectivity of tirapazamine is the result of its intracellular one-electron reduction to a radical anion that is back oxidized to the parent nontoxic molecule by oxygen (7). In the absence of oxygen, however, the radical anion is converted either to a highly toxic hydroxyl radical (8) or to an oxidizing radical by the elimination of water (9), either of which would be sufficient to produce damage to intracellular macromolecules. We have previously shown that DNA double-strand breaks (DSB) are the major contributor to the cytotoxicity of tirapazamine under hypoxic conditions (10) and that these lesions seem to be formed not by the direct action of the oxidizing radical on DNA but rather by an indirect mechanism that, at least in some cell lines, is produced by the action of topoisomerase II (11). In yeast, DSBs generated by topoisomerase II poisons such as etoposide seem to be repaired primarily by a pathway of homologous recombination (12), but nonhomologous end joining (NHEJ) may also play a role (13). In contrast, the removal of topoisomerase II-mediated DSBs induced by etoposide in mammalian cells occurs predominantly by NHEJ (14). However, despite the fact that tirapazamine induces DSBs through topoisomerase II, it does not act like a classic topoisomerase II poison in that the cleavable complexes of topoisomerase II stabilized by tirapazamine are much more difficult to remove than the ones induced by etoposide (11). This is likely to be the result of different mechanisms by which such complexes are formed by tirapazamine and etoposide.

Using a genetic approach, we show in this study that different repair pathways, including homologous recombination, base excision repair (BER), and the XPF/ERCC1 factor of nucleotide excision repair (NER), but not NHEJ and NER *per se*, play a significant role in cell sensitivity to tirapazamine under hypoxia. Such a sensitivity profile of tirapazamine closely resembles that of agents producing DNA interstrand cross-links (15). Because



**Figure 1.** Cells defective in homologous recombination are hypersensitive to tirapazamine. *A*, survival of exponentially growing hamster XRCC3-mutant line irs1SF, wild-type hamster AA8, and wild-type XRCC3-corrected CXR3 cells after 1-h exposure to tirapazamine under hypoxia. *B*, survival of exponentially growing hamster Rad51D-mutant line 51D1, wild-type hamster AA8 cells, and wild-type Rad51D-corrected 51D1.3 cells after 1-h exposure to tirapazamine. *C*, survival of exponentially growing BRCA1-deficient HCC1937 cells and wild-type BRCA1-corrected cells after 1-h exposure to tirapazamine under hypoxia. *D*, survival of exponentially growing BRCA2-deficient EUFA423F and wild-type BRCA2-corrected EUFA423B cells after 1-h exposure to tirapazamine under hypoxia. Survival was measured by colony formation. Points, average from two (*B*) to four independent determinations; bars, SE (or range).

tirapazamine itself is not known to produce DNA interstrand cross-links, we therefore hypothesize that an important lesion, either directly induced by tirapazamine or produced during repair of tirapazamine-induced damage, is repaired by mechanisms similar to the repair of an interstrand cross-link intermediate. Our data support the hypothesis that tirapazamine induces DSBs as a result of replication forks stalled or collapsed at unrepaired base damage, single-strand breaks (SSB), or DNA-protein cross-links (including DNA-topoisomerase II complexes) and which require homologous recombination for their repair. These data emphasize the importance of the choice of patients who might be especially sensitive to tirapazamine. In particular, the striking sensitivity of BRCA-deficient cancer cells to tirapazamine has important implications for optimizing the systemic therapy of these tumors in the clinic.

## Materials and Methods

**Cell culture, plasmids, and transfection.** Rodent AA8, V79, UV41, UV5, UV135, EM9, UV20, Pol $\beta$ (+/+), and Pol $\beta$ (-/-) cell lines were purchased from the American Type Culture Collection (ATCC). Irs1 and irs1SF, as well as complemented cell lines CXR3, generated by the transfection of an expression vector containing wild-type XRCC3 into irs1SF cells, and H9T3, generated by the transfection of an expression vector containing wild-type XRCC3 into EM9 cells, were a generous gift from L.H. Thompson (Lawrence Livermore National Laboratory, Livermore, CA), as were 51D1 (a knockout of the *RAD51D* gene obtained by deletion of exon 4 in AA8 CHO cells) and 51D1.3 (a gene-complemented clone of 51D1 created by transfection of 51D1 cells with a bacterial artificial chromosome containing the complete hamster *RAD51D* gene; ref. 16). Mouse Ku80(-/-) cells were kindly provided by G.C. Li (Memorial Sloan-Kettering Cancer Center, New York, NY). Murine severe combined immunodeficient (SCID) and wild-type CB17 cells were obtained from C.U. Kirchgessner (Stanford University, Stanford, CA). Mag247 (XPC-deficient XP4PA-SV-EB fibroblasts) and mag 249 (XP4PA-SE2 cells created by transfection of mag247 cells with XPC cDNA containing plasmid pXPC3) were obtained from J.M. Ford (Stanford University,

Stanford, CA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator with 5% CO<sub>2</sub> at 37°C. WIL2NS lymphoblast cells (from Jeff Schwartz, University of Washington, Seattle, WA) were grown in RPMI medium containing 10% bovine calf serum.

Human BRCA1-deficient HCC1937 cells, derived from a primary ductal carcinoma of mammary gland arising in a BRCA1 carrier, were obtained from ATCC. HCC1937 cells were cultured in Iscove's medium supplemented with 20% FBS (Sigma) and maintained at 37°C in a humidified incubator with 7.5% CO<sub>2</sub> at 37°C. The pcDNA3 vector expressing hemagglutinin-tagged human wild-type BRCA1 was provided by D. Livingston (Dana-Farber Cancer Institute, Boston, MA). Human BRCA2-deficient EUFA423F cells were obtained from A. D'Andrea (Dana-Farber Cancer Institute, Boston, MA) and maintained in DMEM with 10% FBS and maintained in a humidified incubator with 5% CO<sub>2</sub> at 37°C. The hemagglutinin-tagged BRCA2 plasmid was obtained from Barbara Weber (University of Pennsylvania, Philadelphia, PA) and was transfected into EUFA423F cells to derive the BRCA2-complemented cell clone EUFA423B. Plasmid DNA transfections were done using LipofectAMINE-Plus (Life Technologies) following the manufacturer's instructions.

**Tirapazamine treatment.** Tirapazamine was obtained from Sanofi-Sythelabo. Tirapazamine solutions were freshly prepared before each experiment. For hypoxia treatment, cells were grown in notched glass dishes. On addition of tirapazamine, cells were placed in prewarmed hypoxic jigs, and the jigs were evacuated five times to 0.1 atm with a mixture of N<sub>2</sub> and 5% CO<sub>2</sub> under constant shaking to achieve hypoxia of <200 ppm O<sub>2</sub>. All hypoxia treatments were for 1 h at 37°C. Experiments with BRCA1- and BRCA2-deficient cells and the 51D1 cell lines were carried out in a hypoxic chamber. For WIL2NS lymphoblast cells, hypoxia was achieved with spinner flasks. Cells were incubated with tirapazamine for 30 min under aerobic or anoxic conditions. Flasks were pre-gassed for 30 min with 5% CO<sub>2</sub> in air or 5% CO<sub>2</sub> in N<sub>2</sub>. Cells were incubated with continuous gassing at a cell density of  $8 \times 10^5$ /mL (in 10 mL).

**Measurement of sister chromatid exchange levels.** Following treatments, cells were subcultured into medium containing 5'-bromo-2'-deoxyuridine (Sigma) at a final concentration of  $1 \times 10^{-5}$  mol/L for two

cycles (28 h), with colcemid (0.04  $\mu\text{g}/\text{mL}$ ) being added for the final 3 h. Cultures were harvested and metaphase spreads were prepared on microscope slides, which were then stained with Hoechst 33258 (5  $\mu\text{g}/\text{mL}$ ; Sigma) in  $2\times$  SSC buffer for 15 min at room temperature. Slides were then exposed to 366-nm UV light (UVGL-58 MINERALIGHT Lamp) for 25 to 30 min. Immediately after UV treatment, slides were incubated in  $2\times$  SSC at  $60^\circ\text{C}$  for 30 min. Slides were dried and stained with 5% Giemsa for 15 min. Metaphase spreads were viewed under bright-field microscopy and sister chromatid exchange (SCE) was scored each time a color switch occurred.

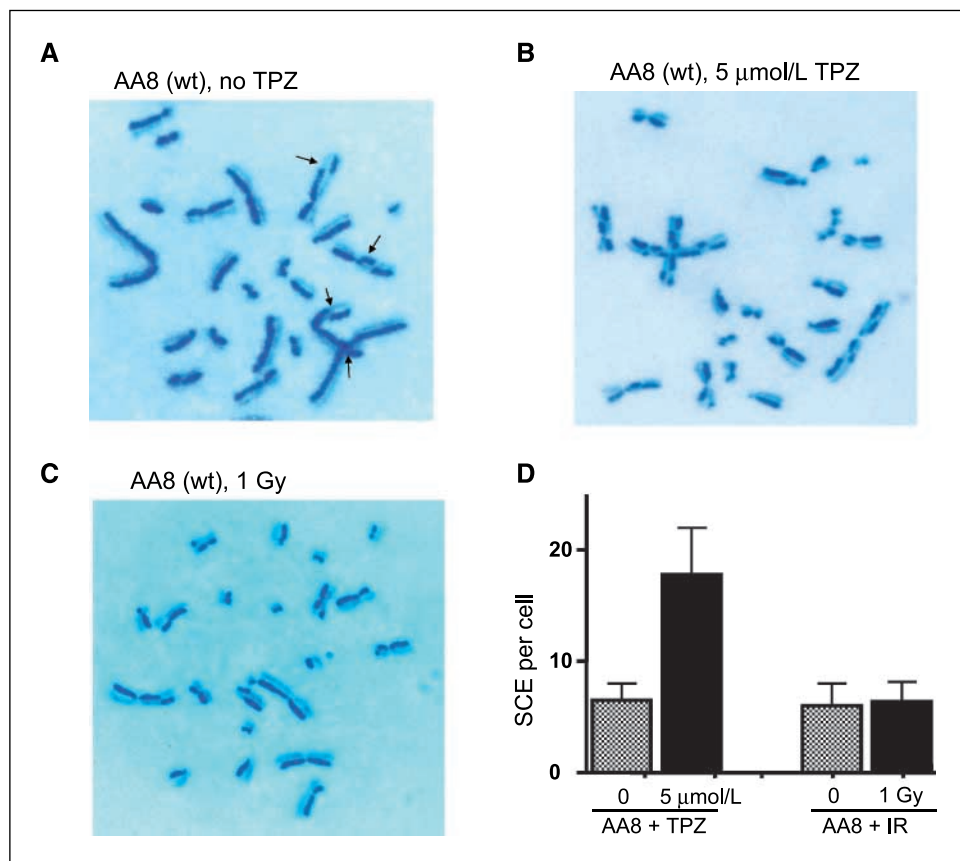
**Detection of base damage after tirapazamine treatment (comet assay).** After incubation, WIL2NS lymphoblast cells were centrifuged, washed, and resuspended in PBS, then embedded in 1% low gelling temperature agarose. Detection of base damage was done as previously described (17) by pipetting 100- $\mu\text{L}$  cell suspension containing 14,000 cells onto an agarose-precoated slide. Coverslips were placed over agarose, which was allowed to gel on an ice-cold block for 2 to 5 min. The coverslip was then removed and slides were submersed horizontally in the permeabilization lysis buffer solution containing 10 mmol/L Tris, 0.1 mol/L  $\text{Na}_2\text{EDTA}$ , 2.5 mol/L NaCl, and 1% Triton X-100 (pH 10). After 1 h, slides were rinsed free of salt and detergent in 10 mmol/L Tris-HCl containing 1 mmol/L EDTA and 100 mmol/L KCl (pH 7.5). Slides were then incubated in 60  $\mu\text{L}$  of the Tris buffer (pH 7.5) containing 85 ng of endonuclease III or 85 ng of formamidopyrimidine-glycosylase (supplied by S.S. Wallace, University of Vermont, Burlington, VT) or the combination of the two for 30 min at  $37^\circ\text{C}$  in a humidified box before proceeding with the alkali comet assay. Slides were placed in lysis solution containing 0.1% sarkosyl, 1.2 mol/L NaCl, and 0.03 mol/L NaOH for 45 min. Lysis was followed by rinsing in 0.03 mol/L NaOH, 2 mmol/L EDTA for  $2 \times 20$  min and then by electrophoresis at 0.6 V/cm for 20 min. After staining DNA with propidium iodide,  $\sim 150$  comet images were analyzed for DNA damage using in-house software. Tail moment was measured as the percentage of DNA in the tail and the distance between the means of the head and tail distributions.

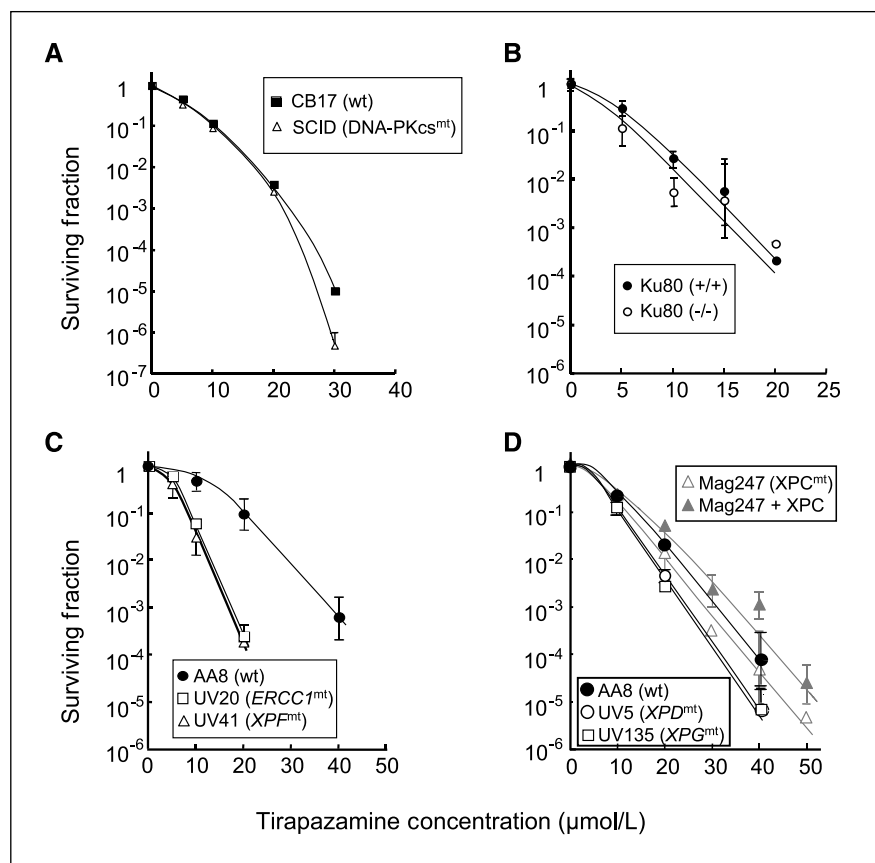
**Flow cytometry for  $\gamma\text{H2AX}$ .** One hour after treatment with tirapazamine, cells were fixed in 70% ethanol. Following washes with PBS and blocking with PBS containing 0.1% Tween 20, 1% bovine serum albumin, and 1% FBS, cells were incubated with FITC-conjugated anti- $\gamma\text{H2AX}$  antibody (Upstate) for 2 h at room temperature. Cells were stained in PBS containing 10  $\mu\text{g}/\text{mL}$  propidium iodide and 10 units/mL RNase A for at least 1 h and were analyzed on FACSCalibur (Becton Dickinson). The populations of  $\gamma\text{H2AX}$ -labeled cells were gated according to control histograms and the average  $\gamma\text{H2AX}$  antibody staining relative to the untreated controls was calculated based on geometric mean fluorescence.

**DNA-protein cross-link measurements.** DNA-protein cross-links were detected by the SDS/potassium precipitation method according to Zhitkovich and Costa (18). Briefly, cellular DNA was labeled with [ $^{14}\text{C}$ ]thymidine for 24 h and subsequently incubated in nonradioactive medium before tirapazamine treatment. After drug treatment, cells were washed and held at  $4^\circ\text{C}$  before processing for DNA-protein cross-links. The cell pellets ( $10^6$  cells) were then resuspended in 1 mL of PBS and were lysed by the addition of 0.5 mL of 2% SDS solution. The lysates were vortexed and heated at  $65^\circ\text{C}$  for 10 min and then mixed with 0.5 mL of precipitation buffer [20 mmol/L Tris (pH 7.5), 200 mmol/L KCl]. DNA was sheared by passing the lysates through a 21-gauge needle. Samples were placed on ice for 5 min to form potassium dodecyl sulfate precipitates. The precipitates were collected by centrifugation at  $6,000 \times g$  for 5 min at  $4^\circ\text{C}$ . After removal of supernatants, the pellets were resuspended in wash buffer [20 mmol/L Tris (pH 7.5), 100 mmol/L KCl], vortexed, and heated for 10 min at  $65^\circ\text{C}$ , and the washing steps as described above were repeated twice. The DNA-protein cross-link coefficient was calculated as the ratio of the percent of labeled DNA in the precipitated pellet of the treated sample divided by that of the untreated control sample.

**Detection of cellular topoisomerase I cleavage complexes.** Topoisomerase I cleavage complexes were detected using the *in vivo* complex of enzyme bioassay as described (19). Briefly, cells were lysed in 1% Sarkosyl

**Figure 2.** Tirapazamine induces high levels of SCEs. A, example of spontaneously induced SCEs in wild-type hamster AA8 cells. Arrows, SCE sites. B, tirapazamine (TPZ) significantly increases numbers of SCEs. C, ionizing radiation (IR) does not increase SCE levels above background (shown in A). D, quantitative comparison of SCE levels in AA8 cells treated with tirapazamine and ionizing radiation. Data from two independent experiments with 30 metaphase spreads counted per sample in each experiment.





**Figure 3.** Cells defective in NHEJ and NER, with exception of XPF/ERCC1, are not sensitive to tirapazamine. *A*, survival of exponentially growing murine SCID cells deficient in DNA-dependent protein kinase catalytic subunit (*DNA-PKcs*) and wild-type (*wt*) CB17 cells exposed to tirapazamine under hypoxia for 1 h. *B*, survival of exponentially growing Ku80(-/-) and Ku80(+/-) cells after 1-h exposure to tirapazamine under hypoxia. *C*, survival of exponentially growing UV20 and UV41 cells deficient in ERCC1 and XPF, respectively, and wild-type AA8 cells after 1-h exposure to tirapazamine under hypoxia. *D*, survival of exponentially growing UV5 cells deficient in XPD and wild-type AA8 cells after 1-h exposure to tirapazamine under hypoxia. Survival of exponentially growing mag247 cells deficient in XPC and wild-type XPC-corrected mag249 cells after 1-h exposure to tirapazamine under hypoxia. Survival was measured by colony formation. *Points*, average from three (with exception of mag247 and mag249 cells) and two (for mag247 and mag249 cells) experiments; *bars*, SE and range, respectively.

and homogenized with a Dounce homogenizer. The cell lysates were centrifuged on cesium chloride step gradients at  $165,000 \times g$  for 20 h at  $20^\circ\text{C}$ . Twenty 0.5-mL fractions were collected and diluted (vol/vol) into 25 mmol/L potassium phosphate buffer (pH 6.6). The DNA-containing fractions (fractions 7–11) were pooled and applied to polyvinylidene difluoride membranes (Immobilon-P, Millipore) using a slot-blot vacuum manifold. Topoisomerase I cleavage complexes were detected by immunoblotting with the C21 Top1 mouse monoclonal antibody (1:1,000 dilution; a kind gift from Dr. Yung-Chi Cheng, Yale University, New Haven, CT).

## Results

**Homologous recombination-defective mutants are sensitive to tirapazamine.** To ascertain the importance of homologous recombination for tirapazamine resistance, we tested a collection of mutants with known defects in homologous recombination for their sensitivity to tirapazamine under hypoxic conditions using clonogenic assay. *Irs1* and *irs1SF* are well-characterized hamster cell lines derived from V79 and AA8 CHO cells, respectively, which are defective in the Rad51 paralogues XRCC2 and XRCC3. In addition, we tested a recently characterized knockout of Rad51D in AA8 cell line (16). We treated these strains with various concentrations of tirapazamine under hypoxia for 1 h and compared their sensitivity to that of their parental cell lines and, in some cases, to the corrected versions of the strains that had been stably transfected with the cloned cDNA for that gene. *Irs1SF* cells had a 3.6-fold increase in sensitivity to tirapazamine (determined as the ratio of doses to give equal survival) compared with wild-type AA8 cells and *irs1SF* cells expressing a wild-type copy of the cDNA for XRCC3 (Fig. 1A). A similar increase in sensitivity to tirapazamine under hypoxia was observed for the Rad51D-knockout cell line

51D1 (Fig. 1B) and the XRCC2-deficient cell line *irs1* (data not shown). BRCA1 and BRCA2 proteins, mutations in which account for the majority of families with hereditary susceptibility to breast and ovarian cancers, play important roles in regulating homologous recombination (20). We tested the sensitivity to tirapazamine of the BRCA1-defective HCC1937 breast cancer cell line with and without correction by the cDNA for BRCA1 and observed a large increase in sensitivity to tirapazamine (by a factor of 6.1; Fig. 1C). BRCA2-deficient EUFA423 cells, derived from a patient with Fanconi anemia complementation group D1, also show an increased sensitivity to tirapazamine relative to a BRCA2-corrected clone although not to the same extent as the BRCA1-deficient cell line (Fig. 1D). Taken together, these data establish homologous recombination as an important determinant of tirapazamine cytotoxicity in mammalian cells.

**SCE levels are significantly elevated in tirapazamine-treated cells.** Homologous recombination has been suggested to be the principal mechanism responsible for SCE formation in vertebrate cells (21). In support of our data and conclusions from Fig. 1, we observed extensive SCE formation after 5  $\mu\text{mol/L}$  tirapazamine under hypoxia in AA8 cells (Fig. 2B and D). In contrast, ionizing radiation, the lesions of which are repaired largely by NHEJ, did not increase SCE levels at equivalent cell killing level of 1 Gy (Fig. 2C and D). As SCEs are the product of repair of DSBs by homologous recombination, the data from Figs. 1 and 2 provide good evidence that homologous recombination is involved in the repair of DSBs generated after exposure to tirapazamine under hypoxia.

**NHEJ mutants are not sensitive to tirapazamine.** The preferred mode for the repair of DSBs in mammalian cells is via NHEJ. To test if NHEJ is important for resistance to tirapazamine in

mammalian cells, we compared two different mouse cell lines, SCID and Ku80(-/-), to their wild-type counterparts. Both of these cell lines are defective in NHEJ and are hypersensitive to agents that kill cells by producing DNA DSBs, such as ionizing radiation. The murine SCID cell line has a well-characterized mutation in a DNA-dependent protein kinase catalytic subunit that significantly impairs DSB rejoining (22, 23), and Ku80(-/-) cells have a knockout mutation of the Ku80 subunit of the Ku70/Ku80 heterodimer that is responsible for the initial binding of the DNA-dependent protein kinase complex to the broken ends of the DNA (24). As shown in Fig. 3A and B, NHEJ plays little or no role in the repair of tirapazamine lesions induced under hypoxia.

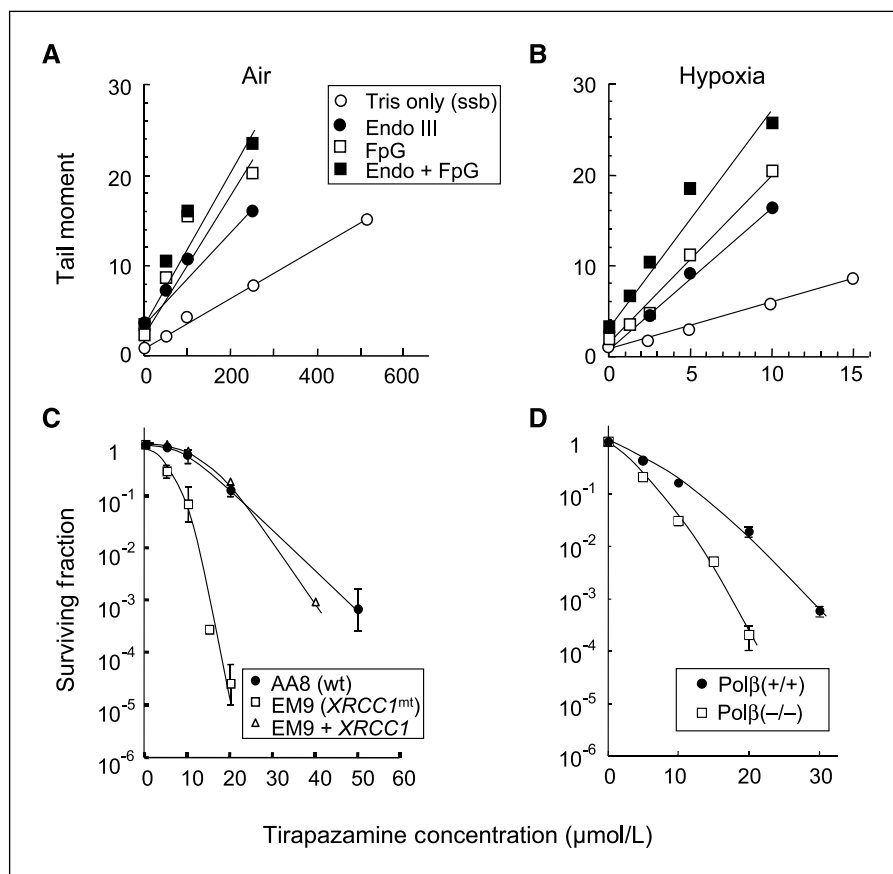
**The structure-specific endonuclease ERCC1-XPF, but not other NER factors, is required for resistance to tirapazamine.** NER is responsible for the removal of bulky lesions in DNA. The process involves ~30 proteins and consists of several steps, which include damage recognition and formation of an incision bubble by XPC-hHR23B, TFIIH (including XPB and XPD), XPA, RPA, and XPG; incision 3' and 5' to the lesion by XPG and XPF/ERCC1; and DNA repair synthesis and ligation mediated by Pol $\delta$  and Pol $\epsilon$ , proliferating cell nuclear antigen, replication factor C, and DNA ligase I. Previous observations in hamster and yeast cells have shown that XPF-ERCC1 has important functions outside of NER, participating in interstrand cross-link repair and homologous recombination (25, 26). Similarly to interstrand cross-link, repair of tirapazamine-induced damage required the non-NER function of XPF/ERCC1. Figure 3C shows that UV41 and UV20 hamster cell lines, deficient in XPF and ERCC1, respectively, were ~2.5-fold more sensitive to tirapazamine than the parental wild-type AA8 cell line, whereas

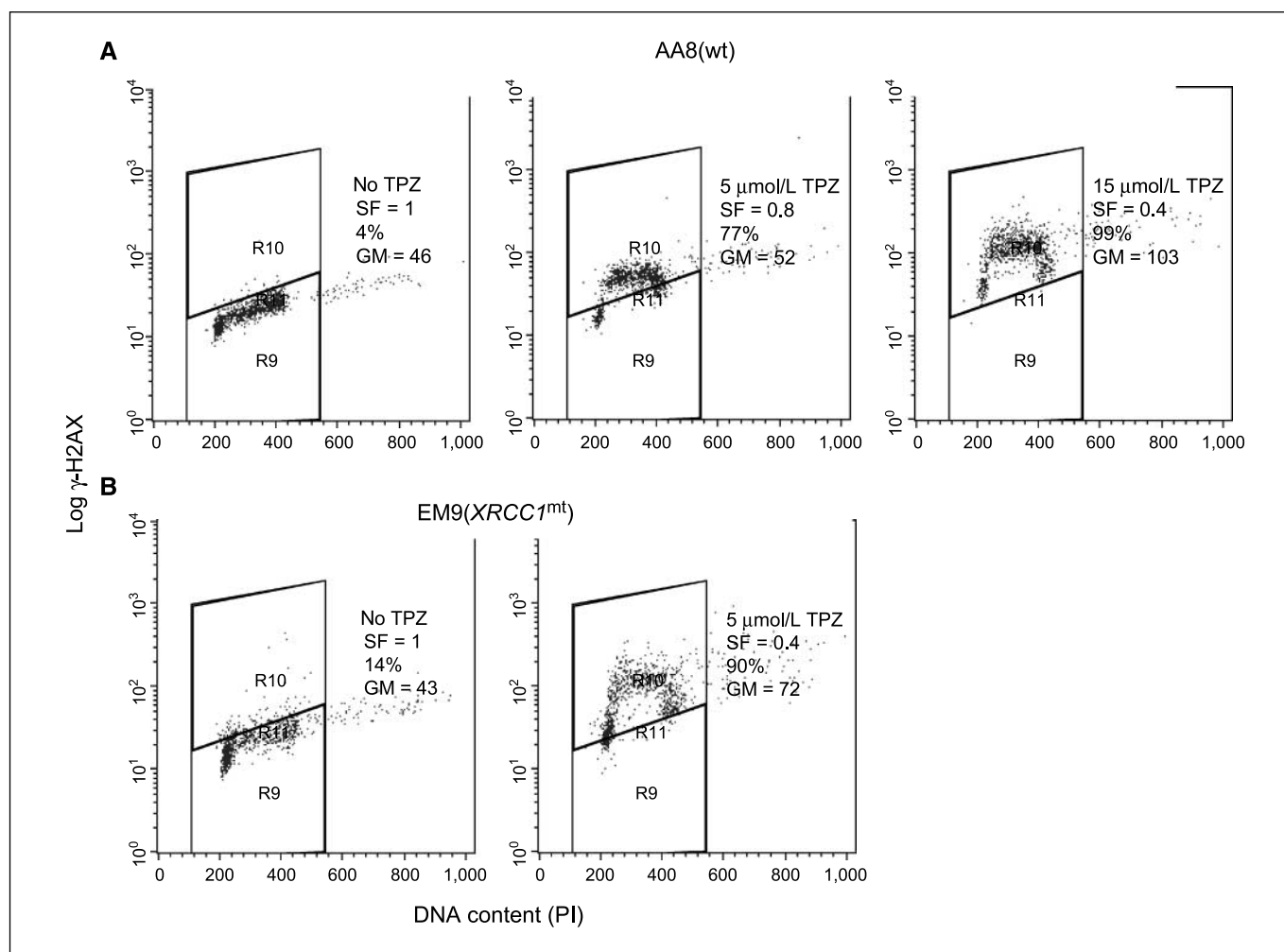
mutants defective in other NER factors, XPD (UV5 cells), XPG (UV135 cells), and XPC (mag247 cell line), showed tirapazamine sensitivity similar to that of wild-type AA8 cells (Fig. 3D) or the wild-type XPC complemented cell line mag249 (Fig. 3D).

**BER is involved in the repair of tirapazamine-induced damage.** Under hypoxic conditions, tirapazamine undergoes one-electron reduction by nuclear reductases, yielding highly reactive radicals producing DNA strand breaks and base damage lesions (27, 28). Normally, hypoxic conditions do not promote radical-mediated breakage of DNA because oxygen is required for the conversion of DNA radicals into strand breaks. However, tirapazamine and its metabolites have been shown to produce oxygenation of these radical sites leading to DNA breaks (29). In addition to DNA strand breaks, tirapazamine under hypoxic conditions has been reported to generate oxidative DNA base damage that is dominated by formamidopyrimidine and 5-hydroxy-6-hydropyrimidine lesions (30). These lesions would be expected to block DNA transcription and replication and thus contribute to the cytotoxicity of the drug (30). In addition, BER intermediates can be substrates for topoisomerase II and poison the enzyme to produce double-strand breaks (31). Consistent with this, we have shown that tirapazamine is a topoisomerase II poison (11).

To quantitatively assess the amount of DNA base damage induced by tirapazamine, we used the alkaline comet assay to detect DNA strand breaks after treatment with endonuclease III and formamidopyrimidine-glycosylase, enzymes known to create DNA strand breaks at enzyme-sensitive sites. Figure 4A and B shows a dose-dependent increase of DNA breaks and base damage after tirapazamine treatment, with >20-fold higher levels of

**Figure 4.** BER plays a significant role in the removal of tirapazamine-induced damage. Tirapazamine-induced base damage under normoxic (A) and hypoxic (B) conditions was measured by treating agarose-embedded cells with endonuclease III (*Endo III*) and formamidopyrimidine-glycosylase (*FpG*), followed by the alkaline comet assay to detect DNA strand breaks. Representative of two independent experiments. C, survival of exponentially growing EM9 hamster cells deficient in XRCC1, EM9 cells corrected with wild-type XRCC1, and wild-type AA8 cells after 1-h exposure to tirapazamine under hypoxia. D, survival of exponentially growing murine Pol $\beta$ (-/-) and Pol $\beta$ (+/+) cells after 1-h exposure to tirapazamine under hypoxia. Survival was measured by colony formation. Points, average from three experiments; bars, SE.





**Figure 5.** Flow cytometry analysis of AA8 (A) and EM9 (B) cells stained for DNA and  $\gamma$ H2AX 1 h after treatment with tirapazamine for 1 h under hypoxia. Note the apparent greater sensitivity of S-phase cells and the increased  $\gamma$ H2AX formation in EM9 cells. The upper quadrangle (R10) in each dot-plot was drawn to encompass <5% of cells of the untreated population, and the percentage of cells within this gate is shown for each dose of tirapazamine. SF, surviving fraction; GM, geometric mean of the  $\gamma$ H2AX signal in the R10 quadrangle. Representative data of two independent experiments.

tirapazamine under normoxia required to achieve the same levels of damage as under hypoxia.

To determine the role in tirapazamine resistance of the BER pathway that deals mainly with base damage and SSB, we used the EM9 hamster cell line, deficient in the *XRCC1* gene, and a mouse Pol $\beta$ ( $-/-$ ) knockout cell line. In the main BER pathway, involving short-patch repair, Pol $\beta$  polymerase incorporates a single nucleotide and removes the abasic site using its AP lyase activity. XRCC1 has a broader role in BER, acting in both short-patch and long-patch BER. XRCC1 coordinates BER through multiple protein-protein interactions stabilizing DNA ligase III, directly detecting breaks, coordinating the binding and polymerase activity of Pol $\beta$ , promoting an S phase-specific mode of SSB repair, and regulating poly(ADP-ribose) polymerase (reviewed in ref. 32). EM9 cells have a frameshift mutation in the *XRCC1* gene that results in truncated polypeptide lacking two thirds of the normal sequence, thus leading to an effectively knockout phenotype of the EM9 cell line. As shown in Fig. 4C, EM9 cell line has a 3-fold increase in sensitivity to tirapazamine compared with the wild-type AA8 strain of Chinese hamster ovary (CHO) cells, from which EM9 clone had originated, and with the H9T3 cell line, which has the cDNA for

XRCC1 transfected into the EM9 cell line. In addition, Pol $\beta$ ( $-/-$ ) cells showed significantly increased (1.7-fold) sensitivity to tirapazamine compared with BER-proficient Pol $\beta$ ( $+/+$ ) (Fig. 4D). These results indicate that the BER pathway plays an important role in the repair of tirapazamine-induced damage.

**Assessment of H2AX phosphorylation induced by tirapazamine.** Phosphorylation of histone H2AX ( $\gamma$ H2AX) has been identified as an early event after the production of DSBs.  $\gamma$ H2AX is detected on Ser<sup>139</sup> at sites flanking DNA DSBs generated either by exogenous stimuli or normally during V(D)J recombination and class-switch recombination, as well as in association with DNA replication.  $\gamma$ H2AX immunofluorescence measured by flow cytometry has been proposed as a surrogate for cell killing by drugs that create DSBs (including tirapazamine; ref. 33). Confirming the data obtained in ref. 33, we found that  $\gamma$ H2AX levels were significantly higher in S-phase cells after tirapazamine treatment (Fig. 5A). The increase in  $\gamma$ H2AX immunofluorescence was dose dependent, and although at higher doses of tirapazamine (15  $\mu$ mol/L) most of the cells were  $\gamma$ H2AX positive, the S-phase cells had the most pronounced increase in the  $\gamma$ H2AX signal (Fig. 5A). Moreover, failure to repair base damage and SSBs resulted in significantly

increased  $\gamma$ H2AX levels in the EM9 mutant of *XRCC1* after 5  $\mu$ mol/L tirapazamine (Fig. 5B), which were similar to the  $\gamma$ H2AX levels in AA8 cells treated with 15  $\mu$ mol/L tirapazamine, a dose that produces the equivalent cell kill (Fig. 5A).

**DNA-protein cross-links.** To determine whether tirapazamine treatment of cells under hypoxia produces DNA-protein cross-links, we used the SDS-potassium precipitation assay following a 1-h exposure to tirapazamine. We found a clear increase of DNA-protein cross-link levels with increasing dose of tirapazamine (Fig. 6A). However, higher levels of tirapazamine were needed to detect a significant increase in DNA-protein cross-links compared with a similar increase of base damage, probably reflecting the low sensitivity of the SDS-potassium assay. The high reactivity of the tirapazamine radical under hypoxia and the selective reduction of tirapazamine by nuclear matrix reductases (27) suggest that proteins cross-linked to DNA must be in close proximity to DNA. Consistent with this, we found earlier that tirapazamine induces irreversible DNA-topoisomerase II cleavable complexes (11) as well as DNA complexes with other nuclear matrix proteins such as nuclear lamin and NUMA1 (data not shown). Importantly, we report here that exposure to tirapazamine under hypoxia results in the formation of DNA-topoisomerase I cleavage complexes (Fig. 6B). Similarly to DNA-topoisomerase II cleavable complexes, DNA-topoisomerase I cleavage complexes are very stable and, in contrast to DNA-topoisomerase I cleavage complexes induced by camptothecin, do not reverse after 3-h incubation in tirapazamine-free medium (Fig. 6B).

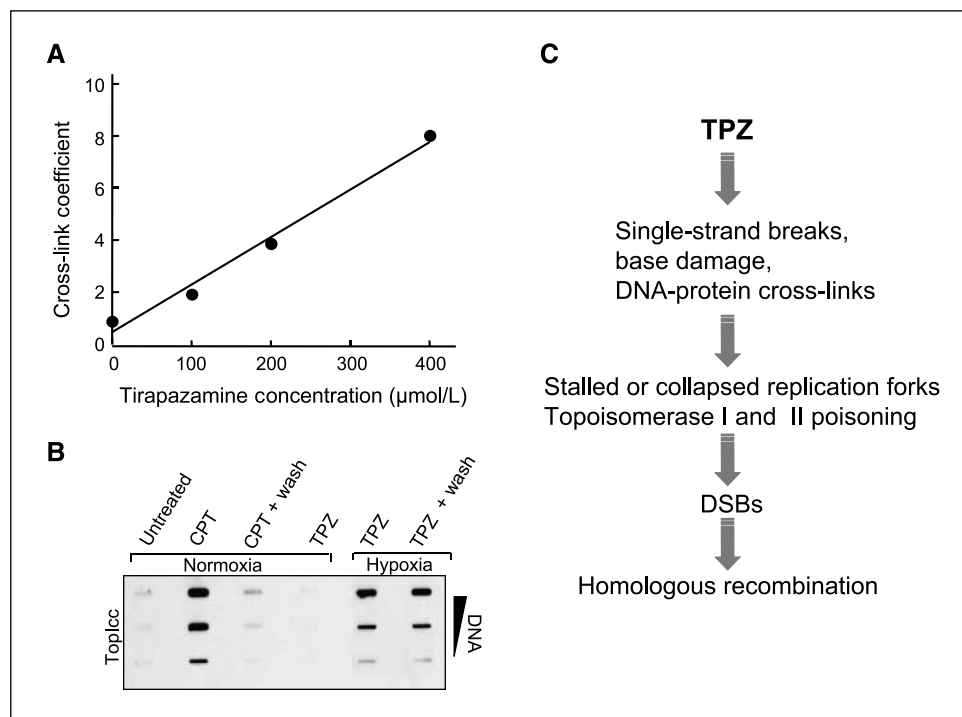
## Discussion

In a recent genome-wide study in *S. cerevisiae*, DNA repair proteins involved in homologous recombination were identified as playing a significant role in protecting against tirapazamine-induced damage (34). However, as homologous recombination is

the main pathway for repair of DSBs in yeast (35) whereas NHEJ is the main pathway in mammalian cells (36), the results of this screen in yeast do not necessarily imply that homologous recombination would be the important repair pathway for the DSBs induced by tirapazamine under hypoxic conditions. Nonetheless, we found that homologous recombination plays a major role in repairing tirapazamine-induced damage in both hamster and human cells. In particular, we show in this study that hamster cells defective in the Rad51 paralogues, Rad51D, XRCC2, and XRCC3, and human cells deficient in BRCA1 and BRCA2, all defective in homologous recombination, are significantly more sensitive to tirapazamine than their normal counterparts (Fig. 1). Consistent with this involvement of homologous recombination in processing tirapazamine-induced damage, we also observed a high incidence of SCEs after tirapazamine treatment (Fig. 2). On the other hand, the NHEJ pathway, which we interrogated using mouse SCID and Ku80(−/−) cells, does not protect cells against tirapazamine-induced damage (Fig. 3A and B). This finding of the importance of homologous recombination in the repair of tirapazamine damage is consistent with our earlier finding that although tirapazamine induces DNA DSBs, these are not formed directly by radical damage to DNA but indirectly by producing cleavable complexes between topoisomerase II and DNA (11). Our finding of the high sensitivity of cells deficient in BRCA1 and BRCA2, proteins involved in homologous recombination, also suggests that tirapazamine might be particularly effective in the therapy of homologous recombination-defective tumors. For breast cancer patients carrying mutations in *BRCA1* and *BRCA2* genes, the use of tirapazamine in neoadjuvant therapy when breast cancers are hypoxic (37) may result in a substantial improvement in response.

The phenotype that characterizes homologous recombination-defective mutants is their extreme sensitivity to DNA interstrand cross-linking agents. The hamster cell lines *irs1*, *irs1SE*, and *51D1* (defective in *XRCC2*, *XRCC3*, and *Rad51D*, respectively) plus

**Figure 6.** A, tirapazamine treatment under hypoxia induces DNA-protein cross-links. DNA-protein cross-link formation after 1-h tirapazamine treatment under hypoxia was measured by the SDS-potassium precipitation method in wild-type AA8 cells. Points, average values from two experiments. B, tirapazamine induces irreversible topoisomerase I cleavage complexes (*Top1cc*) under hypoxia. HeLa cells were treated with tirapazamine (100  $\mu$ mol/L, 1 h). The DNA-containing fractions were pooled and probed at three concentrations (10, 3, and 1  $\mu$ g DNA) with an antibody against Top1. The topoisomerase I cleavage complex induced by tirapazamine did not reverse after washing and incubating the cells in tirapazamine-free medium for 3 h (TPZ + wash). Camptothecin (CPT; 10  $\mu$ mol/L, 1 h) was used as a positive control for the induction of reversible topoisomerase I cleavage complex. C, model explaining why tirapazamine damage repair proceeds via homologous recombination pathway.



BRCA1- and BRCA2-deficient cells all show this marked sensitivity to the DNA cross-linking agent mitomycin C (16, 38). In addition to homologous recombination, however, several lines of evidence suggest that the repair of interstrand cross-links requires an incisional step 5' to the lesion that involves the XPF-ERCC1 component of NER (25, 39, 40). Significantly, our data also show that tirapazamine resistance involves the function of the XPF-ERCC1 complex but not of other components of the NER pathway, as XPD-, XPG-, and XPC-deficient cells are not sensitive to tirapazamine (Fig. 3C and D). Thus, the combination of enzymes involved in the repair of tirapazamine-induced damage closely resembles the one involved in the repair of DNA interstrand cross-links (41) despite the fact that tirapazamine is not known to produce interstrand cross-links.

Recently, it was reported that BRCA1- and BRCA2-deficient cells are highly sensitive to poly(ADP-ribose) polymerase inhibitors (42, 43). The authors propose that the mechanism for this sensitivity to poly(ADP-ribose) polymerase inhibitors is by inhibition of repair of SSBs produced during the removal of endogenously generated base damage. During the S phase of the cell cycle, these unrepaired SSBs are converted to DSBs, which then require the homologous recombination pathway to restart the stalled replication forks. Consistent with this model, Saleh-Gohari et al. (44) have recently shown that XRCC1-deficient EM9 cells have elevated levels of spontaneously formed DSBs and RAD51 foci, again suggesting that stalled replication forks convert SSBs into DSBs that require homologous recombination for their repair. Thus, it seems probable that some of the DSBs produced after treatment with tirapazamine have a similar origin, as we show (Fig. 4C and D) that cells deficient in EM9 and Pol $\beta$ (-/-) cells are significantly more sensitive to tirapazamine than their parental or cDNA-corrected cells and that tirapazamine induces S phase-specific phosphorylation of H2AX, which is especially high in EM9 cells (Fig. 5).

What tirapazamine-induced damage would require a DNA interstrand cross-link-like mechanism of processing? As we show in this study, tirapazamine produces DNA-protein cross-links (Fig. 6A and B) in addition to SSBs and base damage. These DNA-protein cross-links could be formed by reaction of radical-damaged DNA with nearby proteins or by attempts to repair tirapazamine-induced base damage producing DNA-Pol $\beta$  cross-links (45) or stable topoisomerase II-DNA complexes (11, 30, 31). The mechanism of repair of DNA-protein cross-links is not understood, but because many DNA-protein cross-link-inducing agents also generate DNA interstrand cross-links, it has been proposed that both of these types of lesions may be repaired by the same pathway or using

common elements. XPF/ERCC1, for example, seems to be involved in the repair of both DNA-protein cross-links (46, 47) and DNA interstrand cross-links (25, 39, 40).

Based on the results obtained in this study, we propose a model that explains why tirapazamine induces DSBs, yet the repair of the damage proceeds via pathways involved in the repair of SSBs, base damage, and interstrand cross-links. This model is shown in Fig. 6C. Essentially, we propose that the lesions induced by tirapazamine, comprising SSBs (48), base damage (ref. 28; Fig. 4), and DNA-protein cross-links (ref. 11; Fig. 6), produce DNA DSBs when a replication fork encounters them, and these DSBs require homologous recombination for their repair.

The model accounts for the following observations: (a) tirapazamine-induced damage repair depends on XPF/ERCC1, homologous recombination, and BER, but not on NER and NHEJ (which acts on frank DSBs); (b) topoisomerase II-DNA complexes are formed after tirapazamine treatment, which are more stable than those produced by etoposide (11); (c) tirapazamine is a potent inhibitor of DNA replication (49); and (d)  $\gamma$ H2AX levels in tirapazamine-treated cells are significantly higher in S phase, which is not the case for cells treated with etoposide, doxorubicin, bleomycin, or ionizing radiation (ref. 33; Fig. 5). Importantly, this model also provides a ready explanation for the marked time-dependent synergism between tirapazamine and cisplatin (50, 51): the initial tirapazamine treatment would be expected to sequester repair factors, such as XPF-ERCC1, needed for the repair of cisplatin-induced intrastrand and interstrand DNA cross-links.

In summary, we have shown that repair of the damage induced by the hypoxic cytotoxin tirapazamine requires the function of multiple DNA repair enzymes, including those involved in homologous recombination, BER, and the XPF/ERCC1 complex, but not NHEJ and NER *per se*. The involvement of these enzymes fits into a pathway that accounts for the known phenomena associated with this drug and has implications for optimizing the systemic therapy of tumors with defects in those pathways.

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