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Amifostine Metabolite WR-1065 Disrupts Homologous Recombination in Mammalian Cells

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Abstract

Repair of DNA damage through homologous recombination (HR) pathways plays a crucial role in maintaining genome stability. However, overstimulation of HR pathways in response to genotoxic stress may abnormally elevate recombination frequencies, leading to increased mutation rates and delayed genomic instability. Radiation-induced genomic instability has been detected after exposure to both low- and high-linear energy transfer (LET) radiations, but the mechanisms responsible for initiating or propagating genomic instability are not known. We have demonstrated that WR-1065, the active metabolite of amifostine, protects against radiation-induced cell killing and delayed genomic instability. We hypothesize that hyperstimulation of HR pathways plays a mechanistic role in radiation-induced genomic instability and that, in part, WR-1065 exerts it radioprotective effect through suppression of the HR pathway. Results of this study demonstrate that WR-1065 treatment selectively protected against radiation-induced cell killing in HR-proficient cell lines compared to an HR-deficient cell line. Further, WR-1065 treatment decreases HR in response to DNA damage using two different mammalian cell systems. This suppression of hyper-recombination is a previously unrecognized mechanism by which WR-1065 effects radioprotection in mammalian cells.

INTRODUCTION

One of the major concerns regarding the use of radioprotective agents is that, while they protect cells from the acute effects of radiation, the surviving cells and their progeny are at an increased risk for delayed radiation-induced genomic instability. Genomic instability is defined as the increased rate of alterations to the genome, including gene mutations, chromosomal abnormalities, micronucleus formation, reduced plating efficiency and cellular transformation. Genomic instability has been detected after both low- and high-linear energy transfer (LET) radiation exposure (1). The severity of the effect depends on a variety of factors including genetics and radiation quality (LET, dose, dose rate). Currently, the mechanisms responsible for initiating and perpetuating delayed genomic instability are unknown; however, several pathways have been suggested (2,3). We hypothesize that overstimulation of the homologous recombination (HR) DNA double-strand repair pathway leads to hyper-recombination and increased genomic instability in irradiated cells (4).

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We developed and characterized a novel green fluorescence protein (GFP) reporter assay for investigating delayed effects of exposure to ionizing radiation as measured by deletion/mutation events and/or homologous recombination in human cells (4). In this model system a recombination or mutation event in single genetically unstable GFP^+ or GFP^- cells can result in a mixture of green and clear cells within the same colony during clonal expansion ($GFP^{+/-}$). This experimental system was used to measure delayed genomic instability induced by exposure to low-LET X rays (4), high-LET iron ions (5), or UV radiation (6). Results of these studies demonstrated that WR-1065, the active metabolite of amifostine, decreased genetic instability in the progeny of irradiated cells (5).

Amifostine and its dephosphorylated metabolite WR-1065 can protect against the immediate and delayed effects of radiation exposure. Immediate radioprotective effects of WR-1065 include free radical scavenging, auto-oxidation leading to intracellular hypoxia, and chemical repair by donating hydrogen to radiation-damaged DNA (7,8). Delayed radioprotective effects of WR-1065 include up-regulation of manganese superoxide dismutase (MnSOD) protein levels and activity, resulting in free radical scavenging (9,10), and stimulation of endogenous polyamine levels stabilizing chromatin to facilitate DNA repair (11).

However, other WR-1065-mediated mechanisms of protection against genomic instability cannot be ruled out. Pathways other than free radical/reactive oxygen species scavenging could be especially important for protection from high-LET radiations such as those commonly encountered by astronauts in space. DNA damage after high-LET irradiation is caused primarily by direct interactions between high-*Z* and -energy (HZE) charged particles and DNA, not indirectly through production of hydroxyl free radicals. These differences in radiation quality mean that amifostine/WR-1065 is not as effective in protecting against high-LET radiation-induced cellular damage as it is against low-LET radiation effects. Nonetheless, WR-1065 is still capable of significantly reducing the delayed effects of high-LET radiation exposure, including genomic instability as measured by DNA hyper-recombination/mutation (5).

In this study we investigated the influence of WR-1065 on homologous recombination in mammalian cells and the possible role of HR in the radioprotective activity of WR-1065. Using two different mammalian experimental systems, we demonstrate that WR-1065 treatment decreases the frequency of DNA damage-induced homologous recombination, thereby protecting cells from the potentially negative effects of hyper-recombination.

MATERIALS AND METHODS

Cell Culture

The S31WT clone of human 46BR.1G1 cells harboring SCneo construct and corrected for ligase 1 deficiency was kindly provided by Dr. A. Tomkinson (University of Maryland, Baltimore) and was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 0.2 mg/ml of hygromycin and 0.67 μg/ml puromycin (12). Cells of the MCF10A apparently normal human breast epithelial cell line were maintained in DMEM/F12 medium containing 5% horse serum, 20 ng/ml EGF, 1 μg/ml cholera toxin, 10 μg/ml insulin and 10 μg/ml hydrocortisone (13). The SPD8 cell line is a derivative of the V79 Chinese hamster lung cell line. The AA8 (wild-type), irs1SF (Xrcc3-deficient) and CXR3 (Xrcc3-restored) cell lines are Chinese hamster ovary (CHO) derivatives. All four Chinese hamster cell lines were generously provided by Dr. T. Helleday (University of Oxford, UK) and were maintained in DMEM containing 10% FBS and penicillin-streptomycin (100 μg/ml and 100 U/ml, respectively) (14,15). For the SPD8 cells, the medium was supplemented with 6-thioguanine (5 μg/ml) to minimize the frequency of spontaneous mutation reversion prior to treatment (16). All cells were kept at 37°C in a 5% CO_2/95% air incubator. The cells were

routinely tested for mycoplasma (Bionique, Saranac Lake, NY) and showed no evidence of infection.

WR-1065 Treatment

WR-1065 was obtained from Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Institutes of Health (Bethesda, MD). A 1 *M* stock solution was prepared in sterile phosphate-buffered saline (PBS) immediately before use. For the short-term WR-1065 treatment, cells were exposed to 4 m*M* final concentration WR-1065 for 30 min, and then the drug-containing medium was replaced with fresh medium immediately before drug, restriction enzyme or radiation treatment. For the low-dose WR-1065 treatment, cells were treated continuously with 40 μ*M* final concentration WR-1065 for 24 h before drug, restriction enzyme or radiation treatment. For each treatment protocol, control samples were treated with drug vehicle for the same duration.

Irradiation

Cells growing in monolayer cultures were exposed to radiation at ambient temperature. Control samples were sham-treated according to the same protocol, without irradiation. X radiation was delivered using a Pantak HF320 X-ray machine (250 kV peak, 13 mA; half-value layer, 1.65 mm copper) at a dose rate of 2.4 Gy/min (Radiation Oncology Research Laboratory, University of Maryland).

Homologous Recombination in SPD8 Hamster Cells

The assay was conducted according to procedures described previously for SPD8 cells (14). Camptothecin (CPT) was prepared as a 20 m*M* stock solution in dimethyl sulfoxide (DMSO) and stored at −20°C. Hydroxyurea (HU) was dissolved in sterile PBS immediately before use. Briefly, 1×10^6 cells were treated with 100 n*M* CPT for 1 h or 0.2 m*M* HU for 24 h, rinsed three times with PBS, and allowed to recover in fresh medium for 24–48 h. Surviving cells were then trypsinized, counted and plated (0.3×10^6 per plate, in triplicate) in hypoxanthine/aminopterin/thymidine (HAT) selective medium. In addition, two dishes were plated to obtain plating efficiency of the drug-treated cells (200 cells/plate). Colonies were fixed and stained after 7–10 days using 0.1% crystal violet in 20% ethanol. All experiments were repeated independently three times.

Homologous Recombination in S31WT Human Cells

To induce HR, S31WT cells (1×10^6) were transiently transfected with the pCMV3xnlsI-*Sce*I expression vector (30 ng) using Lipofectamine2000 as described previously (17). To test for WR-1065 protection against HR, cells were preincubated with WR-1065, 4 m*M* for 30 min or 40 μ*M* for 24 h prior to transient transfection. At the end of the 6-h transfection, plates were rinsed three times with PBS and allowed to recover for 48 h in fresh DMEM. Surviving cells were then trypsinized, counted and plated (0.3×10^6 per plate, in triplicate) in 1 mg/ml G-418 selective medium. In addition, two dishes were plated to obtain plating efficiency for the transfected cells (200 cells/plate). Cells were allowed to grow for 14–17 days before staining with 0.1% crystal violet in 20% ethanol. All experiments were repeated independently three times.

Reactive Oxygen Species Levels

Changes in cellular reactive oxygen species (ROS) and/or reactive free radical levels for the S31WT cells after transient transfection with the pCMV3xnlsI-*Sce*I expression vector and WR-1065 treatment were measured using the oxidation-specific probe 5′,6′-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) and flow cytometry techniques as described previously (18). Cells (1×10^6 per ml) were incubated with freshly prepared 5 μ*M*

CM-H2DCFDA for 30 min, washed and analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Dead cells were excluded from analysis by propidium iodide staining.

In Vitro Digestion of SCneo Plasmid with I-SceI Restriction Enzyme

The SCneo plasmid (19) (1 μg) was incubated with 1 U of I-*Sce*I (Fermentas) in the manufacturer recommended buffer for 16 h at 37°C. WR-1065 (0.1–10 m*M*) was added to samples 5 min before the enzyme was added and remained present during incubation. After digestion the plasmid was purified by standard phenol/chloroform extraction and ethanol precipitation. Recovered DNA was resolved on 1% agarose gel, stained with ethidium bromide, photographed and analyzed for topologic form distribution (SC, supercoiled circular; OC, open circular; L, linear).

RNA Interference

The siRNA (siGenome SMARTpool) anti-Rad51, -Rad51C, -Xrcc3 and control siRNA (siGenome Non-Targeting siRNA pool #2) were obtained from Dharmacon (Lake Placid, NY). MCF-10A cells were transfected with 20 n*M* siRNA using Oligofectamine according to the manufacturer's instructions. Target protein expression levels were measured 48–72 h after transfection by SDS-PAGE and Western blotting of whole cell extracts (20). The antibodies for Rad51, Rad51C and Xrcc3 were obtained from Upstate Biotechnology (Lake Placid, NY); the antibody for β-actin (mouse monoclonal) and the HRP-conjugated secondary antibodies were from Sigma.

Clonogenic Cell Survival Assay

Cells were treated with WR-1065 or sham-treated and irradiated with X rays as required for each experiment. After irradiation, cells were harvested by trypsinization, resuspended in fresh culture medium, and replated into 100-mm-diameter culture dishes at densities calculated to yield 50–100 cell colonies per dish. After 10–14 days of incubation, colonies were fixed, stained with 0.1% crystal violet in 20% ethanol, and counted (>50 cells/colony). The plating efficiency of controls was calculated as the number of colonies obtained divided by the number of plated cells. The surviving fraction was defined as the number of colonies obtained divided by the number of cells plated multiplied by the plating efficiency.

Cytotoxicity Assay

The cells were seeded in 60-mm-diameter dishes at 100 cells per dish and allowed to attach. Then WR-1065 was added to final concentrations ranging between 0 and 10 m*M*. For some dishes, the drug-containing medium was replaced with fresh, drug-free medium after 30 min. There were no changes in SPD8 or MCF10A cell morphology or attachment as a result of the 30-min drug treatment or medium replacement. After 7–10 days of incubation, surviving colonies were fixed, stained and counted. Surviving fractions for each drug treatment were calculated as described above, and data points were fitted to modified Hill's logistic equation to calculate EC_{50} concentrations (21).

Statistical Analysis

Means and standard errors were calculated for all data from two to three independent experiments. The means were compared between WR-1065-treated and untreated samples by one-way ANOVA.

RESULTS

WR-1065 Reduces Homologous Recombination Frequencies in SPD8 Cells Treated with HU or CPT

We tested the hypothesis that protection against genomic instability by WR-1065 is related at least in part to its ability to reduce the frequency of HR in response to DNA damage using the SPD8 Chinese hamster cell line (14,16). In these cells HR between the 5-kb tandem repeats restores the functional *HPRT* gene that can be selected for in HAT medium (16). HR was induced by treatment with 0.2 m*M* HU for 24 h or 100 n*M* CPT for 1 h, and the frequency of recombination events was assessed. These drug concentrations were selected to induce maximal HR at an acceptable level of cytotoxicity. Surviving fractions for 0.2 m*M* HU and 100 n*M* CPT were ~60% and 30%, respectively (14,17). Both HU and CPT treatments increased the HR frequency to 12–15 times the HR frequency of controls (Fig. 1). Preincubation with WR-1065 significantly reduced the HR frequency in both the HU-treated cells and the CPT-treated cells (*$P < 0.05$ and **$P < 0.01$, respectively). The reduction in HR frequency was similar using either a low concentration of WR-1065 for 24 h prior to HU or CPT treatment or a high concentration of WR-1065 for 30 min prior to HU or CPT treatment. WR-1065 alone did not interfere with the background frequency of HR.

Cellular Activities of WR-1065 Differ in HR-Proficient and Deficient CHO Cell Lines

Next we tested whether the radioprotective properties of WR-1065 against cell killing depend on the HR proficiency of the cell line using the 4 m*M*/30 min WR-1065 treatment protocol (Fig. 2). After irradiation with 2 Gy of X rays, both HR-proficient cell lines (AA8 and CXR3) showed similar levels of protection by WR-1065 against radiation-induced cell killing ($P < 0.01$). However, the WR-1065-treated and irradiated HR-deficient irs1SF cells exhibited no radioprotection ($P > 0.1$). The differences between the irs1SF (HR-deficient) and the AA8 and CXR3 cells (both HR-proficient) were statistically significant (Fig. 2; $P < 0.05$).

To reconcile this observation with the well-documented radioprotective activities of WR-1065, we evaluated the cytotoxicity of WR-1065 in all three CHO cell lines (Fig. 2B). The results of this experiment showed that the HR-deficient irs1SF cell line is approximately five times more sensitive to the cytotoxicity of WR-1065 ($EC_{50} = 0.21$ m*M*) than HR-proficient AA8 and CXR3 lines ($EC_{50} = 1.12$ and 0.83 m*M*, respectively). The decrease in survival for the “protected” and irradiated irs1SF cells relative to the “unprotected” irradiated irs1SF cells is likely a reflection of additive (or synergistic) effects of radiation exposure and WR-1065 cytotoxicity.

WR-1065 Reduces Homologous Recombination Frequencies in SCneo Construct in S31WT Human Cells

We confirmed that WR-1065 treatment reduces the frequency of HR in mammalian cells using an artificial intracellular substrate for HR, SCneo (12,19). Transient transfection of S31WT cells with the pCMV3xnlsI-*Sce*I vector induced an ~40-fold increase in HR events as a result of I-*Sce*I restriction endonuclease activity compared to sham-transfected controls (Fig. 3). Preincubation with WR-1065 significantly reduced the HR frequency in I-*Sce*I-transfected cells ($P < 0.001$). Similar to the results obtained using SPD8 cells and HU or CP treatment, WR-1065 protected equally well against restriction endonuclease-induced hyper-recombination using either the 4 m*M*/30 min or the 40 μ*M*/24 h treatment.

The SCneo/I-*Sce*I HR experimental system depends on the efficiency of transient transfection as well as on appropriate I-*Sce*I digestion of the recombination substrate. To determine whether WR-1065 pretreatment could interfere with transfection, S31WT cells were transfected with a GFP-expressing plasmid using the same method used for the pCMV3xnlsI-*Sce*I plasmid. Flow cytometry for GFP fluorescence 24 h after transient transfection demonstrated that

pretreatment using either WR-1065 protocol had no effect on transfection efficiency (Fig. 4A). *In vitro* digestion of the SCneo recombination substrate by purified I-*Sce*I enzyme demonstrated that WR-1065 did not interfere with restriction endonuclease activity (Fig. 4B). One unit of I-*Sce*I converts 1 μg of SCneo plasmid from supercoiled (lane 1, SC ~90%) to linear form (lane 2, L), and pretreatment with concentrations of WR-1065 up to 4 m*M* (lanes 4–7) does not influence the ability of I-*Sce*I to cut SCneo substrate.

Since WR-1065 imparts most of its biological activity through free radical scavenging, we tested whether transfection with and expression of I-*Sce*I changes the levels of ROS and/or reactive free radicals in S31WT cells. Using an oxidation-specific fluorescent probe and flow cytometry, we demonstrated that expression of I-*Sce*I does not change the cellular levels of ROS/free radicals compared to sham-treated controls or hydrogen peroxide-treated positive controls (Fig. 4C).

Effects of WR-1065 in MCF-10A Cells Partially Depleted of Rad51, Rad51C or Xrcc3

Finally, we tested whether depletion of crucial HR factors in MCF-10A human cells through siRNA knockdown would change the radioprotection or cytotoxicity produced by WR-1065. Figure 5A shows that siRNA treatment reduced target protein expression by ~90% for Rad51, ~50% for Rad51C, and ~70% for Xrcc3. When MCF-10A cells were irradiated with 6 Gy of X rays, WR-1065 pretreatment (4 m*M*/30 min) resulted in a two- to threefold increase in clonogenic survival (Fig. 5B). While knockdown of Rad51 or Rad51C did not alter the radioprotective effects of WR-1065, depletion of Xrcc3 resulted in decreased WR-1065-mediated cell survival. However, the differences between different knockdown cells were not as pronounced as in CHO cell lines (Fig. 2) and were not statistically significant. Evaluation of the cytotoxicity of WR-1065 demonstrated similar sensitivities to cell killing by WR-1065 irrespective of the protein that had been depleted (continuous treatment, Fig. 5C; 30 min treatment, data not shown). Unlike the results obtained for the CHO cell lines, MCF-10A cells depleted of Xrcc3 were as sensitive to the cytotoxicity of WR-1065 as those depleted of Rad51 and Rad51C. One possible explanation for this difference could be the partial depletion of Xrcc3 by the siRNA method as opposed to the complete absence of Xrcc3 in irs1SF cells.

DISCUSSION

We recently reported that WR-1065 treatment reduces the hyper-recombination/mutation frequency in RKO36 human colon carcinoma cells exposed to ionizing radiation, protecting cells from long-term genomic instability (5). Several mechanisms of the radioprotective activity of WR-1065 have been already proposed. Unfortunately, though, our understanding of WR-1065's radioprotective activity is far from complete, especially in the case of long-term protection against mutagenesis and genomic instability. In this work we determined that WR-1065 directly influences HR and that HR deficiency changes the radioprotective effects of WR-1065 in mammalian cells.

HR is an essential cellular process required for proper repair of complex DNA damage, especially double-strand damage such as double-strand breaks and interstrand crosslinks. HR also provides support for DNA replication by recovering stalled or broken replication forks (22). However, overstimulation of HR after DNA damage can result in an increased frequency of inappropriate recombination events such as gene conversion and mutations and give rise to genomic instability (23,24).

In our present work, we observed a significant decrease in HR frequency in mammalian cells pretreated with WR-1065 and challenged with an HR-inducing treatment. Similar responses were registered irrespective of the cell line (both hamster and human) and the method used to induce HR (HU, CPT, restriction enzyme I-*Sce*I). HU inhibits ribonucleotide reductase,

depleting cells of several deoxyribonucleoside triphosphates, which in turn stalls replication forks (25). CPT stabilizes topoisomerase I-cleavable complexes, leaving open single-strand breaks that are converted into double-strand breaks during replication, producing collapsed replication forks (26). The I-*Sce*I rare-cutting restriction enzyme produces sequence-specific "clean" double-strand breaks and is used extensively for direct induction of HR in an artificial recombination substrate (19,27). All three treatments were used previously and showed a high-efficiency stimulation of HR in mammalian cells (17).

It could be argued that the observed reduction in the frequency of HR in cells treated with WR-1065 is due to a decrease in initial DNA damage through well-known activities of WR-1065 such as free radical scavenging or chemical repair of damaged DNA. However, in good agreement with published results on the antimutagenic action of WR-1065, even a low micromolar concentration (40 μ*M*) of the drug was efficient in decreasing recombination frequencies. Such a low drug concentration is known to be ineffective in reducing radiation-related initial DNA damage and preventing cell death (5). We demonstrated that, while 40 μ*M* WR-1065 treatment 24 h prior to irradiation did not protect against the immediate DNA damaging effects of irradiation as measured by micronucleus induction, 4 m*M* WR-1065 treatment 30 min prior to irradiation did reduce the micronucleus frequency. In the case of the I-*Sce*I/SCno system, our control experiments demonstrated that WR-1065, even at the high concentration of 4 m*M*, does not interfere with the stable transfection of cells with the SCneo substrate or transient transfection of the same cells with I-*Sce*I. In addition, transfection and generation of DNA strand breaks in the I-*Sce*I/SCneo system did not change free radical levels in cells, providing evidence that WR-1065 activity in this system is independent of its free radical scavenging properties. Based on these observations, it is plausible that WR-1065 can interact directly with HR machinery in mammalian cells. Similar decreases in hyper-recombination were observed in yeast treated with amifostine prior to exposure to the DNA-damaging radiomimetic agent bleomycin (28).

One of the ways that WR-1065 interferes with HR could be through its ability to change chromatin structure. Under physiological conditions, WR-1065 is a divalent cation with its 1,3-diaminepropane moiety similar to spermine and spermidine, facilitating its binding to DNA in a manner analogous to polyamines (29). This WR-1065-DNA interaction can change chromatin structure by distorting DNA supercoiling without breaking the molecule. This could affect the processes of DNA replication and facilitate repair (11). Additionally, treatment with WR-1065 results in a significant increase in intracellular synthesis of polyamines (11). Polyamines (e.g. putrescine, spermidine, spermine) are an essential part of chromatin structure due to their polycationic properties, stabilizing DNA through electrostatic interactions. The elevated levels of polyamines or polyamine-like molecules observed after WR-1065 treatment may explain its antirecombinogenic and antimutagenic properties.

Another possible mechanism for the interference of WR-1065 with HR is its ability to block mammalian cells in the G_2/M phase of the cell cycle (30), probably through inhibition of DNA topoisomerase II catalytic activity (31). Topoisomerase II is a ubiquitous enzyme that removes knots and tangles from DNA, and it is an essential factor in most DNA processing pathways, including replication and damage repair. WR-1065 inhibits the catalytic activity of topoisomerase II at concentrations as low as 4 μ*M* (32,33). Since HR is most active in G_2/M, prolonging this phase could provide cells with time to correct inappropriate recombination events, reducing hyper-recombination and mutation frequencies. It is also possible that prolonging the G_2 phase of the cell cycle allows additional time for inappropriate *de novo* HR to occur; however, delayed mutagenesis and genomic instability data suggest that this is not the case (5,34,35).

We observed decreased radioprotection and increased cytotoxic activity of WR-1065 in Xrcc3-deficient (HR-deficient) cells. According to current models, the Rad51 family of proteins includes Rad51, Xrcc2, Xrcc3, Rad51B, Rad51C and Rad51D, which have crucial and nonredundant roles in HR repair of DSB inter-strand crosslinks in DNA (36). Rad51 binds to single-stranded DNA to form the Rad51 nucleofilament, the crucial structure in HR, and catalyzes DNA strand exchange. Xrcc3 forms complexes with Rad51C and other paralogs, but not Rad51, and catalyzes the homologous strand pairing (37,38). However, Rad51C is also implicated in nuclear trafficking of Rad51 after DNA damage (39).

It was demonstrated in earlier studies using *E. coli* that the radioprotective effects of aminothiols similar to amifostine are practically absent in cells deficient in homologous recombination factors (e.g. $RecA^{-}$) (40). Similar studies in *S. cerevisiae* confirmed this observation using HR-deficient rad^{-} yeast mutants. Even more significantly, cysteamine and cysteine did not protect haploid yeast cells from the effects of radiation exposure (41). It was concluded that the radioprotective action of aminothiols may be mediated through a recombination-like mechanism, for which the diploid state and HR proficiency are required. A more recent study (42) demonstrated that the RecN gene product is required for amifostine-mediated DNA protection in *E. coli* cells. RecN protein is involved in homologous recombination, DSB repair (43), and structural maintenance of *E. coli* chromosome (44).

In our experiments this phenomenon was especially pronounced in Chinese hamster cells (irs1SF compared to AA8) and less marked in human MCF10A cells knocked down for Xrcc3. SiRNA-directed knockdown of Rad51 and Rad51C did not produce changes in WR-1065-mediated radioprotection or cytotoxicity, possibly indicating a specific role for Xrcc3 in the biological activities of WR-1065.

Similar discrepancies between hamster and human cells in responses to WR-1065 were noted previously. For example, non-homologous end joining (NHEJ)-proficient and deficient human cell lines (MO59K and MO59J, respectively) were protected to a similar extent by pretreatment with 4 m*M* WR-1065 despite their very different base radiosensitivity (45). In contrast, earlier studies using Chinese hamster cells demonstrated that the NHEJ-deficient mutant xrs5 was not protected against the effects of ionizing radiation by WR-1065 (34,46). This discrepancy was related to differences in NHEJ deficiency (DNA-$PKcs^{-/-}$ compared to $Ku^{-/-}$) or other factors such as differences in chromatin structures, which could be related to polyamine content. Similar factors could be responsible for differences observed here between human and hamster cell line responses. In addition, the differences between human and hamster cells in the length of cell cycle phases, which translates directly into the proportion of time the cell spends in the HR-proficient S and G_2 phases, should be also considered.

In summary, we demonstrated that WR-1065, the active metabolite of amifostine, disrupts HR in mammalian cells, reducing recombination induced by DNA double-strand breaks and broken replication forks. This ability could be responsible at least in part for the long-term radioprotective effects of WR-1065, preventing hyper-recombination and mutagenesis in irradiated cells. In addition, it could be crucial for radioprotective activity of amifostine/WR-1065 against high-LET space radiation exposures. Our future studies will investigate the molecular mechanism(s) by which WR-1065 interferes with HR and establish the relationship between this activity and the overall biological activity of WR-1065.

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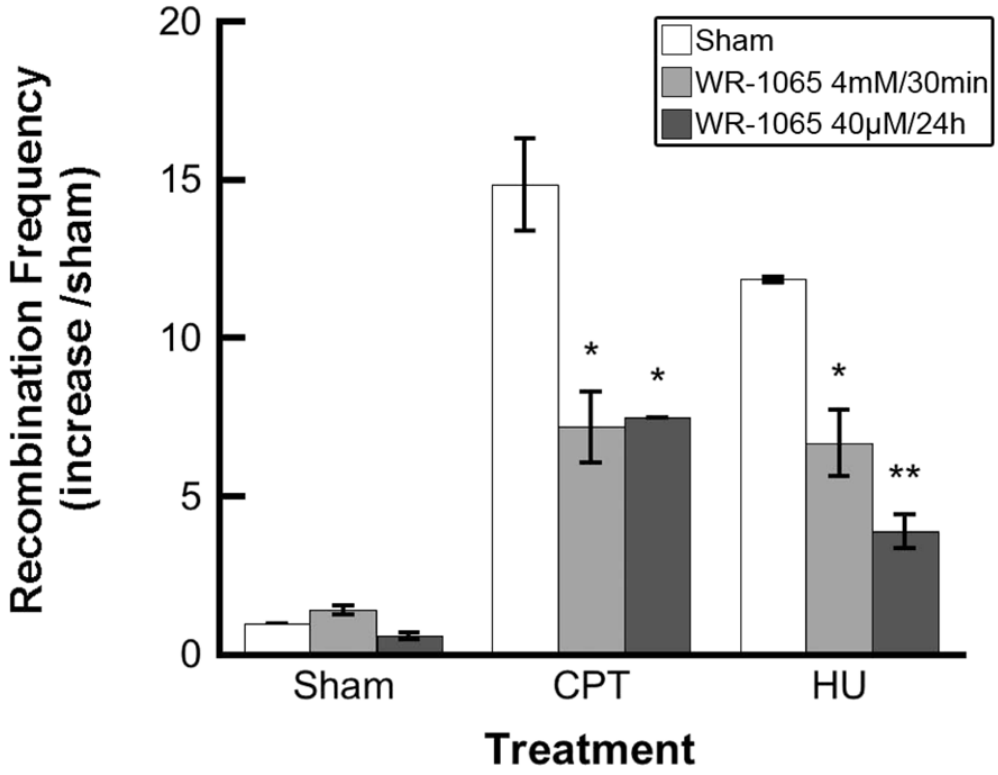

FIG. 1.

Homologous recombination induced in SPD8 cells by camptothecin (CPT) or hydroxyurea (HU) is reduced by treatment with WR-1065. The cells were incubated with WR-1065 for 30 min at 4 m*M* (light gray bars) or 24 h at 40 μ*M* (dark gray bars) and then treated with CPT (100 n*M* for 1 h) or HU (0.2 m*M* for 24 h) and assayed for homologous recombination frequency as described in the Materials and Methods. Results represent means of three independent experiments ± SEM (**P* < 0.05, ***P* < 0.01).

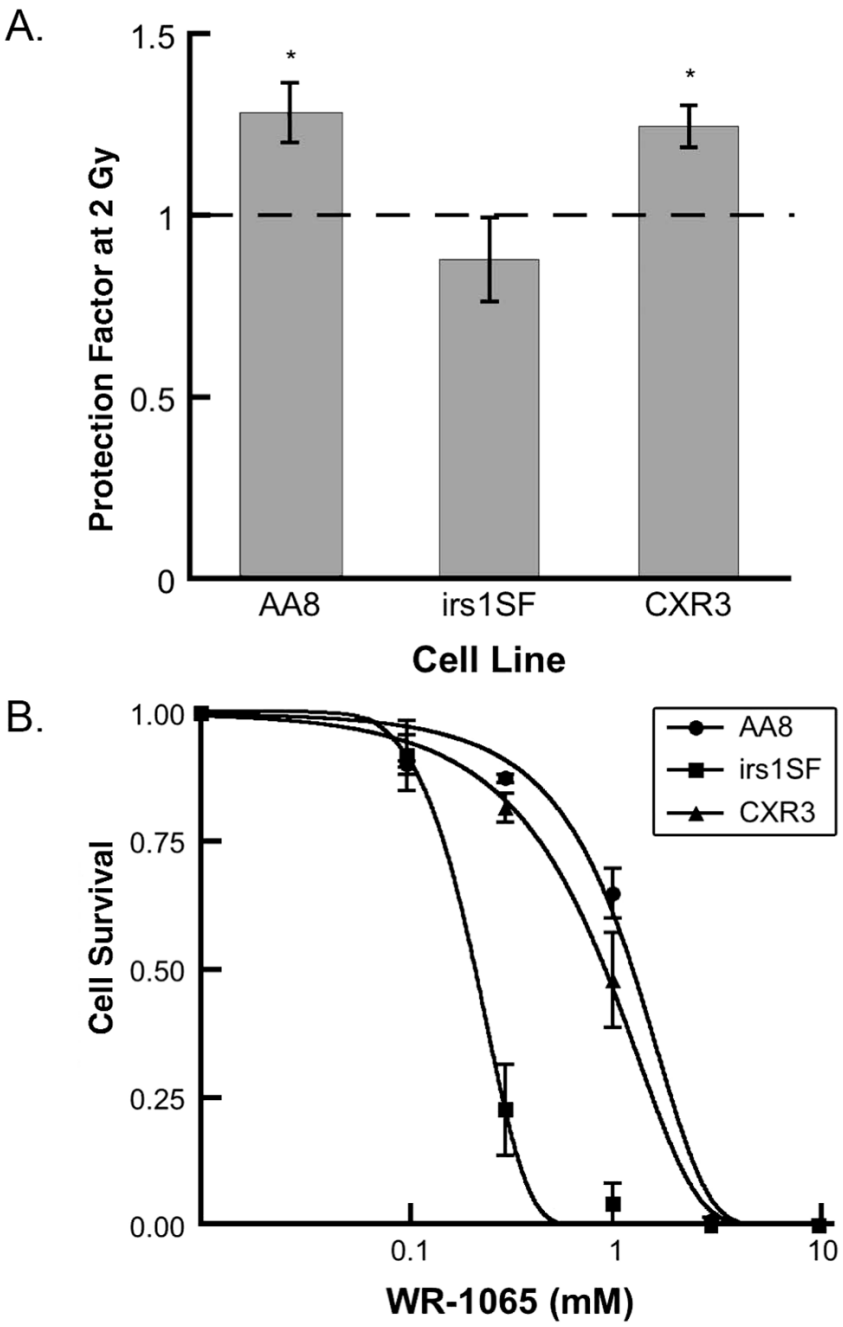

FIG. 2.

WR-1065 effects in CHO cells differ between homologous recombination-proficient (AA8 and CXR3) and deficient (irs1SF) cells. Panel A: Radioprotective effects of WR-1065 at 2 Gy. CHO cells were treated with 4 m*M* WR-1065 for 30 min or sham-treated, then irradiated with 2 Gy and immediately replated for clonogenic survival. Data are normalized to sham-treated controls. Results represent means of five independent experiments ± SEM (**P* < 0.05). Panel B: Cytotoxicity of WR-1065. Exponentially growing CHO cells (●, AA8;■, irs1SF; and ▲, CXR3) were treated with WR-1065 (0–10 m*M*) for 30 min, washed and incubated in drug-free medium for 7 to 10 days. Clonogenic cell survival after drug treatment was measured as

described in the Materials and Methods. Results represent means of two independent experiments ± SEM.

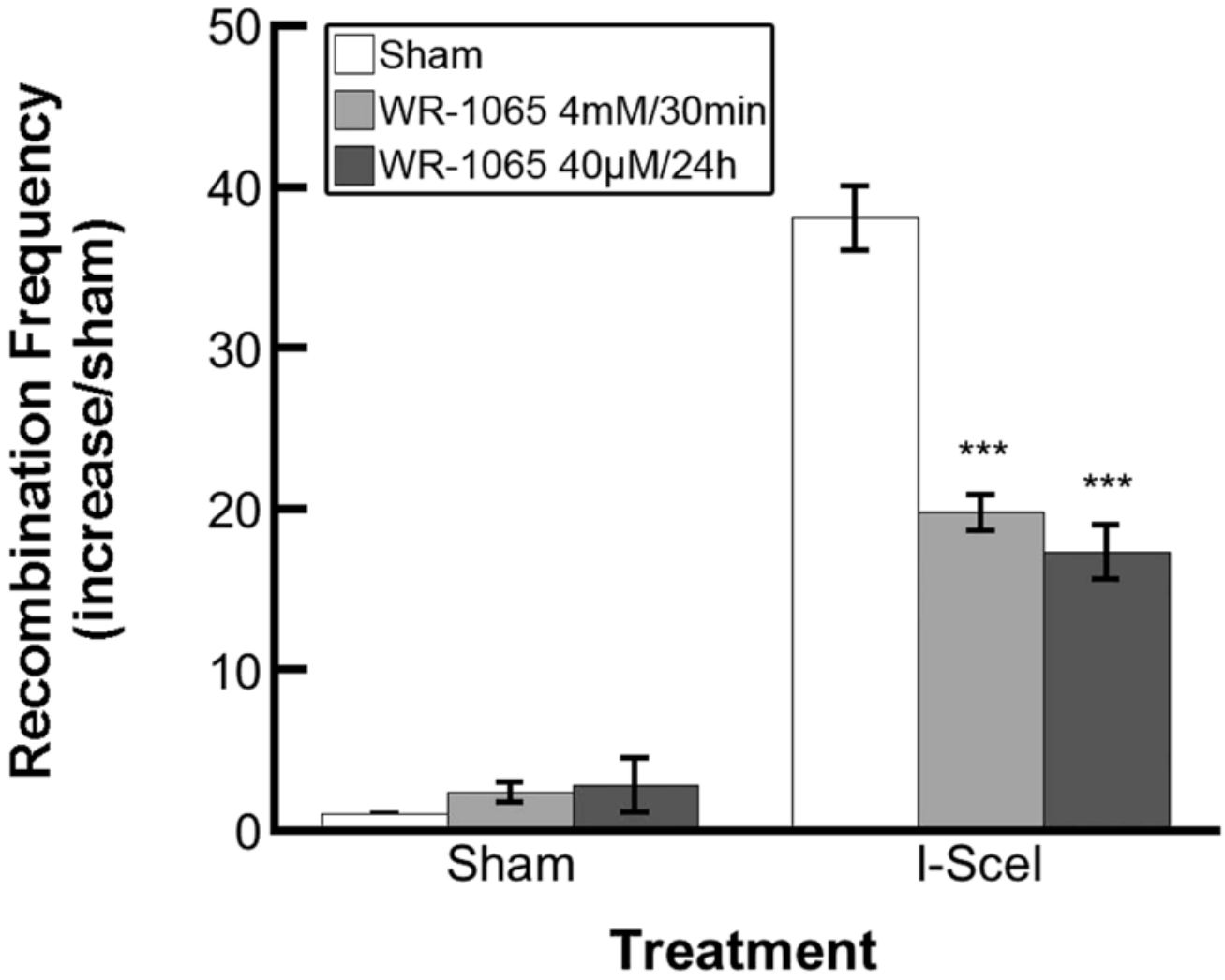

FIG. 3.

Homologous recombination induced in S31WT cells by I-*Sce*I expression is reduced by treatment with WR-1065. The S31WT cells (harboring SCneo construct) were incubated with WR-1065 for 30 min at 4 m*M* (light gray bars) or 24 h at 40 μ*M* (dark gray bars), transfected with I-*Sce*I plasmid, and assayed for homologous recombination frequency as described in the Materials and Methods. Results represent means of three independent experiments ± SEM (***$P < 0.001$).

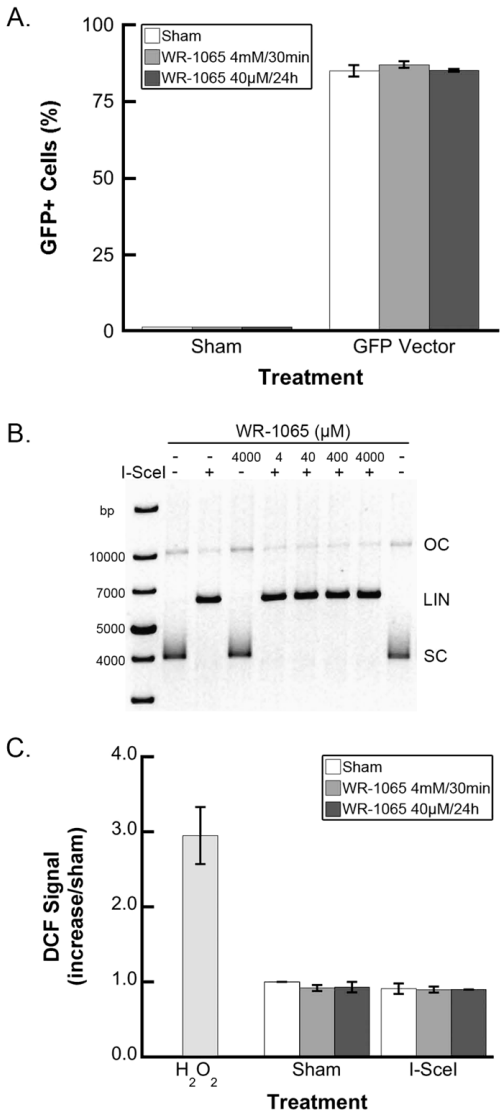

FIG. 4.

Decrease in homologous recombination frequency after WR-1065 treatment is not caused by WR-1065 preventing DNA transfection (panel A), interfering with I-*Sce*I induction of double-strand breaks in substrate DNA (panel B), or changes in reactive oxygen species levels (panel C). Panel A: S31WT cells were treated with WR-1065 for 30 min at 4 m*M* (light gray bars) or 24 h at 40 μ*M* (dark gray bars) or were sham-treated with PBS (white bars) and were transfected with GFP plasmid as outlined in the Materials and Methods for the I-*Sce*I transient transfection. After 24 h recovery, the GFP-positive cells were counted by flow cytometry. Results of two independent experiments (±SEM) are shown. Panel B: SCneo supercoiled plasmid (1 μg, lanes 2 and 9, untreated control) was preincubated with WR-1065 (4 μ*M*, lane 5; 40 μ*M*, lane 6; 400 μ*M*, lane 7; 4 m*M*, lanes 4 and 8) for 5 min followed by 16 h incubation with I-*Sce*I enzyme (1 U, lanes 3 and 5–8). The digestion products were resolved using agarose gel electrophoresis (SC, supercoiled circular; OC, open circular; LIN, linear). Panel C: Changes in cellular reactive oxygen species (ROS) levels after WR-1065 treatment (at 4 m*M* for 30 min, light gray bars; 40 μ*M* for 24 h, dark gray bars; or sham-treated, white bars) and I-*Sce*I transfection were measured using the specific probe CM-H2DCFDA and flow cytometry as described in the

Materials and Methods. H_2O_2-treated cells serve as an experimental positive control. Results represent means of two independent experiments ± SEM.

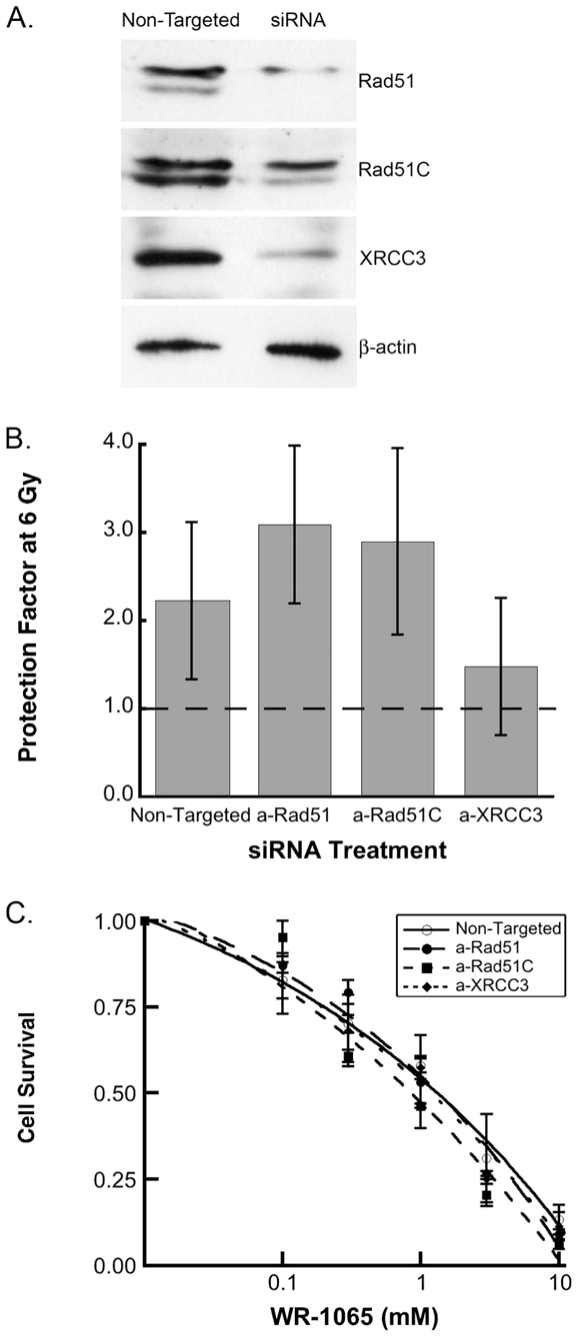

FIG. 5.

siRNA-directed knockdown of critical factors in homologous recombination (Rad51, Rad51C, Xrcc3) does not significantly change the effects of WR-1065 in human MCF-10A cells. Panel A: Western blot demonstrating knockdown efficiency. Panel B: Radio-protective effects of WR-1065 at 6 Gy. MCF-10A cells were treated with 4 m*M* WR-1065 for 30 min (gray bars) or sham-treated, irradiated with 6 Gy, and immediately replated for clonogenic survival. Data are normalized to sham-treated controls. Panel C: Cytotoxicity of WR-1065 in MCF-10A cells. siRNA-transfected cells (○, non-targeted siRNA;●, anti-Rad51;■, anti-Rad51C;◆, anti-

Xrcc3) were treated continuously with WR-1065 (0–10 m*M*) for 7 to 10 days. Results represent means of three independent experiments ± SEM.