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Acquired Resistance to Combination Treatment with Temozolomide and ABT-888 Is Mediated by Both Base Excision Repair and Homologous Recombination DNA Repair Pathways

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Abstract

Many established cancer therapies involve DNA-damaging chemotherapy or radiotherapy. Gain of DNA repair capacity of the tumor represents a common mechanism used by cancer cells to survive DNA-damaging therapy. Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that is activated by DNA damage and plays a critical role in base excision repair. Inhibition of PARP represents an attractive approach for the treatment of cancer. Previously, we have described the discovery and characterization of a potent PARP inhibitor, ABT-888. ABT-888 potentiates the activity of DNA-damaging agents such as temozolomide (TMZ) in a variety of preclinical models. We report here the generation of HCT116 cells resistant to treatment with TMZ and ABT-888 (HCT116R cells). HCT116R cells exhibit decreased H2AX phosphorylation in response to treatment with TMZ and ABT-888 relative to parental HCT116 cells. Microarray and Western blot studies indicate that HCT116R cells have decreased PARP-1 and elevated Rad51 expression levels. HCT116R cells are dependent on Rad51 for proliferation and survival, as shown by inhibition of proliferation and induction of apoptosis upon treatment with Rad51 small interfering RNA. In addition, HCT116R cells are more resistant to radiation than the parental HCT116 cells. Our study suggests that cancer cells upregulate the homologous recombination DNA repair pathway to compensate for the loss of base excision repair, which may account for the observed resistance to treatment with TMZ and ABT-888. (Mol Cancer Res 2009;7(10):1686–92)

Introduction

Poly(ADP-ribose) polymerase-1 (PARP-1) is the founding member of a family of proteins that share a catalytic PARP homology domain and are characterized by their ability to poly (ADP-ribosyl)ate their protein substrates (1-3). Of the 18 PARP family members identified to date (1, 2), PARP-1 and PARP-2 are unique in stimulating catalytic activity in response to DNA damage (4). The activation of PARP-1 and PARP-2 is an immediate eukaryotic cellular response to DNA damage induced by a variety of stimuli including ionizing radiation, alkylating agents, and oxidants (2). Activated PARP-1 and PARP-2 bind to DNA strand breaks, and covalently attach poly(ADP-ribose), or PAR, to nuclear proteins including PARP-1 itself, histones, and transcription factors (1). The enzymatic product of PARP is generated from its substrate β-NAD^+ and consists of linear and branched ADP-ribose units of variable size (5).

Most established cancer therapy regimens involve the utilization of DNA-damaging chemotherapy and/or radiotherapy. Upregulation of DNA repair capacity represents a common mechanism used by cancer cells to survive such therapy. Inhibiting DNA repair systems increases the ability of DNA-damaging agents to eliminate cancer cells and represents an attractive approach for cancer therapy (3). Both PARP-1 and PARP-2 are essential for the repair of single-strand DNA breaks (SSB) through base excision repair (BER; refs. 1, 6, 7); thus, inhibition of PARP-1/PARP-2 will enhance the efficacy of cancer therapeutics that cause DNA damage requiring BER-mediated repair (6, 8). In addition, PARP-1 is overexpressed in a variety of human tumors, including malignant lymphoma (9), hepatocellular carcinomas (10), cervical cancer (11), and non–Hodgkin lymphoma (12).

Mammalian cells have multiple ways to repair damaged DNA. DNA repair pathways can be divided into those that respond to SSBs and those that respond to double-strand breaks (DSB). SSB repair pathways include BER, mismatch repair, and nucleotide excision repair. DSB repair pathways include nonhomologous end joining (NHEJ), and homologous recombination (HR). In response to DSB, the Mre11/Rad50/NBS1 (MRN) complex is recruited to the DNA damage site, triggering the activation of ATM (13). Activated ATM phosphorylates Ser^{139} on the carboxyl tail of H2AX. In addition to ATM, ATR and DNA-PKcs are also able to phosphorylate H2AX (14, 15). Phosphorylated H2AX (γH2AX) plays an important role in the recruitment of DNA repair proteins such as BRCA1, MDC1, and 53BP1, followed by recruitment of Rad51 (15-19). Rad51 is a central DNA recombinase that catalyzes homologous pairing and strand exchange in the HR pathway (20). It

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is well recognized that γH2AX levels correlate with the extent of DNA DSBs (21, 22).

Temozolomide (TMZ) is a monofunctional DNA-alkylating agent that is used to treat malignant glioma and melanoma (23). TMZ forms methyl adducts in DNA at N^7 guanine, O^6 guanine, and N^3 adenine. The O^6 methyl guanine DNA lesion can be repaired by O^6-methylguanine-DNA methyltransferase, whereas the methylpurines (N^7-MeG and N^3-MeA) are repaired by the BER system facilitated by PARP-1 and PARP-2 (24, 25). Inhibition of PARP potentiates the activity of DNA-damaging agents such as TMZ in cancer cells *in vitro* and in preclinical tumor models *in vivo* (6, 23). Interestingly, PARP inhibition has been shown to be synthetically lethal in the context of cancer cells defective in BRCA1 and BRCA2 function (26, 27). In fact, a deficiency of other proteins in the HR DNA repair pathway also sensitizes cancer cells to PARP inhibition (28). This effect is most likely due to the fact that PARP inhibition leads to the persistence of endogenous SSBs that are converted to DSBs during DNA replication. The failure to efficiently repair these DSBs in the absence of functional HR leads to cell lethality.

Previously, we described the discovery and characterization of a potent PARP inhibitor, ABT-888 (29). ABT-888 is a potent inhibitor of both PARP-1 and PARP-2 and is currently being examined in multiple phase I/II clinical trials. ABT-888 potentiates the activity of multiple DNA-damaging agents including TMZ in a variety of preclinical models. We report here the generation of HCT116 cells resistant to TMZ/ABT-888 (HCT116R cells). HCT116R cells exhibit decreased γH2AX levels compared with parental HCT116 cells in response to TMZ/ABT-888. Microarray and Western blot studies indicate that HCT116R cells have decreased levels of PARP-1 and elevated levels of Rad51. Knockdown of Rad51 by small interfering RNA (siRNA) indicates that HCT116R cells are dependent on Rad51 for proliferation and survival. In addition, HCT116R cells are also more resistant to treatment with radiation than parental HCT116 cells. Our study suggests that resistance to treatment with TMZ and PARP inhibitors arises from defective BER and enhanced HR repair capacity.

Results

Characterization of HCT116 Cells Resistant to TMZ and ABT-888 Combination Treatment

We have described previously the *in vivo* activity of ABT-888 in combination with TMZ in a variety of preclinical tumor models (29). In order to study potential molecular mechanisms of resistance to treatment with TMZ/ABT-888, we generated HCT116R cells by incubating parental HCT116 cells with TMZ/ABT-888 for an extended period for selection of resistant clones. Three clones of HCT116R cells, designated HCT116-C1, HCT116-C2, and HCT116-C3, were isolated. As shown in Fig. 1A, ABT-888 potentiated the effect of TMZ in parental HCT116 cells, consistent with our previous data (23). However, the ability of ABT-888 to enhance the cell-killing effects of TMZ was significantly attenuated in all three clones (Fig. 1B, C, and D).

Rad51 Is Upregulated in HCT116R Cells

In order to investigate potential mechanisms for resistance to treatment with TMZ/ABT-888, we examined differences in gene expression pattern between HCT116 and HCT116R cells. Significant changes in gene expression common to all resistant cells compared with the sensitive parental cells could be grouped into several major functional categories including DNA damage repair/response, cell cycle regulation, transcription, signaling, and catalytic processes (Supplemental Fig. S1). The Database for Annotation, Visualization, and Integrated Discovery (DAVID) for Gene Ontologies was used to evaluate these functional classes (30). The analysis indicated that changes of gene expression in DNA damage repair/response and cell cycle regulation were the most significant based on *P* value (data not shown). Given the logical association of potential for changes in gene expression in DNA repair pathways to influence sensitivity to TMZ/ABT-888, we examined this in more detail. The microarray data indicated that all of the HCT116R clones have decreased levels of PARP-1 mRNA and increased levels of mRNA encoding various HR proteins including Rad51, FANCA, FANCG, BLM, BRCA1, and BRCA2 (Table 1). Western blot analysis confirmed that HCT116R cells had decreased levels of PARP-1 and elevated levels of Rad51, BLM, and BRCA2 (Fig. 2). Among these changes, the decrease in PARP1 and increase in Rad51 are the most consistently observed in all resistant clones (Fig. 2).

HCT116R Cells Have Increased Ability to Repair DSB

TMZ is a monofunctional DNA-alkylating agent that induces SSBs in mammalian cells. Previously, we showed that ABT-888 enhances the toxicity of TMZ by converting TMZ-induced SSBs to DSBs (23). The fact that HCT116R cells have elevated levels of Rad51 indicates that HCT116R cells may have elevated ability to repair TMZ/ABT-888–induced DSB through the HR repair pathway. In order to test this, we conducted γH2AX analysis because it is well established that increased levels of the γH2AX correlated with increases in DSB accumulation (23). The cells were analyzed for γH2AX levels 1 hour (Fig. 3A) and 6 hours (Fig. 3B) after combination treatment with TMZ and ABT-888. Consistent with our previous data, TMZ/ABT-888 induced a strong γH2AX signal with 32% and 31% cells positive for γH2AX staining in parental HCT116 cells 1 and 6 hours posttreatment, respectively (Fig. 3), whereas ABT-888 alone did not elicit a significant γH2AX response (Fig. 3). In HCT116R cells, ~10% cells were γH2AX-positive 1 hour posttreatment with TMZ alone or with TMZ plus ABT-888 (Fig. 3A). However, the γH2AX levels in HCT116R cells were reduced to background levels 6 hours posttreatment with TMZ alone or TMZ plus ABT-888 (Fig. 3B).

HCT116R Cells Depend on Rad51 for Proliferation and Survival

In order to determine the role of Rad51 in HCT116R cells, we used RNA interference to selectively knockdown Rad51 in parental HCT116 and HCT116-C3 cells. As shown in Fig. 4A and B, Rad51 siRNA reduced Rad51 protein levels in both HCT116-C3 and HCT116 cells to a similar extent. Knockdown of Rad51 led to a 79% inhibition of proliferation in HCT116-C3 cells, but only a 24% decrease in proliferation in parental HCT116 cells (Fig. 4C). Moreover, Rad51 siRNA also induced significant activation of caspase-3/7 in HCT116-C3 cells, but had no effect on caspase activation in parental HCT116 cells (Fig. 4D).

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HCT116R Cells Are More Resistant to Radiation than Parental HCT116 Cells

Rad51 is known to be a key regulator of cellular response to radiation owing to its critical function in HR (31, 32). In order to examine the functional significance of elevated Rad51 expression in HCT116R cells, we examined the response of these cells to radiation treatment. The HCT116-C3 cells are significantly more resistant to treatment with X-ray radiation than parental HCT116 cells. Following treatment with 10 Gy of radiation, 27% of the HCT116-C3 cells remained viable whereas only 2% viability was observed for parental HCT116 cells (Fig. 5).

HCT116R Cells Fail to Generate PAR in Response to DNA Damage

As shown in Fig. 2, PARP-1 levels were also greatly reduced in all the resistant clones relative to the parental cells. In order to confirm functional inactivation of PARP-1, we examined the levels of PAR, the product of PARP-1, in HCT116 and HCT116-C3 cells with hydrogen peroxide in the presence or absence of ABT-888 (29). As shown in Fig. 6, HCT116 cells had high basal levels of PAR. H_2O_2 further enhanced PAR formation, whereas ABT-888 blocked the process (Fig. 6). However, HCT116-C3 cells, which had inherently low levels of PAR and H_2O_2, failed to induce significant PAR formation (Fig. 6). These results show that HCT116R cells are also functionally defective in BER.

Discussion

ABT-888 is a potent PARP inhibitor that is currently in multiple phase I/II clinical trials, including studies examining combination with TMZ. In order to examine the potential mechanisms of drug resistance that may occur in response to treatment with TMZ/ABT-888 chemotherapy, we generated resistant cells by *in vitro* selection. Our data indicate that HCT116R cells become resistant to TMZ/ABT-888 through enhanced HR capacity and defects in the BER pathway. DSBs in the mammalian genome are repaired through NHEJ and HR. DNA-PKcs, Ku70, and Ku80 are critical components of NHEJ pathway, whereas proteins such as BRCA1/2, MDC1, 53BP1, and Rad51 are critical components of the HR pathway. The HR pathway is mainly active in S phase, whereas NHEJ is active through all phases of the cell cycle (33). HCT116R cells have elevated levels of Rad51, but unchanged levels of DNA-PKcs and Ku70. Rad51 is a central DNA recombinase that catalyzes homologous pairing and strand exchange in the HR pathway (20), whereas other proteins such as BRCA1/2 facilitate the assembly of Rad51 foci at the DSB site (34-36). Previously, we have shown that ABT-888 potentiates the ability of TMZ to kill cancer cells by converting TMZ-induced SSBs to DSBs, as shown by γH2AX assay and neutral comet assay (23). HCT116R cells are able to repair DSBs via the HR pathway more efficiently, as shown by the fact that HCT116R cells exhibited elevated levels of Rad51 (Fig. 2), decreased levels of γH2AX in response to TMZ/ABT-888 (Fig. 3), and resistance to radiation treatment relative to HCT116 parental cells (Fig. 5). In addition, knockdown of Rad51 by siRNA showed that HCT116R cells are more dependent on Rad51 for growth and survival relative to parental HCT116 cells (Fig. 4). Rad51 is overexpressed in a variety of tumor cell lines through transcriptional regulation, contributing to both tumorigenesis and

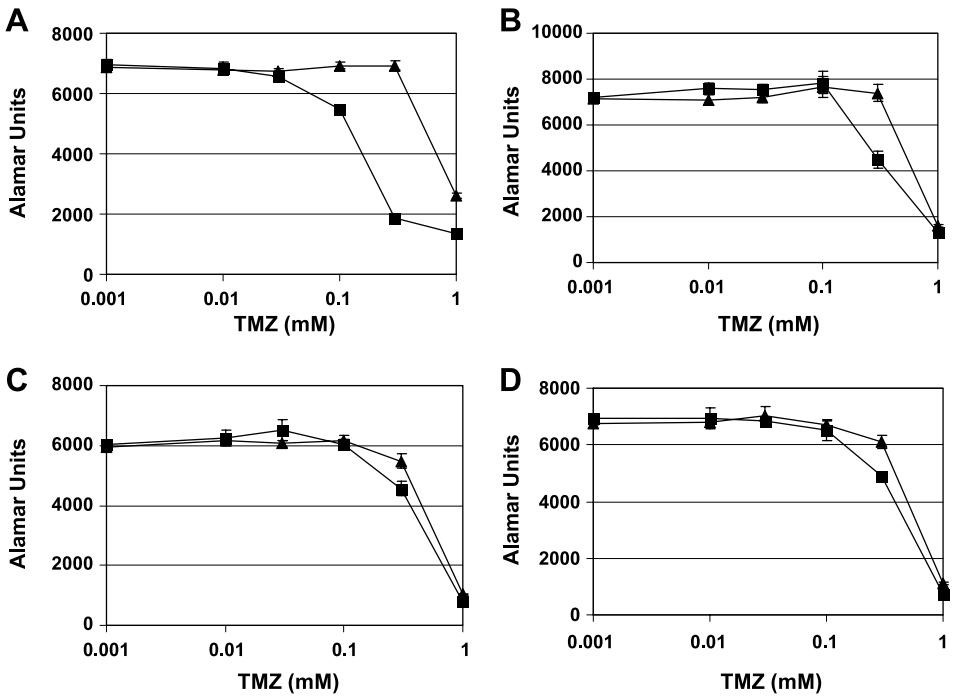

FIGURE 1. Characterization of HCT116R cells. HCT116R cells were generated as described in Materials and Methods. Cells were treated with the indicated concentration of TMZ in the absence (▲) or presence of 5 μmol/L of ABT-888 (■) for 5 d. The AlamarBlue cell proliferation assay was used to assess cell viability. Parental HCT116 (**A**), HCT116-C1 (**B**), HCT116-C2 (**C**), and HCT116-C3 (**D**) cells.

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Table 1. Alteration of DNA Damage Repair/Response Genes in HCT116R Cells

Gene	HCT116-C1	HCT116-C2	HCT116-C3
PARP1	−21.16	−20.77	−17.14
ATM	−1.74	−1.35	−1.76
FANCF	−1.72	−1.49	−1.60
FANCA	2.25	1.57	1.32
PARP2	1.46	1.82	1.64
RAD1	1.56	1.87	1.83
MSH2	1.64	1.80	1.82
RAD51	2.02	2.23	2.43
BARD1	2.04	2.14	1.59
BRCA1	2.1	2.4	1.9
BLM	2.36	2.43	2.02
CHK2	2.60	2.09	2.14
FANCG	2.75	2.16	1.97
BRCA2	3.0	2.4	2.5

NOTE: DNA damage repair/response genes that are differentially regulated in all three resistant clones (>1.5-fold) were shown as fold change to that in parental HCT116 cells.

resistance to DNA-damaging radiation and chemotherapy (32). It is also interesting to note that HCT116R cells are not resistant to treatment with TMZ alone. The expression of O^6-methylguanine-DNA methyltransferase in HCT116R cells is similar to that in HCT116 parental cells (Fig. 2). Our data indicates that HCT116R cells become resistant to combination therapy by mechanisms independent of direct repair of damaged DNA. Our results are consistent with the observation that the sensitivity of various xenograft tumors to combination treatment with TMZ and ABT-888 is independent of the expression levels of O^6-methylguanine-DNA methyltransferase.[1]

Surprisingly, both the mRNA and protein levels of PARP-1 are greatly reduced in HCT116R cells (Fig. 2). As a result, HCT116R cells fail to generate PAR in response to hydrogen peroxide (Fig. 6). Our CGH data (data not shown) also reveals that there is no significant difference in PARP-1 gene copy number between HCT116 and HCT116R cells, indicating that the downregulation of PARP-1 is through transcriptional regulation as shown in our microarray study (Fig. 2). We also examined the methylation status of the PARP-1 gene, and there is no significant difference between HCT116R and parental HCT116 cells (data not shown). It is also interesting to note that the mRNA level of PARP-2 is not downregulated in HCT116R cells even though ABT-888 inhibits PARP-2 to a similar extent *in vitro*. It is possible that the protein level of PARP-2 is so low that its PARP activity is negligible, although we cannot rule out the possibility that PARP-2 may function differently from PARP-1 in BER. The reason why HCT116R cells prefer the absence of PARP-1 is not clear. One possibility is that PARP-1 is recruited to the DNA damage site and has to be removed later in the DNA repair process to allow other DNA repair proteins access to the damage sites. ABT-888 may lock PARP-1 in the DNA damage site and prevent its disassociation, which may hinder the efficient repair of DSB through the HR pathway (37). Alternatively, resistance could be generated through a two-step process. First, cells lose PARP-1 expression which reduces capacity for BER. This, in turn, leads to the overexpression of Rad51 to compensate for the loss of BER.

The breast cancer susceptibility genes *BRCA1* and *BRCA2* are frequently mutated in breast and ovarian cancer and play critical roles in HR by facilitating the assembly of Rad51 at the site of DNA damage (34). Recently, inhibition of PARP-1 has been shown to be synthetically lethal in combination with defects in BRCA1/2 as well as other genes in the HR pathway (26-28), which indicates that simultaneous inhibition of BER and HR is cytotoxic to cancer cells, most likely due to the fact that PARP inhibition leads to the persistence of endogenous SSBs which are converted to DSBs during DNA replication. The failure to efficiently repair these DSBs in the absence of functional HR leads to cell lethality. Restoration of BRCA function in BRCA-defective cancer cells may serve as one way to be resistant to the treatment with PARP inhibitor as a single agent (38). Our data indicates that acquired defects in BER and enhanced capacity for HR repair may mediate the resistance of cancer cells to combination treatment with TMZ and ABT-888. These results suggest a novel mechanism of resistance to combination therapy in which the HR and BER pathways interact to regulate cancer cell viability.

Our microarray data indicate that changes in the expressions of genes involved in pathways other than DNA repair may also contribute to the observed resistance (Supplement Fig. S1), For example, significant changes in gene expression were observed

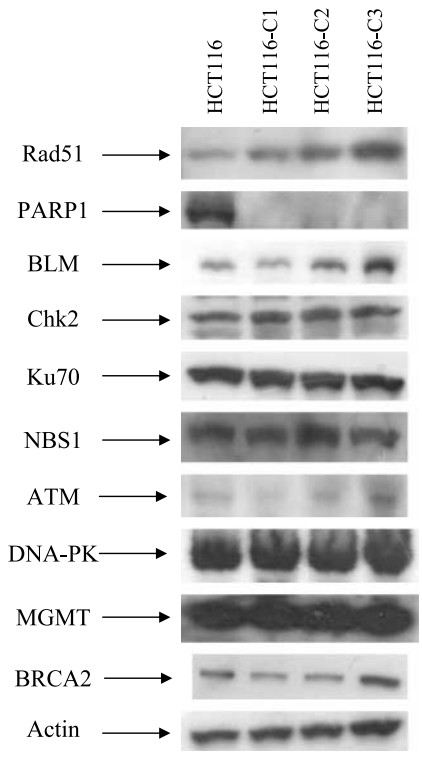

FIGURE 2. Western blot analysis of DNA repair proteins in HCT116 and HCT116R cells. Cells were harvested and the cell lysate (50 μg total protein) was subjected to Western blot analysis for the indicated proteins as described in Materials and Methods.

[1] Palma et al., manuscript submitted.

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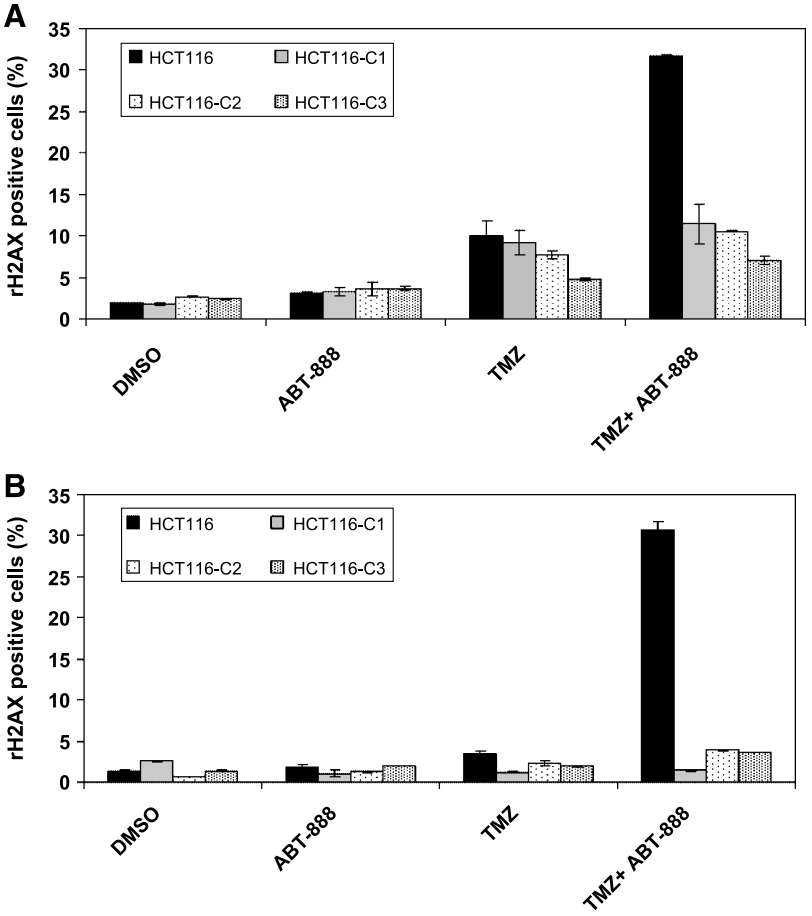

FIGURE 3. HCT116R cells exhibit diminished γH2AX induction in response to TMZ/ABT-888. Cells were pretreated with 5 μmol/L of ABT-888 for 30 min, then 1 mmol/L of TMZ and 5 μmol/L of ABT-888 for 30 min, followed by incubation with ABT-888 for 1 h (**A**) or 6 h (**B**) to allow DNA repair. The γH2AX assay was carried out as described in Materials and Methods.

in genes that regulate cell cycle control such as cyclin A2, centromere protein E/M/N, and p57KIP2. Additional studies will be required to determine if any of these additional factors contribute significantly to the resistance phenotype described here.

Materials and Methods

Chemicals

All chemicals were from Sigma. Protein concentration was determined using the BCA method (Pierce).

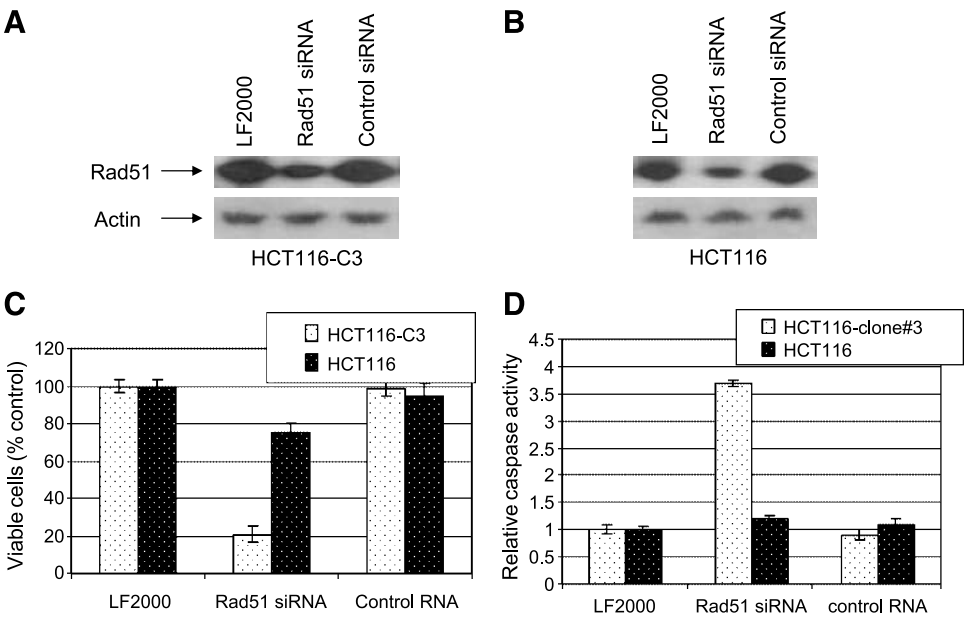

FIGURE 4. Rad51 siRNA inhibits cell proliferation and induces apoptosis in HCT116R cells. Cells were transfected with control siRNA or Rad51 siRNAs as described in Materials and Methods. Ninety-six hours later, cells were harvested. Half of the cells were used for Western blot analyses with anti-Rad51 and anti-actin antibodies (**A** and **B**), the other half of the cells were used for viability and caspase-3/7 activity assays as described in Materials and Methods (**C** and **D**).

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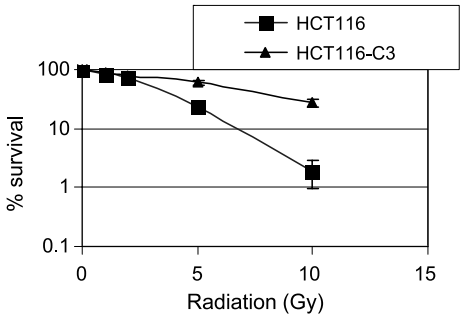

FIGURE 5. HCT116R cells are more resistant to radiation compared with HCT116 cells. On day 1, 1×10^6 cells were treated with the indicated dose of radiation. On day 2, 200 cells were plated into each well of six-well plates in triplicate. Colony formation assays were carried out as described in Materials and Methods.

Cell Lines

HCT116 cells were obtained from the American Type Culture Collection. To generate HCT116 cells resistant to TMZ/ABT-888, 10 million HCT116 cells were incubated with 0.3 mmol/L of TMZ and 10 μmol/L of ABT-888 for 3 wk. Three colonies, designated HCT116-C1, HCT116-C2, and HCT116-C3, were isolated and grown in 96-well plates, 24-well plates, and T-75 flasks sequentially to amplify the cell population. Experiments with these resistant cells were carried out after the cells were grown in the absence of TMZ/ABT-888 for 1 wk.

Western Blot Analysis

Cells from 10 cm dishes were harvested and lysed in 200 μL of buffer A [20 mmol/L Hepes (pH 7.5), 10 mmol/L NaCl, 20 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L sodium pyrophosphate, 2 mmol/L sodium vanadate, 10 mmol/L β-glycerolphosphate, and 1% NP40] on ice for 30 min. The samples were centrifuged at 12,000 × *g* at 4°C for 10 min. Cell lysates were subjected to SDS-PAGE and western analysis. Rabbit anti-BLM antibody was purchased from Bethyl Laboratories. Goat anti-ATM antibody was purchased from Abcam, Inc. Rabbit anti-PARP antibody was purchased from Cell Signaling. Anti-actin, anti-Chk1, anti-Chk2, anti-Rad51, anti-Ku70, anti-NBS1, and anti–DNA-PK antibodies were from Santa Cruz Biotechnology. Polyclonal anti-PAR antibody was from Trevigen. Western blot analysis was done using enhanced chemiluminescence detection reagents (Amersham) according to the instructions of the manufacturer.

Caspase-3 Activity Assay

Cells from each treatment condition (30,000 cells) were harvested and diluted in 100 μL culture medium and placed into a 96-well plate in triplicates. The caspase-3 activity assay was carried out with the caspase-3/7 glow assay (Promega) according to the instructions of the manufacturer.

siRNA Transfection

The on-target plus Rad51 siRNA and luciferase siRNA for control (sequence: AACGUACGCGGAAUACUUCGA) were from Dharmacon. For transfection, 0.6×10^6 cells were plated in a 10-cm dish on day 0. On day 1, 60 μL of LipofectAMINE 2000 (LF2000; Invitrogen) was added to 1.5 mL of Opti-MEM (Invitrogen) and incubated at room temperature for 5 min (solution A). Then the siRNAs were added to 1.5 mL of Opti-MEM (solution B). Solution A and solution B were mixed and incubated at room temperature for 20 min. The medium in the plate was reduced to 1 mL and the LF2000-siRNA mixture was laid onto the cells. Four hours after incubation at 37°C in a CO_2 incubator, medium was replaced with complete medium. The cells were harvested 96 h posttransfection for cell number determination, caspase assay and Western blot analysis.

AlamarBlue Cell Proliferation Assay

Cells were plated at 750 cells per well in a 96-well plate. Cells were then cotreated with TMZ and ABT-888 for 5 d. AlamarBlue assay was carried out according to the instructions of the manufacturer (Biosource, Int., Inc.). Briefly, medium was removed from the plate, and 100 μL of AlamarBlue solution (10% AlamarBlue in complete culture medium) was added to each well. After 3 h, the plate was read on an *fmax* Fluorescence Microplate Reader (Molecular Devices) using an excitation wavelength of 544 nm and an emission wavelength of 595 nm. Data were analyzed using SOFTmax PRO software (Molecular Devices). Each data point represents the average of three determinations.

γH2AX Assay

Cells were fixed with 250 μL of BD Cytofix/Cytoperm solution (BD Bioscience) at room temperature for 20 min followed by washing thrice with 1XBD Perm/Wash Solution (BD Bioscience). After blocking with 3% bovine serum albumin for 30 min, the cells were incubated with the following antibodies in Perm/Wash Solution with washes in between: mouse anti-γH2AX antibody (1:250; Upstate Biotechnologies) and Alexa 488 goat anti-mouse IgG(H+L)F(ab′)2 fragment conjugate (1:200; Invitrogen). The DNA was immunostained with 50 μg/mL of propidium iodide

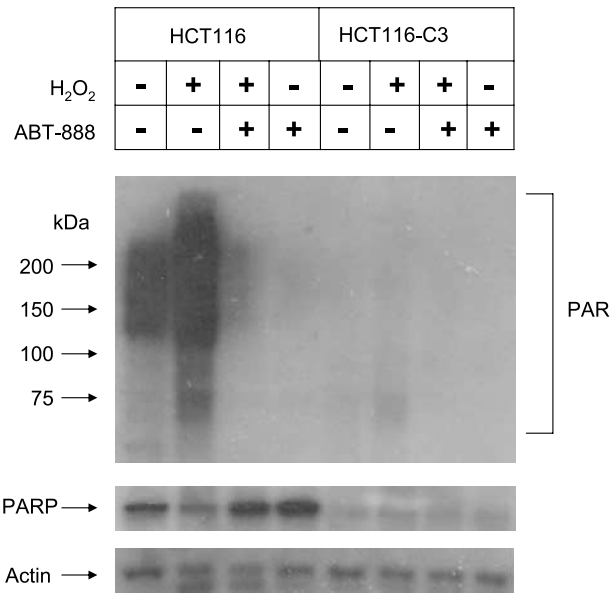

FIGURE 6. H_2O_2 induced PAR formation in parental HCT116 cells but not HCT116R cells. Cells were treated with DMSO or 5 μmol/L of ABT-888 for 30 min prior to cotreatment with 1 mmol/L of H_2O_2 with either DMSO or 5 μmol/L of ABT-888 for another 10 min. Then, the cells were harvested and subjected to Western blot analysis for PAR, PARP1, and actin, respectively.

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in staining buffer [PBS without Mg^{2+} or Ca^{2+}, 1% heat-inactivated FCS, and 0.09% (w/v) sodium azide (pH 7.4-7.6)] for 10 min in the dark. Immuno-flow cytometry was done to quantify the γH2AX signal. Each data point is the average of two values.

Microarray Analysis

Cell samples were lysed and total RNA was isolated using QIAshredder and RNeasy columns (Qiagen). Total RNA (5 μg) was used for microarray analysis. Labeled cRNA was prepared according to the microarray manufacturer's protocol and hybridized to human genome U133A 2.0 arrays (Affymetrix, Inc.). The intensity files were imported into Rosetta Resolver gene expression analysis software version 6.0 (Rosetta Inpharmatics). Resolver's Affymetrix error model was applied, and replicates were combined. Expression profiles were derived from mRNA from three independent samples for each cell line. Significant expression changes were identified using a 5% false discovery rate (39).

Colony Formation Assay

Cells were irradiated with Xrad320 at 320 kV at 1 Gy/min dose rate (Precision X-Ray). Twenty-four hours after radiation, 200 cells were seeded into six-well plates in triplicates. The cells were allowed to grow for 10 d and then fixed with methanol and stained with Giemsa (Sigma) to visualize colonies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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