BRCA1- and BRCA2-Deficient Cells Are Sensitive to Etoposide-Induced DNA Double-Strand Breaks via Topoisomerase II

Alejandro D. Treszczamsky, Lisa A. Kachnic, Zhihui Feng, Junran Zhang, Chake Tokadjian, and Simon N. Powell

Abstract
The function of BRCA1 and BRCA2 in DNA repair could affect the sensitivity of cells to cytotoxic agents, and would therefore be an important component of planning therapy for breast and ovarian cancers. Previously, both BRCA1- and BRCA2-deficient tumors were shown to be sensitive to mitomycin C, and the mechanism was presumed to be a defect in the repair of interstrand crosslinks by homologous recombination. Here, we show that both BRCA1 and BRCA2 determine the sensitivity to the cytotoxic drug, etoposide, using genetic complementation of BRCA-deficient cells. Etoposide is known to bind to topoisomerase II and prevent the resolution of the “cleavable complex,” in which one DNA duplex is passed through a second duplex. The specificity of this BRCA-dependent sensitivity was confirmed by the use of aclarubicin, which is a catalytic inhibitor of topoisomerase II and prevents the formation of the cleavable complex. In the presence of aclarubicin, the differential sensitivity of BRCA-proficient and BRCA-deficient cells was lost. Thus, etoposide requires the presence of topoisomerase II to show specific sensitization in the absence of the function of BRCA1 or BRCA2. We conclude that homologous recombination is used in the repair of DNA damage caused by topoisomerase II poisons. Overall, these results suggest that etoposide is a potentially useful drug in the treatment of BRCA-deficient human cancers.

Introduction
Mutations in BRCA1 and BRCA2 account for the majority of families with hereditary susceptibility to breast and ovarian cancer (1). Although carriers of the BRCA gene mutations are heterozygous (one normal copy and one mutated copy) within the germ line, tumorigenesis in individuals with germ line BRCA mutations requires the inactivation of the remaining wild-type allele. One important role of the BRCA proteins is in the promotion of DNA double-strand break (DSB) repair via homologous recombination (2). Loss of BRCA function results in a marked sensitivity to DNA crosslinking agents, such as mitomycin C (3). The reason for this phenotype is thought to be the requirement of homologous recombination for the repair of the interstrand crosslinks. A genetically engineered deficiency in either BRCA1 or BRCA2 results in early embryonic lethality in mice (4, 5). Cells derived from these knockout mice show increased spontaneous chromosomal aberrations and the impaired formation of Rad51 nuclear foci caused by DNA damage (2, 5). Although BRCA1-deficient cells have a more complex phenotype, including transcriptional and cell cycle checkpoint defects, both BRCA1- and BRCA2-deficient cells showed increased sensitivity to ionizing radiation, bleomycin, mitomycin C, and cisplatinum (3, 7).

Etoposide, an epipodophyllotoxin, is a widely used antitumor agent which acts by the inhibition of topoisomerase II, an enzyme that facilitates the separation of intertwined DNA duplex molecules that arise following DNA replication. Topoisomerase II introduces a transient DSB in one of the DNA duplexes, followed by passing the unbroken DNA duplex through the transient DSB. The enzyme then religates the DNA break and releases both DNA molecules. Etoposide prevents this religation step by covalently binding to topoisomerase II and creating a “frozen cleavable complex” (a stable drug–enzyme–DNA complex; ref. 8). The role of etoposide as a specific topoisomerase II “poison” was further established by showing that its activity was downstream of a step controlled by aclarubicin, a topoisomerase II catalytic inhibitor. Aclarubicin inhibits topoisomerase II before it is able to catalyze the production of these specialized DSBs. Pretreatment with aclarubicin has been previously shown to decrease etoposide-induced DSBs and its resultant cytotoxicity (9).

Here, we provide evidence that BRCA1- and BRCA2-deficient cells are more sensitive to the antineoplastic agent etoposide than their genetically complemented isogenic partner cell. Furthermore, we show that pretreatment of cells with aclarubicin (aclacinomycin A), eliminated the difference between BRCA-deficient and BRCA-proficient cells, proving that the observed etoposide sensitivity was due to the induction of the specialized DNA DSBs that arise by the action of the drug on the enzyme topoisomerase II (10). The marked sensitivity of BRCA-deficient cancer cells to specific cytotoxic agents may have important implications for the optimum systemic therapy of breast and ovarian tumors in the clinic.

Materials and Methods
Cell lines and genetic complementation. HCC1937 cells, derived from a tumor arising in a patient with early onset breast cancer and a germ line mutation in the BRCA1 gene, were obtained from the American Type Culture Collection. The tumor cells have a COOH-terminal truncating mutation (the germ line mutation) and loss of the second BRCA1 allele (11). Stable transfection of wild-type BRCA1 was achieved using an expression plasmid containing a full-length human BRCA1 cDNA inserted into the pcDNA3.1neo vector, using LipofectAMINE Plus (Life Technology) following the manufacturer’s instructions. Cells were grown as described previously (12).

BRCA2-deficient EUFA423 immortalized fibroblasts, with biallelic truncations of the COOH-terminal region of both BRCA2 genes (13), were provided by Alan D’Andrea (Dana-Farber Cancer Institute, Boston, MA). Restoration of BRCA2 wild-type status was achieved by stable transfection of an expression plasmid containing a full-length human BRCA2 cDNA.

Requests for reprints: Simon N. Powell, Department of Radiation Oncology, Washington University School of Medicine, 4511 Forest Park, St. Louis, MO 63108. Phone: 314-362-9700; Fax: 314-747-5498; E-mail: snpowell@radonc.wustl.edu.

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insert in the pcDNA3.1neo vector, again using LipofectAMINE Plus and selected for expression with 500 μg/mL of genetin (G418, Sigma).

The expression of BRCA1 protein in the complemented HCC1937 cells is shown in Fig. 1A; the expression of BRCA2 protein in the complemented EUFA423 cells is shown in Fig. 1B. Both of these cell pairs have been used in previous studies (12, 13).

**Drug treatments.** Etoposide (Sigma) was diluted to experimental concentrations from a 25 mmol/L stock in DMSO. Aclarubicin (Sigma) was prepared for the experiments using a 200 μmol/L solution in PBS and 0.32% ethanol, according to the manufacturer’s guidelines. Etoposide was applied to cells at concentrations up to 30 μmol/L (≈ 17.6 μg/mL) for 1 h and then rinsed thrice in PBS. The cells were then collected by trypsinization, resuspended in ice-cold medium, and plated in 25 cm² flasks for clonogenic survival assays. Aclarubicin was applied to cells at concentrations up to 1 μmol/L for 30 min, with or without a subsequent exposure to etoposide for 1 h.

**Colony-forming assay of cell survival.** Cell killing was measured by clonogenic survival. Cells were harvested from tissue culture flasks using trypsin at 37°C, made into a single cell suspension and were plated in triplicate into T25 flasks at various cell densities, with a target number of surviving colonies at 50 to 100 per flask. Treatment with etoposide was carried out 14 to 18 h after cell plating. After a 1-h exposure to the drug, cells were rinsed thrice with PBS, and then regular medium was added. After 8 to 10 days for EUFA423 cells, or 17 to 20 days for HCC1937 cells, methanol fixation and staining with 0.35% methylene blue was undertaken. Depending on the experiment, the surviving colonies were counted after methanol fixation and staining with 0.35% methylene blue.

**Survival assays** were done in triplicate; therefore, each datapoint represents the mean of three experiments, with error bars in the graphs depicting the SE.

![Figure 1](image_url)

**Results**

**BRCA1- and BRCA2-deficient cells show increased sensitivity to etoposide.** HCC1937 cells (BRCA1-deficient) showed increased cell sensitivity compared with the HCC1937 cells transfected with wild-type BRCA1. The effect was observed for all doses of etoposide tested (see Fig. 2A), reaching a maximum when the etoposide concentration was 30 μmol/L. The doses used in this study were within the expected range to be achieved when patients are treated with conventional doses of etoposide in the clinic. For example, 4.7 μg/mL is the average plasma level after 100 mg of i.v. etoposide, which is equal to ~8 μmol/L, based on the etoposide data sheet. BRCA1-deficient cells showed a surviving fraction 3.24-fold smaller than that observed with the BRCA1-complemented cells.

A similar result was observed for the BRCA2-deficient cells, EUFA423. These fibroblasts revealed a greater sensitivity to etoposide than the cells transfected with wild-type BRCA2. The effect was apparent with doses as low as 0.8 μmol/L and reached a 2.75-fold difference at 10 μmol/L (see Fig. 2B).

**BRCA-deficient cells are equally sensitive to aclarubicin.** The survival of the same cell line pairs to a topoisomerase II inhibitor was assessed by exposing the cells to aclarubicin at doses of 0.5 and 1 μmol/L. Aclarubicin inhibits the topoisomerase II enzyme, and prevents the formation of the cleavable complex, which occurs after the enzymatic process of topoisomerase II is completed. The purpose of these experiments was to determine whether the sensitivity of BRCA-deficient cells was dependent on the formation of the cleavable complex DSB. The survival curves (shown in Fig. 3A and B) reveal that, within the range of doses used in these experiments, the toxic effect of aclarubicin was mild and was unaffected by the BRCA status of the cell. Even at the 1-μmol/L concentration, the cell-killing induced by the topoisomerase II catalytic inhibitor was limited to ~30% (70% survival). By comparison, cell-killing induced by the topoisomerase II poisons, such as etoposide, was <1%, which implies that lethality from etoposide is a consequence of forming the complex DNA DSB.

**Pretreatment with aclarubicin abrogates the differential sensitivity of BRCA-deficient cells to etoposide.** Survival assays after treatment with etoposide were repeated by first treating the cells with increasing doses of aclarubicin for 30 min. With increasing doses of aclarubicin, the survival of all cell lines increased, but the differential sensitivity of the BRCA-deficient cells was eliminated. For the BRCA1 cell pair, using 25 μmol/L of etoposide, which had resulted in 8% and 22% survival for the BRCA1-deficient and complemented cells, respectively, a concentration of 1 μmol/L of aclarubicin increased the surviving fraction of both cell lines to 35% (Fig. 4A). Thus, the differential sensitivity of the BRCA1-deficient cell is caused by the action of etoposide in producing topoisomerase II-dependent DNA lesions.

Similarly, for the BRCA2 cell pair, increasing pretreatment doses of aclarubicin decreased the sensitivity of both cell lines to etoposide and, at 1 μmol/L of aclarubicin, the surviving fractions became similar, independent of the BRCA2 status (Fig. 4B). This cell pair is noted to be more sensitive to etoposide, and 10 μmol/L produces 1% and 3% cell survival for the BRCA2-deficient and BRCA2-complemented cells, respectively. After aclarubicin treatment, the survival of both lines increased to 7%. The implication, again, is that the sensitivity of BRCA2-deficient cells to etoposide is due to the formation of complex double-stranded lesions.
Discussion

We have shown that BRCA1- and BRCA2-deficient cells exhibited increased sensitivity to etoposide compared with isogenic cells in which the deficiency had been corrected. The use of isogenic cell pairs in these experiments is crucial because many other unknown factors could contribute to the differential sensitivity found with these drugs in nonisogenic systems. The same observation for the effect of both BRCA1 and BRCA2 deficiency provides further support to the idea that it is the DNA repair pathways regulated by these proteins which accounts for many of the common features of genomic instability found in familial breast and ovarian cancer.

Whereas sensitivity to mitomycin C and cisplatinum has been reported for BRCA1- or BRCA2-deficient cells (3, 14, 15), sensitivity to etoposide was not necessarily an expected observation. The phenotype of cells deficient in homologous recombination is to show marked sensitivity to interstrand crosslinking agents, and mild sensitivity to agents producing DSBs, such as ionizing radiation and bleomycin. In this context, etoposide produces DSBs via topoisomerase II. However, the DSBs produced by etoposide are S and G2 phase–specific. Thus, it was a reasonable hypothesis to think that the combination of a functional DSB occurring in the context of the S and G2 phases of the cell cycle might reveal the repair deficiency of homologous recombination found in BRCA1- and BRCA2-deficient cells. The experiments reported here have conclusively shown that the etoposide sensitivity of BRCA1- and BRCA2-deficient cells was due to the formation of the cleavable complex because aclorubicin abrogates the effect of etoposide, and eliminates the differential sensitivity of BRCA-deficient cells.

Previous studies (16, 17) reported decreased growth of cells containing a BRCA1 mutation after 3 days of continuous exposure to etoposide. Our results, in line with these studies, were obtained using a colony formation assay as the preferred readout for cytotoxicity, excluding an effect of cell cycle arrest. Increased etoposide sensitivity of the BRCA2-deficient Capan-1 human pancreatic cancer cell line has been reported (18). However, the authors compared its sensitivity to three other human cancer cell lines and with wild-type BRCA2 from different origins, which makes it difficult to exclude the effects of other nonspecific factors. Results with the EUFA423-BRCA2 isogenic cell pair in this study make this observation more secure. In addition, the injection of Capan-1 cells into immunodeficient mice resulted in smaller tumors following etoposide treatment compared with the tumors

![Figure 2. Clonogenic survival assays following treatment with etoposide. A, HCC1937 cells transfected with wt-BRCA1 (▲) show a 3.24-fold greater surviving fraction (compared with HCC1937 with control vector; ■) after exposure to etoposide at 30 μmol/L. B, EUFA423 cells transfected with wt-BRCA2 (▲) show a 2.75-fold greater surviving fraction (compared with EUFA423 cells with control vector; ■) after exposure to etoposide at 10 μmol/L.]

![Figure 3. Clonogenic survival assays following treatment with aclorubicin. A, BRCA1 mutant and BRCA1 wild-type HCC1937 cells show similar sensitivity to the drug in the dose range used. B, BRCA2 mutant and BRCA2 wild-type EUFA423 cells also show similar sensitivities to aclorubicin.]

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resulting from the injection of a different pancreatic cancer cell line expressing wild-type BRCA2, suggesting that the observation can be reproduced in a tumor model (19). The experiments using aclarubicin are important to emphasize the significance of the results presented in this report. The toxicity of aclarubicin in the doses used was mild and independent of BRCA function. Thus, any concern about the complex interactive effects of aclarubicin and etoposide seems unlikely because the toxic effects were dominated by etoposide. The differential sensitivity of BRCA-deficient cells to etoposide was due to the component of etoposide toxicity which operates through topoisomerase II. The combination of experiments shown in this report has clearly shown that topoisomerase II–mediated double-strand damage has a particular sensitivity to tumor cells lacking the function of the BRCA1 or BRCA2 proteins. Whether this is due to the production of DSBs at phases in the cell cycle when BRCA1/BRCA2-mediated repair was prominent, or due to other more specific interactions between BRCA1 or BRCA2 and topoisomerase II, is not yet clear (20). Given the recent report showing the interaction of BRCA1 and topoisomerase IIα and the effect of BRCA1 on topoisomerase II activity, some of the sensitivity of the BRCA1-deficient cells could be due to a direct effect on topoisomerase II. Whatever the detailed mechanism, these results have suggested that drugs such as etoposide can be specifically useful in tumors with BRCA1/BRC2 inactivations.

In summary, our results show that the increased sensitivity of BRCA1- and BRCA2-deficient cells to etoposide was due to the specific DSB created by topoisomerase II. These findings are in line with an increasing body of evidence supporting the notion that BRCA deficiency renders proliferating cells more sensitive to a specific type of DSB. The ability to determine whether or not the BRCA1/BRC2-dependent pathway of DNA repair was functional would be valuable information, as it could determine the choice of therapy in the patient.

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