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X-ray Repair Cross-Complementing Gene I Protein Plays an Important Role in Camptothecin Resistance

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ABSTRACT

X-ray repair cross-complementing gene I protein (XRCC1) in complex with DNA polymerase β, DNA ligase III, and poly(ADP-ribose) polymerase is important in the base excision repair process. Previously, we isolated camptothecin (CPT)-resistant cell lines (KB100 and KB300) from the human epidermoid carcinoma cell line KB by exposure to CPT. From these CPT-resistant cell lines, their revertants (KB100^{rev} and KB300^{rev}), which lost most of their CPT-resistant phenotype during passage in the absence of CPT, were established. In this study, we found the expression levels of XRCC1 protein in KB100 and KB300 were ≥5-fold more than in their respective revertant cell lines, whereas there was no difference in the expression of XRCC1-associated proteins such as DNA polymerase β, DNA ligase III, poly(ADP-ribose) polymerase, and apurinic/apyrimidinic endonuclease. The degree of CPT resistance was relatively correlated with the XRCC1 protein amount. We also found *XRCC1* gene amplification in CPT-resistant KB100 and KB300 cell lines. To confirm a correlation between overexpression of XRCC1 and CPT resistance, we transfected the *XRCC1* gene into KB100^{rev} and obtained two different transfected cell lines (clones 14 and 16). The expression levels of XRCC1 in the transfected cell lines were higher than in KB100^{rev} but lower than in KB100 with no difference in XRCC1-associated protein expression levels. Resistance to CPT in transfected cell lines was 2–2.5-fold higher than in KB100^{rev} in regard to growth inhibition and 4-fold higher with respect to clonogenicity. Transfected cell lines also showed increased resistance to other topoisomerase I poisons. However, the cytotoxicity of VP-16 and cisplatin was similar in both the transfected cells and KB100^{rev}. Similar to our CPT-resistant cell lines, the resistance of transfected cell lines was reversed by treatment with 3-aminobenzamide. These results indicate that CPT resistance in our cells could be partly attributable to the overexpression of XRCC1.

INTRODUCTION

CPT[2] and its analogues make up an important class of anticancer drugs. The molecular target of CPTs is topo I (1–3). Topo I makes a transient single-stranded break in a phosphodiester bond of DNA through a phosphotyrosine linkage (4, 5). CPTs bind to this topo I-DNA complex and prevent the religation step (1, 6), and recently, a binding mode for CPT was proposed on the basis of X-ray crystallographic studies (7). This topo I-DNA-cleavable complex becomes an irreversible DNA lesion by collision with DNA replication fork or RNA polymerase complex (5). The mechanisms of CPT resistance have been divided into three categories: (*a*) pretarget events such as uptake of CPT; (*b*) drug-target events such as altered topo I level, activity, and topo I mutation; and (*c*) post-target events such as cell proliferation and DNA repair/recombination. Post-target events have been shown to play an important role in the sensitivity of topo I poisons (8, 9). The repair process of the topo I-DNA complex likely impacts on the sensitivity to CPT (reviewed in Ref. 10). To date, 3′ specific tyrosyl-DNA phosphodiesterase (11), ubiquitination (12), sumonation (13), nucleotide excision repair (14, 15), and BER (16, 17) have been proposed as possible mechanisms of this repair process. But very little is known about how the cell deals with the topo I-DNA complex.

To understand the mechanisms involved in CPT cytotoxicity, two CPT-resistant cell lines, KB100 and KB300, were established by continuous selection in increasing concentrations of CPT using human epidermoid cell line KB in our laboratory (9). KB100 and KB300 cells were 300- and 500-fold resistant, respectively, in their colony-forming ability as compared with KB. After culturing in CPT-free medium, partially revertant cell lines KB100^{rev} and KB300^{rev} exhibiting respectively 2.5- and 3-fold resistance, were also isolated. It has been shown previously that both the resistant and the partially revertant cell lines have no cross-resistance to VP-16 and cisplatin. The mechanism of CPT resistance was unrelated to the mutation of topo I or uptake of CPT. The difference in sensitivity to CPT between resistant and partially revertant cell lines could not be explained by topo I alteration, because these cell lines had similar topo I levels, topo I activity, and PLDB production by CPT. Thus, these resistant and partially revertant cell lines provide a good model for studying the resistance mechanisms related to post-PLDB events. According to our previous data, the cytotoxicity of CPT in KB100 and KB300 was increased by coincubation with 3AB, an inhibitor of PARP, but not changed in revertant cell lines (9). There was no difference in PARP activity between resistant and their revertant cell lines. On the basis of this finding, we speculated that the resistance may be attributable to alteration of PARP-related DNA repair system.

XRCC1, cloned in 1990, was the first mammalian gene shown to play a role in cellular sensitivity to IR (18). The human *XRCC1* gene is 33 kb in length and encodes a 2.2-kb transcript. XRCC1 protein (69.5 kDa) is a coordinator of single-stranded DNA break and BER (reviewed in Ref. 19). XRCC1 has no known catalytic activity and serves as a scaffolding protein during BER. This protein has been shown to interact with three other proteins, DNA ligase III (20–22), DP β (23, 24), and PARP (24, 25). An additional protein, polynucleotide kinase, was found to interact with XRCC1 recently (26). XRCC1 mutant Chinese hamster ovary cells showed increased sensitivity to alkylating agents, ultraviolet-A, ultraviolet-C, IR, hydrogen peroxide, and mitomycin C (19).

In this work, we report that the XRCC1 expression levels in resistance cells are higher than in revertant cells, and the resistance to CPT was increased by transfection of this gene into KB100^{rev}. In addition, we show that the resistance of transfected cells can be reversed by treatment with a PARP inhibitor. These results suggest that XRCC1 protein is an important key factor for CPT resistance and that its action is related to the PARP-related DNA repair process.

MATERIALS AND METHODS

Cells. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The growth medium used was RPMI 1640 supplemented with 5% fetal bovine serum and 100 μg/ml of kanamycin. The CPT-resistant

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[2] The abbreviations used are: CPT, camptothecin; XRCC1, X-ray repair cross-complementing gene I protein; topo I, topoisomerase I; PARP, poly(ADP-ribose) polymerase; DP β, DNA polymerase β; APE1, apurinic/apyrimidic endonuclease; BER, base excision repair; PLDB, protein-linked DNA break; IR, ionizing radiation; VP-16, etoposide; 3AB, 3-aminobenzamide; TPT, topotecan.

KB100 and KB300 cell lines were maintained in the presence of 100 nM and 300 nM of CPT, respectively. Before each experiment, KB100 and KB300 cell lines were cultured in CPT-free medium for 3 days.

Drugs and Antibodies. CPT was provided by Dr. Zong-Chao Liu, Cancer Institute, Sun Yat-Sen University of Medical Sciences (Guangzhou, China). Cisplatin, VP-16, and 3AB were purchased from Sigma Chemical Co. (St. Louis, MO). TPT was obtained from the National Cancer Institute (Bethesda, MD). CPT, VP-16, and cisplatin were made from a 10 mM stock solution in 100% DMSO stored at −70°C. TPT was prepared from a 5.9-mM stock solution in sterile water stored at −70°C. 3AB was made from a 100-mM stock solution in 27% ethanol/PBS stored in −70°C. Monoclonal anti-topo I antibody (clone 21; IgM) was made in our laboratory, and polyclononal antiligase III antibody (TL 25) was kindly provided by Dr. Tomas Lindahl of Imperial Cancer Research Fund. Anti-PARP monoclonal antibody (SA250) was purchased from Biomol (Plymouth Meeting, PA). Antiactin (AC-40) and antiactinin (BM-75.2) monoclonal antibodies, FITC conjugated antimouse IgG, and peroxidase conjugated secondary antibodies (antimouse IgM, antimouse IgG, and antirabbit IgG) were purchased from Sigma Chemical Co. Anti-XRCC1 (33-2-5) and anti-DP β (18S) antibodies were purchased from Lab Vision (Fremont, CA). APE1 monoclonal antibody (13B8E5C2) was purchased from Novus (Littleton, CO).

DNA Constructs. The mammalian expression construct pcD2EXH, encoding human XRCC1 protein tagged at COOH-terminus with 10 histidine residues (denoted XRCC1-His), was kindly provided by Dr. Larry H. Thompson of Lawrence Livermore National Laboratory, Livermore, CA. The pcDNA3.1-XH was made by isolating the fragment, encoding XRCC1-His (denoted *XRCC1-His*), from pcD2EXH and religating with the pcDNA 3.1 vector (In Vitrogen, Carlsbad, CA).

Establishment of Stable Transfected Cell Lines. KB100^{rev} cells were transfected with pcD2EXH, pcD2E (vector control), pcDNA3.1-XH, and pcDNA3.1 (vector control) by Lipofectamin2000 (Life Technologies, Inc., Rockville, MD). Transfectants were selected in medium containing 400 μg/ml of G418 (Life Technologies, Inc.). Two stable transfectants of KB100^{rev} expressing human XRCC1-His were obtained and named clone 14 and clone 16. These two clones were maintained in the presence of 100 μg/ml of G418.

Growth Inhibition Assay. Exponentially growing cells were plated in a 24-well plate (1×10^4 cells/well), and 24 h later, were treated with drugs in triplicates. After three generation times, cells were stained with 0.5% methylene blue solution in 50% ethanol/H_2O and destained with 1% sarkosyl solution. The absorbances were measured to obtain the percentage of growth relative to untreated control. IC_{50} was defined as the concentration of drug that inhibited cell growth by 50%.

Clonogenic Assay. Mid-log phase cells were exposed to serial dilutions of drugs for one generation time (22 h) or 4 h in triplicates. After drug exposure, cells were harvested and plated in six-well plate (200 cells/well) and cultured for 8–12 generation times. The resulting colonies were stained with methylene blue solution and counted to obtain the surviving fraction (%). LC_{50} was defined as the concentration of drug to give 50% of surviving fraction.

Slot Blotting. DNA in 50% 20× SSC/H_2O solutions were spotted onto the Hybond N^+ membrane (Amersham Life Science, Piscataway, NJ) in duplicates. Membranes were denatured with a solution containing 0.5 M NaOH and 1.5 M NaCl for 30 min at room temperature and neutralized with a solution containing 1 M Tris (pH 8.0) and 1.5 M NaCl for 30 min at room temperature. Air-dried membranes were UV cross-linked to immobilize DNA. Probes were labeled with ^{32}P by random primer labeling kit (Stratagene, La Jolla, CA). For prehybridization, membranes were placed in Quick hybrid solution (Stratagene) containing 2 mg of salmon sperm DNA at 65°C for 1–2 h. Probes were added and hybridized to DNA overnight. Membranes were then washed three times with a solution containing 2× SSC and 0.5% SDS at room temperature, and washed three times again with a solution containing 0.1× SSC and 0.5% SDS at 65°C. Wrapped membranes were exposed to Kodak X-OMAT film at −70°C.

Southern Blotting. Genomic DNA was digested with desired restriction enzyme (5 units/μg DNA) overnight and electrophoresed on 1% agarose gels. Gels were transferred to Hybond N^+ membrane. These membranes were UV cross-linked to immobilize DNA. The hybridization procedure was the same as for slot blotting.

Northern Blotting. Total RNA was extracted by RNA STAT 60 (Tel-Test, Friendswood, TX). Ten μg of RNA per lane were subjected to 1.2% agarose formaldehyde gel electrophoresis and transferred to Hybond N^+ membrane. The hybridization procedure was the same as for slot blotting.

Western Blotting. Cells were washed with PBS at room temperature and detached by scraping. All of the following steps were done on ice. Cells were centrifuged and washed again with PBS. The resulting cell pellets were transferred to microtubes and resuspended in lysis buffer containing 1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors. Cells were disrupted by passing through a 21-gauge needle and incubated on ice for 30 min. After microcentrifugation at 10,000 × *g* for 30 min, the supernatant was moved to new microtubes for quantification of protein content. Proteins were electrophoresed on 7.5% or 12% acrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) using a Mini Trans-Blot cell (Bio-Rad). Filters were blocked with 5% nonfat milk in PBS containing 0.15% Tween 20 for 1 h at room temperature or overnight at 4°C. Filters were incubated with primary antibodies for appropriate times and washed with 5% nonfat milk in PBS containing 0.15% Tween 20. Secondary antibodies were added to the filters and incubated for 1 h at room temperature. After another wash, enhanced chemiluminescence (NEN, Boston, MA) was used to detect the peroxidase conjugate by exposure to X-ray film.

Confocal Microscopy. Briefly, 10^5 cells were seeded onto 22 mm × 22 mm glass coverslip in 35-mm culture dishes and incubated for overnight. Cells were fixed by 4% paraformaldyhde in PBS and then permeabilized by 0.5% Triton X-100 in PBS. To block nonspecific binding, 3% of BSA in PBS was used. XRCC1 protein was targeted by XRCC1 monoclonal antibody (clone 33-2-5) at 1:200 dilution followed by FITC-conjugated antimouse IgG at 1:100 dilution. Cytoplasm was counterstained by 0.25 μg/ml of rhodamine phalloidin (Molecular Probes, Eugene, OR). Cells were sealed in antifade reagent (Molecular Probes). Confocal micrographs were scanned by laser scan confocal microscope, LSM 510 (Zeiss).

RESULTS

XRCC1 and Its Associated Protein Expression Levels in CPT-resistant and Revertant Cell Lines. XRCC1 expression levels in KB100 and KB300 were shown to be higher than in their respective revertant cell lines (Fig. 1*A*). The degree of sensitivity to CPT in KB, resistant, and partially revertant cell lines correlated with XRCC1 expression levels. The largest amount of XRCC1 was found in KB300 followed by KB100. These CPT-resistant cell lines had 5–10-fold more XRCC1 protein than their revertant cell lines. KB100^{rev} and KB300^{rev} with similar sensitivities to CPT were similar in their XRCC1 expression. KB had less XRCC1 protein as compared with revertant cell lines. There was no difference in the expression levels of XRCC1-associated proteins, such as DP β, PARP, ligase III, and APE1, which is also involved in the BER process in resistance, and their revertant cell lines. Confocal microscopy data (Fig. 1*B*) demonstrate the difference in XRCC1 expression in the five cell lines. XRCC1 was localized in nuclear but not in the nucleolus.

KB100 and KB300 Cell Lines Have More *XRCC1* DNA and RNA Than Their Revertants. To elucidate the mechanism responsible for the differences in XRCC1 expression between CPT-resistant and their revertant cell lines, genomic DNA extracted from the five cell lines including KB were analyzed by slot blotting (Fig. 2*A*). DNA, 2.5 μg and 0.5 μg, were loaded onto the membrane in duplicates and hybridized with *XRCC1* probe (1.9 kb). This blot showed that KB100 and KB300 had ~5 times more *XRCC1* DNA than their revertants and 10 times more than KB but no difference between KB100 and KB300 cell lines. These results indicate that gene amplifications occurred in CPT-resistant cell lines. The southern blot data of KB100 and KB100^{rev} cell lines showed that amplification was through the entire *XRCC1* coding sequence (Fig. 2*B*). This *XRCC1* sequence was resistant to *Hpa*II but sensitive to *Msp*I restriction enzyme action in both cell lines. This suggests that *XRCC1* gene be highly methylated because *Hpa*II is unable to cut methylated DNA. The Northern blot data of the five cell lines were similar to that of Western blot

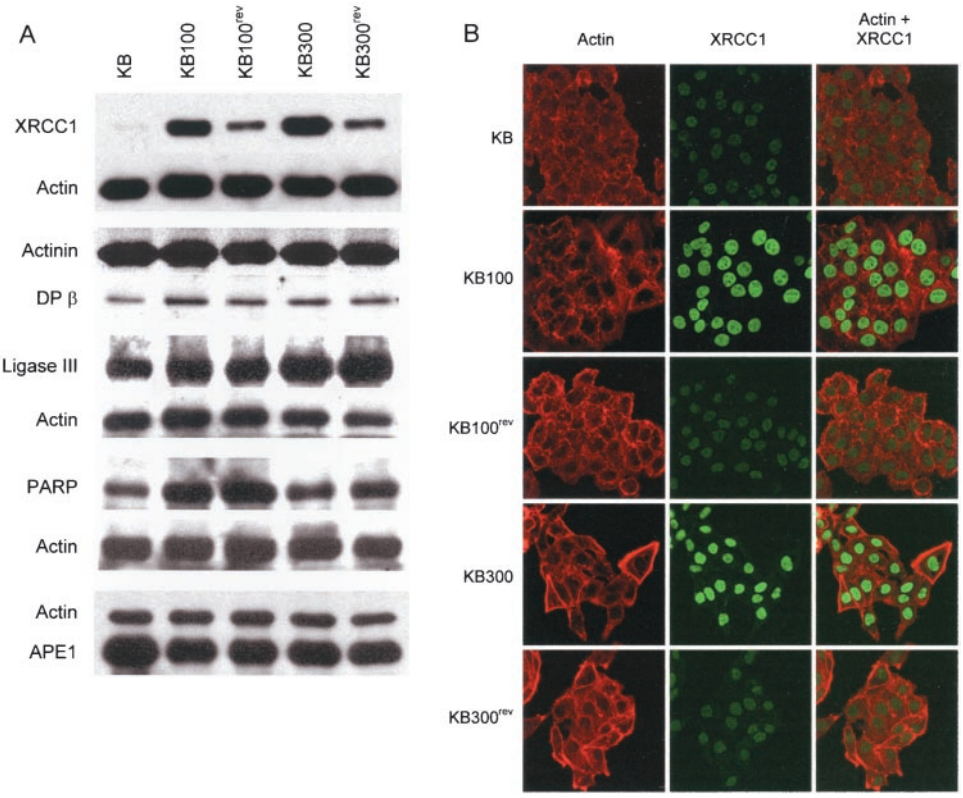

Fig. 1. XRCC1 and its associated protein expression levels in KB, CPT-resistant, and revertant cell lines. *A,* Western blot analysis. Total cell lysates were analyzed by Western blot using specific antibodies as described in "Materials and Methods." *B,* confocal micrographs of XRCC1 protein. Cells were stained with rhodamine phalloidine or/and XRCC1 monoclonal antibody (33–2-5) and imaged using Zeiss LSM 510 laser scan confocal microscope at ×63 objective.

(Fig. 2*C*). The largest amount of *XRCC1* RNA was found in KB300, and CPT-resistant cell lines had about 5–10 times more *XRCC1* RNA than their revertant cell lines.

Establishment of Stable *XRCC1-His* Transfected KB100^{rev} Cell Lines (KB100^{rev}-X). The mammalian expression construct pcD2EXH (20) has SV40 early promoter, and the pcDNA3.1-XH has CMV promoter. These two constructs were transfected into KB100^{rev}, and 1 day later, the XRCC1 expression levels were checked in transient condition by Western blotting. Transfectants with pcDNA3.1-XH resulted in a higher expression of XRCC1 than with pcD2EXH (data not shown). By selection with G418, clone 14 and clone 16 were isolated from pcD2EXH-transfected cells. However, we could not get any clone from pcDNA3.1-XH-transfected cells. XRCC1 expression levels of clone 14 and clone 16 were 2–2.5-fold higher than in KB100^{rev} but lower than in the KB100 cell (Fig. 3). The levels of topo I and XRCC1-associated proteins were not changed by transfection. The growth rates of clone 14 and clone 16 were similar to that of KB100^{rev}. To confirm the independence of these two clones, genomic DNA of KB100^{rev}, clone 14, and clone 16 were digested with *Pst*I and analyzed by Southern blotting using the *XRCC1* probe (Fig. 4). Clone 14 and clone 16 exhibited different *XRCC1* gene fragmentation patterns indicating that they were indeed different clones.

KB100^{rev}-X Cell Lines Are More Resistant to CPT and Its Analogue, TPT, Than KB100^{rev}. The growth inhibition and colony-forming ability of transfected cell lines were assessed after treatment with CPT. By transfecting the *XRCC1-His* gene into KB100^{rev}, the resistance to CPT was increased 2–2.5-fold in regard to IC_{50} (Fig. 5*A*). For clonogenic assay, we used two different incubation times with CPT, one generation time (22 h) and 4 h. In both cases, transfected cells showed a higher surviving fraction than either vector control or KB100^{rev} (Fig. 5, *B* and *C*). LC_{50} obtained from the one-generation time incubation data (Fig. 5*B*) showed that KB100^{rev}-X cell lines had ~4-fold higher values when compared with vector control and KB100^{rev}. The two transfected cell lines were also resistant to TPT in terms of colony-forming ability (Fig. 6).

KB100^{rev}-X and KB100^{rev} Cell Lines Have Similar Sensitivity to Cisplatin and VP-16. To address whether this impact of XRCC1 expression is specific to topo I poisons, cytotoxicities of VP-16, a topoisomerase II inhibitor, and cisplatin, a DNA intercalating agent, were examined in the transfected cell lines (Fig. 7). There was no difference in sensitivities to these two drugs in terms of growth and colony-forming ability between KB100^{rev} and KB100^{rev}-X. In addition, the expression levels of excision repair cross-complementing gene I, a marker of cisplatin resistance (27), were the same in KB, CPT-resistant, and revertant cell lines (data not shown).

Resistance of KB100^{rev}-X Cell Lines Were Decreased by Treatment with PARP Inhibitor, 3AB. Previous studies of our laboratory showed that coincubation with 3AB, an inhibitor of PARP, enhanced the sensitivity to CPT in KB100 but not in KB100^{rev}. To examine whether this feature of resistance is related to XRCC1, vector control and KB100^{rev}-X cell lines were exposed to 1 mM of 3AB and 100 nM of CPT for 4 h and analyzed by clonogenic assay. Because of the toxicity of 3AB, the exposure time was limited to 4 h. Under this condition, the maximum killing fraction was ~50%, and the difference between vector control and transfected cell lines was best appreciated at 100 nM of CPT. Two transfected cell lines treated with 3AB showed enhanced sensitivity to CPT as compared with nontreated cells (Fig. 8). Vector control cell line showed no difference between 3AB-treated and nontreated cells. Therefore, CPT resistance induced by XRCC1 can be reversed by 3AB treatment. This results suggest that XRCC1 effect on CPT resistance is mediated through PARP action.

DISCUSSION

Because of the clinical importance of CPTs, its resistance mechanisms have been studied extensively by using various CPT-resistant

cell lines (28–34). In a previous study with CPT-resistant (KB100 and KB300) and revertant (KB100^{rev} and KB300^{rev}) cell lines, we proposed that a mechanism that is related to the post-PLDB formation could be involved in resistance phenotype. A nuclear enzyme PARP (113 kDa)-related biochemical event was a key factor in this resistance phenotype, because coincubation with a specific inhibitor of PARP, 3AB, enhanced CPT cytotoxicity in resistant cell lines without affecting the partial revertant cell lines (9). This reversal of CPT resistance was also observed in cells treated with isoquinolinediol, which is a PARP inhibitor structurally different from 3AB but not in the cells treated with benzoic acid, a compound structurally similar to 3AB without PARP inhibitory activity.[3] Activation of PARP is one of the immediate cellular responses to DNA damage as inflicted by alkylating agents, IR, or oxidants (35, 36). This highly conserved enzyme uses nicotinamide adenine dinucleotide as substrate to carry out post-translational modifications of a number of nuclear proteins, including itself, with poly(ADP-ribose). PARP is also involved in the BER (19, 37). With this information, we decided to investigate the possibility of BER involvement in the CPT resistance phenotype. First of all, expression levels of five proteins involved in BER were tested in our cell lines. Surprisingly, XRCC1 protein levels in resistant cell lines were at least 5-fold higher than in revertant cell lines, whereas expression levels of other proteins such as DP β, ligase III, PARP, and APE1 were similar in resistant and revertant cell lines.

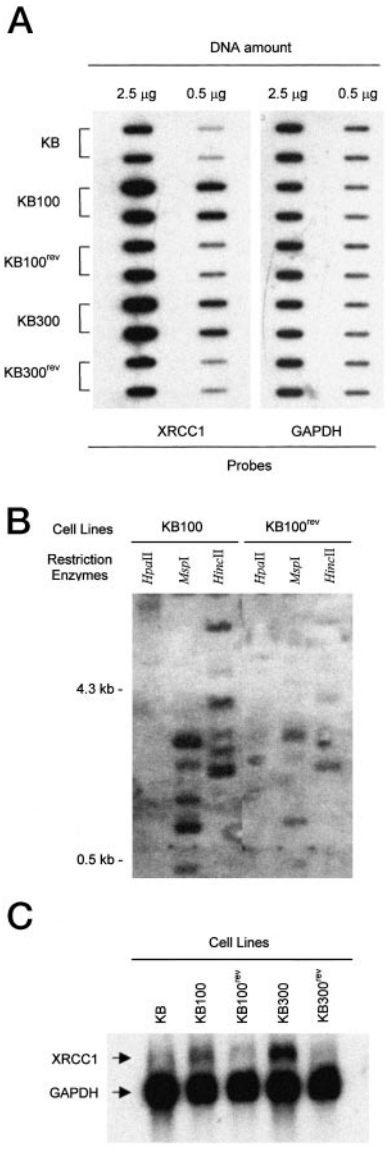

Fig. 2. Comparison of *XRCC1* DNA and RNA levels between CPT-resistant and revertant cell lines. *A*, *XRCC1* gene amplification in CPT-resistant cell lines. Described amounts of DNA from KB, resistant, and revertant cell lines were loaded onto the membrane and hybridized with ^{32}P-labeled *XRCC1* probe (1.9 kb). For control, the membrane was stripped of bound probe and rehybridized with ^{32}P-labeled *GAPDH* probe. *B*, Southern blot analysis of *XRCC1* gene in KB100 and KB100^{rev} cell lines. Ten μg of DNA were digested with described restriction enzymes (5 units/μg DNA) and size-fractioned on 1% agarose gel. The blot was hybridized with ^{32}P-labeled *XRCC1* probe. *C*, Northern blot analysis of *XRCC1* transcripts. Total RNA was isolated and size fractioned on 1.2% agarose/formaldehyde gel. The blot was hybridized with ^{32}P-labeled *XRCC1* and *GAPDH* probes.

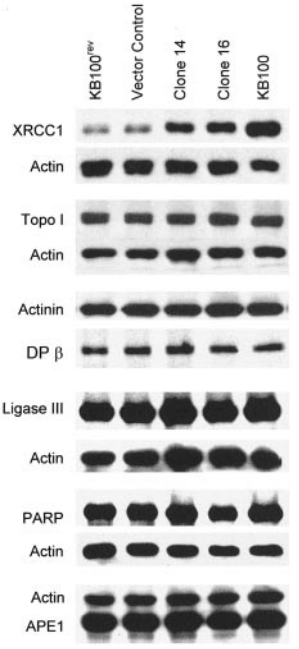

Fig. 3. Topo I, XRCC1, and its associated protein expression levels in KB100, KB100^{rev}, and transfected cell lines. Total cell lysates were analyzed by Western blot using specific antibodies as described in "Materials and Methods."

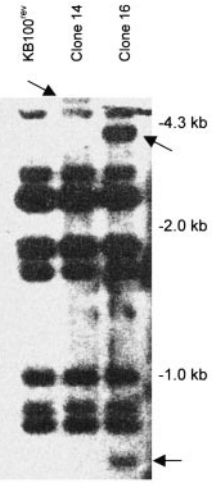

Fig. 4. *XRCC1* gene fragmentation patterns showing independence of two transfected cell lines. Ten μg of DNA from KB100^{rev} and two transfected cell lines were digested with *Pst*I and analyzed by Southern blotting using ^{32}P-labeled *XRCC1* probe. *Arrows* indicate extra fragments of *XRCC1* caused by gene insertion.

[3] David R. Beidler and Yung-chi Cheng, unpublished observations.

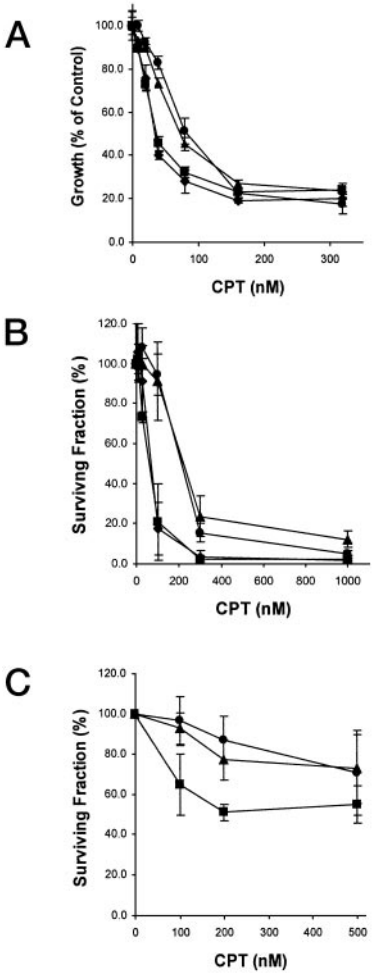

Fig. 5. Determination of CPT sensitivity in KB100^{rev} (♦), vector control (■), clone 14 (▲), and clone 16 (●) cell lines. *A*, growth inhibition induced by CPT. Mid-log phase cells were exposed to various concentration of CPT for three generation times (66 h). Data presented are means from triplicate samples; *bars*, ± SD. *B* and *C*, colony-forming ability of cells treated with CPT for one generation time (*B*) or 4 h (*C*). Data presented are means from three independent experiments; *bars*, ±SD.

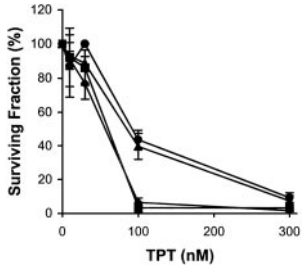

Fig. 6. Determination of TPT sensitivity in KB100^{rev} (♦), vector control (■), clone 14 (▲), and clone 16 (●) cell lines. Cells were treated with TPT for one generation time and the viability was obtained using the clonogenic assay. Data presented are means from three independent experiments; *bars*, ±SD.

To test whether XRCC1 was truly related to our resistance phenotype, the *XRCC1* gene was transfected into KB100^{rev}. The constructs (pcD2EXH and pcDNA3.1-XH) for transfection have a histidine tag at the COOH-terminus of XRCC1, and this COOH-terminal histidine tag has been shown to have no effect on the XRCC1 function (21). As expected, cells transfected with pcDNA3.1-XH expressed a higher level of XRCC1 than cells with pcD2EXH in transient condition given that the CMV promoter of pcDNA3.1-XH was stronger than the SV40 promoter of pcD2EXH. However, none of the pcDNA3.1-XH-transfected cells survived the selection time using G418. Furthermore, two clones transfected with pcD2EXH had only 2–2.5-fold more XRCC1 protein than KB100^{rev}. There were two possible explanations for why the transfected cell lines did not express similar or higher levels of XRCC1 as compared with KB100. First, the continuously overexpressed XRCC1 could be toxic to KB100^{rev}. It has been shown that XRCC1-deficient cells had abnormal biological functions such as BER and maintenance of genomic stability. *XRCC1*−/− knockout proved to be lethal in mice. Abnormally high levels of XRCC1 protein may also disturb the normal efficiency of XRCC1 function (19). It seems likely that the amount of XRCC1 protein is well regulated within a specific range by an unknown mechanism. So, it is possible that KB100^{rev} cells could not maintain the very high levels of XRCC1 protein suddenly introduced by transfection. Secondly, other factors could be involved to stabilize the higher expressed XRCC1 in the KB100 cell line. Although the expression levels of XRCC1 in two transfected cell lines were lower than that of KB100, two transfected cell lines showed clearly increased resistance to CPT by growth inhibition and clonogenic assay. It is likely that XRCC1 is not the sole determinant in the resistance of KB100, but we believe that the modest levels of resistance generated in transfected cell lines is attributable to the lower levels of XRCC1 protein as compared with KB100. Some interesting results were that growth and colony-forming inhibition profile of CPT in KB100^{rev} and vector control cell lines

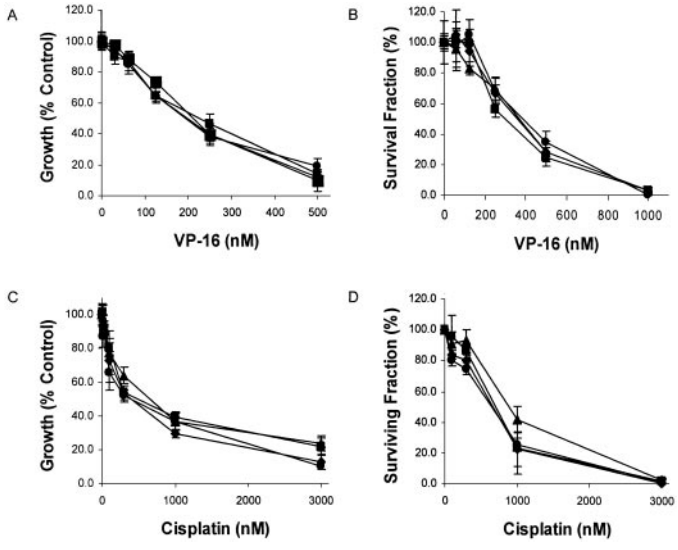

Fig. 7. Determination of sensitivity to VP-16 and cisplatin in KB100^{rev} (♦), vector control (■), clone 14 (▲), and clone 16 (●) cell lines. *A* and *C*, growth inhibition induced by VP-16 (*A*) and cisplatin (*C*). Data presented are means from triplicate samples; *bars*, ± SD. *B* and *D*, colony-forming ability of cells treated with described concentration of VP-16 (*B*) and cisplatin (*D*) for one generation time. Data presented are means from three independent experiments; *bars*, ±SD.

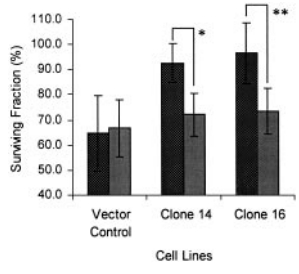

Fig. 8. Reversion of resistance in transfected cell lines by 3AB. Mid-log phase cells were exposed to 100 nM of CPT and 1 mM of 3AB for 4 h before cell survival analysis using clonogenic assay. Data presented are means from three independent experiments; *bars*, ± SD. ■ 3AB−, ▩ 3AB+; *, $P < 0.05$; **, $P < 0.1$ by Student's *t* test.

were similar, whereas those of two transfected cell lines were different. In the cases of KB100^{rev} and vector control cell lines, 80% of cell growth was inhibited at the 80% of cell killing dose (~200 nM CPT), and 70% of cell growth was inhibited at the 60% of cell killing dose (~80 nM CPT). But only 30% of two transfected cells were killed at the 80% of cell growth inhibition dose (200 nM CPT). According to the previous work of our laboratory (9), the LC_{50}:IC_{50} (the ratio of the 50% killing dose *versus* 50% growth inhibition dose) is a useful way to characterize CPT resistance. The LC_{50}:IC_{50} values of KB, KB100^{rev}, and KB300^{rev} were all ~1, whereas those of KB100 and KB300 were ~10, suggesting that these CPT-resistant cells can survive at growth-inhibitory doses, whereas revertant cells cannot. In the case of clones 14 and 16, LC_{50}:IC_{50} values were between 3 and 4. These results indicate that the phenotype of two transfected cell lines become more similar to that of resistant cell lines by increasing the amount of XRCC1.

Slot blotting (Fig. 2*A*) showed that the high expression of XRCC1 resulted from amplified DNA levels. DNA amplification is a flexible, reversible genomic change allowing rapid evolution under stress and escape from growth inhibition (38). We hypothesize that KB cells amplified the *XRCC1* gene as an adaptive response to cytotoxicity of CPT. Gene amplification in part is responsible for the different expression levels of XRCC1 in resistant cell lines.

KB100^{rev} cells showed increased resistance to not only CPT but also its analogues, TPT (Fig. 6) and SN38 (data not shown), by insertion of the *XRCC1* gene. However, there was no change in the sensitivity to VP-16 and cisplatin in KB100^{rev} and KB100^{rev}-X cell lines (Fig. 7). Therefore, we believe that XRCC1 is linked specifically to topo I-mediated DNA damage.

Potentiation of cytotoxicity by coincubation with PARP inhibitors has been observed in alkylating agents, IR, cisplatin, TPT, and CPT (35, 39–41). This activity of the PARP inhibitor (3AB) was also observed in KB100 (9) and KB100^{rev}-X (Fig. 8) but not in KB100^{rev} (9). It has been reported that the PARP inhibitors may also interfere with normal cellular metabolism by affecting other nicotinamide adenine dinucleotide-dependent processes. Although it cannot be ruled out that 3AB might potentiate CPT cytotoxicity through mechanisms other than PARP inhibition, our results (Fig. 8) strongly suggest that the XRCC1 effect on CPT resistance is mediated through PARP action. Differing amounts of XRCC1 protein may account for different effects of 3AB on KB100, KB100^{rev}-X, and KB100^{rev} cell lines, because the expression levels of PARP in these cell lines were the same (Fig. 1*A*), whereas XRCC1 protein levels were different. A threshold amount of XRCC1 may be required for PARP activation after DNA damage induced by CPT.

XRCC1 and PARP are involved in BER. As we know, two groups reported on the possibility of BER involvement in the CPT-induced cytotoxicity. One group observed that XRCC1-mutant Chinese hamster ovary cells exhibiting supersensitivity to CPT became resistant by transfection with the *XRCC1* gene (16). A second group observed that NU1025, a PARP inhibitor, increased CPT-induced DNA strand breakage and cytotoxicity by a similar amount. In contrast to its effect on CPT cytotoxicity, NU1025 had no effect on VP-16 (topo II poison)-mediated cytotoxicity or DNA strand breakage (17). Our results also showed that XRCC1 had no relationship with VP-16.

The repair process of CPT-induced DNA damage is now recognized as an important mechanism of resistance. On the basis of our study, XRCC1 plays a key role in the repair of CPT-induced DNA damage. XRCC1 may serve as a scaffolding protein that recognizes the topo I-linked DNA breaks and brings several proteins together for the purpose of DNA repair, thereby preventing double-stranded DNA breaks. Therefore, a detailed mechanistic study about this repair process and their clinical significance need additional investigation. The combined use of CPT analogues together with PARP inhibitors for cancer treatment should be explored.

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REFERENCES

1. Hsiang, Y. H., Hertzberg, R., Hecht, S., and Liu, L. F. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem., *260:* 14873–14878, 1985.
2. Liu, L. F. DNA topoisomerase poisons as antitumor drugs. Annu. Rev. Biochem., *58:* 351–375, 1989.
3. Schneider, E., Hsiang, Y. H., and Liu, L. F. DNA topoisomerases as anticancer drug targets. Adv. Pharmacol., *21:* 149–183, 1990.
4. Pommier, Y., Pourquier, P., Fan, Y., and Strumberg, D. Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. Biochim. Biophys. Acta, *1400:* 83–106, 1998.
5. Liu, L. F., Desai, S. D., Li, T. K., Mao, Y., Sun, M., and Sim, S. P. Mechanism of action of camptothecin. Ann. N. Y. Acad. Sci., *922:* 1–10, 2000.
6. Hsiang, Y. H., Lihou, M. G., and Liu, L. F. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. Cancer Res., *49:* 5077–5082, 1989.
7. Redinbo, M. R., Stewart, L., Kuhn, P., Champoux, J. J., and Hol, W. G. Crystal structures of human topoisomerase I in covalent and noncovalent complexes with DNA. Science (Wash. DC), *279:* 1504–1513, 1998.
8. Larsen, A. K., and Skladanowski, A. Cellular resistance to topoisomerase-targeted drugs: from drug uptake to cell death. Biochim. Biophys. Acta, *1400:* 257–274, 1998.
9. Beidler, D. R., Chang, J. Y., Zhou, B. S., and Cheng, Y. C. Camptothecin resistance involving steps subsequently to the formation of protein linked DNA breaks in human camptothecin-resistant KB cell lines. Cancer Res., *56:* 345–353, 1996.
10. Pourquier, P., and Pommier, Y. Topoisomerase I-mediated DNA damage. Adv. Cancer Res., *80:* 189–216, 2001.
11. Pouliot, J. J., Yao, K. C., Robertson, C. A., and Nash, H. A. Yeast gene for a tyr-DNA phosphodiesterase that repairs topoisomerase I complexes. Science (Wash. DC), *286:* 552–555, 1999.
12. Desai, S. D., Liu, L. F., Vazquez-Abad, D., and D'Arpa, P. Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. J. Biol. Chem., *272:* 24159–24164, 1997.
13. Mao, Y., Sun, M., Desai, S. D., and Liu, L. F. Sumo-1 conjugation to topoisomerase I: a possible repair response to topoisomerase-mediated DNA damage. Proc. Natl. Acad. Sci. USA, *97:* 4046–4051, 2000.
14. Fujimori, A., Gupta, M., Hoki, Y., and Pommier, Y. Acquired camptothecin resistance of human breast cancer MCF-7/C4 cells with normal topoisomerase I and elevated DNA repair. Mol. Pharmacol., *50:* 1472–1478, 1996.
15. Sastry, S., and Ross, B. M. Mechanisms for the processing of a frozen topoisomerase-DNA conjugate by human cell-free extracts. J. Biol. Chem., *273:* 9942–9950, 1998.
16. Barrows, L. R., Holden, J. A., Anderson, M., and D'Arpa, P. The CHO XRCC1 mutant, EM9, deficient in DNA ligase III activity, exhibits hypersensitivity to camptothecin independent of DNA replication. Mutat. Res., *408:* 103–110, 1998.
17. Bowman, K. J., Newell, D. R., Calvert, A. H., and Curtin, N. J. Differential effects of the poly (ADP-ribose) polymerase (PARP) inhibitor NU1025 on topoisomerase I and II inhibitor cytotoxicity in L1210 cells *in vitro*. Br. J. Cancer, *84:* 106–112, 2001.
18. Thompson, L. H., Brookman, K. W., Jones, N. J., Allen, S. A., and Carrano, A. V. Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand break repair and sister chromatid exchange. Mol. Cell. Biol., *10:* 6160–6171, 1990.
19. Thompson, L. H., and West, M. G. XRCC1 keeps DNA from getting stranded. Mutat. Res., *459:* 1–18, 2000.
20. Caldecott, K. W., Mckeown, C. K., Tucker, J. D., Ljungquist, S., and Thompson, L. H. An interaction between the mammalian DNA repair protein XRCC1 and DNA ligase III. Mol. Cell. Biol., *14:* 68–76, 1994.
21. Caldecott, K. W., Tucker, J. D., Stanker, L. H., and Thompson, L. H. Characterization of the XRCC1-DNA ligase III complex *in vitro* and its absence from mutant hamster cells. Nucleic Acids Res., *23:* 4836–4843, 1995.
22. Nash, R. A., Caldecott, K. W., Barnes, D. E., and Lindahl, T. XRCC1 protein interacts with one of two distinct forms of DNA ligase III. Biochemistry, *36:* 5207–5211, 1997.
23. Kubota, Y., Nash, R. A., Klungland, A., Schär, P., Barnes, D. E., and Lindahl, T. Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase β and the XRCC1 protein. EMBO J., *15:* 6662–6670, 1996.
24. Caldecott, K. W., Aoufouchi, S., Johnson, P., and Shall, S. XRCC1 polypeptide interacts with DNA polymerase β and possibly poly (ADP-ribose) polymerase, and DNA ligase III is a novel molecular 'nick-sensor' *in vitro*. Nucleic Acids Res., *24:* 4387–4394, 1996.
25. Masson, M., Niedergang, C., Schreiber, V., Muller, S., Murcia, J. M., and Murcia, G. XRCC1 is specifically associated with poly (ADP-ribose) polymerase and negatively regulates its activity following DNA damage. Mol. Cell. Biol., *18:* 3563–3571, 1998.
26. Whitehouse, C. J., Taylor, R. M., Thistlethwaite, A., Zhang, H., Karimi-Busheri, F., Lasko, D. D., Weinfeld, M., and Caldecott, K. W. XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair. Cell, *104:* 107–117, 2001.

27. Britten, R. A., Liu, D., Tessier, A., Hutchison, M. J., and Murray, D. ERCC1 expression as a molecular marker of cisplatin resistance in human cervical tumor cells. Int. J. Cancer, *89:* 453–457, 2000.
28. Andoh, T., Ishii, K., Suzuki, Y., Ikegmi, Y., Kusunoki, Y., Takemoto, Y., and Okada, K. Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. Proc. Natl. Acad. Sci. USA, *84:* 5565–5569, 1987.
29. Chang, J. Y., Dethlefsen, L. A., Barley, L. R., Zhou, B. S., and Cheng, Y. C. Characterization of camptothecin-resistant Chinese hamster lung cells. Biochem. Pharmacol., *43:* 2443–2452, 1992.
30. Eng, W. K., Mccabe, F. L., Tan, K. B., Mattern, M. R., Hofmann, G. A., Woessner, R. D., Hertzberg, R. P., and Johnson, R. K. Development of a stable camptothecin-resistant subline of P388 leukemia with reduced topoisomerase I content. Mol. Pharmacol., *38:* 471–480, 1990.
31. Gupta, R. S., Gupta, R., Eng, B., Lock, R. B., Ross, W. E., Hertzberg, R. P., Caranfa, M. J., and Johnson, R. K. Camptothecin-resistant mutants of Chinese hamster ovary cells containing a resistant form of topoisomerase I. Cancer Res., *48:* 6404–6410, 1988.
32. Rubin, E., Pantazis, P., Bharti, A., Toppmeyer, D., Giovanella, B., and Kufe, D. Identification of a mutant human topoisomerase I with intact catalytic activity and resistance to 9-nitro-camptothecin. J. Biol. Chem., *269:* 2433–2439, 1994.
33. Komatani, H., Morita, M., Sakaizumi, N., Fukasawa, K., Yoshida, E., Okura, A., Yoshinari, T., and Nishimura, S. A new mechanism of acquisition of drug resistance by partial duplication of topoisomerase I. Cancer Res., *59:* 2701–2708, 1999.
34. Urasaki, Y., Laco, G. S., Pourquier, P., Takebayashi, Y., Kohlhagen, G., Gioffre, C., Zhang, H., Chatterjee, D., Pantazis, P., and Pommier, Y. Characterization of a novel topoisomerase I mutation from a camptothecin-resistant human prostate cancer cell line. Cancer Res., *61:* 1946–1969, 2001.
35. Bürkle, A. Poly (ADP-ribosyl)ation, a DNA damage-driven protein modification and regulator of genomic instability. Cancer Lett., *163:* 1–5, 2001.
36. Pleschke, J. M., Kleczkowska, H. E., Strohm, M., and Althaus, F. R. Poly (ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. J. Biol. Chem., *275:* 40974–40980, 2000.
37. Lindahl, T., and Wood, R. D. Quality control by DNA repair. Science (Wash. DC), *286:* 1897–1905, 1999.
38. Hasting, P. J., Bull, H. J., Klump, J. R., and Rosenberg, S. M. Adaptive amplification: an inducible chromosomal instability mechanism. Cell, *103:* 723–731, 2000.
39. Chatterjee, S., Cheng, M. F., Trivedi, D., Petzold, S. J., and Berger, N. A. Camptothecin hypersensitivity in poly(adenosine diphosphate-ribose) polymerase-deficient cell lines. Cancer Commun., *1:* 389–394, 1989.
40. Delaney, C. A., Wang, L. Z., Kyle, S., White, A. W., Calvert, A. H., Curtin, N. J., Durkacz, B. W., Hostomsky, Z., and Newell, D. R. Potentiation of temozolomide and topotecan growth inhibition and cytotoxicity by novel poly(adenosine diphosphoribose) polymerase inhibitors in a panel of human tumor cell lines. Clin. Cancer Res., *6:* 2860–2867, 2000.
41. Chen, G., and Zeller, W. J. Reversal of acquired cisplatin resistance by nicotinamide *in vitro* and *in vivo*. Cancer Chemother. Pharmacol., *33:* 157–162, 1993.

X-ray Repair Cross-Complementing Gene I Protein Plays an Important Role in Camptothecin Resistance

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