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Acquired Camptothecin Resistance of Human Breast Cancer MCF-7/C4 Cells with Normal Topoisomerase I and Elevated DNA Repair

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SUMMARY

A camptothecin (CPT)-resistant cell line (MCF-7/C4) was established from MCF-7 cells by mutagenic treatment with methylmethanesulfonate and selection with CPT. MCF-7/C4 is 30-fold resistant to CPT and is cross-resistant to UV and *cis*-dichlorodiammineplatinum(II) but not to VP-16 or ionizing radiation. Topoisomerase I (top1)-mediated cleavable complexes in the presence of CPT, measured by oligonucleotide assay and by alkaline elution, were similar in both cell lines. Other top1 parameters such as top1 protein, RNA levels, and DNA relaxation were also similar in both cell lines. Thus, CPT resistance is not due to alterations in top1 activity but is caused by changes in the downstream pathways from the top1-induced damage. Both cell lines had similar doubling time (22 hr), but MCF-7/C4 cells showed reduced S-phase fraction in the absence of CPT and reduced G_2 delay after CPT treatment. *p53*, *GADD45*, and *p21*$^{WAF1/CIP1}$ were induced similarly by CPT in both cell lines. The overall repair capacity estimated by the ability of cells to reactivate UV-damaged pSV-CAT plasmid was increased in MCF-7/C4 cells. These observations suggest that enhanced DNA repair is one of the factors involved in CPT resistance.

DNA top1 is an essential enzyme that relaxes DNA torsional tension during replication, transcription, recombination, and repair (see Refs. 1 and 2 for reviews). Top1-mediated cleavable complexes are DNA SSBs with the enzyme linked to the 3′-phosphoryl end of the DNA via a phosphotyrosine linkage. CPT and its derivatives poison top1 by inhibiting the religation of cleavable complexes (3, 4). Topotecan, CPT-11, and 9-aminocamptothecin have been introduced recently in cancer chemotherapy and are active in carcinomas (5, 6).

Because top1 exists in both normal and cancerous cells, it is important to understand the mechanisms of selectivity and resistance to top1-targeted drugs (top1 poisons). CPTs induce S-phase-specific DNA damage as a result of collisions of DNA replication forks into cleavable complexes (3, 4). Thus, the higher the top1 and the more active DNA replication, the greater DNA damage and cytotoxicity. This probably is why one of the most common alterations in CPT-resistant cells is decreased top1 activity. In some cases, top1 mutations also can render the enzyme insensitive to CPT (see Refs. 2 and 7 for reviews).

We have observed recently that top1 and DNA damage levels are not always correlated with CPT cytotoxicity (8) in the panel of colon carcinoma cell lines of the National Cancer Institute anticancer drug screen (9). Kauh *et al.* (10) also found that *SCT1* mutants suppress the CPT sensitivity of yeast cells that express wild-type top1. Thus, the influence of defective pathways downstream from the cleavable complexes (including apoptosis, cell-cycle checkpoints, and DNA repair) may play a key role in the selectivity of CPTs toward cancer cells (7). The aim of the present study was to generate CPT-resistant cells with normal cleavable complexes to provide new cellular systems to elucidate the cytotoxicity/resistance pathways downstream from the top1 cleavable complexes and DNA damage induced by CPT.

Materials and Methods

Cell cultures. Human breast cancers (MCF-7) purchased from the American Type Culture Collection (Rockville, MD) were grown in Roswell Park Memorial Institute 1640 medium (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum (Intergen, Purchase, NY), 2 mM L-glutamine (Life Technologies, Grand Island, NY), 1 mM nonessential amino acids, 0.1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human leukemia

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ABBREVIATIONS: top1, topoisomerase I; CAT, chloramphenicol acetyltransferase; CDDP, *cis*-dichlorodiammineplatinum(II); CPT, 20-(*S*)-camptothecin; MMS, methylmethanesulfonate; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SSB, single-strand break; SSC, standard saline citrate; VP-16, etoposide; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

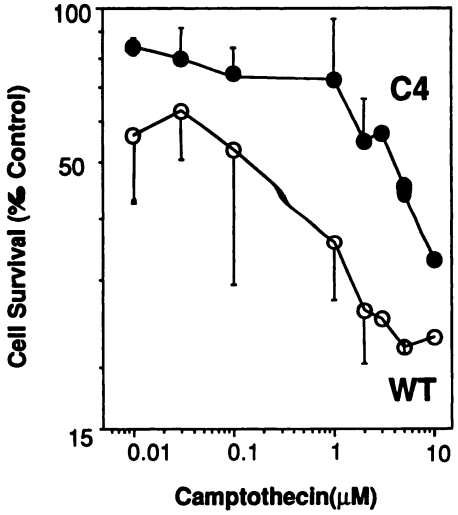

Fig. 1. Cytotoxicity of CPT in MCF-7 (*WT*) and MCF-7/C4 (*C4*) cells. Cytotoxicity was determined by clonogenic assays. *Error bars*, standard deviations (<95%) of five independent experiments.

HL60 cells, used as a calibrator in the alkaline elutions, were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37° in a humidified atmosphere of 95% air and 5% CO_2.

Chemicals. CPT, VP-16, and CDDP were obtained from the Drug Synthesis and Chemistry Branch of the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). CPT was dissolved in dimethylsulfoxide at 10 mM and aliquots were stored at −20°. CDDP was dissolved in PBS at 37~42° immediately before use.

Clonogenic assays. Exponentially growing cells were seeded and treated with drugs in 25-cm^2 flasks. CPT treatments were performed for 8 hr, and CDDP treatments were performed for 1 hr. After drug treatments, cells were trypsinized and plated into 25-cm^2 flasks in triplicate (400 cells per flask). After 14 days, colonies were fixed in 95% methanol, stained with 0.05% methylene blue, and counted. Cloning efficiencies of MCF-7 and MCF-7/C4 cells were 35–45% and 30–40%, respectively. Cytotoxicity was determined as the cloning efficiency ratio of drug-treated and untreated cells.

Northern blotting. Human top1 cDNA (T1B) was provided by Dr. W. C. Earnshaw (Johns Hopkins University School of Medicine, Baltimore, MD). Human β-actin cDNA was purchased from Clontech (Palo Alto, CA). Total RNA was extracted with acid guanidium thiocyanate and phenol-chloroform and was electrophoresed in 1% agarose gels containing 0.66 M formaldehyde in 1× MOPS buffer. After transfer in 10× SSC (1× = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) to duralose-UV membrane (Stratagene, La Jolla, CA), the RNA was fixed by UV cross-linking. Filters were treated at 42° for 20 min in 6× SSC and 50% formamide and hybridized with the human top1 cDNA probe labeled with [α-^{32}P]dATP using a random primer-labeling kit (Boehringer Mannheim, Indianapolis, IN). Filters were washed twice with 2× SSC and 0.1% SDS and twice with 0.5× SSC and 0.1% SDS at 65°. After stripping the top1 probe by boiling for 10 min, filters were hybridized with a labeled β-actin cDNA probe. Quantitations were performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

DNA cleavage assays. An oligonucleotide containing a single top1 cleavage site (11, 12) (see Fig. 5A) was used to assay top1 sensitivity to CPT. The 32-mer DNA was labeled at the 3′ terminus of the scissile strand with [α-^{32}P]-cordycepin and terminal transferase (DuPont, Wilmington, DE), as described previously (11, 12). The oligonucleotide was incubated with nuclear extract at 37° for 10 min in the presence of CPT. Reactions were terminated by adding 1% SDS. Samples were denatured by adding four volumes of loading buffer containing 90% formamide, 0.1% xylene cyanol, 0.1% bromphenol blue, and 10 mM EDTA, pH 8.0, and separated in a denaturing gel (16% polyacrylamide, 7 M urea) run at 52°. Top1-mediated cleavage (Fig. 5A, *caret*) generates a faster DNA band that corresponds to a cleaved (19-mer) product from the uncleaved substrate (33-mer). Radioactivity of the cleaved and uncleaved products was quantitated using a PhosphorImager and ImageQuant software (version 3.22; Molecular Dynamics, Sunnyvale, CA).

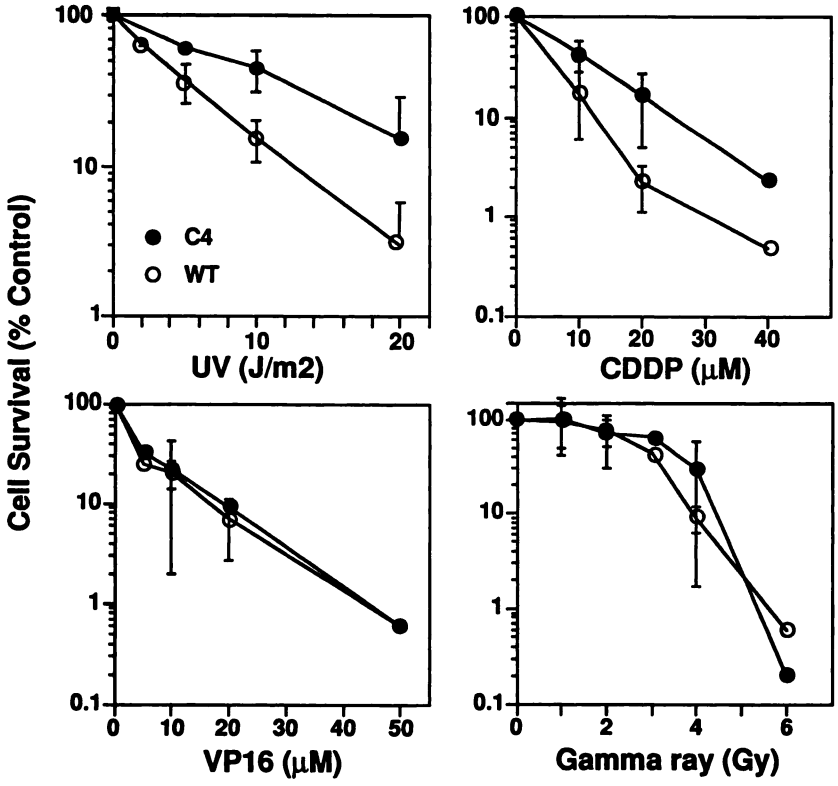

Fig. 2. Cytotoxicity of DNA damaging agents in MCF-7 cells (*WT*) and CPT-resistant MCF-7/C4 cells (*C4*). Cytotoxicity was determined by clonogenic assays. *Error bars*, standard deviations (<95%) of at least three independent experiments.

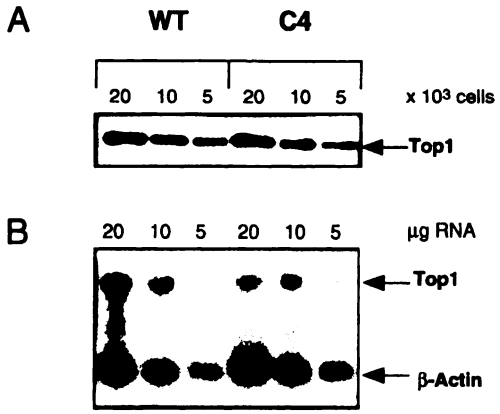

Fig. 3. Expression of top1 in MCF-7 (*WT*) and MCF-7/C4 (*C4*) cells. A, Lysates of the indicated number of cells were resolved on 18% Tris/glycine/SDS gels. Western blotting was performed using monoclonal anti-human top1 antibody. B, Total RNA samples were prepared and Northern blotting was performed using radiolabeled top1 cDNA probe. The filter was also hybridized with a β-actin probe to normalize the RNA amount transferred on the filter. Imaging and measurement of radioactivity were performed with a PhosphorImager.

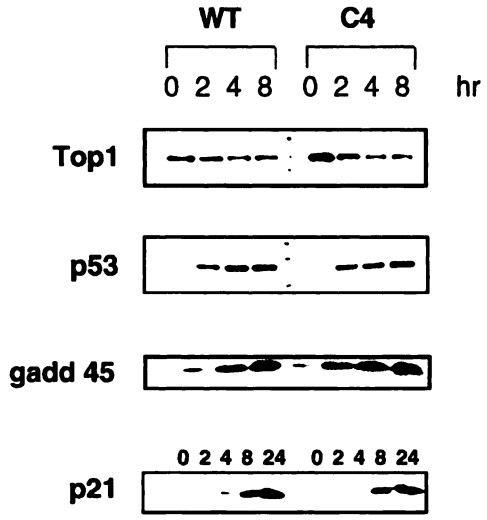

Fig. 4. DNA damage response induced by CPT in MCF-7 (*WT*) and MCF-7/C4 (*C4*) cells. Crude cell lysates of 2× 10^4 cells were resolved on Tris/glycine/SDS gel, and immunoblotting was performed using the indicated antibodies.

Western blot analysis. Cells were suspended in 100 μl of 2× loading buffer (0.125 M Tris·HCl, pH 6.8, 20% glycerol, 4% SDS, 0.005% bromphenol blue) and separated on a precast polyacrylamide Tris-glycine gel (Novex, San Diego, CA) with running buffer (0.1 M Tris·HCl, 0.1 M Tricine, 0.1% SDS, pH 8.3). After electrophoresis, the samples were transferred to Immobilon-*p* membranes (Millipore, Bedford, MA). Monoclonal antihuman top1 antibody was provided by Dr. Y.-C. Cheng (Yale University, New Haven, CT). Anti-p53 mouse monoclonal (DO-1) and anti-GADD45 rabbit polyclonal (H-165) antibodies were purchased from Santa Cruz Biomedicals (Santa Cruz, CA). Anti-$p21^{WAF1/CIP1}$ (6B6) mouse monoclonal antibody was purchased from PharMingen (San Diego, CA). Detection of immunoreactive signals was accomplished with chemiluminescent Western blotting detection reagents (Amersham International, Buckinghamshire, UK). Intensity of top1-specific band was quantitated by using Computing Densitometer and ImageQuant software.

Measurement of DNA Contents by Flow Cytometry. Cells were washed with PBS (150 mM NaCl, 4.3 mM K_2HPO_4, 0.7mM KH_2PO_4, pH 7.4), fixed in 70% ethanol, and treated with RNase for 30 min at 37°. The samples were stained with propidium iodide (Sigma Chemical, St. Louis, MO) for 30 min at 4° and analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA). The percentage of cells in each cell-cycle phase was quantitated using the SFIT model package provided by the manufacturer.

Measurement of DNA-protein cross-links and DNA SSBs. Alkaline elution assays were performed as described previously (13, 14). MCF-7 and MCF-7/C4 cells were labeled with [^{14}C]thymidine (0.04 μCi/ml) for 24 hr. HL60 internal standard cells were labeled with [*methyl*-^{3}H]thymidine (0.2 μCi/ml). Cells were chased for an additional 20 hr with radioisotope-free medium before drug treatments and were harvested after drug treatment into ice-cold Hanks' balanced salt solution containing CPT to avoid reversal of cleavable complexes (15). For the DNA-protein cross-link assays, cells were irradiated on ice with 30 Gy immediately before elution and were kept on ice until loading onto protein-adsorbing filters (Metricel; 0.8-μm pore-size; Gelman Sciences, Ann Arbor, MI). Cells were lysed with SDS lysis solution (0.1 M glycine, 2% SDS, 0.025 M Na_2EDTA, pH 10) without proteinase K. After one wash with 0.02 M EDTA, pH 10, DNA was eluted in tetrapropylammoniumhydroxide, pH 12.1, at

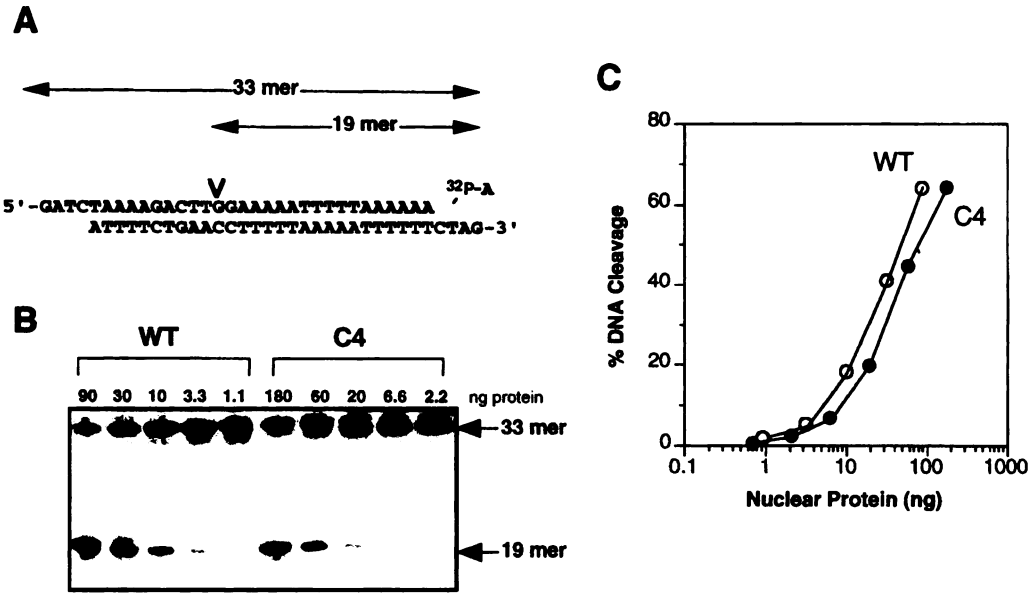

Fig. 5. Top1 DNA cleavage activity in MCF-7 (*WT*) and MCF-7/C4 (*C4*) cells. A, Various amounts of nuclear extracts were tested for cleavage of an oligonucleotide labeled at the 3′-terminus of the upper strand and containing a single top1 cleavage site (*inverted caret*). CPT stabilizes top1-mediated DNA cleavage, resulting in the appearance of a 19-mer cleaved product from the uncleaved substrate DNA (33-mer). B, Gel picture. C, Quantitations were performed with a PhosphorImager, and top1-induced DNA cleavage was determined as the relative intensity of top1 cleavage.

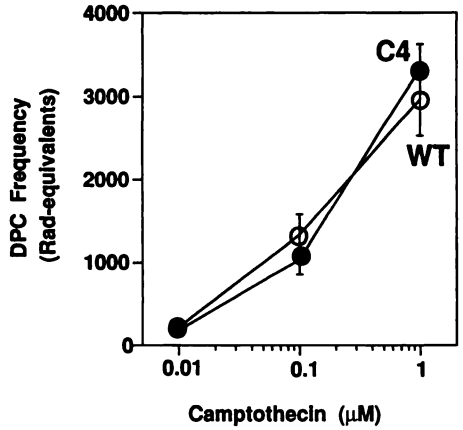

Fig. 6. DNA-protein cross-links (*DPC*) induced by CPT in MCF-7 (*WT*) and MCF-7/C4 (*C4*) cells. DPCs were measured after 8-hr CPT treatments by alkaline elution. DPC frequency is computed in rad-equivalents.

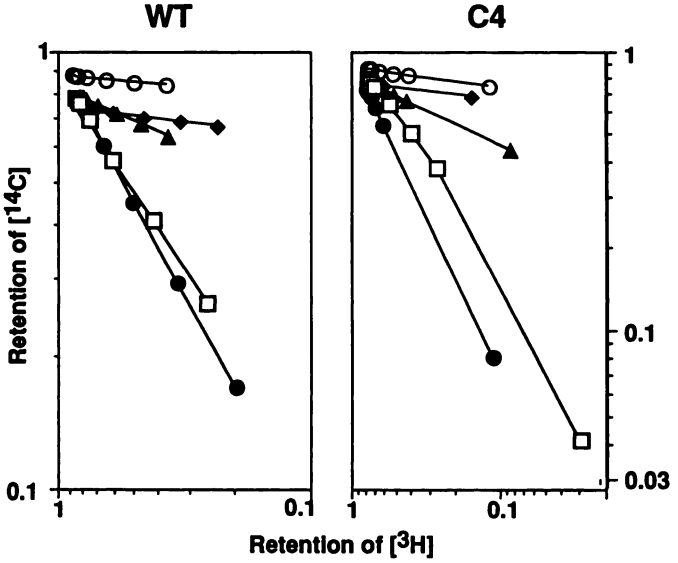

Fig. 7. DNA SSBs induced by CPT in MCF-7 (*WT*) and MCF-7/C4 (*C4*) cells. Cells were treated with 1 μM CPT for 8 hr and DNA SSBs were measured by alkaline elution. ○, Untreated cells; ●, end of CPT treatment; ▲, 15 min after CPT removal; ♦, 60 min after CPT removal; □, γ-irradiation (20 Gy).

a flow rate of 0.03–0.04 ml/min. For measuring DNA SSBs, only internal standard HL60 cells and calibrator cells were irradiated on ice with 3 Gy. Cells were loaded onto polycarbonate filters (2-μm pore-size; Poretics, Livermore, CA) and lysed with SDS lysis solution containing 0.5 mg/ml proteinase K (Boehringer-Mannheim Biochemicals). Elutions were performed with tetrapropylammoniumhydroxide, pH 12.1, containing 0.1% SDS at a flow rate of 0.12–0.16 ml/min.

Host cell reactivation assays. pSV_2-CAT was amplified in *Escherichia coli*, and DNA was prepared by alkaline lysis. Five micrograms of UV-damaged pSV_2-CAT plasmid DNA, 3 μg of pSV-β-galactosidase plasmid, and 10 μg of lipofectin (Life Technologies) were mixed gently in a sterile polystyrene tube in a final volume of 200 μl of serum-free medium and incubated for 30 min at 22°. This mixture was then added to 1.8 ml of serum-free medium and was layered over the cell cultures. After 24 hr, cotransfection was stopped by removing the liposome-DNA mixture, washing cells twice with medium, and adding complete culture medium. After an additional 48 hr of incubation, cells were transferred to microcentrifuge tubes, washed with PBS, and resuspended in 100 μl of 0.25 M Tris·HCl, pH 7.5. They were then lysed by three consecutive freeze-thaw cycles, and lysates were centrifuged at 14,000 × *g* for 5 min at 4°. Supernatant was used to determine protein concentration by BioRad protein assay kit (BioRad, Hercules, CA). Another aliquot was used to determine β-galactosidase activity (16). Transient CAT gene expression was assayed 72 hr after transfection. Quantitation of [^{14}C]chloramphenicol conversion was performed by counting on a PhosphorImager and ImageQuant software.

Results

Generation and drug-resistance profile of MCF-7/C4 cells. Mutagenesis with MMS, followed by selection with CPT, was used to obtain the CPT-resistant clones. Approximately 10^8 MCF-7 cells were treated with MMS (50 μg/ml) for 24 hr. Cytotoxicity of MMS at this concentration was approximately 70%. After 3 days, surviving cells were treated with 0.03 μM CPT. This concentration was maintained for 3 days by supplying fresh CPT-containing medium every day. Resistant cells were allowed to grow in drug-free medium for 3 additional days. This CPT selection schedule was repeated twice. Surviving colonies were picked up and inoculated into microtiter plates. Resulting clones were grown in 25-cm^2 flasks in the presence of 0.03 μM CPT. MCF-7/C4 is one of the CPT-resistant clones generated by this procedure. Monoclonal MCF-7/C4 cells were obtained after recloning and were cultured in the absence of CPT.

Resistance of MCF-7/C4 cells to CPT is shown in Fig. 1. Estimated IC_{50} values for CPT in MCF-7 cells and MCF-7/C4 cells were 0.12 μM and 4 μM, respectively, which indicates that MCF-7/C4 cells are approximately 30-fold resistant to CPT. The cytotoxicity of the top2 inhibitor, VP-16, and γ-radiation was similar in MCF-7/C4 and parental cells, but MCF-7/C4 cells were cross-resistant to UV and CDDP (Fig. 2).

Top1-related parameters in MCF-7/C4 cells. We first examined whether CPT resistance was caused by changes in *top1* gene expression. Western and Northern blottings showed normal size of top1 and similar mRNA levels in both cell lines (Fig. 3). Top1 protein levels were also examined at various times after treatment with CPT (Fig. 4). Top1 protein decreased in both cell lines after CPT treatment. Top1-mediated damage by CPT was determined using an assay based on our previous finding that CPT induces a single top1 cleavage site in a 3′-end-labeled DNA oligonucleotide (17, 18) (Fig. 5A). In the presence of 10 μM CPT, DNA cleavage was not significantly different with MCF-7 and MCF-7/C4 nuclear extracts (Fig. 5, B and C). To exclude the possibility that resistance of MCF-7/C4 might be caused by reduced drug uptake, cleavable complexes were measured directly in CPT-treated cells. Alkaline elution experiments showed that CPT produced similar DNA-protein cross-links in MCF-7 and MCF-7/C4 cells (Fig. 6). Consistently, DNA SSBs were induced and reversed similarly after drug removal in both cell lines (Fig. 7). These results indicate that the CPT resistance of MCF-7/C4 cells cannot be attributed to appreciable top1 activity and expression.

Cell-cycle alterations in MCF-7/C4 cells. Cell-cycle distribution was examined next because CPT has been shown to be most toxic in S phase (2, 4, 7). Flow cytometry analyses demonstrated that the fraction of cells in S phase was half in the resistant MCF-7/C4 cells (Table 1; Fig. 8). However,

TABLE 1
Cell-cycle distribution of MCF-7 and MCF-7/C4 cells

Phase	MCF-7	MCF-7/C4
G_1	45.4 ± 2.8	54.4 ± 2.3
S	31.3 ± 4.9	14.2 ± 5.4
G_2/M	23.5 ± 1.9	31.3 ± 6.1
Doubling time	22 hr	22 hr

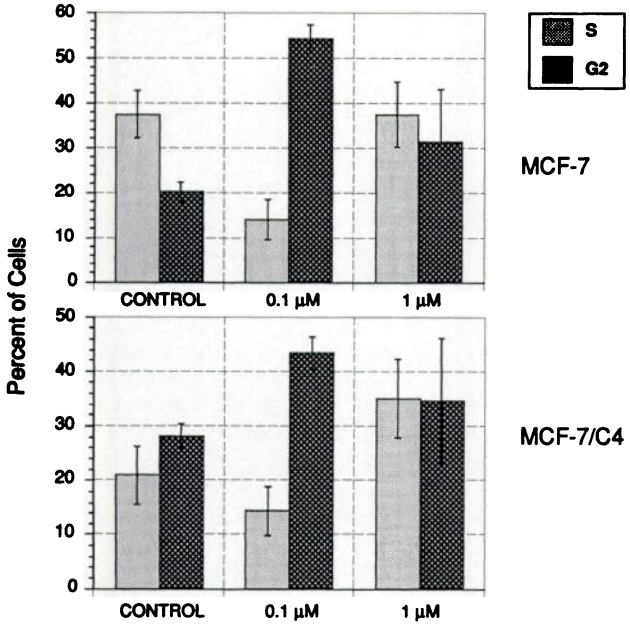

Fig. 8. Cell-cycle effects of CPT treatment in MCF-7 and MCF-7/C4 cells. Cells were treated with CPT for 8 hr and samples were prepared 24 hr after drug removal. The percentage of cells in S phase and G_2 phase was computed using the SFIT program. Shown are mean ± standard error of three independent experiments.

growth curves showed that MCF-7 and MCF-7/C4 had similar doubling time (22 ± 2 hr). Thus, MCF-7/C4 cells probably complete DNA replication more rapidly than MCF-7 cells. This result prompted us to test the cytotoxicity of prolonged CPT exposure. Extending treatments to 24 hr, which is longer than the cell doubling time, did not affect the CPT resistance of MCF-7/C4 cells (Fig. 9). DNA protein cross-links were also similar between the two cell lines after 24-hr treatments (data not shown). These results indicate that CPT resistance of MCF-7/C4 is not caused by reduced top1-mediated DNA damage.

Cellular response to CPT was investigated by flow cytometry (Fig. 8). In parental MCF-7 cells, CPT induced G_2 delay and depletion of S-phase cells at 0.1 μM. At 1 μM, the proportion of cells arrested in G_2 was less and cells tended to be arrested in S. The G_2 delays were less pronounced in the resistant MCF-7/C4 cells (Fig. 8). Together, these results show that MCF-7/C4 cells have fewer G_2 delays in response to CPT than MCF-7 cells, despite similar induction of top1-cleavable complexes.

p53 pathway and DNA damage-inducible genes in MCF-7/C4 cells. CPT has been shown previously to be a potent inducer of p53 and to trigger p53-dependent DNA damage response pathways in wild-type p53 cells (19). MCF-7 cells have wild-type p53, and as expected, p53 protein levels increased rapidly in response to CPT. Similar induction was observed in MCF-7/C4 cells (Fig. 4). The base-line

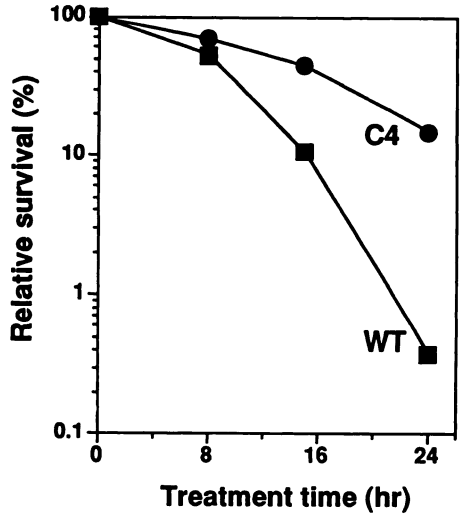

Fig. 9. CPT cytotoxicity with increasing exposure time in MCF-7 (*WT*) and MCF-7/C4 (*C4*) cells. Cells were treated with 1 μM CPT for the indicated times. Cytotoxicity was measured by clonogenic assays.

p53 levels were also slightly but consistently higher in MCF-7/C4 than in MCF-7 cells. The p53-inducible gene products, GADD45 and $p21^{WAF\text{-}1/CIP\text{-}1}$ were, however, similarly induced in both cell lines. These data demonstrate that CPT activates the p53-dependent pathways in both cell lines, consistent with the view that initial DNA damage is comparable in MCF-7/C4 and MCF-7 cells.

Enhanced DNA repair in MCF-7/C4 cells. Because CPT-induced DNA damage and repair are still poorly understood and MCF-7/C4 cells are cross-resistant to UV (Fig. 2), we used UV-damaged DNA to test DNA repair in both cell lines. Repair of UV-induced DNA lesions in a pSV-CAT plasmid restores CAT gene expression (for details, see Ref. 20). Fig. 10 shows the result of a typical experiment. DNA repair was two- to fourfold more efficient in MCF-7/C4 cells than in wild-type MCF-7 cells.

Discussion

As CPTs are introduced in the clinic, it is important to elucidate not only how the drugs interact with their primary target (top1) (12) but also how they kill cells (7). There is evidence that CPT and its derivatives bind at the enzyme-

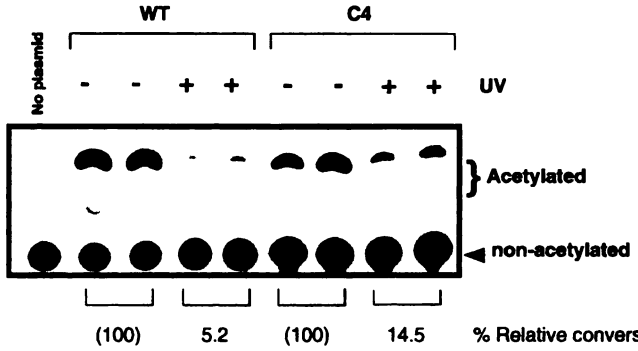

Fig. 10. Nucleotide excision repair in MCF-7 (*WT*) and MCF-7/C4 (*C4*) cells. Cells were transfected with 5 μg of undamaged (−) or UV-damaged pSV-CAT plasmids (+), and crude extracts from MCF-7 and MCF-7/C4 cells were tested for CAT expression. A typical experiment is shown.

DNA interface (7, 12, 21) and stabilize top1 cleavable complexes by inhibiting the religation of top1-mediated DNA breaks (18, 22). However, top1 generally is not qualitatively different in cancer and normal cells. Thus, we must understand the cellular pathways downstream of the cleavable complexes to identify useful cellular markers for drug activity and resistance to CPT and, possibly, novel strategies for cancer chemotherapy. In this context, the pair of MCF-7/C4 and parental MCF-7 cells provides a novel system to elucidate cellular response(s) that determine anticancer activity of and resistance to CPTs because top1-related parameters are not appreciably different in the two cell lines.

Cellular resistance to CPTs can be defined based on two parameters: top1 protein levels and top1 cleavable complexes. Reduction of cleavable complexes is observed commonly in CPT-induced resistant cells (2). This is because CPT inhibits top1 by converting the enzyme into a cellular poison (2, 4, 23). Hence, as CPT-trapped cleavable complexes are reduced, DNA damage is decreased. Reduction of top1 cleavable complexes commonly is caused by reduced top1 levels in CPT-resistant cell lines (for review, see Ref. 2). For instance, we recently observed silencing of the normal *top1* alleles in a CPT-resistant cell line with changes of *top1* gene methylation (24). Top1 cleavable complexes also can be reduced in the absence of profound decreases in top1 expression levels in cells with top1 mutations that render the enzyme insensitive to CPT (17, 25–31) (for review, see Ref. 2).

CPT resistance with normal cleavable complexes and without top1 alterations seems to be the primary mechanism of resistance of MCF-7/C4 cells. This type of CPT resistance has been observed in human colon carcinoma cell lines (8) from the National Cancer Institute anticancer drug screen (32) and in human nasopharyngeal KB cells (33). Conversely, LY-R cells isolated from strains of mouse lymphoma L5178Y are more sensitive to UV radiation and CPT, and this increased sensitivity is not related to top1 activity (34). This type of resistance also has been observed in yeast cells and led to the description of an *SCT1* (suppressor of camptothecin toxicity) gene (10). Therefore, this CPT-resistance phenotype [NCC (normal cleavable complexes) resistance] may not be infrequent and probably is clinically relevant.

The cytotoxicity of CPT-induced cleavable complexes is linked to DNA synthesis in a number of cell lines, because aphidicolin abolishes the cytotoxicity of CPT (35, 36). This defines CPT as an S-phase-specific DNA-damaging agent. The reduced proportion of cells in S phase in MCF-7/C4 cells then could seem to contribute to the resistance of MCF-7/C4 cells. However, the observation that 24-hr CPT treatment (more than a doubling time for either cell line) was still more cytotoxic in MCF-7/C4 cells suggests that S-phase kinetic differences are not sufficient to account for CPT resistance. An S-phase-independent component to the cytotoxicity of CPT also has been observed in some cell lines (8) and at high drug concentrations (36). In MCF-7/C4 and MCF-7 cells, we found that CPT induced expression of *p53* and its downstream genes, *GADD45* and $p21^{WAF\text{-}1/CIP\text{-}1}$, similarly. This suggests that signaling of the *p53* pathway mediated by DNA damage is similar in both cell lines.

DNA repair probably plays a critical role in cellular tolerance to CPT-induced DNA damage. Collision of replication forks into cleavable complexes would be expected to produce DNA double-strand breaks and irreversible top1-DNA adducts (7). Such double-strand breaks have been detected in SV40 (37, 38) and in human cells (39). It is also known that cells with deficient DNA repair are hypersensitive to CPT (40–42). The cross-resistance of MCF-7/C4 cells to UV and CDDP was used to test DNA repair with a cellular reactivation assay of a UV-damaged plasmid with a CAT reporter (20, 43). Our finding that MCF-7/C4 cells repair UV-induced damage more efficiently than MCF-7 cells suggests that enhanced DNA repair contributes to the resistance of MCF-7/C4 cells. Enhanced DNA repair also has been observed in CDDP-resistant cells (43–45). Survival of CPT-induced DNA damage would require at least the DNA repair machinery to excise the irreversible top1-DNA adducts and repair DNA double-strand breaks generated within replicating DNA.

In conclusion, it is tempting to speculate that DNA repair deficiencies might make malignant cells more sensitive to chemotherapeutic agents, including CPTs, that target DNA but do not have a cellular target unique to cancer cells. MCF-7/C4 cells provide a novel experimental system to study genes that are expressed differentially in CPT-resistant cells.

Acknowledgments

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References

1. Champoux, J. Mechanistic aspects of type-I topoisomerases, in *DNA Topology and its Biological Effects* (J. C. Wang and N. R. Cozarelli, eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 217–242 (1990).
2. Gupta, M., A. Fujimori, and Y. Pommier. Eukaryotic DNA topoisomerases I. *Biochim. Biophys. Acta* **1262**:1–14 (1995).
3. Pommier, Y., and A. Tanizawa. Camptothecins: mechanism of action and resistance. *Cancer Invest.* **11**:3–6 (1994).
4. Chen, A. Y., and L. F. Liu. DNA topoisomerases: essential enzymes and lethal targets. *Annu. Rev. Pharmacol. Toxicol.* **94**:194–218 (1994).
5. Slichenmyer, W. J., E. K. Rowinsky, R. C. Donehower, and S. H. Kaufmann. The current status of camptothecin analogs as anticancer agents. *J. Natl. Cancer Inst.* **85**:271–287 (1993).
6. Potmesil, M. Camptothecins: from bench research to hospital wards. *Cancer Res.* **54**:1431–1439 (1994).
7. Pommier, Y., F. Leteurtre, M. R. Fesen, A. Fujimori, R. Bertrand, E. Solary, G. Kohlhagen, and K. W. Kohn. Cellular determinants of sensitivity and resistance to DNA topoisomerase inhibitors. *Cancer Invest.* **12**: 530–542 (1994).
8. Goldwasser, F., T. Shimizu, J. Jackman, Y. Hoki, P. M. O'Connor, K. W. Kohn, and Y. Pommier. Correlations between S- and G2-phase arrest and cytotoxicity of camptothecin in human colon carcinoma cells. *Cancer Res.* **56**:4430–4437 (1996).
9. Goldwasser, F., I. Bae, M. Valenti, K. Torres, and Y. Pommier. Topoisomerase I-related parameters, and camptothecin activity in the colon carcinoma cell lines from the National Cancer Institute anticancer screen. *Cancer Res.* **55**:2116–2121 (1995).
10. Kauh, E. A., and M. -A. Bjornsti. *SCT1* mutants suppress the camptothecin sensitivity of yeast cells expressing wild-type DNA topoisomerase I. *Proc. Natl. Acad. Sci. USA* **92**:6299–6303 (1995).
11. Fujimori, A., W. G. Harker, G. Kohlhagen, Y. Hoki, and Y. Pommier. Mutation at the catalytic site of topoisomerase I in CEM/C2, a human leukemia cell resistant to camptothecin. *Cancer Res.* **55**:1339–1346 (1995).
12. Pommier, Y., G. Kohlhagen, F. Kohn, F. Leteurtre, M. C. Wani, and M. E. Wall. Interaction of an alkylating camptothecin derivative with a DNA base at topoisomerase I-DNA cleavage sites. *Proc. Natl. Acad. Sci. USA* **92**:8861–8865 (1995).
13. Kohn, K. W., R. A. G. Ewig, L. C. Erickson, and L. A. Zwelling. Measurement of strand breaks and crosslinks by alkaline elution, in *DNA Repair: A Laboratory Manual of Research Procedures* (E. C. Friedberg and P. C. Hanawalt, eds.). Marcel Dekker Inc., New York, NY, 379–401 (1981).
14. Bertrand, R., and Y. Pommier. Assessment of DNA damage in mammalian cells by DNA filtration methods, in *Cell Growth and Apoptosis: A Practical Approach* (G. Studzinski, ed.). IRL Press, Oxford, 96–117 (1995).
15. Covey, J. M., C. Jaxel, K. W. Kohn, and Y. Pommier. Protein-linked DNA strand breaks induced in mammalian cells by camptothecin, an inhibitor of topoisomerase I. *Cancer Res.* **49**:5016–5022 (1989).
16. An, G., K. Hidaka, and L. Siminovitch. Expression of bacterial b-galactosidase in animal cells. *Mol. Cell. Biol.* **2**:1628–1632 (1982).

17. Fujimori, A., W. G. Harker, G. Kohlhagen, Y. Hoki, and Y. Pommier. Mutation at the catalytic site of topoisomerase I in CEM/C2, a human leukemia cell line resistant to camptothecin. *Cancer Res.* **55:**1339–1346 (1995).
18. Pommier, Y., J. Jenkins, G. Kohlhagen, and F. Leteurtre. DNA recombinase activity of eukaryotic DNA topoisomerase I: effects of camptothecin and other inhibitors. *Mutat. Res.* **337:**135–145 (1995).
19. Nelson, W. G., and M. B. Kastan. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Mol. Cell. Biol.* **14:**1815–1823 (1994).
20. Smith, M., I. -T. Chen, Q. Zhan, P. O'Connor, and A. Fornace, Jr. Involvement of the p53 tumor suppressor in repair of u.v.-type DNA damage. *Oncogene* **10:**1053–1059 (1995).
21. Jaxel, C., G. Capranico, D. Kerrigan, K. W. Kohn, and Y. Pommier. Effect of local DNA sequence on topoisomerase I cleavage in the presence or absence of camptothecin. *J. Biol. Chem.* **266:**20418–20423 (1991).
22. Svejstrup, J. Q., K. Christiansen, I. I. Gromova, A. H. Andersen, and O. Westergaard. New technique for uncoupling the cleavage and relegation reactions of eukaryotic topoisomerase I. The mode of action of camptothecin at a specific recognition site. *J. Mol. Biol.* **222:**669–678 (1991).
23. Bjornsti, M. -A., P. Benedetti, G. A. Viglianti, and J. C. Wang. Expression of human DNA topoisomerase I in yeast cells lacking yeast DNA topoisomerase I: restoration of sensitivity of the cells to the antitumor drug camptothecin. *Cancer Res.* **49:**6318–6323 (1989).
24. Fujimori, A., Y. Hoki, N. Popescu, and Y. Pommier. Silencing and selective methylation of the normal topoisomerase I gene in camptothecin-resistant CEM/C2 human leukemia cells. *Oncol. Res.* **8:** in press (1996).
25. Tamura, H. -O., C. Kohchi, R. Yamada, T. Ikeda, O. Koiwai, E. Patterson, J. D. Keene, K. Okada, E. Kjeldsen, K. Nishikawa, and T. Andoh. Molecular cloning of a cDNA of a camptothecin-resistant human DNA topoisomerase I and identification of mutation sites. *Nucleic Acids Res.* **19:**69–75 (1991).
26. Kubota, N., F. Kanzawa, K. Nishio, Y. Takeda, T. Ohmori, Y. Fujiwara, Y. Terashima, and N. Saijo. Detection of topoisomerase I gene point mutation in CPT-11 resistant lung cancer cell line. *Biochem. Biophys. Res. Commun.* **188:**571–577 (1992).
27. Knab, A. M., J. Fertala, and M. -A. Bjornsti. Mechanisms of camptothecin resistance in yeast DNA topoisomerase I mutants. *J. Biol. Chem.* **268:** 22322–22330 (1993).
28. Levin, N. A., M. -A. Bjornsti, and G. R. Fink. A novel mutation in DNA topoisomerase I of yeast causes DNA damage and *RAD9*-dependent cell cycle arrest. *Genetics* **133:**799–814 (1993).
29. Tanizawa, A., and Y. Pommier. Topoisomerase I alteration in a camptothecin-resistant cell line derived from chinese hamster DC3F cells in culture. *Cancer Res.* **52:**1848–1854 (1992).
30. Rubin, E., P. Pantazis, A. Bharti, D. Toppmeyer, B. Giovanella, and D. Kufe. Identification of a mutant human topoisomerase I with intact catalytic activity and resistance to 9-nitro-camptothecin. *J. Biol. Chem.* **269:** 2433–2439 (1994).
31. Benedetti, P., P. Fiorani, L. Capuani, and J. C. Wang. Camptothecin resistance from a single mutation changing glycine 363 of human DNA topoisomerase I to cysteine. *Cancer Res.* **53:**4343–4348 (1993).
32. Goldwasser, F., I. S. Bae, K. Torres, and Y. Pommier. Topoisomerase I-related parameters and camptothecin activity in the colon cell lines from the National Cancer Institute anticancer screen. *Cancer Res.* **55:**2116–2121 (1995).
33. Beidler, D. R., and Y. -C. Cheng. Camptothecin induction of a time- and concentration-dependent decrease of topoisomerase I and its implication in camptothecin activity. *Mol. Pharmacol.* **47:**907–914 (1995).
34. Lanza, A., S. Tornaletti, M. Stefanini, H. H. Evans, M. Ricanati, G. C. B. Astaldi Ricotti, and A. M. Pedrini. The sensitivity to DNA topoisomerase inhibitors in L5178Y lymphoma strains is not related to a primary defect of DNA topoisomerases. *Carcinogenesis* **14:**1759–1763 (1993).
35. Hsiang, Y. -H., M. G. Lihou, and L. F. Liu. Arrest of DNA replication by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res.* **49:**5077–5082 (1989).
36. Holm, C., J. M. Covey, D. Kerrigan, and Y. Pommier. Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I and II inhibitors in Chinese hamster DC3F cells. *Cancer Res.* **49:**6365–6368 (1989).
37. Shin, C. -G., and R. M. Snapka. Exposure to camptothecin breaks leading and lagging strand simian virus 40 DNA replication forks. *Biochem. Biophys. Res. Commun.* **168:**135–140 (1990).
38. Tsao, Y. P., A. Russo, G. Nyamusa, R. Silber, and L. F. Liu. Interaction between replication forks and topoisomerase I-DNA cleavable complexes: studies in a cell-free SV40 DNA replication system. *Cancer Res.* **53:**5908–5914 (1993).
39. Ryan, A. J., S. Squires, H. L. Strutt, and R. T. Johnson. Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistent double strand breaks in replicating DNA. *Nucleic Acids Res.* **19:**3295–3300 (1991).
40. Squires, S., A. Ryan, H. Strutt, and R. Johnson. Hypersensitivity of Cockayne's syndrome cells to camptothecins is associated with the generation of abnormally high levels of double strand breaks in nascent DNA. *Cancer Res.* **53:**2012–2019 (1993).
41. Smith, P. J., T. A. Makinson, and J. V. Watson. Enhanced sensitivity to camptothecin in ataxia telangiectasia cells and its relationship with the expression of DNA topoisomerase I. *Int. J. Radiat. Biol.* **55:**217–231 (1989).
42. Jones, N. J., S. Ellard, R. Waters, and E. M. Parry. Cellular and chromosomal hypersensitivity to DNA crosslinking agents and topoisomerase inhibitors in the radiosensitive Chinese hamster irs mutants: phenotypic similarities to ataxia telangiectasia and Fanconi's anaemia cells. *Carcinogenesis* **14:**2487–2494 (1993).
43. Zeng-Rong, N., J. Paterson, L. Alpert, M. -S. Tsao, J. Viallet, and M. A. Alaoui-Jamali. Elevated DNA repair capacity is associated with intrinsic resistance of lung cancer to chemotherapy. *Cancer Res.* **55:**4760–4764 (1995).
44. Dabholkar, M., R. Parker, and E. Reed. Determinants of cisplatin sensitivity in non-malignant non-drug-selected human T cell lines. *Mutat. Res.* **274:**45–46 (1992).
45. Chao, C., Y. Lee, P. Cheng, and S. Lin-Shao. Enhanced host cell reactivation of damaged plasmid DNA in HeLa cells resistant to cis-diamminedichloroplatinum(II). *Cancer Res.* **51:**601–605 (1991).

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