Inactivation of p53 Increases the Cytotoxicity of Camptothecin in Human Colon HCT116 and Breast MCF-7 Cancer Cells

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ABSTRACT
Camptothecin (CPT) derivatives are topoisomerase I (top1) inhibitors recently introduced as clinical agents. To explore the role of p53 in CPT-induced cytotoxicity, we examined CPT effects in two isogenic pairs of human cancer cell lines, MCF-7 breast carcinoma and HCT116 colon carcinoma cells, in which p53 function had been disrupted by transfection with the human papillomavirus type-16 E6 gene. Clonogenic survival assays showed that both MCF-7/E6 and HCT116/E6 cells were more sensitive to CPT. No differences in top1 protein levels and activity analyzed by a novel in vitro oligonucleotide assay were observed in the E6 transfectants. Also, CPT showed comparable top1 cleavable complex formation in vivo, as determined by DNA single-strand breaks and DNA protein cross-links. These results suggest that p53 can protect against CPT-induced cytotoxicity and that this protection is mediated downstream of CPT-induced DNA damage. Flow cytometry analyses showed that CPT can induce G1 arrest in cells with normal p53. This G1 arrest was markedly reduced in the p53-deficient cells. These results demonstrate a critical role of p53 as a G1 checkpoint regulator after CPT-induced DNA damage and suggest a rationale for the selectivity of CPT toward tumors with p53 mutations.

INTRODUCTION
The nuclear protein p53 is important in the etiology of cancer and is mutated in more than half of all human tumors (1). Loss of p53 function leads to gene amplification and the development of aneuploidy (2), suggesting that p53 acts as a “guardian of the genome” (3–5). In some cell types, the G1 arrest induced by p53 in response to DNA damaging agents is believed to allow additional time for the cell to repair its DNA. In addition, DNA repair of at least some types of DNA lesions might also be under the influence of p53 (6, 7). p53 has also been implicated in G2-M checkpoints control (8). Alternatively, p53 can activate an apoptotic response to DNA damage (9, 10). These opposing functions of p53 can influence the response of human cancers to DNA-damaging agents in different ways.

In hematopoietic and lymphoid cells types, the loss of p53 leads to an inability to trigger apoptosis in response to DNA-damaging agents (11–13). In epithelial tumor cell lines, the ability to trigger apoptosis by IR2 is also often lost when p53 is disrupted, whereas in primary mouse fibroblast cells, there are no differences in the radioreistance of the p53 null cells versus the normal cells (14). A relationship between p53 gene status and chemosensitivity of cancer cells has recently been reviewed (15).

CPT is an antitumor drug extracted from the Chinese tree *Camptotheca acuminata* (16) and is a specific inhibitor of DNA top1 (17). CPT derivatives have been approved as clinical agents and are active against many tumors, including colorectal, ovarian, non-small cell lung, breast cancer, and mesothelioma (18). CPT and the derivatives of CPT are also active against multidrug-resistant tumor cell lines (19–21). We examined the effect of CPT on p53 alteration in colon and breast cancer cell lines.

In the present study, we used MCF-7 and HCT116 cell lines, both of which contain functionally intact wt p53 and do not appear to undergo apoptosis following γ-irradiation (7) or CPT treatment. p53 function was disrupted in these cell lines by constitutive high-level expression of the human papillomavirus type-16 E6 gene, which stimulates the degradation of p53 through a ubiquitin pathway (22, 23). We found that cells lacking p53 function showed increased sensitivity to CPT (16). The mechanism of the increased sensitivity in MCF-7/E6 and HCT116/E6 was not related to alterations in top1 protein levels, top1 activity, or initial DNA damage produced by CPT. Differences in cell cycle analyses suggested that G1 delay controlled by p53 plays a role in CPT-induced cytotoxicity.

MATERIALS AND METHODS

Cell Culture and Drugs. The breast carcinoma MCF-7 and colon carcinoma HCT116 cell lines were cultured in RPMI 1640 (Life Technologies, Inc.) containing 10% serum and 2 mm L-glutamine. Cells transfected with either control vector and vector containing overexpressed HPV-16 E6 were grown in the same medium containing G418. CPT was a gift from Drs. Monroe E. Wall and Mansukh C. Wani of the Research Triangle Institute (Research Triangle Park, NC). Nocodazole was purchased by Sigma Chemical Co. (St. Louis, MO). Stock solutions of CPT were prepared in 10 mm DMSO and further diluted in

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2 The abbreviations used are: IR, ionizing radiation; CPT, camptothecin; top1, topoisomerase I; SSB, single-strand break; DPC, DNA protein cross-link; wt, wild-type.
1654 p53 and Camptothecin

Clonogenic Assays. Cells were plated in log phase into 25-cm² culture flasks with 5 ml of medium for 24 h, rinsed with fresh medium, and then treated with various doses of CPT for 8 h. Cells were then washed twice with prewarmed PBS, trypsinized, diluted, and 500 cells were seeded per flask in 8 h.

DNA Cleavage Assays. An oligonucleotide containing a single high-affinity cleavage site for top1 activity was used to assay top1 sensitivity to CPT (24). The 32 mer oligonucleotide was labeled at the 3'-terminus of the scissile strand with α-[32 P] cordycepin and terminal transferase (DuPont, Wilmington, DE) as described previously (24). The oligonucleotide was incubated with nuclear extract at 37°C for 10 min in the presence of CPT. Reactions were terminated by adding 1% SDS. Samples were denatured by adding 4 volumes of loading buffer containing 90% formamide, 0.1% xylene cyanol, 0.1% bromphenol blue, and 10 mM EDTA (pH 8.0) and were separated in a denaturing gel (16% polyacrylamide and 7 M urea) that was run at 52°C. top1-mediated cleavage generated a DNA band corresponding to a cleaved (19 mer) product from the uncleaved substrate (33 mer). The radioactivity of the cleaved and the uncleaved products was quantitated using a PhosphorImager and IMAGE QUANT Ver. 3.22 (Molecular Dynamics, Sunnyvale, CA).

Measurement of DPCs and DNA SSBs. Alkaline elution assays were performed as described (25, 26). Briefly, MCF-7 and HCT116 cell lines were labeled with 0.04 μCi/ml [14C]-thymidine for 24 h. CEM cells were used as internal standard and were labeled with 0.2 μCi/ml methyl-[3H]-thymidine. Cells were then washed with PBS, fixed with methanol (95%), and stained with 0.005% methylene blue (24). Survival fraction for each cell line was expressed as the ratio of plating efficiency of treated cells to that of the untreated control cells. The plating efficiency of the control cells was between 50–60%. The IC50 was calculated as the drug concentration that inhibited colony formation to 50% of the control cells.

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Table 1  IC50 for HCT116 and MCF-7 cell lines treated with CPT

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>CPT (μM)</th>
</tr>
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<tbody>
<tr>
<td>HCT116</td>
<td>0.30</td>
</tr>
<tr>
<td>HCT116/CMV</td>
<td>0.30</td>
</tr>
<tr>
<td>HCT116/E6</td>
<td>0.03</td>
</tr>
<tr>
<td>MCF-7/CMV</td>
<td>0.08</td>
</tr>
<tr>
<td>MCF-7/E6</td>
<td>0.03</td>
</tr>
<tr>
<td>MCF-7/mup53</td>
<td>0.025</td>
</tr>
</tbody>
</table>

IC50 values were determined from the cytotoxicity dose-response curves (shown in Fig. 1) after 8 h drug exposure.

Fig. 1  Clonogenic survival of MCF-7 (A) and HCT116 (B) cell lines transfected with control pCMV.3 vector (●) or the human papilloma virus type-16 E6 (□) containing plasmid after treatment with CPT for 8 h. Mean ±SD of three independent experiments is shown. ●, HCT 116.
Fig. 2 Expression of p53 and top1 in HCT116/CMV and HCT116/E6 cells. Cell lysates from 2 × 10^5 (2) and 1 × 10^5 (1) cells were resolved on 12% Tris/glycine/SDS gels. Western blotting was performed using monoclonal anti-human top1 (B) and p53 mouse (DO-1) (A) antibodies (Santa Cruz Biotechnology, Inc.). B, 0.05 μM and 0.5 μM CPT treatment for 8 h.

The DPCs (in rad = equivalents) are calculated using the formula:

\[ DPC = \left(\frac{1}{1-r} - \frac{1}{1-r_0}\right) \times 3000 \]

where \( r \) is the retention for drug-treated cells and \( r_0 \) the retention for cells that have not been drug-treated.

Filter Elution Assays for the Measurement of DNA Fragmentation. DNA fragmentation was measured by filter elution as described previously (26, 28). Briefly, reaction mixtures containing 0.5 × 10^6 [14C]-thymidine-labeled cells were deposited on protein-adsorbing filter (Metricel, Gelman Science, Ann Harbor, MI) and washed with 5 ml of nucleus buffer. This fraction (W) was collected and lysis was performed with 5 ml LS10 (2 m NaCl, 0.04 m Na2EDTA, 0.2% sarkosyl, pH 10) followed by washing with 5 ml of 0.02 m Na2EDTA, pH 10. The lysis (L) with EDTA (E) fractions were collected. All fractions (W, L, and E) and filter (F) were counted by liquid scintillation. DNA fragmentation was calculated as the percentage of DNA eluting from the filter as follows:

\[ \text{DNA fragmentation} = 100 \times \frac{(W + L + E)(W + L + E + F)}{W + L + E + F} \]

Each value was normalized to the untreated sample. All experiments were repeated at least two or three times.

Flow Cytometry. Samples were prepared for flow cytometry as described previously (7). Briefly, cells were fixed in ice-cold 70% ethanol, washed with PBS, treated with RNase (Boehringer Mannheim) at 37°C for 30 min, and stained with 50 μg/ml propidium iodide (Sigma). Cell cycle analysis was performed using a Becton Dickinson fluorescence-activated cell analyzer. Cells (15,000) were analyzed for each point, and quantitation of cell cycle distribution was performed using the SOBR model program provided by the manufacturer.

RESULTS

Abrogation of wt p53 Function by Human Papillomavirus Type-16 E6 Transfection Sensitizes MCF-7 and HCT116 to CPT. The human papilloma virus type-16 E6 gene product, which stimulates the degradation of p53 through an ubiquitin-dependent pathway (22, 23) was used as a means of inhibiting p53 function in MCF-7 and HCT116 cells (7, 29). The E6 gene was transfected into MCF-7 or HCT116 cells in a plasmid containing the CMV promoter, which allows constitutive high-level expression of the transgene. In additional experiments, we used a dominant-negative mutant p53 transgene (143 Ala to Val cloned into a PCMV plasmid) to inhibit p53 function in MCF-7 cells (7). The generation and characterization of the MCF-7 and HCT116 transfectants have been described previously (7, 29, 30). The survival of MCF-7 and HCT116 cell lines was assessed in clonogenic survival assays after an 8-h treatment with CPT. The cytotoxicity of CPT depends mostly upon
Fig. 3  DNA cleavage activity of top1 from MCF-7 and HCT116 nuclear extracts. A, the oligonucleotide with a single cleavage site for top1. B, PhosphorImager image of a typical experiment of samples electrophoresed into a 16% polyacrylamide denaturing gel. In C and D, the amounts of uncleaved and cleaved bands were quantitated using a PhosphorImager. The percentage of cleavage versus different amounts of nuclear proteins extracted from MCF-7 (C) and HCT116 (D) was plotted.

The percentage of cells in the S phase at the time of drug exposure (31). Fig. 1 shows that at the IC$_{50}$ dose of CPT, MCF-7/E6 cells (Fig. 1A) were approximately 2–3-fold and HCT116/E6 cells (Fig. 1B) were approximately 10-fold more sensitive than control CMV transfectants, whereas differences approximately 2-fold were seen in the plateau levels (Fig. 1) between the E6 and CMV transfectants. The actual IC$_{50}$ values determined from the cytotoxicity curves are shown in Table 1. In both MCF-7/E6 and HCT116/E6 cell lines, 0.03 μM CPT was used to achieve 50% cytotoxicity. Enhanced sensitivity was not limited to the E6-transfected cell lines but was also observed when MCF-7 cells were transfected with a mu-p53 transgene, arguing against a spurious property of the E6 gene product in this sensitization. These results show that disruption of p53 function in MCF-7 and HCT116 cells increases the cytotoxicity of CPT.

top1 Protein Levels Are the Same in HCT116/CMV and HCT116/E6 Cell Lines. The basal amounts of p53 were measured in HCT116/CMV and HCT116/E6 cell lines, and clear differences in the protein levels were seen (Fig 2A). These results are consistent with the ability of the E6 gene to degrade p53 protein. The impaired response to γ-ray-induced p53 by E6 transfection was also seen in MCF-7 (7). We measured top1 protein levels in the transfected cells before and after CPT treatment. HCT116/CMV and HCT116/E6 cells were treated with 0.05 μM and 0.5 μM CPT for 8 h, and top1 protein levels were analyzed by Western blotting (Fig. 2B). top1 protein levels were found not to change significantly with respect to CPT treatment or the expression of the E6 gene product. These results suggest that top1, the cellular target for CPT, in contrast to p53, was not affected by transfection with E6.

CPT-induced top1 DNA Cleavage Activity Was Not Affected by E6 Transfection. An in vitro assay was used to investigate whether E6 affected top1 activity and CPT-induced cleavage. We have reported previously (24) that a 33-mer oligonucleotide containing a high-affinity top1 binding site (Fig. 3A) can be used to determine CPT-induced top1-mediated DNA cleavage activity using crude nuclear extracts. top1 cleaves the labeled 33 mer to produce a 19-mer cleaved product that can be separated by DNA denaturing electrophoresis (Fig. 3B), and
CPT-induced DNA cleavage can be quantitated by PhosphorImager and plotted versus nuclear protein concentration. MCF-7/E6 nuclear extracts produced approximately 1.5-fold more CPT-induced cleavage activity than MCF-7/CMV (Fig. 3C), whereas nuclear extracts from HCT116/E6 cells had approximately 1.5-fold less top1 cleavage activity than HCT116 and HCT116/CMV nuclear extracts (panel 3D). Although differences in top1 cleavage activity were slightly altered in the E6 transfectant, it is clear that such changes did not correlate completely with the survival data.

To assess top1 activity in vivo, CPT-induced SSBs and DPCs were measured by alkaline elution. The induction of SSB by CPT in the MCF-7 cells (Fig. 4A) and HCT116 cell lines pairs (Fig. 4B) was calculated in all of the cell lines and was not significantly different (Table 2). DPCs were measured in HCT116/CMV and HCT116/E6 cells after 1-h treatments with different concentrations of CPT (Fig. 5). HCT116/E6 produced slightly less CPT-induced DPC than HCT116/CMV cells (Table 2). The amount of initial damage induced by CPT was not significantly affected by the p53 status for the MCF-7 and HCT116 cell lines. These results demonstrate that the increased CPT sensitivity of the E6 transfected cells is not a direct result of differences of top1-mediated DNA damage.

DNA Fragmentation Was Not Affected by E6 Transfection. The human papilloma virus 16 E6 gene has been reported to sensitize human epithelial cells to apoptosis by mitomycin C and staurosporine (32). Therefore, the possibility that E6 would sensitize HCT116 cells to CPT-induced apoptosis was investigated using a DNA fragmentation assay (28). Cells were treated for 8 h with CPT, and DNA fragmentation was assayed...
after 24 and 48 h. Neither HCT116/CMV nor HCT116/E6 cells showed any substantial DNA fragmentation for at least 48 h after CPT treatment (Table 3). Morphological studies also failed to show apoptosis in normal, CMV-, and E6-transfected cells (data not shown), indicating that MCF-7 and HCT116 carcinoma cells did not die by rapid apoptosis following CPT treatment.

**Table 2** Measurement of CPT-induced DNA SSBs and DPCs

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>CPT (μM)</th>
<th>SSB (rad equivalent)</th>
<th>DPC (rad equivalent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7/CMV</td>
<td>2</td>
<td>404</td>
<td>ND</td>
</tr>
<tr>
<td>MCF-7/E6</td>
<td>2</td>
<td>409</td>
<td>ND</td>
</tr>
<tr>
<td>HCT116</td>
<td>0.2</td>
<td>485</td>
<td>ND</td>
</tr>
<tr>
<td>HCT116/CMV</td>
<td>1</td>
<td>536</td>
<td>ND</td>
</tr>
<tr>
<td>HCT116/E6</td>
<td>0.2</td>
<td>609</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>628</td>
<td>884</td>
</tr>
</tbody>
</table>

* ND, not determined.

**Fig. 5** Measurement of DPCs induced by CPT in HCT116/CMV and HCT116/E6 after 1 h of CPT treatment. Solid lines and filled symbols, HCT116/CMV cells; dashed lines and open symbols, HCT116/E6 cells.

**DISCUSSION**

To investigate the role of p53 in CPT-induced cytotoxicity, we have examined MCF-7 and HCT116 cell lines in which p53 function was disrupted by transfection with the human papillomavirus type-16 E6 gene. This study shows that these two sets of isogenic cell lines with p53 alteration are more sensitive to CPT when compared to parental- or control-transfected cells. p53 disruption did not affect top1 protein levels or assessable levels of DNA damage (cleavable complexes) in the presence of CPT. These results suggest that p53 plays a role in the downstream events from which CPT-induced top1 DNA damage is translated into cell death (34).

Some cell types with inactivated p53 are more sensitive to other chemotherapeutic agents, including cisplatin and alkylating agents such as melphalan (7, 35). Similar sensitization has been reported for UV (36). One plausible explanation is that the loss of p53 results in impaired repair of DNA damage produced by these agents. Evidence linking p53 to the repair of cisplatin-introduced DNA adducts was shown using a platinated reporter plasmid (7). Also, Smith et al. have shown that RKO cells lacking functional p53 have reduced ability to repair a UV-damaged reporter plasmid. The pathways by which CPT-induced damage is repaired are not known. CPT forms a ternary complex with top1 and DNA that prevents the religation of the top1-induced nicks in DNA. CPT-induced cytotoxicity seems to depend upon ongoing DNA replication. Collision between an advancing replication fork and CPT-induced DNA SSBs probably results in irreversible fork arrest and production of DNA double-strand breaks (16); CPT can then induce p53 only when the DNA breaks are replication associated (37). p53 might be involved in the recognition and repair of CPT-induced damage. In favor of this hypothesis, enhanced binding of wt p53 to its consensus DNA sequence was seen after the exposure of NIH-3T3 mouse fibroblasts to CPT (38). wt p53 and the COOH-terminal 75 amino acid of p53 show an increased binding affinity for damaged DNA by restriction digestion, DNase I treatment, or IR (39, 40). DNA strand breaks may be sufficient to cause an increase in p53 binding activity. The binding of p53 to gene-regulatory sequences activates the transcription of growth-arrest genes e.g., gadd45 (33) and WAF1/CIP1 (41), represses cell cycle genes, and/or halts DNA replication.

A single unpaired lesion (39) may be sufficient to activate the G1 arrest response in human and rodent cells. Huang et al. (39) proposed that the p53-dependent G1 arrest is partly due to the inefficient repair of the double-strand breaks and that this mechanism prevents the proliferation of cells with structural chromosome changes. A breakage of a single dicentric chromosome in a cell expressing wt p53 also produces a prolonged G1 arrest (42). Thus, it is possible that CPT, which is known to be selectively toxic to replicating cells (17, 34), can also directly
activate G1 arrest in cells that are initiating DNA replication. The association of topo1 with replication complexes would be consistent with this possibility (43).

In tumor cells that do not undergo apoptosis, our results indicate that p53 protects cells from DNA damage-induced cell death. Expression of the E6 oncoprotein results in a p53-deficient phenotype with a loss of the CPT-induced G1 checkpoint function. Abrogation of this checkpoint may be responsible for an increase in sensitivity of p53-deficient cells to CPT when compared to cells with normal p53. This observation has implications for the treatment of cancers. Because p53 mutations are most commonly occurring in human malignancies, this provides a rationale for the relative selectivity of CPTs toward malignant cells with altered p53.

### REFERENCES


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**Table 3** DNA fragmentation in HCT116 cell lines treated with CPT

<table>
<thead>
<tr>
<th>CPT (μM)</th>
<th>HCT116</th>
<th>HCT116/CMV</th>
<th>HCT116/E6</th>
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<tr>
<td>24 h</td>
<td></td>
<td></td>
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<tr>
<td>0.05</td>
<td>2.1</td>
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<td>0.2</td>
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<td>48 h</td>
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<td>1.5</td>
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<td>0.2</td>
<td>1.64</td>
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<td>1</td>
<td>10.4</td>
<td>12.5</td>
<td>12.1</td>
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**Fig. 6** Flow cytometry analysis of HCT116/CMV and HCT116/E6 cell lines in the presence of nocodazole and CPT. Cells were treated with CPT for 8 h in the presence of 0.4 μg/ml nocodazole, and CPT was then removed. Cells were washed and resuspended in fresh medium containing nocodazole. Cell cycle profiles were analyzed after an additional 16 h.


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