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Characterization of p53 Wild-Type and Null Isogenic Colorectal Cancer Cell Lines Resistant to 5-Fluorouracil, Oxaliplatin, and Irinotecan

John Boyer,¹ Estelle G. McLean,¹
Somaiah Aroori,¹ Peter Wilson,¹
Andrea McCulla,¹ P. Declan Carey,²
Daniel B. Longley,¹ and Patrick G. Johnston¹

Departments of ¹Oncology, Cancer Research Centre, and ²Surgery, Queen's University Belfast, Belfast, Northern Ireland

ABSTRACT

To elucidate mechanisms of resistance to chemotherapies currently used in the first-line treatment of advanced colorectal cancer, we have developed a panel of HCT116 p53 wild-type (p53^{+/+}) and null (p53^{-/-}) isogenic colorectal cancer cell lines resistant to the antimetabolite 5-fluorouracil (5-FU), topoisomerase I inhibitor irinotecan (CPT-11), and DNA-damaging agent oxaliplatin. These cell lines were generated by repeated exposure to stepwise increasing concentrations of each drug over a period of several months. We have demonstrated a significant decrease in sensitivity to 5-FU, CPT-11, and oxaliplatin in each respective resistant cell line relative to the parental line as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis, with increases in IC₅₀ (72 h) concentrations ranging from 3- to 65-fold. Using flow cytometry, we have also demonstrated compromised apoptosis and cell cycle arrest in 5-FU-, oxaliplatin-, and CPT-11-resistant cell lines compared with the parental lines after exposure to each drug. In addition, we found that resistance to 5-FU and oxaliplatin was higher in parental p53^{-/-} cells compared with parental p53^{+/+} cells, with an ~5-fold increase in IC₅₀ (72 h) for each drug. In contrast, the IC₅₀ (72 h) doses for CPT-11 were identical in the p53 wild-type and null cell lines. Furthermore, apoptosis after treatment with 5-FU and oxaliplatin, but not CPT-11, was significantly reduced in parental p53^{-/-} cells compared with parental p53^{+/+} cells. These data suggest that p53 may be an important determinant of sensitivity to 5-FU and oxaliplatin but not CPT-11. Using

semiquantitative reverse transcription-PCR, we have demonstrated down-regulation of thymidine phosphorylase mRNA in both p53^{+/+} and p53^{-/-} 5-FU-resistant cells, suggesting that decreased production of 5-FU active metabolites may be an important resistance mechanism in these lines. In oxaliplatin-resistant cells, we noted increased mRNA levels of the nucleotide excision repair gene *ERCC1* and ATP-binding cassette transporter breast cancer resistance protein. In CPT-11-resistant cells, we found reduced mRNA levels of carboxylesterase, the enzyme responsible for converting CPT-11 to its active metabolite SN-38, and topoisomerase I, the SN-38 target enzyme. In addition, we noted overexpression of breast cancer resistance protein in the CPT-11-resistant lines. These cell lines are ideal tools with which to identify novel determinants of drug resistance in both the presence and absence of wild-type p53.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the Western world. The number of new cases of CRC worldwide is increasing, and approximately one-half of CRC patients develop metastatic disease. The most active drug against this malignancy, the antimetabolite 5-fluorouracil (5-FU), was developed >40 years ago. In patients with resected stage III CRC, adjuvant 5-FU-based chemotherapy has been demonstrated to improve disease-free and overall survival by 35 and 22%, respectively (1). However, in advanced CRC, 5-FU monotherapy produces response rates of only 10–15% (2). Efforts to improve response rates have led to the combination of 5-FU with the newer cytotoxic drugs CPT-11 and oxaliplatin. This has significantly improved response rates (40–50%) and prolonged progression-free survival (3, 4). Despite these improvements, more than half of patients undergo chemotherapy for advanced CRC without any measurable shrinkage of their disease.

With the increasing number of therapeutic options, predictive marker testing (both in the adjuvant and metastatic setting) could allow selection of chemotherapeutic regimens according to the molecular phenotype of tumor and patient. This would improve response rates and survival and prevent unnecessarily exposing patients to the toxic effects of drugs from which they are unlikely to benefit. Because of the widespread use of 5-FU-based chemotherapy in the treatment of CRC, most predictive data have been reported for this agent. Expression of thymidylate synthase (TS) has been shown to predict for a poor response to 5-FU (5–7). In addition, high expression levels of dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP) have been associated with resistance of metastatic disease to 5-FU (8, 9). Few molecular markers are currently available that would allow the prospective identification of patients most likely to respond to oxaliplatin or CPT-11. High mRNA expres-

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Requests for reprints: Patrick G. Johnston, The Department of Oncology, Cancer Research Centre, Queen's University Belfast, University Floor, Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, Northern Ireland. Phone: 44-2890-263911; Fax: 44-2890-263744; E-mail: oncology@qub.ac.uk.

sion of the *ERCC1* and *TS* genes has been shown to be predictive of poor response in patients treated with oxaliplatin combined with 5-FU (10), suggesting that *ERCC1* may be a determinant of oxaliplatin sensitivity. Reduced TOPO I expression has been demonstrated in CPT-11-resistant cell lines; however, a consistent association between pretreatment TOPO I expression and tumor response to CPT-11 has not been described (11).

The present study describes the development and characterization of a panel of 5-FU-, CPT-11-, and oxaliplatin-resistant p53 wild-type and null cell lines derived from HCT116 colorectal carcinoma cells. These model systems have been used to examine the mRNA expression levels of a number of potentially important mediators of response to these chemotherapies to identify key regulators of resistance or sensitivity that may be of use in the clinical setting.

MATERIALS AND METHODS

Materials. 5-FU was purchased from Sigma Chemical Co. (St. Louis, MO). CPT-11 and oxaliplatin were obtained from Pharmacia and Upjohn (Kalamazoo, MI) and Sanofi-Synthelabo (Malvern, PA), respectively. Stock solutions (1 mM) were prepared in sterile 1 × PBS, with the exception of oxaliplatin, which was prepared in sterile injection water, and stored at 4°C before use. β -tubulin and poly(ADP-ribose) polymerase (PARP) antibodies were purchased from Sigma and PharMingen (San Diego, CA), respectively.

Tissue Culture. HCT116 p53^{+/+} and p53^{-/-} isogenic human colon cancer cells were kindly provided by Professor Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The p53 gene was inactivated in HCT116 p53^{-/-} cells by homologous recombination. Briefly, two promoterless targeting vectors containing either a geneticin or hygromycin resistance gene in place of genomic p53 sequences were sequentially transfected into HCT116 p53^{+/+} cells to disrupt both p53 alleles (12). The phenotype of this cell line is stable as determined periodically by Western blot analysis. Drug-resistant HCT116 sublines were developed in our laboratory by repeated exposure to stepwise increasing concentrations of 5-FU, CPT-11, or oxaliplatin over a period of ~10 months. Parental and drug-resistant HCT116 cell lines were grown in McCoy's 5A medium, supplemented with 10% dialyzed FCS, 50 μ g/ml penicillin-streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen Life Technologies Corp., Paisley, Scotland) and maintained at 37°C in a humidified atmosphere containing 5% CO₂. 5-FU-resistant p53^{+/+} and p53^{-/-} HCT116 cells were maintained in the presence of 2 and 4 μ M 5-FU, respectively. CPT-11-resistant p53^{+/+} and p53^{-/-} HCT116 cells were maintained in the presence of 1 and 3 μ M CPT-11, respectively. Oxaliplatin-resistant p53^{+/+} and p53^{-/-} HCT116 cells were found to be stably resistant and therefore maintained in oxaliplatin-free medium that was spiked every 4 weeks with 8 and 9 μ M oxaliplatin, respectively. Before each experiment, resistant sublines were cultured in the absence of drug for 48 h.

Cytotoxicity Studies. Cells were seeded at 2000 cells/well in 96-well microtiter plates. After 48 h, cells were treated with a range of concentrations of 5-FU, CPT-11, or oxaliplatin.

After 72 h, 25 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) dye (5 mg/ml) were added to each well, and the plates were incubated at 37°C for 3 h. Dark blue formazan crystals formed by live cells were dissolved in 200 μ l of DMSO, and absorbance in individual wells was determined at 570 nm using an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA). Results were expressed in terms of the concentration required to inhibit cell growth by 50% relative to untreated cells [IC_{50} (72 h)].

Flow Cytometry. Cells were seeded at 5×10^4 cells/well in six-well plates. After 48 h, cells were treated with a range of concentrations of 5-FU, CPT-11, or oxaliplatin. Seventy-two h post-treatment, cells were harvested in 5 ml of 1 × PBS/0.5 mM EDTA and pelleted by centrifugation at 1000 rpm/4°C for 5 min. Cell pellets were washed once with 1 × PBS/1% FCS, fixed in 70% ethanol, and stained with propidium iodide. Analyses were performed on a Beckman Coulter Epics XL flow cytometer (Miami, FL).

Immunoblotting. Cells were seeded at 1×10^6 cells/plate in 90-mm tissue culture dishes. Forty-eight h after drug treatment, cells were treated with the described concentrations of 5-FU, CPT-11, or oxaliplatin. After 48 h, cells were harvested and resuspended in 200 μ l of 1 × RIPA buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, and 0.1% SDS]. Cells were lysed and centrifuged at 13,200 rpm/4°C for 15 min to remove cell debris. Protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Each protein sample (20 μ g) was resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane by electroblotting. Immunodetection was performed using anti-PARP or β -tubulin mouse monoclonal antibodies and a 1/2000 dilution of a horseradish peroxidase-conjugated sheep antimouse secondary antibody (Amersham, Buckinghamshire, United Kingdom). The fluorescent signal was detected using the Super Signal chemiluminescent detection system (Pierce) according to the manufacturer's instructions.

Reverse Transcription-PCR Analysis. Total RNA was isolated using the RNA STAT-60 reagent (Biogenesis, Poole, United Kingdom) according to the manufacturer's instructions. Reverse transcription was carried out with 1 μ g of RNA in a total 10- μ l reaction volume containing 4 μ l of RT buffer ($\times 5$), 1 μ l of deoxynucleoside triphosphates (10 mM), 2 μ l of DTT (0.1 M), 1 μ l of oligo (dT)₁₂₋₁₈ primer (500 μ g/ml), 1 μ l of RNase OUT (40 units/ μ l), and 1 μ l of Moloney murine leukemia virus reverse transcriptase (200 units/ μ l; all from Invitrogen Life Technologies). The mixture was incubated for 50 min at 37°C, heated for 10 min at 70°C, and then immediately chilled on ice. The PCR amplification was carried out in a final volume of 50 μ l containing 5 μ l of PCR buffer ($\times 10$), 1 μ l of deoxynucleoside triphosphates (10 mM), 0.5 μ l of TaqDNA polymerase (5 units/ μ l) and 1.5 μ l of MgSO₄ (50 mM; all from Invitrogen Life Technologies), 2.5 μ l of primers (10 μ M), and 2 μ l of cDNA. The primer sequences used in PCR amplification are listed in Table 1.

RESULTS

Cytotoxicity Analyses. By repeated exposure to stepwise increasing concentrations of drug over a period of 10

Table 1 Oligonucleotide primers for PCR amplification

Gene	Primer sequence (5'-3')
Breast Cancer Resistance Protein (BCRP)	Sense: GCCTCACAGTGATAACCAGCT Antisense: ACAGGTGGAGGCAAATCTTCG
Carboxylesterase (CE)	Sense: CGGTGGTGCCTTGTTTTTGG Antisense: GATCCTCATGACCTTGGG
Dihydropyrimidine Dehydrogenase (DPD)	Sense: CGAGAAGCAATGAGATGCC Antisense: ACAGGCGCACATTCCTGC
Excision Repair Cross Complementing Protein 1 (ERCC1)	Sense: CGAATATGCCATCTCACAGCC Antisense: GGGTACTTTCAAGAAGGC
γ -glutamylcysteine Synthetase (γ GCS)	Sense: CATCTACCACGGGTCAAGG Antisense: GCAGGCTTGGAAATGCACC
Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH)	Sense: GTGAAGGTCGGATCAACG Antisense: GGAATTTGCCATGGGTGG
Orotate Phosphoribosyltransferase (OPRT)	Sense: GCGTCTTCTGAGTCAGGTTG Antisense: GCATCTGCTAGCTGCAACAG
Thymidine Kinase (TK)	Sense: GAGCTGCATTAACCTGCC Antisense: TCGACCTCCTTCTCTGTG
Thymidine Phosphorylase (TP)	Sense: CAGCAGCTTGTGGACAAGC Antisense: ACCAGCGTCTTTGCCAGC
Thymidylate Synthase (TS)	Sense: GGAAGGGTGTTTTGGAGGAGTT Antisense: AGATTTTCACTCCCTTGGAGACA
Topoisomerase I (TOPO I)	Sense: CCACCTCCACAACGATTCC Antisense: GGATAGCGCTCTTCTTCCC
Topoisomerase II α (TOPO II α)	Sense: GAAGTGCACCATTGCAGCCT Antisense: TGAGTTCCATCTCACAGCCT
Uridine Kinase (UK)	Sense: CAGGACAGGTTCTACAAGG Antisense: CGATCAGGTTGACAAC
Uridine Phosphorylase (UP)	Sense: CAGTGGATACCTGCTTCAAGG Antisense: TTCTCCGTGTAGGAGCAGAGA
Xeroderma Pigmentosum Group A Complementing Protein (XPA)	Sense: GCTACTGGAGGCATGGCTAAT Antisense: CCCCAAACTTCAAGAGACCTC

Table 2 IC₅₀ (72 h) obtained from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays of 5-FU-, oxaliplatin-, and CPT-11-treated p53^{+/+} and p53^{-/-} HCT116 parental and drug-resistant cells. Values were calculated using Graphpad Prism software (Graphpad Software, Inc., San Diego, CA).

Cell line	IC ₅₀ (72 h) (μ M)		
	5-FU	Oxaliplatin	CPT-11
HCT116 p53 ^{+/+} parental	4.3	0.3	3.2
p53 ^{+/+} 5-FU-resistant	12.7	0.3	7.2
p53 ^{+/+} oxaliplatin-resistant	3.6	9.4	2.9
p53 ^{+/+} CPT-11-resistant	4.2	0.3	30.3
HCT116 p53 ^{-/-} parental	19.7	1.7	3.1
p53 ^{-/-} 5-FU-resistant	178.2	1.9	2.8
p53 ^{-/-} oxaliplatin-resistant	22.0	17.9	3.5
p53 ^{-/-} CPT-11-resistant	47.0	1.7	200.4

months, we generated a panel of isogenic p53^{+/+} and p53^{-/-} HCT116 CRC cell lines resistant to 5-FU, oxaliplatin, and CPT-11. In the p53 wild-type setting, we demonstrated that the IC₅₀ (72 h) for 5-FU, oxaliplatin, and CPT-11 were increased 3-, 31-, and 10-fold in their respective resistant lines compared with sensitive parental cells (Table 2). Interestingly, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis, the p53^{+/+} 5-FU-resistant cell line was shown to be ~2-fold more resistant to CPT-11 than parental cells. However, this cross-resistance was not apparent when further examined using flow cytometry (data not shown). In the p53 null setting, the IC₅₀ (72 h) for 5-FU, oxaliplatin, and CPT-11 were increased by 9-,

10.5-, and 65-fold in their respective resistant lines compared with parental cells (Table 2). In addition, an ~2-fold increase in IC₅₀ (72 h) was noted in p53^{-/-} CPT-11-resistant cells after treatment with 5-FU. However, further examination using cell cycle analyses revealed no evidence of cross-resistance to 5-FU in the CPT-11-resistant cell line (data not shown).

We also found that both the p53^{+/+} and p53^{-/-} CPT-11-resistant cell lines were equally resistant to the CPT-11 active metabolite SN-38 with an ~10- and ~100-fold increase in IC₅₀ (72 h) doses, respectively (Table 3).

Oxaliplatin has shown activity in a number of cell lines which exhibit resistance to cisplatin and carboplatin (13). In accordance with this, we found that neither the p53^{+/+} nor p53^{-/-} oxaliplatin-resistant cell lines were cross-resistant to cisplatin (Table 4). A small increase (~2-fold) in the IC₅₀ (72 h) doses of carboplatin was observed in the oxaliplatin-resistant

Table 3 IC₅₀ (72 h) obtained from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays of SN-38-treated p53^{+/+} and p53^{-/-} HCT116 parental and drug-resistant cells. Values were calculated using Graphpad Prism software (Graphpad Software, Inc.).

Cell line	SN-38 IC ₅₀ (72 h) (nM)	
	p53 ^{+/+}	p53 ^{-/-}
Parental	5.6	4.2
5-FU-resistant	6.5	3.6
Oxaliplatin-resistant	3.7	3.3
CPT-11-resistant	40.0	540.5

Table 4 IC₅₀ (72 h) obtained from 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays of cisplatin- and carboplatin-treated p53^{+/+} and p53^{-/-} HCT116 parental and oxaliplatin-resistant cells. Values were calculated using Graphpad Prism software (Graphpad Software, Inc.)

Cell line	IC ₅₀ (72 h) (μM)	
	Cisplatin	Carboplatin
HCT116 p53 ^{+/+} parental	5.4	76.0
p53 ^{+/+} oxaliplatin-resistant	7.3	176.0
HCT116 p53 ^{-/-} parental	5.7	78.7
p53 ^{-/-} oxaliplatin-resistant	6.7	141.0

cell lines; however, this was significantly less than the increase in resistance to oxaliplatin. These results suggest that oxaliplatin has a different mechanism of action and/or resistance than cisplatin and carboplatin.

Cell Cycle Analyses. Flow cytometry was used to examine the cell cycle distribution of parental and resistant cells after treatment with a range of concentrations of each drug. In the p53 wild-type setting, an S phase arrest and evidence of polyploidy (DNA content > 4N) were observed after treatment of parental cells with 1 μM 5-FU for 72 h (Fig. 1A). After exposure to 5 and 10 μM 5-FU, the majority of p53^{+/+} parental cells had arrested in G₂-M phase, and there was a significant increase in the subG₀-G₁ content (~30–35% compared with ~4% in control samples). In contrast, p53^{+/+} 5-FU-resistant cells showed no

change in cell cycle profile after exposure to 1 μM 5-FU, whereas in response to 5 and 10 μM 5-FU, the majority of cells had arrested at the G₁-S boundary. Furthermore, induction of apoptosis in response to 5 and 10 μM 5-FU was significantly reduced in the 5-FU-resistant subline. When p53^{+/+} parental cells were treated with 0.5 μM oxaliplatin for 72 h, the majority of cells had arrested in G₂-M phase of the cell cycle. This was accompanied by the appearance of a small polyploid peak (Fig. 1B). After treatment of the parental line with 1 and 5 μM oxaliplatin, we noted a significant increase in the proportion of apoptotic cells (~40–50% compared with ~2% in control samples) and number of cells with DNA content > 4N. In contrast, the cell cycle profile of p53^{+/+} oxaliplatin-resistant cells was unaffected by treatment with 0.5 and 1 μM oxaliplatin. After exposure of p53^{+/+} oxaliplatin-resistant cells to 5 μM oxaliplatin, the majority of cells were arrested in S phase. In addition, the induction of apoptosis in the oxaliplatin-resistant subline was dramatically attenuated compared with parental cells. Treatment of p53^{+/+} parental cells with 0.5 μM CPT-11 resulted in accumulation of cells in S phase, and cells with DNA content > 4N were observed (Fig. 1C). Further evidence of polyploidy was demonstrated at 1 μM CPT-11 in the p53^{+/+} parental line, although the majority of cells were now arrested in G₂-M. Treatment with 5 μM CPT-11 resulted in the accumulation of ~40% of cells in the subG₀-G₁ apoptotic phase. The p53^{+/+} CPT-11-resistant cell line was almost completely insensitive to 0.5 and 1 μM CPT-11. However, treatment with 5 μM CPT-11

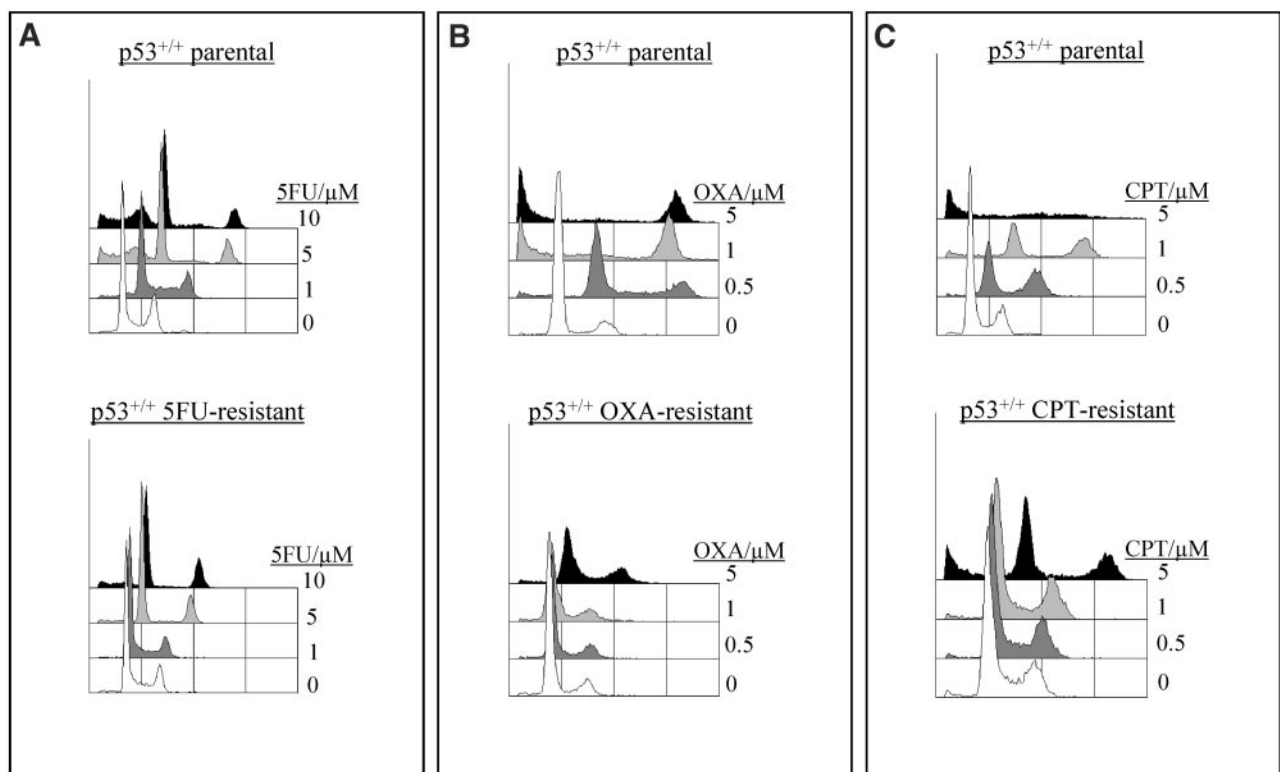


Fig. 1 Cell cycle distribution of p53^{+/+} HCT116 parental and -resistant cells after treatment with 0, 1, 5, and 10 μM 5-FU (A); 0, 0.5, 1, and 5 μM oxaliplatin (B); and 0, 0.5, 1, and 5 μM CPT-11 (C).

did cause a significant G₂-M arrest and accumulation of polyploid cells. A significant degree of apoptosis was also demonstrated (~14%), although this was less than observed in the parental cell line (~40%).

In the p53^{-/-} setting, parental cells treated with 1 μM 5-FU for 72 h had arrested in S phase, and the appearance of a polyploid peak was noted (Fig. 2A). After exposure to 5 and 10 μM 5-FU, the majority of p53^{-/-} parental cells had DNA content > 4N, indicative of polyploid cells. In contrast, p53^{-/-} 5-FU-resistant cells showed no change in cell cycle profile relative to untreated control cells after exposure to 1, 5, and 10 μM 5-FU. When p53^{-/-} parental cells were treated with 1 μM oxaliplatin, we observed an S phase block and a moderate increase in the polyploid fraction (Fig. 2B). In response to 5 μM oxaliplatin, the majority of cells were arrested in G₂-M phase, and a significant percentage had DNA content > 4N. In contrast, p53^{-/-} oxaliplatin-resistant cells exhibited no change in cell cycle distribution after treatment with the same concentrations of oxaliplatin. Treatment of the p53^{-/-} parental cell line with 0.5 and 1 μM CPT-11 resulted in a dramatic G₂-M cell cycle arrest (Fig. 2C). After treatment with 5 μM CPT-11, we noted an increase in the number of apoptotic cells (~35% compared with ~2% in control samples), similar to what was observed in the p53^{+/+} cell line. In contrast, no apoptosis was observed in response to 5 μM CPT-11 in the p53^{-/-} CPT-11-resistant cell line. Furthermore, no G₂-M arrest was observed in response to 0.5 and 1 μM CPT-11 in the resistant subline.

Together, these profiles characterize the differences in cell cycle progression that underlie the resistant phenotypes observed in the growth inhibition analyses.

Role of p53 in Drug Resistance. Flow cytometry was used to compare drug-induced apoptosis in p53^{+/+} and p53^{-/-} parental HCT116 cells after treatment with a range of concentrations of 5-FU, oxaliplatin, or CPT-11. Our results demonstrated significantly less apoptosis in p53^{-/-} cells treated with 5-FU compared with p53^{+/+} cells (Fig. 3A). Similarly, oxaliplatin-induced apoptosis was significantly attenuated in p53^{-/-} cells compared with p53^{+/+} cells (Fig. 3B). In contrast, CPT-11 induced almost identical levels of apoptosis in the p53^{+/+} and p53^{-/-} cell lines (Fig. 3C). These data agree with the cytotoxicity analyses, which generated almost identical IC₅₀ (72 h) for the parental p53^{+/+} and p53^{-/-} cells treated with CPT-11 (Table 2). Furthermore, the IC₅₀ (72 h) doses of SN-38 in the p53^{+/+} and p53^{-/-} cell lines were similar (Table 3). In contrast, the IC₅₀ (72 h) doses for 5-FU and oxaliplatin were increased by 4.6- and 5.7-fold, respectively, in p53^{-/-} compared with p53^{+/+} cells after treatment with 5-FU and oxaliplatin, respectively. In addition, PARP cleavage (a hallmark of apoptosis) was observed in p53^{+/+} cells but not p53^{-/-} cells after treatment with 5 μM 5-FU and 1 μM oxaliplatin for 48 h (Fig. 3D). In contrast, PARP cleavage was evident in both p53^{+/+} and p53^{-/-} cells treated with 5 μM CPT-11 (Fig. 3D). These results suggest that p53 may be an important determinant of the apoptotic response to 5-FU and oxaliplatin but not CPT-11.

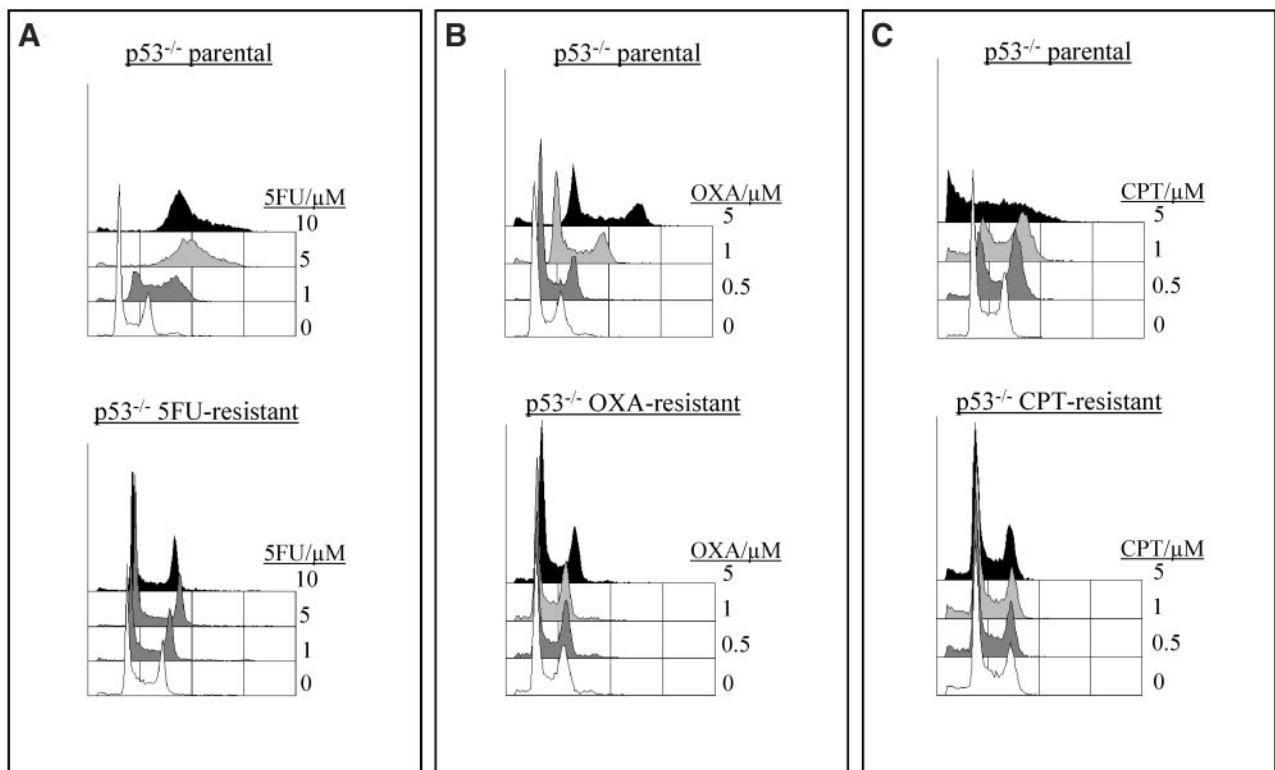


Fig. 2 Cell cycle distribution of p53^{-/-} HCT116 parental and -resistant cells after treatment with 0, 1, 5, and 10 μM 5-FU (A); 0, 0.5, 1, and 5 μM oxaliplatin (B); and 0, 0.5, 1, and 5 μM CPT-11 (C).

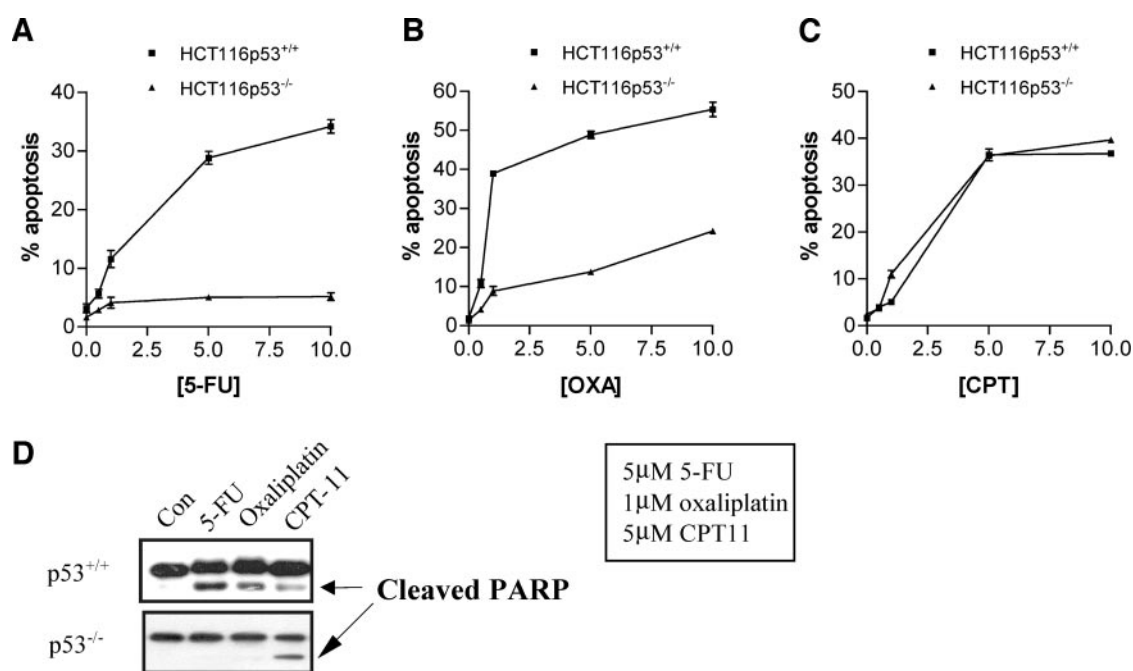


Fig. 3 Reduced levels of apoptosis in p53^{-/-} HCT116 cells treated with a range of concentrations of 5-FU (A) and oxaliplatin (B) for 72 h compared with p53^{+/+} cells. In C, p53^{+/+} and p53^{-/-} cells treated with CPT-11 exhibit identical levels of apoptosis. D, Western blot demonstrating poly(ADP-ribose) polymerase (PARP) cleavage in p53^{+/+} and p53^{-/-} HCT116 cells after treatment with 5 μ M CPT-11 for 48 h. After exposure to 5 μ M 5-FU and 1 μ M oxaliplatin for 48 h, PARP cleavage was only evident in p53^{+/+} cells.

mRNA Expression of Genes Implicated in Drug Resistance. Semiquantitative reverse transcription-PCR analysis was used to analyze the expression levels of a number of genes that have been implicated in determining sensitivity to 5-FU-, oxaliplatin-, and CPT-11-based chemotherapy.

5-FU-Resistant Cells. In both p53^{+/+} and p53^{-/-} 5-FU-resistant cells, we observed significant decreases in the levels of the 5-FU-anabolizing enzyme TP compared with parental cells (Fig. 4A). In addition, we noted that thymidine kinase (TK), which salvages thymidylate from exogenous thymidine, was highly overexpressed in p53^{+/+} 5-FU-resistant cells. Of note, the 5-FU target enzyme TS remained unchanged in parental and resistant cells (Fig. 4A). We also noted that mRNA levels of the 5-FU-catabolizing enzyme DPD and 5-FU-anabolizing enzymes uridine phosphorylase and uridine kinase were comparable in the 5-FU-resistant and parental lines. Interestingly, orotate phosphoribosyltransferase (OPRT) expression was lower in p53^{-/-} 5-FU-resistant cells, whereas in the p53 wild-type setting, the inverse was true. These results suggest that the underlying mechanism of 5-FU resistance in these cell lines may, at least in part, be explained by decreased synthesis of active 5-FU metabolites by TP in both p53^{+/+} and p53^{-/-} cells, down-regulation of OPRT in p53^{-/-} cells, and overexpression of TK in p53^{+/+} cells. These data also imply that TS inhibition is not a key mechanism of action of 5-FU in these cell lines, which is in agreement with the findings of others (14).

Oxaliplatin-Resistant Cells. In p53^{+/+} and p53^{-/-} oxaliplatin-resistant cells, we found significant increases in the mRNA levels of the nucleotide excision repair gene *ERCC1* compared with parental cells (Fig. 4B). Furthermore, we noted

up-regulation of several ERCC1 splice variants in oxaliplatin-resistant cells. In contrast, we saw no modulation of the DNA damage-binding factor xeroderma pigmentosum group A complementing protein or glutathione metabolic enzyme γ -glutamylcysteine synthetase. The ATP-binding cassette transporter breast cancer resistance protein (BCRP), however, was dramatically up-regulated in both the p53^{+/+} and p53^{-/-} oxaliplatin-resistant cell lines compared with the respective parental lines. These data suggest that the oxaliplatin-resistant phenotype, in both p53^{+/+} and p53^{-/-} settings, may at least partially be explained by increased nucleotide excision repair of platinum-DNA adducts. In addition, increased cellular export of oxaliplatin by the multidrug resistance protein BCRP may decrease sensitivity to this chemotherapy.

CPT-11-Resistant Cells. In both p53^{+/+} and p53^{-/-} CPT-11-resistant cells, we noted a marked decrease in the levels of carboxylesterase (CE), the enzyme which converts CPT-11 to SN-38, compared with parental cells (Fig. 4C). The SN-38 target enzyme, TOPO I, was dramatically down-regulated in both the p53^{+/+} and p53^{-/-} CPT-11-resistant cell lines. In contrast, we observed no modulation of TOPO II α mRNA expression. In addition, BCRP expression was increased in both p53^{+/+} and p53^{-/-} CPT-11-resistant cell lines compared with the respective parental cell lines. Together, these data suggest that inhibition of conversion of CPT-11 to SN-38, down-regulation of the SN-38 target enzyme TOPO I, and increased cellular export of SN-38 may contribute to the resistant phenotype in these cells. However, we observed that both p53^{+/+} and p53^{-/-} CPT-11-resistant cell lines were highly cross-resistant

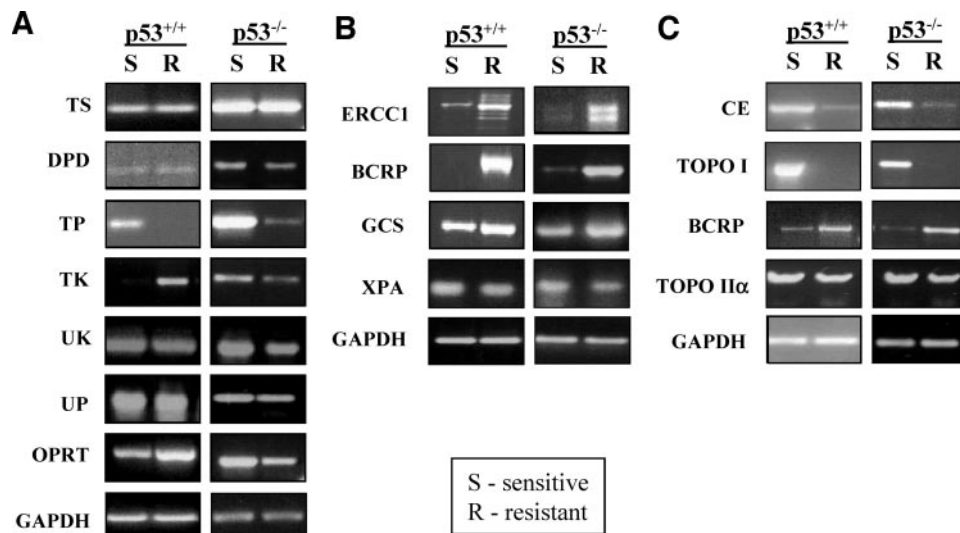


Fig. 4 A, basal mRNA expression levels of thymidylate synthase (*TS*), dihydropyrimidine dehydrogenase (*DPD*), thymidine phosphorylase (*TP*), thymidine kinase (*TK*), orotate phosphoribosyltransferase (*OPRT*), uridine phosphorylase (*UP*), and uridine kinase (*UK*) in $p53^{+/+}$ and $p53^{-/-}$ HCT116 parental and 5-FU-resistant cells. B, basal mRNA expression levels of excision repair cross-complementing protein 1 (*ERCC1*), γ -glutamylcysteine synthetase (γ *GCS*), breast cancer resistance protein (*BCRP*), and xeroderma pigmentosum group A complementing protein (*XPA*) in $p53^{+/+}$ and $p53^{-/-}$ HCT116 parental and oxaliplatin-resistant cells. C, basal mRNA expression levels of carboxylesterase (*CE*), topoisomerase I (*TOPO I*), BCRP, and topoisomerase II α (*TOPO II α*) in $p53^{+/+}$ and $p53^{-/-}$ HCT116 parental and CPT-11-resistant cells. In each case, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression was assessed as a loading control.

to SN-38 (Table 3), suggesting that CE down-regulation is not a primary mechanism of resistance to CPT-11 in these cells.

DISCUSSION

We have developed a panel of $p53^{+/+}$ and $p53^{-/-}$ CRC cell lines resistant to 5-FU, oxaliplatin, or CPT-11 as models with which to study mechanisms of resistance to chemotherapies commonly used in the treatment of CRC. Moreover, we have also used these model systems to examine the relationship between p53 expression and response to 5-FU, oxaliplatin, and CPT-11.

By growing cells in stepwise increasing concentrations of drug, we were able to isolate cells that were between 3- and 65-fold more resistant to their respective chemotherapies than sensitive parental cells as determined by MTT analysis. In addition, using flow cytometric analysis, we demonstrated compromised cell cycle arrest and apoptosis in these resistant cell lines compared with the parental lines after drug treatment. These data indicate that compromised activation of cell cycle checkpoints and cell death pathways underpins the resistant phenotypes observed in each of the newly generated drug-resistant lines.

The p53 tumor suppressor protein plays a key role in coordinating cell cycle arrest, DNA repair, and programmed cell death after DNA damage. Mutations in p53 are seen in 40–50% of CRCs, and several *in vitro* studies have reported that loss of functional p53 reduces cellular sensitivity to 5-FU (15, 16). Results presented in this study concur with these data. We demonstrated a 4.6-fold increase in 5-FU IC_{50} (72 h) dose and significantly less apoptosis in $p53^{-/-}$ HCT116 cells compared with $p53^{+/+}$ cells after treatment with 5-FU. Several clinical

studies have also reported that p53 overexpression, which is often used as a surrogate marker for p53 mutation, correlates with resistance to 5-FU (17–19), although a number of studies have reported no correlation between p53 expression levels and 5-FU response (20, 21). At present, despite compelling *in vitro* data, the clinical usefulness of p53 as a predictive marker for 5-FU-based chemotherapy remains a matter for debate. With regard to oxaliplatin, we noted a decrease in sensitivity to this agent in $p53^{-/-}$ compared with $p53^{+/+}$ cells, as demonstrated by a 5.7-fold increase in IC_{50} (72 h) dose and compromised cell cycle arrest and apoptosis. The bulk of clinical data regarding p53 status and sensitivity to platinum compounds has focused on the first generation compound cisplatin. A study by Houldsworth *et al.* (22) noted that resistance to cisplatin in human male germ cell tumors could be linked to mutations in p53. In addition, Reles *et al.* (23) reported that p53 alterations correlated with resistance to platinum-based chemotherapy, early relapse, and shortened overall survival in ovarian cancer patients. Although oxaliplatin appears to have a different spectrum of activity to cisplatin, a number of *in vitro* studies, including this one, have found that loss of p53 function increases resistance to oxaliplatin (24, 25). At present, the clinical importance of p53 status for oxaliplatin resistance remains to be established. Wild-type p53 has been associated with increased sensitivity to TOPO I inhibitors *in vitro*, although it has also been shown that cells lacking functional p53 can undergo apoptosis after exposure to camptothecins (26, 27). In the present study, we noted equivalent sensitivity to CPT-11, as determined by cytotoxicity analysis, flow cytometric analysis, and PARP cleavage assays in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. Jacob *et al.* (28) also found that p53 status did not correlate with sensitivity to CPT-11 in a

number of CRC cell lines. In the clinical setting, Lansiaux *et al.* (29) demonstrated that levels of DNA-TOPO I complexes correlated with sensitivity to CPT-11, irrespective of their microsatellite instability and p53 phenotypes. Thus, the present study and several others suggest that p53 status may not affect chemosensitivity to CPT-11.

The mechanisms of resistance to antimetabolites frequently involve alterations in drug metabolism or expression of the target protein. Although much is understood about 5-FU, it has a complicated mechanism of action with several enzymes involved in its metabolic activation. Enhanced activities of TS and DPD have been associated with resistance to 5-FU both *in vitro* and in a number of clinical studies (6, 8, 30–32). TS is a major cellular target of 5-FU, and DPD catalyzes the rate-limiting step in the catabolism of 5-FU (33). In this study, we saw no modulation of TS or DPD mRNA expression in either p53^{+/+} or p53^{-/-} 5-FU-resistant cells. In addition to these molecules, reduced activities of 5-FU-anabolizing enzymes, such as OPRT, TP, uridine phosphorylase, and uridine kinase, have been implicated in modulating sensitivity to 5-FU *in vitro* (34). We demonstrated down-regulation of TP mRNA in 5-FU-resistant compared with parental cells. Cell culture and xenograft model systems have indicated that transfection of TP into cancer cells increases their sensitivity to 5-FU, presumably through increased metabolic activation of 5-FU to 5-fluoro-dUMP (35). In contrast, high TP overexpression has been found to be an indicator of poor prognosis in patients with CRC (9). It is thought that these contradictory findings may be attributable to the role of TP as an angiogenic factor, such that *in vivo*, TP expression may be a marker for a more invasive and aggressive tumor phenotype that is less responsive to chemotherapy (36). In addition, we showed down-regulation of OPRT mRNA expression in p53^{-/-} 5-FU-resistant cells. This is consistent with several *in vitro* studies, which have demonstrated a correlation between OPRT levels and 5-FU drug sensitivity (34, 37). Recent clinical data also suggest that OPRT activity can predict sensitivity to 5-FU in CRC patients, with high levels correlating with increased sensitivity (38, 39). Interestingly, OPRT levels appeared to be slightly elevated in p53^{+/+} 5-FU-resistant cells compared with the parental line. Additional studies are required to determine the role of OPRT in mediating the response of HCT116 cells to 5-FU. We have also shown overexpression of TK mRNA in p53^{+/+} 5-FU-resistant cells. This is in agreement with Chung *et al.* (37), who reported increased expression of TK in 5-FU-resistant gastric cancer cells. Furthermore, Oliver *et al.* (40) showed that overexpression of a heterologous TK gene protected murine BAF3 cells from apoptosis induced by inhibitors of nucleotide synthesis, such as methotrexate or fluorodeoxyuridine. The authors suggest that salvaging of thymidine by TK may compensate for inhibition of *de novo* thymidylate synthesis and thereby abrogate thymineless death. In the clinical setting, increased TS and TK activities have been reported to be significant prognostic factors for the overall survival of CRC patients (41). In contrast to these data, we demonstrated moderate down-regulation of TK mRNA levels in p53^{-/-} 5-FU-resistant cells compared with the parental line. Further investigation is necessary to define the role of TK in modulating the response to 5-FU in these cells.

There are relatively few predictive biomarkers currently

available for identification of patients most likely to respond to oxaliplatin. In this study, we demonstrated elevated levels of mRNA encoding the nucleotide excision repair protein ERCC1 in oxaliplatin-resistant cells. Similarly, Hector *et al.* (42) showed that ERCC1 mRNA levels were ~2-fold higher in an oxaliplatin-resistant ovarian carcinoma cell line relative to sensitive parental cells. Arnould *et al.* (43) have also shown that ERCC1 mRNA levels are predictive of oxaliplatin sensitivity. High ERCC1 gene expression has been shown to correlate with poor survival of patients with metastatic CRC after treatment with 5-FU/oxaliplatin (10). It would appear from this study that ERCC1 is an independent predictive marker of response to 5-FU/oxaliplatin-based chemotherapy. In the present study, we demonstrated up-regulation of both full-length ERCC1 and a number of splice variants in oxaliplatin-resistant cells. It has been postulated that the alternatively spliced species may compete with full-length ERCC1 during formation of the DNA damage recognition/excision complex, resulting in inhibition of DNA excision repair (44). Clearly, additional studies are necessary to fully assess the biological role of both full-length and alternatively spliced ERCC1 proteins in determining sensitivity to platinum chemotherapies.

We found no evidence of altered expression of the DNA repair cofactor xeroderma pigmentosum group A complementing protein or glutathione metabolic enzyme γ -glutamylcysteine synthetase in oxaliplatin-resistant cells, despite several clinical and nonclinical studies describing their association with decreased sensitivity to platinum-based chemotherapies (43, 45–47). However, we demonstrated overexpression of the ATP-binding cassette half-transporter BCRP/ATP-binding cassette G2 in both p53^{+/+} and p53^{-/-} oxaliplatin-resistant cell lines relative to parental cells. High expression of BCRP has been demonstrated in a number of drug-resistant cell lines and tumor samples (48–50). A number of chemotherapies have been shown to be substrates for BCRP, including the anthracenedione mitoxantrone; anthracyclines, such as daunorubicin and doxorubicin; topotecan; bisantrene; and the active form of irinotecan, SN-38 (51). To our knowledge, this is the first report of an association between BCRP overexpression and resistance to platinum chemotherapies. Several authors have reported that cisplatin is not a substrate for BCRP (52, 53); however, given the structural differences and lack of cross-resistance between these two molecules, it is possible that they may use different cellular transport mechanisms. In addition, it has been suggested that, unlike other multidrug-resistant proteins, glutathione is not a necessary cofactor for BCRP-mediated transport. These data support our previous observation regarding the lack of modulation of γ -glutamylcysteine synthetase expression in oxaliplatin-resistant cells. Additional studies will be carried out to fully elucidate the biological role of BCRP in oxaliplatin resistance.

A variety of mechanisms of resistance to CPT-11 has been characterized *in vivo*, although relatively little is known about their significance in the clinical setting. Cells lacking CE activity are unable to convert CPT-11 to its active metabolite SN-38 and demonstrate reduced sensitivity to the prodrug *in vitro* (54). We have shown reduced levels of CE mRNA in CPT-11-resistant cells in both the presence and absence of wild-type p53. However, because hepatic conversion is most likely to predominate *in vivo*, CE activity within tumor cells may not play a

major role in determining sensitivity to this agent. Indeed, we have shown that these CPT-11-resistant cells were also resistant to SN-38, indicating that the resistance phenotype is not dependent on the low level of CE expression. As already mentioned, the BCRP transporter has been implicated in the biliary excretion of SN-38 (55). In the present study, we demonstrated significant up-regulation of BCRP mRNA in both p53^{+/+} and p53^{-/-} CPT-11-resistant cells. To date, little information is available regarding the clinical relevance of BCRP-mediated transport of SN-38 and CPT-11 resistance. As TOPO I is the cellular target of SN-38, it is conceivable that the cellular level of TOPO I would be proportional to CPT-11 sensitivity. This notion is supported by experimental evidence from several investigators who reported decreased TOPO I expression in cells rendered resistant to CPT-11, compared with sensitive parental cells (11, 56, 57). In the present study, we demonstrated dramatic down-regulation of TOPO I mRNA in CPT-11-resistant cells in both p53^{+/+} and p53^{-/-} settings. In addition, we examined the mRNA levels of TOPO II α , following reports that decreased TOPO I expression in CPT-11-resistant cells may be compensated for by overproduction of this type II topoisomerase; however, we did not find evidence of altered TOPO II α mRNA expression in our model systems. To date, a consistent association between topoisomerase expression and responsiveness to CPT-11 has not been demonstrated.

In conclusion, we have successfully generated a panel of p53^{+/+} and p53^{-/-} isogenic CRC cell lines resistant to 5-FU, oxaliplatin, and CPT-11. We have used these cell lines to establish the expression levels of a number of markers implicated in predicting response to chemotherapies used in the treatment of advanced CRC. Furthermore, we have demonstrated a potential role for p53 as an important determinant of response to 5-FU and oxaliplatin but not CPT-11. This is an interesting observation given the high incidence of p53 mutations in CRC and suggests that CPT-11 may be equally effective in the treatment of p53 wild-type and mutant tumors. For the purpose of future studies, we plan to use this model system, in conjunction with DNA microarray and proteomic technologies, to identify novel determinants of chemosensitivity in the presence and absence of wild-type p53 and evaluate their usefulness in the clinical setting. The ultimate goal of this research is to identify a cassette of markers that predict how colorectal tumors respond to a particular chemotherapy. This will allow tailoring of treatment regimens to the molecular profile of the tumor and patient. Such an approach has the potential to dramatically improve response rates and would spare patients the harmful side effects of chemotherapeutic treatments from which they are unlikely to benefit.

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