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The Role of the DNA Mismatch Repair System in the Cytotoxicity of the Topoisomerase Inhibitors Camptothecin and Etoposide to Human Colorectal Cancer Cells[1]

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ABSTRACT

The DNA mismatch repair (MMR) system is involved in the correction of base/base mismatches and insertion/deletion loops arising during replication. In addition, some of the MMR components participate in recombination and double-strand break repair as well as cell cycle regulation and apoptosis. The inactivation of *MMR* genes, usually *hMSH2* or *hMLH1*, is associated with human colorectal cancers and is responsible for the characteristic microsatellite instability (MSI)+ phenotype of these tumors. Because MMR is assumed to modulate cytotoxicity to various chemotherapeutic agents that act upon DNA, our objectives have been to define its possible involvement in the cytotoxicity of topoisomerase inhibitors. We have shown that colorectal cancer cell lines defective in DNA MMR exhibit an increased sensitivity to both camptothecin, a topoisomerase I inhibitor, and etoposide, a topoisomerase II inhibitor. Sensitivity to these drugs cannot be predicted by measuring endogenous levels of topoisomerase I and II. Our results also indicate that neither p53 status, nor cell cycle alterations correlate with the sensitivity of colorectal cancer cells to topoisomerase inhibitors. On the other hand, our data showing that resistance to these drugs can be achieved by the functional complementation of hMLH1 in an hMLH1-defective cell line have allowed us to establish that MMR is a critical determinant for chemosensitivity. Interestingly, our observations provide the rationale for the better responsiveness of MSI+ tumors to CPT-11, a camptothecin derivative, which we have observed in patients with metastatic colorectal cancers.

INTRODUCTION

Topoisomerases are nuclear enzymes that modify the topological state of DNA and participate in fundamental metabolic processes such as replication, transcription, repair, recombination, and chromosome segregation (1). Topoisomerases catalyze the relaxation of supercoiled DNA through the transient cleavage of DNA strands by a transesterification reaction, which results in the formation of a covalent bond between the DNA and the enzyme and then religation of the cleaved DNA and dissociation of the topoisomerases. Because topoisomerases play an essential role in many fundamental cellular processes, they have been the targets for many anticancer drugs, such as ETP[3] and CPT (2–4). Both drugs interfere with the catalytic cycle of topoisomerases by reversibly stabilizing the covalent complex formed between the enzymes and the cleaved DNA, usually referred to as cleavage complex (4–6). topoII acts as a dimer and catalyzes the cleavage of both strands of the duplex. By selectively targeting DNA-bound topoII, ETP lengthens the cleavage complex half-life, which increases the number of DSBs (3, 4). topoI, the cellular target of CPT, cleaves only one strand of the double-stranded DNA; thus, stabilization of the topoI-containing cleavage complexes primarily creates SSBs (2, 5). It has been proposed that CPT cytotoxicity relies on the collision of the DNA replication fork with topoI-DNA complexes during the S phase (7, 8). This leads to the conversion of SSBs into harmful DSBs. Cellular sensitivity to CPT is, in fact, reduced by treatment with the polymerase inhibitor aphidicolin (7, 8). In addition, DSB generation may contribute to the G_2-M cell cycle arrest, as well as apoptosis, observed in response to CPT and ETP treatment (4, 9). Consistently, yeast rad52 mutants, deficient in homologous recombination and DSB repair, are hypersensitive to various topoisomerase inhibitors (10).

Several studies have reported that the DNA MMR system is involved in cell response to various chemotherapeutic agents that act on DNA (11, 12). In particular, numerous studies have reported that cells defective in MMR activity are tolerant to the alkylating agents (13–17). MMR recognizes and repairs base-base mispairs, as well as small insertion or deletion loops arising during DNA replication (11, 18–20). In addition to mismatch correction, some MMR proteins (hMSH2, hMSH3, and hMSH6) participate in recombination, DSB repair, and cell cycle regulation (21–23). They also participate in the induction of apoptosis in response to a variety of DNA lesions (11, 17, 24, 25). Mismatch recognition is mainly performed in human cells by MutSα, a heterodimeric complex composed of hMSH2 paired with hMSH6 (26, 27). This complex is able to bind to base-base mismatches and to loops of one or a few nucleotides. A second, less abundant MutS-related complex, consisting of a dimer of hMSH2 and hMSH3, referred to as MutSβ, repairs primarily heteroduplexes with two or more extrahelical bases (26, 27). Once bound to mismatches, MutSα and MutSβ complexes interact with another heterodimeric complex, MutLα, which is composed of two MutL-homologues, hMLH1 and hPMS2. This leads to the excision of a large fragment of the newly synthesized DNA strand containing the mismatch (reviewed in Refs. 18–20). Germ-line mutations of either *hMSH2* or *hMLH1* have been identified in 50–70% of patients with HNPCC (11, 28, 29). Germ-line *hMSH6* mutations are rare in HNPCC patients, but account for some late-onset familial colorectal cancer (30). Nearly all HNPCC colorectal tumors display a high instability in their simple repetitive microsatellite sequences (29, 31–33). This characteristic phenotype, referred to as MSI+, is shared by 15–20% of sporadic colorectal tumors, in the absence of an identified mutation in any of the known MMR genes (29, 34). Inactivation of hMSH6 causes a weaker mutator phenotype primarily confined to base substitutions (30). There are few frameshift mutations, and they are mainly restricted to insertion or deletion in mononucleotide tracts.

The aim of our study was to investigate the possibility that MMR deficiency affects resistance to ETP, a topoII inhibitor, and to CPT, a topoI inhibitor. We have determined the sensitivity of a panel of seven

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[3] The abbreviations used are: ETP, etoposide; CPT, camptothecin; topoII, topoisomerase II; topoI, topoisomerase I; DSB, double-strand break; SSB, single-strand break; MMR, mismatch repair. HNPCC, hereditary non-polyposis colorectal cancer; MSI, microsatellite instability; MNU, *N*-methyl-*N*-nitrosourea; MDR, multidrug resistance; Rh123, rhodamine 123; P-gp, P-glycoprotein; NBS, Nijmegen Breakage Syndrome.

MMR-proficient or -deficient colorectal cell lines to these drugs. Our data indicate that a defect in MMR is associated with a marked increase in sensitivity to both drugs. Functional complementation was obtained in an hMLH1-deficient cell line by stable expression of a cloned wild-type *hMLH1* cDNA. As expected, reexpressing a wild-type allele of *hMLH1* in the hMLH1-deficient HCT116 cell line allowed us to restore resistance to topoisomerase inhibitors. The fact that this also induced alkylation sensitivity showed that the MMR activity was indeed restored in these cell lines. Furthermore, the sensitivity to topoisomerase inhibitors correlates neither with endogenous levels of either topoisomerase I or II, nor with a defect in cell cycle checkpoint controls.

In view of our observations, we have performed a retrospective clinical study of patients with metastatic colorectal cancers treated with irinotecan, one of the CPT analogues that have recently been approved for the treatment of colorectal cancer, and have shown the predictive value of the MSI status (35). Thus, because MSI phenotype occurs in a significant subset of colorectal cancers, we believe that MSI screening of the tumors would be very useful for the selection of patients who are more likely to benefit from chemotherapy with CPT analogues.

MATERIALS AND METHODS

Cell Lines. Colorectal adenocarcinoma cell lines were kindly provided by Dr. Peter Karran (Imperial Cancer Research Fund, Clare Hall, United Kingdom) or obtained from the American Type Culture Collection[4] (36–38). The epidermoid tumor cell line KB3.1 and its Adriamycin-resistant-derived clone KB-A1 were a kind gift of Dr. Anna Starzec (Laboratory of Radiopharmacology, Bobigny, France). All cell lines were grown in monolayer cultures in Dulbecco's modified essential medium supplemented with penicillin (100 μg/ml), streptomycin (100 units/ml), L-glutamine (2 mM), and 10% heat-inactivated FCS.

Drugs. CPT (25 mM), ETP (100 mM), and MNU (20 mg/ml) stock solutions were prepared in DMSO and kept at −20°C until use. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

CPT and ETP Cytotoxicity Assays. Drug cytotoxicity assays were performed using a tetrazolium-based colorimetric assay (39). Cells were seeded in 96-well, flat-bottomed culture plates so that untreated cells grew exponentially for the 5-day culture period. Eight replicates of ten 2- or 3-fold serial dilutions of CPT (10^{-9} M to 10^{-5} M) or ETP (10^{-8} M to 10^{-4} M) were added to the cultures. Cultures in the absence of drugs were used as positive controls, and the absorbance of wells containing no cells was subtracted from each experimental value. On day 5, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (50 μg/well) was added to each well and further incubated at 37°C for 4 h. Absorbance measured at 570 nm represented the mean of a minimum of six wells. The drug concentrations resulting in 50% growth inhibition (IC_{50}) were determined by a curve-fitting analysis of the percentage of absorbance *versus* drug concentrations. Results represent a minimum of five independent experiments.

MNU Cell Sensitivity. Exponentially growing cells were treated with 50 or 100 μg/ml MNU for 1 h at 37°C in serum-free medium. Cells were then washed twice in PBS and plated in complete medium. Surviving cells were counted 3 days later using the trypan blue exclusion test and expressed as the ratio of the number of cells in treated cultures:number of cells in the absence of treatment, as described (15).

MDR Detection. MDR function was assessed by measuring the modulating effect of verapamil on the intracellular retention of Rh123. Exponentially growing cells were incubated for 30 min at 37°C with 10 μg/ml Rh123 after a 30 min of preincubation at 37°C in the presence or absence of 100 μM verapamil, as described (40). After three washings in ice-cold PBS, Rh123 intracellular levels were determined using a fluorescence-activated cell sorter (FACScalibur; Becton Dickinson, San Jose, CA); autofluorescence was quantified on cells not exposed to Rh123.

Cell Cycle Analyses. After a 48-h treatment with CPT (10 and 100 nM) or ETP (1 μM and 10 μM), the cells were trypsinized, washed in PBS, fixed in 70% ethanol, and stained with propidium iodide (50 μg/ml) during 30 min at 37°C in PBS containing 100 μg/ml RNaseA (9, 41). Stained nuclei were analyzed for their distribution in G_0/G_1, S, and G_2-M using a FACScalibur and the Multicycle software (Becton Dickinson). Data are shown as histograms representing the percentage of nuclei in each cell cycle phase of one representative experiment.

Western Blot Analysis. Western blot analyses were performed either on whole-cell lysates or on nuclear extracts, as indicated in the figure legends (41–44). Whole-cell lysates were obtained by boiling cells for 5 min in 1% SDS and further diluted in an equal volume of Laemmli buffer. To prepare nuclear extracts, cells were scraped in ice-cold PBS, lysed on ice for 20 min with a low salt buffer containing 20 mM HEPES (pH 7.9), 10 mM KCl, 0.15 mM EDTA, 0.015 mM EGTA, and 1% NP40 in the presence of pepstatin A, aprotinin, leupeptin, and phenylmethylsulfonide fluoride (Boehringer Mannheim GmbH, Mannheim, Germany). Nuclei were pelleted by centrifugation and incubated for 30 min at room temperature in a hypertonic solution consisting of 10 mM HEPES (pH 8.0), 400 mM NaCl, 0.1 mM EDTA, 25% glycerol, and protease inhibitors. Nuclear proteins were recovered in the supernatant after a 5-min centrifugation at 12000 × *g*. Proteins corresponding to 5×10^5 cells were electrophoresed in 7.5% or 10% SDS-PAGE gels and transferred overnight onto nitrocellulose membranes (Hybond-C; Amersham Pharmacia Biotech, Piscataway, NJ). hMSH2 and hMLH1 were detected using the rabbit polyclonal antibodies PC57 and PC56, respectively (1 μg/ml; Oncogene Research Products, Cambridge, MA); the membranes were subsequently incubated with sheep antirabbit antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). p53 was detected on nuclear extracts using an anti-p53 mouse monoclonal antibody that recognizes both wild-type and mutant p53 (1:1000 Ab-6; clone DO-1; Oncogene Research Products) and an affinity-purified rabbit antimouse IgG-specific antibody conjugated with horseradish peroxidase (1:2000; Zymed, San Francisco, CA). TopoI was detected with polyclonal human antibodies from scleroderma patient serum (Topogen, Colombus, OH) and revealed with sheep antihuman immunoglobulin antibodies coupled to peroxidase. topoII was detected using a mouse monoclonal antibody (NA14; Oncogene Research Products) with subsequent hybridization with rabbit antimouse IgG antibodies coupled to peroxidase. Fluorescence signals obtained using an enhanced chemiluminescence system were visualized by exposure to X-ray films (Hyperfilm MP, Amersham Pharmacia Biotech).

hMLH1 Expression Vector Construction. A full-length, wild-type *hMLH1* cDNA (2314 bp) was amplified from an MMR-proficient cell line. PCR was carried out with a mixture of *Pfu* and *Taq* DNA polymerases (TaqPlus Precision PCR system; Stratagene, La Jolla, CA) using the sense primer 5′-TGGC**GGATCC**GGCGCCAAAA*ATG*TCG-3′, including a *Bam*HI restriction site (in bold) and the translation initiation codon (underlined); and the antisense primer 5′-GAGAA**TCTAGA**ACACATCCCACAGTGC-3′, containing an *Xba*I restriction site (in bold). The hMLH1 expression vector was obtained by cloning PCR fragments under the control of the cytomegalovirus promoter in the pcDNA3.1/Hygro expression vector (Invitrogen, Leek, the Netherlands) and given the name phMLH1. The wild-type status of the *hMLH1* sequence was confirmed by sequencing the insert.

Transfection. The HCT116 cell line was electrotransfected using 4-mm gap cuvettes and an EasyCellJect apparatus (Eurogentec, Seraing, Belgium) delivering a double pulse; the first pulse was performed at 1000 V and at 25 μF capacitance and with a second pulse performed 1 ms later at 100 V and at 2100 μF. Exponentially growing cells (10^7) were transfected with 20 μg of phMLH1 or with pcDNA3.1/Hygro expression vectors previously linearized by digestion with *Bsp*HI (New England Biolabs, Beverly, MA). Transfected cells were selected and grown as clonal cultures in complete medium containing 100 μg/ml hygromycin (Invitrogen).

RESULTS

Expression of hMSH2, hMLH1, and Topoisomerase I and II Proteins, in Colorectal Cancer Cell Lines. The expression of hMSH2 and hMLH1 was examined in exponentially growing cells through a Western blot analysis of nuclear extracts (Fig. 1*A*). The

[4] Internet address: http://www.atcc.org.

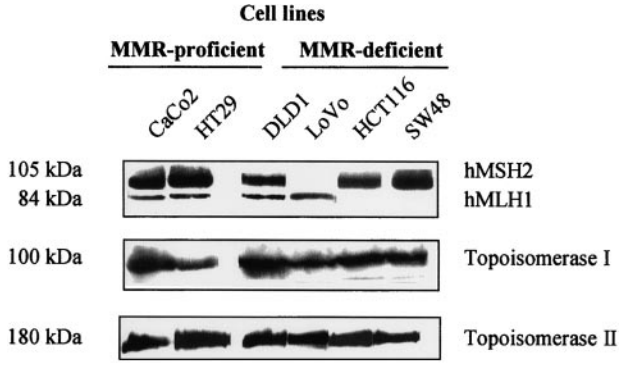

Fig. 1. Expression of MMR and topoisomerase I and II proteins in exponentially growing human colorectal cancer cell lines. The two MMR-proficient cell lines (*CaCo-2* and *HT29*) were loaded on the left side of the gel, whereas the four MMR-deficient cell lines (*DLD1, LoVo, HCT116,* and *SW48*) were loaded on the right of the gel. The expression of hMSH2 (*100 kDa*) and hMLH1 (*84 kDa*) was analyzed in nuclear extracts run on a 7.5% polyacrylamide gel using polyclonal rabbit antibodies. The 100 kDa topoisomerase I protein was detected in whole-cell extracts run on a 10% polyacrylamide gel using polyclonal human antibodies purified from scleroderma patient serum. The α and β isoforms of topoII were analyzed in whole-cell extracts run on a 10% polyacrylamide gel.

hMSH2 protein was expressed at significant levels in all human colorectal carcinoma cell lines except LoVo, a cell line carrying a partial deletion of the *hMSH2* gene (45). The expression of hMLH1 was undetectable in the HCT116 cell line, known to carry a homozygous nonsense mutation at codon 252, as well as in the SW48 cell line, which suffers methylation of the *hMLH1* promoter (45, 46). All other cell lines expressed comparable hMLH1 protein levels. As shown in Fig. 1*B*, topoI was detected in all cell lines as a single band of comparable intensity at the expected 100 kDa apparent molecular mass. The classical 68-kDa proteolytic fragment was not observed in any of the cell lines, because the Western blot analysis was performed on whole-cell extracts rather than on nuclear extracts, which need longer procedures resulting in protein degradation. The expression of topoII analyzed on whole-cell extracts varied slightly among cell lines, but independently of the MMR status of the cells (Fig. 1C).

Cytotoxicity of Topoisomerase Inhibitors to Human Colorectal Cancer Cell Lines. The cytotoxicity of CPT and ETP to colorectal cancer cell lines was determined by a survival assay performed after a 5-day exposure to a wide range of drug concentrations. Histograms shown in Fig. 2 represent the mean concentrations that cause a 50% inhibition of growth (IC_{50}) in at least five independent experiments. As shown in Fig. 2*A*, the three cell lines deficient in either hMSH2 or hMLH1 were highly sensitive to the cytotoxic effects of CPT with IC_{50} values comprised between 5 and 15 nM (mean, 8.7 nM), as compared with the IC_{50} of the three MMR-proficient cell lines, which varied between 58 nM and 153 nM (mean, 105 nM). With an IC_{50} of 35 nM, the sensitivity of the hMSH6-deficient DLD1 cell line was intermediate between these two groups. The range in IC_{50} from most- to least-sensitive cell line for CPT was 30-fold.

Fig. 2*B* shows the IC_{50} range of our cell lines to ETP. As for CPT, the most sensitive group included the three cell lines deficient in either hMSH2 or hMLH1 (IC_{50} values ranging from 0.08 to 0.67 μM), whereas the most resistant cell lines were the three MMR-proficient cell lines. Variations in IC_{50} among MMR-proficient cell lines were higher for ETP than for CPT. Again, the sensitivity of DLD1 to ETP (3.3 μM) was intermediate between the two previous groups and close to that of SW480. The range in IC_{50} from the most- to the least-sensitive cell line was much larger for ETP (384-fold) than for CPT (30-fold).

Functional Assessment of the MDR Phenotype of Colorectal Cancer Cell Lines. To explore the MDR function in the colorectal cancer cell lines used in our study, we have evaluated the uptake of Rh123, a cationic fluorescent dye that binds specifically to P-gp (40). Because the Rh123 efflux by P-gp-associated MDR is inhibited in the presence of the calcium blocker verapamil, the Rh123 fluorescence intensity was determined in the presence or absence of verapamil. As a positive control for MDR function, we have used the KB-A1 Adriamycin-resistant cell line derived from the epithelial carcinoma KB3.1 cell line. As expected, the Rh123 fluorescence intensity was much lower in the MDR-positive KB-A1 cell line, compared with its parental cell line KB3.1 (Fig. 3, *top*). Furthermore, the addition of verapamil, which blocks the P-gp efflux pump, induced a 6-fold increase in the intracellular accumulation of Rh123 in KB-A1, showing that P-gp is active in these cells. In contrast, no increase in Rh123 fluorescence intensity was observed among any of the colorectal cancer cell lines or KB3.1 upon treatment with verapamil (Fig. 3). These observations established that none of the colorectal cancer cell lines used in our study displayed a MDR phenotype.

Cell Cycle Distribution of Colorectal Cancer Cell Lines after Exposure to CPT or ETP. Because the cytotoxicity of CPT is maximal during the S phase, we analyzed the cell cycle distribution patterns, based on DNA content analysis, before and after a drug exposure lasting two generations. Two concentrations of either CPT (10 and 100 nM) or ETP (1 μM and 10 μM) were tested: the lowest concentrations corresponded to the mean IC_{50} observed for the most

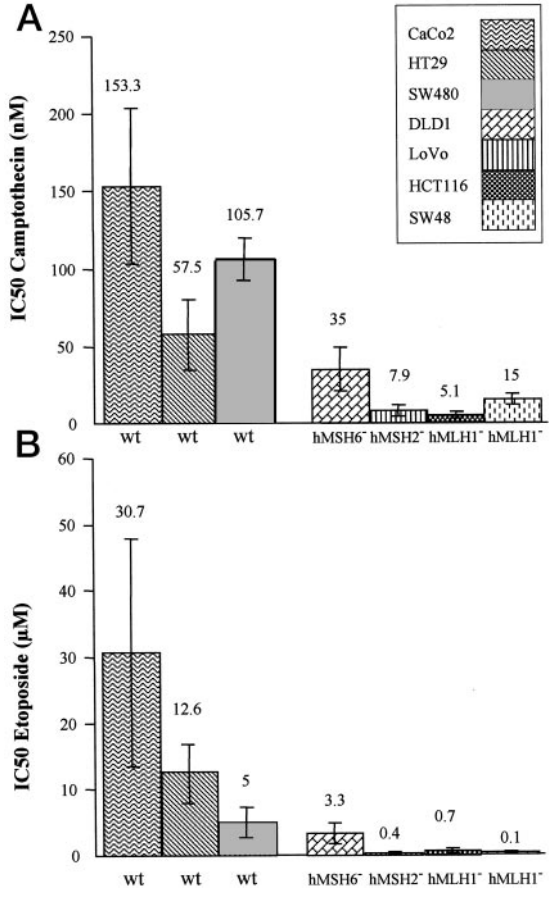

Fig. 2. Cytotoxicity of topoisomerase inhibitors to human colorectal cancer cell lines. Histograms represent the mean IC_{50} values $\pm$ SD determined after 5 days of culturing with continuous exposure to CPT (*A*) or ETP (*B*). The IC_{50} is defined as the concentration of either drug that inhibited growth by 50% relative to drug-free control. *Left* to *right*, the three MMR-proficient cell lines, *CaCo-2, HT29,* and *SW480* and the four MMR-deficient cell lines, *DLD1, LoVo, HCT116,* and *SW48*.

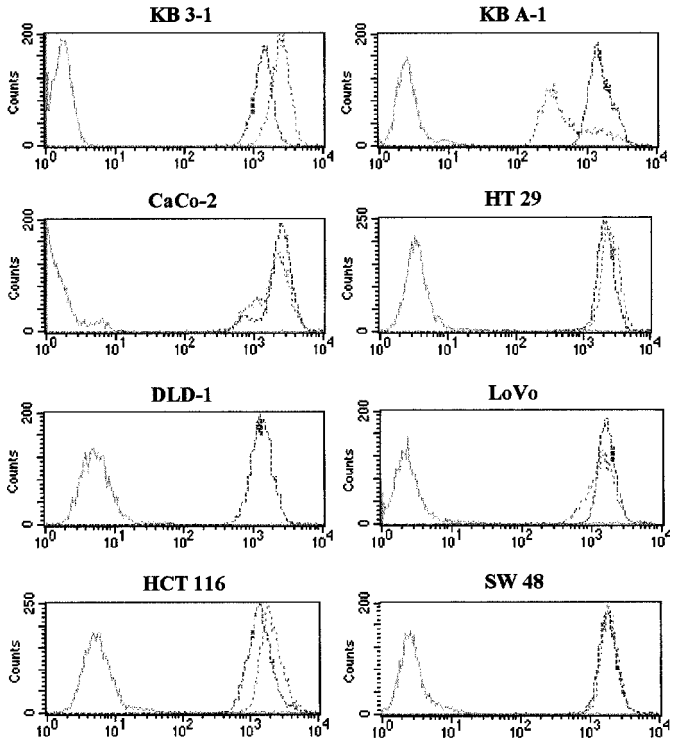

Fig. 3. Rh123 intracellular uptake by the human colorectal cancer cell lines. The intracellular Rh123 fluorescence intensity was measured using a fluorescence-activated cell sorter. Histograms represent the intracellular Rh123 levels obtained in the presence (*black dotted line*) or absence (*gray dotted line*) of the calcium blocker verapamil, a potent MDR inhibitor. Autofluorescence was quantified on cells not exposed to Rh123 (*light gray plain line*). As a positive control for MDR function, we have used the KB-A1 MDR-positive Adriamycin-resistant cell line derived from the epithelial carcinoma MDR-negative KB3.1 cell line.

sensitive cell lines and highest concentrations to the mean IC_{50} of the resistant cell lines. Among our cell lines, untreated asynchronous cell populations displayed similar cell cycle distribution patterns. The percentage of untreated cells in the S phase varied from 20 to 24% for the MMR-deficient cells and from 22 to 26% for MMR-proficient cells. The addition of either drug at its lowest concentration did not significantly affect the cell cycle patterns (data not shown). Only DLD1 slightly accumulated in G_2, with a concomitant loss of cells in G_1, when exposed to 1 μM ETP (data not shown). The addition of high doses of topoisomerase inhibitors resulted in marked alterations in the cell cycle, with similar effects for both drugs. As shown in Fig. 4, all cell lines responded to CPT and ETP by an accumulation of the cells in the G_2-M phase of the cell cycle. The G_2-M arrest observed for HT29 and HCT116 cells was associated with a concomitant complete loss of cells in the G_1 phase and a decrease in the S phase, which was total for HCT116. Whereas all other cell lines (CaCo-2, LoVo, DLD1, and SW48) also accumulated in G_2-M, the loss of cells in the G_1 phase was partial. Upon ETP treatment, the S phase was prolonged for LoVo and DLD1, decreased for SW48, and remained unaffected for CaCo-2. In LoVo, DLD1, and SW48, CPT led to a slight increase in the S phase, but had no effect on CaCo-2 cells. None of the cell cycle pattern alterations observed was correlated with the degree of cytotoxicity of either CPT or ETP to these cell lines. It is striking that LoVo and HCT116, two of the MMR-deficient cell lines, displayed drastically different cell cycle profiles while being equally sensitive to topoisomerase inhibitors.

Functional Complementation of hMLH1 in the hMLH1-deficient HCT 116 Cells. To complement the MMR deficiency of HCT116 cells lacking functional hMLH1, we electrotransfected the human wild-type *hMLH1* cDNA driven by the cytomegalovirus promoter into HCT116 cells. The wild-type status of the *hMLH1* sequence was confirmed by sequencing the insert. As determined by Western blot analysis, hMLH1 expression was restored in four of the five hygromycin-resistant stable transfectants examined, whereas no hMLH1 signal was detectable in five transfectants obtained with the control vector (data not shown). As shown in Fig. 5*A*, relative to the MMR-proficient HT29 cell line, the level of hMLH1 expression was comparable in the mlh1–3 and lower in mlh1–2. The hygromycin-resistant cells transfected with the pcDNA3.1 control vector, mlh0–1, failed to express hMLH1. Cellular resistance to alkylating agents is a constant characteristic of MMR-deficient cells, with complementation of MMR function restoring alkylation sensitivity. To evaluate the MMR function in the *hMLH1*-transfected HCT116 clones that we had established, we have determined their sensitivity to the methylating agent MNU. As shown in Fig. 5B, both hMLH1-expressing mlh1–2 and mlh1–3 clones behaved similarly. The fraction of survival cells in the mlh1–2 and mlh1–3 clones after treatment with 50 μg/ml MNU was only 15 and 20%, respectively, whereas it was 66% for the hMLH1-deficient HCT116 cells. Treatment with a higher dose of MNU (100 μg/ml) killed >90% of the *hMLH1*-transfected clones, whereas half of the parental HCT116 cells still survived (Fig. 5B). Thus, reexpressing wild-type hMLH1 in the MMR-deficient parental HCT116 cell line reverted tolerance to alkylating agents, demonstrating that a normal MMR function was recovered in these clones.

Cytotoxicity of Topoisomerase Inhibitors to hMLH1-expressing HCT116 Transfectants. The cytotoxicity of CPT and ETP toward various HCT116-derived cell lines, transfected with either the *hMLH1*-expression vector or the control vector, was determined and compared with the HT29-resistant cell line and the sensitive HCT116 parental cells. As shown in Fig. 5*C*, the sensitivity to CPT of the two hMLH1-expressing transfectants (mlh1–2 and mlh1–3) was identical and almost fully restored to that of the MMR-proficient resistant HT29 cell line. As expected, cells transfected with the control vector (mlh0–1) remained as sensitive to CPT as the parental HCT116 cell line. The level of hMLH1 expression did not strictly parallel the sensitivity to CPT; even a low level of hMLH1 expression sufficed to restore resistance to this drug. Conversely, the level of hMLH1

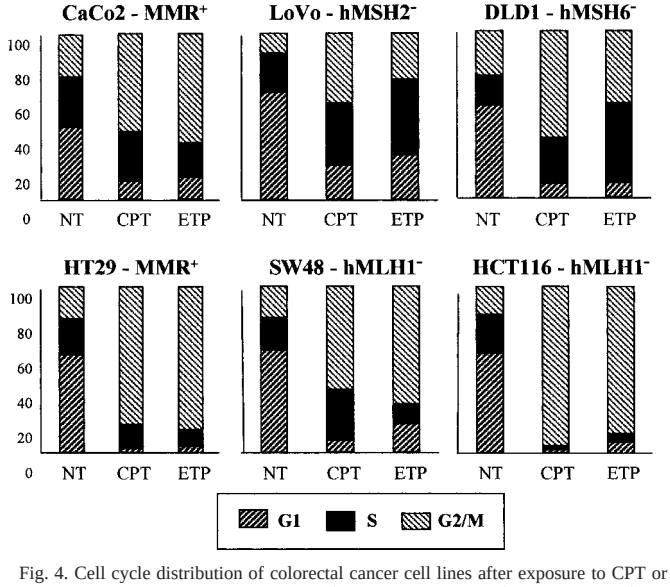

Fig. 4. Cell cycle distribution of colorectal cancer cell lines after exposure to CPT or ETP. Histograms represent the percentage of cells in each phase of the cell cycle after a 48-h culture in absence of drugs (*NT*) or in the presence of high doses of CPT (100 nM) or ETP (10 μM). The two MMR-proficient cell lines (*CaCo-2* and *HT29*) are shown on the *left*; the four MMR-deficient cell lines (*DLD1*, *LoVo*, *HCT116*, and *SW48*) are shown in the *middle* and on the *right*.

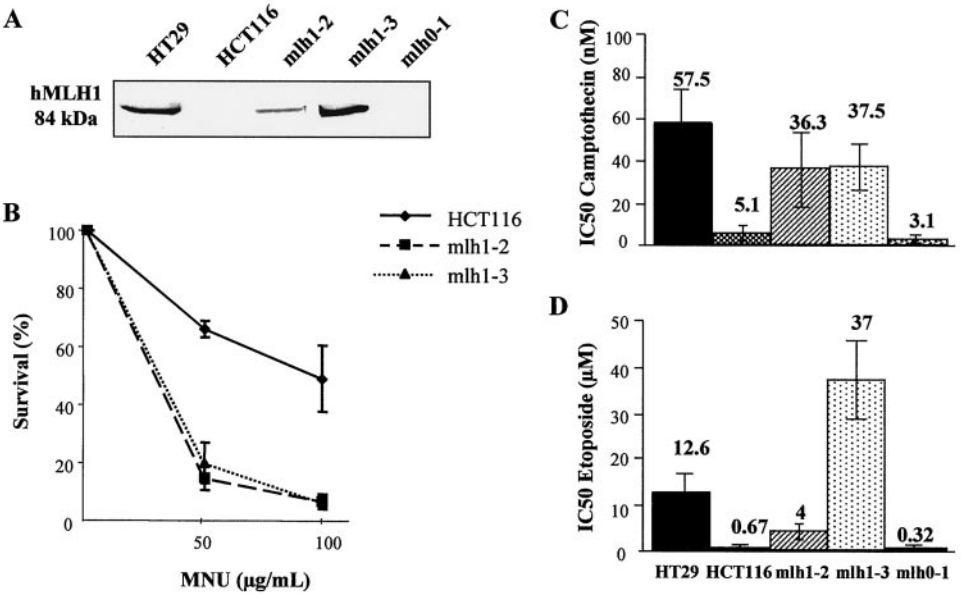

Fig. 5. Cytotoxicity of topoisomerase inhibitors to hMLH1-expressing HCT116 transfectants. *A*, expression of hMLH1 was analyzed on whole-cell lysates prepared on exponentially growing cells. Histograms represent the mean IC_{50} values ± SD determined after 5 days of culturing with continuous exposure to CPT (*B*) or ETP (*C*). The IC_{50} is defined as the concentration of either drug that inhibited growth by 50%, relative to drug-free control. From *left* to *right*, the MMR-proficient HT29 cell line as a control, the hMLH1-deficient parental HCT116 cell line, two hygromycin-resistant HCT116 clones transfected with the hMLH1-expression vector (*mlh1–2* and *mlh1–3*), and one clone transfected with the control vector (*mlh0–1*).

expression seemed to modulate the degree of resistance to ETP. The mlh1–3 clone expressing the highest hMLH1 level was even more resistant to ETP than the MMR-proficient HT29 cell line, whereas resistance to ETP was only partially restored in the mlh1–2 clone expressing low levels of hMLH1 (Fig. 5D). Again, mlh0–1 cells transfected with the control vector remained as sensitive to ETP as the parental HCT116 cell line.

Effects of CPT on p53 Expression by Colorectal Cancer Cells. Because exposure of cells to certain agents that induce DNA damage results in p53 protein stabilization, we have investigated the effects of CPT on p53 levels in various colorectal cancer cell lines. As shown in Fig. 6, a slight increase in p53 was observed upon treatment with CPT in DLD1 cells, which carry both a mutant and a wild-type *p53* allele, and in LoVo cells, which have a functional *p53*. The CPT-induced p53 accumulation was not detectable in CaCo-2 or HCT116 cell lines, two cell lines bearing a functional *p53*. Thus, p53 induction in response to CPT is independent of both the *p53* status and the cellular sensitivity to the toxic effects of this drug.

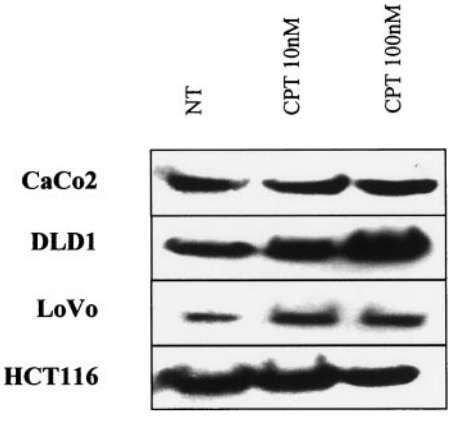

Fig. 6. Effects of CPT on p53 expression by colorectal cancer cells. Western blot analysis of p53 expression was performed on nuclear extracts prepared from cells untreated or treated for 48 h with CPT (10 nM or 100 nM). Equal amounts of proteins were loaded onto the gel. The membrane was probed with an anti-p53 mouse monoclonal antibody that recognizes both wild-type and mutant p53 (clone DO-1).

DISCUSSION

The aim of our study was to determine whether MMR contributes to the cytotoxicity of topoisomerase inhibitors toward colorectal cancer cells. The sensitivity of four colorectal cancer cell lines with a defined genetic defect in MMR was compared with three MMR-proficient cell lines. Our results showed that a defect in MMR was associated with a higher sensitivity to topoisomerase inhibitors, when compared with MMR-proficient cell lines (Table 1). A defect in either hMSH2 or hMLH1, the two key components of MMR, most severely affected the cellular resistance to either drug. The sensitivity of the hMSH6-deficient DLD1 cells was intermediate. This could reflect variability among cell lines and/or a partial functional complementation of hMSH6 by hMSH3 (26, 27).

Because ETP and CPT both stabilize the cleavage complexes, higher levels of endogenous topoisomerases were expected to increase cell killing by these drugs (4–6). *TopoI* gene copy number has been reported to be highly variable in colorectal cancer cell lines; the MMR-deficient LoVo and DLD1 cell lines exhibit normal *topoI* gene copy number, whereas it is amplified in HT29 and CaCo-2, two MMR-proficient cell lines known to be aneuploid (47). We found that endogenous topoisomerase protein levels were comparable in all cell lines, irrespective of the *topoI* gene number, suggesting the existence of a regulatory process. These observations reinforce a previous study performed on seven colorectal cancer cell lines including HT29, HCT116, and HCT15 (derived from the same tumor as DLD1), which reported little variations in topoI mRNA and protein expressions (42). Thus, topoI levels *per se* are not predictive of CPT cytotoxicity.

Because resistance of cancer cells to chemotherapy is often associated with the overexpression of the *MDR* gene, encoding P-gp, a pump that can extrude a large number of hydrophobic agents including ETP, we have ascertained P-gp function in our cell lines. In particular, the overexpression of P-gp frequently occurs in colorectal cancer cells, which leads to resistance toward ETP, hampering its chemotherapeutic use (48). Using flow cytometry analysis, we have quantified the accumulation of Rh123, a fluorescent compound known to be a specific P-gp substrate (40). Because P-gp function could not be detected in any of our cell lines, we could rule out the possibility that cellular resistance to ETP was attributable to the alteration in drug accumulation. Conversely, the various pumps known to expel chem-

Table 1 *Relationship between the sensitivity of human colorectal cancer cells to topoisomerase inhibitors and their status of MMR and p53*

Cell lines	Sensitivity to[a]		MMR status	p53 status	G_2-M arrest[b]
	CPT	ETP			
CaCo2	−	−	Proficient	Wild type	++
HT29	−	−	Proficient	Arg273His	+++
SW480	−	+	Proficient	Arg273His/Pro309Ser	ND[c]
LoVo	+++	+++	hMSH2−	Wild type	+
HCT116	+++	+++	hMLH1−, hMSH3−	Wild type	+++
SW48	++	+++	hMLH1−	Wild type	++
DLD1	+	++	hMSH6−	Wild type/Ser241Phe	++

[a] The sensitivity to CPT was scored as follows: −, $IC_{50} \geq 50$ nM; +, 20 nM $< IC_{50} <$ 50 nM; ++, 10 nM $< IC_{50} <$ 20 nM; +++, $IC_{50} <$ 10 nM. The sensitivity to ETP was scored as follows: −, $IC_{50} \geq 10$ μM; +, 5 μM $\leq IC_{50} <$ 10 μM; ++, 1 μM $< IC_{50} <$ 5 μM; +++, $IC_{50} <$ 1 μM.

[b] G_2-M arrest was scored as the percentage of cells in G_2-M as follows: +, <35%; ++, 35–70%; +++, >70%.

[c] ND, not determined.

otherapy drugs from tumor cells poorly interact with CPT (49, 50). Moreover, variations in CPT uptake by colorectal cells are low, and they are not correlated with CPT sensitivity (42).

Colorectal cancers proceed through a step-by-step progression from colonic epithelium to the malignant phenotype, accumulating a wide spectrum of characteristic genetic alterations (51). Thus, to establish formally the MMR involvement in the sensitivity to topoisomerase inhibitor, it was necessary to compare the response of MMR-deficient and MMR-proficient cells within a constant genetic background. In our experiments, we have constructed an *hMLH1*-expression vector similar to the one that was reported to complement DNA MMR when transferred into mutant tumor cells (43, 44, 52). In these studies, the expression of hMLH1 in the transfectants was determined by Western blot analysis and was shown to be variable among different clones. As expected, transfectants displayed a reduced base substitution and MSI, a restoration of G_2 cell cycle arrest in response to DNA damage, and an increased sensitivity to the toxic effects of 6-thioguanine as well as to alkylating agents such as *N*-methyl-N^1-nitro-*N*-nitrosoguanidine or MNU. The use of cell lines in which complementation has been achieved by introducing the human chromosome that contains the *MMR* gene into an MMR-deficient tumor cell has long been preferred to the use of cDNA expression vectors. In fact, the latter approach often leads to overexpression of the cloned cDNA, which, in the case of hMSH2 or hMLH1, may induce apoptosis (25). Nevertheless, in the approach that we have taken, selection of the cells that stably expressed the transfected *hMLH1* cDNA required growing cells in the presence of hygromycin over three weeks. Thus, it is likely that clones that overexpressed hMLH1 to levels sufficient to induce apoptosis, were counterselected during the period of hygromycin selection, a process that is accompanied by a massive loss of cells. Yet, clones that we have established as long term cultures express hMLH1 levels comparable with that of hMLH1-proficient cells. The level of hMLH1 expression in the mlh1–3 clone is slightly higher that in the MMR-proficient HT29 cell line and significantly higher than in the mlh1–2 clone. The fact that both clones display a resistance to CPT equivalent to that of HT29 may indicate that low levels of hMLH1 are sufficient to restore resistance to this drug. Conversely, higher levels of hMLH1 may be required to allow cells to be fully protected against the cytotoxic effects of ETP. Concerning the mlh1–3 clone, the IC_{50} for ETP is higher than for wild type when compared with HT29, but similar to that of CaCo-2, another MMR-proficient cell line. In that respect, it is noteworthy that variations in IC_{50} are greater for ETP than for CPT, suggesting that other unidentified determinants may also contribute to ETP cytotoxicity. Another explanation could be that the number of DNA-breaks is lower after treatment with CPT than with ETP. Finally, using a similar experimental system, Buermeyer *et al.* (43) also reported that there was no apparent correlation between the level of expression of hMLH1 and the extent of reduction in the mutation rate. This observation led them to conclude that even a low level of constitutively expressed hMLH1 is sufficient to restore hMLH1 function. Because stable expression of wild-type *hMLH1* cDNA in the hMLH1-deficient cell line HCT116 restored resistance to both ETP and CPT, we conclude that hMLH1 definitely participates in the response to these drugs. Furthermore, in addition to hMLH1 inactivation, we and others have shown that HCT116 cells display a secondary homozygous frameshift mutation in the poly(A)8 track present in the coding region of hMSH3 (53). Because this mutation consists of a 1-bp deletion causing a premature stop codon, resulting in the synthesis of an inactive truncated protein, we can conclude that hMSH3 is dispensable for resistance to topoisomerase inhibitors or may be substituted by hMSH6. In this respect, correction of the MSI+ phenotype, the tolerance to alkylating agents, and the G_2 cell cycle cell checkpoint has also been successfully achieved by transferring into HCT116 cells a copy of chromosome 3 containing the wild-type *hMLH1* gene but not *hMSH3* (54).

Apoptosis and cell cycle arrest at the G_2 phase in response to alkylating agents or ionizing radiation have been shown to involve hMLH1 (14, 17, 55). In a previous study performed with SW620 and KM12, two mutant p53 human colorectal cell lines, CPT cytotoxicity was related to a reduced G_2 arrest (9). In contrast, we have observed that the two hMLH1-deficient cell lines used in our study, which both carry wild-type p53, displayed a marked G_2-M cell cycle arrest in response to either SSB- and DSB-inducing drugs. Looking at our panel of colorectal cell lines, we did not observe any correlation between cell cycle perturbations and the cytotoxicity of topoisomerase inhibitors. This led us to conclude that a defect in G_2-M arrest is unlikely to be the major determinant of sensitivity to these drugs (Table 1).

Although several studies have addressed the role of p53 in the sensitivity of tumor cells to topoisomerase inhibitors, the issue is still debatable. It has recently been shown that CPT induces phosphorylation of p53 at both serines 15 and 20, which results in the stabilization of p53 and in the inhibition of its interaction with MDM2 (56). We also observed that treatment of MMR-deficient colorectal cancer cells with CPT slightly increased p53 levels in some cell lines independently of their p53 status or cellular sensitivity to this drug. Stabilization of p53 after treatment with either CPT or ETP downregulates the expression of MDM2 and enhances the expression of $p21^{Waf1/Cip1}$ (56, 57). Thus, one might expect that apoptosis induced by these drugs is impaired in tumor cells with mutant p53. By injecting MSI+ or MSI− human tumors into mice treated with CPT-11, a semi-synthetic water-soluble CPT derivative, it has been shown that a mutant p53 status is associated with a poor response to CPT-11, whereas an MSI+ phenotype moderately increases sensitivity (58). Yet such a predominant role of p53 has not been confirmed in a recent study that defined the effects of SN-38, the active metabolite of CPT-11, on colorectal cancer cell lines expressing wild-type or mutant p53 (59). Indeed, although expression of wild-type p53 led

to a more rapid induction of apoptosis, cytotoxicity was generally greater in cells with mutant p53, inasmuch as cells with wild-type p53 underwent a prolonged cell cycle arrest in G_2-M that allowed them to escape apoptosis (59). In our study, among the three resistant cell lines, HT29 and SW480 displayed only mutant *p53* alleles, whereas CaCo-2 harbored wild-type *p53* (60; Table 1). The three highly sensitive MMR-deficient cell lines also contained wild-type *p53* (Table 1). Taken together these observations indicate that *p53* status may not be a major factor for cellular resistance to topoisomerase inhibitors, and that its role in DNA damage has not yet been fully elucidated.

Both hMSH2 and hMLH1 have recently been shown to be part of a super complex of BRCA1-associated proteins also containing MSH6, ATM, NBS1, and MRE11 (61). This complex is believed to act as a DNA damage sensor and to participate in the recognition and repair of aberrant DNA structures (61). Indeed, cells derived from patients with NBS or ataxia telangiectasia are hypersensitive to ionizing radiation, ETP, and CPT (62–64). NBS is caused by a mutation in the gene encoding NBS1, which is part of the MRE11/RAD50 complex known to participate in DSB repair. NBS1 has been shown to interact with and to be phosphorylated by ATM, whose gene is mutated in patients with ataxia telangiectasia. Although ATM phosphorylation of NBS1 does not affect the NBS1-MRE11-Rad50 complex formation, it is necessary for an appropriate response to DSB (65). Moreover, yeast *rad52* mutants that are deficient in homologous recombination and DSB repair are also hypersensitive to CPT (10). In conclusion, we propose that a defect in DSB repair linked to MMR-deficiency may be responsible for the hypersensitivity of the MSI+ colorectal cancer cells to topoisomerase inhibitors; this hypothesis is currently under investigation in our laboratory.

Given the incidence of the MSI+ phenotype among colorectal tumors, our observations, showing that a defect in MMR results in hypersensitivity to topoisomerase inhibitors are particularly relevant to the treatment of colorectal cancer. Indeed, though CPT derivatives have shown promising results in the treatment of colorectal cancer, benefit from this chemotherapy has been restricted to one group of patients. Interestingly, we have shown that the MSI+ phenotype significantly promotes tumor responsiveness to CPT-11 (35). Therefore, MSI screening should be of great help in the selection of patients with colorectal cancers who are more likely to benefit from chemotherapy with CPT derivatives.

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