Cellular Response to Irinotecan in Colon Cancer Cell Lines Showing Differential Response to 5-Fluorouracil

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Abstract. Background: Use of irinotecan (CPT-11) as second-line therapy for metastatic colorectal cancer has shown some promise in cases where 5-fluorouracil (5-FU) has failed. Cross-resistance to both drugs may however be a potential clinical problem. The cellular response to CPT-11 was investigated in two human colon cancer cell lines that demonstrate a differential response to 5-FU. Materials and Methods: Cell cycle progression, clonogenic survival, DNA damage checkpoint activation, apoptosis induction and senescence development were assessed during 48 hours of treatment and 72 hours of recovery. Results: Both cell lines had similar cellular response patterns to CPT-11. Growth inhibition, loss of clonogenicity, ataxia telangiectasia mutated (ATM) activation, H2AX phosphorylation, TP53 stabilization, CDKN1A induction, G2/M arrests, endoreduplication, negligible cell death and appearance of a senescence-associated beta-galactosidase phenotype were observed. Conclusion: Cross-resistance to 5-FU and CPT-11 was not demonstrated. The appearance of a senescence phenotype in response to CPT-11 treatment may have potential clinical relevance for treatment regimens.

Treatment of advanced colorectal cancer with 5-fluorouracil (5-FU)-based regimens has provided modest survival benefits, despite attempts to increase the efficacy of the drug. Irinotecan (CPT-11), an analog of camptothecin (CPT), is currently used in combination with 5-FU to treat metastatic colorectal cancer. It is also used as a single agent since it shows consistent and reproducible activity after 5-FU failure (1-4). The mechanism of action of the pro-drug CPT-11 involves the inhibition of topoisomerase I, a nuclear enzyme that maintains and modulates DNA structure. The active form of CPT-11, SN-38, binds to and prevents topoisomerase I from rejoining transient DNA breaks during replication, which results in the formation of cleavable complexes (CPT-11-stabilized topoisomerase I-bridged DNA breaks). Replication fork collision with cleavable complexes during S-phase is the major cytotoxic mechanism of topoisomerase inhibitors in dividing cells (5).

The cleavable complexes are converted to permanent DNA double-strand breaks (6), which activate the DNA damage checkpoint response, involving ATM kinase activation and ATM autophosphorylation at Ser1981 (7, 8). Activation of the DNA damage checkpoint response can lead to cell cycle arrest in order to prevent either replication of damaged DNA (G1/S checkpoint) or mitosis in the presence of damaged DNA (G2/M checkpoint). The intra-S-phase checkpoint is activated by S-phase damage or by unrepaired damage that escapes the G1/S checkpoint and leads to a replication block (9, 10). While these checkpoints are distinct from each other, the damage sensors (e.g. ATM, ATR), mediators (e.g. BRCA1, 53BP), transducers (e.g. CHEK1, CHEK2) and effectors (e.g. TP53, CDC25) appear to be shared by all three pathways (9).

We were interested in investigating the cellular and DNA damage responses to CPT-11 in 5-FU-refractory and 5-FU-sensitive colon cancer cell lines, in light of the recent studies showing lack of cross-resistance to CPT-11 and 5-FU (1-4). We were especially interested in determining potential differences in DNA damage checkpoint activation, cell cycle progression and apoptosis induction in response to CPT-11 treatment and during short-term recovery, as such knowledge could have implications for CPT-11 chemotherapeutic regimens. We treated a 5-FU-resistant derivative of the HCT116 colon cancer cell line (HCT116-D) (11) and the HCT116 cell line with clinically relevant concentrations of CPT-11 and monitored their cellular responses to the drug. Both cell lines have a wild-type TP53 gene, but the HCT116-D cell line has reduced apoptosis at 24 hours of 5-FU treatment relative to the HCT116 cell line and recovers exponential growth three times faster than the HCT116 cell line (12).
Materials and Methods

Cell lines, drug treatment, clonogenic survival and detection of senescence. The human colon cancer cell line HCT116 was obtained from the American Type Culture Collection (LGC-Promochem, Wesel, Germany). The HCT116-D cell line has been described elsewhere (11, 12). Both cell lines were maintained as monolayers in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 5 mM L-glutamine and 0.05 mg/ml gentamicin in 25 ml culture flasks placed in a humidified atmosphere at 37°C in 5% CO₂. All experiments were performed on cells in exponential growth phase. HCT116 and HCT116-D cultures were treated with two clinically relevant concentrations of CPT-11 (Sanofi-Aventis, Bridgewater, NJ, USA), 2 μM and 5 μM. For each experiment, 3x10⁵ cells per well were seeded out in 6-well plates 24 hours prior to addition of CPT-11. Cultures were treated for 48 hours with the drug followed by a 72-hour recovery period in drug-free media, then harvested along with corresponding untreated controls at 0, 4, 8, 24, 48, 72, 96 and 120 hours. A standard Trypan blue assay was used to count viable cells and dead cells.

For clonogenic survival assays, drug-treated and untreated control monolayers were trypsinized following 48-hour exposure to 2 BM and 5 BM CPT-11 and re-seeded at a density of 750 cells per well in 6-well plates. The cells were monitored for colony formation over a period of 13 days and only colonies with more than 30 cells were counted and scored as the survival fraction.

The development of a senescence phenotype (a state in which cells neither proliferate nor die, but remain inactive indefinitely in culture (13)) in drug-treated cultures was monitored using a previously published staining procedure for senescence-associated (SA)-beta-galactosidase activity (at pH 6) (13). Exponentially growing HCT116 and HCT116-D monolayers grown on chamber slides were treated for 48 hours with 2 BM or 5 BM CPT-11 and stained for SA-beta-galactosidase activity. SA-beta-galactosidase can be detected in single cells by X-Gal (Sigma Aldrich, Oslo, Norway), which forms a local blue precipitate upon cleavage, independent of DNA synthesis measurements (13). SA-beta-galactosidase-positive cells (dark blue color) were counted and data were presented as percentage positive cells out of 100 cells counted. Additionally, the 6 well plates used for clonogenic survival assays were stained on day 13 for SA-beta-galactosidase in order to determine whether lack of colony formation was correlated with a senescence phenotype.

Cell cycle analysis and BrdU incorporation. Trypsinized monolayers were pooled with floating cells, washed in phosphate-buffered saline (PBS), fixed in 80% ethanol and kept frozen at −20°C until used for cell cycle analyses. Nuclei isolation and subsequent DNA staining with propidium iodide were performed using a standard Vindelov’s procedure (14) and cell cycle analyses performed as described elsewhere (12) using a FACSCalibur laser flow cytometer (BD Biosciences, Palo Alto, CA, USA). For assessment of BrdU incorporation using flow cytometry, monolayers were pulse-labeled with 30 μg/ml BrdU for 1 hour prior to trypsinization at 48, 72 and 120 h. Samples were prepared and stained with an unconjugated anti-BrdU monoclonal antibody (BD Biosciences) as described elsewhere (15) and measured for bivariate BrdU/DNA content on the FACSCalibur.

Detection of apoptosis and necrosis. Apoptosis and necrosis were measured in the HCT116 cell lines in response to CPT-11 treatment using a flow cytometric AnnexinV/ToPro-3 double staining procedure, respectively. Monolayer cells were scraped, pooled with floating cells and washed in cold PBS. Unfixed cell pellets were resuspended in 100 μl Annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4), before 5 μl of AnnexinV-FITC conjugate (Molecular Probes, Eugene, OR, USA) were added. After 15 min incubation in the dark at room temperature, another 400 μl of Annexin-binding buffer were added and the samples were placed on ice. The DNA fluorochrome ToPro-3 (1Bg/ml final concentration) was added to label necrotic cells prior to analysis on a FACS Calibur flow cytometer. Necrotic cells were also quantified using a standard Trypan blue assay.

Immunoblotting. Monolayers were scraped in cold PBS using a rubber policeman, pooled with floating cells, washed in PBS and cell pellets lysed in standard Laemmli buffer and heated at 95°C for 10 min. Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA, USA). Fifteen micrograms of protein per lane were separated on 12%, 10%, 7.5% or 5% SDS-polyacrylamide gels. Immunoblotting was performed as described elsewhere (12). The following antibodies were used: actin (C2), BAX (B9), CCNB1 (GNS1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); BCL-2 (clone 124) (Dako Cytomation, Glostrup, Denmark); phospho-CDC2 (Tyr15), phospho-ATM (Ser1891), phospho-CHEK1 (Ser345), phospho-CHEK2 (Thr68), cleaved PARP (Asp214) (Cell Signaling Technology, Beverly, MA, USA); MCL-1 (BD Biosciences, San Jose, CA, USA); TP53 (Ab-2), CDKN1A, CCND1 (Ab-3) (Oncogene Research Products, Cambridge, MA, USA); PLK-1 (Zymed Laboratories, South San Francisco, CA, USA); and phospho-H2AX (Ser139) (Upstate, Lake Placid, NY, USA). The human lymphoblast Reh cell line irradiated with 4 Gy was used as a positive control for phospho-ATM and phospho-CHEK2 staining. Protein levels were quantified using UnScanIt gel software version 5.1 for Windows (Silk Scientific Inc., Orem, UT, USA).

Statistical analysis. Statistical analyses and plots were generated using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Cell proliferation and clonogenic survival during CPT-11 treatment and recovery. Cell counts for both HCT116 and HCT116-D cultures were decreased already at 24 hours in response to both CPT-11 concentrations relative to their respective untreated controls. By 48 hours, there was nearly complete growth inhibition in the 2 BM-treated cell lines and complete growth inhibition in the 5 BM-treated cell lines (Figure 1a, b), which persisted throughout the recovery period. Untreated controls reached confluence by 120 hours. Colony formation in drug-treated cultures was monitored for 13 days following the shift to drug-free media. HCT116 cultures treated with 2 BM and 5 BM CPT-11 had clonogenic survival fractions of 10% and 0.005% respectively on day 13, whereas HCT116-D cultures treated with 2 BM and 5 BM CPT-11 had clonogenic survival fractions of 5.5% and 0.0005% respectively. Untreated HCT116 and HCT116-D cultures were completely confluent on day 13.
Cell cycle progression during CPT-11 treatment and recovery. To investigate whether growth inhibition in both cell lines was due to alterations in cell cycle progression, cell cycle analyses were performed following CPT-11 addition. Figure 2 shows the cell cycle distributions for HCT116 and HCT116-D cultures treated with 2 µM and 5 µM CPT-11 for the entire treatment and recovery periods relative to their respective untreated controls. At 4 and 8 hours following addition of 2 µM or 5 µM CPT-11 to the media, a population of partially synchronized S-phase cells was seen in both cell lines (data not shown). By 24 hours, a cell cycle arrest in G2/M-phase was seen in both cell lines (Figure 2a, b). The majority of cells had arrested in G2/M at 24 hours in the 5 BM-treated cultures compared to circa 50% of cells in the 2 BM-treated cultures. At 48 hours and at subsequent timepoints, the cell cycle distributions for both 2 BM- and 5 BM-treated cultures demonstrated the presence of cells with 8n DNA content, indicative of endoreduplication/ polyploidization. Fractions of cells with >4n DNA content tended to be higher in 5 BM-treated HCT116 compared to 5 BM-treated HCT116-D.
cultures (Figure 3a, b). There was negligible spontaneous endoreduplication in corresponding untreated cultures for both cell lines. Endoreduplication was also reflected in increases in nuclear size in the drug-treated samples. DNA content distributions were back-gated for forward scatter data which were then used as an indication of nuclear size. Using this gating, 60-70% and 150% increases in nuclear size were measured for HCT116 and HCT116-D cultures treated with 2 µM and 5 BM CPT-11 respectively at 120 hours relative to their respective untreated controls (data not shown).

CCND1 levels continued to increase in drug-treated cultures following drug removal, with highest levels seen at 120 hours (Figure 4). Drug-treated and untreated HCT116 and HCT116-D cultures had mostly similar patterns of CCNB1 and PLK1 expression (data not shown). Levels of phosphorylated CDC2 (Tyr15) were elevated in both drug-treated cell lines from 24 hours of treatment and remained elevated relative to their untreated controls, indicative of lack of progression into mitosis (Figure 4).

BrdU pulse-labeling of both cell lines confirmed the presence of endoreduplicating cell populations in drug-treated cultures (the presence of cells with 16n DNA content can be seen in Figure 5b) and showed that substantial percentages of S-phase cells in these cultures had not incorporated BrdU, compared to untreated controls. The highest levels of non-incorporation were seen in 5 BM-treated cultures. In untreated HCT116 cultures at 72 hours, 6% of S-phase cells had not incorporated BrdU compared to 22% and 25% of S-phase cells in the 2n and 4n cycling populations respectively in 2 µM-treated cultures (data not shown). At 120 hrs, 5% of S-phase cells in untreated HCT116 cultures had not incorporated BrdU (Figure 5a) compared to 40%, 28% and 35% of S-phase cells in the 2n, 4n and 8n cycling populations respectively in 2 BM-treated cultures (Figure 5b) and 40%, 40% and
40% of S-phase cells in the 2n, 4n and 8n cycling populations respectively in 5 BM-treated cultures (data not shown). Similar results were obtained for the HCT116-D cell line (data not shown).

DNA damage checkpoint activation during CPT-11 treatment and recovery. The activation status/expression levels of proteins involved in DNA damage checkpoint pathways and cell cycle progression were measured in HCT116 and HCT116-D cell lines in order to more fully characterize the DNA damage response during CPT-11 treatment and recovery. Drug-treated HCT116 and HCT116-D cultures exhibited the same patterns of DNA damage checkpoint activation; Figure 7 shows immunoblotting data for the HCT116 cell line only. Phosphorylated ATM (Ser1981) was detected at early timepoints following drug addition in both cell lines relative to their respective untreated controls (Figure 7) and remained detectable throughout the experimental period, with highest levels generally seen in 5 BM-treated cultures. Phosphorylated CHEK1 (Ser345) was not detected in either drug-treated cell line at any timepoint (data not shown). Phosphorylated CHEK2 (Thr68) was not detected until 72 hours in drug-treated cultures. Levels of endogenous CHEK2 were detectable at all timepoints measured. In response to DNA double-strand breaks, activated ATM is reported to phosphorylate H2AX at Ser 139 (16, 17). Phosphorylated H2AX (Ser139) levels were slightly elevated at 2 hours in response to 5 BM CPT-11 treatment and continued to increase thereafter for both drug...
concentrations. Untreated cultures also expressed phosphorylated H2AX, consistent with the fact that H2AX is normally phosphorylated during DNA replication (18). Previous studies have shown that upon DNA damage, TP53 is phosphorylated at several sites in its transactivation domain, including at Ser15 and Ser20 (19). ATM and ATR phosphorylate TP53 at Ser15 (20, 21), which inhibits the interaction of TP53 with MDM2 (22). This results in TP53 stabilization since MDM2 phosphorylation by ATM reduces its capability to promote nucleocytoplasmic shuttling and the subsequent degradation of TP53 (23). TP53 stabilization/accumulation were detectable at 2 hours in 5 BM-treated

Figure 4. CCND1 and phospho-CDC2 (Tyr15) levels are increased as a result of CPT-11 treatment. Immunoblotting data shown for the HCT116 cell line. Levels of these proteins increased over the experimental time period in drug-treated cultures. The HCT116-D cell line had similar patterns of expression (data not shown). Actin was used as a loading control.

Figure 5. Lack of BrdU incorporation in a fraction of S-phase cells in CPT-11-treated cultures. (a) At 120 h, 5% of S-phase cells in untreated HCT116 cultures had not incorporated BrdU, compared to (b) 40%, 28% and 35% of S-phase cells in the 2n, 4n and 8n cycling populations, respectively, in 2 µM-treated HCT116 cultures. Note the BrdU(-) S-phase cells in all cycling populations in the drug-treated culture. The same pattern of BrdU non-incorporation was also seen for the drug-treated HCT116-D cell line (not shown).
cultures and remained detectable for the remainder of the experiment for both drug concentrations. CDKN1A induction was weakly detectable at 2 and 4 hours of drug treatment and expression levels continued to increase for the remainder of the experimental period, with highest levels seen in 5 µM-treated cultures, especially at later timepoints.

Cell death in CPT-11-treated cell cultures. Levels of apoptotic cell death were assessed by quantifying the numbers of AnnexinV-positive cells and the levels of cleaved PARP in drug-treated HCT116 and HCT116-D cultures. Percentages of AnnexinV-positive cells in drug-treated HCT116 and HCT116-D cultures remained at untreated control levels throughout the 48-hour treatment period for both cell lines, after which they gradually increased, peaking at 45-50% at 96 hours for both cell lines (Figure 8a, b). Levels of cleaved PARP were 15-30% higher in 2 BM-treated and 19-45% higher in 5 BM-treated HCT116 and HCT116-D cultures (compared to their corresponding untreated controls) starting at 72 hours and continuing throughout the recovery period (Figure 8c). There were no significant changes in the levels of BAX, BCL-2, or MCL-1 in either drug-treated cell line relative to its respective untreated control at any experimental timepoint (data not shown). Levels of necrotic cell death assessed by Trypan blue viability assays or ToPro-3 measurements were ≤10% in CPT-11-treated cultures at all experimental timepoints, regardless of drug concentration used.

Figure 6. Loss of clonogenicity is associated with an SA-beta-galactosidase-positive phenotype in CPT-11-treated HCT116 and HCT116-D cultures. Senescence staining of clonogenic survival plates (day 13) revealed that singly-growing large cells (HCT116) (a), HCT116-D (b) that had not formed colonies were positive for SA-beta-galactosidase (x40 magnification); (c) cells in the center of densely-growing colonies in untreated HCT116 cells were also senescence-positive (x4 magnification).
Discussion

Elevated levels of phosphorylated H2AX (biomarker of DNA double-strand breaks) were detected in both CPT-11-treated cell lines throughout the treatment and recovery periods, in agreement with a previous study that showed that H2AX was phosphorylated in response to DNA double-strand breaks induced by DNA topoisomerase I cleavage complexes (24). ATM autophosphorylation (and delayed CHEK2 activation), TP53 stabilization/accumulation, and
lack of CHEK1 activation indicated that the ATM-CHEK2-TP53 signal transduction pathway was activated in CPT-11-treated cells to prevent DNA-damaged cells from progressing to mitosis, in agreement with two recent studies (25, 26). The DNA double-strand breaks induced by CPT-11 resulted in G2/M arrest, growth inhibition, endoreduplication and subsequent polyplodyization, loss of clonogenicity (especially in 5 BM-treated cultures), negligible cell death (until about 96 hours when levels approached 50%), and the appearance of a senescence phenotype in both cell lines. Continual DNA double-strand breakage was likely due to successive rounds of endoreduplication and attempts by each new endoreduplicated population to replicate DNA. TP53 accumulation and high levels of CDKN1A induction characterized the drug-treated cell cultures throughout treatment and recovery periods, in agreement with earlier studies (27, 28) that suggested that TP53 and CDKN1A were required for maintenance of G2/M arrest in cells with DNA damage.

One of the aims of the study was to ascertain whether there was a differential response to CPT-11 in the 5-FU refractory cell line compared to the parental cell line. The parental HCT116 cell line was earlier shown to respond to 5-FU by inducing three-fold higher levels of apoptosis compared to the HCT116-D derivative cell line (11). Despite its apoptotic resistance and faster recovery in response to 5-FU treatment, the HCT116-D cell line responded to CPT-11 like the parental HCT116 cell line, indicating that it was not cross-resistant to CPT-11. Generally, although there were slight differences in the numbers of endoreduplicating cells and expression levels of some important DNA damage checkpoint proteins between the two cell lines, their patterns of response were mostly similar. Our findings are consistent with those of Rougier (1) who reported that both chemotherapy-naive and pretreated patients with advanced metastatic colorectal cancer who experienced disease progression on 5-FU responded to CPT-11, which suggests a lack of cross-resistance between CPT-11 and 5-FU.

We speculated whether some S-phase cells in drug-treated cultures had in fact become senescent since they did not incorporate BrdU. The most widely used biomarker for senescent cells is SA-beta-galactosidase, defined as beta-galactosidase activity detectable at pH 6.0 in senescent cells, but the origin of SA-beta-galactosidase and its cellular roles in senescence are not known (29). Besides expression of SA-beta-galactosidase, TP53-dependent expression of CDKN1A, inhibition of expression of CCNA, CCNB1, and CDK1, activation of Rb, lack of BrdU incorporation into DNA and loss of clonogenicity were shown to be characteristic of senescent HCT116 cells treated with camptothecin in a recent study (30) and to collectively characterize cell senescence (31-33). Our overall results suggest that clinically-relevant CPT-11 treatment induces a senescence phenotype in colon cancer cell lines in agreement with these studies. The role of SA-beta-galactosidase as a marker of senescence is however controversial, with some authors suggesting that it is rather a marker for cellular age, cellular stress, or confluence/ quiescence (29, 34). In the present work, highly-confluent monolayers and the centers of very dense cell colonies demonstrated the presence of some senescent cells, consistent with these studies. However, a clear positive association between cells that had lost clonogenicity and SA-beta-galactosidase positivity was demonstrated. Furthermore, the majority of cells that stained positive for SA-beta-galactosidase in drug-treated cultures were considerably larger than non-senescent cells, indicating that it was the endoreduplicated cells that had become senescent. Studies are now underway to assess the importance of some of the DNA damage checkpoint proteins in the cellular response to CPT-11 as well as to determine whether the observed G2/M arrests are TP53-dependent.

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