Epigenetic mechanisms of irinotecan sensitivity in colorectal cancer cell lines

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Abstract

Irinotecan is a topoisomerase-I (Top-I) inhibitor used for the treatment of colorectal cancer. DNA demethylating agents, including 5-azacytidine (5-aza), display synergistic antitumor activity with several chemotherapy drugs. 5-Aza may enhance irinotecan cytotoxicity by at least one of the following mechanisms: (a) Top-I promoter demethylation, (b) activation of genes involved in Top-I transcriptional regulation (p16 or Sp1), and (c) modulation of the cell cycle and apoptosis after DNA damage. The growth-inhibitory effects of SN38, the active metabolite of irinotecan, 5-aza, and their combinations, were studied in four colorectal cancer cell lines. The effects of treatments on cell cycle were analyzed by flow cytometry, and apoptosis was measured by fluorescence microscopy. Top-I, Sp1, and p53 expression modulated by 5-aza were measured by real-time PCR. Methylation of Top-I, p16, 14-3-3σ, and hMLH1 promoters before and after 5-aza treatment were measured by MethylLight PCR and DNA bisulfite sequencing. Low-dose 5-aza significantly enhanced the apoptotic effect of irinotecan in all colorectal cancer cells, whereas a synergistic cytotoxic effect was observed only in p53-mutated cells (HT29, SW620, and WiDr). This synergistic effect was significantly correlated with Top-I up-regulation by 5-aza, and coupled to p16 demethylation and Sp1 up-regulation. p16 demethylation was also associated with enhanced cell cycle arrest after irinotecan treatment. In contrast, 5-aza down-regulated Top-I expression in the p53 wild-type LS174T cells in a p53-dependent manner, thereby reducing SN38 cytotoxicity. In conclusion, 5-aza modulates Top-I expression by several mechanisms involving Sp1, p16, and p53. If confirmed in other models, these results suggest that p16 and p53 status affects the 5-aza–irinotecan interaction. [Mol Cancer Ther 2009;8(7):1964–73]

Introduction

For many years, cancer has been viewed as a disease driven by progressive genetic alterations involving oncogenes and tumor suppressor genes, but only recently, it has become apparent that cancer is also driven by epigenetic alterations, i.e., changes in gene expression patterns not dependent on changes in DNA sequence. Among epigenetic events, abnormal methylation of cytosine residues by DNA methyltransferases (DNMT) contributes to the development of many cancers (1). Cytosine methylation occurs in genomic regions called CpG islands, and it is known to alter the chromatin structure leading to gene silencing. During colorectal cancer progression, a global genome demethylation is observed, coupled with selective hypermethylation of tumor suppressor, cell cycle regulator, and proapoptotic genes (2).

DNMT inhibitors, including 5-azacytidine (5-aza) and decitabine, may reverse these epigenetic alterations by reactivating silenced genes, blocking cancer cell proliferation, and/or inducing apoptosis. DNMT inhibitors showed a relevant antitumor activity in many preclinical models of solid tumors and leukemias (3). 5-Aza has been approved for the treatment of myelodysplastic syndromes, whereas decitabine showed promising activity against chronic myelogenous leukemia (4, 5). Several studies suggested that demethylating agents might be synergistic with classic chemotherapy drugs, inducing a global change in gene expression and reversing acquired or constitutive drug resistance (6). However, most of these studies did not clarify the relative contribution of apoptosis, growth arrest, and modulation of drug target–genes in determining this synergism.

Irinotecan is a chemotherapeutic prodrug that is converted to its most active metabolite (SN38) by carboxylesterases (7). During the S phase, SN38 binds the transient cleavable complex between DNA and topoisomerase I (Top-I), preventing the dissociation of the DNA–Top-I complex and thereby inducing DNA damage, G2 arrest, or apoptosis (8). Irinotecan is one of the most frequently used drugs for the treatment of metastatic colorectal cancer because its combination with fluoropyrimidines significantly improves survival (9). Nevertheless, constitutive or acquired resistance to irinotecan may occur, leading to tumor progression (10). Many hypotheses have been proposed about the mechanisms of resistance to irinotecan, including activity of detoxifying enzymes and somatic mutations in the Top-I locus which prevent SN38 from binding to Top-I.
(11). Moreover, Top-I mRNA levels showed a wide interindividual variability among colorectal cancer specimens, prompting the hypothesis that this phenomenon may be responsible for the different drug sensitivities (12). In particular, Top-I gene silencing is a feature of some irinotecan-resistant cells (13, 14), whereas Top-I up-regulation favors irinotecan cytotoxic activity (15). It is not clear which mechanisms are involved in Top-I transcriptional control, although the elucidation of such phenomena may help to overcome acquired and constitutive resistance to Top-I-targeting drugs. One hypothesis is that Top-I expression may be controlled by DNMTs through two distinct mechanisms: (a) direct methylation of Top-I promoter (which harbors a CpG island), and (b) silencing of genes controlling Top-I transcription. DNMT inhibitors may therefore directly demethylate the Top-I locus, increasing Top-I expression. Moreover, they have been shown to activate the cell cycle control gene p16 (3) and the transcription factor Sp1 (16), both of which may increase Top-I expression.

In addition to its effects on Top-I, 5-aza could favor SN38-induced growth arrest through reactivation of cell cycle control genes. For example, p16 is involved in G1-S and G2-M cell cycle arrest after DNA damage (17, 18), and it is frequently methylated in colorectal cancers (19). 14-3-3σ is a G2-M checkpoint control gene activated by DNA damage. It was shown to be silenced due to promoter methylation in some cancer cell lines, including one colorectal cancer cell line (20). Moreover, epigenetic reactivation of some pro-apoptotic genes, such as hMLH1, has been shown to favor the proapoptotic effect of several anticancer drugs in cancer cells, and to reverse chemoresistance (3, 21). Methylation of hMLH1 occurs in ~15% of colorectal cancers (22) leading to the microsatellite-instable phenotype. Finally, DNA demethylating agents may up-regulate p53 expression in cancer cells (23), a mechanism that may trigger chemotherapy-dependent apoptosis (24).

Thus, the aim of the present study was to investigate whether pretreatment of colorectal cancer cells with 5-aza may enhance SN38 antitumor activity through at least one of the following mechanisms: (a) demethylation of Top-I promoter, (b) indirect activation of Top-I expression, (c) modulation of cell cycle progression and/or apoptosis after DNA damage.

Materials and Methods

Drugs and Chemicals

SN38, 5-aza, and pifithrin-α (PFT), an inhibitor of p53 transcriptional activity (25), were purchased from Sigma-Aldrich. Drugs were dissolved in sterile distilled water (5-aza) or DMSO (SN38 and PFT) and diluted in culture medium immediately before use. McCoy’s, Leibovitz’s, and minimal essential media; fetal bovine serum, t-glutamine (2 mmol/L), penicillin (50 IU/mL), and streptomycin (50 μg/mL) were from Invitrogen. All other chemicals were from Sigma-Aldrich.

Cell Culture

The colorectal cancer cell lines HT29, SW620, WiDr, and LS174T were obtained from American Type Culture Collection. HT29, SW620, and WiDr harbor a p53 missense mutation which inactivates the protein, whereas LS174T is p53 wild-type (wt; ref. 26). Cells were maintained as monolayer cultures, respectively, in McCoy’s (HT29), Leibovitz’s (SW620), and minimal essential medium (WiDr and LS174T) with 10% fetal bovine serum, glutamine, and penicillin streptomycin. Cells were cultivated in 75 cm² tissue culture flasks (Costar) at 37°C in 5% CO₂ and 95% air, and were harvested with trypsin/EDTA when they were in logarithmic growth.

Assay of Cell Proliferation

Cells were plated in 24-well sterile plastic plates (Costar) and were allowed to attach for 24 h. The number of plated cells per well was such that at the end of the treatment, control cells were 60% to 70% confluent. Cells were treated with (a) SN38 0.0001 to 1,000 nmol/L for 48 h; (b) 0.01 to 100 μmol/L of 5-aza for 7 days; (c) 1 μmol/L of 5-aza for 7 days followed by medium replacement and SN38 treatment at 50% inhibiting concentration (IC₅₀) for 48 h. The 1 μmol/L 5-Aza concentration was less cytotoxic, showing a significant DNA demethylating activity. After drug treatments were completed, cells were cultured for an additional 24 h in drug-free medium, and the growth inhibition by drugs was assessed by counting cells. The IC₅₀ values relative to untreated cultures were calculated by nonlinear least-squares curve-fitting.

Drug interaction between 5-aza and SN38 was assessed through the combination index (CI, ref. 27), where CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively. The dose reduction index (DRI) was also calculated. DRI determines the magnitude of dose reduction allowed for each drug when given in synergistic combination, as compared with the concentration of a single agent that is needed to achieve the same effect level (28). In this case, DRI >1 indicates synergism. CI and DRI were calculated on the basis of the analysis for mutually exclusive effects. Data analysis was done by the CalcuSyn software (Biosoft) as previously described (29).

Cell Cycle Analysis

Cells were plated at 1 x 10⁶ in 100 mm plastic dishes (Costar) and were allowed to attach for 24 h. After treatment with SN38 (IC₅₀ concentration, 48 h), 5-aza (1 μmol/L for 7 days), and their combination followed by a 24-h washout, cells were harvested with trypsin/EDTA, and the growth inhibition by drugs was measured by flow cytometry. The IC₅₀ and DRI values were calculated using the Probit analysis tool of the statistical software package, GraphPad Primier. Data analysis was done by the CalcuSyn software (Biosoft) as previously described (29).
Quantitative PCR Analysis
To establish a correlation between 5-aza treatment and modulation of Top-1, p53, and Sp1, cells were treated with 5-aza (1 μmol/L, for 7 days). To investigate the role of p53 in 5-aza-induced Top-1 modulation, cells were incubated with 5-aza (1 μmol/L, one dose at day 0) and PFT (30 μmol/L, two doses at days 0 and 3.5) for 7 days. The PFT concentration used was shown to efficiently inhibit p53 activity in colorectal cancer cells (30). Total RNA was extracted from treated and untreated cells using the TRI REAGENT LS (Sigma-Aldrich). RNA was dissolved in 10 mmol/L of DTT and 200 units/mL of RNase inhibitor in RNase-free water and measured at 260 nm. One microgram of RNA was reverse-transcribed at 37°C for 1 h in a 100 μL reaction volume containing 0.8 mmol/L of deoxynucleotide triphosphates, 200 units of Moloney murine leukemia virus reverse transcriptase, 40 units of RNase inhibitor, and 0.05 μg/mL of random primers. The cDNA was amplified by quantitative, real-time PCR with the Applied Biosystems 7900HT sequence detection system (Applied Biosystems). Quantitative PCR reactions were done in triplicate using 5 μL of cDNA, 12.5 μL of TaqMan Universal PCR Master Mix, 2.5 μL of probe, and 2.5 μL of forward and reverse primers in a final volume of 25 μL. Samples were amplified using the same thermal profile described under quantitative-PCR analysis. Two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest (Top-1, p16, hMLH1, 14-3-3σ, and LINE-1) and a reference set, β-actin (ACTB), to normalize for input DNA.

P16 was analyzed because previous studies showed its role in Top-1 transcriptional regulation (15); hMLH1 is one of the proapoptotic genes most frequently methylated in colorectal cancer (22), whereas 14-3-3σ is involved in G2-M checkpoint activation after DNA damage (20) and its methylation status had not yet been assessed in HT29, SW620, WiDr, and LS174T cell lines. LINE-1 repetitive element methylation was assessed as a measure of global DNA methylation, as previously described (32).

Forward and reverse primers and probes were obtained from Applied Biosystems Assay-by-Design Gene expression products. For ACTB, p16, hMLH1, 14-3-3σ, and LINE-1, we selected previously described primers and probe sequences (31–34). For Top-1, we selected the following sequences, designed by Methyl Primer Express v1.0 software (Applied Biosystems), based on Top-1 promoter sequence (GenBank accession no. X52601). Forward primer, 5′ TCG TAT AGG TCG GTT CGT C 3′; reverse primer, 5′ CGA ACC GTA CCT AAA AAT CG 3′; probe, 5′ AAC GCC ACG ACA AAC GAA ACC AGC 3′. The specificity of the reactions for methylated DNA were confirmed separately using CpGenome Universal Methylated DNA (Chemicon International, Inc.), as described previously (35). The percentages of fully methylated molecules at a specific locus were calculated by dividing the GENE:ACTB ratio of a sample by the GENE:ACTB ratio of Universal Methylated DNA and multiplying by 100. We use the abbreviation PMR (percentage of methylated reference) to indicate this measurement. The PMR values obtained by MethylLight were dichotomized at 4 PMR for statistical purposes, as described previously (31). A dichotomization point of 4 PMR was selected because it was highly correlated with loss of protein expression.

Bisulfite DNA Sequencing
Genomic DNA (1 μg) from the colorectal cancer cells was bisulfite-converted and recovered as described above. For the conventional bisulfite DNA sequencing approach, we amplified a 470 bp portion of the Top-1 promoter CpG island (GenBank accession no. X52610), including the transcription-initiation site.

The forward and reverse primers were designed by Methyl Primer Express v1.0 software (Applied Biosystems), as follows: forward primer, 5′ TAG GTT GTT ATA TAA TTG TTG GGG 3′; reverse primer, 5′ AAA ACA ACC ATC CCT ACC TC 3′.

The PCR was done on a Gene Amp PCR System 9770 (Applied Biosystems) containing 2.5 μL of 10 mmol/L deoxynucleotide triphosphates, 1.5 μL of 25 mmol/L of MgCl2, 0.5 μmol/L of forward and reverse primers, and 0.25 units of FidelIq polymerase (USB Corporation). The PCR conditions were as follows: 94°C for 2 min, then 35 cycles of 94°C for 30 s, 61°C for 30 s, and 68°C for 1 min. A final incubation
at 68°C for 5 min concluded the PCR. PCR products were verified by gel electrophoresis, and excess primer and nucleotides were removed by SAP/Exo treatment following the protocols of the manufacturer (USB Corporation). The purified products (5 μL) were subsequently sequenced using the forward primer and the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in an ABI Prism 3130 Sequencer (Applied Biosystems).

**Statistical Analysis**

All experiments were done in duplicate and were repeated at least twice. Data were expressed as mean values ± SD and were analyzed by Student’s t-test, ANOVA followed by Bonferroni’s multiple comparisons or linear regression. The level of significance was set at P < 0.05.

**Results**

**Growth Inhibition by 5-aza, SN38, and Their Combination**

Treatment of colorectal cancer cells with 1 μmol/L of 5-aza resulted in a minimal growth inhibition of WiDr and HT29 cells, and in a more relevant effect on SW620 and LS174T cells [i.e., the growth-inhibitory effect ranged from 4.3 ± 3.9% (WiDr) to 23.1 ± 1.0% (SW620; Supplementary Fig. S1)].

On the contrary, SN38 induced a marked dose-dependent inhibition of growth in all cell lines, with IC$_{50}$ values of 0.53 ± 0.08 nmol/L (HT29), 0.85 ± 0.18 nmol/L (SW620), 1.14 ± 0.17 nmol/L (LS174T), and 2.74 ± 0.30 nmol/L (WiDr). On the basis of these results, combination studies were done to test whether pretreatment with 5-aza enhanced the antitumor activity of SN38. As shown in Fig. 1, pretreatment with 5-aza reduced the IC$_{50}$ of SN38 to 0.024 ± 0.003 nmol/L in HT-29, to 0.082 ± 0.004 nmol/L in SW620, and to 0.95 ± 0.03 nmol/L in WiDr cells; on the contrary, the IC$_{50}$ value was increased to 3.3 ± 0.2 nmol/L in LS174T. A synergistic interaction between the two drugs was observed in p53-mutated cell lines, with CI < 1 at fraction affected (FA) of 0.50 and 0.75 (Table 1). For LS174T, a synergistic activity was observed only at low FA, whereas at the most relevant FA levels (i.e., higher SN38 concentrations), the two drugs showed a clear antagonism. These data were confirmed by DRI values which showed that 5-aza pretreatment remarkably reduced the SN38 cytotoxic concentration in p53-mutated cells.

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4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Modulation of Top-I, Sp1, and p53 Expression by 5-aza
To assess the status of Top-I mRNA expression after treatment with 5-aza, quantitative PCR assay was done (Fig. 2A). The demethylating agent significantly (P < 0.05) increased Top-I expression in HT29 and SW620 cells, whereas Top-I expression was not affected in WiDr cells. In contrast, Top-I expression was strongly inhibited by 5-aza in LS174T cells, a result that was in line with the antagonism of the drugs in this cell line. Thus, 5-aza could affect SN38 activity through Top-I transcriptional control, which is supported by the significant (P = 0.021) correlation between Top-I modulation and CI at FA of 0.50 (Table 1). In particular, increased Top-I expression was associated with enhanced 5-aza–SN38 synergism. Remarkably, SN38 DRI and Top-I modulation showed an even stronger correlation (P = 0.008, linear regression). These results suggest that Top-I modulation plays a key role in 5-aza–SN38 interaction.

To test whether Top-I up-regulation was due to an increase in Sp1 transcription factor, Sp1 mRNA was quantified in cells treated with 5-aza, and a statistically significant increase in Sp1 mRNA in HT29 cells was observed (Fig. 2B). However, no correlation was found between Sp1 and Top-I modulation in the other cell lines. Thus, Sp1 overexpression may explain Top-I up-regulation in HT29, but not in SW620 cells.

Intriguingly, 5-aza down-regulated Top-I mRNA only in the p53-wt cell line. Because demethylating agents can up-regulate p53 in cancer cells (23), we investigated the role of p53 in 5-aza–dependent Top-I down-regulation. We found that 5-aza treatment induced a significant p53 up-regulation in all cell lines (Fig. 2C). Thus, we treated HT29 and LS174T cells with PFT, a well known inhibitor of p53 transcriptional activity. Concurrent treatment with 5-aza and PFT did not affect Top-I up-regulation in HT29 cells. On the contrary, PFT treatment was able to completely abrogate Top-I down-regulation in LS174T cells (Fig. 2D). Thus, 5-aza may induce Top-I down-regulation in a p53-dependent fashion.

Table 1. CI and DRI values for 5-aza–SN38 treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Fraction affected</th>
<th>CI</th>
<th>DRI SN38</th>
<th>DRI 5-Aza</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>0.25</td>
<td>0.780</td>
<td>58.226</td>
<td>1.113</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>0.680*</td>
<td>11.850(^{3})</td>
<td>1.680</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>0.876</td>
<td>2.236</td>
<td>2.146</td>
</tr>
<tr>
<td>SW-620</td>
<td>0.25</td>
<td>0.890</td>
<td>4.557</td>
<td>1.496</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>0.736*</td>
<td>8.244(^{3})</td>
<td>1.217</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>0.943</td>
<td>6.058</td>
<td>1.072</td>
</tr>
<tr>
<td>WiDr</td>
<td>0.25</td>
<td>0.658</td>
<td>84.631</td>
<td>1.517</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>0.958*</td>
<td>4.557(^{3})</td>
<td>2.279</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>0.734</td>
<td>11.424</td>
<td>2.889</td>
</tr>
<tr>
<td>LS-174T</td>
<td>0.25</td>
<td>0.906</td>
<td>4.647</td>
<td>1.648</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>1.234*</td>
<td>1.168(^{3})</td>
<td>1.285</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>9.390</td>
<td>0.118</td>
<td>1.056</td>
</tr>
</tbody>
</table>

NOTE: Top-I expression after 5-aza treatment was calculated with the \(^{2}\Delta\Delta\text{CT}\) method, as indicated in Materials and Methods, and as shown in Fig. 3A.

*Significantly correlated with Top-I expression after 5-aza treatment (P = 0.021, linear regression).

\(^{3}\)Significantly correlated with Top-I expression after 5-aza treatment (P = 0.008, linear regression).

Promoter Methylation of LINE-1, Top-I, 14-3-3\(^{\star}\), hMLH1, and p16
To confirm the DNA demethylating activity of 5-aza, we did a MethyLight assay on LINE-1 repetitive elements. As it is shown in Fig. 3A, 1 \(\mu\)mol/L of 5-aza induced a robust and reproducible reduction of LINE-1 methylation in all cell lines. MethyLight was also used to measure Top-I promoter methylation. All colorectal cancer cells exhibited a basal PMR < 4, showing an unmethylated Top-I promoter. This value was not changed by 5-aza treatment. Among cell cycle control genes, 14-3-3\(^{\star}\) was unmethylated in all cell lines. On the contrary, the p16 gene was methylated in HT29 and SW620 cells, whereas the proapoptotic hMLH1 gene was methylated in LS174T cells. After 5-aza treatment, the PMR value of methylated genes decreased to <1 (Fig. 3B). To validate MethyLight data, HT29, SW620, WiDr, and LS174T cells were also subjected to direct bisulfite sequencing of Top-I promoter. With this method, original 5-methyl cytosine can be detected as cytosine in the sequence, whereas unmethylated cytosine is converted to uracil and amplified as thymine. In all the cell lines, there was a complete concordance between methylation status, as detected by MethyLight Q-MSP analysis and bisulfite sequencing, showing an unmethylated Top-I promoter. A representative electropherogram is shown in Fig. 3C.

Cell Cycle Effects of 5-aza, SN38, and Their Combination
Both SN38 and the combination were able to affect the cell cycle distribution of colorectal cancer cells (Table 2). On the contrary, 1 \(\mu\)mol/L of 5-aza alone had no effect on cell cycle. The main effect of SN38 was the activation of the G2 checkpoint after DNA damage. Pretreatment with 5-aza elicited opposite effects by abrogating the G2 checkpoint in WiDr and LS174T cells, whereas in HT-29 cells, the combination induced a relevant G1 arrest, and in SW620, it resulted in enhanced G2 arrest.

Induction of Apoptosis by 5-aza and SN38
Cells exposed to SN38, 5-aza, or to their combination (i.e., 5-aza followed by SN38) presented typical apoptotic morphological with cell shrinkage, nuclear condensation and fragmentation, and rupture into debris. In all cell lines, 18% to 31% of apoptotic cells were observed after SN38 treatment (Fig. 4). 5-aza induced a clear proapoptotic effect in LS174T cells only. However, whereas the apoptotic index of 5-aza–SN38 in p53-mutated cells was significantly higher than expected by a simply additive effect (P < 0.05, t test), the two drugs seemed to be simply additive in LS174T cells. In this cell line, no significant difference was reported between the sum of SN38 and 5-aza apoptotic indices and the apoptotic index of the combination (35.4 ± 5.5% versus 45.0 ± 4.9%).

Discussion
The present study shows that pretreatment of the p53-mutated colorectal cancer cells HT29, SW620, and WiDr with 5-aza resulted in increased growth inhibition by SN38. 5-aza and its analogue decitabine were originally developed and tested as nucleoside antimetabolites with clinical specificity for acute myelogenous leukemia (36). Because 5-aza could...
be activated to the deoxynucleoside triphosphate and incorporated into DNA and RNA, treatment of cells with this drug leads to the inhibition of DNA, RNA, and protein synthesis, eventually inducing cell death. At doses low enough to avoid triggering cell death, incorporation of 5-aza into DNA leads to rapid loss of DNMT activity because the enzyme becomes irreversibly bound to 5-aza cytosine residues. DNA demethylation activates many genes and induces different effects, including cell cycle arrest, apoptosis, and differentiation, depending on cell type, drug, and dose used (37). Because these phenomena are also triggered by cytotoxic drugs, it is still difficult to clarify whether 5-aza and its analogue act primarily through cytotoxicity or DNA demethylation. For this reason, in the present study, we used a drug exposure schedule that showed minimal cytotoxic activity, despite its ability to efficiently inhibit DNA methylation, as shown by LINE-1 methylation data. Thus, the key mechanism by which 5-aza enhances irinotecan efficacy is mediated by DNA demethylation.

Several pathways seem to affect 5-aza–SN38 interaction, including cell cycle control, apoptosis, and epigenetic regulation of Top-I expression. Our data support the idea that p53 mutational status and p16 promoter methylation are key determinants in this process. The main difference between p53-mutated and p53-wt cells is the apoptotic response to 5-aza. In line with previous evidence (38), we found that 5-aza induced apoptosis only in p53-wt LS174T cells, whereas the 5-aza pretreatment significantly enhanced the SN38 apoptotic effect in p53-mutated cells, where this interaction was more than additive. These results are consistent with the CI and DRI data, showing a clear synergism only in p53-mutated cells. In HT29 and SW620 cells, this effect is associated with Top-I up-regulation and enhancement of cell cycle arrest. It is well known that Top-I up-regulation results in increased SN38-dependent DNA damage and apoptosis (15), and that many irinotecan-resistant cancer cells have reduced or absent Top-I protein expression (13, 14). Thus, it is not surprising that 5-aza–induced Top-I up-regulation was coupled with enhanced SN38 sensitivity in HT29 and SW620 cells. In addition, these two cell lines showed a methylated p16 promoter, a condition reversible upon 5-aza treatment. Reactivation of p16 may contribute to the G1 (HT29) and G2 (SW620) arrest observed after 5-aza–SN38 treatment. These results are in agreement with previous evidence, indicating that p16 is involved in p53-independent G1 and G2 arrest after DNA damage, and that the effects of p16 the on cell cycle are cell line–dependent (17, 18). It is interesting to note that WiDr and LS174T cells, harboring an unmethylated p16 promoter, elicited an opposite cell cycle pattern: 5-aza pretreatment abrogated SN38-dependent G2 arrest. This phenomenon leads to mitotic catastrophe and apoptosis, but is not always coupled with increased irinotecan antitumor activity (39). In keeping with this evidence, we found that 5-aza–SN38 combination was synergistic in WiDr cells, probably due to the enhanced apoptotic response.

In contrast, in LS174T cells, the two drugs seemed synergistic for $FA < 0.50$, and antagonistic for $FA \geq 0.50$. This apparent dichotomy could be solved considering the two main effects of 5-aza on this cell line: a general reactivation of

![Figure 2](https://mct.aacrjournals.org/issue). Gene expression modulation induced by 5-aza. Top-I(A), Sp1 (B), and p53 (C) modulation in 5-aza-treated cells. Columns, mean; bars, SE; white columns, untreated controls; black columns, 5-aza–treated cells (*, $P < 0.05$, Student’s t test). D, effect of concurrent PFT–5-aza treatment on HT29 and LS174T cells. White columns, untreated controls; black columns, PFT–5-aza–treated cells (*, $P < 0.05$, Student’s t test).
proapoptotic genes (hMLH1 and p53) and a significant Top-1 down-regulation. As shown by linear regression analysis, Top-1 modulation affected SN38 activity at FA = 0.50, with a significant proportional correlation between Top-1 levels and CI values. Our data showed an even stronger correlation between DRI and Top-1 modulation. Importantly, at the most relevant FA levels (i.e., for higher SN38 concentration), 5-aza strongly reduced the SN38 effective dose in p53-mutated cells. Taken together, these results support the notion that p53 mutational status is a major determinant of 5-aza-SN38 activity in colorectal cancer cells.

We did several analyses to further understand the role of p53, as well as of other molecular mechanisms, in the differential modulation of Top-1 expression by 5-aza in colorectal cancer cells. Despite the fact that Top-1 promoter harbors a CpG island, we found that this region is normally unmethylated in the four colorectal cancer cells. Thus, 5-aza does not increase Top-1 mRNA synthesis through direct demethylation of its promoter. It has been shown that in addition to gene expression changes, occurring as a direct consequence of promoter demethylation, indirect gene expression changes may also result from inhibiting DNA methylation. There are several mechanisms by which indirect transcriptional changes may occur, including gene regulation by a reactivated transcription factor or modulation of specific signal transduction pathways (37).

Top-1 transcription is regulated by a complex interplay between many factors, and these mechanisms are only partially understood. Hence, based on our own and other previous studies, we propose a model for epigenetic regulation of Top-1 transcription in colorectal cancer cells (see Fig. 5), which may explain the dissimilar effects of 5-aza treatment in different colorectal cancer cell lines. In addition, it may provide a tool to predict the synergism between irinotecan and DNMT inhibitors in patients with colorectal cancer. The present results suggest that 5-aza may activate at least three genes involved in Top-1 modulation: p16, p53, and p53.

Table 2. Cell cycle modulation by 5-azacytidine, SN38, and their combination

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>G1 phase (%)</th>
<th>S phase (%)</th>
<th>G2-M phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>5-Azacytidine</td>
<td>61.39 ± 3.4</td>
<td>37.61 ± 2.5</td>
<td>1.00 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>61.27 ± 2.1</td>
<td>37.05 ± 1.9</td>
<td>1.68 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>SN38</td>
<td>50.43 ± 5.0</td>
<td>37.09 ± 2.2</td>
<td>12.48 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>53.86 ± 1.7</td>
<td>36.18 ± 1.6</td>
<td>9.98 ± 1.7</td>
</tr>
<tr>
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<td>30.36 ± 4.0</td>
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Sp1. It is well known that p16 gene is methylated in many colorectal cancer cells, and that p16 transfection in cancer cells causes Top-I up-regulation and increases the sensitivity to irinotecan (15). Thus, p16 reactivation by DNMT inhibitors may support Top-I expression, thereby enhancing SN38 cytotoxicity.

Moreover, Top-I promoter harbors several Sp1 binding sites, which play a significant role in the initiation of gene transcription (40, 41). It has been shown that DNMT inhibitors may activate Sp1 transcription (16), which in turn, up-regulates the promoter activity of many Sp1-dependent genes (42). Thus, Sp1 overexpression may be an additional mechanism of Top-I up-regulation by 5-aza. Finally, p53 may indirectly regulate Top-I transcription because it binds Sp1, inhibiting its transcriptional activity (43). Although the p53 promoter does not harbor a CpG island, methylation of single CpG sites within this region may reduce p53 mRNA levels (44), and this phenomenon seems to contribute to carcinogenesis in some human malignancies (45). Hodge et al. (23) showed that p53 promoter demethylation by DNMT inhibitors up-regulates p53. Thus, 5-aza could activate p53 transcription, thereby reducing Top-I expression. Consistent with this model, we found that 5-aza up-regulates Top-I in HT29/SW620 cells (p16 methylated), but not in WiDr/LS174T cells (p16 unmethylated). The highest Top-I expression after 5-aza treatment was observed in HT29 cells, which showed Sp1 up-regulation, in addition to p16 demethylation (Fig. 5).

Three out of four colorectal cancer cells used in this study harbored an inactivated p53 protein, due to a biallelic missense mutation (26). In these cells, we found no p53 effect on Top-I modulation. The only p53-wt cell line (LS174T), which also harbored an unmethylated p16 promoter, showed Top-I down-regulation after 5-aza treatment. Hence, we provide evidence that p53 inhibition completely abrogates Top-I
down-regulation in LS174T cells. This result further strengthens our model, and suggests a novel link between DNA methylation, p53, and Top-1 transcriptional control. More importantly, this p53-dependent Top-1 down-regulation could explain the different effects of 5-aza–SN38 combination in p53-mutated and p53-wt cells.

Future studies are needed to dissect the specific role of Sp1, p16, and p53 in Top-1 promoter regulation. However, such experiments are beyond the purpose of the present work, which is aimed at identifying the molecular basis of a drug interaction.

The chemosensitizing effect of demethylating agents has been previously described in several cancer cell lines (46–48). These studies showed that 5-aza and its analogues increase the apoptotic index of cancer cells exposed to various DNA-damaging agents, including irinotecan. Other studies addressed the role of DNA methylation in irinotecan metabolism (49), but they did not evaluate the contribution of cell cycle, apoptosis, and Top-1 modulation to the chemosensitizing effect of epigenetic therapy. Ishiguro et al. (46) showed that decitabine may reactivate p16 but they found no Top-1 modulation in HCT-15 colorectal cancer cells. This is probably due to the fact that they measured gene expression through a standard reverse transcription-PCR that is far less sensitive than the real-time PCR used in this study.

In conclusion, we found that 5-aza enhanced irinotecan growth-inhibitory effect and Top-1 expression only in p53-mutated colorectal cancer cells. In addition, p16 demethylation may contribute to 5-aza–dependent cell cycle arrest and Top-1 up-regulation. Of note, p16 methylation and p53 mutation are common features of human colorectal cancers, both occurring at a frequency of ~50% (19, 50). Thus, our results may have implications for the rational design of future drug regimens incorporating irinotecan and demethylating agents for the treatment of colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Epigenetic mechanisms of irinotecan sensitivity in colorectal cancer cell lines

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