5-aza-Cytidine Is a Potent Inhibitor of DNA Methyltransferase 3a and Induces Apoptosis in HCT-116 Colon Cancer Cells via Gadd45- and p53-Dependent Mechanisms

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ABSTRACT

Methyltransferase inhibitors commonly used in clinical trials to promote tumor cell death, but their detailed cytotoxic action, are not yet fully understood. A deeper knowledge about the mechanisms of apoptosis-inducing mechanisms and the interaction with DNA methyltransferases (DNMTs) DNMT1, DNMT3a, and DNMT3b might allow the design of more effective drugs with lower cytotoxicity. 5-aza-cytidine (5-aza-CR), a potent inhibitor of DNMT1, is known to induce demethylation and reactivation of silenced genes. In this study, we investigated the p53 dependence of apoptotic, cell cycle, and growth inhibitory effects of 5-aza-CR, as well as the influence on the expression level of DNMT1, DNMT3a, and DNMT3b in the colon cancer cell line HCT-116. Exposure to 5-aza-CR induced the up-regulation of genes promoting cell cycle arrest and DNA repair (p21WAF1 and GADD45) and apoptosis (p53, RIPK2, Bak1, caspase 2, caspase 6). In parallel, there was a down-regulation of anti-apoptotic Bcl2 protein and the E2F/mediator cyclin B1. Co-incubation with pifithrin-alpha (PFT-α), a selective p53 inhibitor, restored GADD45, Bcl2, cyclin B1, and p21WAF1 expression. There was an almost complete reversal of the growth inhibitory, cell cycle, and apoptotic effects of 5-aza-CR. 5-aza-CR treatment led to re-expression of DNMT1 mRNA and demethylation and reactivation of p16INK4a expression. The resulting DNA hypomethylation has been linked to the induction of cellular differentiation in vitro (Petti et al., 2002). 5-aza-CR incorporates into DNA forming covalent adducts with cellular DNMT1, thereby depleting the methyl group transferred to the carbon position of the cytosine from the methyl donor S-adenosyl-L-methionine (Okano et al., 1997). To date, 5-aza-cytidine (5-aza-CR) and its deoxyribosyl analog 5-aza-2′-deoxycytidine are the DNMT inhibitors that have undergone the most preclinical and clinical testing (Santini et al., 2001). 5-aza-CR was evaluated in clinical trials as a cancer therapeutic agent for the treatment of patients with acute myeloid leukemia and myelodysplastic syndrome (Santini et al., 2001; Kornblith et al., 2002; Silverman et al., 2002). 5-aza-CR incorporates into DNA forming covalent adducts with cellular DNMT1, thereby depleting the cells from enzyme activity and causing demethylation of genomic DNA as a secondary consequence (Christman, 2002). In various in vitro experiments, 5-aza-CR treatment leads to re-expression of former silenced genes (Christman, 2002). The resulting DNA hypomethylation has been linked to the induction of cellular differentiation in vitro (Petti et al., 1993) and altered expression of genes involved in tumor suppression (Christian, 2002). In addition, it has been dem-
onstrated that 5-aza-CR inhibits telomerase activity via transcriptional repression of hTERT in prostate cancer cell lines (Kitagawa et al., 2000). Recent studies have demonstrated that DNMT1-5-aza-2′-deoxycytidine adducts in DNA can activate a p53 DNA damage response pathway in the colon cancer cell line HCT-116 (Karpf et al., 2001, 2004). However, the mechanism responsible for this drug's inhibition of cell growth and its other biological effects remain unclear.

In the present study, we examined the effects of 5-aza-CR on apoptosis, cell growth, global methylation status, and the expression of the methyltransferases DNMT1, DNMT3a, and DNMT3b in the human colon cancer cell line HCT-116. To develop a mechanistic model of 5-aza-CR action, we investigated the observed effects before and after treatment with the selective p53 inhibitor, pifithrin-α (PPT-α), as well as in HCT (−/− p53) and the regulation of methyltransferases after 5-aza-CR treatment.

Materials and Methods

Reagents and Drugs. Prolong Antifade and and trypan blue were obtained from Sigma-Aldrich (St. Louis, MO). RPMI 1640, fetal bovine serum, and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA).

Methylation-Specific PCR. Genomic DNA was prepared using the standard proteinase K-phenol-chloroform extraction method. The methylation status of the p16INK4a gene was monitored by methylation-specific PCR. Bisulfite modification was performed using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Digested DNA (500 ng) was incubated, in triplicate, according to the recommendations of the manufacturer (AGS, Heidelberg, Germany). The reaction mixture was incubated at 37°C for 2 h. The reaction was stopped by heating at 65°C for 10 min. Reactions without DNA were used as background controls.

Global Genomic Methylation Status. The methylation status of CpG islands in genomic DNA is determined by the reaction of methylated DNA with Sadenosyl-L-[methyl-3H]methionine (SADEM; Roche Diagnostics, Mannheim, Germany), and the assay was performed according to the manufacturer's suggestions.

4. Treatment. Cells were treated with 0.5 or 5 μM of 5-aza-CR for 12 to 24 h, or with PPT-α (1 μM) for 12 to 24 h. Assays were conducted at least in duplicate. After the treatment, a fraction of cells (the selective p53 inhibitor, PPT-α) was harvested, and the inhibition of p53-dependent apoptosis was determined by counting the number of cells that survived in the absence of p53.

To determine whether cells recover from 5-aza-CR treatment, cells were replated with fresh medium without PPT-α at 96 h post-treatment and were allowed to grow for a further 48 h (total of 144 h).

Flow Cytometric Analysis of DNA Content. Cells were seeded in 10-cm dishes at a density of 5 × 10⁶ cells per well. They were incubated and allowed to grow to 50% confluence after which they were treated with 1 μM 5-aza-CR and incubated for further 24 h. They were then harvested, centrifuged, and washed twice with phosphate-buffered saline (PBS) and permeabilized with 70% ethanol, treated with RNase, and finally stained with propidium iodide solution (10 μg/ml, final concentration). Distribution of cell cycle phases with different DNA contents was determined using a flow cytometer (BD Biosciences, San Jose, CA). Cells less intensely stained than G1 cells (sub G1 cells) in flow cytometric histograms were considered as apoptotic cells and cell debris. Analysis of cycle distribution and the percentage of cells in the G1, S, and G2/M phases of the cell cycle were determined using Cell QuestPro and FlowJo (Verity Software House, Topsham, ME) software.

Annexin V Assays. TUNEL assay. Apoptosis was scored either by assessing the fraction of cells with a sub-G0/G1 DNA content by flow cytometry (see above) or by estimating the extent of DNA fragmentation using the TUNEL assay. Briefly, cells were plated on a 24-well plate and treated with 1 μM 5-aza-CR or PPT-α as described earlier. The medium was then aspirated and cells were washed twice with warm PBS. Cellular DNA was stained with the In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany), and the assay was performed according to the recommendations of the manufacturer (Roche Diagnostics). Cytospin preparations were fixed and labeled and four independent ×100 fields containing a minimum of 300 cells on each of three replicate slides were evaluated for nuclear labeling by fluorescence microscopy (Axiocam 200; Carl Zeiss GmbH, Jena, Germany) for each treatment. Nuclear chromatin condensation was observed by fluorescence microscopy (LSM 410, Carl Zeiss GmbH, Jena, Germany).

Annexin-V-FITC binding assay. After exposure to 10 μM 5-aza-CR for 6 h, cells were washed twice with cold PBS. Binding to annexin V-FITC to the cell surface, which is an early marker of apoptosis, was determined according to the instruction of the manufacturer (BD Biosciences PharMingen, San Diego, CA). The slides were viewed immediately on a fluorescence microscope (Leica DM2500; Leica, Wetzlar, Germany) equipped with a SpotRT camera (Diagnostics Instruments, Burroughs, MI). Images were captured and pseudocolored using Spot camera Software. For each of the three replicate experiments, three randomly selected microscopic fields were examined at 400× magnification, and the annexin V-FITC cells (green fluorescence) were counted.

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Real-Time RT-PCR for Detection of mRNA Expression. For mRNA expression studies, total RNA was extracted using TRIzol reagent (Invitrogen). cDNA synthesis was done in a 20-μl reaction mix starting with 1 μg of total RNA using the reverse transcription system of Promega (Madison, WI; 42°C for 30 min, 99°C for 5 min, and 4°C for 5 min). Real-time PCR was performed using a LightCycler (Roche Diagnostics), and threshold cycle numbers were determined using the LightCycler software, version 3.5. Each sample was run twice, and the threshold cycle numbers were averaged. In addition, all samples showing >10% deviation of their values were tested in a third run. For amplification detection, the LightCycler DNA Master Hybridization Probes Kit was used as described by the manufacturer (Roche Diagnostics). Briefly, in addition to the two primers, two different oligonucleotides hybridize each to internal sequence of the experimental genes or the β2-microglobulin (β2-M) gene. PCR assays were carried out in a 20-μl reaction volume containing 1 μl of cDNAs, 0.5 μM of each primer, 2 pmol LCRed 640 probe, 4 pmol fluorescein hybridization probe, and 2.5 mM MgCl₂. Annealing temperatures are given in Table 1. All genes examined were normalized to a housekeeping gene encoding β2-M.

Fold induction was calculated according to the formula 2ΔΔCt (Saha et al., 2001), where R is the threshold cycle number for the β2-M gene observed in the cell line before 5-aza-CR treatment; ΔCt is the threshold cycle number for the experimental genes observed before and after 5-aza-CR treatment; En is the threshold cycle number for the experimental gene observed in the cell line after 5-aza-CR treatment, and ΔE is the threshold cycle number for the experimental gene observed in the cell line before 5-aza-CR treatment. Primers and oligonucleotide probes were chosen using the TIB MOLBIOL computer program (Berlin, Germany; http://www.tibmolbio.de/oligoag.html) to ensure their specificity to individual genes involved in apoptosis (Superarray Bioscience Corp., Bethesda, MD). Hybridization with the housekeeping protein β2-M was used as a control of equal loading and mRNA integrity.

cDNA Array Analysis. To identify apoptosis-associated genes that are up-regulated by 5-aza-CR, we used GEArray Q series Human Apoptosis GeneChip designed to profile the expression of 96 key genes involved in apoptosis (Superarray Bioscience Corp., Bethesda, MD). The complete gene list can be found at http://www.superarray.com. The cDNA fragments are printed on a 3.8-× 4.8-cm glass microscope slide. Total RNA was prepared by TRIzol Reagent (Invitrogen) and homogenized in a homogenizer at pH 7.4 containing 50 mM sodium phosphate, 0.2 M NaCl, 10 mM EDTA, 100 mM E-64, and 1 mM phenylmethylsulfonyl fluoride by sonication (Mandelin, Berlin, Germany). Homogenates were centrifuged for 10 min at 4°C and 12,000 rpm (Eppendorf, Hamburg, Germany). The supernatants were subjected to SDS-PAGE followed by immunoblotting.

Protein Extraction and Quantification. Cell samples were homogenized in a buffer at pH 7.4 containing 50 mM sodium phosphate, 0.2 M NaCl, 10 mM EDTA, 100 mM E-64, and 1 mM phenylmethylsulfonyl fluoride by sonication. The cell lysates were subjected to SDS-PAGE using Laemmli buffer containing 20% dithiothreitol and boiled. Protein measurements were carried out in all samples according to the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA), and equal protein amounts were subjected to SDS-PAGE followed by immunoblotting. Western Blotting. After homogenizing to a polyvinylidene difluoride membrane (Perkin-Elmer Enzyme Analytical Sciences, Boston, MA) and blocking the nonspecific binding with 5% milk, it was incubated with the primary antibodies allowed to react with the secondary antibodies conjugated to either anti-rabbit or anti-mouse (3,000) for 30 min. We used following human recombinant monoclonal or polyclonal antibodies: anti-p53 (clone 64B 1446, Biocarta), anti-actin (clone 3A, Biocarta), anti-cyclin B1 (clone 7A9, Novocastra), anti-p21WAF1 (clone EA10, Oncogene Science), anti-p16INK4a (clone SC-6850, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-DNMT1 (clone 1A9, Biocarta), anti-DNMT3a (clone 52A 1018, Biocarta), anti-DNMT3b (clone HU25, Roche Diagnostics) as primary antibodies and the secondary peroxidase-conjugated goat anti-rabbit antibody (1:1000 dilution, Novocastra). After washing, the membrane was incubated with the horseradish peroxidase-labeled anti-goat secondary antibody followed by incubation with the substrate, West Pico Supersignal (Pierce Chemical, Rockford, IL), and the image was scanned using Syngene Bio-Imaging System, Synoptics (LaJolla, MD). Hybridization with the housekeeping protein β2-M was used as a control of equal loading and mRNA integrity.

Table 1. Sequences of primers, hybridization probes, annealing temperature, and lengths of PCR products.

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<tr>
<th>Gene</th>
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<th>Probes</th>
<th>Annealing Temperature</th>
<th>Length</th>
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<td>DNMT1 A 1</td>
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<td>DNMT3a LC</td>
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<td>DNMT3a FL</td>
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<td>P16 F</td>
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LC, LightCycler Red 640; X, fluorescein; p, phosphate; hyb, hybridization probe.

GenBank accession number.
Solution followed by hybridization at 68°C overnight with continuous agitation. The membrane was washed at 68°C twice with 2× standard saline citrate, 1% SDS and 0.1× standard saline citrate, 0.5% SDS, respectively. For detection of chemiluminescence, the membrane was blocked using the alkaline phosphatase-conjugated streptavidin method in combination with CPD star. All signals were measured using the Syngene BIO Imaging system. The raw signal intensities were corrected for background by subtracting the signal intensity of the average of the three negative controls (pUC18 cDNA) and the four blanks and were expressed as fold changes given as the relative expression ratio: gene/housekeeping gene. Any signal whose raw intensity was less than 150% of the background was treated as a background signal and thus interpreted to be not detectable in the sample.

Results

5-aza-CR Induces Demethylation and Significant Down-Regulation of DNMT1 and DNMT3a Gene Transcription in HCT-116 Cells. We determined the time-course effects of 5-aza-CR on the level of global DNA methylation in p53 (+/+/-) HCT-116 cells by incubating DNA with [3H]SAM in the presence of bacterial SssI methylase. In this assay, the number of methyl groups incorporated into DNA is inversely proportional to the original number of CpG sites available for methylation. Thus, it is inversely proportional to the prior methylation status of DNA. The DNA isolated from HCT-116 cells treated with 1 μM 5-aza-CR was significantly hypomethylated after 72 (p < 0.001) and 96 h (p < 0.001) of treatment compared with cells kept 5 days without the drug, indicating that 5-aza-CR decreased the global level of DNA methylation in a time-dependent manner (Fig. 1A). Considering the fact that hemimethylation of the p16INK4a promoter is observed in HCT-116 cells (presence of both methylated and unmethylated bands in the untreated control), we assessed the effect of 5-aza-CR on the methylation status of the p16INK4a gene promoter. One day post-treatment with 5-aza-CR (1 μM) for 3 days, the methyl band almost completely disappeared after 96 h (Fig. 1B), corresponding to the disappearance of the methylation-specific band at the time-dependent expression of p16INK4a mRNA in HCT-116 cells (Fig. 1C). Re-expression of p16INK4a transcript levels occurred when cells were allowed to recover 5-aza-CR treatment.

We then determined the effect of 5-aza-CR on the expression of human DNMT1, DNMT3a, and DNMT3b. Real-time PCR revealed a 3-fold down-regulation in DNMT1 mRNA expression levels following treatment with 5-aza-CR for 4 days (Fig. 1D). A significant down-regulation in DNMT3a expression levels occurred already 24 h after post-treatment, peaked at 48 h, and persisted even after 4 days of treatment with 5-aza-CR (Fig. 1E). Conversely, 5-aza-CR treatment did not affect the mRNA expression levels of DNMT3b (Fig. 1F).

5-aza-CR Treatment Induces Growth Inhibition and G2/M Arrest in HCT-116 Cells. The time of exposure to 5-aza-CR required to inhibit cell growth was evaluated in p53 (+/+/-) HCT-116 cancer cells. Cells were treated with 5-aza-CR, and cell morphology and viability were monitored for 96 h using the MTT assay. The addition of 5-aza-CR resulted in a significant decrease in cell viability, with a time-dependent decrease in cell proliferation. The addition of 5-aza-CR resulted in a significant decrease in cell viability, with a time-dependent decrease in cell proliferation.

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Fig. 1. A, time-course analysis of the global DNA methylation status in HCT-116 (p53 +/+/-) cells cultured in the presence of 0 or 1 μM 5-aza-CR. The extent of global DNA methylation is inversely proportional to the incorporation of methyl groups by bacterial SssI methylase in the presence of [3H]SAM. Values are means ± S.E.M., n = 3. ANOVA, Aza, 72 h, p < 0.001; Aza, 96 h, p < 0.001. B, effect of 5-aza-CR on p16INK4a gene methylation. Methylation-specific PCR was performed using the unmethylated (u) and methylated (m) primer sets. C, time-course analysis of mRNA re-expression of p16INK4a. D to F, expression of the DNA methyltransferases DNMT1, DNMT3a, and DNMT3b in HCT-116 cells cultured in the presence of 1 μM 5-aza-CR.
in the appearance of many damaged cells at 72 h after treatment and to a lesser extent at 48 h (Fig. 2A). A time-dependent inhibition of cell viability was observed upon treatment with 5-aza-CR; 1 μM decreased cell viability by 65% at 72 h post-treatment (Fig. 2A). At 96 h, the viability of HCT-116 cells was most severely affected presumably because growth inhibition requires both the incorporation of 5-aza-CR into genomic DNA and time for the alteration of DNA methylation patterns and the accumulation of enzyme DNA adducts. In addition, the kinetics of the induction of methylation-silenced genes (Fig. 1C) and cell growth inhibition after 5-aza-CR was consistent with this time frame of treatment.

To dissect the mechanism of the antiproliferative effects of 5-aza-CR, we determined whether growth inhibition is associated with specific cell cycle changes. Exponentially growing HCT-116 (+/+) cells were treated with 5-aza-CR for 48 or 72 h and harvested for flow cytometric analysis of DNA content by PI staining (Fig. 2B). Cell cycle distribution analysis showed an increase, within 48 h, in the number of cells in the G₂ phase of the cell cycle following treatment with 1 μM 5-aza-CR, providing evidence of G₂/M arrest. By 72 h, 50% of the cells arrested at G₂ phase, and the percentage of cells in S phase decreased by more than 50% compared with the untreated control. Furthermore, the accumulation of 5-aza-CR for 72 h induced an accumulation of sub-G₀/G₁ (apoptotic) DNA. Thus, the growth/inhibitory effects of 5-aza-CR (Fig. 2A) are due to induction of G₂/M arrest and programmed cell death (Fig. 2B).

5-aza-CR Induces Apoptosis in HCT-116 Cells. To understand and confirm the nature of cell death, we used the TUNEL and Annexin-V flow cytometric assay methods (Fig. 3). Numerous TUNEL-positive cells with apoptotic characteristics, based on the typical shrunken shape of the nucleus and on intense staining of FITC-conjugated dUTP, appeared in the 5-aza-CR-treated cultures (Fig. 3A, left). A time-dependent increase up to 65% in apoptotic cells occurred in 5-aza-CR-treated cultures (Fig. 3A, right). Drug exposure also caused a strong increase in Annexin-V staining, a classic feature of early apoptosis (Fig. 3B). The proportion of apoptotic cells at 72 h post-treatment was significantly higher than that of necrotic cells (Fig. 3B, right), indicating that apoptosis rather than necrosis is the mechanism of the 5-aza-CR-induced cell death in HCT-116 (+/+) cells. These results collectively confirm that 5-aza-CR-induced increases in the sub-G₀/G₁ fraction (Fig. 2B) are due to apoptosis.

Inhibition of p53 Activation Abrogates 5-aza-CR-Induced Cell Cycle Arrest and Apoptosis. To assess the contribution of p53 activation in 5-aza-CR-induced growth inhibition, cell cycle arrest, and apoptosis, we employed the strategy of using PFT-α, a selective inhibitor of p53 function. The concentration of PFT-α that does not inhibit basal levels of p53 activation in HCT-116 cells was determined to be 1 μM. The growth inhibition and cell cycle arrest effects of 5-aza-CR were significantly reduced (not shown) when HCT-116 cells were pre-treated with PFT-α. In parallel, the proportion of apoptotic cells at 72 h post-treatment was significantly reduced when PFT-α was employed (Fig. 3B). These results suggest that p53 activation is required for 5-aza-CR-induced growth inhibition and apoptosis in HCT-116 cells.

Fig. 2. Induction of growth/cell cycle arrest by 5-aza-CR in HCT-116 (+/+) human colon cancer cells. A, cell growth determined by the MTT assay. Controls were treated with ethanol. Values represent the means of duplicates from two separate experiments. B, effects on cell cycle profiles of HCT-116 cells after treatment with 1 μM 5-aza-CR. Cells in active growth were treated with 5-aza-CR for 48 or 72 h and then fixed, and the DNA content was determined by flow cytometric analysis by PI staining, analyzing 20,000 events per sample. The percentages of cells in PreG₁, G₁/G₂, S, and G₂/M are shown. The data shown are typical of one of three independent experiments.
of p53 protein expression in control untreated cells and is not toxic to the cells was found to be 30 μM. Seventy-two hours after addition of 5-aza-CR (1 μM), the percentage of viable cells increased from 24% in 5-aza-CR-treated cells to 86% in cells treated with combination of 5-aza-CR and PFTα (Fig. 4A). The fraction of cells in the G1 phase increased from 17% in 5-aza-CR-treated cells to 58% in cells treated with a combination of 5-aza-CR and PFTα, whereas the fraction of cells in the S phase decreased from 7 to 38% (Fig. 4B). The cell cycle kinetics of exponentially growing cells were not affected by the addition of PFTα alone (Fig. 4B).

Furthermore, the number of apoptotic cells decreased significantly to reach levels of the untreated control when cultures were treated with a combination of 5-aza-CR and PFTα (Fig. 4C). In agreement with these results, the fraction of cells in G1 phase decreased to 39% and apoptosis was reduced in 5-aza-CR-treated p53−/− cells (data not shown). These results demonstrate clearly that functional p53 is required for induction of apoptosis and cell cycle arrest in the G2 phase by 5-aza-CR.

5 Aza-CR Treatment Results in Activation of p53 and Its Downstream Effectors p21WAF1 and Gadd45. Growth arrest of cells by DNA damage and by other stress signals that arrest cells in G2 is frequently associated with induction of Gadd45 (Taylor and Stark, 2001), and this was also found in HCT-116 (+/−) cells treated with 5-aza-CR (Fig. 5). Gadd45 can be induced by various mechanisms, including the p53 pathway, which is activated by phosphorylation on serine 15 in response to DNA damage and various other signals that induce G2 cell cycle arrest (Taylor and Stark, 2001). Therefore we tested whether 5-aza-CR treatment resulted in an altered expression of p53. We observed a 2- to 5-fold induction of p53 protein expression in HCT-116 cells after 5-aza-CR treatment (Fig. 5A). Similar to Gadd45 and p53 activation, we observed a time-dependent increase of p21WAF1 protein and transcript levels after 5-aza-CR treatment (Fig. 5, A and B). At 72 h post-treatment, a significant decrease in Bcl2 protein content to almost undetectable levels occurred in drug-treated cells (Fig. 5A), a time when apoptosis was extensive (Fig. 3). Despite the decrease in Bax protein expression, the Bax/Bcl2 ratio increased (48 h, 1.8-fold; 72 h, 2.4-fold) (Fig. 5A). Furthermore, treatment with 5-aza-CR resulted in a reduction in Bcl2 mRNA expression (Fig. 5C). Interestingly, when cells were allowed to recover from drug effects, the protein levels of p53, p21WAP1, cyclin B1, Gadd45, and Bcl2 returned to levels of untreated control cells (Fig. 5A).

Role of p53 Activation in 5-aza-CR-Altered Protein Expression in HCT-116 Cells. To characterize the role of p53 in mediating 5-aza-CR-induced changes in protein ex-
pression, we used PFT-α to investigate whether the abrogation of p53 induction in HCT-116 (+/+)
cells treated with 5-aza-CR affects the level of expression of p21WAF1, Bcl2, Bax, and Gadd45. Our results indicate that the induction of Gadd45 by 5-aza-CR treatment has a p53-dependent component because its expression was reduced by PFT-α (Fig. 5A). For illustration, approximately a 5-fold induction of Gadd45 protein seen in 5-aza-CR-treated HCT-116 cells at 72 h disappeared when cells were exposed to the p53 inhibitor (Fig. 5A). A similar reduction in the magnitude of 5-aza-CR-induced Gadd45 mRNA expression was observed in HCT-116 cells treated with the inhibitor (Fig. 5D). On the other hand, PFT-α treatment did not significantly affect 5-aza-CR-induced p21WAF1 transcript levels (Fig. 5B). Interestingly,

Fig. 4. Reversal of the growth inhibitory (A), cell cycle (B), and apoptotic (C) effects of 5-aza-CR by the p53 inhibitor PFT-α. HCT-116 cells were pretreated with 30 μM PFT-α for 5 h before treatment with 1 μM 5-aza-CR for 72 h. A, cell growth determined by the MTT assay. B, DNA content determined by flow cytometric analysis following PI staining. Numerical data corresponding to cell cycle analysis are presented in boxes. C, DNA fragmentation determined by TUNEL assay in fixed and labeled cytospin preparations using fluorescence microscopy.

TABLE 2
Profile of dysregulated genes after treatment of HCT-116 cells with 5-aza-CR

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*p* Treatment with 1 μM 5-aza-CR after 48 h.
PFT-H9251 reversed the 5-aza-CR-elicited decrease of Bcl2 protein expression (Fig. 5A) but not that of Bcl2 mRNA (Fig. 5C). In conclusion, this inhibitor experiment signified that 5-aza-CR-induced accumulation of Gadd45 is mediated by p53.

Reversal of 5-aza-CR-Induced Hypomethylation by the p53-Selective Inhibitor PFT-H9251. To investigate the role of p53 activation in 5-aza-CR-induced hypomethylation, we determined the effect of PFT-H9251 on the DNA methylation status and on the expression of methyltransferases in drug-treated cells and studied the effect of 5-aza-CR on methyltransferase activity in HCT-116 cells lacking the p53 gene (Figs. 6 and 7).

DNA isolated from HCT-116 cells treated with the p53 inhibitor plus 5-aza-CR showed a greater degree of global genomic methylation compared with cells treated with the drug alone, suggesting that PFT-H9251 prevented global DNA hypomethylation elicited by 5-aza-CR treatment (Fig. 6A). After cell recovery from 5-aza-CR exposure, the methylation-specific band generated by methylation-specific PCR appeared again (Fig. 6B). We then determined the effect of PFT-α on the expression of the human DNA methyltransferases DNMT1, DNMT3a, and DNMT3b in drug-treated HCT-116 cells. The inhibition of p53 activation restored the expression of DNMT3a protein (Fig. 6C) and the transcript levels of DNMT1 and DNMT3a (Fig. 6, D and E), suggesting that DNA demethylation by 5-aza-CR is related to p53 activity. DNMT1 mRNA expression was also down-regulated in 5-aza-CR-treated HCT-116 p53 (-/-) cells, in contrast to the DNMT3a mRNA expression level, which was not reduced. PFT-α treatment as well as the p53 knockout status of HCT-116 cells had no effect on DNMT3b mRNA expression (Figs. 6F and 7C). Although DNMT3a protein expression was greatly reduced in 5-aza-CR-treated HCT-116 (p53 +/+ ) cells, this protein did not completely disappear after 48 and 72 h of 5-aza-CR treatment in p53 -/- HCT-116 cells (Fig. 7D). This indicates a lack of p53 involvement and/or dissociation of DNMT3b response from DNMT1 and DNMT3a.

Discussion

In this study, we sought to elucidate the mechanism of 5-aza-cytidine-induced apoptosis, and, in addition, the possible link between demethylation and p53 induction in the p53 wild-type colorectal carcinoma cell line HCT-116. We observed that 5-aza-CR treatment arrests the cell cycle at G2/M phase and induces apoptosis most likely by a joined activation of p53 protein and its downstream effectors GADD45 and p21WAF1. This study investigates for the first time the expression of all three DNMTs in response to 5-aza-CR and...
its dependence on p53. We report further that 5-aza-CR caused a marked down-regulation of DNMT1 and DNMT3a mRNA levels, in contrast to a null effect on DNMT3b. The down-regulation of DNMT1 resulted in global demethylation, which was confirmed by methylation-specific PCR showing reactivation of a silenced hemimethylated p16INK4a gene in cells. P53 dependence was confirmed by the finding that the DNA demethylation status was reversible upon treatment with the p53 inhibitor. Thus, p53 protein rendered HCT-116 cells less sensitive to 5-aza-CR-induced cell cycle- and apoptosis-related effects and restored the expression of GADD45, p21WAF1, Bcl2, DNMT1, and DNMT3a proteins to normal levels in the control. Furthermore, the absence of p53 diminished the response of HCT-116 cells to the apoptotic and cell cycle modulatory effects of 5-aza-CR. P53 −/− cells did not differ between p53 +/+ and p53 −/− cells in methylation-dependent and -independent mechanisms involved in 5-aza-CR action on HCT-116 cells.

For many years, 5-aza-CR has been used as a potent anticancer agent for the treatment of several hematopoietic neoplasms (Wijermans et al., 2000; Santini et al., 2001). However, to date, the mechanisms of cellular responses of this drug are not well understood. Treatment with 5-aza-CR causes a variety of changes in cells, including decondensation of chromatin (Haaf and Schmidt, 1989), the activation of silenced genes and global genomic hypomethylation (Christman, 2002), and alterations in DNA replication timing (Jablonska et al., 1985), all of which are believed to be consequences of drug-induced demethylation. 5-aza-CR-induced cytotoxicity may be also related to enzyme adduct formation (Juttermann et al., 1994) whereby the incorporation of 5-aza-CR into DNA leads to the irreversible binding of DNMT1 to incorporated 5-aza-CR residues and the rapid loss of DNMT1 activity (Christman, 2002). To date, no studies have been conducted to investigate 5-aza-CR effects on the activity of DNMT3a and DNMT3b. These recently identified de novo methyltransferases are predicted to have the same response to this inhibitor as DNMT1, yet this has not been proven. There is reason to suspect that DNMT3a and DNMT3b respond much more to the inhibitory effects of 5-aza-CR residues incorporated into DNA since they are randomly incorporated in place of cytidine, and, unlike DNMT1, DNMT3a and DNMT3b are capable of methylating cytidine residues that are not in CpG islands (Aoki et al., 2001). The p53 dependency of the DNMT3a expression, but not that of DNMT3b, is surprising when considering the fact that both enzymes are de novo methyltransferases. It is noteworthy to mention that mice embryos lacking both copies of DNMT3b

Fig. 6. Reversal of 5-aza-CR-induced hypomethylation by the p53-selective inhibitor PFT-a. A, effect of PFT-a on global DNA methylation status in 5-aza-CR-treated HCT-116 (+/+). The extent of global DNA methylation is inversely proportional to the incorporation of methyl groups by SssI methylase in the presence of SAM. Values are means ± S.E.M., n = 3. ANOVA, 5-aza-CR, 96 h, p < 0.05; Aza + PFT, 96 h, p < 10−2. 5-aza-CR was performed using the unmethylated (u) and methylated (m) primer sets. C, effect of PFT-a on protein expression levels of the DNA methyltransferase DNMT3a and the transcript levels of DNMT1 (D), DNMT3a (E), and DNMT3b (F) in 5-aza-CR-treated HCT-116 cells.
die before birth, whereas DNMT3a-null mice survive for about 4 weeks (Okano et al., 1999). DNMT3b targets especially centromeric satellite repeats and is linked to lymphocyte-mediated immunological defects (Hansen et al., 1999). In addition, DNMT3a and DNMT3b have overlapping functions in global methylation during early embryogenesis (Reik et al., 1999). However, the enzymes may have distinct cell- or tissue-specific functions during later embryogenesis or tumorigenesis (Bestor, 2000). In this respect, more studies are needed to prove if the observed effect on DNMT3a expression is specific for colon cancer cell lines. The mechanism by which p53 regulates DNMT3a expression is still unknown.

p53 has been shown to play a critical role in growth arrest and apoptosis in response to DNA damage by chemotherapeutic agents (Lakin and Jackson, 1999). The p53 status has a significant impact on the drug sensitivity to 5-aza-CR (Karpf et al., 2001, 2004). The role of p53 in the apoptotic efficacy of 5-aza-CR is controversial. In some systems (HCT-116), the presence of wild-type p53 is essential for apoptosis induction, whereas in other systems (mouse embryonic fibroblasts), the absence of p53 determines a higher chemotherapeutic sensitivity for 5-aza-CR (Karpf et al., 2001; Nieto et al., 2004). Wild-type p53 colon cancer cells were more sensitive to 5-aza-CR-induced growth arrest and cytotoxicity, whereas cells bearing mutant p53 protein were not affected by 5-aza-CR (Karpf et al., 2001; Nieto et al., 2004). In agreement with the latter studies, we show an induction of p53 protein and a significant decrease in the antiapoptotic Bcl2 protein in response to 5-aza-CR. p53 protein expression increased about 4-fold, whereas Bcl2 protein expression was almost undetectable after 72 h, an incubation period when apoptosis was extensive. This supports other reports demonstrating that overexpression of Bcl2 protein inhibits p53-mediated apoptosis and p53-mediated transcriptional activation (Shen and White, 2001). After drug recovery, p53 protein expression declined to control levels. Upon treatment with PFTα or in p53−/− cells, apoptosis induction was markedly reduced, indicating that p53 is a major modulator of 5-aza-CR-induced apoptosis. Our findings are in agreement with the hypothesis that 5AzA-CR induces DNA damage post-translationally by stabilizing the p53 protein (Lakin and Jackson, 1999). Normally, p53 protein has a relatively short half-life, being rapidly targeted for ubiquitination and degradation. Following cellular stress, the p53 protein is phosphorylated, increasing its half-life and transactivation activity (Meek, 1994). In our study, the increase in p53 protein expression does not appear to be transcriptionally induced as measured by real-time RT-PCR. This finding is in accordance with that reported by Karpf et al. (2001) using northern blotting analysis. Thus, we cannot suggest a possible demethylation of the p53 promoter by 5-aza-CR.

To further understand the mechanism of 5-aza-CR-mediated p53 activation, we investigated the DNA damage-inducible gene, GADD45 (Wang et al., 1999). It is known that p53 is a transcriptional activator of GADD45 (Wang et al., 1999). Nevertheless, the mechanisms for p53 dependence of the GADD45-induced cell cycle arrest are not clear at the present time (Jin et al., 2002). Earlier studies have shown that the microinjection of a GADD45 expression vector into normal fibroblasts resulted in arrest of G2/M, which could be attenuated by cyclin B1 overexpression (Wang et al., 1999). In our study, a dose-dependent accumulation of cells in G2/M
after 5-aza-CR treatment was indeed confirmed by down-regulation of cyclin B1 expression. We also demonstrate that 5-aza-CR treatment remarkably up-regulated GADD45 expression by an intracellular pathway via p53 induction. Recovering from 5-aza-CR treatment as well as PFT-α treatment restored the GADD45 protein levels to untreated controls and inhibited GADD45 mRNA expression. However, the induction in the transcript level of GADD45 upon 5-aza-CR treatment did not change upon recovery. This discrepancy suggests that both transcriptional and post-transcriptional mechanisms contribute to the GADD45 expression after 5-aza-CR treatment. p53-dependent and -independent mechanisms have been reported to play a role in the regulation of GADD45 expression (Lakin and Jackson, 1999). In addition, the pathway down stream from GADD45 observed in 5-aza-CR-treated cells remains to be elucidated.

The up-regulation of p21WAF1 is believed to be a main mediator of p53-dependent G1 arrest, causes cells to accumulate in both G1 and G2 after DNA damage, and is associated with a reduction of cyclin B1 expression (Medema et al., 1998). Inhibition of DNMT1 can induce p21WAF1 protein levels (Bestor TH (2000) The DNA methyltransferases of mammals. Hum Mol Genet 9:2395–2402).

References


In conclusion, this study demonstrates that 5-aza-CR inhibits DNMT1 and DNMT3 activity in HCT-116 colorectal cancer cells through a cell cycle regulatory protein which interacts with p21Cip1. Oncogene 11:1675–1683.


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31 August 2016

RETRACTED
Correction to “5-aza-Cytidine Is a Potent Inhibitor of DNA Methyltransferase 3a and Induces Apoptosis in HCT-116 Colon Cancer Cells via Gadd45- and p53-Dependent Mechanisms”

In the above article [Schneider-Stock R, Diab-Assef M, Rohrbeck A, Foltzer-Jourdainne C, Boltze C, Hartig R, Schonfeld P, Roessner A, and Gali-Muhtasib H (2005) J Pharmacol Exp Ther 312:525–536], similar groups of cells appear at different time points in Figures 2A and 3A that have raised suspicion regarding the original preparation of these figures. Given the length of time since original publication, the exact reason for this discrepancy could not be determined. These experiments have been repeated and the new data clearly support the main message of the manuscript on the pro-apoptotic effects of 5-aza-cytidine (5-aza-CR). Corrected figure panels are reprinted below with new legends. A Methods for the repeated experiments is also provided.

The authors regret this error and any inconvenience it may have caused.

**Fig. 2A.** Induction of growth arrest by 5-aza-CR (Aza) in HCT-116 (+/+) human colon cancer cells. Cell were seeded and treated with 1 μM 5-aza-CR for 48 and 72 hours. Representative bright field images were taken of two separate experiments. Ctrl, control.

**Fig. 3A.** The apoptotic effects of 5-aza-CR (Aza) in HCT-116 (+/+) cells. Cells treated for 48, 72, and 96 hours with 1 μM 5-aza-CR and the extent of DNA fragmentation determined by TUNEL assay (green) in fixed cells using fluorescence microscopy. Bar, 20μm; Ctrl, control.
Methods

Cell Growth and Treatment. The human colon cancer HCT-116 cells were cultured in RPMI 1640 medium. Cells were grown at 37°C in an atmosphere of 5% CO2 supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% fetal calf serum. For experiments, cells were seeded on six-well plates at a density of 40,000 cells per well. Cells were treated with 1 μM of freshly prepared 5-aza-CR (Sigma-Aldrich, St. Louis, MO) 24 hours after plating, and treatment was replenished every 48 hours. Randomly selected bright field microscopic fields were acquired on an inverted microscope Leica DMi1 (Wetzlar, Germany) using 4× and 10× air objective lenses.

Apoptosis: Terminal Deoxyribonucleotidyl Transferase–Mediated dUTP Nick-End Labeling Assay. Apoptosis was scored by estimating the extent of DNA fragmentation using the terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay. Briefly, cells were plated on autoclaved glass coverslips in six-well culture plates and treated with 1 μM 5-aza-CR. The medium was then aspirated and cells were washed twice with warm phosphate-buffered saline. Cellular DNA was stained with the In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany), and the assay was performed according to the recommendations of the manufacturer. For each of three replicated experiments four randomly selected microscopic fields were acquired on an inverted microscope Nikon eclipse Ti-U (Tokyo, Japan) using a 20× air objective lens (Nikon). The same microscopy setup was used for all representative images. Bar, 20μm.