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 promote tumor cell death, but their detailed cytotoxic action is not yet fully understood. A deeper knowledge about their apoptosis-inducing mechanisms and their interaction with DNA methyltransferases 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-dependency 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, GADD45) or apoptosis (p53, RIPK2, Bak1, caspase 5, and caspase 6). In parallel, there was a down-regulation of anti-apoptotic Bcl2 protein and the G2/M-mediator cyclin B1. Coincubation with pifithrin-alpha (PFT-α), a selective p53 inhibitor, restored GADD45, Bcl2, cyclin B1, and p21WAF1 expression levels and almost completely reversed the growth inhibitory, cell cycle, and apoptotic effects of 5-aza-CR. 5-aza-CR treatment caused global demethylation and reactivation of p16INK4 expression. There was a marked decrease in DNMT1 and DNMT3a mRNA expression, with PFT-α reversing these effects. However, 5-aza-CR treatment did not modulate DNMT3b expression. Our data demonstrate that 5-aza-CR action in HCT-116 is mediated by p53 and its down-stream effectors p21WAF1 and GADD45. This is the first report to show a link between p53 and regulation of DNMT1 and de novo methyltransferase DNMT3a.
INTRODUCTION

Since the causal relationship between hypermethylation of the promotor of tumor suppressor genes and the development of cancer has been clearly demonstrated, specific demethylating agents are of interest for use in molecular targeted therapeutics (Zhu and Otterson, 2003). Methylation of cytosines within CpG dinucleotides is associated with transcriptional silencing during mammalian development and tumorigenesis (Bird, 1996). The main enzyme responsible for replicating the DNA methylation pattern is the DNA methyltransferase 1 (DNMT1). In contrast, DNMT3a and DMMT3b are responsible for de novo methylation, in which a methyl-group is transferred to the carbon position of the cytosine from the methyl donor S-adenosyl-L-methionine (Okano et al., 1999).

To date, 5-aza-cytidine (5-aza-CR) and its deoxyribose analog 5-aza-2′-deoxycytidine (5-azaCdR) 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 DNA methyltransferase I (DNMT1) thereby depleting the cells from enzyme activity and causing demethylation of genomic DNA as a secondary consequence (Christman et al., 2002). In various in vitro experiments, 5-aza-CR treatment leads to reexpression of former silenced genes (Christman et al., 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 (Christman et al., 2002). In addition, it has been demonstrated that 5-aza-CR inhibits telomerase activity via transcriptional repression of hTERT in prostate cancer cell lines (Kitagawa et al.
Recent studies have demonstrated that DNMT1-5-AzaC 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. In order 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 (PFT-α). We found a possible link between p53 and the regulation of methyltransferases after 5-aza-CR treatment.

MATERIAL and METHODS

Reagents and Drugs

Prolong Antifade and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR). RNase, and trypan blue were obtained from Sigma Chemical Co. (St Louis, MO). RPMI 1640, Fetal Bovine Serum (FBS) and penicillin-streptomycin were obtained from GIBCO-BRL (Gaithersburg, MD).

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 (MSP). Bisulfite modification was performed using the CpGenome modification kit (QBiogene, Heidelberg, Germany). Two µl of bisulfite-modified DNA was amplified by PCR, using primers that were specific for methylated
or unmethylated sequences of p16\textsuperscript{INK4a} gene as described previously (Schneider-Stock et al., 2003). PCR-amplified products were separated by electrophoresis on 8% polyacrylamide gels and visualized by silver-staining.

**Global genomic methylation status**

The methylation status of CpG sites in genomic DNA was determined by the *in vitro* methyl acceptance capacity of DNA by using S-adenosyl-L-[methyl-3H]methionine ([3H]SAM, Amersham Pharmacia Biotech) as a methyl donor and a prokaryotic CpG DNA methyltransferase (SssI methylase, New England Biolabs). The manner in which this assay is performed produces a reciprocal relationship between the endogeneous DNA methylation status and the exogeneous methyl-3H methyl incorporation. Briefly, 2\mu g of genomic DNA was digested to completion overnight with EcoRI (10u/\mu g of DNA) according to the recommendations of the manufacturer (AGS, Heidelberg, Germany). Digested DNA (500ng) was incubated, in triplicate, in 25\mu l containing 2u SssI methylase, 2\mu M [3H]SAM and 2\mu M non-radioactive S-adenosylmethionine (SAM, New England Biolabs) in the buffer supplied by the manufacturer. The reaction mixture was incubated at 37\degree C for 2h. The reaction was stopped by heating at 65\degree C for 10min. Reaction mixtures containing no enzyme were used as background controls for each DNA sample. The incubation mixtures were applied onto disks of Whatman DE-81 ion exchange filters (Fisher Scientific, Springfield, NJ) by using a vacuum filtration apparatus; the disks were then washed with 0.35M Na\textsubscript{2}HPO\textsubscript{4} for 45min. The disks were dried at 95\degree C for 30min, and the resulting radioactivity of the DNA retained on the disks was measured by scintillation counting with Tri-Carb Liquid Scintillation Analyzer 2100 TR (Parkard, USA).
Cell growth and treatment

The human colon cancer cells HCT-116 (+/+ and -/- p53) were cultured in RPMI 1640 medium. Cells were grown at 37°C in an atmosphere of 5% CO_2_ supplemented with penicillin (100U/ml), streptomycin (100mg/ml) and 10% fetal calf serum. For experiments, cells seeded on 6-well plates at a density of 4x10^4 cells per well, were left to grow. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Calbiochem, San Diego, CA) reduction by cells was used to assess drug-induced cell growth inhibition and cytotoxicity. The proliferation assay used was a MTT-based method to measure the ability of metabolically active cells converting tetrazolium salt into a cleavage product. The absorbance was recorded at 405nm. Cell proliferation and cytotoxicity were determined using the Cell Titer96™ Non-radioactive cell proliferation assay (Sigma, Taufkirchen, Germany) according to the manufacturer’s suggestions.

Treatment

Cells were treated with 1 or 5µM freshly prepared 5-aza-CR (Sigma, St. Louis, MO) 24h after plating and treatment was replenished every 48h. Assays were conducted and/or extracts were harvested 1, 2, 3, and 4 days after treatment. Treatment of cells with the selective p53 inhibitor, PFT-α, was performed by pre-treating the cells with the inhibitor 5h prior to addition of 5-aza-CR.

To determine whether cells recover from 5-aza-CR treatment, cells were replenished with fresh medium without drug at 96hr post-treatment and were allowed to recover for further 48h (total 144h).
Flow cytometric analysis of DNA content

Cells were seeded in 100mm dishes at a density of $7.5 \times 10^5$ cells per well. They were incubated and allowed to grow to 40-50% confluence after which they were treated with 1µM 5-aza-CR and incubated for further 72h. They were then harvested by trypsin release, washed twice with phosphate buffered saline (PBS), permeabilized with 70% ethanol, treated with 1% RNase and finally stained with propidium iodide solution (100µg/ml final concentration). Distribution of cell cycle phases with different DNA contents was determined using a flow cytometer LSR (Becton-Dickinson, 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 cell 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 ModfitLT (Verity Software House) software.

Apoptosis: TUNEL and 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 terminal deoxy-transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. Briefly, cells were plated on autoclaved glass coverslips in 6-well culture plates and treated with 1µM 5-aza-CR or PFT-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 performed according to the recommendations of the manufacturer (Boehringer Mannheim, Germany). Cytospin preparations were fixed and labeled and four independent x 100 fields containing a
minimum of 300 cells on each of three replicate slides were evaluated for nuclear labeling by fluorescence microscopy (Axiovert 200, Zeiss, Intermedic, Germany) for each treatment. Nuclear chromatin condensation was observed by fluorescence microscopy (LSM 410, Zeiss, Germany).

Annexin-V-FITC binding assay

After exposure to 10µM 5-aza-CR for 6h, 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 (Pharmingen, San Diego, CA). The slides were viewed immediately on a fluorescence microscope (Leica DMRE7 (Leica, Germany) equipped with 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 400x magnification and the annexin V-FITC cells (green fluorescence) were counted.

Real time RT-PCR for detection of mRNA expression

For mRNA expression studies, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA). cDNA synthesis was done in a 20µl reaction mix starting with 1µg of total RNA using the reverse transcription system of Promega (Madison, Wis.; 42°C for 30min, 99°C for 5min, and 4°C for 5min). Real-time PCR was performed using a LightCycler (Roche Diagnostics, Mannheim, Germany) 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 amplicon detection, the LightCycler DNA Master Hybridization Probes Kit
was used as described by the manufacturer (Roche Diagnostics, Mannheim, Germany). Briefly, besides the two primers, two different oligonucleotides hybridize each to internal sequence of the experimental genes or the β2-M gene. PCR assays were carried out in 20 µl reaction volume containing 1 µl cDNAs, 0.5 µM of each primer, 2 pmol LCRed 640 probe, 4 pmol Fluorescein hybridization probe and 2.5 mM MgCl₂. Annealing temperatures were given in Table 1. All genes examined were normalized to a house-keeping gene encoding β2-microglobulin (β2-M).

Fold induction was calculated according to the formula (Saha et al., 2001) \( 2^{(R_t-E_t)/2(R_n-E_n)} \) where \( R_t \) is the threshold cycle number for the β2-M gene observed in the cell line after 5-aza-CR treatment, \( E_t \) is the threshold cycle number for the experimental gene observed in the cell line after 5-aza-CR-treatment, \( R_n \) is the threshold cycle number for the β2-M gene observed in the cell line before 5-aza-CR-treatment, and \( E_n \) 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: www.TIBMOLBIOL.de/oligoag.html) to ensure their total gene specificity (Table 1). Primer sequences for p53, Bcl2, p21\(^{WA1}\), and β2-M have been previously described (Gali-Muhtasib et al., 2004). Primers for DNMT1, DNMT3a, DNMT3b, GADD45, and p16\(^{INK4a}\) were summarized in Table 1.

**Protein Extraction and quantification**

Cell samples were homogenized in a buffer at pH 6.0 containing 50 mM sodium phosphate, 0.2 M NaCl, 5 mM EDTA, 100 μM E-64, and 1 mM PMSF by sonication (Bandelin, Berlin, Germany). Homogenates were centrifuged for 10 min at 4°C at 10000 rpm (Eppendorf, Hamburg, Germany). The supernatants were stored in 1/3 aliquots at -20°C or immediately reduced by adding 5x Laemmli buffer containing
20% DTT and boiled. Protein measurements were carried out in all samples according to the BioRad DC Protein Assay (BioRad, Hercules, Ca), and equal protein amounts were subjected to SDS-PAGE followed by immunoblotting.

**Western Blotting**

After transferring to a PVDF membrane (NEN Life Sciences Products, Boston, MA) and blocking the non-specific binding with 5% milk, it was incubated with the specific antibodies followed by incubation with the secondary peroxidase conjugated goat anti-rabbit antibody (1:30000) for 30min. We used the following human reactive monoclonal or polyclonal antibodies: anti-p21\(^{WAF1}\) (clone EA10, Oncogene, Boston, MA), anti-p53 (clone DO-1, Oncogene, Boston, MA), anti-Bcl2 (DAKO, Denmark), anti-Gadd45 (clone SC-6850, Santa Cruz Biotech), anti-cyclin B1 (clone 7A9, Novocastra Lab., New Castle, UK), anti-DNMT1 (clone 60 B1220, Biocarta, Hamburg, Germany), DNMT3a (clone 64B 1446, Biocarta, Hamburg, Germany), and DNMT3b (clone 52A 1018, Biocarta, Hamburg, Germany). \(\beta\)-actin antibody (clone HHF35) was purchased from Novocastra Lab., New Castle, UK). The substrate, West Pico Supersignal (Pierce, USA), was left on the membrane until distinct bands had developed (max. 30min). The ECL membrane images were evaluated for the quantification of the protein expression, using the GeneGnome and GeneTools image scanning and analysis package (Syngene BioImaging Systems, Synoptics Ltd.). Hybridization with the house-keeping protein \(\beta\)-actin was used as a control of equal loading and protein quality.

**cDNA array analysis**

To identify apoptosis-associated genes that are dysregulated by 5-aza-CR we used GEArray Q series Human Apoptosis Gene Array 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 3.8x4.8cm nylon membrane. Total RNA was prepared by TRIzol Reagent (Invitrogen/Life Technologies). An aliquot of 4µg of total RNA was reversely transcribed (Promega, Madison, WI, USA) and the probe was simultaneously labeled using the GEA labeling buffer mix (Biotin-16-dUTP, Roche Diagnostics) according to the instructions of the manufacturer. The membrane was prehybridized with sheared salmon sperm DNA in GEAhyb Hybridization Solution followed by hybridization at 68°C overnight with continuous agitation. The membrane was washed at 68°C twice with 2xSSC, 1%SDS and 0.1xSSC, 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 substracting 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 downregulation of DNMT1 and DNMT3a gene transcription in HCT-116 cells

We determined the time-course effects of 5-aza-cytidine (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 in the presence of \(^3\)H-SAM and SssI methylase is 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\(\mu\)M of 5-aza-CR was significantly hypomethylated following 72h (p<0.05) and 96h (p<0.01) of treatment compared with cells not treated with 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 \(p16^{\text{INK4a}}\) promoter is observed in HCT-116 cells (presence of both, methylated and unmethylated band in the untreated control), we assessed the effect of 5-aza-CR on the methylation status of the \(p16^{\text{INK4a}}\) gene promoter. Following treatment with 5-aza-CR (1\(\mu\)M) for 3 days, the methylated band almost completely disappeared after 96h (Fig. 1B). Corresponding to the disappearance of the methylation-specific band was the time-dependent reexpression of \(p16^{\text{INK4a}}\) mRNA in HCT-116 cells (Fig. 1C); up-regulation of \(p16^{\text{INK4a}}\) transcript levels occurred even when cells were allowed to recover from 5-aza-CR treatment.

We then determined the effect of 5-aza-CR on the expression of human DNA methyltransferases (DNMTs: DNMT1, DNMT3a and DNMT3b). Real-time PCR revealed a 3-fold downregulation in DNMT1 mRNA expression levels following treatment with 5-aza-CR for 4 days (Fig. 1D). A significant downregulation in DNMT3a expression levels occurred as early as 24h post-treatment, peaked at 48h, and persisted even after 4-day 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 96h using the MTT assay. The addition of 5-aza-CR resulted in the appearance of many damaged cells at 72h after treatment and to a lesser extent at 48h (Fig. 2A). A time-dependent inhibition of cell viability was observed upon treatment with 5-aza-CR; 1µM decreased cell viability by 65% at 72h post-treatment (Fig. 2A). At 96h, 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 anti-proliferative 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 72h and harvested for flow cytometric analysis of DNA content by PI staining (Fig 2B). Cell cycle distribution analysis showed an increase, within 48h, in the number of cells in the G2 phase of the cell cycle following treatment with 1µM of 5-aza-CR, providing evidence of G2/M arrest. By 72h, 50% of the cells arrested at G2 phase and the percentage of cells in S phase decreased by more than 50 % (Fig. 2B) as compared to the untreated control. Furthermore, the addition of 5-aza-CR for 72h 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 utilized the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and Annexin-V flow cytometric assay methods (Fig. 3). Numerous TUNEL-positive cells with apoptotic characteristics, based on the rounded, shrunken shape of the nucleus and on intense staining of FITC-conjugated dUTP, appeared in the 5-aza-CR-treated cultures (Fig. 3A - left panel). A time-dependent increase of up to 65% in apoptotic cells occurred in 5-aza-CR-treated cell cultures (Fig. 3A - right panel). Drug exposure also caused a strong increase of Annexin-V staining, typical feature of early apoptosis (Fig. 3B - left panel). The proportion of apoptotic cells at 72h post-treatment was significantly higher than that of necrotic cells (Fig. 3B – right panel), indicating that apoptosis rather than necrosis is the mechanism of 5-aza-CR-induced cell death in HCT-116 (+/+) cells. These findings collectively confirm that 5-aza-CR–induced increases in the sub-G_0-G_1 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 protein expression in control untreated cells and is not toxic to the cells was found to be 30µM. Seventy-two hours following addition of 5-aza-CR (1µM), 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 PFT-α (Fig. 4A). The fraction of cells in the G_1 phase increased from 17% in 5-aza-CR -treated cells to 58% in cells treated
with combination of 5-aza-CR and PFT-α, while the fraction of cells in the S phase increased from 7% to 38% (Figs. 2B and 4B). The cell cycle kinetics of exponentially growing cells was not affected by the addition of PFT-α alone (Fig. 4B). Furthermore, the number of apoptotic cells decreased significantly to reach levels of the untreated control when cultures were treated with combination of 5-aza-CR and PFT-α (Fig. 4C). In agreement with these findings, the fraction of cells in G₁ phase decreased to 42% 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.

Several apoptotic genes are induced in cells after treatment with 5-aza-CR

We used GEArray Q series cDNA microarray to analyze expression changes of 96 key apoptotic genes in HCT-116 cells after treatment with 5-aza-CR. Cells were treated with 1µM of 5-aza-CR for 48h and their mRNA was harvested for microarray analyses. A total of 5 genes (5.2%) was differentially expressed (>5-fold induction) after 5-aza-CR treatment. The upregulated genes include RIPK2 (10-fold), Gadd45 (17-fold), BAK1 (16-fold), caspase 6 (20-fold) and caspase 5 (25-fold) (Table 2). These genes are associated with the regulation of either apoptosis (caspases, RIPK2 and BAK) or DNA damage (Gadd45) (Table 2). Real time RT-PCR and Western blot analyses confirmed the induction of Gadd45 by 5-aza-CR treatment (Fig. 5A and D).

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
2-5-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. 5A and B). At 72h 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 (48h: 1.8fold, 72h: 2.4fold) (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,
p21WAF1, 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
expression, 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 and D). For illustration, approximately a 5-fold induction of Gadd45
protein seen in 5-aza-CR-treated HCT-116 cells at 72h 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, PFT-α 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-α**

To investigate the role of p53 activation in 5-aza-CR-induced hypomethylation, we determined the effect of PFT-α on the DNA methylation status and on the expression of methyltransferases in drug-treated cells and studied the effect of 5-aza-CR on methyltransferases in HCT-116 cells lacking the p53 gene (Fig. 6 and 7). DNA isolated from HCT-116 (+/+) cells treated with the p53 inhibitor plus 5-aza-CR showed greater degree of global genomic methylation compared with cells treated with the drug alone, suggesting that PFT-α 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. 6D 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 knock out status of HCT-116 cells had no effect on
DNMT3b mRNA expression (Fig. 6F, Fig. 7C). Although DNMT3a protein expression was greatly reduced in 5-aza-CR-treated HCT-116 (p53 +/-) cells, this protein did not completely disappear after 48h and 72h of 5-aza-CR treatment in p53 -/- HCT-116 cells (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–wildtype 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 down-stream 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 dependency on p53. We report further that 5-aza-CR caused a marked downregulation 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 MSP showing reactivation of silenced hemimethylated p16INK4a gene in cells. P53 dependency was confirmed by the finding that the DNA demethylation status was reversible upon treatment with the p53 inhibitor. The inhibition of 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 untreated control. Furthermore, the absence of p53 gene diminished the response of HCT-116 cells to the apoptotic and cell cycle modulatory effects of 5-aza-CR. P53 -/- cells showed no DNMT3a down-regulation (mRNA and protein level) after 5-aza-CR-treatment, DNMT1 and DNMT3b levels did not differ between p53 +/- and p53 -/-
cells. Thus, methylation-dependent and independent mechanisms are involved in 5-aza-CR action on HCT-116 cells.

For many years, 5-aza-CR has been utilized as a potent anticancer agent for the treatment of several hematopoietic neoplasms (Santini et al., 2001; Wijermans et al., 2000). 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 et al., 2002) and alterations in DNA replication timing (Jablonka 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 (Jutterman 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 die before birth, while 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, 1999). In addition, DNMT3a and DNMT3b, have overlapping functions in global remethylation during early embryogenesis (Reik et al., 1999). But the enzymes may have distinct cell- or tissue specific functions during later embryogenesis or tumorigenesis (Bestor, 2002). 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 cancer cell 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 wildtype p53 is essential for apoptosis induction while in other systems (mouse embryonic fibroblasts) the absence of p53 determines a higher chemotherapeutic index 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-mediated growth arrest and cytotoxicity, while 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 anti-apoptotic Bcl2 protein in response to 5-aza-CR. p53 protein expression increased about 4-fold, while Bcl2 protein expression was almost undetectable after 72h, an incubation period when apoptosis was extensive. This supports other reports demonstrating that the 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
PFT-α 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 et al., 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 promotor 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 G2/M 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 G2/M arrest, 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 downregulation of cyclin B1 expression. We also demonstrate that 5-aza-CR treatment remarkably upregulated 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 upregulation of p21WAF1 is believed to be a major 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, 1998; Jin et al., 2002). We determined whether p21WAF1 is required for GADD45-mediated G2/M arrest, since p21WAF1 binding to GADD45 in vivo has been previously documented (Chen et al., 1995). We show that initiation of DNA damage by 5-aza-CR is accompanied by a time-dependent induction of p21WAF1 protein. Furthermore, p21WAF1 and cyclin B1 protein expression were inversely correlated upon drug treatment. Abrogation of p53 after PFT-α reduced p21WAF1 protein expression to untreated control levels, but did not alter the mRNA expression level. Thus, p21WAF1 induction after 5-aza-CR has a p53-dependent component and can be modulated on the transcriptional level. Such uncoupling of p21WAF1 mRNA and protein expression was found to occur in several cell lines upon exposure to genotoxic stress (Butz et al., 1998). The induction of p21WAF1 protein has been shown after treatment of tumor cells with antisense oligonucleotides directed against DNMT1 (Fournel et al., 1999) or oligonucleotide-based substrate inhibitors of DNMT1 (Milutinovic et al., 2000). Inhibition of DNMT1 can induce p21WAF1 protein levels by a post-translational mechanism as recently suggested by Fournel et al. (1999). Recent studies demonstrated the ability of p21WAF1 to interact with GADD45 (Kearsey et al.,
1995) as well as DNMT1 competing for association with proliferating cell nuclear antigen (PCNA) (Chuang et al., 1997). Further experiments are required to clarify this association.

In conclusion, this study demonstrates that 5-aza-CR inhibits DNMT1 and DNMT3a activity in HCT-116 colorectal cancer cells, and causes CpG demethylation and reactivation of methylation-silenced genes. To our knowledge, this is the first demonstration of p53-dependent 5-aza-CR action on de novo methyltransferase DNMT3a. The effects on apoptosis and cell cycle arrest, as well the inhibitory effects on cell growth, initiated by 5-aza-CR in HCT-116 cells, are caused by methylation-dependent and independent pathways.

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REFERENCES


FIGURE LEGENDS

Figure 1. (A) Time course analysis of the global DNA methylation status in HCT-116 (p53 +/+) cells cultured in the presence of 0 or 1µM 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 S-adenosyl-L-[methyl-3H]methionine ([3H]SAM). Values are means ± SEM, n = 3. ANOVA: Aza-72h, P < 0.001; Aza-96h, P < 0.006. (B) Effect of 5-aza-CR on p16INK4a gene methylation. Methylation-specific PCR was performed using the u (unmethylated) and m (methylated) primer sets. (C) Time course analysis of mRNA reexpression of p16INK4a. (D-F) Expression of the DNA methyltransferases DNMT1, DNMT3a and DNMT3b in HCT-116 cells cultured in the presence of 1µM 5-aza-CR.

Figure 2. Induction of growth/cell cycle arrest by 5-aza-CR in HCT-116 (+/+) human colon cancer cells. (A) Cell growth was 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 1µM of 5-aza-CR. Cells in active growth were treated with 5-aza-CR for 48 or 72hrs, then fixed and the DNA content determined by flow cytometric analysis by PI staining, analyzing 20,000 events per sample. The percentage of cells in PreG1, G0/G1, S and G2/M are shown. The data shown are typical of one of three independent experiments.

Figure 3. The apoptotic effects of 5-aza-CR in HCT-116 (+/+) cells (A) Cells were treated with 5-aza-CR for 72h and the extent of DNA fragmentation was determined by TUNEL assay in fixed and labeled cytospin preparations using fluorescence
microscopy. (B) The percentage of apoptotic cells was also scored by the Annexin-V flow cytometric assay method which can detect cells in an earlier stage of the apoptotic pathway and distinguish among apoptotic and necrotic cells.

**Figure 4.** Reversal of the (A) growth inhibitory (B) cell cycle and (C) apoptotic effects of 5-aza-CR by the p53 inhibitor, PFT-α. HCT-116 were pretreated with 30 µM PFT-α for 5h before treatment with 1µM of 5-aza-CR for 72h. (A) Cell growth was determined by the MTT assay. (B) DNA content was determined by flow cytometric analysis following PI staining. Numerical data corresponding to cell cycle analysis are presented in boxes. (C) DNA fragmentation was determined by TUNEL assay in fixed and labeled cytospin preparations using fluorescence microscopy. YOU DO NOT MENTION ANYTHING ABOUT THE P53 (-/-) CELLS, ALTHOUGH YOU DO IN THE TEXT?

**Figure 5.** (A) Western Blotting showing the expression of Gadd45, p21^{WAF1}, p53, cyclin B1, and Bcl2 proteins in 5-aza-CR-treated (Aza), control (C) cells and PFT-α-treated cells (PFT). HCT-116 (+/+ ) cells were treated with 30µM PFT-α for 5h prior to addition of 1µM 5-aza-CR. Total proteins were extracted and the levels of protein were analyzed as described in Materials and Methods. The blot was reprobed with an antibody directed against β-actin to confirm equivalent protein loading. The immunoblots shown here represent the typical result from three independent experiments. (B-D) The effects of treatment of cells with 5-aza-CR (Aza) and the p53 selective inhibitor PFT-α (Aza+PFT) on the transcript levels of p21^{WAF1}, Bcl2 and Gadd45, respectively. Expression levels of mRNA were determined by quantitative RT-PCR using a LightCycler. Relative mRNA values depicted are means ± SD of two independent experiments each performed in duplicates. Rec (Recovery): After 96h,
5-aza-CR treatment was removed and fresh medium added to allow the cells to recover from drug effects for 48h.

**Figure 6.** Reversal of 5-aza-CR-induced hypomethylation by the p53 selective inhibitor PFT-α.

(A) The effect of PFT-α on global DNA methylation status in 5-aza-CR-treated HCT-116 (+/+) cells. The extent of global DNA methylation is inversely proportional to the incorporation of methyl groups by SssI methylase in the presence of [3H-methyl]S-adenosyl-methionine (SAM). Values are means ± SEM, n = 3. ANOVA: PFT-α, 96h, p<0.05; Aza+PFT-96h, p<0.01. (B) Effect of 5-aza-CR on p16INK4a promoter methylation. Methylation-specific PCR was performed using the u (unmethylated) and m (methylated) primer sets. (C) The effect of PFT-α on protein expression levels of the DNA methyltransferase DNMT3a and on the transcript levels of (D) DNMT1, (E) DNMT3a and (F) DNMT3b in 5-aza-CR-treated HCT-116 cells.

**Figure 7**

(A-D) Time course analysis of the mRNA expression (A-C) and the protein expression (D) of the DNA methyltransferases DNMT1, DNMT3a and DNMT3b in HCT-116 (-/-) cells in the presence of 1µM 5-aza-CR.
Table 1
Sequences of primers, hybridization probes, annealing temperature (Tm), and lengths of PCR Products

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LC, LightCycler Red 640; X, fluorescein; p, phosphate; hyb, hybridization probe
* Gene bank accession number
Table 2
Profile of dysregulated genes after treatment of HCT116 cells with 5-AzaCR*

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<td>NM005658</td>
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<td>NM001066</td>
<td>pro-apoptotic</td>
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<td>CASP14</td>
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<td>NM012114</td>
<td>unspecific in apoptosis</td>
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<td>Casp6</td>
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* treatment with 1uM 5-AzaCR after 48hrs
the complete gene list of the GEArray Q series Human Apoptosis Gene Array can be found at http://www.superarray.com. All signals were measured using the Syngene BIO Imaging System. Common gene names are indicated. Expression ratios (gene/house keeping gene GAPDH) were calculated per blot and final results are expressed as fold changes given as a relative gene expression (according to the formula: ratio treatment/ratio control). Any signal whose raw intensity was less than 150% of the background was interpreted to be ‘no detectable’ in the sample.
Fig. 1

A. Incorporation of methyl-3H Cpm $10^3$ (H-SAM/0.5 µg of DNA) over time after Aza treatment (h).

B. MSP p16${}^{INK4a}$ expression at 72h and 96h.

C. Ratio p16/ß2M expression over time after Aza treatment (h).

D. DNMT1 expression with 1 µM Aza.

E. DNMT3a expression with 1 µM Aza.

F. DNMT3b expression with 1 µM Aza.

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Fig. 2

A) Comparison of cell morphology between control (Ctrl) and Aza-treated cells at 48h and 72h.

B) Flow cytometry analysis showing cell cycle distribution.

Viability (% control) vs. Time (h):

- Ctrl 48h: Pre G1: 0.69, G0/G1: 70.26, S: 13.23, G2/M: 15.82
- Aza 48h: Pre G1: 7.58, G0/G1: 49.92, S: 16.23, G2/M: 26.27
- Ctrl 72h: Pre G1: 0.45, G0/G1: 71.16, S: 14.63, G2/M: 13.76
Fig. 3

A

Ctrl

Aza 48h

Aza 72h

Aza 96h

% TUNEL positive cells

Time (hrs)

0 24 72 96

**

B

a: vital cells

b: apoptotic cells
c: mixed cells
d: necrotic cells

a b
d c
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Fig. 4
Fig. 5
**Fig. 6**

This article has not been copyedited and formatted. The final version may differ from this version.
Fig. 7