Growth inhibition of human cancer cells by 5-aza-2′-deoxycytidine does not correlate with its effects on *INK4a/ARF* expression or initial promoter methylation status

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Abstract

The cytotoxicity of 5-aza-2′-deoxycytidine (DAC) has been linked to demethylation of the *INK4a/ARF* tumor suppressor gene locus in various cell systems, but the causality of this association remains unproven. To test this assumption, we have examined the effects of DAC in two human cancer cell lines of differing *INK4a/ARF* promoter methylation status: MDA-MB-468 breast cancer cells in which *INK4a/ARF* is unmethylated and normally expressed, and DLD-1 colorectal cancer cells in which *INK4a/ARF* is methylated and repressed. In MDA-MB-468 cells, DAC induces cytotoxicity in the absence of any detectable increase of p14 or p16 expression, whereas small interfering RNA knockdown of p16/p14 expression fails to attenuate DAC cytotoxicity. In DLD-1 cells, DAC demethylates *INK4a/ARF* and restores both p16 and p14 expression at concentrations that fail to cause detectable growth inhibition or apoptosis; moreover, neither *ARF* nor *INK4a* transgene expression inhibits DLD-1 cell growth despite normalization of p14 and p16 expression. These data imply that neither of these cell lines depends on up-regulated expression of *INK4a/ARF* for DAC cytotoxicity. We propose that optimal anticancer use of this drug will await unambiguous identification of those DAC target genes primarily responsible for triggering growth inhibition, followed by clarification as to whether these upstream events are caused by hypomethylation or DNA damage. [Mol Cancer Ther 2009;8(4):779–85]

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Introduction

Several reports have linked the anticancer effects of 5-aza-2′-deoxycytidine (DAC) to demethylation-dependent up-regulation of *INK4a/ARF* (1–4), a unique tumor-suppressive two-gene locus (5) that is often epigenetically inactivated in common solid tumor types such as colorectal cancer (6, 7) and breast cancer (6, 8). Casting doubt on this hypothesis, however, are many reports attributing DAC cytotoxicity either to nondemethylating mechanisms of action (9–11), including direct induction of DNA damage (12–15), or to demethylation-dependent induction of downstream proapoptotic pathways not known to be solely controlled by *INK4a/ARF* (3).

To resolve this important debate, we have selected two cell lines of opposite *INK4a/ARF* promoter methylation status: (*a*) MDA-MB-468 human breast cancer cells, which normally express the unmethylated gene (16), and (*b*) DLD-1 human colorectal cancer cells, which are characterized by *INK4a/ARF* gene hypermethylation and associated absence of p16 and p14 protein expression (17). Using this defined experimental system, we show here that *INK4a/ARF* promoter demethylation is neither necessary nor sufficient for the cytotoxicity of DAC in human tumor cells.

Materials and Methods

Cell Culture and DAC Treatment

DLD-1 human colorectal cancer cells and MDA-MB-468 human breast cancer cells were purchased from the American Type Culture Collection. DLD-1 and MDA-MB-468 cells were maintained in RPMI 1640 or DMEM, respectively, with 10% fetal bovine serum (BioWest), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine at 37°C in 5% CO_2. Because DAC is not stable in medium, cells treated with DAC were changed fresh medium and DAC each day within the incubation period. DAC stocks were dissolved in DMSO, the top concentration of which in culture medium (0.1%) was used in experiments as a control.

Methylation-Specific PCR

Genomic DNA of control or treated cell samples was isolated by precipitation (Cells and Tissue DNA Isolation kit; GE). Bisulfite modification of genomic DNA was carried out as described previously (7, 18). In brief, 2 μg genomic DNA was denatured by adding freshly prepared NaOH (0.3 mol/L final concentration) for 15 min at 37°C, and 30 μL of freshly prepared 10 mmol/L hydroquinone (Sigma) and 520 μL of 3.6 mol/L sodium bisulfite (pH 5; Sigma) were added to the reaction mixture. These samples were incubated under mineral oil for 17 h at 50°C. The modified

DNA was then purified (Gel Extraction System; Viogene) and extracted (DNA/RNA extraction kit; Viogene) and further treated with NaOH at a concentration of 0.3 mol/L for 15 min at 37°C. The DNA was ethanol-precipitated and resuspended in 50 μL water, 5 μL of which were used for monoplex methylation-specific PCR (MSP) analysis (17). Primers for MSP and PCR conditions are as follows: primers for methylated promoter of *INK4a*, sense primer TTATTAGAGGGTGGGGCGGATCGC, antisense primer CCACCTAAATCGACCTCCGACCG, running cycles 35, annealing temperature 65°C, and length of PCR products 234 bp; primers for unmethylated promoter of *INK4a* sense primer TTATTAGAGGGTGGGGTGGATTGT, antisense primer CCACCTAAATCAACCTCCAACCA, running cycles 35, annealing temperature 65°C, and length of PCR products 151 bp; primers for methylated promoter of *ARF* sense primer TTATTAGAGGGTGGGGCGGATCGC, antisense primer CCACCTAAATCGACCTCCGACCG, running cycles 35, annealing temperature 62°C, and length of PCR products 122 bp; and primers for unmethylated promoter of *ARF* sense primer TTTTTGGTGTTAAAGGGTGGTGTAGT, antisense primer CACAAAAACCCTCACTCACAACAA, running cycles 35, annealing temperature 62°C, and length of PCR products 132 bp.

MTT Assay of Cell Viability

For cell viability assays, trypsinized DLD-1 or MDA-MB-468 cells were seeded in 96-well plates at a density of 8,000 per well. Twenty-four hours later, cells were treated with DAC for the period indicated, and medium containing DAC was replenished daily. To assay the growth rate of DLD-1 transfectants, cells were seeded at a density of 500 per well. After DAC treatment for 4 days, or at the time points as indicated, cells in 100 μL medium/well received 20 μL of 5 mg/mL MTT (Sigma) in PBS solution and were then incubated in a CO_2 incubator at 37°C for 3 h. After incubation, medium was removed and 100 μL DMSO was added to each well. Culture dishes were then placed on a shaker at 150 rpm for 5 min to mix the formazan into the solvent. Plates were then incubated at 37°C for 5 min, and absorbance was read at 550 nm to measure cell quantity. All experiments were conducted in triplicate.

Extraction of Total RNA and Reverse-Transcription PCR

Total cellular RNA was purified from cells using Trizol reagent (Invitrogen). Following reverse transcription of 1 μg total RNA by random hexamer, the resulting single-strand cDNA was amplified using Taq DNA polymerase (Applied Biosystems) and specific primers directed against human *INK4a* and *ARF*; the PCR conditions were individually optimized for each gene product studied, and the cycle number was adjusted so that the reactions fell within the linear range of product amplification (Table 1). The β-actin gene was used as an internal standard. Aliquots (10 μL) of the amplified cDNA were separated by 1.5% agarose gel electrophoresis and subsequently visualized by ethidium bromide staining.

Western Blotting

Whole-cell protein lysates were prepared as described (19). Antibodies used for Western blotting were obtained from Santa Cruz Biotechnology (p16, Bax, and BcL-XL), Lab Vision (p14), Cell Signaling (phospho-cdc2, Tyr^{15}), or Sigma (actin).

Knockdown of p16/*INK4a* and *p14/ARF*

MDA-MB-468 cells were plated in 6-well dishes at 5×10^5 per well. Knockdown experiments were done 24 h post-seeding. Negative control small interfering RNA (siRNA; scrambled siRNA, NC siRNA), p16 siRNA, or p14 siRNA duplex oligonucleotides (300 pmol) were transfected into MDA-MB-468 cells with Lipofectin 2000 (Invitrogen) followed by 24 h incubation. Whole-cell protein lysates were extracted for Western blot evaluation of knockdown efficiency. siRNA sequences used in the experiments were as reported previously (20, 21). p16 siRNA sequences were CGCACCGAAUAGUUACGGUtt (sense) and ACCGUAACUAUUCGGUGCGtt (antisense), whereas p14 siRNA sequences were GAACAUGGUGCGCAGGUUCtt (sense) and GAACCUGCGCACCAUGUUCtt (antisense).

Quantification of Apoptosis and Cell Cycle Distribution Using Flow Cytometry

Untreated control, DMSO-treated control, or DAC-treated cell monolayers were harvested and fixed with ice-cold 70% (v/v) ethanol for 24 h. After centrifugation at $200 \times g$ for 5 min, cell pellets were washed twice with PBS (pH 7.4) and resuspended in PBS containing propidium iodide (Sigma; 10 μg/mL) and RNase (50 μg/mL). Cells were then incubated at 37°C for 30 min. Flow cytometric analysis was done using Cytomics FC 500 MPL (Beckman Coulter). Apoptotic cell and cell cycle distribution was analyzed using ModFit 3.1 software.

Establishment of DLD-1 Cell Sublines Stably Expressing p16/*INK4a* or p14/ARF

DLD-1 cells were transfected with retrovirus vectors using Lipofectamine 2000 (Invitrogen). Vectors pWZL-hygro

Table 1. Sequences of PCR primers and reaction condition

Primers	Sequences (5′-3′)	No. cycles	Annealing temperature (°C)	Length of PCR products (bp)
ARF forward	TTCTTGGTGACCCTCCGGATT	35	53	150
ARF reverse	TGCCCATCATCATGACCTGG			
INK4a forward	GGAGCAGCATGGAGCCTT	35	53	197
INK4a reverse	TGCCCATCATCATGACCTGG			
β-actin forward	CAAGAGATGGCCACGGCTGCT	28	55	257
β-actin reverse	TCCTTCTGCATCCTGTCGGCA			

and pWZL-hygro-p14 were kindly provided by Prof. Androphy (University of Massachusetts Medical School), pBabe-puro-p16 by Prof. Brugge (Harvard Medical School), and pBabe-puro-EGFP by Dr. Zhang RX (Li Ka Shing Faculty of Medicine, The University of Hong Kong). After transfection, cells were screened with hygromycin B1 (100 μg/mL; Merck) or puromycin (20 μg/mL; Merck) for 10 days. Survival clones were used to assay cell growth rate in the presence of hygromycin B1 (50 μg/mL) or puromycin (10 μg/mL).

Results

DAC Treatment of MDA-MB-468 Breast Cancer Cells Triggers G2-M Arrest, Apoptotic Signaling, and Growth Inhibition without Increasing Either p14 or p16 Expression

Because *INK4a/ARF* is unmethylated and normally expressed in MDA-MB-468 cells (16), and because DAC can increase expression of some unmethylated genes (10), we initially used this cell system to assess the consequences of DAC treatment. G_2-M arrest and proapoptotic signaling were efficiently induced by DAC (0.1 μmol/L) as shown by increased phospho-cdc2 (22), Bax, and repression of Bcl-xL, and this was accompanied by G_2-M arrest, apoptosis, and growth inhibition; however, neither p14 nor p16 expression was enhanced by DAC (Fig. 1). These findings indicate that DAC induces cytotoxicity in this cell line via a pathway independent of p14 and p16 up-regulation.

Knockdown of Either p14/*ARF* or p16/*INK4a* Expression in MDA-MB-468 Cells Fails to Attenuate Dose-Dependent DAC Cytotoxicity

Notwithstanding that absolute levels of p14 and p16 expression are not enhanced by DAC treatment (Fig. 1), others have reported that the proapoptotic function of these proteins may be regulated by DNA damage-dependent induction of nuclear localization (23). Hence, to test the possibility that *INK4a/ARF* plays a necessary but not sufficient role in DAC-dependent cytotoxicity, we next examined the effect of siRNA knockdown of p14 and p16 expression on dose-dependent DAC growth inhibition of MDA-MB-468 cells. Figure 2A and B confirms that siRNA knockdown potently reduced p14 and p16 protein expression in these cells. Despite this, neither knockdown phenotype abrogated the dose-dependent cytotoxicity of DAC; rather, an unexpected (albeit slight) enhancement of DAC cytotoxicity was evident in p14/p16 knockdowns (Fig. 2C). We conclude from these experiments that physiologic levels of *INK4a/ARF* expression may not only be insufficient (Fig. 1) but also unnecessary for DAC-induced growth inhibition in this cell system.

Both *ARF* and *INK4a* Gene Expression Levels Are Efficiently Restored by DAC Treatment in DLD-1 Human Colorectal Cancer Cells

Unlike MDA-MB-468 cells, which exhibit normal basal levels of *INK4a/ARF* expression associated with promoter demethylation (16), the DLD-1 cell line is characterized by *INK4a/ARF* promoter hypermethylation and gene repression (17). To interpret the relationship of DAC cytotoxicity in this cell system to its effects, if any, on p14/p16 expression, we first assessed the influence of DAC treatment on *INK4a/ARF* gene expression. As expected, basal levels of *INK4a* and *ARF* gene expression are undetectable in control cells, DMSO-treated cells, and cells treated with the lowest concentration of DAC (0.01 μmol/L). However, DAC concentrations of ≥0.1 μmol/L induced maximal expression of *INK4a/ARF* after 3 to 4 days of continuous treatment

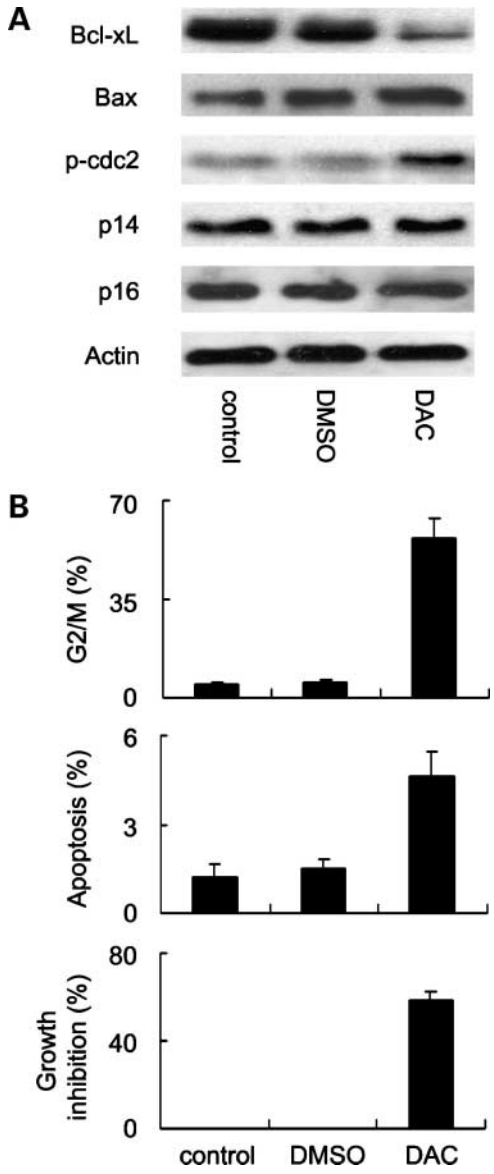

Figure 1. Effects of DAC (0.1 μmol/L) for 4 d on MDA-MB-468 cells expressing hypomethylated *INK4a/ARF*. **A,** effects on protein and phosphoprotein expression. Control MDA-MB-468 cells (*lane 1*) were treated for 4 d with 0.1% DMSO (*lane 2*) or 0.1 μmol/L DAC (*lane 3*) and then Western-blotted using antibodies to the proteins (*left*). *p-cdc2,* phospho-cdc2 (see Materials and Methods). **B,** effects on growth. MDA-MB-468 cells were grown (*column 1*) and treated with either 0.1% DMSO (*column 2*) or 0.1 μmol/L DAC (*column 3*). After 4 d, the percentage of flow cytometric G_2-M-arrested cells (*top*), apoptotic cells (*middle*), or growth-inhibited cells (*bottom*) was measured as detailed in Materials and Methods. SEs are based on triplicate samples.

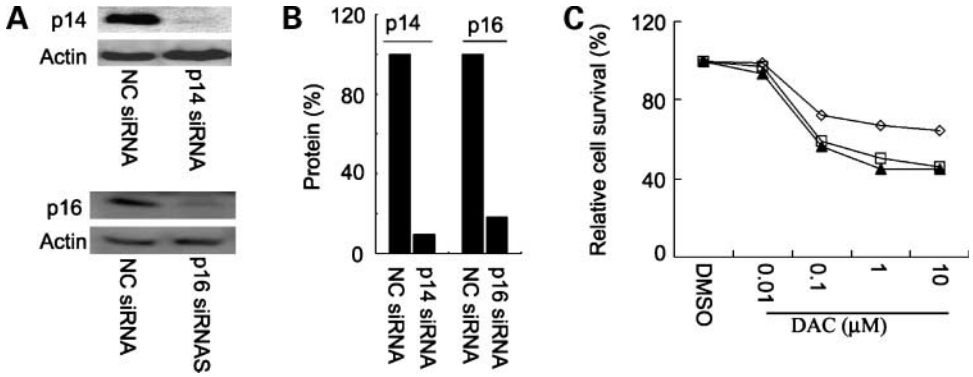

Figure 2. Failure of p14/*ARF* and p16/*INK4a* knockdown to abrogate DAC-dependent growth inhibition in MDA-MB-468 cells expressing hypomethylated *INK4a/ARF*. **A,** Western blot confirmation of p16 and p14 knockdown efficiency. **B,** densitometric quantitation of p14 and p16 protein expression (Western blot) levels in negative control (*NC, left columns*) and experimental (*siRNA, right columns*) *ARF* and *INK4a* knockdowns, respectively. **C,** dose-dependent DAC growth inhibition of MDA-MB-468 cells with scrambled siRNA, *INK4a* siRNA, or *ARF* siRNA. *Open diamonds,* cells treated with scrambled siRNA; *open squares,* cells with knockdown of p14/*ARF*; *filled triangles,* cells with knockdown of p16/*INK4a*.

(Fig. 3). These data confirm that DAC restores expression of this gene in cells with promoter hypermethylation and absent protein levels.

Constitutive *INK4a/ARF* Expression Fails to Inhibit DLD-1 Cell Growth

To test whether restoration of *INK4a/ARF* expression affects DLD-1 cell growth without confounding by (*a*) DAC-induced DNA damage, (*b*) DAC-dependent demethylation of other gene promoters, or (*c*) concomitant (synergistic) inhibition of cell growth by both *INK4a* and *ARF* genes within single cells, stably transfected cell sublines were first created using each one of the two gene constructs in isolation. As shown in Fig. 4A, these *ARF* and *INK4a* transfectants expressed constitutive levels of the respectively encoded proteins, p14 (*left*) and p16 (*right*). Despite this, no effect of either *ARF* or *INK4a* gene expression alone was detectable in DLD-1 cells (Fig. 4B and C, respectively), consistent with earlier findings in p53-mutated cell systems (24, 25). These findings imply that any DAC-dependent inhibition of DLD-1 growth may not be attributable to demethylation-dependent *INK4a/ARF* gene induction alone.

Dose Dependency of DAC-Inducible p16 and p14 Up-Regulation Differs from That of Both *INK4a/ARF* Promoter Demethylation and Growth Inhibition in DLD-1 Cells

To determine the dose-dependent relationship between DAC-inducible *INK4a/ARF* promoter hypomethylation, p16/p14 protein induction, and growth dynamics in DLD-1 cells, these variables were cross-correlated. As shown in Fig. 5, and consistent with Fig. 3, MSP confirms that DAC concentrations of ≥0.1 μmol/L maximally induce both *ARF* (*row 1*) and *INK4a* (*row 3*) hypomethylation. Unexpectedly, however, DAC-induced p14 and p16 up-regulation is maximal at 0.1 μmol/L and declined at higher concentrations (*rows 5* and *6*). Moreover, when growth inhibition, apoptosis, or cell cycle arrest in S phase (26) are quantified, all parameters are maximal at the highest dose of 10 μmol/L yet unmeasurable at the most potent *INK4a/ARF*-inducing

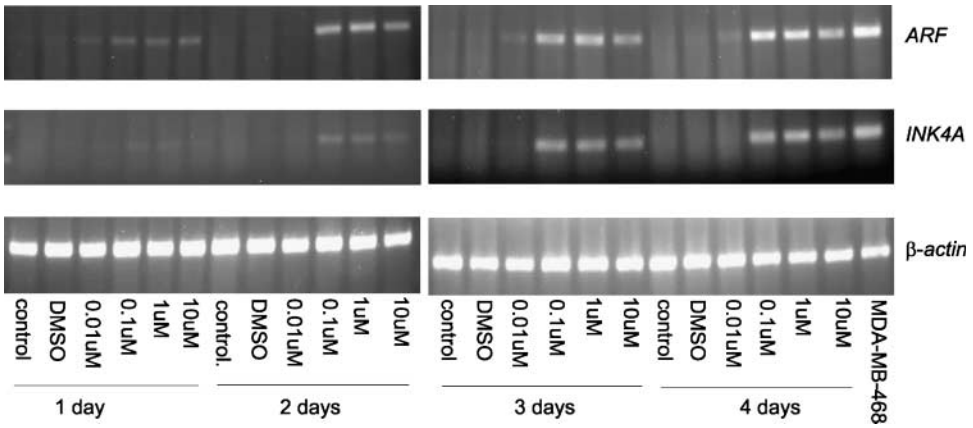

Figure 3. DAC inducibility of *ARF* (*top row*) and *INK4a* (*middle row*) mRNA expression in DLD-1 cells with hypermethylated *INK4a/ARF*. DLD-1 cells were treated with varying concentrations of DAC for 1 to 4 d, and total RNA was extracted for semiquantitative reverse-transcription PCR. β-Actin mRNA expression is shown as internal standard (*bottom row*). MDA-MB-468 cells expressing hypomethylated *INK4a/ARF* are included as a positive control (*far right*).

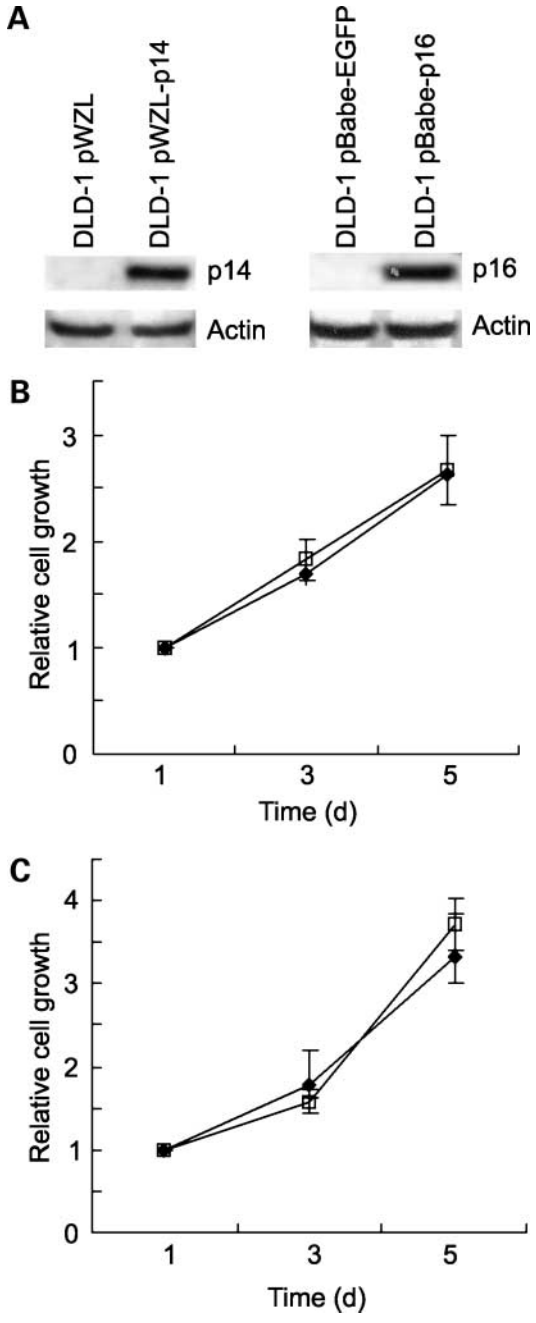

Figure 4. Growth effects of constitutive p14/*ARF* or p16/*INK4a* expression in transfected DLD-1 cells with hypermethylated *INK4a/ARF* promoters. **A,** Western blot confirmation of expression of p14 (*left*) and p16 (*right*). DLD-1 cells were stably transfected with vectors pWZL-hygro, pWZL-hygro-p14, pBabe-puro-EGFP, and pBabe-puro-p16 as described in Materials and Methods. **B,** growth effects of constitutive p14/*ARF* expression in transfected DLD-1 cells. Cell number was assayed by MTT after seeding for 1, 3, or 5 days. *Open squares,* DLD-1 with stable transfection of pWZL-hygro-p14; *solid diamonds,* DLD-1 with stable transfection of control vector pWZL-hygro. **C,** growth effects of constitutive p16/*INK4a* expression in transfected DLD-1 cells. Cell number was assayed by MTT after seeding for 1, 3, or 5 d. *Open squares,* DLD-1 with stable transfection of pBabe-puro-p16; *solid diamonds,* DLD-1 with stable transfection of control vector pBabe-puro-EGFP.

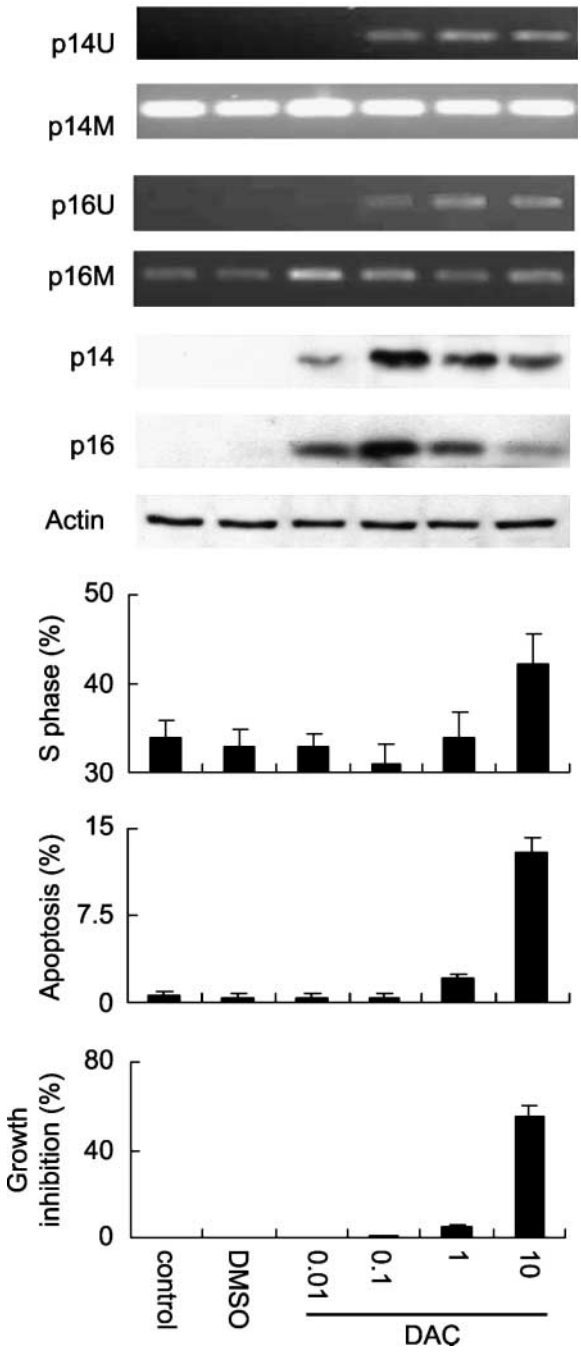

Figure 5. Comparative DAC dose-response of DLD-1 *INK4a/ARF* parameters (*top*) and growth (*bottom*). No treatment and DMSO controls, and DAC concentrations (0.01-10 μmol/L for 4 d), are indicated (*bottom*). MSP was used as described in Materials and Methods to measure genomic promoter DNA in either unmethylated (*ARF,* p14U, *row 1*; *INK4a*, p16U, *row 3*) or methylated (*ARF,* p14M, *row 2*; *INK4a*, p16M, *row 4*) genes. Western blotting was used to quantify dose-dependent p14 (*row 5*), p16 (*row 6*), or control β-actin protein expression (*row 7*). The percentage of cells recruited into flow cytometric S-phase arrest (*top*), apoptosis (*middle*), or growth inhibition (*bottom*) was measured as detailed in Materials and Methods. SEs are based on triplicate samples.

concentration of 0.1 μmol/L (Fig. 5, *bottom three graphs*). These results confirm that neither DAC-dependent *INK4a/ARF* demethylation nor p14/p16 up-regulation plausibly underlies DLD-1 growth inhibition.

Discussion

The central finding of this study is that DAC-dependent demethylation of an important tumor-suppressive gene locus makes no discernible contribution to the impressive cell growth inhibition in DAC-treated DLD-1 human colorectal cancer cells, in which these gene promoters are usually hypermethylated. This finding casts doubt on earlier correlative studies, which raised the possibility of a causal connection between these two endpoints (1–4). The latter is a plausible enough hypothesis based on *in vitro* findings of p16 induction by DAC and could neatly account for the chemosensitizing effects of both DAC (27) and p16 up-regulation (28). Yet, although it is possible that the specific cell system (and associated variations in integrity of p53-dependent apoptosis; refs. 29–31) contribute to the confusion in the literature, our findings raise alternative hypotheses relevant to more rational future use of hypomethylating agents.

One key possibility raised by our study is that DAC triggers human cancer cell growth inhibition at least in part via the hypomethylation-induced up-regulation of genes other than *INK4a/ARF*, such as those encoding p73 or retinoic acid receptor β2 (29, 32). In this respect, it is also pertinent to note that fluoropyrimidine cytotoxicity is potentiated by DAC (33), perhaps reflecting a chemosensitizing role for up-regulation of the *hMLH1* mismatch repair gene (34), which is often methylated in colorectal cancer (35, 36). This latter possibility is supported by the clinical observation that methylation of *hMLH1* and other genes also predicts drug resistance and poor prognosis in colorectal cancer (37) and other tumor types (38), consistent with the notion that mismatch repair gene function may lower apoptotic thresholds (39).

There are important limitations of the present study. First, we used qualitative monoplex MSP rather than quantitative MSP. Relevant to this, we note that although there is a diminished gene reexpression at high doses of DAC, there is still abundant overall expression. Our conclusions are therefore qualitative rather than quantitative, and negative (supporting the lack of a causal relationship) rather than positive (establishing a clear causal mechanism).

Second, although our data reduce the plausibility that *INK4a/ARF* is the sole target gene for the cytotoxic action of DAC in DLD-1 cells, we have not excluded the proapoptotic contribution of DNA damage induced by this pyrimidine antimetabolite (15, 40, 41). Hence, more quantitative future studies will be needed to establish the causal mechanism of DAC-induced cytotoxicity in normal and cancer cell systems.

Finally, the role of p53 in DAC-induced cytotoxicity is a further variable relevant to the present study, because we note that both experimental cell lines happen to express mutant p53 (42-44). Although several groups have shown that DAC induces apoptosis in p53-wild type cells (13, 45), others have implicated defective p53 function in DAC-dependent apoptosis (46). Future experiments will thus be needed to clarify this complex question by direct comparison of p53 wild-type and mutant cell lines.

In conclusion, our study shows that demethylation of the silenced tumor suppressor gene *INK4a/ARF* by DAC may be neither necessary nor sufficient for human tumor cell growth inhibition by this drug. We conclude that in these cell systems the growth-inhibitory activity of DAC arises from mechanisms other than demethylation of *INK4a/ARF*. Improved anticancer use of such drugs in the clinic will await predictive identification of DAC-inducible genes triggering apoptosis and cell cycle arrest, permitting in turn the use of biomarkers to identify patients most likely to benefit from the use of this important class of drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Bender CM, Pao MM, Jones PA. Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res 1998;58:95–101.

2. Timmermann S, Hinds PW, Munger K. Re-expression of endogenous p16INK4a in oral squamous cell carcinoma lines by 5-aza-2′-deoxycytidine treatment induces a senescence-like state. Oncogene 1998;17:3445–53.

3. Missiaglia E, Donadelli M, Palmieri M, et al. Growth delay of human pancreatic cancer cells by methylase inhibitor 5-aza-2′-deoxycytidine treatment is associated with activation of the interferon signalling pathway. Oncogene 2005;24:199–211.

4. Badal V, Menendez S, Coomber D, et al. Regulation of the p14ARF promoter by DNA methylation. Cell Cycle 2008;7:112–9.

5. Sherr CJ. The INK4a/ARF network in tumour suppression. Nat Rev Mol Cell Biol 2001;2:731–7.

6. Herman JG, Merlo A, Mao L, et al. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 1995;55:4525–30.

7. Esteller M, Tortola S, Toyota M, et al. Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. Cancer Res 2000;60:129–33.

8. Nielsen NH, Roos G, Emdin SO, et al. Methylation of the p16(INK4a) tumor suppressor gene 5′-CpG island in breast cancer. Cancer Lett 2001;163:59–69.

9. Schmelz K, Sattler N, Wagner M, et al. Induction of gene expression by 5-aza-2′-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and -independent mechanisms. Leukemia 2005;19:103–11.

10. Zhu WG, Dai Z, Ding H, et al. Increased expression of unmethylated CDKN2D by 5-aza-2′-deoxycytidine in human lung cancer cells. Oncogene 2001;20:7787–96.

11. Soengas MS, Capodieci P, Polsky D, et al. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 2001;409:207–11.

12. Juttermann R, Li E, Jaenisch R. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. Proc Natl Acad Sci U S A 1994;91:11797–801.

13. Zhu WG, Hileman T, Ke Y, et al. 5-Aza-2′-deoxycytidine activates the p53/p21WAF1/CIP1 pathway to inhibit cell proliferation. J Biol Chem 2004;279:15161–6.

14. Covey JM, D'Incalci M, Tilchen EJ, et al. Differences in DNA damage produced by incorporation of 5-aza-2′-deoxycytidine or 5,6-dihydro-5-azacytidine into DNA of mammalian cells. Cancer Res 1986;46:5511–7.

15. Palii SS, Van Emburgh BO, Sankpal UT, et al. DNA methylation inhibitor 5-aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3b. Mol Cell Biol 2008;28:752–71.

16. Paz MF, Fraga MF, Avila S, et al. A systematic profile of DNA methylation in human cancer cell lines. Cancer Res 2003;63:1114–21.

17. Zheng S, Chen P, McMillan A, et al. Correlations of partial and extensive methylation at the p14(ARF) locus with reduced mRNA expression in colorectal cancer cell lines and clinicopathological features in primary tumors. Carcinogenesis 2000;21:2057–64.

18. Herman JG, Graff JR, Myohanen S, et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–6.

19. Xiong J, Fan S, Meng Q, et al. BRCA1 inhibition of telomerase activity in cultured cells. Mol Cell Biol 2003;23:8668–90.

20. Oruetxebarria I, Venturini F, Kekarainen T, et al. p16INK4a is required for HSNF5 chromatin remodeler-induced cellular senescence in malignant rhabdoid tumor cells. J Biol Chem 2004;279:3807–16.

21. Eymin B, Claverie P, Salon C, et al. p14ARF activates a TIP60-dependent and p53-independent ATM/ATR/CHK pathway in response to genotoxic stress. Mol Cell Biol 2006;26:4339–50.

22. O'Connor PM. Mammalian G_1 and G_2 phase checkpoints. Cancer Surv 1997;29:151–82.

23. Yogev O, Saadon K, Anzi S, et al. DNA damage-dependent translocation of B23 and p19 ARF is regulated by the Jun N-terminal kinase pathway. Cancer Res 2008;68:1398–406.

24. Schmitt CA, Fridman JS, Yang M, et al. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. Cell 2002;109:335–46.

25. Bardeesy N, Bastian BC, Hezel A, et al. Dual inactivation of Rb and p53 pathways in Ras-induced melanomas. Mol Cell Biol 2001; 21:2144–53.

26. Zhang Y, Wang Z, Ahmed F, et al. Down-regulation of jagged-1 induces cell growth inhibition and S phase arrest in prostate cancer cells. Int J Cancer 2006;119:2071–7.

27. Kanda T, Tada M, Imazeki F, et al. 5-Aza-2′-deoxycytidine sensitizes hepatoma and pancreatic cancer cell lines. Oncol Rep 2005;14:975–9.

28. Halloran CM, Ghaneh P, Shore S, et al. 5-Fluorouracil or gemcitabine combined with adenoviral-mediated reintroduction of p16INK4a greatly enhanced cytotoxicity in Panc-1 pancreatic adenocarcinoma cells. J Gene Med 2004;6:514–25.

29. Schmelz K, Wagner M, Dorken B, et al. 5-Aza-2′-deoxycytidine induces p21WAF expression by demethylation of p73 leading to p53-independent apoptosis in myeloid leukemia. Int J Cancer 2005;114: 683–95.

30. Schneider-Stock R, Diab-Assef M, Rohrbeck A, et al. 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. J Pharmacol Exp Ther 2005;312:525–36.

31. Sherr CJ, Bertwistle D, DEN Besten W, et al. p53-dependent and -independent functions of the ARF tumor suppressor. Cold Spring Harb Symp Quant Biol 2005;70:129–37.

32. Yang Q, Shan L, Yoshimura G, et al. 5-Aza-2′-deoxycytidine induces retinoic acid receptor β2 demethylation, cell cycle arrest and growth inhibition in breast carcinoma cells. Anticancer Res 2002;22:2753–6.

33. Morita S, Iida S, Kato K, et al. The synergistic effect of 5-aza-2′-deoxycytidine and 5-fluorouracil on drug-resistant tumors. Oncology 2006;71: 437–45.

34. Plumb JA, Strathdee G, Sludden J, et al. Reversal of drug resistance in human tumor xenografts by 2′-deoxy-5-azacytidine-induced demethylation of the HMHL1 gene promoter. Cancer Res 2000;60:6039–44.

35. Arnold CN, Goel A, Boland CR. Role of HMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. Int J Cancer 2003;106:66–73.

36. Fujita H, Kato J, Horii J, et al. Decreased expression of HMLH1 correlates with reduced 5-fluorouracil-mediated apoptosis in colon cancer cells. Oncol Rep 2007;18:1129–37.

37. Van Rijnsoever M, Elsaleh H, Joseph D, et al. CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. Clin Cancer Res 2003;9:2898–903.

38. Gifford G, Paul J, Vasey PA, et al. The acquisition of HMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. Clin Cancer Res 2004;10:4420–6.

39. Casorelli I, Russo MT, Bignami M. Role of mismatch repair and mgmt in response to anticancer therapies. Anticancer Agents Med Chem 2008; 8:368–80.

40. Wang H, Zhao Y, Li L, et al. An ATM- and Rad3-related (ATR) signaling pathway and a phosphorylation-acetylation cascade are involved in activation of p53/p21WAF1/CIP1 in response to 5-aza-2′-deoxycytidine treatment. J Biol Chem 2008;283:2564–74.

41. Karpf AR, Moore BC, Ririe TO, et al. Activation of the p53 DNA damage response pathway after inhibition of DNA methyltransferase by 5-aza-2′-deoxycytidine. Mol Pharmacol 2001;59:751–7.

42. Tomita Y, Marchenko N, Erster S, et al. WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization. J Biol Chem 2006;281:8600–6.

43. Macip S, Igarashi M, Berggren P, et al. Influence of induced reactive oxygen species in p53-mediated cell fate decisions. Mol Cell Biol 2003;23: 8576–85.

44. Rodrigues NR, Rowan A, Smith ME, et al. p53 mutations in colorectal cancer. Proc Natl Acad Sci U S A 1990;87:7555–9.

45. Jiemjit A, Fandy TE, Carraway H, et al. p21(WAF1/CIP1) induction by 5-azacytosine nucleosides requires DNA damage. Oncogene 2008;27: 3615–23.

46. Nieto M, Samper E, Fraga MF, et al. The absence of p53 is critical for the induction of apoptosis by 5-aza-2′-deoxycytidine. Oncogene 2004;23: 735–43.

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