

Azacitidine and decitabine have different mechanisms of action in non-small cell lung cancer cell lines

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Abstract: Azacitidine (AZA) and decitabine (DAC) are cytidine azanucleoside analogs with clinical activity in myelodysplastic syndromes (MDS) and potential activity in solid tumors. To better understand the mechanism of action of these drugs, we examined the effects of AZA and DAC in a panel of non-small cell lung cancer (NSCLC) cell lines. Of 5 NSCLC lines tested in a cell viability assay, all were sensitive to AZA (EC₅₀ of 1.8–10.5 μM), while only H1299 cells were equally sensitive to DAC (EC₅₀ of 5.1 μM). In the relatively DAC-insensitive cell line A549, both AZA and DAC caused DNA methyltransferase I depletion and DNA hypomethylation; however, only AZA significantly induced markers of DNA damage and apoptosis, suggesting that mechanisms in addition to, or other than, DNA hypomethylation are important for AZA-induced cell death. Cell cycle analysis indicated that AZA induced an accumulation of cells in sub-G1 phase, whereas DAC mainly caused an increase of cells in G2/M. Gene expression analysis of AZA- and DAC-treated cells revealed strikingly different profiles, with many genes distinctly regulated by each drug. In summary, while both AZA and DAC caused DNA hypomethylation, distinct effects were demonstrated on regulation of gene expression, cell cycle, DNA damage, and apoptosis.

Keywords: apoptosis, azacitidine, decitabine, gene expression, non-small cell lung cancer

Introduction

Azacitidine (AZA) (5-azacytidine, Vidaza[®]; Celgene Corporation, Summit, NJ) and decitabine (DAC) (2'-deoxy-5-azacytidine, Dacogen[®]; Eisai Inc., Woodcliff Lake, NJ) are used clinically for the treatment of myelodysplastic syndromes (MDS), a heterogeneous group of bone marrow stem cell disorders.^{1,2} Both AZA and DAC are cytidine nucleoside analogs that become incorporated into newly synthesized DNA, where they bind DNA methyltransferases (DNMTs) in an irreversible, covalent manner.^{3,4} The sequestration of DNMTs prevents maintenance of the methylation state of DNA, leading to DNA hypomethylation.^{5,6} As a consequence, genes previously silenced by DNA hypermethylation can be re-expressed upon treating cancer cell lines with these DNMT inhibitors.^{7,8} Re-expression of aberrantly methylated genes involved in normal cell cycle control, differentiation, and apoptotic pathways is believed to contribute to the anticancer effects of these drugs.⁹

Clinical activities of AZA and DAC are best established in the hematological malignancies MDS and acute myeloid leukemia (AML), cancers with a high frequency of aberrantly methylated genes.¹⁰ Aberrant DNA methylation of genes involved in DNA repair, cell adhesion, cell cycle, and cell death has also been reported in multiple types of solid cancers, including colon, stomach, breast, ovary, kidney, and lung.¹¹

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For example, in non-small cell lung cancer (NSCLC), hypermethylation of tumor suppressor genes RAS association domain family 1A (*RASSF1A*), adenomatous polyposis coli (*APC*), fragile histidine triad (*FHIT*), and *p16^{INK4A}* has been associated with poor survival.^{12–15} Clinical trials investigating the use of AZA and DAC in solid tumors have been reported, although response rates were poor. In a Phase I study of DAC in patients with cancers involving the lungs, esophagus, and pleura, no objective responses were observed.¹⁶ Similar outcomes were obtained with DAC in patients with other forms of solid tumors.¹⁷ In a Phase II trial of AZA in patients with solid tumors, the responses were minimal and transient.¹⁸ The clinical response rate was also low for the combination of AZA and phenylbutyrate in patients with refractory solid tumors.¹⁹

A better understanding of the mechanistic activities of AZA and DAC will provide insights into rational use of these agents as therapies for solid tumor patients, including potential uses as combination therapies, adjuvant therapies, and maintenance therapies. Here, we directly compared

the *in vitro* effects of AZA and DAC on cell viability, DNMT1 protein levels, DNA methylation, DNA damage, apoptosis, cell cycle, and gene expression in NSCLC cell lines. Although AZA and DAC caused similar effects on DNA-mediated markers such as DNMT1 depletion and DNA methylation, the drugs showed very different effects on cell viability, DNA damage, apoptosis, cell cycle, and gene expression.

Results

AZA and DAC have differential effects on NSCLC cell viability

AZA and DAC were compared in a panel of 5 NSCLC cell lines (A549, H1975, H460, H23, and H1299) for their effects on cell viability (Figure 1 and Supporting Information Figure 1). AZA reduced cell viability by at least 75% at high concentrations, with EC₅₀ values of 1.8–10.5 μM (Table 1). In contrast, DAC did not reduce cell viability more than 55%, and EC₅₀ values were not reached in 4 (A549, H1975, H460, and H23) of the 5 NSCLC cell lines tested. In H1299

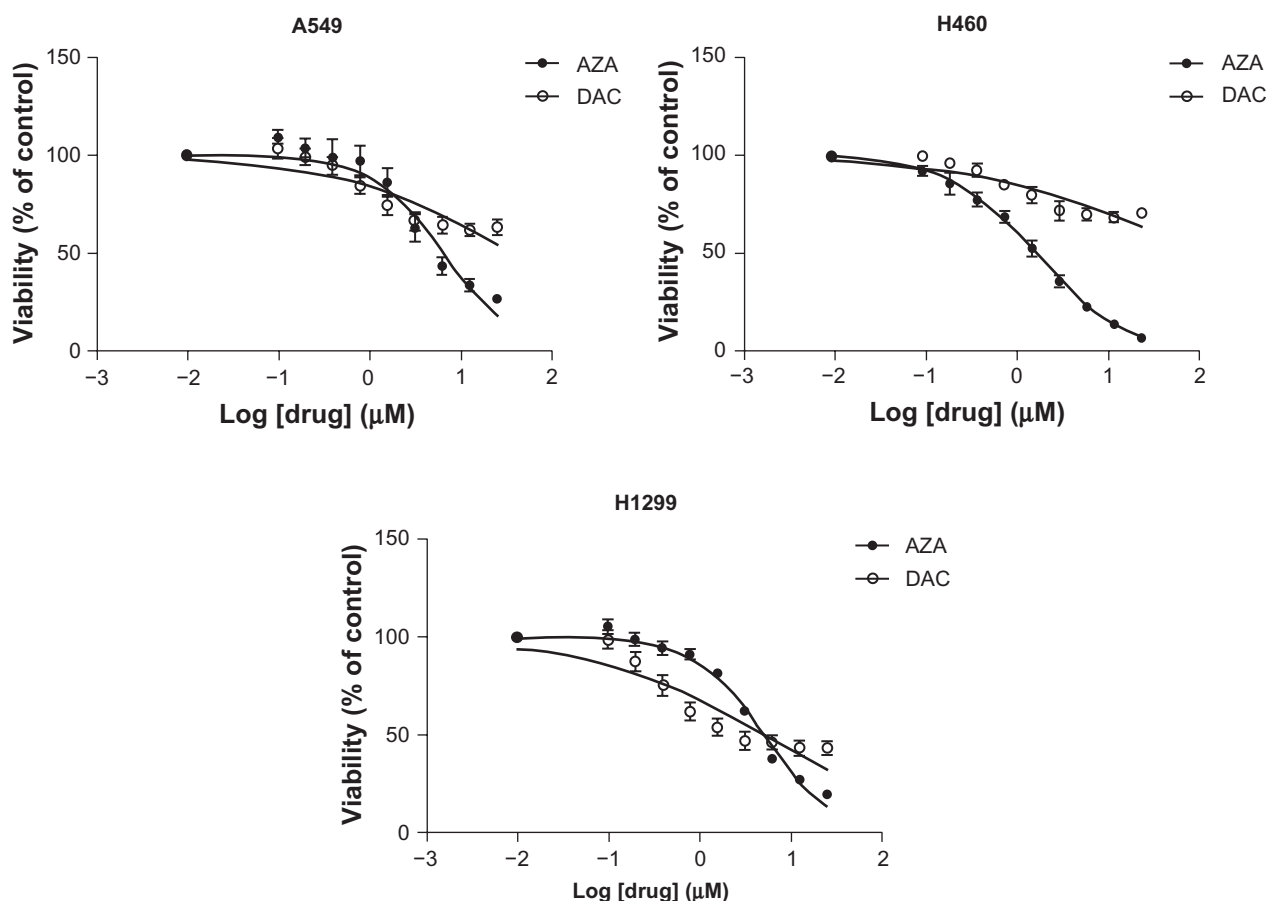


Figure 1 AZA and DAC differentially affect cell viability in a panel of NSCLC cell lines. Viability of A549, H460, and H1299 cells was assessed after 72 hours of treatment with AZA or DAC (0–25 μM). Error bars represent the standard error of mean of 3 independent experiments, with triplicate wells per experiment. **Abbreviations:** AZA, azacitidine; DAC, decitabine; NSCLC, non-small cell lung cancer.

Table 1 EC₅₀ values for AZA and DAC on NSCLC cell viability

	AZA EC ₅₀ ± SEM (μM)	DAC EC ₅₀ ± SEM (μM)
A549	6.3 ± 1.1	>25
H1975	8.6 ± 2.9	>25
H460	1.8 ± 0.3	>25
H23	10.5 ± 1.8	>25
H1299	5.1 ± 0.2	5.9 ± 2.1

Note: EC₅₀ values were calculated from 3 independent experiments using Graphpad Prism software.

Abbreviations: AZA, azacitidine; DAC, decitabine; NSCLC, non-small cell lung cancer.

cells, DAC EC₅₀ values were calculated; however, the 95% confidence intervals for the EC₅₀ values were poor (data not shown). The EC₅₀ values for AZA and DAC are similar to those reported for drugs commonly used in NSCLC, including gemcitabine, cisplatin, and carboplatin.^{20–22} The distinct dose-response curves and EC₅₀ values indicate differential sensitivities of these NSCLC cell lines to AZA and DAC.

AZA and DAC cause DNMT1 depletion and DNA hypomethylation

To determine whether the differential sensitivities of NSCLC cell lines to AZA versus DAC in cell viability assays reflected differences in the incorporation of each drug into DNA, DNMT1 protein depletion and DNA hypomethylation were evaluated as indirect measures of drug incorporation into DNA. When A549 and H1299 cells were treated with AZA or DAC for 20 hours, DNMT1 protein levels were reduced (Figure 2). Dose-dependent decreases in DNMT1 protein were observed

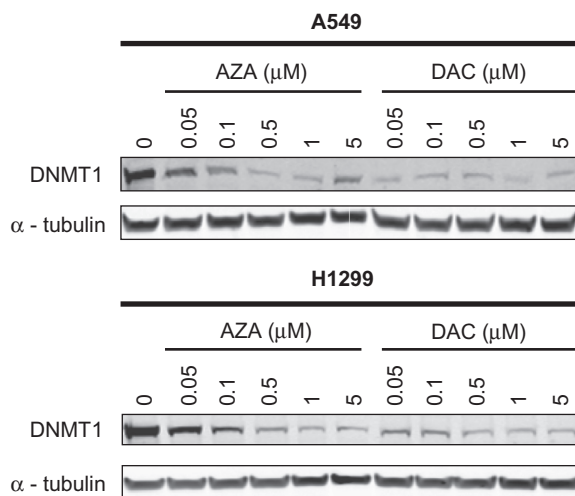


Figure 2 AZA and DAC cause DNMT1 depletion in NSCLC cell lines. A549 and H1299 cells were treated with AZA or DAC (0–5 μM) for 20 hours and DNMT1 protein was detected by Western blotting of cell extracts. Alpha-tubulin was used as a loading control.

Abbreviations: AZA, azacitidine; DAC, decitabine; DNMT1, DNA methyltransferase I; NSCLC, non-small cell lung cancer.

with AZA, while near-maximal reduction of DNMT1 protein was observed at the lowest concentration (0.05 μM) of DAC. In A549 cells, DNMT1 depletion caused by 5 μM AZA was not as much as that caused by 0.5 or 1 μM AZA, possibly as a consequence of cell growth inhibition at the higher AZA concentration.²³ Reduced DNMT1 levels were detected as early as 4 hours after drug treatment (Supporting Information Figure 2). Similar results were obtained in the H460 and H23 cell lines (data not shown).

We next determined whether AZA and DAC caused DNA hypomethylation by examining the methylation status of LINE-1 elements in A549 and H1299 cells treated for 48 hours (Figure 3) or 72 hours (Supporting Information Figure 3). Both AZA and DAC decreased LINE-1 methylation; however, DAC was 3- to 10-fold more potent. Peak hypomethylation was observed at 0.3–1.0 μM AZA and 0.1 μM DAC. LINE-1 methylation was unaffected at the highest DAC

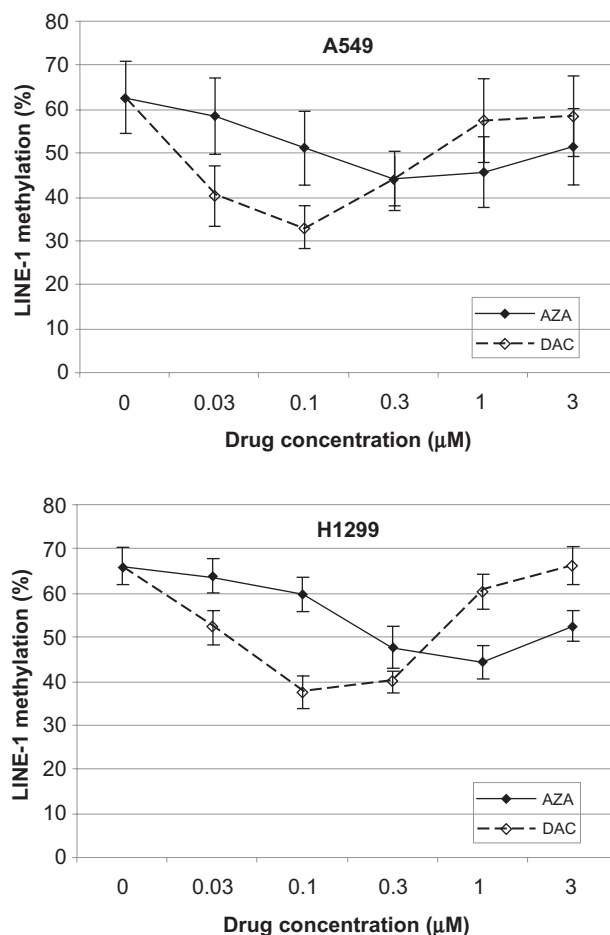


Figure 3 AZA and DAC reduce DNA methylation in A549 and H1299 cells. LINE-1 DNA methylation was assessed in A549 and H1299 cells after 48 hours of treatment with AZA or DAC (0–3 μM). Percentage LINE-1 methylation represents the average percentage methylation of 4 CpG sites in duplicate samples, with error bars representing the standard deviation.

Abbreviations: AZA, azacitidine; DAC, decitabine.

concentration tested, possibly as a consequence of cell growth inhibition.²³ DAC modulated the DNA-mediated markers (DNMT1 depletion and DNA hypomethylation) in both cell lines, suggesting that the relative insensitivity to DAC in cell viability assays cannot be attributed to a lack of drug uptake, phosphorylation, and DNA incorporation. These findings rule out dysfunctional deoxycytidine kinase, the rate-limiting kinase in the phosphorylation of DAC, as a possible mechanism of relative DAC-insensitivity,²⁴ and suggest that mechanisms in addition to DNA incorporation are responsible for the greater sensitivity of NSCLC cell viability to AZA.

AZA, but not DAC, robustly induces markers of DNA damage and apoptosis

Phase contrast images of A549 cell cultures after 3 days of drug treatment showed reduced cell numbers and increased debris in AZA-treated cell cultures, but healthy-looking cells in DAC-treated cultures (Figure 4). These findings confirmed results of the cell viability assays (Figure 1). To examine the mechanism(s) of drug-induced cell death, A549 and H1299 NSCLC cell lines were treated with AZA or DAC for 24 or 48 hours, and markers of double-strand DNA (dsDNA) damage (histone-H2AX(ser139) phosphorylation) and apoptosis (PARP cleavage) were evaluated by Western blot (Figure 5 and data not shown). AZA dose-dependently induced histone-H2AX(ser139) phosphorylation and PARP cleavage in A549 cells. Similar results were observed in the H460 cell line (data not shown). There was relatively high basal phosphorylation of histone-H2AX(ser139) in H1299 cells, which was further increased by 10 μ M AZA.

High concentrations of AZA also induced PARP cleavage in H1299 cells. In A549 and H1299 cells, DNMT1 protein was completely depleted by DAC treatment; however, neither histone-H2AX(ser139) phosphorylation nor PARP cleavage were induced.

As AZA induced PARP cleavage, we further examined early-apoptotic (AnnexinV-FITC⁺ and 7-AAD⁻) and late-apoptotic (AnnexinV-FITC⁺ and 7-AAD⁺) cell populations by flow cytometry in A549 and H1299 cells treated with AZA (3 μ M) or DAC (3 μ M) for 72 hours (Figure 6). AZA (3 μ M) treatment of A549 and H1299 cells caused a significant increase in the early- and late-apoptotic populations (Figures 6B and 6C). DAC did not significantly cause an increase in these populations. These results demonstrated that AZA, but not DAC, induced dsDNA damage and apoptosis in NSCLC cell lines.

DAC-treated H1299 cells show delayed DNA damage response

AZA and DAC appear to be incorporated into DNA of NSCLC cell lines, as both drugs induced DNMT1 depletion (Figure 2) and DNA hypomethylation (Figure 3). It was therefore surprising that 48-hour treatment with DAC did not induce dsDNA damage (histone-H2AX(ser139) phosphorylation) in A549 and H1299 cells (Figure 5). To better define the DNA damage response of NSCLC cell lines treated with AZA and DAC, we treated NSCLC cell lines with the drugs for an extended period of time. A549 and H1299 cells were treated with AZA or DAC for 6 days and lysates were collected on days 3 and 6 (Figure 7). At the 3-day time point in both cell lines, the results were

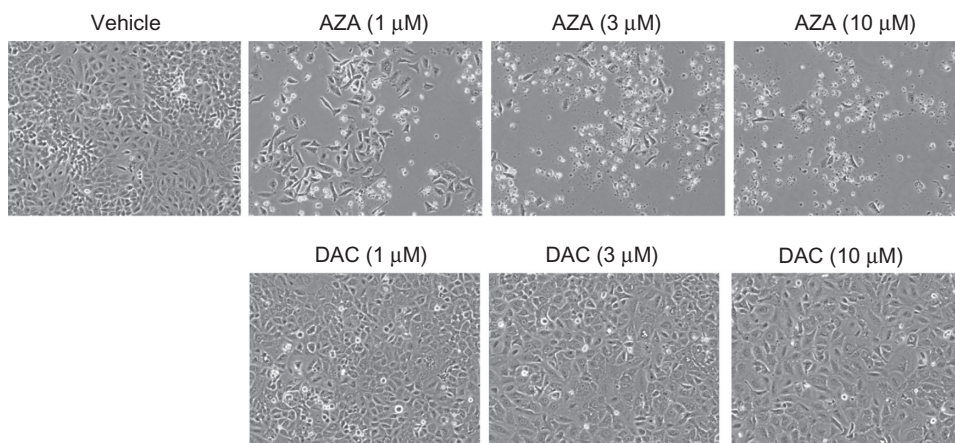


Figure 4 AZA-treated A549 cultures show reduced cell numbers. A549 cells, seeded in 6-well plates, were treated with vehicle or 1, 3, and 10 μ M AZA or DAC for 72 hours. The CoolSNAP ES2 CCD camera (Photometrics) was used to take phase-contrast images of cells under the Plan Fluor 10X objective (Nikon) on the Eclipse Ti-S microscope (Nikon).

Abbreviations: AZA, azacitidine; DAC, decitabine.

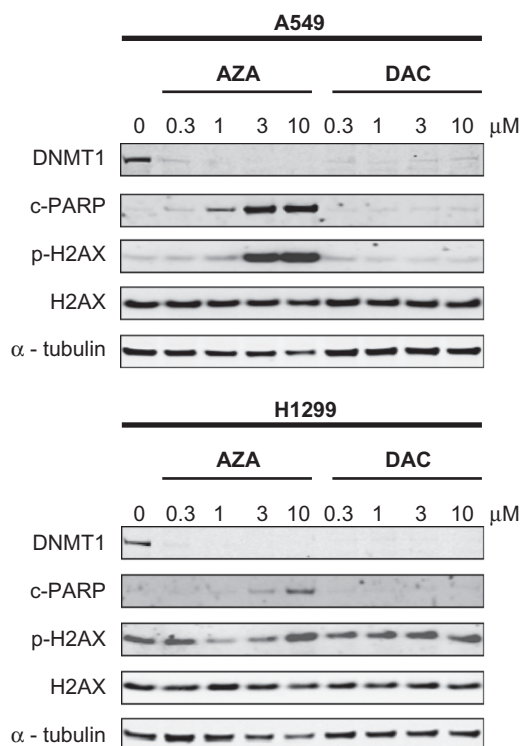


Figure 5 AZA, but not DAC, induces markers of DNA damage and apoptosis in NSCLC cell lines. A549 and H1299 cells were treated with AZA or DAC (0–10 μM) for 48 hours and Western blotting of cell extracts was used to detect DNMT1, cleaved-PARP, phospho-histone-H2AX(ser139), and total histone-H2AX. alpha-tubulin was used as a loading control.

Abbreviations: AZA, azacitidine; DAC, decitabine; DNMT1, DNA methyltransferase I; NSCLC, non-small cell lung cancer.

similar to those at the 24- and 48-hour time points; AZA, but not DAC, induced histone-H2AX(ser139) phosphorylation and PARP cleavage. In A549 cells, even after 6 days of daily treatment with DAC, there was no induction of histone-H2AX(ser139) phosphorylation and PARP cleavage (Figure 7). The EC_{50} values for AZA and DAC were 4.4 μM and 2.5 μM , respectively, for A549 cells after 6 days of treatment (Supporting Information Table 1). Although the calculated EC_{50} value for DAC was lower than that of AZA, DAC did not reduce cell viability more than 75%, while AZA almost completely inhibited cell viability (Supporting Information Figure 4). In H1299 cells, substantial histone-H2AX(ser139) phosphorylation, without much effect on PARP cleavage, was observed after 6 days of DAC treatment (Figure 7). Consistent with these results, phase contrast images of H1299 cells treated with DAC for a prolonged period did not show many cells undergoing apoptosis. Rather, prolonged treatment of H1299 cells resulted in fewer cells that are enlarged (data not shown). These results suggest that DAC may have a delayed effect on inducing DNA damage in NSCLC cell lines.

AZA and DAC differentially affect the cell cycle

The effects of AZA and DAC on cell cycle distribution were evaluated in A549 and H1299 cells treated for 72 hours (Figure 8). AZA dose-dependently increased the sub-G1 population in A549 cells, consistent with the induction of apoptosis (Figures 4–6). AZA also caused a minor increase in the sub-G1 population in H1299 cells (Figure 8), consistent with the induction of early-, rather than late-, apoptotic cell population at this time point (Figure 6). DAC also caused a minor increase in the sub-G1 population in these cell lines; however, the more prominent effect of DAC was an increase in the G2/M population.

AZA and DAC modulate expression of different sets of genes

Although both AZA and DAC caused DNMT1 depletion and DNA hypomethylation in NSCLC cell lines, the drugs had very different effects on cell viability, DNA damage, apoptosis, and cell cycle. To better understand the molecular pathways regulated by each drug, A549 and H1299 cells were treated with a dose range (0.3–3.0 μM) of AZA or DAC for 48 hours, and effects on gene expression were assessed by microarray analysis. The total number of genes regulated by AZA or DAC, and the overlap of regulated genes, are presented in Table 2. At the lower drug concentration (0.3 μM), AZA and DAC modulated few genes, with DAC modulating 4- to 20-fold more genes than AZA. At the higher drug concentrations (1 and 3 μM), many more genes were modulated, with AZA typically modulating 2- to 5-fold more genes than DAC. Interestingly, the number of genes modulated in common between the 2 drugs was low (6%–22%). For example, in A549 cells, AZA (3 μM) and DAC (3 μM) commonly upregulated 66 genes, while AZA uniquely upregulated 636 genes and DAC uniquely upregulated 413 genes (Table 2).

Functional groupings of the modulated genes were determined using Gene Ontology classifications in NextBio. Different biogroups were regulated by each drug. The top 200 biogroups most significantly regulated by each drug (at 3 μM) are shown in Supporting Information Tables 2–5. In H1299 cells, AZA treatment caused a general downregulation of genes within the “cell cycle”, “metabolic process”, and “biosynthetic process” biogroups. DAC treatment of H1299 cells caused a general upregulation of genes within the “cell differentiation” biogroup. In A549 cells, AZA treatment

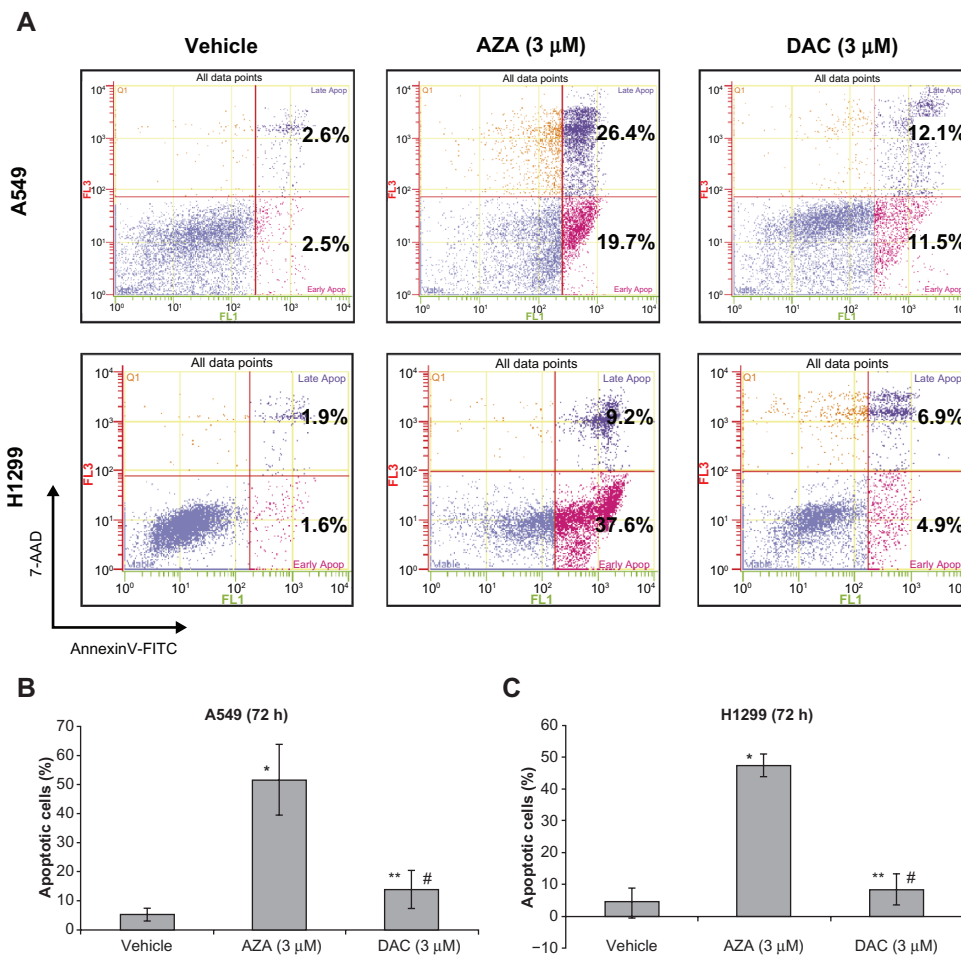


Figure 6 AZA, but not DAC, strongly induces apoptosis in NSCLC cell lines. A549 and H1299 cells were treated with AZA or DAC (3 μM) for 72 hours, and staining for AnnexinV-FITC (x-axis) and 7-AAD (y-axis) was detected by flow cytometry. **A**) The percentages of early apoptotic cells and late apoptotic cells are represented in the lower right and upper right quadrants, respectively. Representative data of 4 independent experiments are shown. **B**) Percentage (mean ± SD; n = 4) of apoptotic (early and late) cells with AZA or DAC treatment of A549 cells. *P < 0.001 versus “vehicle”. **P = 0.328 versus “vehicle”. #P < 0.001 versus “AZA (3 μM)”. **C**) Percentage (mean ± SD; n = 4) of apoptotic (early and late) cells with AZA or DAC treatment of H1299 cells. *P < 0.001 versus “vehicle”. **P = 0.442 versus “vehicle”. #P < 0.001 versus “AZA (3 μM)”.

Abbreviations: AZA, azacitidine; DAC, decitabine; NSCLC, non-small cell lung cancer.

caused downregulation of genes involved in extracellular matrix, while DAC treatment caused downregulation of genes involved in cell cycle. Aside from the regulation of genes related to extracellular matrix, these results are similar to the gene expression data from AML cell lines treated with AZA and DAC.²⁵ Interestingly, AZA treatment of A549 and H1299 cells caused a general upregulation of genes within the “response to DNA damage stimulus” and “DNA repair” biogroups (Figure 9, Supporting Information Tables 2 and 4). These results are consistent with the induction of the dsDNA damage marker (histone-H2AX(ser139) phosphorylation) by AZA in these cells (Figure 5). On the contrary, DAC treatment caused a general downregulation of genes within these biogroups in A549 cells (Figure 9, Supporting Information Table 3), and DAC did not significantly modulate these biogroups in H1299 cells (Supporting Information Table 5). Collectively,

these results indicate that AZA and DAC regulate different cellular pathways.

Discussion

In this study, we revealed differential effects of AZA and DAC on cell viability in a panel of NSCLC cell lines, with AZA inducing greater cellular toxicity and markers of apoptosis (PARP cleavage and AnnexinV staining) in comparison to DAC. Furthermore, AZA induced phosphorylation of histone-H2AX(ser139), a marker of dsDNA damage, while DAC had no, or delayed, effect on this endpoint. The striking differences in the response of NSCLC cell lines to these structurally similar cytidine nucleoside analogs further support emerging evidence that the common perception of these agents as mechanistically interchangeable DNA hypomethylating agents should be reconsidered.^{25,26}

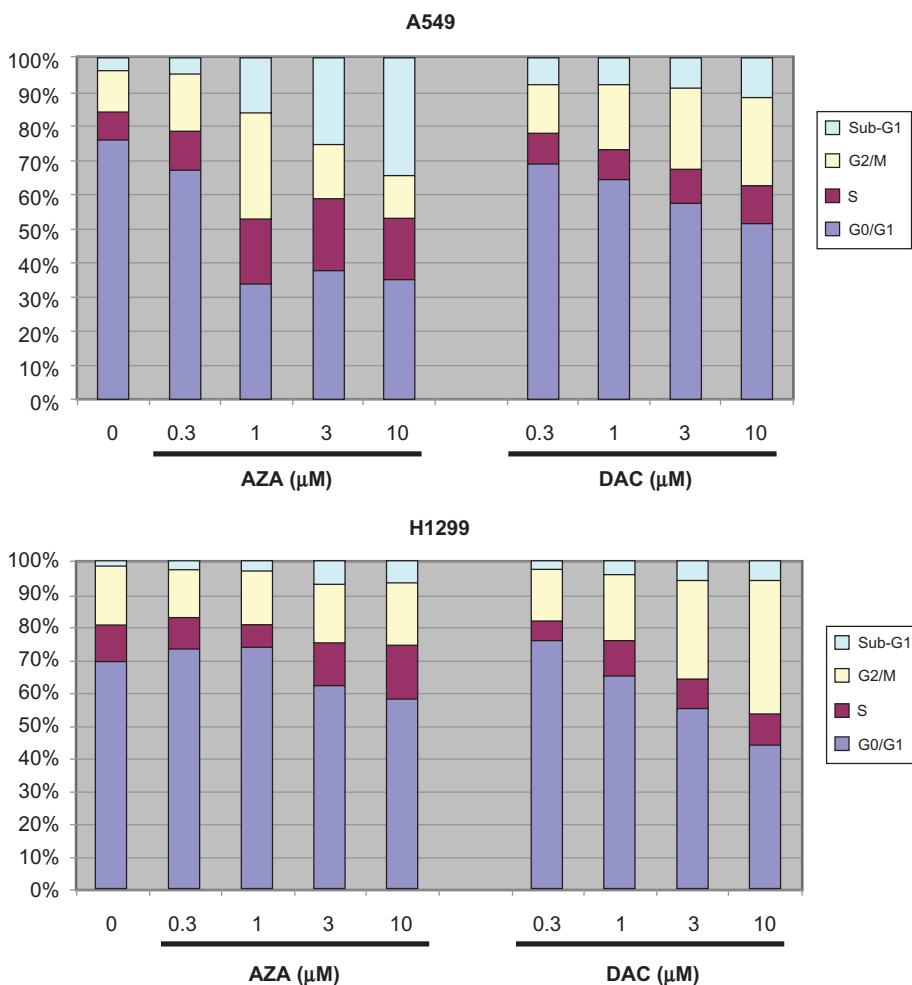


Figure 8 AZA increases the sub-G1 population of cells, while DAC increases the G2/M population. A549 and H1299 cells were stained with NIM-DAPI after 72 hours of treatment with AZA or DAC at 0, 0.3, 1, 3, and 10 μM. The percentage of cells in sub-G1, G2/M, S, and G0/G1 was quantified by flow cytometry. Representative data of 3 independent experiments are shown.

Abbreviations: AZA, azacitidine; DAC, decitabine.

synthesis.^{25,38,42–45} The *in vitro* anticancer activity of AZA in NSCLC models warrants its evaluation in the clinic. It will be important to consider the multiple mechanisms of AZA activity when selecting therapies for use in combination.

Materials and methods

Cell culture and drug treatments

NSCLC cell lines (H460, H1299, A549, and H1975) were purchased from American Type Culture Collection (ATCC, Manassas, VA). The H23 NSCLC cell line

Table 2 Number of genes regulated by AZA and/or DAC

Cell line	[Drug] (μM)	Upregulated genes			Downregulated genes		
		AZA-specific genes	Genes in common	DAC-specific genes	AZA-specific genes	Genes in common	DAC-specific genes
A549	0.3	16	17	139	14	11	55
	1.0	279	45	261	273	30	111
	3.0	636	66	413	560	55	239
H1299	0.3	10	55	214	33	45	121
	1.0	435	135	238	393	107	170
	3.0	1368	173	303	991	153	257

Notes: A549 and H1299 cells were treated with AZA or DAC (0–3.0 μM) for 48 hours, and RNA was isolated for evaluation of gene expression using Affymetrix human U133A 2.0 gene chipset. The table shows the number of genes regulated by AZA and DAC at different drug concentrations. Duplicate samples of each were averaged and compared with untreated samples. A fold change of ≥1.7 in gene expression was considered as regulated.

Abbreviations: AZA, azacitidine; DAC, decitabine.

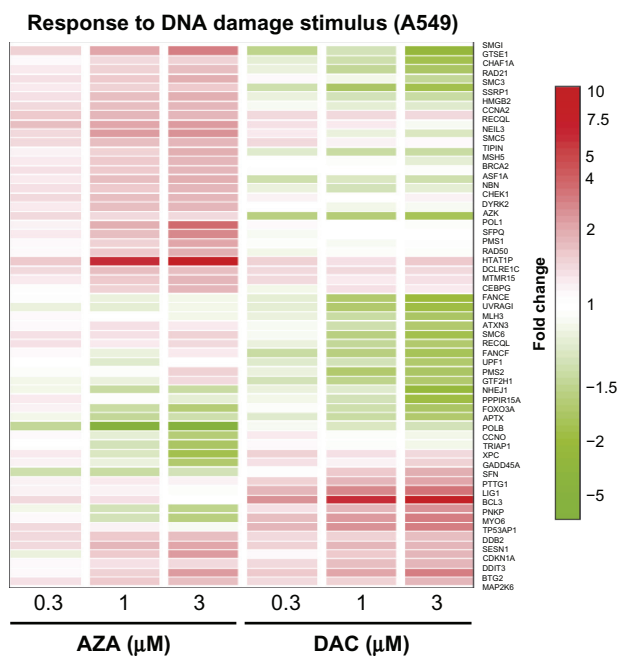


Figure 9 AZA upregulates, while DAC downregulates, genes important in the response to DNA damage stimulus. Gene expression profiling was performed in A549 cells after 48 hours of treatment with AZA or DAC at 0, 0.3, 1, and 3 μ M. NextBio (<http://www.nextbio.com/>) was used to identify regulated Gene Ontology biogroups from lists of regulated genes. The genes displayed represent all genes within the “response to DNA damage stimulus” biogroup that were modulated 1.7-fold or greater by AZA or DAC.

Abbreviations: AZA, azacitidine; DAC, decitabine.

was obtained from the National Cancer Institute (NCI) (Bethesda, MD). Cell lines were cultured in their respective media, as recommended by ATCC and NCI. AZA was manufactured at Aptuit (Greenwich, CT) for Celgene Corporation, while DAC was purchased from Sigma-Aldrich (St Louis, MO). In all experiments, cells were seeded 24 hours before drug treatment and incubated at 37°C and 5% CO₂. For cell viability assays, H460, H1299, A549, H23, and H1975 cells were seeded in triplicate at 1×10^3 , 1×10^3 , 1×10^3 , 4×10^3 , and 4×10^3 cells per well, respectively, in 96-well plates using 200 μ L of medium per well. As the half-lives of AZA and DAC in cell culture are short (~8–12 hours) (data not shown), fresh drug was added every 24 hours by replacing medium with drug-containing medium. For all other assays, cells were seeded at 0.6 – 1.2×10^5 cells per well, in 6-well plates, using 4 mL of medium per well, with fresh drug added directly to the medium every 24 hours. At this seeding density, cells are 30%–40% confluent at the start of drug treatments. The concentrations of AZA and DAC used in these experiments are similar to the maximum concentrations (C_{max}) achieved in human plasma at clinically used dosages and schedules of administration (3–11 μ M AZA and 0.3–1.6 μ M DAC).^{28,46,47}

Cell viability

Cell viability was assessed 72 hours after the initial drug treatment, using the CyQUANT assay (Life Technologies Corporation, Carlsbad, CA). Fluorescence was measured with a spectrophotometer (Molecular Devices, Sunnyvale, CA), and EC₅₀ values were calculated from three independent experiments using Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA).

Western analysis

For Western analyses of protein levels, cells were washed with phosphate-buffered saline (PBS) and lysed with radio immuno precipitation assay (RIPA) buffer (Cell Signaling Technology, Inc., Danvers, MA) supplemented with 350 mM NaCl and 0.1% sodium dodecyl sulfate (SDS). Cell lysates were sonicated with two 5-second bursts under low amplitude (20%) using the Digital Sonic Dismembrator (ThermoFisher Scientific, Inc., Waltham, MA). Proteins were separated on 4%–12% Bis-Tris NuPAGE gels (Life Technologies Corporation) and transferred to nitrocellulose membranes. DNMT1, phospho-histone-H2AX(ser139), total histone-H2AX, cleaved-PARP, and alpha-tubulin were detected using the Li-Cor Odyssey imaging system (Li-Cor Biotechnology, Lincoln, NE), following incubation with the appropriate primary and secondary antibodies. The phospho-histone-H2AX(ser139) and cleaved-PARP antibodies were obtained from Cell Signaling Technology, Inc. The total histone-H2AX (C-20) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The alpha-tubulin and DNMT1 antibodies were purchased from EMD Chemicals, Inc. (Gibbstown, NJ) and Abcam, Inc. (Cambridge, MA), respectively. The goat anti-Rabbit IRDye 680, goat anti-Mouse IRDye 800CW, and donkey anti-Goat IRDye 800CW secondary antibodies were obtained from Li-Cor Biotechnology.

DNA methylation analysis

Genomic DNA was purified from cells using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. DNA yield was quantitated with a NanoDrop 8000 spectrophotometer (ThermoFisher Scientific, Inc.). Genomic DNA (1 μ g/sample) was submitted to EpigenDx (Worcester, MA) for bisulfite conversion and pyrosequencing of LINE-1 elements. Briefly, 1 μ g of DNA was bisulfite treated using the Zymo DNA Methylation Kit (Zymo Research, Orange, CA) and eluted in 10 μ L volume. DNA eluate (1 μ L) was used for polymerase chain reaction (PCR) with biotinylated primers to the LINE-1 locus, converting the PCR product to single-stranded DNA

templates. PCR products (each 10 μ L) were sequenced by the Pyrosequencing PSQ96 HS System (Biotage AB), following the manufacturer's instructions (Biotage, Kungsgatan, Sweden). The methylation status of each locus was analyzed individually as a T/C SNP using QCpG software (Biotage). Percentage LINE-1 methylation represents the average percentage methylation of 4 CpG sites in duplicate samples. Epi-genDx provided 3 controls for the LINE-1 methylation assay: (1) low methylated DNA control, which is human genomic DNA that has been chemically and enzymatically treated to remove the methyl groups; (2) high methylated DNA control, which is human genomic DNA that has been methylated *in vitro*; and (3) 50/50 mix control, which is an equal mixture of the low methylated DNA and high methylated DNA controls. The percentages of LINE-1 methylation for the low methylated DNA control, the 50/50 mix control, and the high methylated DNA control were 25.8 ± 8.1 , 56.2 ± 4.6 , and 86.3 ± 6.5 , respectively (data not shown).

Flow cytometry

For cell cycle distribution, cells were stained with the NIM-DAPI reagent (Beckman Coulter, Inc., Fullerton, CA). For measurement of early- and late-apoptotic cell populations, cells were stained with AnnexinV-FITC and 7-AAD reagents (Beckman Coulter, Inc.). Samples were processed according to manufacturer's instructions and analyzed on a Cell Lab Quanta MPL flow cytometer (Beckman Coulter, Inc.). The effects of treatment were compared using one-way ANOVA, followed by single step method for adjusting *P*-values in multiple testing with the bioconductor package multcomp.⁴⁸

Gene expression analysis

Cells were lysed using the TRIzol reagent (Life Technologies Corporation), and total RNA was isolated using the miRNeasy kit (Qiagen). Double-stranded cDNA and biotin-labeled cRNA were synthesized using 100 ng of total RNA with Ambion's MessageAmp Premier RNA Amplification Kit (ABI, Foster City, CA). Biotin-labeled cRNA (10 μ g) was fragmented and hybridized to each human U133A 2.0 genechip (Affymetrix, Santa Clara, CA). The GC-RMA algorithm was used for normalization, and all analyses were done using GeneSpring 7.3 (Agilent, Santa Clara, CA). Averaged signals from biological duplicate samples were used to determine fold change (treated versus untreated), with an absolute fold change of ≥ 1.7 defining regulated genes. NextBio (<http://www.nextbio.com/>) was used to identify regulated gene ontology biogroups from lists of regulated

genes. The top 200 biogroups are those with the lowest *P*-values calculated within NextBio.

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Disclosure

ANN, PWH, NR, AL-M, HB, CH, and KJM are employees of Celgene and as such own stock in the company.

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Supporting information figures and tables

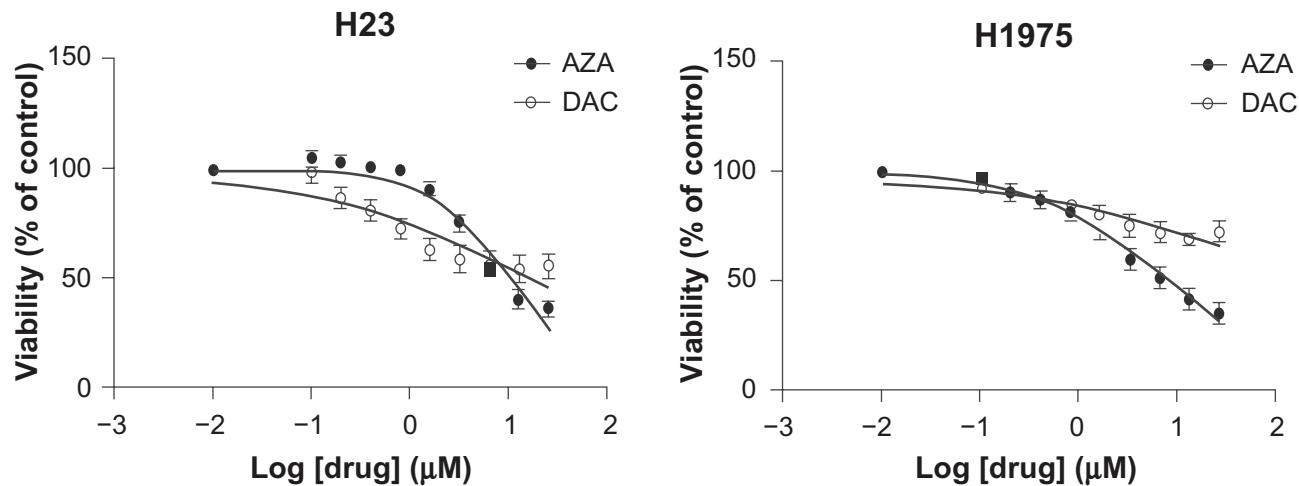


Figure S1 Viability of H23 and H1975 cells was assessed after 72 hours of treatment with AZA or DAC (0–25 µM). Error bars represent the standard error of mean of three independent experiments, with triplicate wells per experiment.

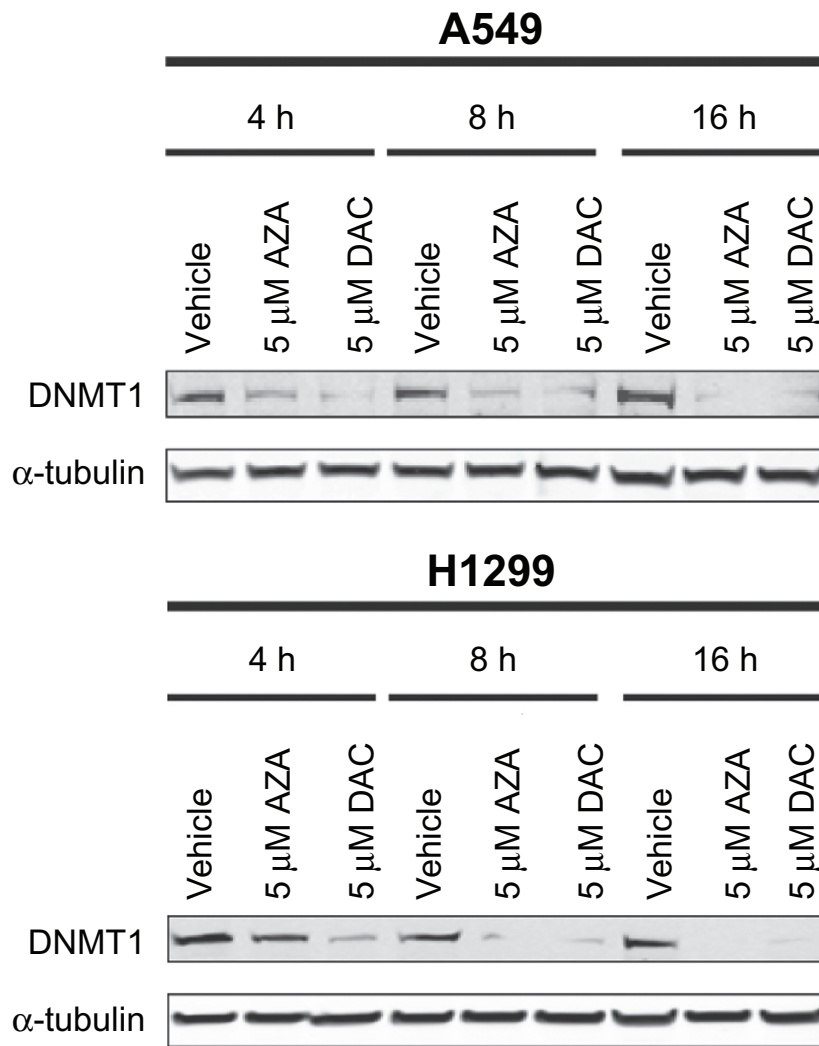


Figure S2 AZA and DAC cause DNMT1 depletion in NSCLC cell lines. A549 and H1299 cells were treated with AZA or DAC (5 µM) for 4, 8, or 16 hours and DNMT1 protein was detected by Western blotting of cell extracts. Alpha-tubulin was used as a loading control.

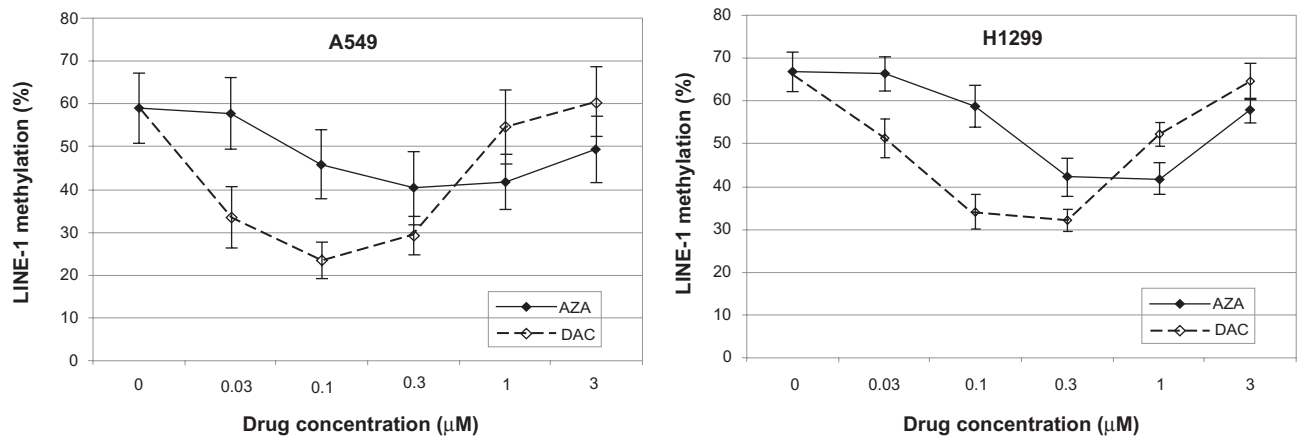


Figure S3 AZA and DAC reduce DNA methylation in A549 and H1299 cells. LINE-1 DNA methylation was assessed in A549 and H1299 cells after 72 hours of treatment with AZA or DAC (0–3 µM). Percentage LINE-1 methylation represents the average percentage methylation of 4 CpG sites in duplicate samples, with error bars representing the standard deviation.

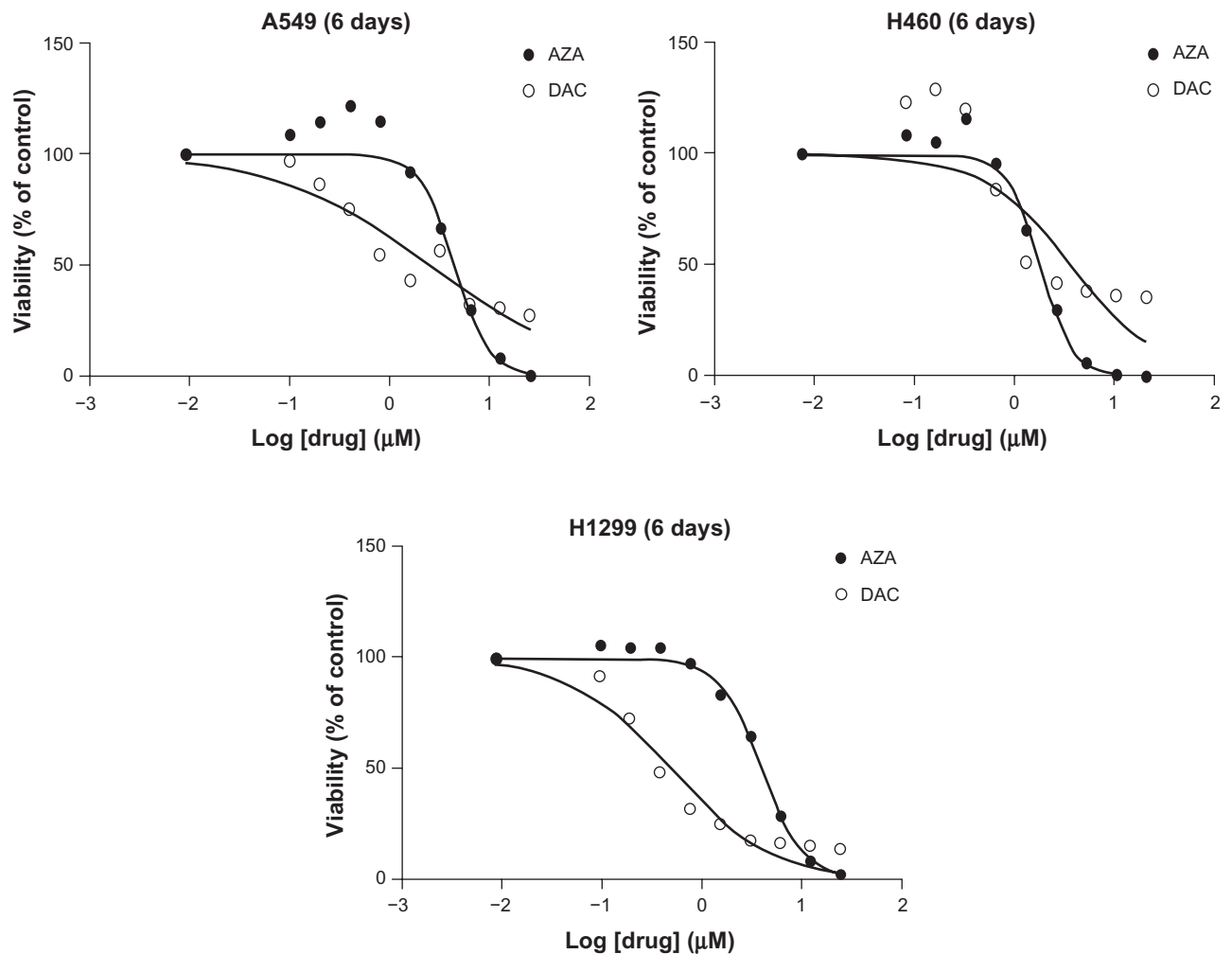


Figure S4 Viability of A549, H460, and H1299 cells was assessed after 6 days of treatment with AZA or DAC (0–25 µM).

Table S1 EC₅₀ values for AZA and DAC on NSCLC cell viability (6 days)

	AZA EC ₅₀ (μM)	DAC EC ₅₀ (μM)
A549	4.4	2.5
H460	2.2	4.4
HI299	4.1	0.5

Abbreviations: AZA, azacitidine; DAC, decitabine; NSCLC, non-small cell lung cancer.

Table S2 Top 200 biogroups modulated by azacitidine (AZA) in A549 cells

A549 cells treated with 3 μM AZA (48 hours)		
Biogroup name	Direction	P value
Proteinaceous extracellular matrix	down	3.40E-18
Extracellular matrix	down	4.70E-18
Transcription	up	2.50E-16
Extracellular matrix structural constituent	down	4.90E-16
Glycosaminoglycan binding	down	7.30E-15
Polysaccharide binding	down	9.60E-15
Pattern binding	down	2.80E-14
Lipid biosynthetic process	down	3.60E-14
Fibrillar collagen	down	1.60E-13
Calcium ion binding	down	2.20E-13
Fibrinogen complex	down	7.20E-13
Humoral immune response	down	8.90E-12
Protein binding, bridging	down	9.00E-12
Collagen	down	1.70E-11
Response to wounding	down	6.00E-11
Response to external stimulus	down	1.50E-10
Platelet activation	down	2.20E-10
Ligase activity, forming aminoacyl-tRNA and related compounds	up	3.30E-10
Ligase activity, forming carbon-oxygen bonds	up	3.30E-10
Response to nutrient	up	3.70E-10
Carbohydrate binding	down	3.80E-10
Basement membrane	down	1.00E-09
Lipid metabolic process	down	2.20E-09
Response to nutrient levels	up	2.30E-09
Steroid biosynthetic process	down	2.30E-09
Collagen binding	down	2.40E-09
Response to extracellular stimulus	up	3.70E-09
Inflammatory response	down	7.80E-09
Nucleoplasm	up	8.70E-09
Acute inflammatory response	down	1.30E-08
Response to stress	down	1.50E-08
Blood pressure regulation	down	1.50E-08
RNA binding	up	1.60E-08
Cell motility	down	3.40E-08
Localization of cell	down	3.40E-08
Epithelial cell differentiation	down	4.70E-08
tRNA binding	up	4.80E-08
Steroid metabolic process	down	7.20E-08
Endoplasmic reticulum	down	8.00E-08
Translation	up	1.30E-07
Fatty acid biosynthetic process	down	1.90E-07
Parturition	down	2.50E-07
Sterol metabolic process	down	2.50E-07

(Continued)

Table S2 (Continued)

A549 cells treated with 3 μM AZA (48 hours)		
Biogroup name	Direction	P value
Blood coagulation	down	2.90E-07
Coagulation	down	3.10E-07
Humoral immune response mediated by circulating immunoglobulin	down	3.60E-07
Hemostasis	down	3.60E-07
Organic acid biosynthetic process	down	4.40E-07
Regulation of body fluids	down	6.70E-07
Wound healing	down	6.90E-07
ER-Golgi intermediate compartment	down	8.00E-07
Actin binding	down	9.70E-07
Anion transport	down	1.10E-06
Extracellular structure organization and biogenesis	down	1.10E-06
Transaminase activity	up	1.10E-06
Complement activation	down	1.20E-06
Extracellular matrix organization and biogenesis	down	1.20E-06
Calmodulin binding	down	1.90E-06
Circulation	down	1.90E-06
Female pregnancy	down	2.50E-06
Cellular homeostasis	down	2.60E-06
Morphogenesis of an epithelium	down	2.70E-06
Cell proliferation	down	3.10E-06
Alkene metabolic process	down	3.10E-06
Ribosome biogenesis and assembly	up	3.20E-06
Complement activation, classical pathway	down	3.50E-06
Ribonucleoprotein complex biogenesis and assembly	up	4.90E-06
Sodium:potassium-exchanging ATPase complex	down	5.20E-06
Transferase activity, transferring nitrogenous groups	up	5.80E-06
Cell activation	down	6.10E-06
Endoplasmic reticulum lumen	down	6.60E-06
Fatty acid metabolic process	down	6.90E-06
Vesicular fraction	down	8.20E-06
Cellular ion homeostasis	down	1.10E-05
Cellular chemical homeostasis	down	1.10E-05
Positive regulation of immune system process	down	1.10E-05
Positive regulation of immune response	down	1.10E-05
Phosphoinositide binding	down	1.40E-05
Activation of immune response	down	1.50E-05
Positive regulation of multicellular organismal process	down	1.60E-05
Cofactor transporter activity	up	1.60E-05
Soluble fraction	up	1.70E-05
Enzyme inhibitor activity	down	1.70E-05
Development of primary sexual characteristics	up	1.80E-05
NAD binding	down	1.90E-05
Amine biosynthetic process	up	1.90E-05
Cytoskeleton	down	2.20E-05
Lymphocyte mediated immunity	down	2.40E-05
Receptor binding	down	2.50E-05
Transcription corepressor activity	up	2.50E-05

(Continued)

Table S2 (Continued)**A549 cells treated with 3 μ M AZA (48 hours)**

Biogroup name	Direction	P value
Response to DNA damage stimulus	up	2.60E-05
Cartilage development	down	2.70E-05
SNARE complex	up	2.80E-05
Gastrulation	up	3.20E-05
mRNA transport	up	3.50E-05
Epidermis development	down	3.80E-05
Cell migration	down	4.20E-05
Immune effector process	down	4.20E-05
Response to hypoxia	down	4.30E-05
Leukocyte mediated immunity	down	4.50E-05
Adaptive immune response	down	4.90E-05
Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	down	4.90E-05
Endopeptidase inhibitor activity	down	5.00E-05
Protease inhibitor activity	down	5.00E-05
Oxidoreductase activity, acting on heme group of donors	down	5.30E-05
Oxidoreductase activity, acting on heme group of donors, oxygen as acceptor	down	5.30E-05
Cytochrome-c oxidase activity	down	5.30E-05
Heme-copper terminal oxidase activity	down	5.30E-05
Germ cell migration	up	6.00E-05
Coenzyme binding	down	6.20E-05
Regulation of translation	up	6.90E-05
Cytokine biosynthetic process	up	7.30E-05
Neurotransmitter:sodium symporter activity	up	7.40E-05
Ectoderm development	down	7.50E-05
Establishment of RNA localization	up	8.00E-05
RNA transport	up	8.00E-05
Nucleic acid transport	up	8.00E-05
Transcription factor binding	up	8.30E-05
Regulation of immune response	down	8.50E-05
Regulation of immune system process	down	8.50E-05
Ligase activity	up	8.60E-05
RNA localization	up	9.10E-05
Neurotransmitter transporter activity	up	9.30E-05
RNA export from nucleus	up	9.80E-05
Phospholipid binding	down	9.90E-05
Cell cycle	up	0.0001
Cytosol	down	0.0001
Cytoskeletal protein binding	down	0.0001
Response to endogenous stimulus	up	0.0001
Gonad development	up	0.0001
Nucleobase, nucleoside, nucleotide and nucleic acid transport	up	0.0001
Nucleolus	up	0.0001
Regulation of cytokine biosynthetic process	up	0.0001
Rhythmic process	up	0.0001
Reproductive structure development	up	0.0002
Mitochondrion organization and biogenesis	up	0.0002
Structural constituent of cytoskeleton	down	0.0002
Sex differentiation	up	0.0002
Transcription repressor activity	up	0.0002
Peroxidase activity	down	0.0002
Oxidoreductase activity, acting on peroxide as acceptor	down	0.0002

(Continued)

Table S2 (Continued)**A549 cells treated with 3 μ M AZA (48 hours)**

Biogroup name	Direction	P value
Laminin-I complex	down	0.0002
Transcription cofactor activity	up	0.0002
Female sex differentiation	up	0.0002
Development of primary female sexual characteristics	up	0.0002
Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	down	0.0002
Nitrogen compound biosynthetic process	up	0.0002
Cell structure disassembly during apoptosis	up	0.0003
Amino acid transport	up	0.0003
Acyl-CoA binding	down	0.0003
Response to dsRNA	up	0.0003
Neuron development	down	0.0003
Integrator complex	up	0.0003
Immune response	down	0.0003
Protein dimerization activity	up	0.0004
Laminin complex	down	0.0004
Cofactor binding	down	0.0004
Germ-line sex determination	down	0.0004
Intramolecular oxidoreductase activity	down	0.0004
DNA repair	up	0.0004
Cell soma	down	0.0004
Cellular morphogenesis during differentiation	down	0.0004
RNA polymerase II transcription factor activity	up	0.0005
Regulation of epithelial cell proliferation	down	0.0005
Regulation of biosynthetic process	up	0.0005
UDP-glycosyltransferase activity	up	0.0005
Pyridoxal phosphate binding	up	0.0005
Lipid binding	down	0.0005
Positive regulation of programmed cell death	up	0.0005
Helicase activity	up	0.0006
Cell redox homeostasis	down	0.0006
Cell death	up	0.0006
Death	up	0.0006
Epithelial cell proliferation	down	0.0006
Mesenchymal cell development	down	0.0006
Ovulation	up	0.0006
Positive regulation of locomotion	down	0.0006
Positive regulation of cell motility	down	0.0006
DNA catabolic process	up	0.0006
Cell differentiation	down	0.0006
Basal lamina	down	0.0007
Insulin-like growth factor binding mesenchymal cell differentiation	down	0.0007
Sequestering of metal ion	down	0.0007
Neurotransmitter transport	up	0.0007
Specific RNA polymerase II transcription factor activity	up	0.0007
Intramolecular oxidoreductase activity, transposing C=C bonds	down	0.0007
Cellular component disassembly	up	0.0008
Heme binding	down	0.0008
Tetrapyrrole binding	down	0.0008
Presynaptic active zone	up	0.0008
Amine transport	up	0.0009

(Continued)

Table S2 (Continued)

A549 cells treated with 3 μM AZA (48 hours)		
Biogroup name	Direction	P value
Sequestering of calcium ion	down	0.0009
Cell recognition	down	0.0009
Endoplasmic reticulum part	down	0.0009
Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	down	0.001
Myosin binding	down	0.001
Lyase activity	up	0.001
Transferase activity, transferring hexosyl groups	down	0.001
Neuron differentiation	down	0.001

Notes: Functional groupings of the modulated genes were determined using Gene Ontology classifications in NextBio. The top 200 biogroups most significantly regulated by AZA (at 3 μM) are shown.

Table S3 Top 196 biogroups modulated by decitabine (DAC) in A549 cells

A549 cells treated with 3 μM DAC (48 hours)		
Biogroup name	Direction	P value
Mitosis	down	2.00E-09
Cell cycle	down	2.90E-09
Cell division	down	5.70E-08
Transferase activity, transferring sulfur-containing groups	up	5.80E-08
Meiosis	down	1.50E-07
Meiotic cell cycle	down	1.70E-07
Response to DNA damage stimulus	down	1.80E-07
Male gamete generation	up	6.10E-07
Response to endogenous stimulus	down	7.60E-07
Chromosome segregation	down	2.10E-06
Aromatic compound metabolic process	up	2.70E-06
Phenol metabolic process	up	2.70E-06
Structural constituent of cytoskeleton	up	5.10E-06
Sister chromatid cohesion	down	5.90E-06
Cellular lipid catabolic process	up	6.70E-06
DNA repair	down	7.20E-06
Alkali metal ion binding	up	9.00E-06
Regulation of neurotransmitter levels	up	1.10E-05
DNA damage response, signal transduction	down	1.30E-05
Cofactor transporter activity	up	1.60E-05
Sulfotransferase activity	up	1.70E-05
Intermediate filament	up	3.20E-05
Neurotransmitter:sodium symporter activity	up	3.40E-05
Chromatin assembly	down	3.90E-05
Neurotransmitter transporter activity	up	4.60E-05
Cytokinesis	down	5.10E-05
Chromosome	down	5.30E-05
Mitotic spindle organization and biogenesis	down	5.30E-05
Negative regulation of enzyme activity	up	5.60E-05
Establishment of mitotic spindle localization	down	6.50E-05
Establishment of spindle localization	down	6.50E-05
Spindle localization	down	6.50E-05
Retinol binding	up	6.50E-05
Microtubule organizing center part	down	7.60E-05
Mitotic sister chromatid segregation	down	8.30E-05
Alcohol metabolic process	up	8.60E-05

(Continued)

Table S3 (Continued)

A549 cells treated with 3 μM DAC (48 hours)		
Biogroup name	Direction	P value
Sister chromatid segregation	down	9.30E-05
Catabolic process	up	0.0001
Soluble fraction	up	0.0001
Retinal binding	up	0.0001
Positive regulation of programmed cell death	up	0.0001
Steroid biosynthetic process	up	0.0002
Response to stress	down	0.0002
Gamma-tubulin complex	down	0.0002
Mitotic chromosome condensation	down	0.0002
Transporter activity	up	0.0002
Phosphopyruvate hydratase complex	up	0.0002
Amino acid derivative metabolic process	up	0.0003
Vitamin binding	up	0.0003
Lipid catabolic process	up	0.0003
Nuclear chromosome	down	0.0003
Retinoid binding	up	0.0003
Isoprenoid binding	up	0.0003
Homologous chromosome segregation	down	0.0003
Meiotic chromosome segregation	down	0.0003
Meiotic spindle organization and biogenesis	down	0.0003
Cell differentiation	up	0.0003
NADP binding	down	0.0004
Steroid metabolic process	up	0.0004
Lipid raft	up	0.0004
Cohesin complex	down	0.0004
Meiosis I	down	0.0004
Sodium:potassium-exchanging ATPase complex	up	0.0004
Negative regulation of cell proliferation	up	0.0004
Actin binding	down	0.0005
Nuclear matrix	down	0.0005
Cytoskeletal protein binding	down	0.0005
Protein kinase inhibitor activity	up	0.0005
Cell proliferation	up	0.0006
Cytoskeleton	down	0.0006
Cytoskeleton organization and biogenesis	down	0.0006
Fat cell differentiation	down	0.0006
Hormone metabolic process	up	0.0006
Positive regulation of progression through cell cycle	down	0.0006
Kinase inhibitor activity	up	0.0006
Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	down	0.0007
Neurotransmitter transport	up	0.0007
Membrane invagination	down	0.0008
Endocytosis	down	0.0008
Amide metabolic process	up	0.0008
Spindle	down	0.0008
Ion transport	up	0.0009
Blastocyst growth	down	0.0009
Interleukin binding	down	0.0009
RNA export from nucleus	down	0.0009
Tubulin binding	down	0.0009
Epidermis development	up	0.0009
Neurotransmitter metabolic process	up	0.0011
Translation activator activity	up	0.0011
Spindle pole	down	0.0011
Synaptic transmission	up	0.0012
Intracellular cyclic nucleotide activated cation channel complex	up	0.0012

(Continued)

Table S3 (Continued)**A549 cells treated with 3 μ M DAC (48 hours)**

Biogroup name	Direction	P value
Biogenic amine metabolic process	up	0.0012
Cell fate determination	up	0.0013
Oxidoreductase activity, acting on iron-sulfur proteins as donors	up	0.0013
Ion channel activity	up	0.0013
Lipoprotein binding	down	0.0014
Positive regulation of neurogenesis	down	0.0014
Cytosol	up	0.0018
Microtubule organizing center	down	0.002
Microtubule	down	0.002
Glutathione peroxidase activity	up	0.0021
Odontogenesis	down	0.0022
Passive transmembrane transporter activity	up	0.0022
Transmission of nerve impulse	up	0.0023
Oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor	down	0.0024
Dynein binding	down	0.0024
Humoral immune response	down	0.0024
Ectoderm development	up	0.0025
Arginine metabolic process	up	0.0025
Myosin binding	down	0.0025
Lipid biosynthetic process	up	0.0026
Muscle contraction	up	0.0027
Mitochondrion organization and biogenesis	up	0.0027
Fat-soluble vitamin metabolic process	up	0.0028
Female gamete generation	down	0.0028
Urea cycle intermediate metabolic process	up	0.0029
Inclusion body	down	0.0029
Folic acid transporter activity	down	0.0029
Protein heterodimerization activity	down	0.003
Angiogenesis	up	0.003
Replication fork	down	0.0031
Nucleoside metabolic process	down	0.0031
Regulation of axonogenesis	down	0.0032
Anatomical structure formation	up	0.0033
Protein kinase regulator activity	up	0.0034
Lipid metabolic process	up	0.0037
Glycoprotein binding	up	0.0037
Pyridoxal phosphate binding	up	0.0037
Blood vessel morphogenesis	up	0.004
Carbohydrate metabolic process	up	0.0041
Tissue regeneration	up	0.0041
Regeneration	up	0.0041
Germ cell development	up	0.0042
Growth factor binding	down	0.0042
Peptide transporter activity	up	0.0043
Nitrogen compound biosynthetic process	up	0.0044
Cytokine binding	down	0.0044
Nitric oxide metabolic process	up	0.0047
Nitric oxide biosynthetic process	up	0.0047
Centrosome	down	0.0047
Embryonic morphogenesis	down	0.0048
Regulation of neurogenesis	down	0.0048
Oxidoreductase activity, acting on the aldehyde or oxo group of donors	up	0.0048
Cytokinesis during cell cycle	down	0.0049
Cell-cell signaling	up	0.005
Calmodulin binding	up	0.005

(Continued)

Table S3 (Continued)**A549 cells treated with 3 μ M DAC (48 hours)**

Biogroup name	Direction	P value
Structure-specific DNA binding	down	0.0051
Oxidoreductase activity, acting on the CH-CH group of donors	down	0.0051
Peroxidase activity	up	0.0051
Oxidoreductase activity, acting on peroxide as acceptor	up	0.0051
Microfibril	up	0.0052
Protein-DNA complex assembly	down	0.0052
Vasculature development	up	0.0054
Excretion	up	0.0055
mRNA transport	down	0.0056
Identical protein binding	up	0.0056
Vitamin transporter activity	down	0.0057
Response to organic cyclic substance	up	0.0059
Response to alkaloid	up	0.0059
Kinase regulator activity	up	0.006
Chromatin	down	0.006
Electron carrier activity	up	0.0061
Vitamin biosynthetic process	down	0.0062
RNA transport	down	0.0067
Nucleic acid transport	down	0.0067
Establishment of RNA localization	down	0.0067
Protein domain specific binding	up	0.0068
Homophilic cell adhesion	down	0.0068
RNA localization	down	0.0069
Hormone biosynthetic process	up	0.007
Protein dimerization activity	down	0.0071
RNA binding	down	0.0073
Blastocyst development	down	0.0074
Cyclin binding	up	0.0075
Nucleobase, nucleoside, nucleotide and nucleic acid transport	down	0.0077
Cartilage development	down	0.0077
Folic acid binding	down	0.0079
Positive regulation of developmental process	down	0.0081
Chordate embryonic development	down	0.0082
NAD binding	up	0.0082
Cofactor binding	up	0.0082
Vesicle docking during exocytosis	up	0.0084
Developmental maturation	up	0.0085
Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amidines	up	0.0086
Lysosome	up	0.0086
Embryonic digit morphogenesis	down	0.0086
DNA helicase activity	down	0.0089
Axon guidance	down	0.0091
Membrane docking	up	0.0093
Vesicle docking	up	0.0093
Voltage-gated sodium channel complex	down	0.0093
mRNA binding	up	0.0094
Establishment of organelle localization	down	0.0096
Vitamin metabolic process	up	0.0096
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	down	0.0099

Notes: Functional groupings of the modulated genes were determined using Gene Ontology classifications in NextBio. The top 196 biogroups most significantly regulated by DAC (at 3 μ M) are shown.

Table S4 Top 200 biogroups modulated by azacitidine (AZA) in H1299 cells

H1299 cells treated with 3 μM AZA (48 hours)		
Biogroup name	Direction	P value
Transcription	up	1.90E-25
Cell cycle	down	7.60E-25
Mitosis	down	8.00E-24
Cell division	down	1.00E-22
Cytoskeleton	down	5.60E-14
Microtubule	down	1.30E-13
Spindle	down	1.80E-13
Mitochondrion	down	1.80E-12
Sterol metabolic process	down	1.40E-11
Chromosome	down	1.20E-10
Alcohol metabolic process	down	2.20E-10
Ligase activity	up	2.40E-10
Lipid biosynthetic process	down	2.70E-10
Steroid biosynthetic process	down	3.00E-10
Mitotic sister chromatid segregation	down	1.90E-09
Steroid metabolic process	down	2.50E-09
Sister chromatid segregation	down	2.90E-09
Endoplasmic reticulum	down	3.60E-09
Envelope	down	4.70E-09
Lipid metabolic process	down	2.60E-08
Response to nutrient	down	7.00E-08
Collagen binding	down	1.60E-07
Centrosome	down	1.70E-07
Wound healing	down	1.90E-07
Response to nutrient levels	down	2.00E-07
Intramolecular oxidoreductase activity	down	2.50E-07
Response to extracellular stimulus	down	2.70E-07
Mitochondrial membrane	down	2.80E-07
Microtubule organizing center	down	2.80E-07
Acid-amino acid ligase activity	up	3.10E-07
Cell proliferation	down	3.60E-07
Blood coagulation	down	3.70E-07
Establishment of chromosome localization	down	4.40E-07
Coagulation	down	4.90E-07
Chromosome segregation	down	5.00E-07
Nitrogen compound catabolic process	down	5.20E-07
Kinase binding	up	5.30E-07
Beta-catenin binding	down	5.50E-07
Nucleoplasm	up	6.10E-07
Enzyme inhibitor activity	down	8.80E-07
Alcohol catabolic process	down	9.80E-07
Hemostasis	down	1.10E-06
Transcription cofactor activity	up	1.10E-06
Transcription repressor activity	up	1.20E-06
Midbody	down	1.30E-06
Ligase activity, forming carbon-nitrogen bonds	up	1.70E-06
Establishment of organelle localization	down	2.00E-06
Germ-line sex determination	down	2.00E-06
Oligosaccharyl transferase complex	down	2.10E-06
Response to external stimulus	down	2.20E-06
Oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor	down	2.20E-06
Amine catabolic process	down	2.20E-06
Cytoskeleton organization and biogenesis	down	2.20E-06
Cofactor binding	down	2.30E-06

(Continued)

Table S4 (Continued)

H1299 cells treated with 3 μM AZA (48 hours)		
Biogroup name	Direction	P value
Coenzyme binding	down	2.70E-06
Transcription corepressor activity	up	3.00E-06
Cell differentiation	up	3.20E-06
mRNA binding	down	3.30E-06
Meiotic chromosome segregation	down	3.60E-06
Homologous chromosome segregation	down	3.60E-06
Nuclear envelope-endoplasmic reticulum network	down	3.90E-06
Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	down	5.00E-06
Intramolecular oxidoreductase activity, transposing C=C bonds	down	5.30E-06
Mitotic chromosome condensation	down	5.50E-06
Endoplasmic reticulum part	down	5.50E-06
Transcription factor binding	up	5.60E-06
Organic acid transport	up	5.70E-06
Carboxylic acid transport	up	5.70E-06
Acyl-CoA binding	down	5.70E-06
DNA-directed RNA polymerase II, holoenzyme	up	5.80E-06
Interphase of mitotic cell cycle	down	6.00E-06
Primary sex determination	down	6.00E-06
Cell-matrix adhesion	down	6.20E-06
Hormone activity	down	6.30E-06
Organic acid transmembrane transporter activity	up	6.30E-06
Mitochondrial inner membrane	down	7.30E-06
Cell-substrate adhesion	down	7.60E-06
Transcription activator activity	up	7.70E-06
Mitotic spindle organization and biogenesis	down	7.90E-06
Lyase activity	down	8.10E-06
Sterol transport	down	8.70E-06
Arginine metabolic process	down	8.70E-06
Chromatin assembly	down	1.00E-05
Nitrogen compound biosynthetic process	down	1.10E-05
RNA polymerase II transcription factor activity	up	1.10E-05
Transaminase activity	up	1.20E-05
Meiotic spindle organization and biogenesis	down	1.30E-05
Organelle localization	down	1.40E-05
Isomerase activity	down	1.40E-05
Interphase	down	1.40E-05
Urea cycle intermediate metabolic process	down	1.50E-05
Fatty acid biosynthetic process	down	1.70E-05
Receptor binding	down	1.70E-05
Regulation of body fluids	down	1.80E-05
Condensin complex	down	1.80E-05
Regulation of coagulation	down	1.90E-05
Nuclear envelope	down	2.20E-05
Caveola	down	2.30E-05
Organic acid biosynthetic process	down	2.30E-05
Meiotic cell cycle	down	2.30E-05
Amino acid transport	up	2.50E-05
NADP binding	down	2.80E-05
Protein dimerization activity	up	2.90E-05
Spindle pole	down	3.40E-05

(Continued)

Table S4 (Continued)

H1299 cells treated with 3 μ M AZA (48 hours)		
Biogroup name	Direction	P value
Ubiquitin–protein ligase activity	up	3.50E-05
Transferase activity, transferring nitrogenous groups	up	3.60E-05
Nucleoside metabolic process	down	3.80E-05
Structural constituent of cytoskeleton	down	3.90E-05
Carbohydrate catabolic process	down	4.10E-05
Endoplasmic reticulum membrane	down	4.30E-05
Nucleosome	down	4.40E-05
Nucleotide catabolic process	down	4.60E-05
Cellular chemical homeostasis	down	4.70E-05
Cellular ion homeostasis	down	4.70E-05
Cytokinesis	down	4.90E-05
Muscle cell differentiation	up	5.00E-05
Myeloid cell differentiation	up	5.20E-05
Catabolic process	down	5.20E-05
Oxygen and reactive oxygen species metabolic process	down	5.20E-05
Chromatin	down	5.30E-05
Epidermis development	down	5.40E-05
Oxidoreductase activity, acting on the CH–NH group of donors	down	5.60E-05
SNARE complex	up	5.60E-05
Ligase activity, forming carbon–oxygen bonds	up	5.70E-05
Ligase activity, forming aminoacyl–tRNA and related compounds	up	5.70E-05
Soluble fraction	up	5.80E-05
Endopeptidase inhibitor activity	down	5.90E-05
Protease inhibitor activity	down	5.90E-05
Small protein conjugating enzyme activity	up	6.00E-05
AP-type membrane coat adaptor complex	down	6.40E-05
Cell–cell signaling	down	6.70E-05
Amine transport	up	6.80E-05
Response to DNA damage stimulus	up	7.20E-05
Male sex determination	down	7.20E-05
Cell–cell adhesion	down	8.00E-05
Protein heterodimerization activity	down	8.00E-05
Enzyme binding	up	8.40E-05
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	down	9.10E-05
Ectoderm development	down	9.40E-05
Positive regulation of progression through cell cycle	down	0.0001
One-carbon compound metabolic process	down	0.0001
Heterogeneous nuclear ribonucleoprotein complex	down	0.0001
Response to stress	down	0.0001
Response to endogenous stimulus	up	0.0001
Cell death	up	0.0001
Death	up	0.0001
Meiosis	down	0.0001
Dioxygenase activity	up	0.0001
Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	up	0.0001
Isoprenoid biosynthetic process	down	0.0001

(Continued)

Table S4 (Continued)

H1299 cells treated with 3 μ M AZA (48 hours)		
Biogroup name	Direction	P value
Carbon–carbon lyase activity	down	0.0001
Lipid digestion	down	0.0001
Nitric oxide metabolic process	down	0.0001
Nitric oxide biosynthetic process	down	0.0001
Cellular homeostasis	down	0.0001
Positive regulation of locomotion	down	0.0001
Positive regulation of cell motility	down	0.0001
Sulfur compound biosynthetic process	up	0.0001
Proton-transporting ATP synthase complex, catalytic core F(1)	down	0.0001
Mitochondrial proton-transporting ATP synthase complex	down	0.0002
Muscle development	up	0.0002
Integrator complex	up	0.0002
Caspase inhibitor activity	down	0.0002
Fatty acid binding	down	0.0002
Isoprenoid metabolic process	down	0.0002
Blood vessel morphogenesis	up	0.0002
Vasculogenesis	up	0.0002
Kinetochore	down	0.0002
Low-density lipoprotein binding	down	0.0002
Cell structure disassembly during apoptosis	up	0.0002
Endoplasmic reticulum lumen	down	0.0002
Negative regulation of multicellular organismal process	down	0.0002
Intestinal absorption	down	0.0002
Organelle outer membrane	down	0.0002
Proton-transporting two-sector ATPase complex, catalytic domain	down	0.0002
Regulation of transforming growth factor beta receptor signaling pathway	down	0.0002
Clathrin adaptor complex	down	0.0002
Fatty acid metabolic process	down	0.0003
Replication fork	down	0.0003
Lipoprotein binding	down	0.0003
Insemination	up	0.0003
Behavior	down	0.0003
Leukocyte differentiation	up	0.0003
Single-stranded RNA binding	down	0.0003
Histone acetyltransferase complex	up	0.0003
Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	up	0.0003
Hemopoiesis	up	0.0003
Outer kinetochore of condensed chromosome	down	0.0003
Response to virus	up	0.0003
Vasculature development	up	0.0003
DNA-directed RNA polymerase complex	up	0.0003
Perinuclear region of cytoplasm	down	0.0003
Sterol binding	down	0.0003
Generation of precursor metabolites and energy	down	0.0003
Copulation	up	0.0004
Spindle localization	down	0.0004

(Continued)

Table S4 (Continued)

H1299 cells treated with 3 μM AZA (48 hours)		
Biogroup name	Direction	P value
Establishment of mitotic spindle localization	down	0.0004
Establishment of spindle localization	down	0.0004
Neural crest cell development	down	0.0004
GTPase inhibitor activity	down	0.0004

Notes: Functional groupings of the modulated genes were determined using Gene Ontology classifications in NextBio. The top 200 biogroups most significantly regulated by AZA (at 3 μM) are shown.

Table S5 Top 200 biogroups modulated by decitabine (DAC) in H1299 cells

H1299 cells treated with 3 μM DAC (48 hours)		
Biogroup name	Direction	P value
Cofactor binding	down	4.70E-12
Lipid metabolic process	down	5.10E-12
Cell differentiation	up	2.50E-08
Coenzyme binding	down	3.70E-08
Transcription	up	1.00E-07
Inner ear development	up	2.20E-07
Cell fate determination	up	2.50E-07
Fatty acid metabolic process	down	2.90E-07
Collagen binding	up	4.90E-07
Oxidoreductase activity, acting on the CH–OH group of donors, NAD or NADP as acceptor	down	1.20E-06
Enzyme inhibitor activity	up	1.30E-06
Nucleosome	up	1.30E-06
Aldehyde metabolic process	down	1.40E-06
Sensory organ development	up	2.30E-06
Oxidoreductase activity, acting on CH–OH group of donors	down	2.50E-06
Response to external stimulus	up	2.80E-06
Hormone biosynthetic process	up	3.00E-06
CoA–ligase activity	down	4.00E-06
Insulin-like growth factor binding	up	4.20E-06
Response to stress	up	4.40E-06
Mitochondrion	down	5.20E-06
Acid–thiol ligase activity	down	6.30E-06
Proteinaceous extracellular matrix	up	7.40E-06
Transcription repressor activity	up	7.90E-06
Extracellular matrix	up	8.60E-06
Muscle cell differentiation	up	9.40E-06
Response to wounding	up	9.50E-06
Alcohol metabolic process	down	9.90E-06
Enzyme regulator activity	up	9.90E-06
Neurotransmitter metabolic process	up	1.00E-05
Muscle fiber development	up	1.10E-05
Skeletal muscle fiber development	up	1.10E-05
Peroxisome	down	1.10E-05
Microbody	down	1.10E-05
Ligase activity, forming carbon-sulfur bonds	down	1.40E-05
Regulation of epidermis development	up	2.00E-05
Cell fate commitment	up	2.20E-05
Hormone metabolic process	up	2.20E-05
Sterol metabolic process	down	2.30E-05
Inflammatory response	up	2.30E-05

(Continued)

Table S5 (Continued)

H1299 cells treated with 3 μM DAC (48 hours)		
Biogroup name	Direction	P value
Oxidoreductase activity, acting on the CH–CH group of donors, NAD or NADP as acceptor	down	2.60E-05
Death	up	3.20E-05
Cell death	up	3.20E-05
Steroid metabolic process	down	3.50E-05
Lipid biosynthetic process	down	3.80E-05
Lyase activity	down	4.10E-05
Glycosaminoglycan binding	up	4.50E-05
Polysaccharide binding	up	5.30E-05
Pyridoxal phosphate binding	down	5.50E-05
Muscle development	up	6.70E-05
Nitrogen compound biosynthetic process	down	6.90E-05
Protease inhibitor activity	up	7.60E-05
Endopeptidase inhibitor activity	up	7.60E-05
Phosphatase activator activity	up	8.00E-05
Phenol metabolic process	up	8.40E-05
Epidermis development	up	8.50E-05
Regulation of neurotransmitter levels	up	9.20E-05
Pattern binding	up	9.60E-05
Cofactor catabolic process	down	9.80E-05
Positive regulation of developmental process	up	1.00E-04
Vitamin binding	down	0.0001
Oxidoreductase activity, acting on the CH–CH group of donors	down	0.0001
Amino acid derivative metabolic process	up	0.0001
Chromatin assembly	up	0.0001
Protein–DNA complex assembly	up	0.0001
Amino acid derivative biosynthetic process	up	0.0001
Positive regulation of cell differentiation	up	0.0001
Acute inflammatory response	up	0.0001
Dopamine metabolic process	up	0.0001
Growth factor binding	up	0.0002
Endothelial cell development	down	0.0002
Transcription corepressor activity	up	0.0002
Keratinocyte differentiation	up	0.0002
Ectoderm development	up	0.0002
Cellular respiration	down	0.0002
RNA polymerase II transcription elongation factor activity	up	0.0002
Angiogenesis	up	0.0003
Calcium-dependent phospholipid binding	down	0.0003
Suckling behavior	down	0.0003
Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	down	0.0003
Germ-line sex determination	down	0.0003
RNA polymerase II transcription factor activity	up	0.0003
Protein kinase inhibitor activity	up	0.0004
Translation activator activity	up	0.0004
Regulation of Notch signaling pathway	up	0.0004
Fatty acid biosynthetic process	down	0.0004
Kinase inhibitor activity	up	0.0004
Regulation of cell differentiation	up	0.0004
ER–Golgi intermediate compartment	up	0.0005
UDP–glycosyltransferase activity	down	0.0005
Cell maturation	up	0.0005

(Continued)

Table S5 (Continued)

H1299 cells treated with 3 μ M DAC (48 hours)		
Biogroup name	Direction	P value
Cell surface	down	0.0005
Inner ear receptor cell fate commitment	up	0.0005
Organic acid biosynthetic process	down	0.0005
Negative regulation of signal transduction	up	0.0006
Hydro-Lyase activity	down	0.0006
Epidermal cell differentiation	up	0.0007
Inner ear morphogenesis	up	0.0007
Regulation of cell growth	up	0.0007
Response to bacterium	up	0.0007
Blood vessel morphogenesis	up	0.0007
Carbohydrate metabolic process	down	0.0007
Lipoprotein binding	down	0.0007
Multicellular organismal movement	down	0.0008
Aromatic compound metabolic process	up	0.0008
Amine biosynthetic process	down	0.0008
tRNA binding	down	0.0008
Cell migration	up	0.0008
Transcription elongation factor complex	up	0.0009
Glutathione peroxidase activity	up	0.0009
Oxidoreductase activity, acting on the CH–NH group of donors, NAD or NADP as acceptor	down	0.0009
Primary sex determination	down	0.0009
Negative regulation of cell differentiation	up	0.001
Defense response to bacterium	up	0.001
Nuclear envelope	down	0.001
Ear morphogenesis	up	0.0011
Ligase activity, forming carbon–oxygen bonds	down	0.0011
Ligase activity, forming aminoacyl–tRNA and related compounds	down	0.0011
Generation of precursor metabolites and energy	down	0.0011
Developmental maturation	up	0.0011
Peripheral nervous system development	down	0.0011
Extracellular matrix structural constituent	up	0.0012
Oxidoreductase activity, acting on the aldehyde or oxo group of donors	down	0.0012
Biogenic amine metabolic process	up	0.0012
Epidermis morphogenesis	up	0.0013
Endothelial cell differentiation	down	0.0013
Endoplasmic reticulum	down	0.0013
Carbon–oxygen lyase activity	down	0.0013
Germ cell development	up	0.0014
Cell proliferation	down	0.0015
Peroxidase activity	up	0.0015
Oxidoreductase activity, acting on peroxide as acceptor	up	0.0015
Specific RNA polymerase II transcription factor activity	up	0.0015
Positive regulation of programmed cell death	up	0.0016
Response to nutrient levels	down	0.0016
Myeloid cell differentiation	up	0.0016
Lung development	up	0.0017
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	down	0.0017
Vasculature development	up	0.0017

(Continued)

Table S5 (Continued)

H1299 cells treated with 3 μ M DAC (48 hours)		
Biogroup name	Direction	P value
Respiratory tube development	up	0.0018
Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	down	0.0018
Dioxygenase activity	down	0.0018
Negative regulation of enzyme activity	up	0.0019
Odontogenesis	up	0.0019
Positive regulation of locomotion	down	0.0019
Positive regulation of cell motility	down	0.0019
Cell cycle	up	0.0019
Anatomical structure formation	up	0.002
Cell growth	up	0.0021
Regulation of cell size	up	0.0021
NAD binding	down	0.0021
Steroid biosynthetic process	down	0.0022
Response to extracellular stimulus	down	0.0022
Endocytosis	down	0.0023
Membrane invagination	down	0.0023
Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	down	0.0025
Musculoskeletal movement	down	0.0025
Brain development	up	0.0026
Immune system development	up	0.0027
Negative regulation of cell growth	down	0.0028
Negative regulation of cell size	down	0.0028
Envelope	down	0.0029
Negative regulation of developmental process	up	0.0029
Chromatin	up	0.003
mRNA binding	up	0.0032
Antioxidant activity	up	0.0033
Selenium binding	up	0.0035
Receptor signaling protein serine/threonine kinase activity	down	0.0036
Outer membrane-bounded periplasmic space	down	0.0036
Cell envelope	down	0.0036
Axon	up	0.0036
Transferase activity, transferring hexosyl groups	down	0.0038
Energy derivation by oxidation of organic compounds	down	0.0038
Response to reactive oxygen species	up	0.0038
Meiosis	up	0.0038
Meiotic cell cycle	up	0.0038
Enzyme activator activity	up	0.0039
Hemopoiesis	up	0.0043
Positive regulation of biosynthetic process	up	0.0043
Regulation of transforming growth factor beta receptor signaling pathway	down	0.0043
FAD binding	down	0.0044
Negative regulation of growth	down	0.0045
Defense response to Gram-positive bacterium	up	0.0048
Sensory perception of light stimulus	down	0.0048
Catabolic process	down	0.0049
Hemopoietic or lymphoid organ development	up	0.005
Positive regulation of protein metabolic process	up	0.0051
Intermediate filament	up	0.0051
Ras GTPase activator activity	down	0.0052

(Continued)

Table S5 (Continued)

HI299 cells treated with 3 μM DAC (48 hours)		
Biogroup name	Direction	P value
Response to nutrient	down	0.0053
Protein kinase regulator activity	up	0.0053
Carbohydrate binding	up	0.0054
Calcium ion binding	down	0.0055
Oxidoreductase activity, acting on sulfur group of donors	down	0.0056
Response to hypoxia	down	0.0057
Transferase activity, transferring aldehyde or ketonic groups	up	0.0058
Positive regulation of multicellular organismal process	up	0.0059
Xenobiotic transporter activity	up	0.0063
Xenobiotic-transporting ATPase activity	up	0.0063
Phospholipase inhibitor activity	up	0.0064

Notes: Functional groupings of the modulated genes were determined using Gene Ontology classifications in NextBio. The top 200 biogroups most significantly regulated by DAC (at 3 μ M) are shown.

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