Preclinical Development

Combinations of DNA Methyltransferase and Histone Deacetylase Inhibitors Induce DNA Damage in Small Cell Lung Cancer Cells: Correlation of Resistance with IFN-Stimulated Gene Expression

Wioleta Luszczek, Venugopalan Cheriyath, Tarek M. Mekhail, and Ernest C. Borden

Abstract

Because epigenetic inhibitors can reduce cancer cell proliferation, we tested the hypothesis that concurrent inhibition of histone acetylation and DNA methylation could synergistically reduce the viability of small cell lung cancer (SCLC) cells. Sub-IC$_{50}$ concentrations of the DNA methyltransferase (DNMT) inhibitor decitabine (5-AZA-dC) and the histone deacetylase (HDAC) inhibitors (LBH589 or MGCD0103) synergistically reduced the proliferation of five of nine SCLC cell lines. Loss of viability of sensitive SCLC cells did not correlate with the inhibition of either DNMT1 or HDACs, suggesting nonepigenetic mechanisms for synergy between these two classes of epigenetic modulators. Because combinations of 5-AZA-dC and HDAC inhibitors had marginal effects on the apoptosis index, Comet assay was undertaken to assess DNA damage. MGCD0103 and 5AZA-dC cotreatment augmented DNA damage in SCLC cells, resulting in increased tail length and moment in Comet assays by 24 hours in sensitive cell lines ($P < 0.01$). Consistent with augmented DNA damage, combination of a DNMT and HDAC inhibitor markedly increased the levels of phospho-H2A.X in sensitive cells but not in resistant ones. Comparison of basal gene expression between resistant and sensitive cells identified markedly higher basal expression of IFN-stimulated genes in the resistant cell lines, suggesting that IFN-stimulated gene expression may determine SCLC cell sensitivity to epigenetic modulators or other DNA damaging agents. 

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Introduction

Epigenetic inactivation of genes essential for normal cell growth control is a frequent event in carcinogenesis. These epigenetic mechanisms involve cross-talk between DNA methylation and histone modifications that regulate gene transcription (1, 2). The ability of DNA-demethylating agents and histone deacetylase (HDAC) inhibitors to reverse epigenetic changes has stimulated interest in them as anticancer drugs (3–6).

Decitabine (5-AZA-dC) is a potent and specific inhibitor of a maintenance DNA methyltransferase (DNMT1), leading to reduction in DNA methylation and activation of the expression of transcriptionally silenced genes (1–3, 7, 8). This in turn can result in the induction of tumor cell differentiation, cell cycle arrest, DNA damage, and apoptosis (9–12). Acetylation of histones affects the transcription of genes by modification of chromatin structure and is dependent on the contrasting actions of histone acetyltransferase and HDAC (13). Like DNMT inhibitors, HDAC inhibitors exhibit their antineoplastic effects by enhancing differentiation, apoptosis, growth inhibition, cell cycle arrest, and cell death through DNA damage by transcriptional modulation of antiapoptotic and proapoptotic genes (14–18).

Combining 5-AZA-dC with the HDAC inhibitor trichostatin A or phenylbutyrate synergistically restored mRNA expression of silenced tumor suppressor genes (19). In studies using a human lung carcinoma cell line, the combination resulted in greater inhibition of DNA synthesis and loss of clonogenicity than with either drug alone (20). Sequential treatment of lung cancer cells with 5-AZA-dC and the HDAC inhibitors depsipeptide or trichostatin A markedly enhanced apoptosis induced by HDAC inhibitors (21). 5-AZA-dC can also initiate cytotoxicity by its incorporation into DNA with the induction of DNA damage (10, 22, 23). When incorporated into DNA, 5-AZA-dC covalently links with DNMT causing DNA damage either by structural instability at its incorporation site or by obstructing DNA synthesis (23). The mechanism underlying HDAC inhibitor-induced DNA damage is still not entirely understood. Possibly, it occurs through changes in chromatin structure and is associated

Authors’ Affiliation: Translational Hematology/Oncology Research, Taussig Cancer Institute, The Cleveland Clinic, Cleveland, Ohio

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Current address for T.M. Mekhail: Hematology and Oncology/Florida Hospital Cancer Institute, Orlando, FL.

Corresponding Author: Ernest C Borden, Taussig Cancer Institute, The Cleveland Clinic, 9500 Euclid Avenue/R40, Cleveland, OH 44195. Phone: 216 444-8183; Fax: 216 636-2498. E-mail: bordene@ccf.org

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with the acetylation of histone proteins (15). Moreover, HDAC inhibitors may suppress the removal of the incorporated 5-AZA-dC from DNA and significantly increase DNA damage induced by 5-AZA-dC (10).

We thus investigated whether a methylation inhibitor in combination with HDAC inhibitors might have potentiating effects on cell death of human small cell lung cancer (SCLC) cell lines. We have identified a synergistic

Figure 1. Effect of 5-AZA-dC and HDAC inhibitors on SCLC cells viability. Cells were treated with increasing concentrations of (A) 5-AZA-dC or LBH589 alone and in combination, and (B) 5-AZA-dC or MGCD0103 alone or in combination for 72 h. Graphs represent viability assay results from an experiment done with six replicates and with subsequent repeat. The repeat experiment also yielded similar results. Points, mean; bars, SEM. Method of Chou-Talalay was used to assess combination index (CI) for synergistic effect.
effect of 5-AZA-dC and HDAC inhibitors, one isotype selective for HDAC class and one pan inhibitory, in the reduction of SCLC cells viability. This resulted primarily from DNA damage rather than apoptosis. Unexpected was a correlation between resistance to the synergistic effects of the combination and a basal expression pattern of IFN-stimulated genes (ISG).

**Materials and Methods**

**Cell lines and culture conditions**

Human SCLC cell lines H82, H146, H196, H526, DMS114 SW1271, H1688, H1048, and H2195 (American Type Culture Collection) were cultured in 5% CO₂ using RPMI 1640 with 2 mmol/L L-glutamine, adjusted to...
contain 1.5 g/L sodium bicarbonate, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 10% fetal bovine serum, penicillin G (50 U/mL), and streptomycin (50 μg/mL; Cellgro). H1048 and H2195 cells were maintained in DMEM/F12 50:50 medium containing 10% fetal bovine serum; insulin, transferrin, and selenium; 10 mmol/L estradiol; 10 mmol/L hydrocortisone; 1.5 g/L sodium bicarbonate; 10 mmol/L HEPES; additional 2 mmol/L of 1-glutamine; and antibiotics.

Drug treatments
5-AZA-dC (Sigma; 1 mmol/L) stock solution in DMSO and working solution for one-time use were stored at −20°C. The isotype-selective HDAC inhibitor (MGCD0103) was provided by Methylgene and stored as a 30-mmol/L stock in DMSO at −20°C, and pan-HDAC inhibitor (LBH589) from Novartis was stored at −80°C. Cisplatin (CDDP; APP Pharmaceuticals, LLC) was stored at 4°C. Drugs were added fresh to medium. Different concentrations and different time courses of treatment (24, 48, and 72 h) were tested.

Viability assay
Viability of SCLC cells was assessed using the Alamar Blue assay (Invitrogen). For the Alamar blue assay, SCLC cells were seeded in 96-well plates at a density of 3 × 10³ cells per well and incubated for 72 hours with various concentrations of 5-AZA-dC, LBH589, MGCD0103, or CDDP alone or in combination. At the end of incubation, the viability of cells was measured according to the manufacturer’s protocol. Cell numbers before and after the treatment were determined by generating standard curves with known numbers of nontreated cells. An IC₅₀ value was calculated based on the viability curve. The multiple drug effect analysis of Chou and Talalay (24) was used to determine the pharmacologic interaction between 5-AZA-dC and HDAC inhibitors. This analysis defines synergism or antagonism by determining how much the combination effect differs from the expected additive effect of the two therapeutic agents. A combination index (CI) of <1 is consistent with synergy, and a CI of >1 is consistent with antagonism.

Quantification of apoptotic, necrotic, and healthy cells
For quantification of apoptotic, necrotic, and healthy cells, cells were seeded in six-well plates at a concentration of 2.5 × 10⁵ per well and treated as described above. After 72 hours, cells were harvested, washed with 1× PBS, and stained with Annexin V, Ethidium Homodimer III, and Hoechst 33342 (Apoptotic & Necrotic & Healthy Cells Quantification Kit, Biotium, Inc.). Apoptotic cells were stained green; necrotic cells were stained red; and healthy cells were stained blue. Slides were examined by fluorescence microscopy. Total number of cells, necrotic cells, and apoptotic cells were counted in 10 fields then averaged. Percentage of live cells or fold change of dead cells was evaluated compared with nontreated cells. Fold change was calculated as follows: % of nonviable cells in treated sample/% of nonviable cells in nontreated sample.

Cell cycle profile
Cell cycle analysis was done by propidium iodide staining using FACSscan flow cytometer (BD). Briefly, 3 to 5 × 10⁵ cells treated with 5-AZA-dC and HDAC inhibitors alone and in combination were washed with 1× PBS and stained with propidium iodide for 2 hours at 4°C in the dark. Changes in percentage of cells in different phases of cell cycle were analyzed with ModFit software.

Western blots
Whole-cell extracts were obtained from cell pellets lysed for 15 minutes on ice in radioimmunoprecipitation assay buffer (Sigma) supplemented with 1× protease inhibitors (Calbiochem), nuclear and cellular debris cleared by centrifugation, and total cellular proteins quantified using Bradford protein assay (Bio-Rad). Thirty micrograms of cell lysates were separated on SDS-PAGE and transferred to nitrocellulose membranes (Whatman). Membranes were blocked 1 hour in blocking solution (5% nonfat dry milk, 1× TBST) and incubated at 4°C overnight with primary antibodies. The primary antibodies and dilutions were DNMT1 (1:200; Abcam), Caspase-3 (1:1,000) and phosphorylated H2A-X (1:1,000; Cell Signaling), signal transducers and activators of transcription (STAT)1 and STAT2 (1:1,000; BD), IFITM1 (1:200; R&D Systems, Inc.), and ISG15 (1:5,000; ref. 25). After primary antibody incubation, membranes were washed 2 × 15 minutes and 1 × 10 minutes in 1× TBST wash buffer. Membranes were then incubated with horseradish peroxidase–tagged goat antimouse, goat anti-rabbit, or rabbit anti-goat secondary antibodies (Bio-Rad) at room temperature for 1 hour, washed 2 × 15 minutes and 1 × 10 minutes in 1× TBST, and developed by using Western Lightening Chemiluminescence Reagent (Perkin-Elmer). Equal protein loading was confirmed by probing with β-actin monoclonal antibody (Sigma).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay
DNA fragmentation was detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (APO-BRDU, BD) in cells treated with 5-AZA-dC or MGCD0103 alone or in combination. Cells were fixed with 1% paraformaldehyde for 15 minutes on ice, washed twice with 1× PBS, pelleted and suspended in 70% alcohol, and apoptosis detection assay carried out following the manufacturer’s instruction. This system end labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. All samples were analyzed using FACSscan flow cytometer.

Comet assay
Cells were harvested and washed with 1× PBS after 24, 36, and 48 hours of treatment with 5-AZA-dC or...
MGCD0103 alone or in combination. Fifty microliters of cell suspension (1 × 10^5/mL) was mixed with 500 μL of 0.5% low-melting-point agarose at 37°C, and 75 μL was immediately added to Comet Slides (Trevigen). After hardening, slides were immersed in prechilled lysis buffer [2.5 mol/L NaCl, 100 mmol/L EDTA (pH 10), 10 mmol/L Tris, containing 1% Triton X-100 and 10% DMSO] at 4°C for 1 h. Slides were washed with distilled water, placed side by side on a horizontal gel chamber, and submerged in freshly made alkali buffer [300 mmol/L NaOH/1 mmol/L EDTA (pH 13)] for 40 minutes followed by electrophoresis at 25 V (0.86 V/cm), 300 mA for 25 minutes at 4°C to detect both single- and double-stranded DNA breaks. Slides were then neutralized in 0.4 mol/L Tris-HCl (pH 7.4), rinsed with distilled water, placed in prechilled 70% ethanol for 5 minutes, and air dried overnight at room temperature. After staining with ethidium bromide (10 μg/mL for 5 min), comets were assessed by microscopy (×10 objective) using a Leica DMI 4000B microscope equipped with a digital camera (QImaging) and analyzed using the TriTek CometScore software. The percentage of DNA in the tail (tail DNA%) and tail moment (% of DNA in the tail × tail length) was determined for 50 comets in each sample.

**Phospho-H2A.X assay**

DNA double-strand breaks in treated cells were detected by phospho-H2A.X staining and flow cytometry. Briefly, cells were fixed with 1% paraformaldehyde, permeabilized by ice-cold methanol addition, stained with antiphospho-H2A.X (1:20) for 1 hour, and analyzed by flow cytometry. Nontreated negative controls were used to mark the lower limit of background level of positive H2A.X-staining.

**RNA isolation, cDNA synthesis, semiquantitative real-time PCR, and Illumina microarray**

Total RNA was isolated from SCLC cell lines using reagents and protocols provided in the SV Total RNA Isolation System (Promega). RNA samples from cells treated with 5-AZA-dC or MGCD0103 alone or in combination, along with nontreated controls, were checked for purity and quality and reverse-transcribed into cDNA using 1 μg total RNA and Moloney murine leukemia virus reverse transcriptase (Promega).

**Microarray analysis**

Microarray analysis was undertaken using Sentrix Human-6 V2 Expression Bead Chip (Illumina). The analysis was carried out on single samples, each in triplicate. The differentially expressed genes were identified by using the Bead Studio expression analysis software. Signals that changed by >2-fold, with differential P values of ≤0.05 in resistant H196 compared with sensitive H526, and with an average signal of >25 in H196 or H526 were analyzed for differential gene expression. Once the differentially expressed genes were identified, functional interpretation of significant gene relevance to canonical pathways was done by the Ingenuity Pathway analysis software (IPA 7.0 software, Ingenuity System).

**Semiquantitative real-time PCR**

Taqman expression primers and ABI PRISM Sequence Detection Instrument 7500 (Applied Biosystem) were used for real-time PCR. Thermal cycling was done for each gene in triplicate in 25 μL finale volume for quantification. Target gene expression was normalized by comparison with the expression of glyceraldehyde-3-phosphate dehydrogenase. The JMP8 software was used for comparison of expression profiles of target genes between SCLC cell lines. Relative change in the expression of tested genes within cell lines was defined as ΔCT values. ΔCT values were generated as (the average Ct of the reference gene − the average Ct of the gene of interest) + a constant (26) to move all of the data into positive range.

**Statistical analysis**

One-way ANOVA followed by pairwise multiple comparison procedures (Tukey HSD Test) were done using the VassarStats Statistical Computation Web Site (27) for the experiments with more than two groups. A P value of <0.05 was considered statistically significant.

![Figure 2](image-url)

Figure 2. Effect of 5-AZA-dC and LBH589 on the level of DNMT1 protein in SCLC cell lines. 5-AZA-dC (400 nmol/L) reduced the level of DNMT1 in SCLC after 24 h. LBH589 did not have an effect on protein level. The levels of β-actin served as loading control. Results shown are a representative of two independent experiments in which the repeat experiment also yielded similar results.
Figure 3. 5-AZA-dC and MGCD0103 treatment results in the formation of DNA strand breaks in sensitive but not in resistant SCLC after 24 h of treatment assayed by Comet assay. A, comet images of H526 cells; B, comet images of H196 cells; C, percentage of DNA in a tail in cells treated with 5-AZA-dC or MGCD0103 alone or in combination; D, comet tail moment = tail DNA% × tail distance. Columns, mean of the measurement of 50 randomly selected comets from two slides from each experimental point; bars, SEM. Statistical significance of differences in DNA damage induction by different treatments was evaluated by one-way ANOVA. ***, significantly different from all other treatments ($P < 0.01$); •••, significantly different from control ($P < 0.01$); ••, significantly different from control ($P < 0.05$).
Results

Effect of a DNA methylation inhibitor (5-AZA-dC) and HDAC inhibitors (LBH589 or MGCD0103) on the viability of SCLC cells

5-AZA-dC alone (up to 1 μmol/L concentration) resulted in the growth inhibition of only two of nine cell lines, whereas LBH589 (up to 12.8 μmol/L) or MGCD0103 (up to 1.28 mmol/L) resulted in growth inhibition of four of nine cell lines in viability assays after 72 hours. Simultaneous treatment of H526, H82, H146, and DMS114 cells with individual concentrations of 5-AZA-dC and the pan-HDAC inhibitor (LBH589) resulted in the synergistic inhibition of cell growth with CIs of 0.34 to 0.91 and decreased viability of ≤40%. The greatest synergism was in H82 cells with 100 nmol/L of 5-AZA-dC and 0.3 nmol/L of LBH589. Five of nine cell lines, H1048, H2195, SW1271, H196, and H1688 cells, were resistant to the combination (CI, 0.25). Combined treatment of DMS114 cells was synergistic in cell growth inhibition with 200 nmol/L of 5-AZA-dC and 380 nmol/L of MGCD0103. H2195, H196, SW1271, and H1688 cells were resistant to 5-AZA-dC plus MGCD0103 combinations (Fig. 1B).

To explore the contribution of each drug to the synergism, a fixed concentration of one agent and altering concentrations of the second agent and vice versa were used for the treatment of H526 (sensitive) and H196 (resistant) cells (Supplementary Fig. S1). Strong synergistic effects were observed in H526 cells treated with a constant concentration of 5-AZA-dC (400 nmol/L) while varying the concentration of LBH589 (0.05–6.4 nmol/L) or with a steady concentration of LBH589 (6.4 nmol/L) in combination with changing concentrations of 5-AZA-dC (25–800 nmol/L). Similar results were obtained for 5-AZA-dC and MGCD0103 (Supplementary Fig. S1A). There was no synergistic effect in the resistant H196 cells (Supplementary Fig. S1B).

To further confirm the synergistic interaction between 5-AZA-dC and HDAC inhibitors in SCLC, the percentage...
of apoptotic, necrotic, and healthy cells was quantified in two sensitive (H526 and H82) and one resistant (H196) cell lines after 72 hours of treatment using Annexin V, Ethidium Homodimer III, and Hoechst 33342 staining. As expected, there was a marked reduction in the percentage of live cells in H526 and H82 after treatment with 5-AZA-dC or MGCD0103 alone or in combination (Supplementary Fig. S2). Concurrently, there was a significant increase in the percentage of necrotic cells. Apoptotic cells were not identified. Treatment of resistant H196 cells did not have any effect.

SCLC cell lines that were sensitive to the combination of 5-AZA-dC and HDAC inhibitors (LBH589 and MGCD0103) were also sensitive to the combination of HDAC inhibitor (LBH589) and cisplatin, a DNA-damaging agent. (Supplementary Fig. S3). Notably, the combination treatment induced synergistic effects in H526, H146, and H82 cells with a CI of 0.52 to 0.94. Similarly to 5-AZA-dC in combination with HDAC inhibitors, H196 cells remained resistant to the combination of LBH589 and cisplatin.

5-AZA-dC inhibits DNMT1 expression in SCLC cells

To confirm that 5-AZA-dC was inhibiting DNMT1 in SCLC cells, the level of DNMT1 protein in extracts of cells treated with 5-AZA-dC and HDAC inhibitors alone and in combination was assessed by Western blot analysis (Supplementary Fig. S4; Fig. 2). The DNMT1 protein level was markedly reduced in all SCLC cell lines after 24 hours of treatment with 400 nmol/L of 5-AZA-dC compared with nontreated control. Treatment with LBH589 or MGCD0103 either had no effect or slightly depleted DNMT1 after cotreatment with 5-AZA-dC (Supplementary Fig. S4; Fig. 2). Similar results were observed after 48 and 72 hours of treatment (data not shown), suggesting a lack of correlation between cell viability and DNMT1 inhibition.

Analysis of cell cycle inhibition and apoptosis

To assess whether antiproliferative effects of 5-AZA-dC and HDAC inhibitors resulted from cell cycle arrest or apoptosis, cell cycle profile and TUNEL assays were carried out after 72 hours of treatment. 5-AZA-dC alone resulted in accumulation of the sensitive H526, H146, H82, and DMS114 cells in the G2-M phase and induced sub-G0 in H526 cells. LBH589 alone induced sub-G0 population in H526 and H146 cells, whereas MGCD0103 alone induced sub-G0 population in H146 and DMS114 cells and arrested the latter in G2-M phase. Nevertheless, addition of LBH589 or MGCD0103 to 5-AZA-dC increased the percentage of sensitive cells (H526, H146, H82, and DMS114) in the sub-G0 phase. There was no change in the cell cycle profiles of the resistant cell lines H196 and SW1271 (Supplementary Fig. S5A and B). Shorter treatments (24 and 48 h) did not result in significant changes in cell cycle phase distributions in tested cell lines (data not shown).

When assessed by TUNEL assay, treatment of H526 cells with 400 nmol/L of 5-AZA-dC or 300 nmol/L of MGCD0103 alone resulted in 10.6% and 13% of apoptotic cells, respectively, with maximal increase in apoptosis to 19.5% from the combination (Supplementary Fig. S6A). The apoptotic index although modest was significantly different ($P < 0.01$) in combination treatment from all other treatments. Consistent with the marginal increase in apoptosis, there was no marked increase in the cleavage of caspase-3 when H526 or other SCLC cells were treated with 5-AZA-dC or MGCD0103 alone for 72 hours (Supplementary Fig. S6B). Minor induction of apoptosis in H526 cells or its lack in H146 and H82 cells was also observed after treatment with LBH589 in combination with CDDP (data not shown). Together, these data suggested that apoptosis was not a primary mechanism of SCLC cell growth inhibition identified in the viability assay.

5-AZA-dC and MGCD0103 induce DNA damage in sensitive SCLC cells

Because LBH589 had similar effects on SCLC cells as MGCD0103, studies were narrowed to an investigation of effects of 5-AZA-dC combined with MGCD0103. Significant inhibition of SCLC cell viability with only modest apoptosis led to investigations of DNA damage by Comet assay and phospho-H2A.X staining. Treatment of H526, H146, H82, and DMS114 cells with 400 nmol/L of 5-AZA-dC or 70 or 300 nmol/L of MGCD0103 for 24 hours resulted in the DNA fragmentation characteristic of DNA strand breaks, as assessed by the formation of comet-like tails following single cell gel electrophoresis (Fig. 3). Comets were identified after treatment with 5-AZA-dC plus MGCD0103 in sensitive cells (Fig. 3A) but not in resistant cells (Fig. 3B). The amount of DNA in a tail after treatment with the combination was two times greater compared with cells treated with 5-AZA-dC or MGCD0103 alone in H526, H146, H82, and DMS114 cells ($P < 0.01$; Fig. 3C). In cells treated with 5-AZA-dC in combination with the HDAC inhibitor, tail moment was 7-, 4-, 9-, and 3-fold higher than that in cells treated with 5-AZA-dC ($P < 0.01$) and 3-, 5-, and 2-fold greater compared with MGCD0103 treatment ($P < 0.01$) in H526, H146, H82, and DMS114 cells, respectively (Fig. 3D). Longer treatments did not cause more DNA damage (data not shown). No comets were observed in the resistant H196 and SW1271 cells. These results suggested that combination treatment increased DNA damage in sensitive SCLC cells.

The above results were supported by the assessment of phosphorylation of histone H2A.X, an early marker of DNA double-strand breaks. The level of phosphorylated histone H2A.X (γH2A.X) was evaluated by phospho-H2A.X staining and flow cytometry in SCLC cells after treatment with 5-AZA-dC, MGCD0103, or their combination. 5-AZA-dC or MGCD0103 alone increased the phosphorylation of H2A.X in sensitive H526 and H82 cells (Fig. 4). The percentage of γH2A.X was 20.9 and 28.1 in H526 cells, and 7.3 and 15.9 in H82 cells for 5-AZA-dC or MGCD0103, respectively.
H526 and H82 cells with both drugs together further increased the phosphorylation of H2A.X (40.86% in H526, \(P < 0.05\); 22.8% in H82, \(P < 0.01\)). No significant increase in \(\gamma\)H2A.X was observed in resistant H196 and SW1271 cells from treatment with 5-AZA-dC, HDAC inhibitor, or their combination.

**Expression of ISGs in resistant H196 SCLC cells**

5-AZA-dC and HDAC inhibitors reverse the expression of epigenetically silenced tumor suppressor genes (1, 18). Because sensitivity of SCLC did not correlate with DNMT1 inhibition, we postulated that basal gene expression in H526 and H196 cells might be a basis for understanding intrinsic resistance to epigenetic inhibitors. Comparison of ratio of basal expression profiles of resistant H196 to sensitive H526 cells identified expressed (\(\geq 2\times\)) mRNA differences of 2197 genes in DNMT1 and HDAC inhibitor–resistant H196 cells. Of these, the expression of 273 genes was \(> 10\times\) in resistant H196 cells compared with sensitive H526 (Supplementary Table S2). An ontology analysis identified cell development, proliferation, differentiation and IFN signaling as pathways that had many genes intrinsically upregulated in H196 cells (data not shown). More than 17% of genes from highly expressed genes (>60-fold; Table 1) belonged to IFN signaling pathways. \(IFI27\) was the most highly expressed gene (379-fold difference) in resistant H196 cells compared with sensitive H526 cells (Table 1). In sensitive H526 cells, expression levels of ISGs were markedly lower, suggesting a correlation between resistance to DNA damage and loss of viability to expression of ISGs.

To confirm gene expression array results, 15 genes that were differentially expressed (\(\geq 21\times\)) were compared in H196 and H526 cells using semiquantitative real-time PCR (qRT-PCR; Supplementary Table S3). Consistent with the microarray results, qRT-PCR identified marked upregulation of all 15 selected genes in H196 cells. To further confirm the correlation between upregulation of ISGs with DNA damage resistance, basal expression of ISGs in sensitive (H526, H82, H146, DMS114, and H1048) and resistant (H2195, H196, SW1271, and H1688) cells were compared. Box and Whiskers analysis identified markedly higher median mRNA levels for most ISGs in the resistant group (Fig. 5A). The microarray and qRT-PCR data were additionally validated by assessing the basal expression of STAT1, STAT2, STAT3, IFITM1, and ISG15 proteins in SCLC cell lines (Fig. 5B). Consistent with gene array and qRT-PCR results, basal levels of these genes were higher in the resistant cells compared with sensitive ones. Together, these results suggest that basal expression of the ISGs *IFI27, IFI44, IFI35, BST2, MX1, ISG15, STAT1, STAT2, and THBS1* may serve as predictors of SCLC cell resistance to epigenetic modulators or other agents inducing DNA damage.

**Discussion**

SCLC represents approximately 15% to 20% of all bronchogenic malignancies and is biologically and clinically...
distinct from non–small cell types. Despite a good initial response to chemotherapy, most SCLC patients ultimately develop resistance and relapse. Because available therapies have little effect on survival, new therapeutic approaches are needed (28, 29). Epigenetic changes, such as DNA methylation and histone acetylation, have a central role in the control of gene transcription and are early events in lung tumorigenesis (1, 26, 30, 31). 5-AZA-dC and HDAC inhibitors can reactivate aberrantly hypermethylated or acetylated genes, thereby inhibiting tumor cells by inducing apoptosis, cell cycle arrest, and differentiation (32–35). In addition to its demethylating function, 5-AZA-dC may also play an antineoplastic role that is methylation independent (36, 37).

HDAC inhibitors alone inhibited growth in four of nine SCLC cell lines, whereas 5-AZA-dC alone inhibited growth in two cell lines (Supplementary Table S1). Combining 5-AZA-dC either with LBH589 or MGCD0103 resulted in synergistic cell growth inhibition in five of nine cell lines (Supplementary Figs. S1 and S2; Fig. 1). The significant decrease in the viability of cells that resulted from the combination was not from apoptosis. Cell cycle analysis showed an increase in sub-G0 population and G2-M arrest in sensitive cell lines after

**Table 1.** Genes upregulated in H196 relative to H526 cells assessed by Microarray analysis using Sentrix Human-6_V2 Expression Bead Chip, Illumina, and analyzed by the Bead Studio software

<table>
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<tr>
<th>Gene Symbol</th>
<th>Entrez gene ID</th>
<th>Description</th>
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<th>Function</th>
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<td>Cell proliferation</td>
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<tr>
<td><strong>CTGF</strong></td>
<td>1490</td>
<td>Connective tissue growth factor</td>
<td>102.0</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td><strong>CRYAB</strong> †</td>
<td>1410</td>
<td>Crystallin α B</td>
<td>96.2</td>
<td>Visual perception</td>
</tr>
<tr>
<td><strong>COL5A2</strong></td>
<td>1290</td>
<td>Collagen, type V, α 2</td>
<td>92.7</td>
<td>Phosphate transport</td>
</tr>
<tr>
<td><strong>P COLCE</strong></td>
<td>5118</td>
<td>Procollagen C-endopeptidase enhancer</td>
<td>90.8</td>
<td>Development</td>
</tr>
<tr>
<td><strong>DCN</strong> †</td>
<td>1634</td>
<td>Decorin</td>
<td>85.7</td>
<td>Matrix assembly</td>
</tr>
<tr>
<td><strong>VCAN</strong></td>
<td>1462</td>
<td>Versican</td>
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<td>Cell recognition</td>
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<tr>
<td><strong>IFITM1</strong> †</td>
<td>8519</td>
<td>IFN-induced transmembrane protein 1</td>
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<td>Immune response</td>
</tr>
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<td><strong>MGP</strong></td>
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<td>Matrix Gla protein</td>
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<tr>
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<td>10581</td>
<td>IFN-induced transmembrane protein 2</td>
<td>72.0</td>
<td>Immune response</td>
</tr>
<tr>
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<td>Actin γ 2</td>
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<td>1601</td>
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<tr>
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<tr>
<td><strong>FBN2</strong></td>
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<td>Fibrillin 2</td>
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</tr>
<tr>
<td><strong>TWIST1</strong> †</td>
<td>7291</td>
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<tr>
<td><strong>OAS2</strong></td>
<td>4939</td>
<td>2-′,5-′-Oligoadenylate synthetase 2, 69/71 kDa</td>
<td>65.1</td>
<td>Immune response</td>
</tr>
<tr>
<td><strong>BST2</strong> †</td>
<td>684</td>
<td>Bone marrow stromal cell antigen 2</td>
<td>64.1</td>
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</tr>
<tr>
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<td>Transgelin</td>
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<td>Muscle development</td>
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<tr>
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<td>Serglycin</td>
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<td>Unknown</td>
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<tr>
<td><strong>IFITM3</strong></td>
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<td>IFN-induced transmembrane protein 3</td>
<td>60.0</td>
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</tr>
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</table>

*Genes upregulated ≥60-fold in resistant H196. Numbers represent the fold difference of expression of genes in H196 cells compared with sensitive H526.
†Validated by qRT-PC; results shown in Supplementary Table S3.
‡Not expressed in sensitive H526 cells, Ct = 40.
treatment with HDAC inhibitors alone or in combination with 5-AZA-dC. Accumulation of cells in G2-M phase after treatment of SCLC cells with either LBH589 alone or in combination with etoposide has also been reported (38). Longer exposure (60 h) to the HDAC inhibitor resulted in the increase of dead cells. In the latter case, LBH589 was potent not only in cell lines but in tumor xenografts as well (38).

Although it is commonly assumed that all sub-G0 cells are apoptotic, the population contains, in fact, necrotic cells and cells undergoing mitotic catastrophe, which could result in a sub-G0 peak. Based on the results of TUNEL assay, caspase-3 cleavage, and ethidium bromide staining (see Supplementary Figs. S2 and S6), the sub-G0 peak might have resulted from necrosis and not from apoptosis. Moreover, significant reduction of DNMT1

Figure 5. Expression ranges for ISGs in SCLC cell lines. A, Taqman qRT-PCR was used to measure relative change in gene expression as described in Materials and Methods and relative to glyceraldehyde-3-phosphate dehydrogenase. Result shown is a representative of three independent experiments in which the repeat experiments also yielded similar results. Median, 25th, 75th percentiles, and range are shown. B, basal levels of STAT1, STAT2, STAT3, ISG15, and IFITM1 proteins in SCLC cells. Protein concentration was normalized to β-actin. Result shown is a representative of three independent experiments in which the repeat experiments also yielded similar results.
protein level did not correlate with SCLC loss of viability (Supplementary Fig. S4; Fig. 2). Despite evidence that apoptosis induced by HDAC inhibitors can be increased by 5-AZA-dC (16, 21, 39), our data suggested that induction of apoptosis was not the primary mechanism for synergy between 5-AZA-dC and MGCD0103 in SCLC cells.

Combining 5-AZA-dC with HDAC inhibitors has resulted in a synergistic activation of silenced tumor suppressor genes and synergistic antineoplastic effects on breast and lung carcinomas (19, 20, 40). However, lack of correlation between DNMT1 inhibition by 5AZA-dC and SCLC viability suggested that the synergistic effect between 5AZA-dC and HDAC inhibitors might result from mechanisms other than transcriptional reactivation of proapoptotic genes.

Reduction of cell viability by both 5-AZA-dC and HDAC inhibitors could also result from their genotoxic effects (10, 15, 37, 41, 42). 5-AZA-dC is incorporated into DNA and covalently binds with DNMTs with the 5-AZA-DNMT-DNA adducts causing DNA damage (43). An increase in histone acetylation followed by increased accessibility of genomic DNA to transcription factors may result from HDAC inhibitors (44). Moreover, acetylation of histones induced by HDAC inhibitors can induce an open chromatin structure more accessible not only to transcription complexes but also to therapeutic agents that affect DNA. HDAC inhibitors are also able to induce DNA damage by themselves by changing chromatin structure and activation of ataxia telangiectasia mutated kinase and histone H2AX (15). Consistent with these reports, we identified DNA damage in sensitive H526, H146, H82, and DMS114 cells from 5-AZA-dC or MGCD0103 alone. This was markedly increased by the combination (Fig. 3). 5-AZA-dC-induced DNA damage was mostly single-strand breaks as detected by alkaline electrophoresis in Comet assay and the absence of formation of γH2AX (except in H526 cells).

Global gene expression profiling unexpectedly identified a strong signature of IFN signaling pathway in resistant H196 cells accounting for 10.6% of constitutively upregulated genes. Recent studies have identified a strong correlation between the constitutive expression of ISGs, and radiotherapy and chemotherapy resistance (45). Constitutive high expression of IFN/STAT1-regulated genes protected tumor cells from the cytotoxic effect of cisplatin and fludarabine (46, 47). In human head and neck squamous, radiation-resistant tumors overexpressed a substantial number of genes inducible by IFNs or related to their signaling pathway (48–50). The IFN-related DNA damage resistance signature was also associated with resistance to chemotherapy. IFN-related DNA damage resistance signature (+) xenografts were more resistant to doxorubicin chemotherapy compared with IFN-related DNA damage resistance signature (−) tumors (45). Moreover, a signature of IFN-related genes was associated with prediction of resistance across cell lines and primary tumors of different cancer types and was associated with a clinically adverse outcome after radiotherapy and chemotherapy for breast carcinoma (45). This signature of an IFN-induced pathway correlated with resistance to 5-AZA-dC and HDAC inhibitor-induced DNA damage in SCLC cells.

In summary, the synergistic effects of 5-AZA-dC and MGCD0103 in the reduction of viability of SCLC cells resulted from DNA damage. Compared with sensitive cells, resistance was associated with constitutive expression of ISGs, which has been correlated with drug- and radiation-induced DNA damage resistance. Rather than IFNs per se, this effect may result from as yet to be identified common upstream transcriptional regulators such as STAT, IRF, or NF-κB family member(s). For example, unphosphorylated STAT1 increased the expression of many IFN-induced genes in human fibroblasts (51), which were also increased in resistant H196 cells. With further understanding of resistance mechanisms, targeting epigenetic and DNA damage and repair pathways may be of benefit for designing new approaches to overcome resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

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