ORIGINAL ARTICLE

Antineoplastic Effects of Decitabine, an Inhibitor of DNA Promoter Methylation, in Adrenocortical Carcinoma Cells

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Hypotheses: Decitabine recovers expression of silenced genes on chromosome 11q13 and has antineoplastic effects in adrenocortical carcinoma (ACC) cells.

Design: NCI-H295R cells were treated with decitabine (0.1-1.0μM) over 5 days. Cells were evaluated at 24-hour intervals for the effects of decitabine on ACC cell proliferation, cortisol secretion, and cell invasion. Expression was quantified for 6 genes on 11q13 (*DDB1*, *MRPL48*, *NDUFS8*, *PRDX5*, *SERPING1*, and *TM7SF2*) that were previously shown to be underexpressed in ACC.

Setting: Academic research.

Study Specimen: Human ACC cell line.

Main Outcome Measures: Adrenocortical carcinoma cell proliferation, cortisol secretion, and cell invasion were measured using immunometric assays. Quantitative reverse transcription–polymerase chain reaction was used to measure gene expression relative to *GAPDH*.

Results: Decitabine inhibited ACC cell proliferation by 39% to 47% at 5 days after treatment compared with control specimens ($P<.001$). The inhibitory effect was cytostatic, time dependent, and dose dependent. Decitabine decreased cortisol secretion by 56% to 58% at 5 days after treatment ($P=.02$) and inhibited cell invasion by 64% at 24 hours after treatment ($P=.03$). Of 6 downregulated genes on 11q13, decitabine recovered expression of *NDUFS8* (OMIM 602141)($P<.001$) and *PRDX5* (OMIM 606583) ($P=.006$).

Conclusions: Decitabine exhibits antitumoral properties in ACC cells at clinically achievable doses and may be an effective adjuvant therapy in patients with advanced disease. Decitabine recovers expression of silenced genes on 11q13, which suggests a possible role of epigenetic gene silencing in adrenocortical carcinogenesis.

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Adrenocortical carcinoma (ACC) is a rare malignant neoplasm involving the adrenal cortex, affecting 2 persons per 1 million per year.[1,2] These are aggressive tumors, with a 5-year survival of 50% among patients with surgically resectable disease and with a median survival of less than 1 year among patients with metastases.[3,4] In addition to its high mortality, ACC causes significant detriment to the quality of life of affected patients because of tumoral hypersecretion of hormones, particularly cortisol.[5] Most patients require systemic adjuvant chemotherapy and mitotane treatment to attempt to limit tumor progression and hormonal hypersecretion.[6-8] However, these agents have poor efficacy in most patients.

The molecular pathogenesis of sporadic ACC is poorly understood, but several factors seem important, including insulin growth factor 2 overexpression.[9] In particular, loss of heterozygosity at chromosome 11q13 has been reported in up to 70% to 100% of ACCs,[10,11] suggesting that inactivation of genes on 11q13 could contribute to adrenocortical carcinogenesis. Six significantly underexpressed genes on 11q13 in ACC were recently identified using genome-wide expression microarray analysis.[12] Although the specific mechanisms behind silencing of these genes are unknown, the concept of using therapies to recover expression of these candidate tumor suppressor genes and to possibly halt cancer progression is promising.

Recently, there is increasing evidence that reversible, or epigenetic, mechanisms in gene silencing have an important role in cancer. In turn, this has prompted enthusiasm for the possible usefulness of drugs that specifically target these epigenetic alterations.[13,14] Decitabine (5-aza-2′-deoxycytidine) reverses the classic epigenetic alteration, DNA promoter methylation. It acts by removing inhibitory methyl groups from the cytosine residues of promoter sequences, which presumably restores downstream gene transcription. Compared with high-dose regimens that were used in the

1970s, lower in vitro (≤10μM) and in vivo (15-20 mg/m^2/d for 3-5 days) doses of decitabine have demonstrated growth inhibitory properties in several cancer models with fewer adverse effects.[15,16] Decitabine is approved by the Food and Drug Administration as first-line treatment of myelodysplastic syndromes.[14,17]

Although a few studies[18,19] previously examined the effect of decitabine on cell proliferation and cortisol secretion in human ACC cells, the current emphasis on lower-dose clinical regimens warranted a more specific study on the functional influence of low-dose decitabine. We set out to determine the effects of low-dose decitabine (0.1μM and 1.0μM) on a human ACC cell line. We examined ACC cell proliferation and cortisol secretion using immunometric assays and estimated invasive potential using a model of cell migration through a synthetic polycarbonate membrane. In addition, we determined the effects of decitabine on expression of underexpressed genes at 11q13.

METHODS

CELL CULTURE AND REAGENTS

NCI-H295R cells (ATCC, Rockville, Maryland) were grown and maintained in a 1:1 solution of Dulbecco modified Eagle medium (DMEM) and F12 (DMEM:F12) supplemented with premix (ITS$^+$; BD Biosciences, San Jose, California), serum (Nu-Serum I [2.5%], BD Biosciences), and 10 000 U/mL of penicillin-streptomycin in a standard humidified incubator at 37°C in a 5% CO_2 atmosphere. Decitabine (Sigma, St Louis, Missouri) was dissolved in dimethyl sulfoxide (DMSO) vehicle and prepared to 2 clinically achievable doses (0.1μM in 0.025% DMSO and 1.0μM in 0.25% DMSO). In addition, DMSO vehicle without decitabine was prepared at analogous doses for control specimens. Treatments were initiated 48 hours after trypsin splitting and reseeding of cells onto new plates.

QUANTIFICATION OF ACC CELL PROLIFERATION

Cells were reseeded onto a 96-well plate at a concentration of 1×10^5 cells/200 μL of culture medium. After treatment administration, cells were incubated for 1, 2, 3, 4, and 5 days at 37°C in 5% CO_2. For each specimen group and time point, culture medium was aspirated from the well, and the cells were lysed and immediately frozen at –80°C for 24 hours. The plates were thawed at room temperature and prepared for cell count quantification using an assay kit (CyQUANT; Invitrogen, Carlsbad, California) that measures total nucleic acid content from lysed cells. The plates were processed according to the manufacturer's instructions and analyzed on a fluorometric plate reader at 480-nm and 520-nm wavelengths.

CORTISOL SECRETION MEASUREMENT

Cells were reseeded onto a 12-well plate at a concentration of 5×10^5 cells/mL of culture medium. After treatment administration, cells were incubated for 5 days at 37°C in 5% CO_2. At 24-hour intervals during the 5-day incubation, 30-μL aliquots of culture medium were taken from each well. To remove any cells in suspension, the aliquots were centrifuged, and the supernatants were aspirated off and stored at –80°C until the time of measurement. Cortisol concentration was measured using an immunometric assay kit (Parameter; R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions. The assayed samples were analyzed on a colorimetric plate reader at 480-nm and 520-nm wavelengths.

CELL INVASION ASSAY

Determination of cell invasion was performed with an assay (QCM ECMatrix Cell Invasion Assay; Millipore, Billerica, Massachusetts) that uses a modified Boyden chamber technique. Briefly, cells were starved for 48 hours with serum-free DMEM:F12 before reseeding onto a manufacturer-supplied 96-well upper chamber at a concentration of 1×10^5 cells/100 μL of DMEM:F12. The bottom of the upper chamber consists of a porous polycarbonate membrane with a layer of extracellular matrix (ECM). The upper chamber was placed into a lower 96-well plate with normal serum-containing culture medium, and the cells were incubated for 24 hours at 37°C in 5% CO_2. Cells that invaded through the membrane were detached and lysed, and the cell count was quantified using the kit described in the "Quantification of ACC Cell Proliferation" subsection.

RNA PREPARATION AND QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

After treatment and incubation, total RNA was isolated from cells using a reagent (TRIzol, Invitrogen) and purified using a kit (RNeasy Mini Kit; Qiagen, Valencia, California). At a concentration of 125 ng/μL, total RNA was reverse transcribed using a complementary DNA (cDNA) synthesis kit (RT Script; USB, Cleveland, Ohio). Quantitative polymerase chain reaction (PCR) was performed on the cDNA samples to measure expression levels of 6 genes on 11q13 (*DDB1* [OMIM 600045], *MRPL48* [OMIM 611853], *NDUFS8*, *PRDX5*, *SERPING1* [OMIM 606860], and *TM7SF2* [OMIM 603414]) that were previously found to be underexpressed in ACC.[12] The expression level of each gene was normalized to that of the *GAPDH* housekeeping gene. The PCR primers and probes for the genes were premade (TaqMan Assay-on-Demand kit; Applied Biosystems, Foster City, California). The manufacturer's inventory numbers for the primer-probe sets were Hs01096554_g1 (for *DDB1*), Hs99999905_m1 (*GAPDH*), Hs00740658_m1 (*MRPL48*), Hs00159597_m1 (*NDUFS8*), Hs00201536_m1 (*PRDX5*), Hs00163781_m1 (*SERPING1*), and Hs00162807_m1 (*TM7SF2*). The PCR reactions were performed (ABI PRISM 7900 Sequence Detection System, Applied Biosystems) with 1 μL of cDNA in a final volume of 20 μL for 12 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression was quantified using the following equation:

$$\text{Normalized Gene Expression} = 2^{-(Ct \text{ for Gene of Interest} - Ct \text{ for } GAPDH)} \times 100\%,$$

where *Ct* is the PCR cycle threshold.

STATISTICAL ANALYSIS

Two-tailed *t* test was used to compare outcome variables between study specimens vs controls. The outcome variables were cell count, cortisol concentration, and percentage gene expression.

RESULTS

DECITABINE INHIBITS ACC CELL PROLIFERATION IN NCI-H295R CELLS

The untreated control specimen of NCI-H295R cells had a doubling time of 2 days. No significant difference was

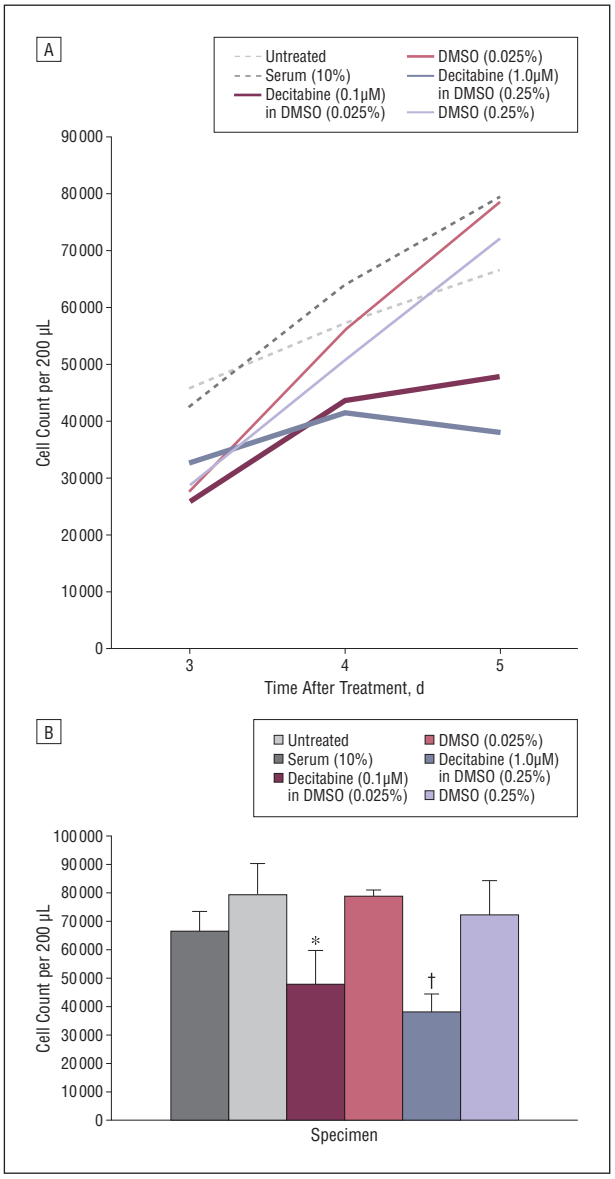

Figure 1. Effects of decitabine on cortical secretion in NCI-H295R cells (ATCC, Rockville, Maryland). Cells were treated with decitabine (in dimethyl sulfoxide [DMSO] vehicle) at 0.1μM and 1.0μM doses for 5 days, and cell counts were measured using an assay kit (CyQUANT; Invitrogen, Carlsbad, California). Compared with control specimens, decitabine caused time-dependent (A) and dose-dependent (B) inhibitory effects on adrenocortical carcinoma cell proliferation, with significant 39% (*P=.02) and 47% (†P<.001) decreases in cell counts observed at 5 days after treatment. Serum (10%) is Nu-Serum (BD Biosciences, San Jose, California).

noted in ACC cell proliferation between the untreated control specimen and the DMSO vehicle control specimen. Decitabine (0.1μM and 1.0μM doses) caused significant time-dependent cytostatic attenuation of ACC cell proliferation (**Figure 1**A). The differences in ACC cell proliferation were significant at 5 days after treatment, with 39% (P=.02) and 47% (P<.001) decreases in cell counts for the 0.1μM and 1.0μM doses, respectively (Figure 1B). The higher dose of decitabine led to greater inhibition of ACC cell proliferation than the lower dose, although the difference was not statistically significant.

DECITABINE LEADS TO DECREASED CORTISOL SECRETION IN NCI-H295R CELLS

Cortisol concentrations did not differ significantly between the untreated control specimen and the DMSO vehicle control specimen of NCI-H295R cells. Treatment with either dose of decitabine led to a significant decrease in cortisol concentration at 5 days after treatment, with 58% (P=.001) and 56% (P=.02) decreases for the 0.1μM and 1.0μM doses, respectively (**Figure 2**A and B). However, only the 0.1μM dose caused a significant decrease in cortisol concentration relative to cell concentration (calculated by dividing cortisol concentration by absolute cell count) (P=.03) (Figure 2C).

DECITABINE INHIBITS NCI-H295R CELL INVASION

The inhibitory effects of decitabine on the invasive potential of NCI-H295R cells were observed at 24 hours after treatment. Compared with controls, decitabine-treated cells showed significantly attenuated cell invasion through the ECM at 24 hours (**Figure 3**). As with ACC cell proliferation, the effects of decitabine on cell invasion were dose dependent, with a 64% decrease in cell invasion at 24 hours using the 1.0μM dose (P=.03).

DECITABINE RECOVERS EXPRESSION OF UNDEREXPRESSED GENES ON 11q13

As already described, quantitative reverse transcription–PCR (RT-PCR) analysis was performed for 6 underexpressed genes at 11q13 on NCI-H295R cells after decitabine treatment. The RT-PCR was performed at 3 days after decitabine (1.0μM) treatment (along with respective controls). After decitabine treatment, expression was significantly altered in 4 of 6 genes (*DDB1*, *NDUFS8*, *PRDX5*, and *TM7SF2*). Of these, only *NDUFS8* (P<.001) and *PRDX5* (P=.006) showed significantly recovered expression after decitabine treatment (**Table**).

COMMENT

Treatment strategies for advanced-stage or recurrent ACC predominantly rely on adjuvant chemotherapeutic regimens that include mitotane. Mitotane remains the most significant single agent shown to affect the disease course of patients with metastatic ACC, with useful clinical remissions of 10 months in up to 30% of patients.[6] Perhaps most important, mitotane leads to significantly diminished hormonal hypersecretion in 80% of patients with functional tumors, thereby improving the quality of life for affected patients.[5] Furthermore, these effects seem optimal at lower-dosing regimens (≤3 g/d of mitotane), which have the dual benefit of achieving therapeutic serum concentrations (10-14 mg/L) and minimizing adverse reactions.[20] However, 70% of patients with ACC do not respond to mitotane treatment, and many patients develop debilitating neurologic and gastrointestinal toxic effects, which attest to the limitations of mitotane therapy in controlling the spread of this aggressive cancer. The

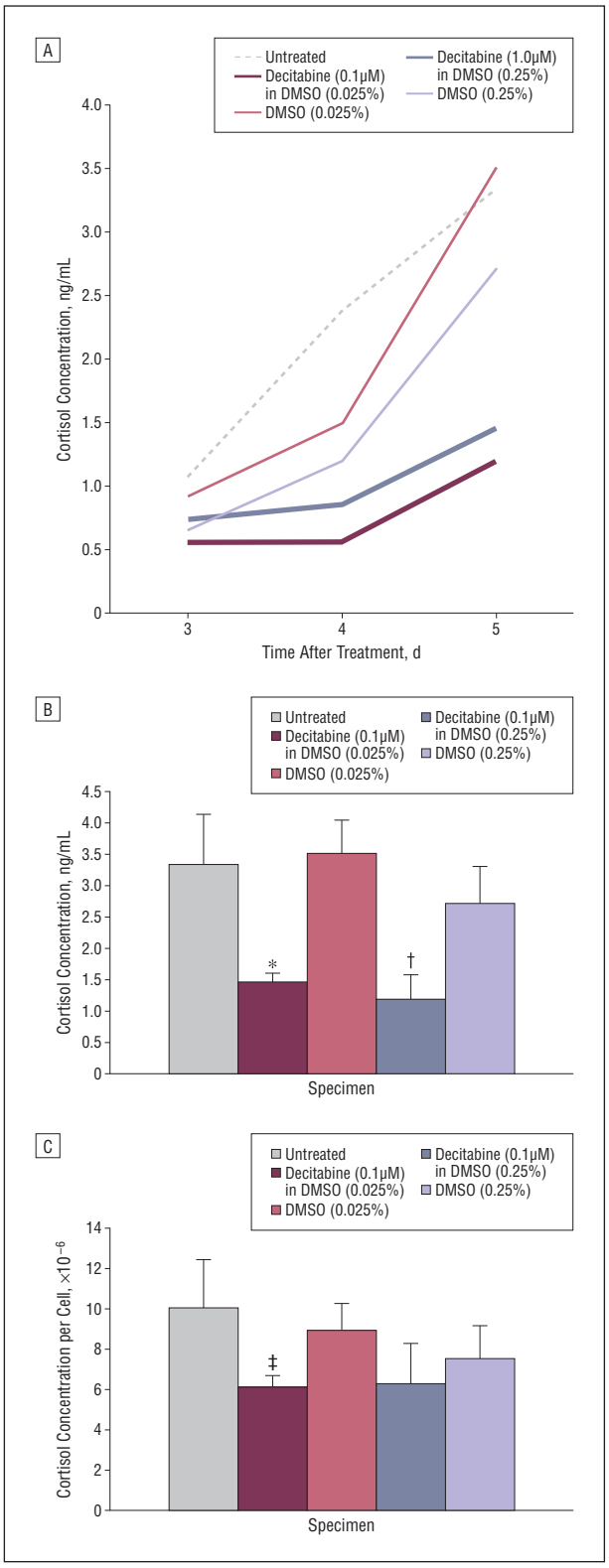

Figure 2. Effects of decitabine on cortisol concentration in NCI-H295R cells (ATCC, Rockville, Maryland). Cells were treated with decitabine (in dimethyl sulfoxide [DMSO] vehicle) at 0.1μM and 1.0μM doses for 5 days, and cortisol concentrations in cell media were measured using an immunometric assay kit (Parameter; R&D Systems, Minneapolis, Minnesota). Media taken from decitabine-treated cells had decreased cortisol concentration over time (A), with significant decreases at 5 days after treatment of 58% (*P=.001) and 56% (†P=.02) for the 0.1μM and 1.0μM doses, respectively (B). The inhibitory effect of decitabine on cortisol concentration per cell (C) was significant only with the lower 0.1μM dose (‡P=.03).

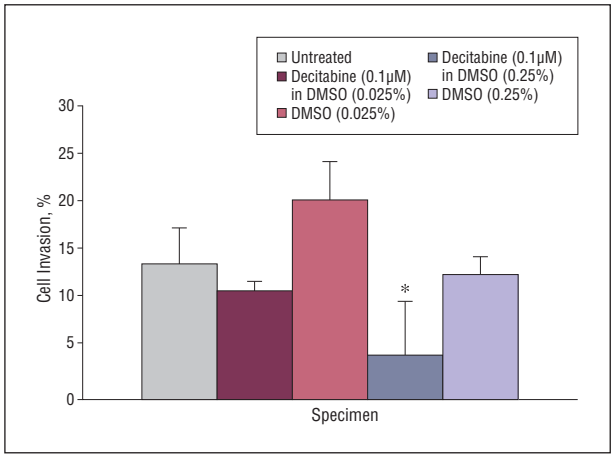

Figure 3. Effects of decitabine on NCI-H295R cell (ATCC, Rockville, Maryland) invasion. Cells were starved of serum for 48 hours and then placed in a modified Boyden chamber for 24 hours. Cells that invaded through the extracellular matrix barrier were counted using an assay kit (CyQUANT; Invitrogen, Carlsbad, California). Decitabine had a dose-dependent inhibitory effect on NCI-H295R cell invasion, with a 64% decrease in cell invasion using the 1.0μM dose (*P=.03). DMSO indicates dimethyl sulfoxide.

use of mitotane in combination with other agents, most notably cisplatin, has only slightly improved outcomes.[7]

Recently, mounting evidence about the role of epigenetic mechanisms in carcinogenesis has prompted interest in whether agents that reverse these processes can be effective anticancer treatments. Epigenetics refers to reversible changes in gene expression that do not fundamentally mutate the genomic DNA sequence. Hypermethylation of DNA promoter sequences is a classic example of these changes, which usually cause silencing of genes downstream of the affected region.[13] Theoretically, the reversal of these changes could lead to reexpression of silenced tumor suppressor genes and inhibit cancer cell progression. Indeed, the relevance of activated DNA methyltransferase to adrenocortical carcinogenesis was first established in mouse models more than a decade ago.[21]

Decitabine is an inhibitor of DNA methyltransferase, which effectively removes methyl groups from silenced promoter sequences. Decitabine has a dual dose-dependent mechanism of action. Lower doses (≤5μM to 10μM) of decitabine inhibit methylation and reactivate gene expression, whereas higher doses (≥10μM to 100μM) induce cytotoxic effects via covalent trapping of DNA methyltransferase into DNA.[16] Even at low doses, decitabine has been shown to inhibit in vitro growth in several human cancers, including colorectal carcinoma, melanoma, renal cell carcinoma, lung carcinoma, and hepatocellular carcinoma.[22-25] Its benefits have been most apparent in hematologic malignant neoplasms, especially myelodysplastic syndrome, for which decitabine has recently been approved by the Food and Drug Administration as first-line therapy.[17]

We hypothesized that decitabine treatment of NCI-H295R cells would have a significant antineoplastic effect. To this aim, we examined the effects of decitabine on ACC cell proliferation, cortisol secretion, and cell invasion,

Table. Quantitative RT-PCR Analysis of 4 Genes on Chromosome 11q13 That Are Underexpressed at Baseline in ACC and Differentially Expressed in NCI-H295R Cells at 3 Days After Decitabine (1.0μM) Treatment

Gene Symbol	Gene Name	Fold Change in Gene Expression in ACC[a]	Change in Gene Expression at 3 Days After Decitabine (1.0μM) Treatment, %[b]	*P* Value[c]
DDB1	Damage-specific DNA binding protein 1	−2.85	−20.9	.02
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8	−2.00	33.1	<.001
PRDX5	Peroxiredoxin 5	−2.40	50.3	.006
TM7SF2	Transmembrane 7 superfamily member 2	−4.28	−42.0	.04

Abbreviations: ACC, adrenocortical carcinoma; NADH, nicotinamide adenine dinucleotide; RT-PCR, reverse transcription–polymerase chain reaction.
[a] From the microarray analysis by Fernandez-Ranvier et al.[12]
[b] Based on RT-PCR performed herein in NCI-H295R cells (ATCC, Rockville, Maryland).
[c] Two-tailed *t* test.

which are 3 hallmarks causing clinical morbidity and mortality in this disease. Using 2 lower doses (0.1μM and 1.0μM), we examined the effects of decitabine on ACC cells at daily intervals for up to 5 days. Decitabine cytostatically inhibited ACC cell proliferation in a time-dependent and dose-dependent manner. Cortisol secretion was also attenuated at 5 days after treatment, although only the lower 0.1μM dose seemed to act via a mechanism independent of its growth inhibitory effects. As opposed to the long incubation time that is necessary to observe differences in cell count (probably because of a long doubling time for NCI-H295R cells), the effects of decitabine on invasive behavior were seen after only 24 hours. These findings support the idea that the functional effects of decitabine can be immediate, regardless of the basal growth rate of the target cell.

Our findings about ACC cell proliferation are in agreement with the results of other studies.[18,19,26] To our knowledge, we are the first group to demonstrate inhibition of ACC cell invasion by decitabine. Our modified Boyden chamber technique used a commercially available kit with a 2-dimensional barrier composed of reconstituted basement membrane and ECM components. Despite its widespread acceptance, this method for determining cell invasion has well-known limitations. First, despite improved standardization of preparation techniques by manufacturers, reconstituted matrices may contain "contaminants" (such as metalloproteinases) that could affect experimental results. Second, individual tumor cells display significant variability in their ability to adhere to and migrate through ECM, which may not be taken into account by quantifying cells in aggregate. Third, with its 2-dimensional design and components within reconstituted matrices, the Boyden chamber model does not necessarily recapitulate the tumor microenvironment. Newer techniques offer 3-dimensional models or tissue-based cell invasion barriers, which may address these limitations but need to be validated in future experiments.[27]

At first glance, our findings about cortisol secretion seem to contradict those in a prior study by Liu et al,[18] who found that decitabine increased cortisol secretion in NCI-H295R cells, possibly via selective regulation of steroidogenic gene expression. However, 2 primary differences between our studies may explain these divergent results. First, Liu et al used a higher dose of decitabine (10μM). The differing mechanism of action at higher doses (as already described) could at least partially account for the discrepancies in our results. Indeed, our results show that only the lower 0.1μM dose led to decreased cortisol secretion independent of decreased cell count. Furthermore, our studies seem to agree that the relative trend of cortisol secretion decreases in proportion to decitabine dose. We speculate that the lower dose of decitabine may affect cortisol expression and secretion, while the higher dose of decitabine may have only a cytotoxic effect, reducing cell count and cortisol secretion.

Second, our preparation of decitabine (unlike that by Liu et al) involved the use of DMSO vehicle to prevent rapid degradation of the unstable molecule. In theory, the benefit of our approach was longer duration of decitabine action, which may be more clinically relevant to longer-course treatment protocols used in patients. The disadvantage was the incorporation of another drug into our experiments and the associated possibility of a confounding effect, despite our use of DMSO concentrations well below thresholds that are known to have functional effects in other in vitro models.[28-30] We believe that we effectively controlled for this factor by standardizing decitabine to proper DMSO vehicle control specimens in our experiments.

In addition to demonstrating the functional effects of low-dose decitabine on NCI-H295R cells, we also sought to evaluate its effects on several genes at 11q13. This chromosomal region seems relevant in adrenocortical carcinogenesis, with studies[10,11] showing that loss of heterozygosity at 11q13 is found in 70% to 100% of sporadic ACCs. A previous microarray-based study[12] identified 6 genes on 11q13 that were underexpressed in ACC and demonstrated high diagnostic accuracy for distinguishing benign from malignant tumors. We tested the effects of decitabine on these genes using quantitative RT-PCR, with the hypothesis that decitabine could recover gene expression if methylation had a role in silencing any of these genes. For these experiments, we used decitabine at 1.0μM because of the marked effects on ACC cell proliferation and cell invasion that we observed at this dose, along with the indirect inhibitory effects on cortisol secretion. Of 6 genes, *NDUFS8* and *PRDX5* showed

significantly recovered expression after decitabine treatment, suggesting that hypermethylation may have a gene silencing role in ACC. However, future studies using methylation-specific techniques are needed to definitively establish methylation patterns of these genes in tumor samples.

The clinical relevance of *NDUFS8* and *PRDX5* remains unknown. *NDUFS8* encodes a subunit protein of a critical enzyme in the mitochondrial respiratory chain, and mutations of this gene are associated with Leigh disease.[31,32] *PRDX5* encodes the antioxidant enzyme peroxiredoxin, which has genome-protective properties in response to oxidative stressors.[33] Underexpression of neither *NDUFS8* nor *PRDX5* has been shown in other cancer models,[34-38] suggesting that dysregulation of these genes may be specific to adrenocortical carcinogenesis. However, further study is needed to determine the functional effects of modifying expression of these genes individually in the absence of other confounding factors. Moreover, the mechanisms behind the seemingly paradoxical downregulation of gene expression after decitabine treatment (such as that of *DDB1* and *TM7SF2* in our experiments), as well as the ways in which restoration of other genes interacts with these inhibitory effects, are unknown and require further investigation.

A cautionary note must be given about attempts to mold the results of our in vitro experiments to clinical relevance. The effects of decitabine on ACC cells are undoubtedly different depending on whether the drug is administered via culture medium or via in vivo intravenous or subcutaneous routes. Decitabine's success in inhibiting hematogenous cancers both in patients as well as in cell cultures may in part be due to similar immersive drug exposures in the bloodstream and in culture medium. This similarity may not necessarily translate as well for solid organ tumors. Indeed, early evidence in the 1980s showed a disappointing lack of demethylating agent activity on solid organ cancers.[16] Nevertheless, we believe that decitabine holds promise as a therapy for patients with ACC for several reasons. First, the earlier findings were limited by adverse effects secondary to higher drug doses and by limited treatment durations.[16] More recent trials[13,17] in other cancers reported superior results using low-dose and longer-duration drug regimens. Second, studies[18,19,21,26,39,40] using in vitro and in vivo models demonstrated the relevance of DNA promoter methylation in adrenocortical carcinogenesis. Third, decitabine has already been approved for use in humans, which should theoretically streamline its path to clinical trials.

In conclusion, low-dose decitabine exhibits significant antineoplastic effects in human ACC cells, possibly by recovering expression of *NDUFS8* and *PRDX5*. Considering that decitabine is already approved by the Food and Drug Administration for hematologic malignant neoplasms and because ACC is an orphan disease for which there is no effective chemotherapy to date for locally advanced and metastatic ACC, future studies evaluating the clinical efficacy of decitabine should be considered based on our results.

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Previous Presentation: This study was presented at the 80th Annual Meeting of the Pacific Coast Surgical Association; February 15, 2009; San Francisco, California.

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