Expression of nucleoside-metabolizing enzymes in myelodysplastic syndromes and modulation of response to azacitidine

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INTRODUCTION

The hypomethylating agent azacitidine (AZA) is the standard therapy for the treatment of myelodysplastic syndromes (MDS) with higher-risk myelodysplastic syndromes (MDS) (International Prognostic Scoring System (IPSS) intermediate-2 or high) and patients with acute myeloid leukemia (AML) with 20–30% bone marrow (BM) blasts in Europe, while in the United States it is approved for all French–American–British (FAB) MDS subtypes. Although AZA is extensively used, 30–40% of patients fail to respond or relapse after treatment.1,2 There have been efforts to characterize biological and clinical parameters predictive of response or resistance to AZA, and although some have been identified, they have not yet been validated to support clinical decisions.3–6 The concept that level of DNA methylation may correlate with response to treatment has been considered for some time, but there is not sufficient evidence to fully confirm this hypothesis as few data are available. For example, an initial study focused on CDKN2B methylation, but this did not correlate with response.5 Similarly, phosphoinositide/phospholipase C beta 1 was shown to be hypermethylated in patients with MDS and reduction of methylation was correlated with response to AZA.4,5 Moreover, Shen et al.6 showed that reduced global methylation over time correlates with survival and response to therapy in patients with MDS.

Several gene mutations that correlate with prognosis in patients with MDS have been evaluated for their power to predict response to AZA. For example, TET2 mutations correlated with response to AZA but without impacting response duration or survival.8 Mutations in other genes involved in epigenetic regulation, including DNMT3A, ASXL1, TET2 and IDH1/IDH2, may impact response to AZA.9 It has also been shown that resistance to AZA significantly correlated with the percentage of MDS or AML cells expressing the BCL-2-like protein 10.9 Finally, clinical parameters have been identified that independently predicted lower response rates (previous low-dose cytosine arabinoside (Ara-C), BM blasts >15% and abnormal karyotype) and reduced overall survival (OS) (performance status >2, intermediate- and poor-risk cytogenetics, presence of circulating blasts and red blood cell transfusion dependency with AZA) in patients with higher-risk MDS.3

AZA is a nucleoside analog (NA) that mimics physiological cytidine in terms of uptake into the cell and activation to exert its pharmacological action.12,13 The concentrative nucleoside transporter and equilibrative nucleoside transporter proteins mediate the transport of NAs such as AZA across membranes.14,15 Inside cells, AZA is activated by the enzyme uridine-cytidine kinase (UCK).13,16 The majority (80–90%) of activated AZA is incorporated into RNA resulting in mRNA and protein metabolism disruption.17–19 A smaller fraction (10–20%) of AZA is converted to decitabine (DAC) by ribonucleotide reductase (RR) and incorporated into newly synthesized DNA, depleting DNA methyltransferase 1 and leading to DNA hypomethylation.15,17

Keywords: myelodysplastic syndromes; azacitidine; resistance; UCK
Many of the mechanisms of primary resistance to NAs are related to alterations in components that impact their metabolism.\textsuperscript{20–24} Mutations in and/or expression levels of nucleoside-activating enzymes have been correlated with tumor response or patient outcome with Ara-C.\textsuperscript{22–26} A recent work indicated that increased cytidine deaminase (CDM; enzyme activating NA) expression/activity in plasma of male MDS patients contributes to decreased drug plasma half-life and could account for worse outcomes with NA therapy.\textsuperscript{27} We hypothesized that alterations in AZA cellular uptake and metabolism could be a mechanism for relapse or refractoriness to treatment.

We studied the expression of genes encoding nucleoside transporter proteins and enzymes involved in the metabolism of AZA: UCK1 and UCK2, deoxycytidine kinase (DCK), human equilibrative nucleoside transporter 1 (hENT1) and the two subunits of the RR gene (RRM1 and RRM2) in samples from primary MDS patients and correlated these data with response to AZA and clinical outcomes.

PATIENTS AND METHODS

Patients

The study population included 57 patients diagnosed with MDS or AML with 20–30% blasts between June 2006 and August 2012. MDS and AML diagnosis was made in accordance with the World Health Organization (WHO) criteria.\textsuperscript{28} Three chronic myelomonocytic leukemia cases were diagnosed according to FAB morphological and cytogenetic criteria\textsuperscript{29} and were included in the study. BM mononuclear cells were collected at diagnosis or immediately before AZA treatment separated on Ficoll Hypaque density gradient (Lympholyte-H; Cedarlane Labs, Hornby, ON, Canada). Informed consent was provided in accordance with the Declaration of Helsinki Principles and local Ethics Committee’s approval. BM mononuclear cells from 10 healthy donors obtained after informed consent were also separated as negative controls.

AZA was given subcutaneously following the schedule approved by the US Food and Drug Administration and European Medicines Agency of 75 mg/m\textsuperscript{2} per day for 7 days every 28 days. Response was analyzed on an intention-to-treat basis. Patients received at least two cycles of AZA and underwent full BM and hematological evaluation to determine response according to the International Working Group criteria for MDS\textsuperscript{30} and AML.\textsuperscript{31} Patients were considered responders when in complete remission (CR), partial remission (PR) or hematological improvement (HI) and non-responders when in stable disease (SD) or progressive disease (PD). Characteristics of all patients are described in Table 1. Though the IPSS score validation was stated for untreated patients with primary MDS, patients who had received growth factors or short courses (< 3 months) of low-dose chemotherapy were included in the analysis.\textsuperscript{32} Herein, IPSS score was recalculated before the onset of AZA therapy.

Expression analysis

Quantitative PCR (qPCR) was carried out with the ABI GeneAmp 5700 instrument and software (Applied Biosystems, Foster City, CA, USA). Specific oligonucleotides and TaqMan probes were available from Applied Biosystems (catalog no: UCK1: Hs01075618_m1, UCK2: Hs00367072_m1, DCK: Hs00176127_m1, hENT1: Hs01085706_m, RRM1: Hs01040698_m1, RRM2: Hs01072069_g1). All qPCR experiments were normalized to the housekeeping gene glyceraldehyde-2-phosphate dehydrogenase (GAPDH). Each reaction was performed in duplicate.

The mean value of the replicates for each sample was calculated and expressed as threshold cycle (CT) values. The amount of gene expression was measured as the difference (ΔCT) between the CT value of the sample and the CT value of the sample for the GAPDH endogenous control gene (ACT = CT(Target) – CT(GAPDH)). The results were then expressed as 2$^{-\Delta\Delta CT}$.

Methylation-specific PCR

One microgram of genomic DNA was converted with bisulfite before amplification. After conversion, methylation of UCK1 was assessed by methylation-specific PCR using specific primers (Supplementary Table S1). DNA from mononuclear cells from healthy donors was used as a negative control and methylated control DNA (CpGenome Universal Methylated DNA; Intergen, Purchase, NY, USA) was used as positive control. The PCR conditions were: hot start at 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 56 °C for 1 min, and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min.

Sequencing analysis

The complete coding sequence of the UCK1 gene was sequenced by the Sanger method. Five PCR amplicons were designed in order to encompass all the exonic regions within the UCK1 gene and its complete coding sequence. Each reaction was performed in duplicate. The mean value of the replicates for each sample was calculated and expressed as threshold cycle (CT) values. The amount of gene expression was measured as the difference (ΔCT) between the CT value of the sample and the CT value of the sample for the GAPDH endogenous control gene (ACT = CT(Target) – CT(GAPDH)). The results were then expressed as 2$^{-\Delta\Delta CT}$.

Table 1. Patient characteristics

| Characteristics | (n, %) |
|-----------------|-------|
| Age (years), n  |       |
| <70             | 24 (42) |
| >70             | 33 (58) |
| Sex, n          |       |
| Male            | 36 (63) |
| Female          | 21 (37) |
| WHO Classification, n |     |
| RA/RAES/RCMD    | 4 (7)  |
| RAEB-1          | 12 (22) |
| RAEB-2          | 25 (46) |
| RAEB-t/AML      | 10 (18) |
| CMML            | 3 (7)  |
| Cyogenetic risk groups, n |   |
| Low             | 35 (64) |
| Intermediate    | 6 (11)  |
| High            | 14 (25) |
| IPSS risk group, n |     |
| Low             | 0 (0)   |
| Intermediate-1  | 7 (13)  |
| Intermediate-2  | 33 (62) |
| High            | 13 (24) |
| BM Blasts, n    |       |
| <5%             | 9 (17)  |
| >5%             | 45 (83) |

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; CMML, chronic myelomonocytic leukemia; IPSS, International Prognostic Score System; RA, refractory anemia; RAEB, RA with excess of blasts; RAEB-t, RA with excess of blasts in transformation; RARS, RA with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; WHO, World Health Organization.
were treated for further 24 h with AZA at the optimal concentration of 1 μM as previously established in our laboratory. After treatment, cells were washed, and RNA and proteins were extracted. Each experiment was performed in duplicate. Effective UCK1 silencing was evaluated by qPCR with the method described above.

Western blotting

The effective presence and downregulation of the UCK1 protein was evaluated by western blotting. The protein extracts of the K562 cultures were studied using the following antibodies: anti-UCK1 mouse monoclonal antibody (1:1000, OriGene Technologies) and the antibody against α-tubulin (1:1000; Sigma-Aldrich, St Louis, MO, USA) used to confirm equal loading of extracts.

Annexin-V-binding assay

To quantify apoptosis, cells were washed twice with phosphate-buffered saline, and the cell pellet was resuspended in 100 ml of Annexin-V-FLUOS (HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered saline solution with 2.5 mM CaCl2). Phycocyanin-labeled Annexin-V (Roche Diagnostics GmbH, Mannheim, Germany) and propidium iodide (PI) were added, and cells were incubated for 15 min in the dark at room temperature. After the addition of 400 ml of binding buffer and agitation, flow cytometer analysis was performed using a FACScant (Becton Dickinson, Franklin Lakes, NJ, USA). Cells positively stained with Annexin-V and PI-negative were considered apoptotic.

Statistical analysis

All descriptive statistics and tests (Mann–Whitney nonparametric U-test, Kruskal–Wallis test, Chi-square test and Fisher’s exact test) were performed using the statistical package SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). Regarding the polymorphic loci of UCK1, unconditional logistic regression was used to assess genotype distributions between patients with or without response to AZA, considering the homozygotes for the more frequent allele as the reference class. Due to the small numbers of samples available, heterozygous and homozygous subjects for the variant alleles were grouped together. A P-value < 0.05 was considered statistically significant.

OS was defined as the time from initiation of AZA treatment to death from any cause. Patients who remained alive were censored at the time of last follow-up. Survival from time of MDS diagnosis was also evaluated in patients with or without response to AZA. Time-to-event curves were estimated by the Kaplan–Meier method and compared by stratified log rank tests. Stratified Cox proportional hazards regression models were used to estimate hazard ratios (HRs) and associated 95% confidence intervals. The primary analysis of OS for patients with or without response to AZA used the stratified Cox proportional hazards model without any covariate adjustments to estimate the HR. All P-values reported are two sided.

RESULTS

Patient characteristics

This study included samples from 57 patients recruited from three different centres in Italy (36/21 M/F with a median age at diagnosis of 70 years (range, 37–86 years). At baseline, median hemoglobin level, absolute neutrophil count and platelet count were 8.9 g/dl (range, 6–14.1), 2 × 10⁹ cells/l (range, 1 × 10⁹–15 × 10⁹) and 48 × 10⁹/l (range, 3 × 10⁹–267 × 10⁹), respectively. Median BM blast count was 13.2%. Cytogenetic risk group, TET2 mutations, IPSS risk groups or BM blast percentage were not significantly different in patients with or without response to AZA.

**Table 2.** Responses to azacitidine according IWG 2006 criteria

| IWG 2006 responses | n | % | Groups |
|-------------------|---|---|--------|
| Complete response  | 6 | 11| Responders (40%) |
| Partial response   | 6 | 11|          |
| Hematological improvement | 8 | 15|          |
| Marrow complete response | 3 | 5|          |
| Stable disease     | 23| 38| Non-responders (60%) |
| Progressive disease| 11| 20|          |

Abbreviation: IWG, International Working Group.

(CR, PR, HI or mCR) and 34 (60%) did not (SD or PD) (Table 2). Overall, median OS from initiation of AZA therapy was 27.2 months; patients who achieved CR/mCR, PR or HI had a mean OS of 54.1, 38 and 23.1 months, respectively, confirming that OS was longer in patients with CR/PR/HI, compared with patients with no response, as previously published. In fact, the median OS from diagnosis in patients with or without a response to AZA was 72.3 and 35.1 months, respectively. When the scoring system designed by Izzykson et al. for predicting OS after AZA was applied to our MDS patients, 18% of them were included in the low-, 79% in the intermediate- and 3% in the high-risk category.

Analysis of TET2 mutations

Eleven TET2 mutations were found in eight of 35 patients, including three frameshift mutations (all inducing a premature STOP codon), three nonsense and five missense mutations, all five in the first conserved domain and one of them was found in homozygosity. Three patients showed two different mutations in the gene. Among patients with TET2 mutations, three patients showed a response to AZA (CR, n = 1; PR, n = 1; and HI, n = 1) while five patients did not (SD, n = 3; PD, n = 2) (P = 0.7).

Expression levels of enzymes involved in the metabolism of AZA in MDS patients

Gene expression of the studied enzymes was heterogeneous among MDS cases, and the level of mRNA expression was different for the various genes (UCK1, median 0.25, Q1: 0.15–Q3: 0.55; UCK2, median 0.45, Q1: 0.25–Q3: 0.76; DCK, median 5.2, Q1: 3.15–Q3: 10.9; hENT1, median 5.9, Q1: 2.7–Q3: 12.6; RMM1 median 1.3, Q1: 0.6–Q3: 2.6; and RRMD2 median 9.2, Q1: 3.7–Q3: 20.1; Figure 1a). We investigated the association between the expression of each gene and the response to AZA treatment as well as other clinical characteristics. Generally, the expression level of each gene was not significantly different in patients with or without response to AZA. However, lower expression of the UCK1 gene was shown in patients without response to AZA (median 0.23 (Q1: 0.15–Q3: 0.40) vs median 0.49 (Q1: 1.7–Q3: 9.3); P = 0.07) (Figure 1b). We also evaluated the expression of UCK1 in the three different groups of the AZA scoring system,11 observing no differences among them. Mean UCK1 expression level in the low-risk group was 0.28 ± 0.13, in the intermediate 0.45 ± 0.38 and in the high 0.15 (P = 0.22).

Correlation was found with age, sex, hemoglobin, absolute neutrophil count, platelet count, WHO classification, cytogenetic risk group, TET2 mutations, IPSS risk groups or BM blast percentage for expression of UCK1, UCK2, DCK, hENT1, RMM1 or RRMD2 (data not shown).

Molecular alterations of UCK1 in MDS patients

In order to investigate the molecular events leading to differential expression of UCK1, we measured the methylation status of the CpG island at the UCK1 promoter. The CpG island was unmethylated in all the samples analyzed and in healthy controls (Figure 2a). To assess whether the difference in UCK1 expression could be due to the presence of genetic variants, we sequenced
the UCK1 coding sequence in a subset of 42 patients for which genomic DNA was available. By this method, seven polymorphic loci were identified. The position of the loci was compared with resequencing data reported in the 1000 Genomes project (http://www.1000genomes.org/). All seven loci corresponded to known single-nucleotide polymorphism, three of which fell into intronic sequences (rs3904960 in Intron 1; rs2296956 and rs189473964 in Intron 3), two in regulatory sequences (rs7041225 in the 5’ untranslated region; rs150900763 in the 3’ untranslated region) and two in the coding sequences of exon 2 (rs7867616, Glu29Gln) and exon 6 (rs2296957, Ala180Ser) (Figure 2b). For the SNPs in the untranslated region, rs2296956 and rs189473964, the variant allele was present in 95% of the samples. For the SNPs in the coding sequences, rs7867616 and rs150900763, the variant allele was present in 60% of the samples. The positions of the SNPs in the coding sequences were 13 amino acids downstream of the sites coding for the variant sequences (Figure 2b). For the SNPs in the untranslated region, the positions of the SNPs were 224 and 1000 nucleotides upstream of the TATA box, respectively.

Silencing of UCK1 gene expression

To understand the effects of UCK1 on the sensitivity to AZA, we carried out a knockdown experiment using two synthetic siRNAs against the UCK1 gene. K562 cells cultured in the presence of two siRNAs for 48 h plus 24 h of exposure to AZA showed a significant downregulation of the UCK1 mRNA compared with control cultures with only one of the two siRNA tested (siUCK1-1 vs control, \( P = 0.046 \); siUCK1-1 + AZA vs control + AZA, \( P = 0.021 \); Figure 3a). UCK1 protein expression was robustly diminished in K562 cells exposed to siUCK1-1 (Figure 3b). When UCK1 was downregulated, a reduction of AZA-induced apoptosis was observed (siUCK1-1: Annexin V-positive cells 11% ± 0.34% vs non-silenced control cultures: Annexin V-positive cells 31% ± 0.65%, \( P = 0.0015 \); Figure 3c). siUCK1-2 was not able to silence the gene, and in that case, there was no variation in the sensitivity to AZA with respect to control or mock transfected cells (Supplementary Figure S1).

Prognostic impact of clinical characteristics and correlation with UCK1 expression

To assess the variables predictive of OS from onset of AZA treatment, we analyzed the baseline characteristics of sex, age, BM blast percentage, IPSS risk score, cytogenetics (within IPSS subgroups), score according to ‘AZA scoring system’ \(^{11} \), TET2 mutations and the different genotypes found in the UCK1 gene. Univariate analysis showed that OS was significantly influenced only by IPSS risk score (\( P = 0.02 \)) and achievement of response to AZA (\( P = 0.001 \)). Median OS by the ‘AZA scoring system’ was not reached in the low-risk group vs 44.7 months in the intermediate-risk group vs 14.9 months in the high-risk group (\( P = 0.18 \)). We also evaluated in the univariate analysis the prognostic impact of the expression of UCK1, UCK2, DCK, hENT1, RRM1 and RRM2. The median levels of gene expression were used to divide cases with low (below median) or high (above median) expression for each gene. We demonstrated that patients with lower expression of UCK1 had shorter OS than patients with higher expression of UCK1 (median OS values: 19 months vs 49 months; \( P = 0.041 \); Figure 4). Levels of UCK2, DCK, hENT1, RRM1 and RRM2 expression did not significantly impact OS. Multivariate analysis showed that the independent factors influencing OS were achievement of response to AZA (HR, 0.23; \( P = 0.0016 \)) and higher expression of UCK1 (HR, 0.38; \( P = 0.049 \)).

DISCUSSION

This study suggests that the expression of UCK1, involved in the activation of AZA, may influence the clinical response to AZA treatment in patients with MDS. The biological regulation of UCK1 expression was not due to methylation status of the UCK1 promoter nor to the presence of genetic polymorphisms in the UCK1 sequence. Prolonged OS in this series of AZA-treated patients with MDS was associated with higher expression of UCK1.

Hypomethylating agents AZA and DA have been shown to induce overall responses in approximately 40–60% of patients with MDS. \(^{1,2,35,36} \) However, only AZA has been shown to significantly prolong OS in patients with IPSS higher-risk MDS. In our cohort, we confirmed that response to AZA is correlated with prolonged survival. There have been efforts to elucidate factors predictive of response to AZA, in hopes of selecting candidates most likely to respond to treatment. We applied the ‘AZA scoring system’ \(^{11} \) to our cohort of MDS patients, and although we could stratify them for OS, only a trend to longer survival was observed for low scoring ones (9/57), the vast majority belonging to intermediate scoring group. Both AZA and DAC inhibit DNA methyltransferase activity and methylation of new DNA strands.

MDS is clinically the most responsive disease to new DNA strands. MDS is clinically the most responsive disease to hypomethylating agents, and although azacitidine has been shown to improve OS, it can be associated with significant toxicity. In this study, we aimed to identify factors predictive of response to AZA and whether these factors could be used to identify patients who may have a more favorable outcome. We found that patients with higher expression of UCK1 had a significantly longer OS than patients with lower expression of UCK1 (median OS values: 19 months vs 49 months; \( P = 0.041 \)).

To understand the effects of UCK1 on the sensitivity to AZA, we carried out a knockdown experiment using two synthetic siRNAs against the UCK1 gene. K562 cells cultured in the presence of two siRNAs for 48 h plus 24 h of exposure to AZA showed a significant downregulation of the UCK1 mRNA compared with control cultures with only one of the two siRNA tested (siUCK1-1 vs control, \( P = 0.046 \); siUCK1-1 + AZA vs control + AZA, \( P = 0.021 \); Figure 3a). UCK1 protein expression was robustly diminished in K562 cells exposed to siUCK1-1 (Figure 3b). When UCK1 was downregulated, a reduction of AZA-induced apoptosis was observed (siUCK1-1: Annexin V-positive cells 11% ± 0.34% vs non-silenced control cultures: Annexin V-positive cells 31% ± 0.65%, \( P = 0.0015 \); Figure 3c). siUCK1-2 was not able to silence the gene, and in that case, there was no variation in the sensitivity to AZA with respect to control or mock transfected cells (Supplementary Figure S1).

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The correlation between the presence of mutations in several epigenetic interfering genes (for example, *TET2*, *EZH2*, *ASXL1* and *DNMT3*) and response to AZA has been shown but must be further confirmed to determine clinical applicability. Only eight of the 35 MDS patients analyzed in our study had mutated *TET2*. As expected, there was no correlation with mutational status and OS, but we could not observe higher rates of response to AZA in mutated cases, most probably because of numerical limitations.

Although AZA and DAC have been shown to induce DNA hypomethylation in vitro and in vivo, despite some reports, research has failed to elucidate the correlation between baseline DNA hypermethylation and response to AZA or DAC. Therefore, the identification of biological markers that permit the selection of patients with MDS unlikely to respond to AZA therapy is of great importance.

The cytosine NAs AZA and DAC are transported inside the cell via specific proteins and then converted into active nucleotides to induce DNA hypomethylation and cytotoxicity. An important mechanism of resistance to NAs may be low levels of active intracellular agent resulting from insufficient cellular uptake, reduced levels of activating enzymes and increased degradation of the agents. Therefore, we speculated that similar mechanisms could account for resistance to AZA. The first ATP-dependent phosphorylation, catalyzed by the enzyme UCK for AZA and DCK for DAC, is a limiting step for AZA and DAC activation. In particular, DCK deficiency has been reported as a major mechanism of resistance to the NAs DAC, Ara-C and gemcitabine. Recently, it has been reported that higher plasma levels of CDA may account for worse outcome after NA treatment in male MDS patients. In that study, CDA production by the liver and its presence in plasma were evaluated, analyzing its impact on DAC pharmacokinetics. We did not observe any difference in AZA response rates between male and female patients. On the other hand, there is still limited information regarding the relationship between alterations in the components of cellular AZA metabolism and the resistance seen in 30–40% of AZA-treated patients with MDS. AZA incorporation into RNA...
may enhance its clinical activity in MDS, as survival data suggest superiority to DAC, which only undergoes DNA incorporation. We compared the expression of UCK1, UCK2, DCK, hENT1, RRM1 and RRM2 in MDS patients with or without clinical response to AZA. Lower expression levels of UCK1 were shown in patients resistant to AZA. In contrast to UCK1, expression levels of UCK2 were not significantly different in responsive vs non-responsive patients. One may presume that both proteins phosphorylate AZA to its nucleoside monophosphate, but our results suggest that these two enzymes could act independently and do not seem to have the same relevance in AZA metabolism. In MDS patients with low expression of UCK1, we did not observe a compensatory higher expression of UCK2. Consistently with our finding, it has been demonstrated that the phosphorylation efficiency of the enzymes UCK1 and UCK2 on the same substrate can be different. Levels of DCK, hENT1, RRM1 and RRM2 expression were not associated with achievement of response to AZA. Additionally, we also found that OS from onset of AZA treatment was significantly shorter in patients with lower levels of UCK1 expression. The prognostic weight of UCK1 may reflect a mechanism of action specific to AZA, which modulates protein synthesis via RNA incorporation. RRM1 and RRM2 did not show lower expression levels in patients without response to AZA in this study. This could suggest that the step catalyzed by RRM1 and RRM2, which converts AZA to DAC and leads to DNA incorporation, is not discriminative for the clinical activity of this agent.

Aberrant hypermethylation of UCK1 was not present in any patient examined, including healthy controls. In addition, the various polymorphisms found in the UCK1 sequence were not related to its expression level nor with response to AZA treatment, alternate to what has been demonstrated for DCK. We did not explore other possible mechanisms of gene expression regulation, such as miRNA or splicing variants. However, UCK1 has several regulating miRNAs, and future studies are warranted, because miRNAs could be helpful tools to modulate sensitivity to AZA. In fact, in order to explore this hypothesis and support our findings, we elaborated a

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**Figure 3.** Silencing of the UCK1 gene. Effect of siUCK1-1 on the expression of the UCK1 gene (relative to GAPDH) was evaluated using quantitative PCR (a) and western blotting analysis (b). Downregulation of the UCK1 mRNA by siUCK1-1 was observed in non-transfected compared with mock transfected control cells, both not treated (\( ^{*} P = 0.046 \)) and treated with AZA (\( ^{**} P = 0.021 \)) (a). Mean of two different experiments is shown. UCK1 protein expression was equally diminished in K562 cells exposed to siUCK1-1 (b). (c) Apoptosis (Annexin V-FITC (fluorescein isothiocyanate) and PI staining) was measured in K562 cells cultured with SRC, TKO and siUCK1-1, without AZA and with 24-h AZA. AZA induced an increase in Annexin V-positive cells, but apoptosis was blunted in K562 cells when siUCK1-1 was present and UCK1, as well as UCK1 protein, were downexpressed (\( ^{*} P = 0.0015 \), siUCK1-1 transfected vs control).
We may envisage a future where selection of AB and VS supervised the study. All authors read the article.

Although other variables may impact response in the clinical setting, these results highlight the importance of UCK1 for AZA activity. Although we demonstrated that level of UCK1 expression is correlated with outcome to AZA therapy, low expression of DCK correlates with outcome to AZA therapy, low expression of UCK1 shows decreased its otherwise significant AZA-induced apoptosis. The reduced expression of UCK1 in the leukemic cell line K562 decreased its otherwise significant AZA-induced apoptosis. The reduced expression of UCK1 shows decreased its otherwise significant AZA-induced apoptosis.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)