Increased CDA Expression/Activity in Males Contributes to Decreased Cytidine Analog Half-Life and Likely Contributes to Worse Outcomes with 5-Azacytidine or Decitabine Therapy

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Abstract

**Purpose:** The cytidine analogs 5-azacytidine and decitabine, used to treat myelodysplastic syndromes (MDS), produce a molecular epigenetic effect, depletion of DNA-methyltransferase 1 (DNMT1). This action is S-phase dependent. Hence, genetic factors that decrease the half-lives of these drugs could impact efficacy. Documentation of such impact, and elucidation of underlying mechanisms, could lead to improved clinical application.

**Experimental design:** Cytidine deaminase (CDA) rapidly inactivates 5-azacytidine/decitabine. The effect of CDA SNP A79C and gender on CDA expression, enzyme activity, and drug pharmacokinetics/pharmacodynamics was examined in mice and humans, and the impact on overall survival (OS) was evaluated in 5-azacytidine/decitabine-treated patients with MDS (n = 90) and cytarabine-treated patients with acute myeloid leukemia (AML) (n = 76).

**Results:** By high-performance liquid chromatography (HPLC), plasma CDA activity was decreased as expected in individuals with the SNP A79C. Interestingly and significantly, there was an even larger decrease in females than in males. Explaining this decrease, liver CDA expression was significantly lower in female versus male mice. As expected, decitabine plasma levels, measured by mass spectrometry, were significantly higher in females. In mathematical modeling, the detrimental impact of shorter drug half-life (e.g., in males) was greater in low compared with high S-phase fraction disease (e.g., MDS vs. AML), because in high S-phase fraction disease, even a short exposure treats a major portion of cells. Accordingly, in multivariate analysis, OS was significantly worse in male versus female patients with MDS treated with 5-azacytidine/decitabine.

**Conclusions:** Increased CDA expression/activity in males contributes to decreased cytidine analog half-life and likely contributes to worse outcomes with 5-azacytidine or decitabine therapy. Clin Cancer Res; 19(4); 938–48. ©2012 AACR.

Introduction

The cytidine analog drugs 5-azacytidine and decitabine are unique oncotherapeutics by virtue of a powerful molecular epigenetic effect, depletion of DNA methyltransferase 1 (DNMT1) after incorporation into DNA (1). Levels of these drugs well below 0.5 µmol/L are sufficient to deplete DNMT1 (2–8). Hence, in contrast to the cytidine analogs cytarabine and gemcitabine that are administered at high doses that are close to maximum tolerated levels (100–3,000 mg/m2) intended for antimetabolite cytotoxic effects, 5-azacytidine and decitabine are administered at relatively low dosages (5–75 mg/m2). Another crucial consideration in the clinical application of 5-azacytidine/decitabine is that DNMT1 depletion requires incorporation of drug into DNA during DNA replication (S-phase–dependent mechanism of action; ref. 5). Hence, drug exposure time and schedule are likely to influence treatment efficacy (5, 9–10). Supporting this premise, reduction of decitabine doses to 20 mg/m2/d from the 45 mg/m2/d dose originally approved by the U.S. Food and Drug Administration (FDA) but administration on more days (5 days every 4 weeks instead of 3 days...
CDA enzyme activity is the nonsynonymous (15, 18–19). A pharmacogenetic factor known to decrease (17), a drastic reduction largely attributable to CDA (16), whereas, the half-life of gemcitabine therapy (20–24).

A79C (Lys27Gln, rs2072671); approximately 60% of Cau-

cated in severe toxic events occurring with cytarabine and 27 to cause as much as a 3-fold decrease in CDA enzyme activity/expression on 5-azacytidine/decitabine treatment outcomes. Interestingly and significantly, we found that gender has a substantially greater influence on CDA enzyme activity/expression than the well-known CDA SNP A79C, with a corresponding impact on overall survival (OS) in 5-azacytidine/decitabine-treated patients with MDS. Most importantly, the identification of this pharmacogenetic factor and the mechanism by which it affects outcomes suggests rational methods for optimizing the clinical application of these unique oncotherapeutics.

The impact of A79C on 5-azacytidine or decitabine treatment outcomes has not previously been evaluated. Such an impact, if it exists, could be different from that reported with gemcitabine or cytarabine: 5-azacytidine/decitabine are administered at relatively low doses, therefore, an increase in 5-azacytidine/decitabine levels from the A79C SNP might not produce a clinically significant increase in toxicity. Instead, genetic factors that increase CDA activity might decrease efficacy by lowering drug levels and shortening half-lives. In this regard, another genetic variable relevant to cytidine analog metabolism and half-life is gender: for both gemcitabine and cytarabine, higher clearance and shorter half-life have been described in males compared with females (25–26), and in a murine model of colon cancer, the cytidine analog zebularine has showed decreased pharmacodynamic and chemopreven-
tive efficacy in males (27). However, the mechanisms underlying these gender differences in cytidine analog levels and actions have not been elucidated.

Thus, given the prominence of CDA in cytidine analog metabolism and clearance, we examined the connections between gender and CDA enzyme activity, CDA expression, and decitabine pharmacokinetics and pharmacodynamics. To better understand how differences in treatment exposure time resulting from differences in CDA activity might impact less aggressive versus more aggressive malignant disease, we mathematically modeled the relation between disease S-phase fraction and treatment exposure time. Interestingly and significantly, we found that gender has a substantially greater influence on CDA enzyme activity/expression than the well-known CDA SNP A79C, with a corresponding impact on overall survival (OS) in 5-azacytidine/decitabine-treated patients with MDS. The mechanistic insights suggest rational methods for optimizing the clinical application of these important oncotherapeutics.

**Materials and Methods**

**Patients and treatment**

The analysis of MDS and AML patient data and samples was approved by the Cleveland Clinic/Case Comprehensive Cancer Center Institutional Review Board (Cleveland, OH). Patients were diagnosed with MDS (*n* = 90) and AML (*n* = 76) per WHO criteria, and initiated on treatment between January 2002 and December 2007, with tissue-banked samples obtained with written informed consent available for SNP analysis by sequencing, and with verifiable follow-up and survival annotation. Date of death was based on physician documentation in the medical record, or on the social security death index, and a phone call to the emergency contact. Other variables analyzed were those known to have major prognostic importance in patients with MDS and AML (bone marrow myeloblast%, karyotype, age; ref. 28). Patients were analyzed in 2 groups: (i) Ninety patients with MDS treated either with 5-azacytidine 75 mg/m²/d D1-7 (s.c. or i.v.) or decitabine 20 mg/m²/d D1-5 (i.v. over 1 hour) in 28-day cycles; and (ii) Seventy-six patients with AML treated with cytarabine-based

**Translational Relevance**

The cytidine analogs 5-azacytidine and decitabine have a powerful molecular epigenetic effect, depletion of DNA methyltransferase 1 (DNMT1). This is an S-phase, DNA replication-dependent action, so treatment exposure time is likely a crucial determinant of efficacy, and genetic factors that influence cytidine analog metabolism could potentially impact treatment outcomes. The ubiquitously expressed enzyme cytidine deaminase (CDA) rapidly inactivates 5-azacytidine/decitabine. We evaluate here, for the first time, the impact of pharmacogenetic factors that affects CDA enzyme activity/expression on 5-azacytidine/decitabine treatment outcomes. Interestingly and significantly, we found that gender has a substantially greater influence on CDA enzyme activity/expression than the well-known CDA SNP A79C, with a corresponding impact on overall survival (OS) in 5-azacytidine/decitabine-treated patients with MDS. Most importantly, the identification of this pharmacogenetic factor and the mechanism by which it affects outcomes suggests rational methods for optimizing the clinical application of these unique oncotherapeutics.
induction chemotherapy: cytarabine dose 100 to 200 mg/m²/d, days 1 to 7 continuous infusion and anthracycline on days 1 to 3.

Measurement of CDA enzyme activity by an HPLC assay
Conversion of cytidine into uridine by plasma at 37°C was measured by high-performance liquid chromatography (HPLC) based on published methods (29). Reaction buffer 0.1 mol/L Tris/HCL pH 7.5 (265 μL) was added to 25 μL of human plasma followed by addition of cytidine to a final concentration of 4.1 mmol/L and 5-flourouridine 0.381 mmol/L (not metabolized by CDA) as an internal control. After incubation at 37°C for 60 minutes, the reaction was terminated with 50 μL of hydrochloric acid 1N. Blanks used in calculations consisted of the above but with cytidine substrate added at the end of the 60-minute incubation. After reaction termination, protein was precipitated with trichloroacetic acid (2%). A total of 20 μL of supernatant was injected for HPLC using ammonium acetate (15 mmol/L) as the mobile phase with a flow rate of 0.35 mL/min through Xbridge OST C18, 2.5 μm, 4.6 × 50 mm column on Dionex UltiMate 3000 μ-HPLC system supported with Chromeleon 7.1 data system (Dionex Corporation). Retention time and peak area of uridine at 260 nm were compared with the internal control for each test sample. Known concentrations of uridine were used to construct a standard curve to calculate the amount of uridine based on the net uridine peak area. One unit of CDA enzyme activity is defined as the amount of enzyme needed to produce 1 μmol of uridine in 1 minute. The specific activity of purified CDA (A79A) was 308.9 U/mg. Purified CDA was used for calibration and for quality control (gift of Professor Silvia Vincenzetti, Universita di Camerino, Italy). Multiple runs with known concentrations of uridine were used to confirm accuracy and precision: between run variability was less than 5%.

Sequencing for the A79C SNP in CDA (Lys27Gln, rs2072671) and measurement of CDA mRNA
The ArchivePure Kit (5Prime) was used to extract DNA mononuclear cells isolated by Ficoll-Hypaque density centrifugation of bone marrow or peripheral blood. Primers 5'-GTTCCCTAAGGAGAGTGTGAAGCA-3' (forward), 5'-gcctcttcctgtacatcttcctct-3' (reverse; accession number NM_001785), Expand High Fidelity PLUS PCR System (Roche Applied Science) and PCR conditions: initial denaturation 94°C × 4 minutes, 30 cycles with denaturation at 94°C × 30 seconds, annealing at 56°C × 30 seconds, and elongation at 72°C × 40 seconds were applied to 50 ng DNA template. Amplicons were purified using the Montage PCR96 Cleanup Kit (Millipore) and sequenced using Big Dye Terminator v3.1 (Applied Biosystems) with forward primer. Sequencing reactions were purified using the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore) and run on a 3100-Avant Genetic Analyzer (Applied Biosystems).

Quantitative real-time PCR (qRT-PCR; accession # NM_028176.1) primers were 5'-CDAAAGGTGAACTTGATTCCACACACCA-3' (forward) and 5'-CDATGGAA-TACCCGTGTCTTGGAGTACC-3' (reverse).

Decitabine pharmacokinetic studies in CD-1 mice
Procedures with CD-1 strain mice were approved by the Institutional Animal Care and Use Committee (IACUC) of Avanza (Gaithersburg, MD). Animals were dosed with decitabine or its vehicle via oral gavage at a dose volume of 10 mL/kg (based on most recent body weight). Blood samples (~0.5 mL or maximum possible) were collected via intracardiac puncture from non-fasted, anesthetized (70% CO2/30% O2) animals 15, 30, 60, 90, 120, and 180 minutes after administration of decitabine. Sample collection tubes were prepared before each collection day by adding 10 μL/tube of a 10 mg/mL tetrahydouridine (THU) solution. This THU solution was prepared by adding sodium phosphate dibasic (1.5 mg/mL), sodium phosphate monobasic (0.4 mg/mL), and THU (10 mg/mL) to sterile water for injection and mixing until visually clear. Samples were collected from the first available 3 animals per time point. All samples were collected within 5 minutes of the target time. Two-way (gender vs. time) ANOVA tests (this is reasonable because each measurement was obtained from a separate mouse and is thus independent) were used to compare drug levels in female versus male mice: the R function lm() was used with time as a factor. In the high dose group, the interaction terms were not significant, so an additive model was used. In the low dose group, gender–time interaction terms were significant at 30 and 60 minutes, so interactions were kept.

Decitabine pharmacodynamic studies in NSG mice
Procedures with NOD-scid-gamma (NSG) mice were approved by the IACUC of Cleveland Clinic. NSG mice were administered subcutaneous decitabine 0.1 mg/kg or 0.4 mg/kg on day 1, 3, and 4, and bone marrow was harvested on day 5 for Western blot analysis of DNMT1 levels in the nuclear fraction (n = 12, evenly divided between male and female mice).

Western blot analysis for DNMT1 levels
After washing twice with 10 mL ice-cold 1 × PBS containing protease inhibitors (Sigma-Aldrich, A8340), bone marrow cells were resuspended in 500 μL of 1 × hypotonic buffer containing 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 10 mmol/L phenylmethylsulfonylfluoride (PMSF), and protease inhibitors (Sigma-Aldrich, A8340), then 20 μL of 10% NP-40 was added to break cell membranes. After 5-minute incubation on ice, the suspensions were centrifuged at 344 × g for 10 minutes. The supernatant was transferred to clean 1.5 mL Eppendorf tubes (labeled cytoplasmic fraction). Nuclear pellets were washed twice with ice-cold 1 × PBS, resuspended in 100 μL of 50 mmol/L Tris-HCL, pH 8.0, 1 mmol/L MgCl2, 10
mmol/L PMSF, protease inhibitor cocktail (Sigma-Aldrich, A8340), and Benzonase (Sigma-Aldrich, D5915, 250 U) and incubated on ice for 90 minutes with vigorous vortex every 5 minutes. Then, 500 μL protein extraction buffer containing 1.5% NP-40, 500 mmol/L NaCl, 5 mmol/L diithiothreitol, 10 mmol/L PMSF, and 5 μL of protease inhibitor cocktail (Sigma-Aldrich, A8340) in 50 mmol/L phosphate buffer (pH 7.4) was added. After 30-minute incubation on ice with vortexing every 5 minutes, the mixture was centrifuged at 12,396 × g for 15 minutes. The same extraction process was repeated 2 more times with 300 and 200 μL of extraction buffer. The supernatant containing nuclear proteins was combined and transferred to clean tubes, and protein concentration was determined by bicinchoninic acid assay. A total of 100 μg of nuclear protein extracts were subjected to one-dimensional SDS-PAGE on precast 4% to 12% NuPAGE gels (Invitrogen) for gel electrophoresis as per manufacturer’s instructions (Invitrogen) and then transferred to polyvinylidene difluoride membranes (Millipore) at 35 constant volts for 1 hour (Invitrogen’s XCell II Blot module). Primary antibodies were anti-DNMT1 (ab92453, ABCAM) with anti-H3 to control for loading.

Overlap probability

The y-axis in Fig. 4 represents the probability "P" of overlap between the fraction of time of adequate drug exposure "D" and the fraction of time of cell susceptibility "S". The following R code snippet generates P:D = seq(0,1,0.01); S = seq(0,1,0.01); P = outer(D,S,FUN = "+"); P[P>1] = 1. Algebraically, one derives P by letting D occupy positions 0 to D on the perimeter of a circle indexed 0 to 1, by noting that S and D miss each other if the beginning of S lies between D and 1−S, by noting that this happens with probability 1−S−D, and by noting that P is 1 minus this, that is, P = S + D, unless S > 1−D, in which case P = 1.

Statistical analysis of clinical data

SAS was used for all statistical analyses except those conducted in R. Sample characteristics between patients of different gender were compared using the Wilcoxon test or χ² test. PROC LIFETEST to generate Kaplan–Meier product limit survival estimates was used to model time from diagnosis to death (OS), and PROC PHREG (Cox proportional hazards regression analysis) was used to assess the impact of multiple variables on time to death. All statistical significance levels were α = 0.05. In the Cox model, variables assessed for influence on time from diagnosis to death were age, myeloblast% at diagnosis, metaphase karyotyping result [categorical variable: (i) chromosome 7 or ≥3 chromosome abnormalities, (ii) other chromosome abnormalities, (iii) normal cytogenetics), and CDA genotype [categorical variable: (i) homozygous ancestral allele—AA, (ii) heterozygous ancestral allele—AC, (iii) homozygous variant allele—CC]. Univariate variables with an α ≤ 0.05 were retained in the multivariate model.

Results

The A79C SNP and female gender are associated with decreased plasma CDA enzyme activity

DNA from normal volunteers and patients with MDS and AML was sequenced to identify cases with the A79C SNP. Plasma samples from equal numbers of individuals homozygous for the ancestral allele (AA, n = 32), heterozygous for the A79C SNP (AC, n = 32), and homozygous for the A79C SNP (CC, n = 32) were then examined for CDA enzyme activity using an HPLC-based method (29). Consistent with observations from other groups (20–21, 23, 30), plasma CDA activity was significantly decreased in samples homozygous for the A79C SNP (CC) compared with samples homozygous for the ancestral allele (AA; P < 0.01; Fig. 1A).

Plasma levels of cytidine analog drugs have been shown to be lower in males (25–26); however, the reason for this has not been elucidated. To see whether gender difference in CDA enzyme activity could be a cause, the plasma samples were further subclassified by gender. Male gender was associated with significantly higher CDA enzyme activity (P < 0.001; Fig. 1B and C). Notably, the difference in plasma CDA activity between male and female gender (>2.5-fold and Fig. 1C) was more than the difference in activity between AA and CC genotype within each gender (>1.5-fold; Fig. 1B).

Gender difference in CDA mRNA expression in the liver and in leukemic cells

The liver is the organ which is most enriched in CDA (Supplementary Fig. S1). Therefore, to evaluate the basis for higher CDA enzyme activity in males compared with females, CDA gene expression was measured in murine liver by qRT-PCR. CDA expression was more than 3-fold higher in male than female liver tissue (n = 6/group, P = 0.01, and Fig. 2A). Because higher CDA expression within malignant cells themselves is a possible mechanism of resistance to cytidine analogs (31–37), we examined public databases of microarray gene expression in primary cancer cells: CDA expression was significantly higher in cancer cells from males compared with females for a number of cancers, including myeloid leukemia (ref. 38; Supplementary Fig. S2).

Decitabine pharmacokinetics and pharmacodynamics in female and in male mice

Higher CDA expression and enzyme activity in males compared with females can be expected to produce lower plasma cytidine analog levels in males. To confirm this, decitabine 0.4 mg/kg (Fig. 2B) or 1.0 mg/kg (Fig. 2C) was administered to CD1 mice by oral gavage, and plasma drug levels were measured by liquid chromatography/tandem mass spectrometry (LC/MS-MS) at 15, 30, 90, 120, and 180 minutes (n = 36 for each dose level). At both dose levels, plasma drug levels were decreased in males compared with females: this decrease was significant collectively across all time points in the higher dose group (P = 0.005, gender main effect in a gender × time
2-way ANOVA analysis; Fig. 2C) and at 30 minutes ($P = 0.0003$) and 60 minutes ($P = 0.01$) in the lower dose group (Fig. 2B, $P$ values for 2-way ANOVA interaction terms). The intended molecular pharmacodynamic effect of decitabine treatment is depletion of DNMT1. DNMT1 depletion in bone marrow cells was measured by Western blot analysis on day 5 after 3 days (day 1, 3, and 4) of subcutaneous decitabine 0.1 mg/kg/d or 0.4 mg/kg/d. Decitabine produced greater depletion of DNMT1 in the cells from females (Fig. 2D).

Overall survival was significantly worse in male MDS patients treated with 5-azacytidine/decitabine

Higher CDA enzyme activity and expression in males, and hence lower 5-azacytidine or decitabine drug levels, could affect treatment outcomes. OS stratified by gender was evaluated in patient with MDS treated with 5-azacytidine or decitabine but not in patients with AML treated with cytarabine is an interaction between treatment exposure time and S-phase fraction of disease: even a short exposure to S-phase–specific therapy may treat a major portion of high S-phase fraction-aggressive AML cells (39), but only a small fraction of more indolent disease (relatively low S-phase fraction has been documented in MDS; ref. 40). This notion was mathematically modeled as follows: let $D$ be the proportion of time that a cell has intracellular drug concentrations that are above the efficacy threshold (the threshold of 5-azacytidine or decitabine required to deplete DNMT1), and let $S$ be the fraction of time that cells are susceptible to drugs (i.e., in S-phase with DNA replication that incorporates sufficient amounts of drug to achieve DNMT1 depletion). The output $P$ is then the probability of overlap between $D$ and $S$: higher values of $P$ predict pharmacodynamic effect in a larger fraction of the malignant clone ($P = 1.0$ predicts a pharmacodynamic effect in 100% of the malignant clone). This modeling suggested that a male gender-related decrease in drug half-life (i.e., decrease in $D$) would produce a substantially greater decrease in efficacy in disease with a low S-phase fraction (Fig. 4).
Discussion

DNMT1 depletion by 5-azacytidine or decitabine is S-phase dependent. Because longer drug exposure time increases the likelihood that susceptible S-phases coincide with effective levels of drug, shortened 5-azacytidine, or decitabine half-lives caused by higher CDA expression and enzyme activity in males could plausibly contribute to the worse outcomes observed in male patients with MDS.

Figure 2. A difference in CDA expression between males and females affects decitabine drug levels and pharmacodynamic effects. A, liver CDA expression in female and male mice. CDA expression measured by qRT-PCR. P value Wilcoxon test. B, decitabine plasma drug levels were decreased in male compared with female mice at multiple time points after administration of decitabine by oral gavage. Mice administered decitabine 0.4 mg/kg. Drug levels measured by LC/MS-MS. Levels from 3 mice per gender for each time point (n = 36). C, decitabine levels in mice administered decitabine 1.0 mg/kg (n = 36). D, DNMT1 depletion in bone marrow cells after decitabine treatment of female and male mice. Decitabine (DAC) 0.1 mg/kg or 0.4 mg/kg or PBS (vehicle control) was administered subcutaneously on day 1, 3, and 4 and bone marrow obtained on day 5. Western blot analysis was used to measure DNMT1 levels in the nuclear fraction with histone 3 levels (H3) used to control for loading.
treated with these drugs. Independent of gender effects on plasma drug levels, higher CDA expression within malignant myeloid cells from males could also contribute to poorer outcomes (upregulation of CDA expression in malignant cells can lower intracellular cytidine analog levels and has been implicated as a mechanism of resistance; refs. 31–37). Furthermore, our mathematical modeling suggests that treatment exposure time is especially pertinent in low S-phase fraction disease. Supporting this inference, in clinical trials from the same institution, administration of decitabine on a greater number of days (10–20 mg/m² for 5–10 days every 4 weeks) produced a response rate of 50% in the lowest-risk MDS category and 28% in the higher risk categories (intermediate-2 and high), whereas administration on fewer days (45 mg/m²/d for 3 days every 6 weeks) produced a response rate of 14% in the lowest risk MDS category and a response rate of 18% in the higher risk categories (11, 13). That is, the largest detrimental impact of fewer days of treatment exposure was in low-risk (more indolent) MDS. Another factor contributing to gender bias in 5-azacytidine/decitabine efficacy could be the relatively low dosages of 5-azacytidine or decitabine used: CDA can more easily drive these lower levels of drug below minimum thresholds required for efficacy. This contrasts with high-dose therapy with cytarabine or gemcitabine, where the concern is that pharmacogenetic factors that decrease drug metabolism and thereby increase drug levels may increase the risk for toxic death because drug levels may already be close to maximum tolerated thresholds. Accordingly, female gender has been associated with higher drug levels and greater toxicity in studies of gemcitabine (23, 25, 41). In other words, female gender could be favorable for efficacy with relatively low-dose cytidine analog therapy (e.g., with 5-azacytidine or decitabine), but conversely, may increase the risk of toxicity with high-dose therapy, especially if these individuals concurrently carry SNPs in CDA (such as A79C) that additionally decrease enzyme activity (23).

Table 1. Pretreatment characteristics of female and male patients with MDS treated with 5-azacytidine or decitabine

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Female patients</th>
<th>Male patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median ± IQR)</td>
<td>63 ± 13</td>
<td>67 ± 12</td>
<td>0.04a</td>
</tr>
<tr>
<td>Bone marrow myeloblasts &lt;5%</td>
<td>10/21 (48%)</td>
<td>30/69 (43%)</td>
<td>0.74b</td>
</tr>
<tr>
<td>Bone marrow myeloblasts ≥20%</td>
<td>2/21 (10%)</td>
<td>9/69 (13%)</td>
<td>0.66b</td>
</tr>
<tr>
<td>Bone marrow myeloblast% (median ± IQR)</td>
<td>8 ± 9</td>
<td>6.5 ± 11</td>
<td>0.41a</td>
</tr>
<tr>
<td>≥3 chr. abnormalities or chr. 7 deletion</td>
<td>5/18 (27%)</td>
<td>23/63 (37%)</td>
<td>0.65b</td>
</tr>
<tr>
<td>Other abnormal cytogenetics</td>
<td>4/18 (22%)</td>
<td>9/63 (14%)</td>
<td></td>
</tr>
<tr>
<td>Normal cytogenetics</td>
<td>9/18 (50%)</td>
<td>31/63 (49%)</td>
<td></td>
</tr>
<tr>
<td>CDA genotype AA</td>
<td>8/17 (47%)</td>
<td>21/60 (35%)</td>
<td>0.40b</td>
</tr>
<tr>
<td>CDA genotype AC</td>
<td>8/17 (47%)</td>
<td>28/60 (47%)</td>
<td></td>
</tr>
<tr>
<td>CDA genotype CC</td>
<td>1/17 (6%)</td>
<td>11/60 (18%)</td>
<td></td>
</tr>
<tr>
<td>Number of treatment cycles (median ± IQR)</td>
<td>7 ± 14</td>
<td>7 ± 8</td>
<td>0.42a</td>
</tr>
</tbody>
</table>

Abbreviation: IQR, interquartile range.

Table 2. Multivariate analysis (Cox Proportional Hazards model) of overall survival in patients with MDS treated with 5-azacytidine or decitabine (n = 90)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Univariate P</th>
<th>HR (95% confidence limits)</th>
<th>Multivariate P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male vs. female)</td>
<td>0.0129</td>
<td>0.356 (0.165–0.766)</td>
<td>0.0083</td>
</tr>
<tr>
<td>CDA genotype (AA, AC, CC)</td>
<td>0.7336</td>
<td>1.038 (1.002–1.075)</td>
<td>0.0382</td>
</tr>
<tr>
<td>Age (continuous)</td>
<td>0.0004</td>
<td>1.038 (1.002–1.075)</td>
<td>0.0382</td>
</tr>
<tr>
<td>BM blasts (continuous)</td>
<td>0.2044</td>
<td>1.992 (1.435–2.764)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Abbreviation: BM, bone marrow.
MDS, OS in males compared with females was 14 versus 17 months (statistically nonsignificant; ref. 43). Why was the negative impact of male gender not as marked as that observed in our cohort? Our computational modeling underscored that a decrease in 5-azacytidine/decitabine half-life is more detrimental in less aggressive, low S-phase fraction disease; most of the patients in these cohorts had aggressive disease: more than 80% of the patients had ≥5% bone marrow myeloblasts and 29% to 39% had ≥20% bone marrow myeloblasts. In contrast, in our series, only 12% of the patients had ≥20% bone marrow myeloblasts and 56% of the patients had ≥5% bone marrow myeloblasts. In 856 mostly untreated patients with MDS (50% did not receive any therapy, 17% received therapy such as hypomethylating agents), significantly worse OS was observed in males (44). In another cohort of 897 untreated patients with MDS, significantly poorer OS was again observed in males (45). The gender differences in OS documented in untreated patients and in MDS incidence (Fig. S3), indicate that additional unknown factors beyond higher CDA expression contribute to poorer OS in males.

This is the first demonstration of a gender difference in CDA expression and its impact on 5-azacytidine or decitabine treatment outcomes; however, not only greater efficacy but also greater toxicity in females for a number of other cancer drugs has been documented, associated with lower expression in females of liver or kidney metabolic enzymes and transporters relevant to metabolism of these drugs (reviewed in ref. (46). The specific mechanisms or reasons underlying this broad gender bias in expression of metabolic enzymes have not been characterized. Speculatively, these differences could relate to the specialized physiologic functions of females. In contrast to other cancer drugs, the
Figure 4. A gender difference in drug exposure time is expected to produce a greater impact in low S-phase fraction disease. D is the fraction of the time that drug levels are above the efficacy threshold (e.g., minimum levels of 5-azacytidine or decitabine required to deplete DNMT1). S is the fraction of time over which malignant cells are susceptible to therapy because they are in S-phase. P is the probability of overlap between D and S; higher values of P predict pharmacodynamic effect in a larger fraction of the malignant clone (y-axis value of 1.0 predicts a pharmacodynamic effect in 100% of the malignant clone). Two plots are shown, each one for different values of D (0.1 and 0.2) that might predict a pharmacodynamic effect in 100% of the malignant clone. Two plots are shown, each one for different values of D (0.1 and 0.2) that might result from gender differences in cytidine analog metabolism. Shorter drug exposure time (D = 0.1) produces a greater relative reduction in P (double-headed black arrow height over double-headed clear arrow height) in low S-phase fraction (e.g., S = 0.1) disease than in high S-phase fraction disease (e.g., S = 0.3).

relatively low clinical dosage of 5-azacytidine and decitabine, and the application of these drugs in MDS, some cases of which are relatively indolent, may amplify gender differences in efficacy without necessarily affecting toxicity.

To address decreased treatment efficacy from higher CDA enzyme activity, one option could be to empirically administer higher doses of 5-azacytidine or decitabine to males. It may be more ideal, however, to use biomarkers of the intended pharmacodynamic effect (e.g., DNA methylation or DNMT1 levels), to guide adjustments to therapy. Pharmacodynamic biomarkers could simultaneously account for the effects of other pharmacogenetic factors, for example, A79C. Measuring CDA enzyme activity has also been proposed as a guide to dose modification (30). A complementary approach might be to dampen the influence of CDA altogether, by combination therapy with a CDA inhibitor (e.g., tetrahydrouridine; refs. 2, 15, 47). Combination with a CDA inhibitor may also attenuate CDA-mediated cancer resistance at the cellular level (48, 49), and cancer cell sanctuary from cytidine analog effects in tissues expressing high levels of CDA (47).

Decitabine and 5-azacytidine have a unique and evolving role in oncotherapy. However, a crucial determinant of the molecular epigenetic effect of these agents is treatment exposure time, which can be significantly influenced by gender differences in CDA expression and activity: higher CDA expression and activity in males likely contributes to inferior outcomes in male patients with MDS treated with 5-azacytidine or decitabine. Importantly, this mechanism for worse outcomes should be amenable to rational modifications to treatment dose, schedule, or formulation.

Disclosure of Potential Conflicts of Interest
R. Tiu has a honoraria from speakers’ bureau from Alexion and is a consultant/advisory board member of INCYTE and Alexion. No potential conflicts of interest were disclosed by the other authors.

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Increased CDA Expression/Activity in Males Contributes to Decreased Cytidine Analog Half-Life and Likely Contributes to Worse Outcomes with 5-Azacytidine or Decitabine Therapy

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