TITLE: Increased CDA expression/activity in males contributes to decreased cytidine analogue half-life and likely contributes to worse outcomes with 5-azacytidine or decitabine therapy

Running title: Gender, cytidine deaminase and 5-aza/decitabine

Authors and Institutions:  Reda Z Mahfouz, MD, PhD1, Ania Jankowska, PhD1, Quteba Ebrahem, MD1, Xiaorong Gu, PhD2, Valeria Visconte, PhD1, Ali Tabarroki, MD1, Pramod Terse, PhD2, Joseph Covey, PhD3, Kenneth Chan, PhD4, Yonghua Ling, PhD4, Kory J. Engelke, PhD5, Mikkael A. Sekeres, MD6, Ramon Tiu, MD1,6, Jaroslaw Maciejewski, MD, PhD1,6, Tomas Radivoyevitch, PhD7, Yogen Saunthararajah, MD1,6

1 Department of Translational Hematology & Oncology Research, Taussig Cancer Institute, Cleveland Clinic, Cleveland, Ohio
2 NCTT, Division of Pre-Clinical Innovations, National Center for Advancing Translational Sciences, NIH, Bethesda, Maryland
3 Toxicology and Pharmacology Branch, Developmental Therapeutics Program, Division of Cancer Treatment and diagnosis, NCI, NIH , Bethesda, Maryland
4 College of Pharmacy, The Ohio State University, Columbus, Ohio
5 AVANZA Laboratories, Gaithersburg, Maryland
6 Department of Hematologic Oncology and Blood Disorders, Taussig Cancer Institute, Cleveland Clinic, Cleveland, Ohio
7 Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio

Correspondence: Yogen Saunthararajah, MD, Taussig Cancer Institute, 9500 Euclid Avenue R40, Cleveland, OH 44195, tel: 216 444 8170, email: saunthy@ccf.org

Word and other counts:
Abstract: 246 words
Text (introduction, materials and methods, results and discussion): 3,947 words
Tables: 2 tables
Figures: 4 figures
References: 49 references.
Supplementary material: 3 supplementary figures
Key words: Cytidine deaminase, Decitabine, 5-azacytidine, pharmacogenomics, S-phase specific therapy
STATEMENT OF TRANSLATIONAL RELEVANCE

The cytidine analogues 5-azacytidine and decitabine have a powerful molecular epigenetic effect, depletion of DNA methyl-transferase (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 analogue 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 affect 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 in 5-azacytidine/decitabine-treated MDS patients. 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.
ABSTRACT

Purpose: The cytidine analogues 5-azacytidine and decitabine, used to treat myelodysplastic syndromes (MDS), produce a molecular epigenetic effect, depletion of DNA-methyltransferase (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.

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 MDS patients (n=90) and cytarabine-treated acute myeloid leukemia (AML) patients (n=76).

Results: By 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 compared to 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 to high S-phase fraction disease (e.g., MDS versus AML), since 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 MDS patients treated with 5-azacytidine/decitabine.

Conclusions: Increased CDA expression/activity in males contributes to decreased cytidine analogue half-life and likely contributes to worse outcomes with 5-azacytidine or decitabine therapy.
INTRODUCTION

The cytidine analogue drugs 5-azacytidine and decitabine are unique oncotherapeutics by virtue of a powerful molecular epigenetic effect, depletion of DNA methyl-transferase 1 (DNMT1) after incorporation into DNA(1). Levels of these drugs well below 0.5 µM are sufficient to deplete DNMT1(2-8). Hence, in contrast to the cytidine analogues cytarabine and gemcitabine that are administered at high doses that are close to maximum tolerated levels (100-3000 mg/m²) intended for anti-metabolite 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)(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/day from the 45 mg/m²/day dose originally approved by the FDA but administration on more days (5 days every 4 weeks instead of 3 days every 6 weeks) increased the overall response rate in myelodysplastic syndromes (MDS) to 32-73% from 17%(11-13). It is possible that exposure time is even more pertinent in relatively indolent MDS than in aggressive acute myeloid leukemia (AML): even a short treatment exposure may be effective treatment for high S-phase fraction malignant disease, since the majority of cells may enter S-phase in the treatment window. Conversely, in disease with a low S-phase fraction, short exposure time may only treat a minor portion of the malignant cells.

Given the importance of treatment exposure time, genetic factors that influence the in vivo half-life of 5-azacytidine and decitabine could significantly impact treatment outcome. In vivo, cytidine, deoxycytidine, and analogues thereof are rapidly deaminated to uracil base moiety counterparts by the ubiquitously expressed enzyme cytidine deaminase (CDA)(14-15). The clinical relevance of CDA is suggested by its effect on cytidine analogue half-life: the half-life of decitabine in buffer in vitro at 37°C is >10 hours(16), by contrast, the half-life in vivo is <10 minutes(17), a drastic reduction largely attributable to CDA(15, 18-19). A pharmacogenetic factor known to decrease CDA enzyme activity is the non-synonymous CDA single nucleotide polymorphism (SNP) A79C (Lys27Gln, rs2072671); ~60% of Caucasians are heterozygous or homozygous for this SNP which changes lysine to glutamine at amino-acid position 27 to cause as much as a 3-fold decrease in CDA enzyme activity.
activity(20-21). The decrease in enzyme activity caused by this SNP has been implicated in severe toxic events occurring with cytarabine and gemcitabine therapy(20-24).

However, 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 analogue metabolism and half-life is gender: for both gemcitabine and cytarabine, higher clearance and shorter half-life has been described in males compared to females(25-26), and in a murine model of colon cancer, the cytidine analogue zebularine has demonstrated decreased pharmacodynamic and chemo preventive efficacy in males(27). However, the mechanisms underlying these gender differences in cytidine analogue levels and actions have not been elucidated.

Thus, given the prominence of CDA in cytidine analogue 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 in 5-azacytidine/decitabine-treated MDS patients. 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 (IRB). 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) (28). Patients were analyzed in two groups: (i) 90 MDS patients treated either with 5-azacytidine 75 mg/m²/day D1-7 (sub-cutaneous or intravenous [IV]) or decitabine 20 mg/m²/day D1-5 (IV over 1 hour) in 28 day cycles; and (ii) 76 AML patients treated with cytarabine-based induction chemotherapy: cytarabine dose 100–200 mg/m²/day days 1-7 continuous infusion and an anthracycline days 1-3.

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 M Tris/HCL pH 7.5 (265µl) was added to 25µl of human plasma followed by addition of cytidine to a final concentration of 4.1 mM and 5-flourouridine 0.381 mM (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 (TCA, 2%). 20 µl of supernatant was injected for HPLC using ammonium acetate (15 mM) as the mobile phase with a flow rate of 0.35 mL/min through Xbridge™ OST C18, 2.5 µm, 4.6x50mm column on Dionex UltiMate® 3000 μ-HPLC system supported with Chromeleon® 7.1 data system (Dionex Corporation, Sunnyvale, CA). Retention time and peak area of uridine at 260 nm were compared to the internal control for each injection. The average net uridine peak area of test minus blank was calculated for each test sample. Known concentrations of uridine (0.0 to 95.8 µM) were used to construct a standard curve to calculate the amount of uridine based on the net uridine peak area. One unit (U) of CDA enzyme activity is defined as the amount of enzyme needed to produce 1 µmole 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 < 5%.

Sequencing for the A79C SNP in CDA (Lys27Gln, rs2072671) and measurement of CDA mRNA. The ArchivePure Kit (5Prime, Gaithersburg, MD) was used to extract DNA mononuclear cells isolated by Ficoll-Hyphaque density centrifugation of bone marrow or peripheral blood. Primers 5'-GTTCCTAAGGGAGAGTGTGAAGCA-3'(forward), 5'-GCCTCTTCTGTACATCTTCCTCTCTCTCTCT-3(reverse) (accession number NM_001785), Expand High Fidelity PLUS PCR System (Roche Applied Science, Indianapolis, IN) and PCR conditions: initial denaturation 94°Cx4 minutes, 30 cycles with denaturation at 94°Cx30s, annealing at 56°Cx30s, and elongation at 72°Cx40s were applied to 50ng DNA template. Amplicons were purified using the Montage PCR96 Cleanup Kit (Millipore, Billerica, MA) and sequenced using Big DyeTerminator v3·1 (Applied Biosystems, Foster City, CA) 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).

QRT-PCR (accession # NM_028176.1) primers were 5’-CDAAGGGTGACCTTGATTCACACACCA-3’(forward); 5’-CDATGGAATACCCGTGTCTTTGGGAGTACC-3’(reverse).

Decitabine pharmacokinetic studies in CD-1 mice: Procedures with CD-1 strain mice were approved by the IACUC of Avanza. 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 intra-cardiac 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 prior to each collection day by adding 10 μL/tube of a 10 mg/mL 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 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 1X PBS containing protease inhibitors (Sigma-Aldrich, A8340), bone marrow cells were resuspended in 500 μL of 1X hypotonic buffer containing 10mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 1.5mM MgCl₂, 10mM KCl, 0.5mM dithiothreitol, 10mM 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 344g 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 1X PBS, resuspended in 100 μL of 50mM Tris-HCl, pH 8.0, 1mM MgCl₂, 10mM PMSF, protease inhibitor cocktail (Sigma-Aldrich, A8340) and Benzonase (Sigma-Aldrich, D5915, 250 units) and incubated on ice for 90 minutes with vigorous vortex every 5 minutes. Then, 500 μL protein extraction buffer containing 1.5% NP-40, 500mM NaCl, 5mM dithiothreitol, 10mM PMSF, and 5 μL of protease inhibitor cocktail (Sigma-Aldrich, A8340) in 50mM phosphate buffer (pH 7.4) was added. After 30-minute incubation on ice with vortexing every 5 minutes, the mixture was centrifuged at 12396g for 15 minutes. The same extraction process was repeated two more time with 300 μL and 200 μL of extraction buffer. The supernatant containing nuclear proteins was combined and transferred to clean tubes, and protein concentration was determined by BCA assay. 100 μg of nuclear protein extracts were subjected to 1D SDS-polyacrylamide gel electrophoresis on precast 4% to 12% NuPAGE gels (Invitrogen) for gel-electrophoresis per manufacturer's instructions (Invitrogen), then transferred to polyvinylidene difluoride membranes (Millipore) at
35 constant voltage 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 Figure 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:

```r
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, i.e. P=S+D, unless S>1-D, in which case P=1.

**Statistical Analysis of Clinical Data.** SAS (Cary, NC) was used for all statistical analyses save those performed in R (above). Sample characteristics between patients of different gender were compared using the Wilcoxon test or Chi-square test. PROC LIFETEST to generate Kaplan-Meier product-limit survival estimates was used to model time from diagnosis to death (overall survival) 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 \( \alpha = 0.05 \). 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 \( \alpha \leq 0.05 \) 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, MDS and AML patients 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 to samples homozygous for the ancestral allele (AA) (p<0.01) (figure 1A).

Plasma levels of cytidine analogue drugs have been shown to be lower in males(25-26), however, the reason for this has not been elucidated. To see if a gender difference in CDA enzyme activity could be a cause, the plasma samples were further sub-classified by gender. Male gender was associated with significantly higher CDA enzyme activity (p<0.001) (figure 1B, C). Notably, the difference in plasma CDA activity between male and female gender (>2.5-fold, figure 1C) was greater than the difference in activity between AA and CC genotype within each gender (<1.5-fold) (figure 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 (figure S1). Therefore, to evaluate the basis for higher CDA enzyme activity in males compared to females, CDA gene expression was measured in murine liver by QRT-PCR. CDA expression was >3-fold higher in male versus female liver tissue (n=6/group, p=0.01, figure 2A). Because higher CDA expression within malignant cells themselves is a possible mechanism of resistance to cytidine analogues(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 to females for a number of cancers, including myeloid leukemia (38)(figure S2).

Decitabine pharmacokinetics and pharmacodynamics in female and in male mice. Higher CDA expression and enzyme activity in males compared to females can be expected to produce lower plasma
cytidine analogue levels in males. To confirm this, decitabine 0.4mg/kg (figure 2B) or 1.0mg/kg (figure 2C) was administered to CD1 mice by oral gavage and plasma drug levels were measured by 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 to females: this decrease was significant collectively across all time-points in the higher dose group (p=0.005, gender main effect in a gender x time two-way ANOVA analysis) (figure 2C) and at 30 minutes (p=0.0003) and at 60 minutes (p=0.01) in the lower dose group (figure 2B, p-values for two-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 on day 5 after 3 days (day 1, 3, 4) of subcutaneous decitabine 0.1 mg/kg/day or 0.4 mg/kg/day. Decitabine produced greater depletion of DNMT1 in the cells from females (figure 2D).

Overall survival (OS) 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 MDS and AML patients treated with 5-azacytidine or decitabine or induction cytarabine between January 2002 and December 2007 at Cleveland Clinic, with bone marrow samples available for sequencing analysis for the A79C SNP, and verifiable survival annotation. Prognostically relevant pre-treatment characteristics (karyotype, bone marrow myeloblast percentage, A79C status) except for higher age in males, were similar in male and female MDS patients treated with 5-azacytidine or decitabine (table 1). OS was significantly worse in males (n=69, median 563 days) compared to females (n=21, median 1033 days)(Log-Rank p=0.01)(figure 4A); this difference remained significant in a multivariate Cox model analysis that controlled for age, karyotype, myeloblast% and the A79C SNP by including them as covariates (table 2). In AML patients treated with induction cytarabine (n=76) and stratified by gender or by A79C, there was no difference in OS (figure 4B and data not shown).

The interaction between treatment exposure time and S-phase fraction of disease. A potential explanation for the significant association of gender with OS in MDS patients treated with 5-azacytidine or decitabine but not in AML patients 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(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 (figure 5).

DISCUSSION

DNMT1-depletion by 5-azacytidine or decitabine is S-phase dependent. Since 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 MDS patients 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 analogue levels and has been implicated as a mechanism of resistance(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 (10-20mg/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 (45mg/m²/day 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, since 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 analogue 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).

Gender differences in OS have been observed in other MDS patient cohorts: in 99 decitabine-treated MDS patients median OS in males was 399 compared to 529 days in females (p-value not provided)(42). In a study of 177 decitabine-treated MDS patients, OS in males versus females was 14 versus 17 months (statistically non-significant)(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: >80% of the patients had ≥5% bone marrow myeloblasts and 29-39% 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 MDS patients (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 MDS patients, significantly poorer OS was again observed in males(45). The gender differences in OS documented in untreated patients, and in MDS incidence (figureS3), 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, 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(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 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, e.g., 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)(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 analogue 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 MDS patients treated with 5-azacytidine or decitabine. Importantly, this mechanism for worse outcomes should be amenable to rational modifications to treatment dose, schedule or formulation.

ACKNOWLEDGEMENTS

Purified human CDA was a generous gift from Professor Silvia Vincenzetti, Universita di Camerino, Italy. This work was supported by grants to YS from NIH (1R01CA138858) and CDMRP (PR081404)
and by NCI Contracts (N01-CM-52205 and N01-CM-42204), NHLBI and NIDDK under the NIH-RAID Pilot Program.

COMPETING FINANCIAL INTERESTS

The authors have no competing financial interests in relation to this work, although in interests of disclosure of all information, we have submitted a patent application for combination therapy with oral tetrahydouridine (an inhibitor of CDA) and decitabine, and have a patent for the use of non-cytotoxic concentrations of decitabine to expand normal hematopoietic stem cells ex vivo.
REFERENCES


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

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

*Wilcoxon test; #Chi-Square test;
Table 2: Multivariate analysis (Cox Proportional Hazards model) of overall survival in MDS patients treated with 5-azacytidine or decitabine (n=90).

<table>
<thead>
<tr>
<th></th>
<th>Univariate p-value</th>
<th>Hazard Ratio (95% confidence limits)</th>
<th>Multivariate p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (m v fm)</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></td>
<td></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></td>
<td></td>
</tr>
<tr>
<td>Cytogenetics (deletion 7 or complex vs. other)</td>
<td>&lt;0.0001</td>
<td>1.992 (1.435-2.764)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1:  **A) Plasma CDA enzyme activity by CDA genotype.** DNA sequencing was used to identify individuals homozygous for the A79C SNP in CDA (CC genotype), heterozygous for A79C (AC genotype) or homozygous for the ancestral allele (AA genotype). Plasma samples from equal numbers of individuals with these genotypes were assessed for CDA enzyme activity using an HPLC-based assay. Box-plot boundaries = inter-quartile range, horizontal line = median, whiskers = range of values. p-value from Wilcoxon test.  **B) Samples were sub-classified by CDA genotype within gender grouping.** p-value Wilcoxon test.  **C) Plasma CDA enzyme activity by gender.** p-value Wilcoxon test.

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 to 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 three 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 was used to measure DNMT1 levels in the nuclear fraction with histone 3 levels (H3) used to control for loading.

Figure 3: **Overall survival (OS) was significantly worse in male MDS/AML patients treated with 5-azacytidine/decitabine.** 90 MDS and 76 AML patients initiated on treatment between January 2002 and December 2007 at Cleveland Clinic with DNA available for sequencing analysis for the A79C SNP and verifiable survival annotation.  **A) OS in MDS/AML patients treated with 5-azacytidine or decitabine.**  **B) OS in AML patients treated with cytarabine.** These patients received induction cytarabine in combination with anthracycline, some received additional cycles of consolidation that included cytarabine.
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, one each for different values of D (0.1 and 0.2) that might result from gender differences in cytidine analogue 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).
**Figure 2**

(A) Box plot showing the distribution of CDA expression (RQ) for female and male groups. The p-value is 0.0125.

(B) Graph showing the decitabine concentration (μM) over time (minutes) for female and male groups.

(C) Graph showing the decitabine concentration (μM) over time (minutes) for female and male groups.

(D) Western blot images of DNMT1 and H3 for female and male groups under different conditions (PBS treated control, 0.1 mg/kg DAC treated, 0.4 mg/kg DAC treated).
Figure 3

(A) Survival distribution function for 5-azacytidine or decitabine treated MDS patients. The table summarizes the data:

<table>
<thead>
<tr>
<th>Gender</th>
<th>n</th>
<th>Deaths</th>
<th>Median Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>69</td>
<td>55</td>
<td>563</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>10</td>
<td>1033</td>
</tr>
</tbody>
</table>

Log-Rank p = 0.0104

(B) Survival distribution function for cytarabine treated AML patients. The table summarizes the data:

<table>
<thead>
<tr>
<th>Gender</th>
<th>n</th>
<th>Deaths</th>
<th>Median Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>43</td>
<td>36</td>
<td>286</td>
</tr>
<tr>
<td>Female</td>
<td>33</td>
<td>27</td>
<td>412</td>
</tr>
</tbody>
</table>

Log-Rank p = 0.4043
Increased CDA expression/activity in males contributes to decreased cytidine analogue half-life and likely contributes to worse outcomes with 5-azacytidine or decitabine therapy

Reda Z Mahfouz, Ania Jankowska, Quteba Ebrahem, et al.

Clin Cancer Res  Published OnlineFirst January 3, 2013.

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Access the most recent version of this article at:
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