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Yogen Saunthararajah, … , Matt Kalaycio, Jaroslaw Maciejewski

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METHODS. In this clinical trial, patients with myelodysplastic syndrome (n = 25) received reduced decitabine dosages (0.1–0.2 mg/kg/day compared with the FDA-approved 20–45 mg/m^2/day dosage, a 75%–90% reduction) to avoid cytotoxicity. These well-tolerated doses were frequently administered 1–3 days per week, instead of pulse cycled for 3 to 5 days over a 4- to 6-week period, to increase the probability that cancer S-phase entries would coincide with drug exposure, which is required for S-phase–dependent DNMT1 depletion.

RESULTS. The median subject age was 73 years (range, 46–85 years), 9 subjects had relapsed disease or were refractory to 5-azacytidine and/or lenalidomide, and 3 had received intensive chemoradiation to treat other cancers. Adverse events were related to neutropenia present at baseline: neutropenic fever (13 of 25 subjects) and septic death (1 […]

Evaluation of noncytotoxic DNMT1-depleting therapy in patients with myelodysplastic syndromes

Yogen Saunthararajah,[1,2] Mikkael Sekeres,[1,2] Anjali Advani,[1] Reda Mahfouz,[2] Lisa Durkin,[3] Tomas Radivoyevitch,[4] Ricki Englehaupt,[1] Joy Juersivich,[1] Kathleen Cooper,[1] Holleh Husseinzadeh,[2] Bartlomiej Przychodzen,[2] Matthew Rump,[1] Sean Hobson,[1] Marc Earl,[1] Ronald Sobecks,[1] Robert Dean,[1] Frederic Reu,[1,2] Ramon Tiu,[1,2] Betty Hamilton,[1,2] Edward Copelan,[5] Alan Lichtin,[1] Eric Hsi,[3] Matt Kalaycio,[1] and Jaroslaw Maciejewski[1,2]

[1]Department of Hematology and Oncology, [2]Department of Translational Hematology and Oncology Research, Taussig Cancer Institute, [3]Department of Clinical Pathology, Tomsich Pathology Institute, and [4]Department of Quantitative Health Sciences, Cleveland Clinic, Cleveland, Ohio, USA. [5]Levine Cancer Institute, Carolinas HealthCare System, Charlotte, North Carolina, USA.

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CONCLUSION. Decitabine regimens can be redesigned to minimize cytotoxicity and increase exposure time for DNMT1 depletion, to safely and effectively circumvent mutational apoptotic defects.

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Introduction

Although conventional cytotoxic treatments for myeloid cancers can have differing proximal actions, e.g., topoisomerase inhibition (daunorubicin) or termination of DNA chain synthesis (cytarabine), a final common pathway converges onto p53 (*TP53*), a master regulator of apoptosis (cytotoxicity) (reviewed in ref. 1). As such, *TP53* mutation or deletion is associated with resistance to cytotoxic treatments in vitro (2, 3) and in vivo: *TP53*-mutated acute myeloid leukemia (AML) treated with daunorubicin and/or cytarabine had a response rate of 33% compared with 81% for *TP53* WT AML, while *TP53*-mutated myelodysplastic syndromes (MDS) had a response rate of 8% versus 60% for MDS with WT *TP53* (4). The poorest-risk MDS and AML subtypes, e.g., MDS and AML with complex cytogenetic abnormalities, have the highest rate of *TP53* mutations, exceeding 70% in some series (5). Even if *TP53* itself is not mutated, alterations in other key p53-system

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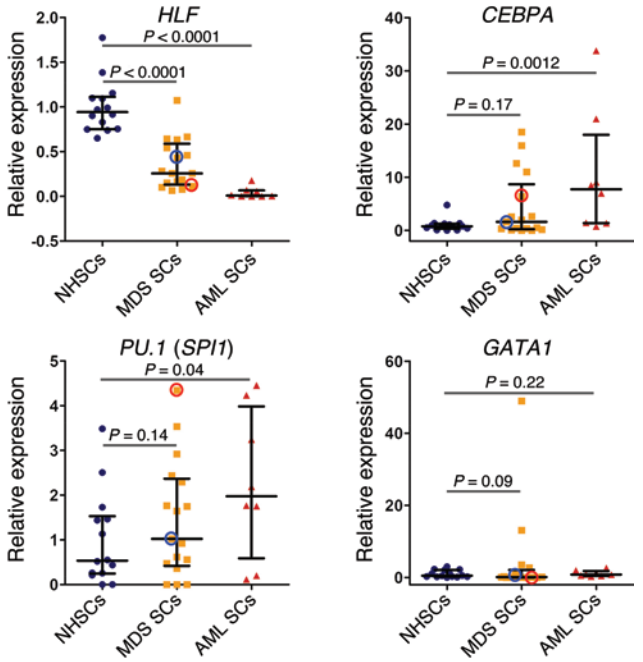

Figure 1. Master transcription factor expression in NHSCs versus MDS and AML stem cells. HLF drives stem cell fate, and CEBPA, PU.1, and GATA1 drive myeloid lineage fates, as demonstrated in lineage conversion and murine knockout studies (25, 47). Surface phenotypes used for flow purification reproducibly identify cell fractions with the capacity to reconstitute long-term myelopoiesis in immunocompromised mice (29, 46) (GEO GSE55689 and GSE24006). These phenotypes were DAPI$^-$Lin$^-$CD34$^+$CD38$^{-/lo}$CD90$^+$CD45RA$^-$ for NHSCs and MDS stem cells (MDS SCs) and DAPI$^-$Lin$^-$CD34$^+$CD38$^{-/lo}$CD90$^-$ for AML SCs (AML engrafting cells differ from NHSCs and MDS SCs in being CD90$^-$ and can also be CD38$^+$, a fraction not analyzed here) (29, 46). MDS and AML cases were representative of the morphologic and genetic spectrum of disease, and in MDS, included low- and intermediate-risk cases (29, 46). MDS cases with *TP53* mutation or deletion are indicated (blue and red circles, respectively). Gene expression is shown relative to the mean expression of the same gene in simultaneously analyzed NHSCs. Error bars represent the median ± interquartile range (IQR). *P* values were determined by the Mann-Whitney *U* test.

genes are frequent in poor-risk myeloid malignancies; e.g., gains in *MDM4,* which inactivates p53, are very common in transforming myeloproliferative neoplasms (6, 7). Meanwhile, cytotoxic treatments damage residual normal hematopoietic stem cells (NHSCs) and stroma (8–10), causing significant toxicities including fatal exacerbations of low blood counts in as many as 29% of patients treated (11, 12), with the elderly and those with AML evolved from MDS being particularly vulnerable (11, 12).

Hence, especially for some subtypes of myeloid malignancy, there is a need for treatments that are not mediated through p53 and apoptosis (noncytotoxic treatments). Several groups have observed that terminal differentiation is induced in vitro when treating myeloid and other cancer cells with drugs or conditions that inhibit gene-silencing, chromatin-modifying enzymes (chromatin relaxation) (reviewed in ref. 13). These differentiation-mediated cell-cycle exits, like those that occur during normal tissue differentiation, do not require p53 and are readily induced in p53/p16-null cancer cells (10, 14–16). The same chromatin-relaxing conditions increase the differentiation of normal progenitors as well (17, 18) but, in contrast, increase self-renewal of NHSCs (10, 14, 17, 19–24). The reasons for this cell context–dependent response have been evaluated: differentiation is driven by relatively few master transcription factors (25). Myeloid cancer cells express master myeloid differentiation–driven transcription factors (e.g., CEBPA and PU.1) at high levels, yet the target genes of these transcription factors are epigenetically silenced (10, 13, 14, 20, 26–29) because of aberrant recruitment of silencing — instead of activating — chromatin-modifying enzymes to the transcription factors (20, 27, 30). Inhibition of silencing enzymes with drugs such as decitabine restores expression of numerous target genes of the transcription factors, including MYC antagonists (e.g., CEBPE and p27/CDKN1B), that terminate proliferation (10, 14, 17, 20–22, 27, 30–32). Normal stem cells, on the other hand, express master stem cell transcription factors (e.g., HLF and HOXB4) and activate stem cell genes and stem cell fate in response to the same treatments (good therapeutic index) (10, 14, 17, 20–23).

To translate these observations into the clinic, decitabine possesses useful properties. Unlike cytidine analogs such as cytarabine, the sugar moiety of decitabine is unmodified, permitting incorporation into DNA without the termination of chain elongation (33, 34). The pyrimidine ring modification of decitabine subsequently depletes DNMT1 and can also directly hypomethylate DNA, since it cannot accept a methyl group (33, 34). High decitabine concentrations, however, do have antimetabolic, DNA-damaging cytotoxic effects (35–37). Moreover, aggressive DNA-damaging cytotoxic therapy, despite the risk of treatment-related mortality, cures some myeloid cancers (e.g., AML subtypes that do not have p53 system abnormalities); this suggested that cytotoxicity could be the key to durable remissions. Decitabine development accordingly conformed to historical precedent, and FDA-approved decitabine regimens to treat MDS (20–45 mg/m^2/day for 3 to 5 days every 4 to 6 weeks) can have cytotoxic effects requiring pulse-cycled administration (38–41), and controversy persists as to the relative importance of cytotoxicity and differentiation, or even the role of DNMT1 depletion, in the clinical actions of decitabine and the related drug 5-azacytidine (42).

Thus, whether the limitations and disadvantages of cytotoxic therapy can be bypassed without loss of anticancer activity by redesigning the decitabine dose, schedule, and route of administration to minimize cytotoxicity and increase DNMT1 depletion is a question that has not been answered. The reasons to evaluate such an approach are that cytotoxicity can negatively impact effectiveness by: (a) limiting the feasible frequency and distribution of drug administration that are essential for S-phase–dependent DNMT1 depletion and p53-independent effects; and (b) destroying the NHSCS and stroma needed to reverse low blood counts (8,9,11,12), the cause of morbidity and death in MDS (43). Hence,

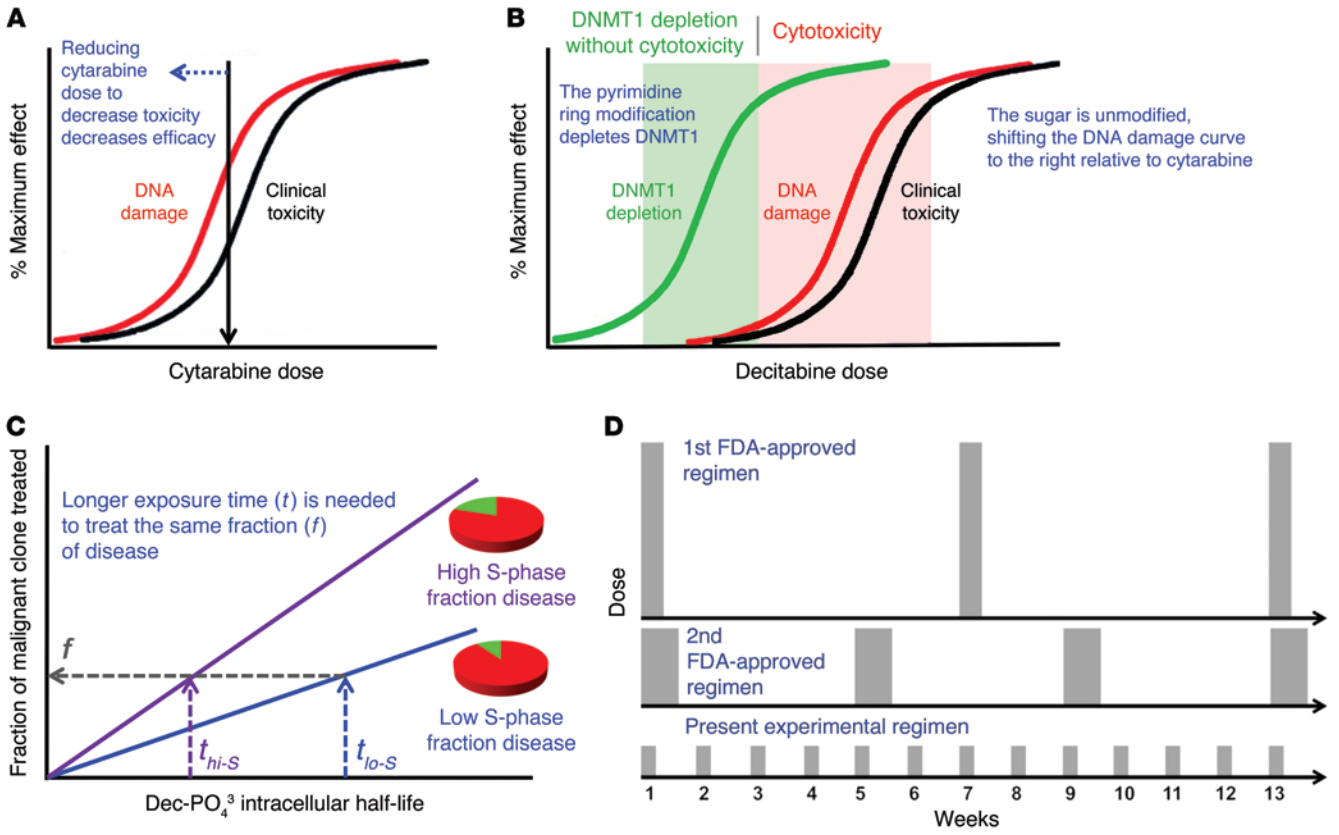

Figure 2. Chemical properties of decitabine and S-phase–dependent mechanism-of-action considerations that influenced regimen design. (**A**) The sugar moiety of the cytidine analog cytarabine is unnatural and thus terminates DNA chain elongation. This is the intended molecular pharmacodynamic effect. Decreasing the cytarabine dose to improve safety decreases this intended effect (reduces efficacy). (**B**) The sugar in decitabine is natural, enabling DNA incorporation without terminating chain elongation. This shifts the DNA damage curve to the right compared with cytarabine (10, 14, 33, 34), even though the decitabine-DNMT1 DNA-protein cross-link requires repair by homologous recombination (35–37). The DNA repair delays cell-cycle progression; however, concentrations can be found that deplete DNMT1 without cytotoxicity (green zone), the intent in this clinical trial (unlike with cytarabine, a lower dose need not imply lower efficacy). (**C**) Since DNMT1 depletion is S-phase dependent, for an equivalent tumor burden, a lower S-phase fraction (green in pie chart) may require more drug exposure time ($t_{lo\text{-}S}$) (e.g., more frequent administration) to treat the same fraction (f) of disease. That is, counterintuitively, less aggressive disease may require more frequent drug administration. More intuitive is that a similar S-phase fraction, but a higher total tumor burden (e.g., 10^{12} versus 10^{11} cancer cells, i.e., more advanced disease), may require greater exposure time to reduce the tumor burden to a level that permits functional hematopoiesis. (**D**) These considerations guided protocol regimen design. Top: FDA-approved regimen of decitabine 45 mg/m^2/day i.v. on days 1–3 every 6 weeks. Middle: FDA-approved regimen of decitabine 20 mg/m^2/day i.v. on days 1–5 every 4 weeks. Bottom: Protocol regimen 0.1–0.2 mg/kg/day (~3.5–7 mg/m^2/day) s.c. 1–3 days per week (shown is 0.2 mg/kg 2 days/week). All treatment regimens may be continued indefinitely.

the present clinical trial selected decitabine doses that have been shown to be noncytotoxic but DNMT1-depleting in nonhuman primates and hemoglobinopathy clinical trials (44, 45), administered s.c. to avoid high peak drug levels that might cause apoptosis and frequently in distributed fashion to increase the possibility of overlap with cancer S-phase entries. Additional hoped-for benefits were better tolerance in order to widen treatment eligibility to include patients with relapsed disease, the elderly, and those with comorbidities. Scientific correlative studies evaluated noncytotoxic DNMT1 depletion and determinants of response.

Results

Master transcription factor difference and decitabine repositioning

Few of the hundreds of transcription factors expressed in cells are master regulators that drive cell lineage fate (25). A premise for this clinical trial is that a difference between MDS/AML stem cells (MDS- and AML-initiating cells) and NHSCs in their expression of such master regulators, common despite the genetic heterogeneity of disease, drives contrasting cell fate responses to chromatin relaxation (10, 14, 17, 19, 20, 27, 28, 30). This transcription factor difference was previously observed when gene and protein expression levels in various high-risk MDS and AML stem cells were compared with levels in NHSCs (10, 14, 17, 19, 20, 27, 28, 30). Here, gene expression evaluation is extended to MDS stem cells from patients with low- to intermediate-risk MDS (29, 46) (public data): HLF, a master transcription factor that drives HSC fate (47), was expressed at less than 10% to 50% of the levels observed in NHSCs (Figure 1). In contrast, the master myeloid differentiation–driving transcription factors CEBPA, GATA1, and SPI1 (PU.1) were expressed at higher levels in MDS stem cells, with many MDS stem cells expressing greater than 10-fold higher levels of CEBPA or GATA1 (Figure 1). Functional behavior accorded with the master transcription factor content: NHSCs produced a mixture of lineages with a majority

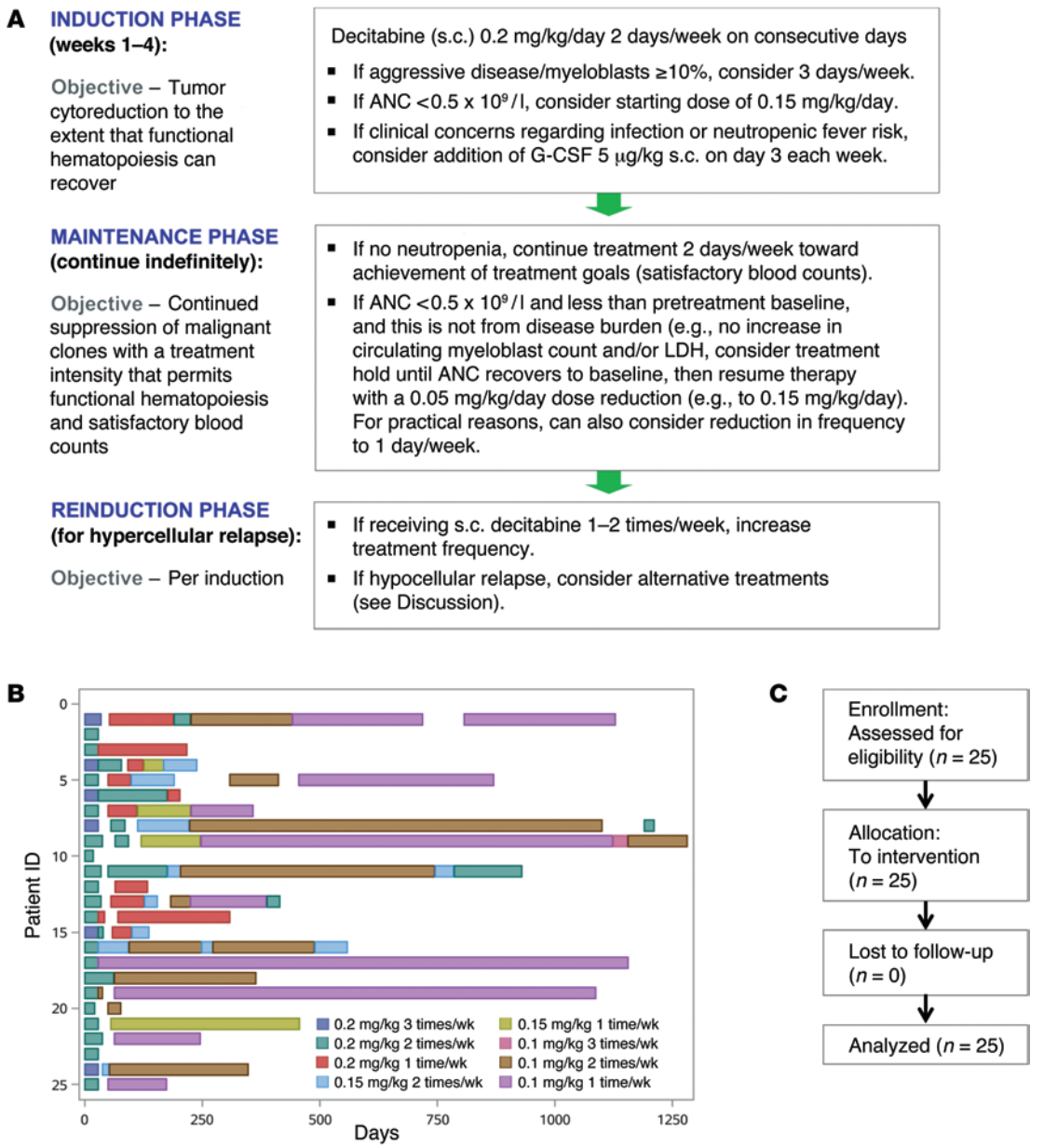

Figure 3. Treatment schema and dosages actually administered to individual subjects. (**A**) Protocol treatment schema. (**B**) Decitabine dose and frequency of administration for individual subjects. After an initial 4-week induction phase, decitabine dose and schedule modifications were protocol mandated to adapt to known inter- and intraindividual differences in decitabine metabolism, cancer S-phase fraction, cancer burden, blood counts, and hematopoietic reserve. (**C**) CONSORT diagram for enrollment and analysis.

of B cells (50%–90%) upon xenotransplantation into immunocompromised mice; in contrast, MDS and AML stem cells produced in vast majority myeloid cells, with less than 5% B cells (10, 29, 48).

Decitabine dose and schedule

Decitabine doses were derived from nonhuman primate studies and hemoglobinopathy clinical trials that intended noncytotoxic DNMT1 depletion (44, 45). The chemical properties of decitabine that permit noncytotoxic DNMT1 depletion are illustrated in Figure 2, A and B, as are S-phase–dependent mechanism-of-action considerations that influenced regimen design (Figure 2, C and D). Treatment was divided into induction and maintenance phases. The induction phase objective was noncytotoxic cytoreduction of the malignant clone such that functional hematopoiesis could recover and relieve cytopenias (Figure 3A). The maintenance phase objective was continued noncytotoxic suppression of the malignant clone with a treatment intensity that permitted functional hematopoiesis and maintenance of satisfactory blood counts (Figure 3A). For the 4-week induction phase, s.c. decitabine 0.2 mg/kg/day was administered 2 days per week, with 3-days-per-week administration to be considered if BM myeloblasts were greater than or equal to 10%, or if there was clinical concern for rapid progression to AML. During the maintenance phase, dose and schedule modifications, including treatment holds, were based on blood counts and marrow cellularity. Regular administration at a lower dose was to be preferred to infrequent administration of a higher dose (Figure 3B). Differ-

Table 1. Individual subject characteristics and response

No.	WHO classification	IPSS-R[A]	Age/sex	Previous treatments (for other cancers/ MDS)	Gene mutations (selected[B])	Before blast %[C]	After blast %[C]	Before cytogenetics Abnormalities [metaphases]	After cytogenetics (cytogenetic responders)	Hematological responders
Responders										
1	CMML2	NA	73/M	Len (2)	*SRSF2, TET2, RUNX1, ASXL1, JAK2*	12	0	20q- [6/20]	Normal [20/20] (CcyR)	CR
4	RAEB1	Hi	83/F	EPO	*TP73, TET2, SF3B1, BCOR, DNMT3A, RUNX1, SUZ12*	9	3	Normal [20/20]	Normal [20/20]	HI
8	RAEB1	NA	67/F	VCR, DOX, melphalan	*STXBP4, CASP2* (del)	6	1	del3, -5, -7, +22 [4/20]	Normal [20/20] (CcyR)	HI
9	CMML1	Int	66/M	None	*ATM, SRSF2, MLL2, ARID2, TET2, KRAS*	4	0	+mar [2/20]	Normal [20/20] (CcyR)	CR
11	RCMD	v. Hi	78/F	Pred	*TP53* (mutations and deletions)	1	0	−4, del5, −17, +19, −6, +inv6, −7, −10, +del10, −14, +add14, −15, −22, t(15;22) [16/20]	Normal [20/20] (CcyR)	CR
13	CMML1	NA	70/M	Mustargen, VCR, procarbazine, pred, len (4)	*BCOR, BCL11A, EZH2, U2AF1*	2	1	Normal [20/20]	Normal [20/20]	HI
14	RCMDRS	NA	72/M	5-Aza (13), Len (24)	*SF3B1*	2	0	−Y [20/20]	−Y [16/20]	HI
17	RCMD	Lo	84/F	Romiplostim	*FASLG*	2	0	del20 [19/20]	del20 [8/20] (PcyR)	HI
18	CMML1	Lo	70/M	None	*TP53AIP1, TET2, SRSF2, RUNX1, CBL*	2	1	Normal [20/20]	Normal [20/20]	HI
19	RCMD	Int	84/F	Romiplostim		1	0	del20,t(1;15) [18/20]	del20 [6/6] (no t[1;15])	HI
21	RCMD	v. Hi	65/M	EPO	*DNAH10, CASP2* (del)	3	1	del5,−6,del7,+8,−13,i(17),+3 [4/7]	Normal [20/20] (CcyR)	CR
Nonresponders										
2	RAEB1	v. Hi	61/F	ATG + CSA	*DNMT3B*	8	9	-7 [19/20]	−7 [6/20] (PcyR)	PD
3	RCMD	NA	77/F	Len (5),EPO	*SF3B1*	1	1	inv10 (constitutive)	inv10	SD
5	RCMD	Int	83/M	EPO, danazol	*ASXL1*	3	3	Normal [20/20]	Normal [20/20]	SD
6	RAEB2	NA	63/M	5-Aza + len (31)	*TP53BP1, SF3B1, FLT3, RUNX1*	10	10	Normal [20/20]	Normal [20/20]	SD
7	RARS	Lo	58/M	EPO, erzatiostat	*SF3B1*	1	1	Normal [20/20]	No growth	SD
10	RAEB2	NA	72/M	5-Aza + len (7), 5-Aza(4), AsO3 + GTO	*TP53, ASXL1, PRPF8*	10	Not done	Normal [20/20]	Not done	PD
12	MDSu	Int	46/M	Danazol	*PAX5*	1	0	+1, der(1;7) [9/12]	No growth	SD
15	RAEB1	NA	75/M	5-Aza (12)	*NPM1, FLT3, SRSF2*	6	1	−Y [3/20]	−Y [7/20]	PD
16	RCMD	Hi	81/M	EPO	*SRSF2*	1	0	t(1;19), del20, t(X;3) [20/20]	t(1;19),del20,t(X;3) [20/20]	SD
20	RAEB1	Hi	63/F	None	*MUC4, CASP8AP2*	7	17	inv3 [12/20]	inv3 [2/5]	PD
22	RCMD	NA	68/F	Len (6)	*SF3B1*	2	1	Normal [20/20]	No growth	SD
23	MDSu	NA	78/F	DOX, VCR, pred, ifos, CTX, etop, busulph, XRT, 5-Aza (11)		6 (22[D])	Not done	−7, del20 [13/17]	Not done	PD
24	RAEB2	v. Hi	85/F	None	*DNMT3A, CBFA2T3*	15	9	Normal [20/20]	Normal [20/20]	SD
25	RCMD	Lo	74/F	None	*ATM, TET2, SRSF2*	2	1	Normal [11/11]	Normal [14/14]	SD

[A]IPSS-R score (if no previous intensive chemotherapy, 5-azacytidine, or lenalidomide); [B]full mutation list is provided in Supplemental Table 1; [C]BM myeloblast percentage before and during study treatment; [D]22% myeloblasts if erythroids excluded. 5-Aza, 5-azacytidine (number of cycles); AsO3, arsenic; busulph, busulphan; CTX, cytoxan; DOX, doxorubicin; etop, etoposide; F, female; GTO, gemtuzamab; ifos, ifosfamide; Int, intermediate; len, lenalidomide (number of months); M, male; NA, not applicable; PcyR, partial cytogenetic remission; PD, progressive disease; pred, prednisone; SD, stable disease; VCR, vincristine; v., very; WHO, World Health Organization. Blood counts are shown in Figure 3.

ences between individuals and over time in cancer burden, cancer S-phase fraction, cytidine analog metabolism, and hematopoietic reserve were expected to require flexibility in dose and schedule of administration (Figure 2C and ref. 49). Re-escalation to an induction phase was to be considered for hypercellular relapse, which could reflect neoplastic cells growing in gaps between treatment exposures rather than true resistance to decitabine pharmacodynamic effects. Detailed information is provided in the Methods and Supplemental material. Therapy according to the protocol was provided for up to 1 year (52 weeks) (Figure 3A).

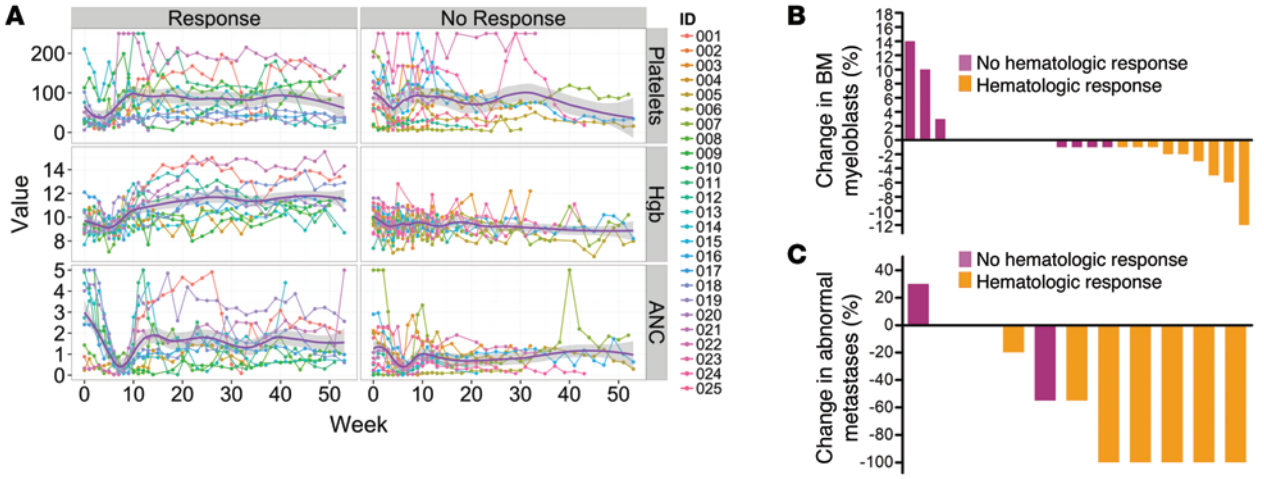

Figure 4. Serial blood counts, myeloblasts, and cytogenetics results in responders and nonresponders. (**A**) Profile of blood counts between baseline and week 52. Left: Responders (those with CR and/or HI). Right: Nonresponders (those with stable disease or progressive disease). Protected data from the Oncore database. Values only for subjects actively receiving drug (Figure 3B) (even some nonresponders continued to receive drug because of stable disease or other treatment benefits). Mean values are indicated with a purple line, and 95% CI is indicated by gray shading. Platelet and ANC values × 10^9/l. Hemoglobin (Hgb) values in g/dl. Values were clipped if they exceeded the depicted *y* axis scales. (**B**) Change in BM myeloblast percentages between weeks 0 and 12. (**C**) Change in the percentage of abnormal metaphases between weeks 0 and week 12 (abnormal metaphases are listed in Table 1).

Subject characteristics

Twenty-five subjects with a median age of 72 years (range, 46–85 years) were enrolled (Figure 3C). Nine subjects (36%) had disease that had relapsed or progressed while on 5-azacytidine and/or lenalidomide (first-line MDS treatments), and 3 had received radiation and/or intensive chemotherapy to treat other cancers (therapy-related MDS) (Table 1). Non-normal cytogenetics were present in 13 of 25 (52%) subjects, with poor or very poor-risk changes (chromosome 7 deletions and/or ≥3 chromosomal abnormalities) in 6 of 25 (24%) subjects (50). Mutation frequency detected by whole-exome sequencing was especially high in subjects with normal cytogenetics and in those previously treated with intensive chemotherapy and radiation for other cancers (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI78789DS1). Mutations and/or deletions in known major p53 system genes were detected in 11 of 25 (44%) subjects (Table 1 and Supplemental Table 1). Revised International Prognostic Scoring System (IPSS-R) risk scores were calculated in the 15 subjects who had not been treated previously with 5-azacytidine, lenalidomide, or other chemotherapies: 4 had very-high, 3 high, 4 intermediate, and 4 low-risk disease, and none had very low-risk disease (Table 1 and ref. 50).

Adverse events

Neutrophil count nadirs occurred between weeks 5 and 11 (Figure 4A) and were complicated by neutropenic fever in 13 of 25 (53%) subjects and by 1 septic death (previous treatments in this subject were cyclophosphamide, doxorubicin, vincristine, ifosfamide, etoposide, busulphan, radiation, and 5-azacytidine) (Table 1). In responding subjects, the median time to neutrophil nadir was 49 days (range, 24–100), and the median time to neutrophil counts greater than 0.5 × 10^9/l was 63 days (range, 56–141) (Figure 4A). Certain pretreatment characteristics predicted neutropenic fever: prestudy neutropenia of less than 1.5 × 10^9/l (10 of 13 with prestudy neutropenia versus 3 of 12 without; $P = 0.013$); neutropenic fever prior to starting study treatment (5 of 5 with prestudy fever versus 8 of 20 without; $P = 0.024$); and prior treatment with 5-azacytidine and/or lenalidomide (7 of 9 of previously treated subjects vs. 6 of 16 of those not treated; $P = 0.05$ by χ^2 test). Eight subjects with neutropenia received granulocyte CSF (G-CSF). No adverse events related to G-CSF use were observed. There were no other grade 3 or 4 treatment–related adverse events.

Antiemetics were not needed. Comorbidities present at baseline included congestive heart failure in 3 subjects, acute renal failure in 1 subject, and iron overload with liver function test abnormalities in another. Other than iron overload, these comorbidities improved during protocol treatment.

Response

Low blood counts cause MDS morbidity and death (43), and improving blood counts (hematologic improvement [HI]) produces better overall survival (51). Therefore, HI or better that met International Working Group (IWG) 2006 Criteria for Response in MDS Clinical Trials was the the primary endpoint (52). Complete remission (CR) (normalization of blood counts) occurred in 4 of 25 and HI in 7 of 25 (overall response rate, 44%) subjects, and these were were highly durable (Figure 4A): in responding subjects who required a transfusion prior to protocol therapy, the median duration of treatment-induced freedom from transfusion was 999 days for platelets (range, 186–1,120; 2 of 5 ongoing as of May 31, 2014) and 695 days for rbc (range, 251–1152; 2 of 8 ongoing) (Figure 4A and Supplemental Figure 1). In all subjects, the median number of days on the protocol regimen was 347 (range, 18–1,281; 3 ongoing). Five of 25 subjects (20%) exceeded 3 years of effective therapy (including 2 subjects >80 years of age) (Figure 3B).

Of the 3 responding subjects who were no longer on protocol therapy at week 52, one had sudden death of unknown

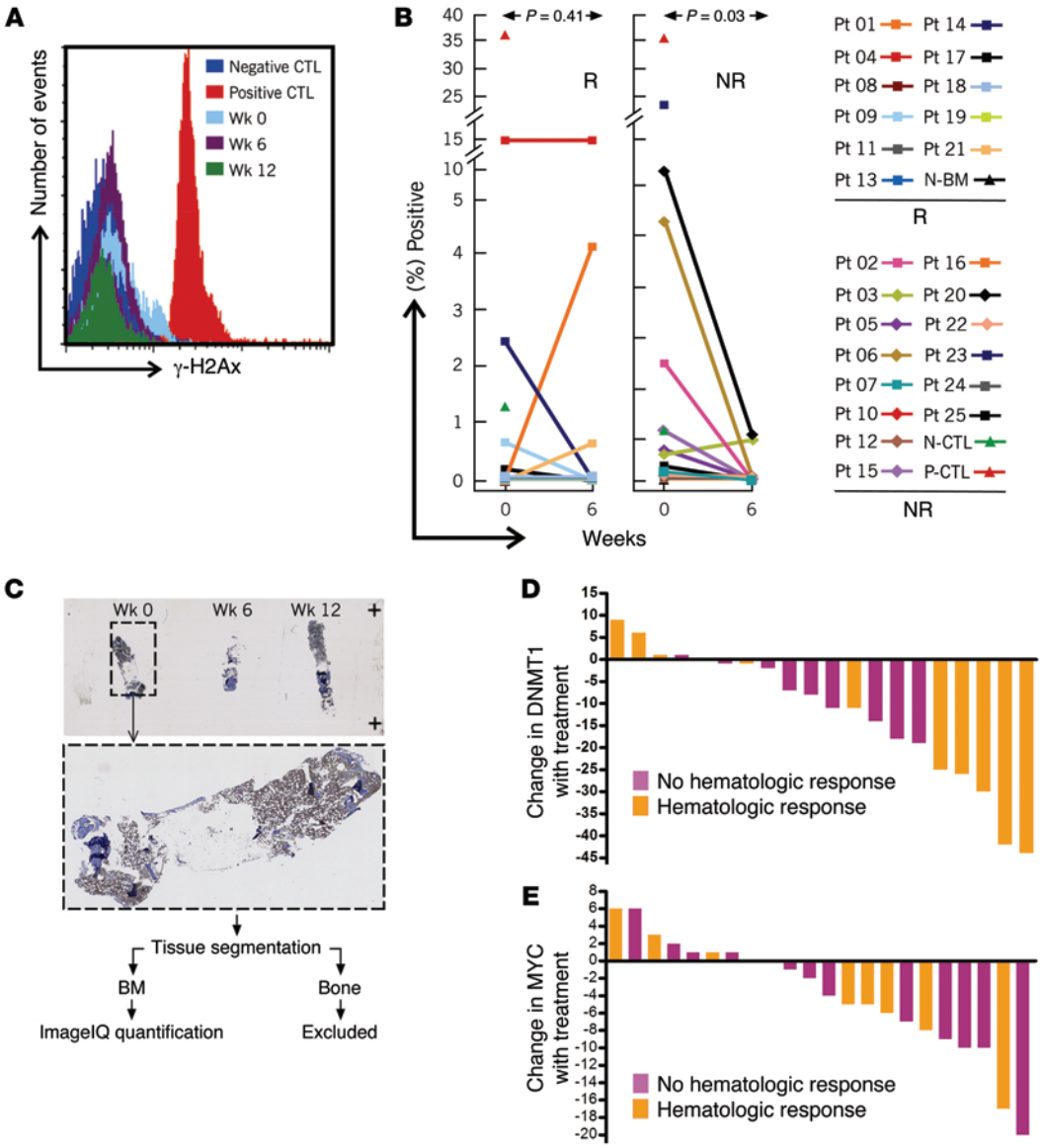

Figure 5. Noncytotoxic DNMT1 depletion and decrease in MYC. (**A**) γH2AX (DNA damage/repair/apoptosis marker) was measured by flow cytometric analysis of BM (an example is shown here). Data for all subjects are provided in the Supplemental material. (**B**) γH2AX expression in BM cells before treatment (week 0) and at week 6. R, responders; NR, nonresponders. *P* values were determined by a paired 1-tailed *t* test. (**C**) Objective quantification of immunohistochemical data. Decalcified and formalin-fixed, paraffin-embedded sections (4 μm thick) of BM biopsies from different time points were immunostained on the same slide. Normal BM and positive and negative controls were concurrently stained. ImageIQ software was used to segment the image, and positive nuclei were objectively quantified in cellular segments. Raw data from software quantification of positive nuclei are provided in Supplemental Table 2. (**D** and **E**) Change in DNMT1 and change in MYC between baseline and week 6. Week 6 corresponded to the nadir (suppression of clonal hematopoiesis). A mean of 3 to 25 tissue segments was quantified per time point per biopsy. Additional graphical representation is provided in Supplemental Figure 7.

cause at week 41 (while transfusion independent with a normal neutrophil count), and 2 relapsed, 1 at week 22 and the other at week 43. Other responding patients continued on the same regimen off-protocol after week 52. Because of stable disease and/or improvements in some disease-related complications, many nonresponders also remained on protocol treatment for 52 weeks or longer (Figure 3B and Figure 4A). For descriptive purposes, overall survival rates, stratified by the IPSS-R (50), are shown in Supplemental Figure 2.

In responders and some nonresponders, the percentage of BM myeloblasts decreased with therapy (Figure 4B). Of 13 cases with chromosomal abnormalities (Table 1), 11 were evaluable with follow-up metaphase karyotyping: complete cytogenetic remission (CyR) was produced in 5 (45%) subjects and partial CyR in 2 (18%) subjects (overall CyRs = 63%, IWG criteria; ref. 52 and Figure 4C). Complete CyR occurred even when myeloid cancer clones contained extensive, highly complex chromosomal abnormalities and multiple single-base mutations (Table 1 and Supplemental Table 1A). All subjects with complete CyR also had HI or better according to IWG criteria.

Leukemia cutis in 1 subject and symptomatic splenomegaly (>10 cm below the costal margin) in another resolved with therapy.

Table 2. Comparison of clinical and pharmacologic characteristics in responders and nonresponders

Parameter	Responders	Nonresponders	*P* value
Clinical			
Age (yr) (median/range)	72 (65–84)	73 (46–85)	0.25[A]
<6 months between diagnosis and treatment	3/11 (27%)	3/14 (21%)	0.73[B]
Relapse/progression on 5-Aza or len	3/11 (27%)	6/14 (43%)	0.42[B]
Previous cancer treated with chemotherapy or radiation	2/11 (18%)	1/14 (7%)	0.39[B]
Pretreatment BM myeloblast % (range)	2 (1–12)	3.5 (1–22)	0.33[A]
RAEB or CMML	6/11 (55%)	7/14 (50%)	0.82[B]
Pretreatment ANC ($\times 10^9$/l) (median/range)	**3.5 (0.28–19.8)**	**0.95 (0.04–5.5)**	**0.03**[A]
Pretreatment platelets ($\times 10^9$/l) (median/range)	**24 (8–174)**	**86 (6–333)**	**0.07**[A]
Pretreatment reticulocytes ($\times 10^9$/l) (median/range)	33 (8–141)	29 (0–89)	0.25[A]
Disease genetics			
High-risk cytogenetics (chromosome 7 deletions and/or ≥3 chromosomal abnormalities)	3/11 (27%)	3/14 (21%)	0.73[B]
PRC2 mutation/deletion	7/11 (64%)	7/14 (50%)	0.49[B]
Splicing factor mutation	6/11 (55%)	8/14 (57%)	0.9[B]
p53 system mutation/deletion	6/11 (55%)	5/14 (36%)	0.35[B]
TET2 system mutation	4/11 (36%)	2/14 (14%)	0.2[B]
Pharmacodynamics and mechanism of action			
γH2AX change between wk 0 and 6 (median/range)	0 (–2.2–3.5)	–0.4 (–6.9–0.3)	0.13[A]
DNMT1 change between wk 0 and 6 (median/range)	–18 (–44–9)	–8 (–19–0)	0.24[A]
MYC change between wk 0 and 6 (median/range)	–3 (–17–6)	–3 (–20–5)	0.33[A]
Number of decitabine shots between wk 0–6 (median/range)	**12 (10–16)**	**10 (6–18)**	**0.04**[A]
Number of wk on regimen (median/range)	**133 (34–161)**	**27 (3–124)**	**0.001**[A]
Hematopoietic capacity			
Pretreatment ATL (median/range)	**3,464**	**1,078**	**0.07**[A]
Pretreatment BM cellularity	**70 (10–95)**	**35 (5–100)**	**0.08**[A]

Significantly different parameters are in bold text. RAEB, refractory anemia with excess blasts; CMML, chronic myelomonocytic leukemia; PRC2, polycomb repressor complex 2 components (EZH2, ASXL1, DNMT3A). [A]Wilcoxon test; [B]χ^2 test or Fisher's exact test.

Molecular pharmacodynamic effects

The molecular pharmacodynamic objectives of treatment were to deplete DNMT1 with minimal DNA damage and cytotoxicity. The biomarker γH2AX increases with DNA repair/damage and is informative for this purpose, whether measured in neoplastic or normal BM cells. Week 6 was selected as the post-treatment time point since it corresponded to nadir, that is, a maximal treatment effect on neoplastic cells and prior to full recovery by more functional hematopoiesis. Comparison with pretreatment marrow was in batched analyses. Minor rises in $H2AX^+$ expression levels occurred in 2 HI/CR subjects (from 0.1% to 3.6% and from 0% to 0.7%), however, no γH2AX increases occurred in the other 9 subjects who achieved HI/CR. A minor increase occurred in 1 of 14 nonresponding subjects (no significant increase in γH2AX was observed in either responders or nonresponders) (Figure 5, A and B, and Table 2). Raw flow cytometric and immunohistochemical quantification data for all biomarkers are provided in Supplemental Figures 3–6 and Supplemental Table 2, A–C.

DNMT1 protein was measured by immunohistochemistry and objectively quantified by ImageIQ imaging and software (Figure 5C). DNMT1 protein levels significantly decreased in both responders and nonresponders on therapy ($P < 0.01$, paired t test) (median decrease of –9.5 between weeks 0 and 6; range, –44 to +9) (Table 2, Figure 5D, and Supplemental Figure 7). The biological objective of noncytotoxic DNMT1 depletion was to antagonize MYC, the master oncoprotein driver of cell growth and division, by restoring expression of maturation-related MYC antagonists (e.g., p27 [CDKN1B]). The levels of p27 protein significantly increased in responders between weeks 0 and 6 ($P = 0.02$, paired t test), but not in nonresponders (Supplemental Figure 7). MYC protein levels decreased in both responders and nonresponders (range, –20 to +6), but this decrease occurred to a significant extent only in nonresponders ($P = 0.02$, paired t test) (Table 2, Figure 5E, and Supplemental Figure 7).

Determinants of hematologic response

To additionally evaluate a noncytotoxic mechanism of action, the impact of apoptotic gene alterations on response was measured. Complex chromosomal and apoptotic gene alterations did not prevent hematologic and/or cytogenetic responses, including complete hematologic and/or cytogenetic responses (Table 2 and Supplemental Table 1). The frequency of key p53 system gene mutations (e.g., *TP53*) and of high-risk structural chromosomal alterations was similar in responders and nonresponders (Table 2 and Supplemental Table 1).

Hematologic response requires recovery by functionally normal hematopoiesis, the capacity for which can be diminished by separate pathophysiologies such as immune-mediated marrow failure that can precede MDS and AML (53, 54). One way of assessing hematopoietic capacity is to measure telomere length, since

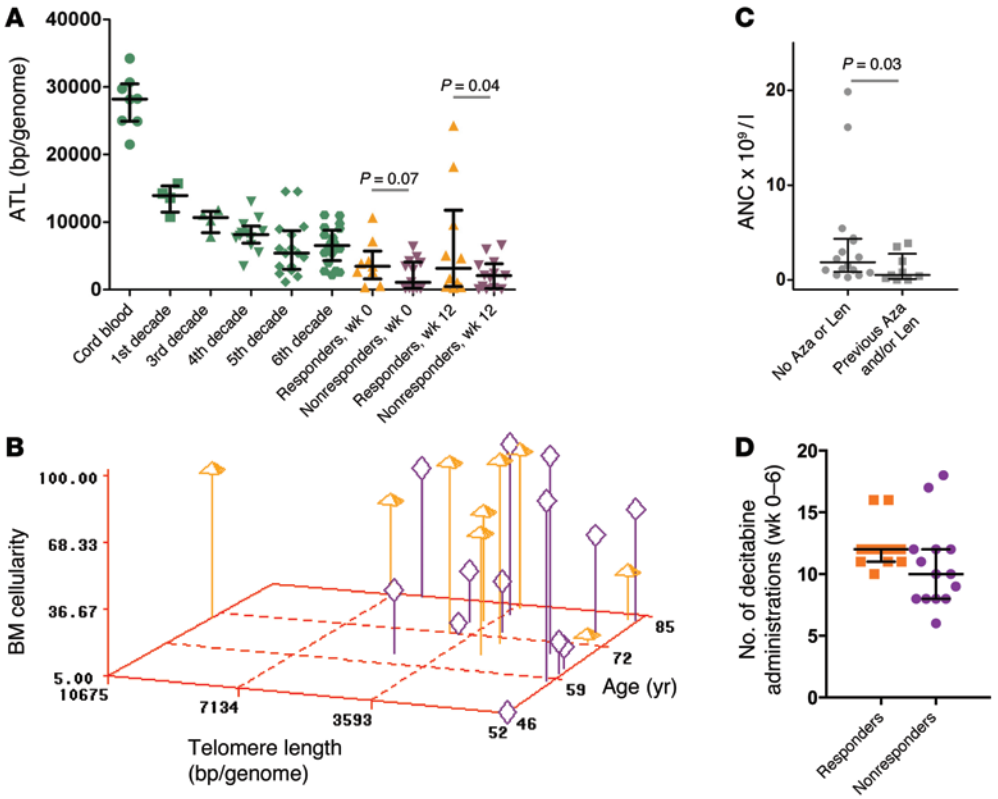

Figure 6. Functional hematopoietic capacity. (**A**) Shorter ATLs in MDS subjects compared with those observed in age-categorized normal controls and especially in nonresponders compared with responders. Shorter telomere length implies greater replications by fewer hematopoietic clones. Telomere length was measured by qRT-PCR. The standard curve is shown in Supplemental Figure 10. Error bars represent the median ± IQR. *P* values were determined by the Mann-Whitney *U* test. (**B**) Pretreatment BM cellularity was especially decreased in subjects with short telomere lengths, including in a young subject with a previous diagnosis of aplastic anemia and cytogenetic response without hematologic response on this protocol. Orange pyramids denote responders; purple diamonds denote nonresponders. (**C**) Pretreatment ANCs were significantly decreased in subjects previously treated with 5-azacytidine (Aza) and/or lenalidomide (Len). Error bars represent the median ± IQR. *P* values were determined by the Mann-Whitney *U* test. (**D**) Lower pretreatment neutrophil counts in nonresponders resulted in fewer decitabine administrations during the induction period (weeks 0–6). Error bars represent the IQR. *P* values were determined by the Mann-Whitney *U* test.

shorter telomere lengths imply greater replication demands on fewer stem cell clones (reviewed in ref. 55). Telomere lengths were decreased in MDS subjects compared with what was found in normal BM, and in nonresponders compared with responders (Figure 6A). Telomere lengths were especially decreased in 2 subjects with severe marrow hypocellularity (~5%) and trilineage pancytopenia despite their relatively young age (Figure 6B and Supplemental Figure 8), 1 of whom had a previous diagnosis of aplastic anemia treated with ATG and cyclosporine. This subject had a partial cytogenetic response without a hematologic response to the protocol treatment (Figure 6B).

In comparing baseline clinical characteristics of responders versus nonresponders, a lower absolute neutrophil count (ANC) in nonresponders was the only significant difference (median of 3.5 versus 0.9×10^9/l) (Table 2). One possible cause of a lower ANC was previous treatment: pretreatment ANC was found to be significantly lower in subjects previously treated with 5-azacytidine or lenalidomide (Figure 6C). Another possible cause was intrinsic disease subtype, since specific genetic alterations in myeloid cancer stem cells have been linked to lower neutrophil and higher platelet counts or vice versa (ref. 32 and Supplemental Figure 9), and nonresponding subjects had higher platelet counts (Table 2). Why were there fewer responses in subjects with baseline neutropenia? For safety reasons, a protocol criterion for reducing the dose and/or frequency of decitabine administration was an ANC below 0.5×10^9/l and below the pretreatment baseline level. Thus, a lower baseline ANC in nonresponders resulted in significantly fewer decitabine administrations around the induction phase (between weeks 0 and 6) (Figure 6D) and possibly less DNMT1 depletion (Figure 5D).

Two distinct patterns of relapse

There were 2 distinct patterns of relapse:

Hypercellular relapse. After achieving CR, subject 1 experienced deteriorating blood counts at week 34, accompanied by an increase in BM cellularity (90%). Per protocol, this was managed by an increase in the frequency of decitabine administration to treat hypercellular relapse caused by malignant cells growing in the gaps between decitabine administrations. More frequent drug administration salvaged the response without toxicity, and this subject continued on therapy beyond week 52 (Figure 7).

Hypocellular relapse. Subjects 4 and 13 who achieved HI and CR, respectively, experienced deteriorating blood counts at weeks 22 and 43. BM evaluation demonstrated a decrease in cellularity

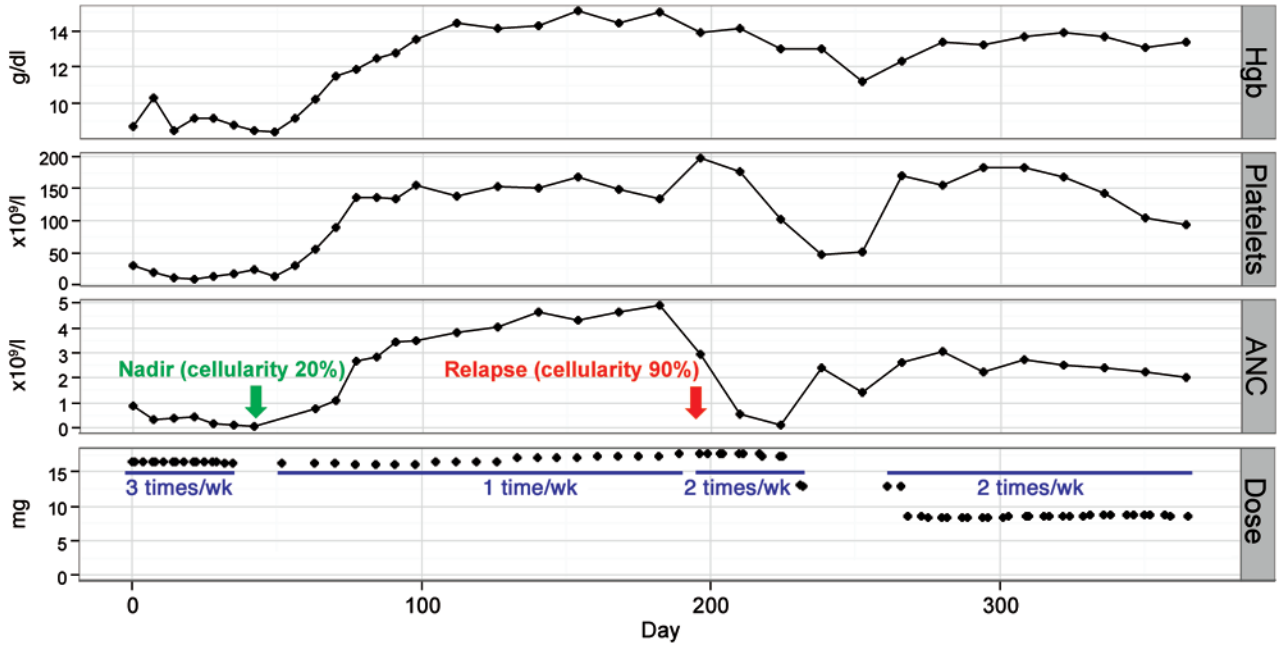

Figure 7. Increase in frequency of drug administration, though at a lower dose, salvaged response, consistent with the importance of exposure time with an S-phase–dependent mode of therapy. Blood count profiles over a 52-week period in a study subject. Green arrow denotes BM evaluation at nadir after the initial induction phase; red arrow denotes BM evaluation of relapsing disease. Cellularity denotes BM cellularity. Each diamond mark in the bottom panel indicates a decitabine administration, with the dose indicated on the *y* axis.

from baseline (from 10% to 50% to 20% to 30% and from 80% to 10%), without an increase in myeloblasts, and resulted in reductions in the dose and frequency of decitabine administration (Figure 3B). Approximately 16 weeks after treatment interruption, there was no improvement in blood counts, but frank progressive disease (increasing BM and peripheral myeloblasts).

Discussion

This clinical trial in MDS redesigned the decitabine dose, schedule, and route of administration to translate preclinical observations regarding a p53-independent mechanism of action, and favorable therapeutic index, of noncytotoxic DNMT1 depletion (8–10, 14, 22). Treatment was well tolerated, without need for antiemetics, including in elderly subjects with organ comorbidities. The overall response rate was 44% in a study population that included 36% of subjects with relapsed or refractory disease following first-line 5-azacytidine and/or lenalidomide treatment. Twenty percent of subjects remained transfusion free on therapy for more than 3 years, including 2 subjects approaching 85 years of age at the time of enrollment. The non- or minimally cytotoxic character of the regimen was supported by: no increases in BM expression of the DNA repair/damage marker γH2AX in the majority of subjects achieving CR and HI; blood count recovery from nadir while actively receiving drug (also supports differential actions of the regimen on malignant and normal hematopoiesis); CR even in disease containing complex chromosomal alterations and p53 mutations and/or deletions; no treatment-related hair loss; no need for antiemetics; and multiyear tolerance and response to therapy. Notably, the identical regimen was evaluated with erythrocyte micronucleus assay, VDJ recombination assays, and sub-G1 fraction analyses in hemoglobinopathy clinical trials, also with the conclusion that the regimen is noncytotoxic (44, 45). Several in vitro studies have detailed that low-range decitabine concentrations can deplete DNMT1 without cytotoxicity or γH2AX increases (reviewed in ref. 13). One caveat is that DNMT1 depletion by shRNA has been shown to decrease mismatch repair (MMR) protein levels and destabilize unmethylated microsatellite repeats in vitro (56), and such a mechanism for genetic instability would be expected to operate even with noncytotoxic DNMT1 depletion. Suppression of myeloid cancer clones, even if by noncytotoxic differentiation (28, 57), suppresses contributions of cancer clones to circulating blood counts and causes nadirs, which occurred between weeks 6 and 11 in the present study.

MDS is genetically heterogeneous. Thus, in the effort to identify pretreatment characteristics that predict response to therapy, there has been a focus on potential mutational predictors, but without a strong mechanistic rationale (58–61). From a mechanistic perspective, however, a minimum requirement for response is achievement of the intended molecular pharmacodynamic effect (DNMT1 depletion), whatever the disease mutations. DNMT1 depletion is S-phase dependent and hence drug exposure time dependent: effectiveness meriting FDA approval was achieved when decitabine doses were reduced to less than 10% of those initially evaluated but administered more often (lower doses caused less toxicity that enabled more frequent administration) (62). A further decrease in this initial FDA-approved dose from 45 mg/m^2/day to 20 mg/m^2/day and a further increase in frequency of administration (5 days every 4 weeks instead of 3 days every 6 weeks) doubled the overall response rate from 30% to 63% (39, 40, 63). Underscoring the importance of frequency of administration, giving this latter lower dose less often (3 days/28-day cycle) decreased overall responses to 23% (64). Hence, the present clinical trial builds on clinical experience, in addition to preclinical mechanism-of-action data (8–10, 14,

22), in selecting doses that minimize or avoid cytotoxicity altogether and in administering these relatively nontoxic, but DNMT1-depleting, doses much more frequently and in a distributed, sustained fashion. The overall response rate of 44% in this trial that had 36% subjects who relapsed or progressed through first-line 5-azacytidine and/or lenalidomide treatment compares favorably to the response rates in previous trials that only enrolled treatment-naive subjects (39, 63, 64). Definitive determination that one regimen or another is superior will require prospective, randomized evaluation in a very large number of patients. It will be necessary to consider in the next steps that disease burden, disease S-phase fraction, and drug half-lives vary among individuals and even in the same individual over time (reviewed in ref. 65). This suggests that flexibility and adaptability in dose and schedule selection toward achievement of pharmacodynamic and clinical objectives is perhaps a more rational approach than is rigid adherence to a particular regimen. The lack of toxicity of the decitabine dose used here permitted such flexibility and adaptability. The experience of the subject with hypercellular relapse, in whom an increasing frequency of drug administration salvaged the response, illustrates this point. Continuous infusion of decitabine causes accumulation of decitabine triphosphate in normal cells up to levels that are cytotoxic (66), hence, the use of intermittent s.c. administration in the present trial.

Responders were more likely than nonresponders to have needed pretreatment platelet transfusions, but had adequate neutrophil counts (32). DNMT1 depletion, whether by decitabine treatment or genetic means, has been shown to shift hematopoietic differentiation to favor the production of megakaryocyte and erythroid precursors over granulocyte and monocyte precursors (19, 44, 45, 67). In subjects with low neutrophil counts at baseline, exacerbation of neutropenia with nadir and by these differentiation shifts deters drug administration, as seen in this and other clinical trials involving decitabine (39). In this way, baseline neutropenia places a practical limit on the frequency of drug administration and thus on pharmacodynamic effect and response. One method of addressing this treatment confounder is to combine therapy with G-CSF, as has been done in other clinical trials with decitabine (39, 40).

Pathologies other than myeloid cancer clone outgrowth, such as autoimmunity, germline mutations in telomere maintenance genes, previous toxic exposures, and simply age-related attrition, can contract the functional HSC pool (53, 54). These additional factors could underlie the poorer outcomes widely observed in older versus younger MDS or AML patients, even after controlling for myeloid cancer clone genetics (11, 12), and may also explain the phenomena of cytogenetic response (implying successful suppression of malignant clones) without the hematologic response observed clinically (68). These pathologies emphasize the need for a good therapeutic index in treatments used to suppress myeloid cancer clones (8–12). Additional treatments that boost functional hematopoiesis and that are FDA approved to treat BM failure, e.g., thrombopoietin receptor (MPL) agonists, can potentially complement clone-suppressing treatments (69, 70).

Mutational apoptosis defects are very common in myeloid and other neoplasms and confound the therapeutic index of apoptosis-intending treatments. This clinical trial showed that a clinically available drug, decitabine, can be redesigned for a noncytotoxic, epigenetic differentiation treatment that circumvents p53 and apoptosis. This approach to the problem of apoptotic defects in cancer merits further mechanism-based evaluation and development.

Methods

Study design and patients. This was a single-arm, open-label phase I/II study (NCT01165996; https://clinicaltrials.gov). HI or better was the primary endpoint according to the IWG 2006 Criteria for Response in MDS Clinical Trials (52), defined as an increase in hemoglobin of greater than or equal to 1.5 g/dl; a reduction in rbc transfusions by at least 4 transfusions over an 8-week period compared with pretreatment transfusions; an increase in platelets by greater than or equal to 30×10^9/l (if baseline platelets were $>20 \times 10^9$/l); an increase in platelets from less than 20×10^9/l to greater than 20×10^9/l and by at least 100%; and at least a 100% increase in neutrophils and an absolute increase of greater than 0.5×10^9/l (if baseline neutrophils were $<0.5 \times 10^9$/l). Secondary endpoints included greater than grade 2 toxicity (National Cancer Institute [NCI]/Cancer Therapy Evaluation Program [CTEP] version 4.0 criteria); other IWG response criteria (52); and a mechanism-of-action and prediction biomarker correlation with response. In addition to MDS confirmed by hematopathological review, subjects were required to have symptomatic anemia or thrombocytopenia with a platelet count of less than 100×10^9/l, a transfusion dependence for rbc or platelets, or an absolute neutrophil count below 1×10^9/l. Previous 5-azacytidine, lenalidomide, and other treatments were permitted, but previous decitabine treatment was an exclusion criterion.

Study drug and regimen. The study drug (Eisai Inc.), supplied as a lyophilized powder for injection (50 mg), was reconstituted with 5 ml sterile water to facilitate s.c. administration (Figure 2). Interindividual differences and differences in the same individual over time in terms of cancer burden, cancer S-phase fraction, cytidine analog metabolism, and hematopoietic reserve were anticipated to require flexibility in dose and frequency of decitabine administration (49). The treatment was divided into induction and maintenance phases. The induction phase objective was noncytotoxic cytoreduction of the malignant clone sufficient to enable normal hematopoiesis and relief of cytopenia. Decitabine 0.2 mg/kg/day was administered 2 times per week during the 4-week induction phase, with 3-times-per-week administration to be considered if BM myeloblasts were greater than or equal to 10% or if there was clinical concern of rapid progression to AML. During the subsequent maintenance phase, the objective was continued noncytotoxic suppression of the malignant clone to the extent that satisfactory blood counts were maintained.

Management of neutropenia. Neutrophil count nadirs occurring 5–8 weeks after initiation of therapy or after any increase in dose or treatment frequency were managed by temporary withholding the drug for 1 to 2 weeks and then resuming at the same dose or reducing the dose by no more than 0.05 mg/kg/day, with a minimum dose of 0.1 mg/kg/day administered once per week. G-CSF support was permitted if neutrophil counts were below 0.5×10^9/l. The overall goal was to relieve cytopenia while maintaining malignant clone suppression with administration of at least 0.1 mg/kg decitabine once per week. Regular administration, at a lower dose if necessary, was always preferred to infrequent administration of a higher dose.

BM aspirate and biopsies. Worsening cytopenias concurrent with increasing BM cellularity (hypercellular relapse) was an indication of progressive disease that could be managed with increasing the fre-

quency of drug administration (maximum of 0.2 mg/kg/day 3 days/week). Worsening peripheral cytopenias concurrent with a decrease in BM cellularity (hypocellular relapse) could reflect nadir or overtreatment to be managed as described for neutropenia.

Immunodetection and quantitation. Immunostaining was performed on decalcified and formalin-fixed, paraffin-embedded BM biopsy sections (4 μm thick) and on positive and negative controls. Nuclei positive for the targeted biomarker were identified and quantified in high-resolution, large field-of-view images according to ImageIQ algorithms (ImageIQ Inc.) after subtraction of bone from the original image. γH2AX was detected and quantified by flow cytometric analyses of fixed and permeabilized BM aspirates and controls.

Whole-exome sequencing. Exome targets captured from BM aspirate and paired $CD3^+$ T cell (germline control) DNA were subjected to massive parallel sequencing using an Illumina HiSeq 2000 system.

Telomere length measurement. Absolute telomere length (ATL) was measured by quantitative reverse transcriptase PCR (RT-PCR), as described previously (Supplemental Table 3) (71, 72).

Data collection and statistics. Data were collected in a protected OnCore database. The sample size (n = 25) was based on a 2-stage design with a null hypothesis of 30% of the patients having hematological improvement or better versus an alternative hypothesis of 60%, using a 1-sided α of 5% and a power of 90%. After 15 patients, at least 5 responses were needed for the trial to proceed, at which point 10 additional patients were enrolled. The nonparametric Wilcoxon signed-rank test was used to determine the significance of within-patient differences between time points. The Fisher's exact or χ^2 test was used to compute P values for differences in proportions of adverse events or positive biomarkers. P values of less than 0.05 were considered statistically significant. The censorship date for overall survival and transfusion independence calculations was May 28, 2014.

Study approval. This study was approved by the IRBs of the Cleveland Clinic and Case Western Reserve University (principal investigator, Yogen Saunthararajah). Written informed consent was obtained from all patients prior to treatment, and all research was conducted according to the principles set forth by the Declaration of Helsinki.

Detailed clinical, statistical, and scientific correlative study methods are provided in the Supplemental Methods.

Acknowledgments

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Address correspondence to: Yogen Saunthararajah, Taussig Cancer Institute, 9500 Euclid Avenue R40, Cleveland, Ohio 44195, USA. Phone: 216.444.8170; E-mail: saunthy@ccf.org.

1. Kinzler KW, Vogelstein B. Cancer therapy meets p53. *N Engl J Med*. 1994;331(1):49–50.
2. Suarez L, et al. $CD34^+$ cells from acute myeloid leukemia, myelodysplastic syndromes, and normal bone marrow display different apoptosis and drug resistance-associated phenotypes. *Clin Cancer Res*. 2004;10(22):7599–7606.
3. Yin B, Kogan SC, Dickins RA, Lowe SW, Largaespada DA. Trp53 loss during in vitro selection contributes to acquired Ara-C resistance in acute myeloid leukemia. *Exp Hematol*. 2006;34(5):631–641.
4. Wattel E, et al. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. *Blood*. 1994;84(9):3148–3157.
5. Haferlach C, Dicker F, Herholz H, Schnittger S, Kern W, Haferlach T. Mutations of the TP53 gene in acute myeloid leukemia are strongly associated with a complex aberrant karyotype. *Leukemia*. 2008;22(8):1539–1541.
6. Toledo F, Wahl GM. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer*. 2006;6(12):909–923.
7. Harutyunyan A, Klampfl T, Cazzola M, Kralovics R. p53 lesions in leukemic transformation. *N Engl J Med*. 2011;364(5):488–490.
8. Brennig S, Rattmann I, Lachmann N, Schambach A, Williams DA, Moritz T. In vivo enrichment of cytidine deaminase gene-modified hematopoietic cells by prolonged cytosine-arabinoside application. *Cytotherapy*. 2012;14(4):451–460.
9. Ben-Ishay Z, Barak V. Bone marrow stromal dysfunction in mice administered cytosine arabinoside. *Eur J Haematol*. 2001;66(4):230–237.
10. Ng KP, et al. p53 independent epigenetic-differentiation treatment in xenotransplant models of acute myeloid leukemia. *Leukemia*. 2011;25(11):1739–1750.
11. Mandelli F, et al. A randomised clinical trial comparing idarubicin and cytarabine to daunorubicin and cytarabine in the treatment of acute non-lymphoid leukaemia. A multicentric study from the Italian Co-operative Group GIMEMA. *Eur J Cancer*. 1991;27(6):750–755.
12. Schoch C, Kern W, Schnittger S, Buchner T, Hiddemann W, Haferlach T. The influence of age on prognosis of de novo acute myeloid leukemia differs according to cytogenetic subgroups. *Haematologica*. 2004;89(9):1082–1090.
13. Saunthararajah Y, et al. p53-Independent, normal stem cell sparing epigenetic differentiation therapy for myeloid and other malignancies. *Semin Oncol*. 2012;39(1):97–108.
14. Negrotto S, et al. CpG methylation patterns and decitabine treatment response in acute myeloid leukemia cells and normal hematopoietic precursors. *Leukemia*. 2012;26(2):244–254.
15. Negrotto S, et al. Noncytotoxic differentiation treatment of renal cell cancer. *Cancer Res*. 2011;71(4):1431–1441.
16. Alcazar O, et al. Epigenetic regulation by decitabine of melanoma differentiation in vitro and in vivo. *Int J Cancer*. 2012;131(1):18–29.
17. Hu Z, et al. Decitabine maintains hematopoietic precursor self-renewal by preventing repression of stem cell genes by a differentiation-inducing stimulus. *Mol Cancer Ther*. 2010;9(6):1536–1543.
18. Trowbridge JJ, Snow JW, Kim J, Orkin SH. DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells. *Cell Stem Cell*. 2009;5(4):442–449.
19. Milhem M, et al. Modification of hematopoietic stem cell fate by 5aza 2′ deoxycytidine and trichostatin A. *Blood*. 2004;103(11):4102–4110.
20. Hu Z, et al. RUNX1 regulates corepressor interactions of PU.1. *Blood*. 2011;117(24):6498–6508.
21. Tsai HC, et al. Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. *Cancer Cell*. 2012;21(3):430–446.
22. Momparler RL, Cote S, Momparler LF. Epigenetic action of decitabine (5-aza-2′-deoxycytidine) is more effective against acute myeloid leukemia than cytotoxic action of cytarabine (ARA-C). *Leuk Res*. 2013;37(8):980–984.
23. Chaurasia P, Gajzer DC, Schaniel C, D'Souza S, Hoffman R. Epigenetic reprogramming induces the expansion of cord blood stem cells. *J Clin Invest*. 2014;124(6):2378–2395.
24. Trowbridge JJ, Sinha AU, Zhu N, Li M, Armstrong SA, Orkin SH. Haploinsufficiency of Dnmt1 impairs leukemia stem cell function through derepression of bivalent chromatin domains. *Genes Dev*. 2012;26(4):344–349.
25. Vierbuchen T, Wernig M. Molecular roadblocks for cellular reprogramming. *Mol Cell*. 2012;47(6):827–838.
26. Iida H, et al. Protein expression and constitutive phosphorylation of hematopoietic transcription factors PU.1 and C/EBP β in acute myeloid leukemia blasts. *Int J Hematol*. 2000;71(2):153–158.

27. Gu X, et al. Runx1 regulation of Pu.1 corepressor/coactivator exchange identifies specific molecular targets for leukemia differentiation therapy. *J Biol Chem*. 2014;289(21):14881–14895.
28. Hu Z, Saunthararajah Y. CEBPE activation in PML-RARA cells by arsenic. *Blood*. 2012;119(9):2177–2179.
29. Woll PS, et al. Myelodysplastic syndromes are propagated by rare and distinct human cancer stem cells in vivo. *Cancer Cell*. 2014;25(6):794–808.
30. Ng KP, Hu Z, Ebrahem Q, Negrotto S, Lausen J, Saunthararajah Y. Runx1 deficiency permits granulocyte lineage commitment but impairs subsequent maturation. *Oncogenesis*. 2013;2:e78.
31. Agrawal S, et al. The C/EBPΔ tumor suppressor is silenced by hypermethylation in acute myeloid leukemia. *Blood*. 2007;109(9):3895–3905.
32. Saunthararajah Y, Maciejewski J. Polycomb segment myeloid malignancies. *Blood*. 2012;119(5):1097–1098.
33. Covey JM, D'Incalci M, Tilchen EJ, Zaharko DS, Kohn KW. Differences in DNA damage produced by incorporation of 5-aza-2′-deoxycytidine or 5,6-dihydro-5-azacytidine into DNA of mammalian cells. *Cancer Res*. 1986;46(11):5511–5517.
34. Schermelleh L, et al. Dynamics of Dnmt1 interaction with the replication machinery and its role in postreplicative maintenance of DNA methylation. *Nucleic Acids Res*. 2007;35(13):4301–4312.
35. Nakano T, et al. Homologous recombination but not nucleotide excision repair plays a pivotal role in tolerance of DNA-protein cross-links in mammalian cells. *J Biol Chem*. 2009;284(40):27065–27076.
36. Palii SS, Van Emburgh BO, Sankpal UT, Brown KD, Robertson KD. DNA methylation inhibitor 5-Aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B. *Mol Cell Biol*. 2008;28(2):752–771.
37. Orta ML, et al. 5-Aza-2′-deoxycytidine causes replication lesions that require Fanconi anemia-dependent homologous recombination for repair. *Nucleic Acids Res*. 2013;41(11):5827–5836.
38. Kantarjian H, et al. Decitabine improves patient outcomes in myelodysplastic syndromes — Resuits of a Phase III randomized study. *Cancer*. 2006;106(8):1794–1803.
39. Steensma DP, et al. Multicenter study of decitabine administered daily for 5 days every 4 weeks to adults with myelodysplastic syndromes: the alternative dosing for outpatient treatment (ADOPT) trial. *JClinOncol*. 2009;27(23):3842–3848.
40. Kantarjian H, et al. Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood*. 2007;109(1):52–57.
41. Kantarjian HM, et al. Update of the decitabine experience in higher risk myelodysplastic syndrome and analysis of prognostic factors associated with outcome. *Cancer*. 2007;109(2):265–273.
42. Tuma RS. Epigenetic therapies move into new territory, but how exactly do they work? *J Natl Cancer Inst*. 2009;101(19):1300–1301.
43. Dayyani F, et al. Cause of death in patients with lower-risk myelodysplastic syndrome. *Cancer*. 2010;116(9):2174–2179.
44. Saunthararajah Y, et al. Effects of 5-aza-2′-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease. *Blood*. 2003;102(12):3865–3870.
45. Olivieri NF, et al. A pilot study of subcutaneous decitabine in beta-thalassemia intermedia. *Blood*. 2011;118(10):2708–2711.
46. Gentles AJ, Plevritis SK, Majeti R, Alizadeh AA. Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia. *JAMA*. 2010;304(24):2706–2715.
47. Riddell J, et al. Reprogramming committed murine blood cells to induced hematopoietic stem cells with defined factors. *Cell*. 2014;157(3):549–564.
48. Herrmann H, et al. Dipeptidylpeptidase IV (CD26) defines leukemic stem cells (LSC) in chronic myeloid leukemia. *Blood*. 2014;123(25):3951–3962.
49. Mahfouz RZ, et al. 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*. 2013;19(4):938–948.
50. Greenberg PL, et al. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood*. 2012;120(12):2454–2465.
51. Itzykson R, et al. Prognostic factors for response and overall survival in 282 patients with higher-risk myelodysplastic syndromes treated with azacitidine. *Blood*. 2011;117(2):403–411.
52. Cheson BD, et al. Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia. *Blood*. 2006;108(2):419–425.
53. Van Zant G, Liang Y. Concise review: hematopoietic stem cell aging, life span, and transplantation. *Stem Cells Transl Med*. 2012;1(9):651–657.
54. Gadalla SM, Savage SA. Telomere biology in hematopoiesis and stem cell transplantation. *Blood Rev*. 2011;25(6):261–269.
55. Calado RT, Young NS. Telomere maintenance and human bone marrow failure. *Blood*. 2008;111(9):4446–4455.
56. Loughery JE, Dunne PD, O'Neill KM, Meehan RR, McDaid JR, Walsh CP. DNMT1 deficiency triggers mismatch repair defects in human cells through depletion of repair protein levels in a process involving the DNA damage response. *Hum Mol Genet*. 2011;20(16):3241–3255.
57. Lo-Coco F, et al. Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *N Engl J Med*. 2013;369(2):111–121.
58. Traina F, et al. Impact of molecular mutations on treatment response to DNMT inhibitors in myelodysplasia and related neoplasms. *Leukemia*. 2014;28(1):78–87.
59. Bejar R, et al. TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. *Blood*. 2014;124(17):2705–2712.
60. Braun T, et al. Molecular predictors of response to decitabine in advanced chronic myelomonocytic leukemia: a phase 2 trial. *Blood*. 2011;118(14):3824–3831.
61. Metzeler KH, et al. DNMT3A mutations and response to the hypomethylating agent decitabine in acute myeloid leukemia. *Leukemia*. 2012;26(5):1106–1107.
62. Rivard GE, et al. Phase I study on 5-aza-2′-deoxycytidine in children with acute leukemia. *LeukRes*. 1981;5(6):453–462.
63. Kantarjian HM, et al. Survival advantage with decitabine versus intensive chemotherapy in patients with higher risk myelodysplastic syndrome: comparison with historical experience. *Cancer*. 2007;109(6):1133–1137.
64. Garcia-Manero G, et al. Randomized open-label phase II study of decitabine in patients with low- or intermediate-risk myelodysplastic syndromes. *J Clin Oncol*. 2013;31(20):2548–2553.
65. Saunthararajah Y. Key clinical observations after 5-azacytidine and decitabine treatment of myelodysplastic syndromes suggest practical solutions for better outcomes. *Hematology Am Soc Hematol Educ Program*. 2013;2013:511–521.
66. Jansen RS, Rosing H, Wijermans PW, Keizer RJ, Schellens JH, Beijnen JH. Decitabine triphosphate levels in peripheral blood mononuclear cells from patients receiving prolonged low-dose decitabine administration: a pilot study. *Cancer Chemother Pharmacol*. 2012;69(6):1457–1466.
67. Broske AM, et al. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. *Nat Genet*. 2009;41(11):1207–1215.
68. Raj K, Mufti GJ. Azacytidine (Vidaza(R)) in the treatment of myelodysplastic syndromes. *Ther Clin Risk Manag*. 2006;2(4):377–388.
69. Kantarjian HM, et al. Phase 2 study of romiplostim in patients with low- or intermediate-risk myelodysplastic syndrome receiving azacitidine therapy. *Blood*. 2010;116(17):3163–3170.
70. Greenberg PL, et al. A randomized controlled trial of romiplostim in patients with low- or intermediate-risk myelodysplastic syndrome receiving decitabine. *Leuk Lymphoma*. 2013;54(2):321–328.
71. O'Callaghan NJ, Fenech M. A quantitative PCR method for measuring absolute telomere length. *Biol Proced Online*. 2011;13:3.
72. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res*. 2002;30(10):e47.