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OIRIGINAL ARTICLE

p53 Independent epigenetic-differentiation treatment in xenotransplant models of acute myeloid leukemia

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Suppression of apoptosis by *TP53* mutation contributes to resistance of acute myeloid leukemia (AML) to conventional cytotoxic treatment. Using differentiation to induce irreversible cell cycle exit in AML cells could be a p53-independent treatment alternative, however, this possibility requires evaluation. *In vitro* and *in vivo* regimens of the deoxycytidine analogue decitabine that deplete the chromatin-modifying enzyme DNA methyl-transferase 1 without phosphorylating p53 or inducing early apoptosis were determined. These decitabine regimens but not equimolar DNA-damaging cytarabine upregulated the key late differentiation factors CCAAT enhancer-binding protein ε and p27/cyclin dependent kinase inhibitor 1B (CDKN1B), induced cellular differentiation and terminated AML cell cycle, even in cytarabine-resistant p53- and p16/CDKN2A-null AML cells. Leukemia initiation by xenotransplanted AML cells was abrogated but normal hematopoietic stem cell engraftment was preserved. *In vivo*, the low toxicity allowed frequent drug administration to increase exposure, an important consideration for S phase specific decitabine therapy. In xenotransplant models of p53-null and relapsed/refractory AML, the non-cytotoxic regimen significantly extended survival compared with conventional cytotoxic cytarabine. Modifying *in vivo* dose and schedule to emphasize this pathway of decitabine action can bypass a mechanism of resistance to standard therapy.
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Introduction

Although conventional chemotherapeutics for acute myeloid leukemia (AML) can have differing proximal mechanisms of action, such as topoisomerase inhibition or termination of DNA-chain synthesis, a final common pathway converges onto p53, a stress and DNA damage sensor and a master regulator of apoptosis (reviewed in Vazquez[1]). Therefore, mutation and chromosome deletion at the *TP53* locus is associated with treatment resistance both *in vitro*[2,3] and *in vivo*: partial or complete remissions in response to intensive chemotherapy were achieved in 81% of AML cases without, and 33% of cases with *TP53* mutations.[4] Similarly, responses in patients with myelodysplastic syndrome (MDS) treated with intensive chemotherapy or low-dose cytarabine were 60% of cases without, and 8% of cases with *TP53* mutations.[4] Even in cases in which *TP53* itself is not directly mutated or deleted, the p53 pathway may be targeted by genetic abnormalities in p53 cofactors: *TP53* defects or *MDM4* gain was noted in 45.5% of patients with blast transformed myeloproliferative disease, which responds only transiently if at all to conventional cytotoxic therapy.[5,6] The rate of *TP53* mutation can exceed 70% in MDS and AML cases with complex cytogenetic abnormalities, another group of patients with very poor treatment outcomes.[7]

Hence, especially for certain subtypes of MDS and AML, there is a need for treatment that is not mediated through p53 and apoptosis. Interestingly, although p53-null mice are cancer prone, the development of these mice is essentially normal, with normal patterns of differentiation in almost all tissues (reviewed in Attardi and Donehower[8]). This suggests that differentiation-mediated cell cycle exit is usually p53-independent. Using differentiation to terminate cancer cell proliferation was proposed more than 50 years ago,[9–11] and differentiation has been observed in AML and cancer cells treated with drugs that inhibit chromatin-modifying enzymes, such as histone deacetylase inhibitors (HDACi), and 5-azacytidine and decitabine that deplete DNA methyl-transferase 1 (DNMT1).[12–18] However, the effects of HDACi are not confined to histones; HDACi can alter the acetylation status of structural, signaling and transcription factor proteins, producing wide-spread cellular effects including apoptosis. Similarly, high concentrations of the nucleoside analogues 5-azacytidine and decitabine have anti-metabolite and DNA-damaging effects, and can trigger apoptosis. Furthermore, DNA hypomethylation by decitabine can induce the expression of apoptosis mediators such as p73, that can mediate apoptosis independent of p53.[19] Hence, it has not been clear that differentiation-mediated cell cycle exit is the most important therapeutic action of 5-azacytidine or decitabine (reviewed in Tuma[20]). There has even been debate about whether inhibition of chromatin-modifying enzymes is the most important effect of these drugs.[20] Therefore, demonstration of a p53-independent non-cytotoxic mechanism of action, rationalization of dose and schedule to emphasize this pathway of action, and demonstration of *in vivo* efficacy, are important translational challenges.

Unlike the cytidine analogues cytarabine or gemcitabine, the sugar moiety of decitabine is unmodified. Therefore, at low concentrations, decitabine can incorporate into the newly synthesized DNA strand during S phase without terminating chain elongation.[21,22] These non-DNA damaging, non-cytotoxic

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concentrations of decitabine can nonetheless deplete DNMT1, both *in vitro* and *in vivo*.[12,21–25] The first objective was to determine if such concentrations of decitabine, which do not kill normal hematopoietic stem cells (HSCs) (low concentrations of decitabine have increased normal HSC self-renewal in a number of studies[26–29]), and which may not induce early apoptosis, can nonetheless induce irreversible cell cycle exit in AML cells. Cell cycle exit is mediated by a family of highly conserved cyclin dependent kinase inhibitors (CDKN): p16/CDKN2A is implicated in apoptotic cell cycle exit, whereas p27/CDKN1B is known to mediate cell cycle exit with differentiation.[30–33] Therefore, the second objective was to measure changes in CDKN protein expression in response to decitabine and equimolar cytarabine, to differentiate between apoptosis and differentiation-mediated cell cycle exit, and to determine if these changes were dependent on p53. To this end, *in vitro* experiments were conducted in p53 wild-type and p53-null MLL-AF9 AML cells: treatment effects on p53 upregulation and phosphorylation could be measured in p53 wild-type cells, whereas p53-null cells could be used to confirm p53-independence of observed effects. Finally, the efficacy of a decitabine dose, schedule and route of administration rationalized for a non-cytotoxic mechanism of action *in vivo* was examined in murine xenotransplantation models of p53-null and relapsed/refractory human AML, to evaluate the translational potential of this alternative, non-cytotoxic treatment approach.

Materials and methods

Healthy volunteer and patient samples

Umbilical cord blood was collected during normal full-term deliveries, and bone marrow aspirates were collected from AML patients and healthy volunteers. All collections occurred after written informed consent of the mother, patient or volunteer as per Case Western Reserve University and Cleveland Clinic IRB approved protocols. Anonymized clinical hematopathology data were associated with patient samples.

Isolation of CD34+ cells

CD34+ cells from umbilical cord blood or bone marrow aspirates were purified using a magnetic cell sorting system (CD34 MicroBead Kit #130-046-702, Miltenyl Biotec Inc., Auburn, CA, USA) according to manufacturer instructions. The purity of the CD34+ population (typically from 95 to 99%) was determined by flow cytometry with a FITC-conjugated monoclonal antibodies against CD34 (Clone 581, Beckman Coulter, Miami, FL, USA).

AML cells analyzed

Three types of AML cells analyzed were: (i) p53 wild-type MLL-AF9 cells were generated as described.[34] These cells have a gene-expression profile similar to primary human MLL-AF9 leukemia cells,[34] and self-renew indefinitely both *in vitro* and *in vivo*, initiating invasive AML in transplanted mice.[34] (ii) p53-null THP1 cells were purchased from ATCC (Manassas, VA, USA). This morphologically monocytoid AML cell line (M5 morphology) contains an MLL-AF9 fusion and is homozygously mutated at the *TP53* and *CDKN2A* loci. The cells used for xenotransplantation were transfected to express luciferase. (iii) Primary (fresh) AML cells from a patient with relapsed/refractory AML. These cells had myelomonocytic morphology (M4) and contained t(8;18)(q22:q23) and t(11;13)(q21:q12).

Human hematopoietic cell culture

Normal human hematopoietic cells and p53 wild-type MLL-AF9 cells were cultured in IMDM supplemented with 10% fetal bovine serum and 10 ng/ml of the following human cytokines: stem cell factor, FLT3 ligand, thrombopoietin, interleukin-3 and interleukin-6. THP1 cells were cultured in RPMI 1640 media without cytokine supplementation.

Treatment of cells with decitabine

Decitabine stock solution (5 mM) was generated by reconstituting lyophilized decitabine in 100% methanol. Stock solution was stored at −20 °C for up to 3 weeks. Similar amounts of methanol are added to untreated control cells. For *in vitro* experiments, cells were treated with decitabine (0.5 μM) on day 1 and 4 unless otherwise specified. For *in vitro* treatment followed by transplantation of cells into mice by tail vein injection, cells were treated with decitabine 0.5 μM on day 1, 0.2 μM on day 2, 0.5 μM on day 5, 0.2 μM on day 6 and transplantation on day 7. This alternating schedule was used to increase *in vitro* decitabine exposure of the normal and leukemia cells without exceeding a decitabine concentration of 0.5 μM (as it was possible that up to 30% of the previous day decitabine could be present in the culture media after 24 h[35,36]).

Apoptosis detection

Apoptosis was detected by Annexin-V and 7AAD (or PI) co-staining using the APOAF commercial kit (Sigma-Aldrich, St Louis, MO, USA).

Clonogenic progenitor assays

p53 Wild-type and p53-null MLL-AF9 cells in liquid culture were treated with decitabine or cytarabine 0.5 μM on day 1 and 4. On day 5, identical numbers of cells from vehicle and decitabine-treated cultures were plated in decitabine-free semisolid media (MethoCult H4434, Stem Cell Technology, Vancouver, BC, Canada, 2000 cells/ml). Ten days after plating, colony-forming units (CFU) were identified by morphology and counted under an inverted microscope.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting

Approximately 100 μg of cytoplasmic and nuclear protein extracts from cells, together with molecular weight markers, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4–12% gradient gels (Invitrogen, Carlsbad, CA, USA) followed by transfer to PVDF membranes (Invitrogen). Blots were probed using antibodies for DNMT1 (Abcam ab16632, Cambridge, MA, USA), phospho-p53 (Cell Signaling, Danvers, MA, USA, #9286), p53 (Sigma P6874), p15 (Cell Signaling #4822), p21 (Cell Signaling #2946), p16 (Santa Cruz Biotech sc-81613, Santa Cruz, CA, USA; Cell Signaling #4824), CCAAT enhancer-binding protein (CEBPα, Santa Cruz Biotech sc-166258), CEPBε (Santa Cruz Biotech sc-25570), PU.1 (Santa Cruz Biotech sc-352), p27 (Cell Signaling #3686) and anti-β-actin peroxidase (Sigma-Aldrich #A3854).

Murine studies

All experiments were approved by the Cleveland Clinic and Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committees (IACUC). Cultured normal HSC, MLL-AF9 cells, p53-null MLL-AF9 cells (THP1) cells or fresh patient bone marrow AML cells were transplanted by

intravenous (IV) injection into non-irradiated 6–8 week old NOD/SCID or NSG mice. Mice were anesthetized with isofluorane before transplantation. By extrapolation from previous studies of decitabine pharmacokinetics in mice, a dose of decitabine 0.2 mg/kg was selected, for the objective of decitabine peak plasma concentrations lower than 0.2 μM. Animals were checked daily and were euthanized by an IACUC approved method for signs of distress. Bone marrow was analyzed by flow cytometry, Giemsa stain and western blot. The proportion of human hematopoietic cells in bone marrow was determined by positive staining with PC5-conjugated anti-human CD45 mAb (BD, Sparks, MD, USA) with isotype-matched immunoglobulin as a control in all experiments. Livers and spleens were weighed and fixed. To anatomically localize THP1 cells in living mice, the substrate D-luciferin (15 mg/ml D-luciferin in sterile phosphate buffered saline (Promega, Madison, WI, USA) was injected IP and mice were imaged after 10 min using an IVIS-200 CCD camera imaging system (Xenogen, Alameda, CA, USA).

Correlation of KI67 gene expression with GI50

Quality controlled raw data (Affymetrix CEL files, SOFT files) from previously published experiments (GSE5846 (ref. 37)) were downloaded from Gene Expression Omnibus datasets (www.ncbi.nlm.nih.gov/geo). KI67 gene expression data in six leukemia cell lines (CCRF-CEM, HL60, K562, MOLT4, RPMI8226, SR) were correlated with the decitabine concentration that produced 50% growth inhibition (GI50) (data from Developmental Therapeutics Program of the NCI (http://dtp.nci.nih.gov/index.html)). Scatter's plots, Spearman and Pearson correlation coefficients were generated using SAS statistical analysis software (SAS Institute Inc., Cary, NC, USA).

Results

Equimolar decitabine or cytarabine in p53 wild-type MLL-AF9 cells

Identical concentrations of decitabine and cytarabine (cytidine analogues metabolized through the same nucleotide pathways) were added to p53 wild-type MLL-AF9 AML cells.[34] Decitabine 0.5 μM depleted DNMT1 (Figure 1a) without causing significant apoptosis (Figure 1b) (annexin staining quantified by flow cytometry 24 h after decitabine or cytarabine treatment). In contrast, an equimolar concentration of cytarabine caused substantial apoptosis (Figure 1b). The decrease in DNMT1 protein in response to cytarabine (Figure 1a) was likely secondary to apoptosis (DNMT1 levels are increased in actively proliferating cells), as cytarabine, unlike decitabine, does not directly bind and deplete DNMT1. Both decitabine and cytarabine 0.5 μM added on day 1 and 4 decreased AML cell proliferation (Figure 1c). Colony formation in methyl-cellulose is an assay for stem and progenitor cell activity. Both decitabine and cytarabine substantially decreased colony formation by p53 wild-type MLL-AF9 cells (Figure 1c). However, at day 4, only decitabine-treated MLL-AF9 cells displayed morphologic changes of monocyte differentiation (increased cell size, decreased nuclear–cytoplasmic ratio, granulation and vacuolization of the cytoplasm) (Figure 1d). Cytarabine-treated cells were small and disrupted, suggesting apoptosis and necrosis (Figure 1d). Both decitabine and cytarabine treatment increased the expression of the monocyte marker CD14 (measured by flow cytometry on day 4), although the increase produced by decitabine was greater ((mean fluorescence intensity decitabine 7.85, cytarabine 6.44, vehicle 3.88), quantified by flow cytometry 96 h after decitabine or cytarabine treatment) (Figure 1e). Neither drug increased the expression of the granulocyte marker CD11b.

Equimolar decitabine or cytarabine in p53-null MLL-AF9 cells

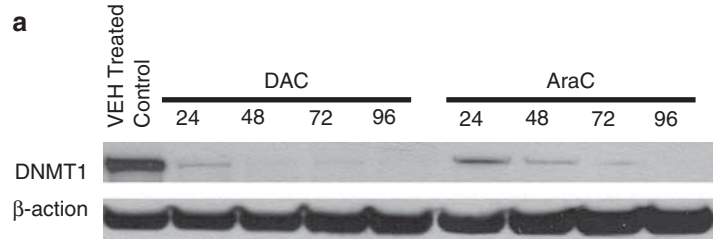
The effects of equimolar decitabine and cytarabine were then compared in p53-null MLL-AF9 AML cells (the THP1 AML cell line[38]). The concentration of decitabine used depleted DNMT1 without causing significant apoptosis (Figures 2a and b). Unlike p53 wild-type MLL-AF9 cells, only decitabine treatment impaired proliferation and decreased colony formation, while cells treated with cytarabine continued to proliferate, after a small initial decrease (Figure 2c). Similar to p53 wild-type cells, only decitabine treatment induced morphologic features of myeloid differentiation (decreased nuclear–cytoplasmic ratio, increased cell size, granulation and vacuolization of the cytoplasm). Cytarabine-treated cells retained immature morphology (Figure 2d). Decitabine treatment markedly increased the expression of the granulocyte marker CD11b, with slight effects on CD14 expression. Cytarabine treatment did not affect these differentiation markers (Figure 2e).

Differential regulation of apoptosis and differentiation protein expression by decitabine and cytarabine

Key events associated with apoptosis and cell cycle exit are known; these include p53 serine-15 phosphorylation, upregulation of p53, p16/CDKN2A and possibly p21/CDKN1A (reviewed in Kim and Sharpless[39]). Myeloid lineage-commitment and differentiation requires lineage-specifying transcription factors such as CEBPα and PU.1 (reviewed in Iwasaki and Akashi[40]). Late myeloid differentiation and cell cycle exit is associated with upregulation of the key late transcription factor CEBPε[41–44] and p27/CDKN1B (p27),[30–33] and possibly p21/CDKN1A and p15/CDKN2B.[45,46] The regulation of these apoptosis and differentiation events by decitabine and cytarabine was examined in p53 wild-type and p53-null AML cells.

In p53 wild-type MLL-AF9 cells, cytarabine and to a lesser extent decitabine increased p53 serine-15 phosphorylation, p53 and p21/CDKN1A levels (Figure 3a). p16/CDKN2A protein was not detected in these cells using two different antibodies (Figure 3a), although p16 mRNA was detected (data not shown). With regard to differentiation events, in p53 wild-type cells, CEBPα protein was decreased by both drugs, whereas no change was detected in PU.1 levels. The most striking change was in CEBPε protein, which peaked at 72 h after decitabine treatment, and p27/CDKN1B protein, which peaked 96 h after decitabine treatment (Figure 3a). Cytarabine had minimal effects on the expression of these key late differentiation proteins (Figure 3a). Despite use of two separate antibodies from different manufacturers, we were unable to detect p15/CDKN2B protein. However, decitabine treatment did increase CDKN2B mRNA by >2-fold measured by quantitative reverse transcription-PCR (data not shown).

Similar to p53 wild-type cells, the most striking change in p53-null THP1 cells was a decitabine-induced increase in CEBPε and p27/CDKN1B protein levels (Figure 3b). Cytarabine did not produce these effects (Figure 3b). p21/CDKN1A levels were also increased by decitabine but not by cytarabine. CEBPα levels decreased with both decitabine and cytarabine, whereas PU.1 levels decreased only in decitabine-treated cells. Neither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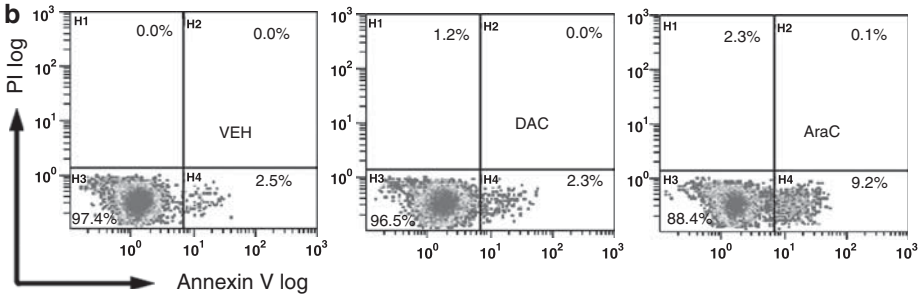
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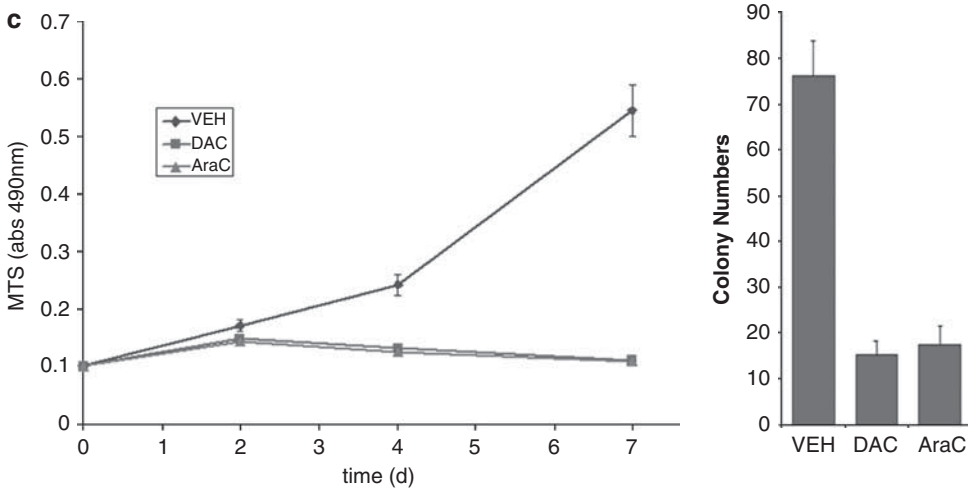
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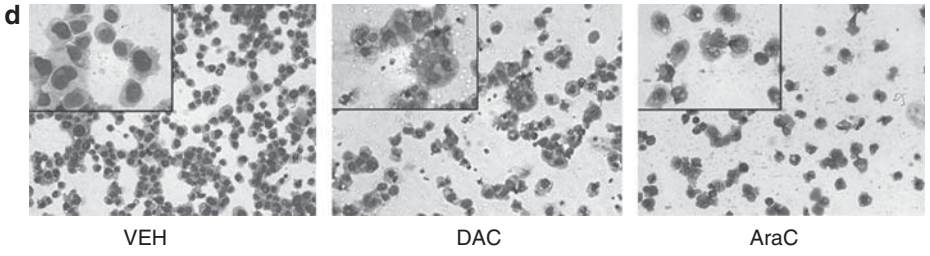
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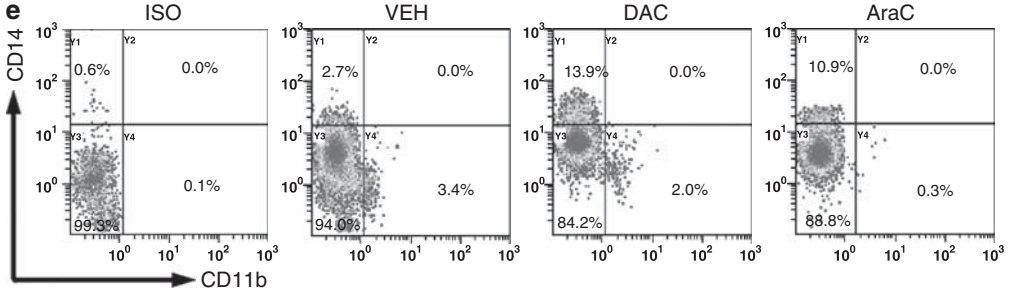
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3.4%
94.0%
13.9%
0.0%
2.0%
84.2%
10.9%
0.0%
0.3%
88.8%

p15 nor p16 mRNA and protein were detected in the THP1 cells (Figure 3b, data not shown).

Differential effect of decitabine on AML leukemia initiating cells and normal HSCs

Engraftment in an immuno-compromised murine host is a functional assay for both normal HSC and leukemia initiating cells.[47,48] Normal CD34+ hematopoietic cells and p53 wild-type MLL-AF9 cells were treated with the identical regimen of decitabine 0.5 μM (day 1 and 5) and decitabine 0.2 μM (day 2 and 6) (the objective was to maximize *in vitro* exposure to decitabine but without reaching concentrations that produce early apoptosis). On day 7, equal numbers (3×10^5 cells each) of viable normal and MLL-AF9 cells were combined and transplanted into sub-lethally irradiated NOD/SCID recipient mice. The mice receiving the combination of mock-treated normal and mock-treated MLL-AF9 cells required euthanasia by week 6 and demonstrated extensive bone marrow engraftment with human leukemia cells (Figures 4a, b and Supplementary Figure S1). Mice receiving the combination of decitabine-treated normal and decitabine-treated MLL-AF9 cells remained healthy and were killed at week 13 (greater than twice the period of survival of the control group) (Figure 4a, Supplementary Figure S1). These mice demonstrated normal human hematopoietic cell engraftment, comparable to that seen in mice receiving 4×10^6 normal human CD34+ cells without leukemia cells. Analysis of their bone marrow showed no morphologic or flow-cytometric evidence of leukemia cell engraftment (Figure 4b, Supplementary Figure S1).

Sensitivity of leukemia cell lines to decitabine inversely correlates with the growth fraction

As decitabine is S phase specific in its mechanism of action, sensitivity to decitabine treatment may relate to the fraction of the leukemia cell population that is proliferating (growth fraction) during treatment exposure (Supplementary Figure S2). KI67 is expressed only in cycling cells, and is used clinically to estimate growth fraction. Consistent with the importance of growth fraction to decitabine effects, in six leukemia cell lines, the concentration of decitabine that produced GI50 inversely correlated with KI67 expression (Supplementary Figure S3).

Better survival with non-cytotoxic decitabine than with cytotoxic cytarabine in murine xenotransplant models of p53-null human AML

As DNMT1 can be depleted at low doses that cause minimal or no measurable cytotoxicity,[14,24,25,49,50] it is feasible to increase and distribute windows of drug exposure in order to capture AML cells entering S phase at different points in time (Supplementary Figure S2). Also, for the objective of DNMT1 depletion with minimal cytotoxicity, subcutaneous (SC) administration could have advantages over IV or intraperitoneal (IP) administration, by producing lower peak levels but longer half-life. Consistent with a non-cytotoxic mechanism of action of decitabine 0.2 mg/kg administered SC 1–2 ×/week for 8 weeks to non-transplanted NSG mice ($n=4$), there was no treatment associated cytopenia (Supplementary Figure S4A), and DNMT1 was depleted without an increased phosphatidylserine exposure in bone marrow cells (Supplementary Figure S4B and C), although a small increase in γH2AX (DNA repair marker) occurred (Supplementary Figure S4D).

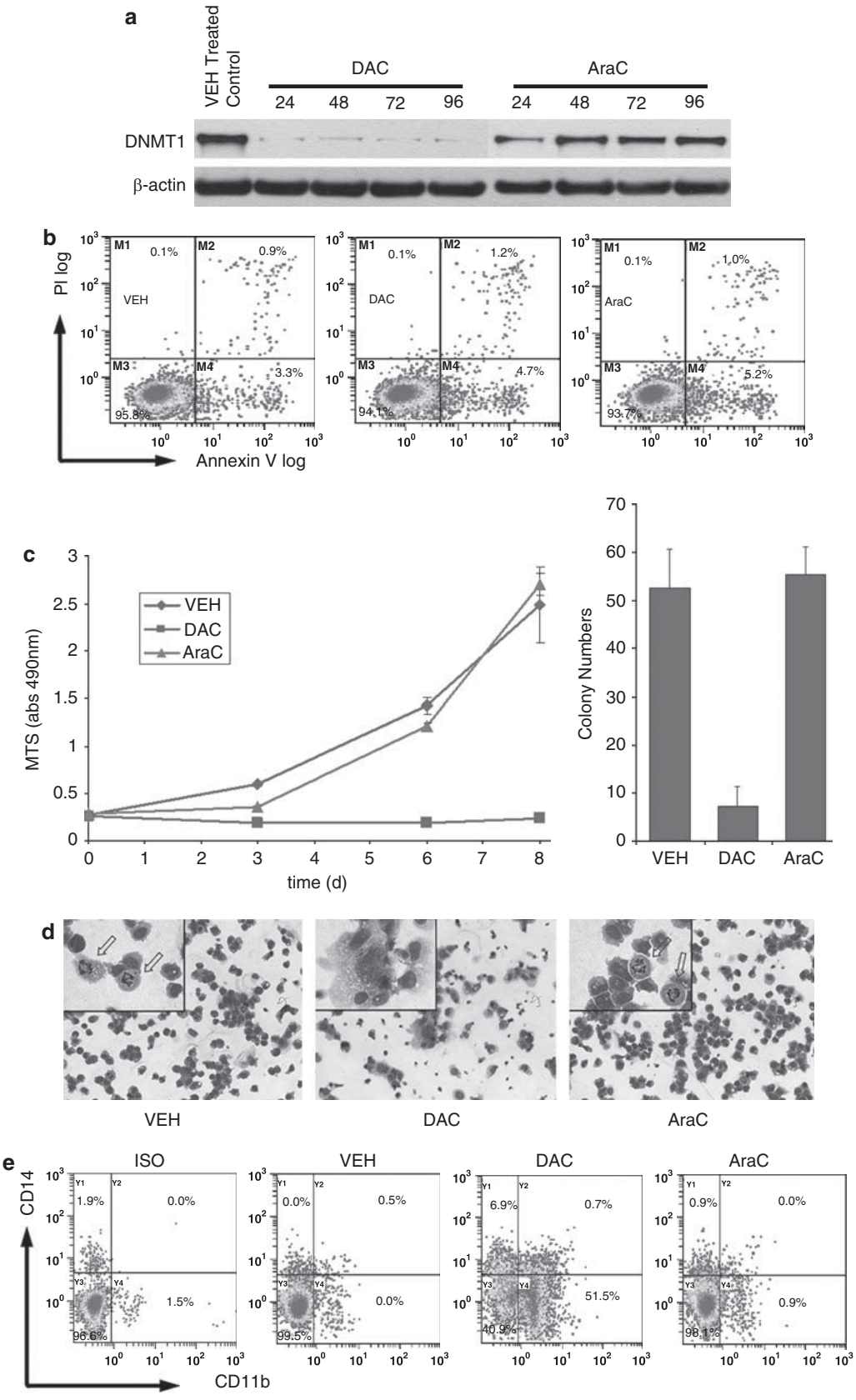
Weekly SC decitabine and conventional cytotoxic cytarabine were then compared in a xenotransplant model of p53-null human MLL-AF9 AML. Non-irradiated NSG mice were transplanted with 3×10^6 THP1 cells by tail vein injection. Starting at day 5 after transplant, mice were treated with vehicle (phosphate buffered saline), cytarabine 75 mg/kg per day IP for 5 consecutive days (to model conventional chemotherapy[51]), or decitabine 0.2 mg/kg SC 3 ×/week for 2 weeks then 2 ×/week for 2 weeks then 1 ×/week thereafter. Mice treated with decitabine had significantly longer median survival (>20% increase) than cytarabine and vehicle-treated mice (median survival 51, 45 and 42 days, respectively, log-rank $P=0.0004$) (Figure 5a). *In vivo* luminescence imaging on day 28 of therapy demonstrated disseminated disease in vehicle-treated mice and disease concentrated in the region of the liver in decitabine and cytarabine-treated mice (Figure 5b). This pattern was confirmed at the time of euthanasia: vehicle-treated mice demonstrated disseminated tumor masses, but disease was concentrated in the liver in cytarabine and decitabine-treated mice (Figure 5c) (the liver, and to a lesser extent the spleen, express high levels of cytidine deaminase, the enzyme that rapidly metabolizes cytidine analogues). Similar results were seen when cytarabine was administered at the same low dose, route of administration and schedule as the decitabine (0.2 mg/kg SC 2 ×/week). Again, mice treated with decitabine had significantly better survival (median survival 48, 39 and 36 days, respectively, log-rank $P=0.0096$) (Supplementary Figure S5A), and significantly lower extra-hepatic tumor burden, than cytarabine or vehicle-treated mice (Supplementary Figure S5B). However, liver and spleen size and weight were significantly greater in decitabine compared with cytarabine and vehicle-treated mice (organs and tumors were harvested at later time points in decitabine-treated mice as indicated by the Kaplan–Meier curves) (Supplementary Figure S5C and D).

Better survival with non-cytotoxic decitabine than with cytotoxic cytarabine in a murine xenotransplant model of refractory/relapsed human AML

To complement the above experiments in which AML cell lines were used, a xenotransplant model was established using fresh AML cells from a patient with relapsed/refractory AML. These AML cells contained multiple chromosome abnormalities including a t(8;18)(q22:q23) and t(11;13)(q21:q12). Non-irradiated NSG mice were transplanted with 1×10^6 patient cells by tail vein injection. Starting at day 5 after transplant, mice were treated with vehicle (phosphate buffered saline), cytarabine 75 mg/kg per day IP for 5 consecutive days,[51] or

Figure 1 Non-cytotoxic concentrations of decitabine (DAC) induce differentiation and terminate proliferation of p53 wild-type MLL-AF9 cells. (**a**) DNMT1 protein levels in DAC and cytarabine (AraC)-treated cells. DNMT1 measured by western blot at different time points. DAC or AraC 0.5 μM added at 0 and 72 h. (**b**) DAC 0.5 μM did not cause significant early apoptosis. Annexin staining measured by flow cytometry 24 h after DAC treatment. Positive control AraC treatment. (**c**) DAC and AraC treatment were anti-proliferative. Cell quantity estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) assay. at day 2, 4 and 7. DAC or AraC 0.5 μM added to cells on day 1 and 4. Cells were plated into methylcellulose (2000 viable cells/ml) on day 5, to assay for stem/progenitor activity. (**d**) DAC-induced morphologic changes of monocytic differentiation (decreased nuclear–cytoplasmic ratio, increased cell size, granulation and vacuolization of the cytoplasm). AraC-treated cells were small and disrupted, suggesting apoptosis and necrosis. Day 4 Giemsa stained cytospins. (**e**) DAC and AraC treatment increased the expression of CD14. CD14 (monocyte marker) and CD11b (granulocytic marker) measured by flow cytometry on day 4. ISO, isotype control.

decitabine 0.2 mg/kg SC 3 ×/week for 2 weeks, then 2 ×/week for 2 weeks, then 1 ×/week thereafter. Mice treated with decitabine had significantly longer median survival (>100% increase) than cytarabine or vehicle-treated mice (median survival 113, 56 and 50 days, respectively, log-rank $P<0.0001$) (Figure 6a). At euthanasia (at different time-points



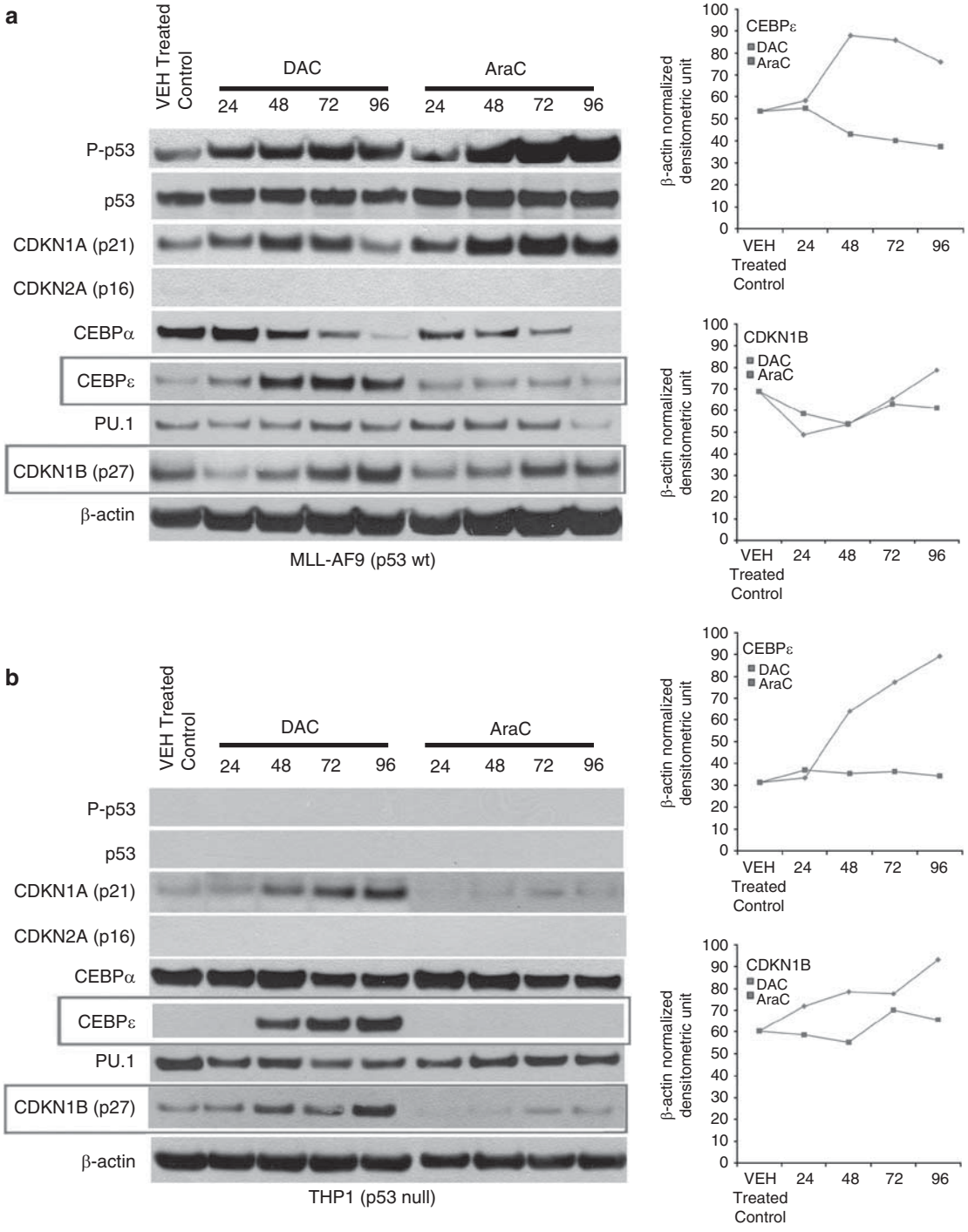


Figure 3 Decitabine (DAC) and cytarabine (AraC) differentially regulate apoptosis and differentiation of proteins in p53 wild-type and p53-null MLL-AF9 cells. Key events associated with apoptosis and differentiation-mediated cell cycle exit were examined in p53 wild-type and p53-null MLL-AF9 cells treated with DAC or AraC 0.5 μM on day 1 and 4. (**a**) Western blots of p53 wild-type MLL-AF9 cells treated with DAC or AraC. Numbers, hours after initiation of treatment. Boxes, protein upregulated by DAC but not by AraC. Graphs depict results of densitometry analysis. (**b**) Western blots of p53-null MLL-AF9 cells (THP1 cells) treated with DAC or AraC. The color reproduction of this figure is available on the *Leukemia* Journal online.

Figure 2 Decitabine (DAC), but not an identical regimen of cytarabine (AraC), terminated proliferation of p53-null MLL-AF9 cells (THP1 cells[38]). (**a**) DAC 0.5 μM depleted DNMT1. DNMT1 measured by western blot. (**b**) Neither DAC nor AraC 0.5 μM caused early apoptosis. Annexin staining measured by flow cytometry 24 h after DAC or AraC treatment. (**c**) DAC, but not AraC treatment, was anti-proliferative. Cell quantity estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) assay at day 1, 3, 6 and 8. DAC or AraC 0.5 μM added to cells on day 1 and 4. Cells were plated into methylcellulose (2000 viable cells/ml) on day 5, to assay for stem/progenitor activity. (**d**) DAC-induced morphologic changes of myeloid differentiation (decreased nuclear–cytoplasmic ratio, increased cell size, granulation and vacuolization of the cytoplasm). Arrows, mitotic cells. Day 4 Giemsa stained cytospins. (**e**) DAC, but not AraC, increased the expression of CD11b, and to a lesser extent CD14. CD11b and CD14 measured by flow cytometry on day 4. VEH, vehicle. ISO, isotype control.

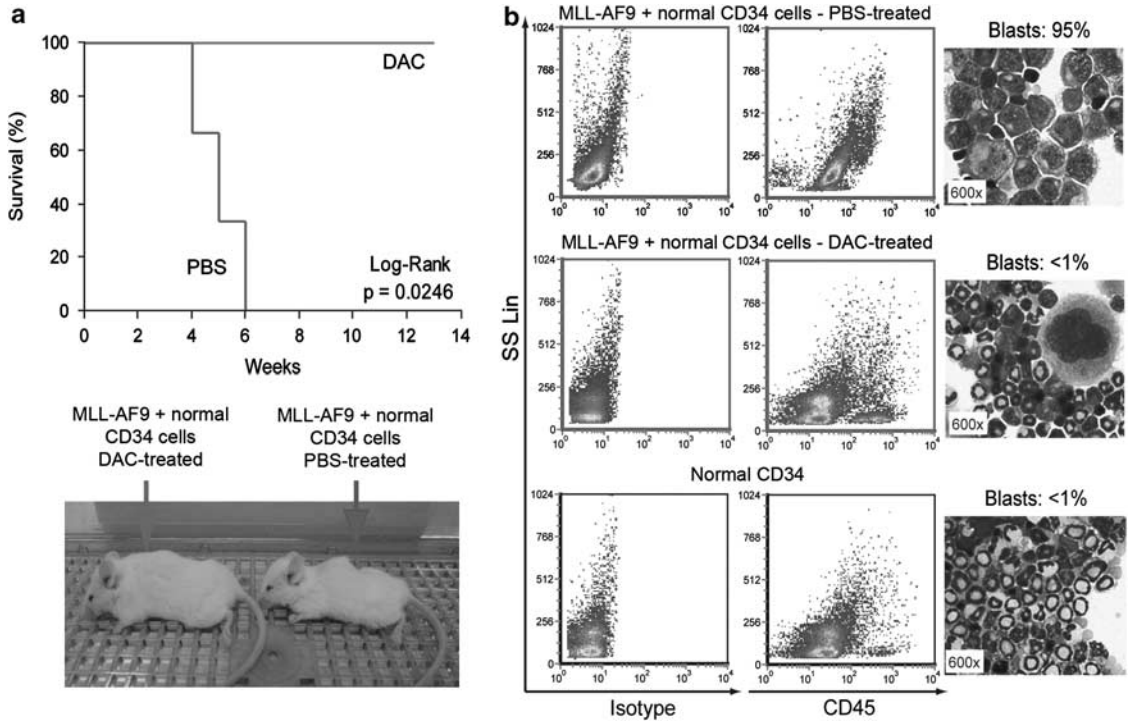


Figure 4 Low concentration decitabine (DAC) inhibits AML leukemia initiating cells (LIC) but spared normal HSC. Engraftment in an immunocompromised murine host is a functional assay for both normal HSC and LIC.[47,48] Normal CD34+ HSC and MLL-AF9 leukemia cells were treated *in vitro* with the identical DAC regimen (0.5 μM on day 1, 0.2 μM on day 2, 0.5 μM on day 5, 0.2 μM on day 6) or mock treated with PBS (phosphate buffered saline). Cells harvested on day 7 were combined (3×10^5 each MLL-AF9 + normal cells), then transplanted by tail vein injection into sublethally irradiated NOD/SCID recipient mice ($n=6$). Additional controls: mice transplanted with PBS-treated normal CD34+ cells alone (4×10^6 cells/mouse). (**a**) Significant survival difference between groups. Surviving mice did not demonstrate distress but were killed for analysis at week 13. (**b**) Engraftment of >90% AML cells in bone marrow of mice receiving PBS-treated cells; ~80% normal, multi-lineage, human hematopoietic cell engraftment in mice receiving DAC-treated cells. Additional engraftment data and controls in Supplementary Figure S1. Cytospin preparations of cells flushed from bone marrow were stained with Giemsa. Measurement of blast percentage in cytospins was blinded to treatment status.

per the Kaplan–Meier plot), the bone marrow of all mice was replaced by human leukemia cells (Figure 6b, Supplementary Figure S6A and B). Despite the later time point of harvest, spleens of decitabine-treated mice were significantly decreased in size compared with cytarabine or vehicle-treated mice (Figure 6c, Supplementary Figure S6C). Unlike the THP1 cells, these primary AML cells did not demonstrate liver tropism (livers were not increased in size or weight at death).

Discussion

Both *in vitro* and *in vivo*, a DNMT1 depleting, but non-cytotoxic, dose and schedule of decitabine was nonetheless able to induce cell cycle exit in AML cells. The absence of early apoptosis or phosphorylation of p53, efficacy in p53- and p16/CDKN2A-null backgrounds, the major increase in CEBPε and p27/CDKN1B, increase in the myeloid membrane differentiation markers CD11b or CD14, and cellular differentiation, were consistent with p53-independent differentiation-mediated cell cycle exit. Hence, this approach to treatment could provide a useful alternative or complement to conventional apoptosis-based therapy. The efficacy of this treatment *in vivo* against cytarabine-resistant p53-null cells and primary cells from a patient with refractory/relapsed AML, supports the translational possibilities.

Why does DNMT1 depletion, or histone deacetylase inhibition, induce differentiation of AML cells? One insight comes from experiments with normal HSC. In normal HSC, DNMT1 depletion by shRNA or by decitabine maintains stem cell phenotype even in differentiation promoting conditions, by preventing repression of key stem cell genes by the differentiation stimuli.[26–29,52] However, after the repression of stem cell genes that occurs with lineage-commitment, decitabine can augment the expression of late differentiation genes and accelerate differentiation instead.[52] Therefore, baseline differentiation stage is a major determinant of the cell fate response to decitabine treatment. The earliest studies suggested that AML cells with leukemia initiating capacity (measured by xenotransplantation) had a surface phenotype resembling that of normal HSC (CD34+38−).[48,53] This suggested that AML cell populations might recapitulate the hierarchical structure of normal hematopoiesis, with a small subset of AML cells with a stem cell phenotype sustaining the bulk cell population.[53] Recently, it has been reported that the antibodies used to sort CD38+ cells can inhibit proliferation and might have technically influenced the earliest studies.[54] Accordingly, in a number of other studies, AML initiating cells had a surface phenotype suggesting lineage-commitment (CD34+38+, CLL-1+, CD71+, CD90−, c-Kit−).[54–60] Cross-species barriers also impact leukemia or cancer initiating capacity.[61] With use of more immunocompromised mice, or mice that express human cytokines, AML initiating cells have surface features of lineage-commitment.[61–63] Differentiation absolutely requires and is driven by lineage-specifying transcription factors such as CEBPA. AML cells, including CD34+ and CD34+38− subsets, express high

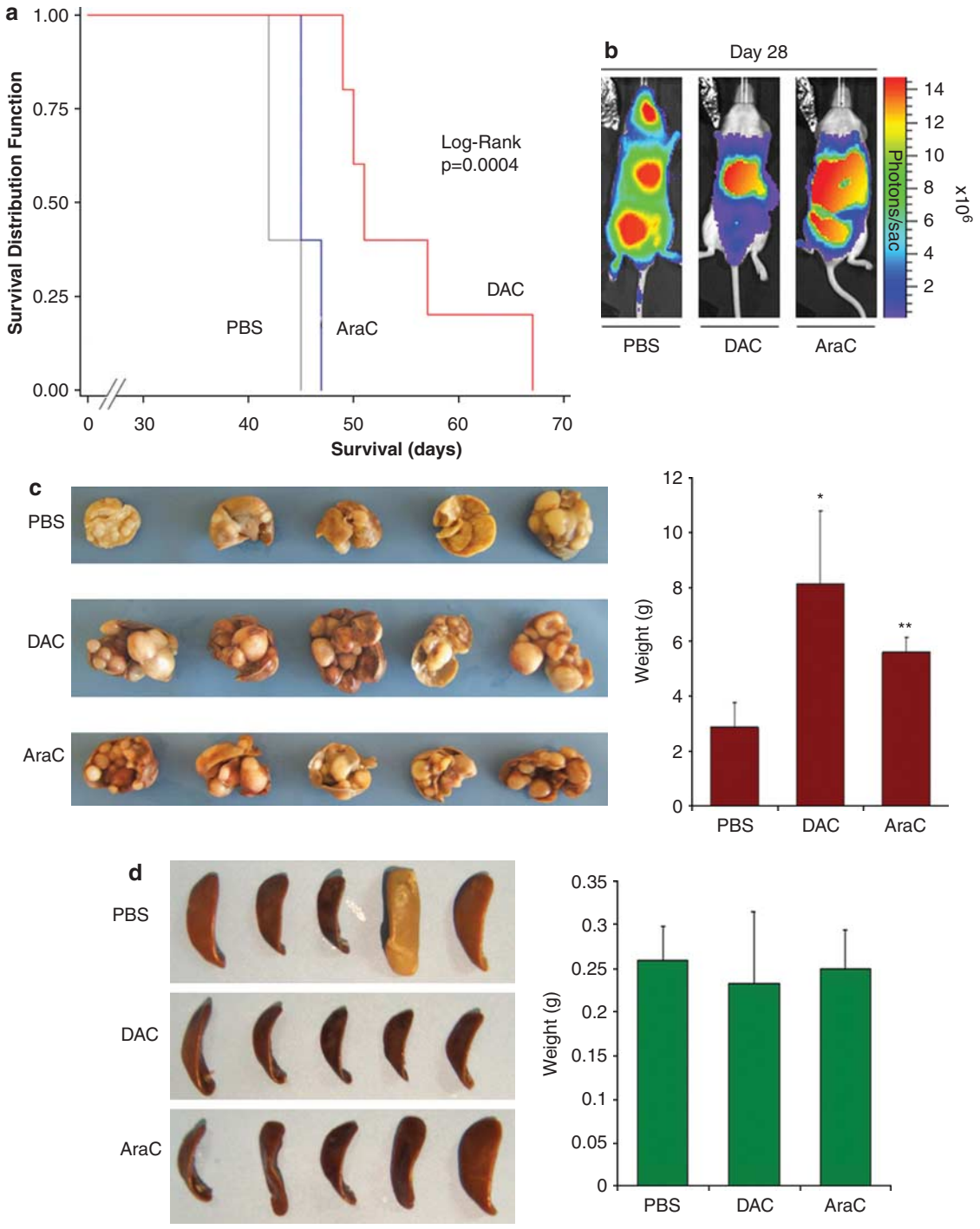


Figure 5 Better survival with non-cytotoxic decitabine (DAC) than with cytotoxic cytarabine (AraC) in a murine xenotransplantation model of p53-null human AML. (**a**) Kaplan–Meier plot of survival distribution function. *x* axis: days after xenotransplant of AML cells. Non-irradiated NSG mice were transplanted with 3×10^6 THP1 cells (p53-null MLL-AF9 AML cells) by tail vein injection. At the starting day 5 after transplantation, mice were treated with vehicle (phosphate buffered saline (PBS), $n=5$), AraC 75 mg/kg per day IP for 5 days ($n=5$) (to model conventional chemotherapy[51]), or DAC 0.2 mg/kg SC 3 × /week for 2 weeks then 1 × /week ($n=5$). (**b**) Bioluminescent imaging to show anatomic localization of engrafted THP1 cells. Images from dorsal perspective obtained 10 min after IP administration of luciferin substrate, on day 28 after the transplant of 3×10^6 THP1 cells transfected to express luciferase. PBS, AraC and DAC treatment started on day 3. Red, most intense; blue, least intense bioluminescence/engraftment. (**c**) Liver focus of disease in AraC and DAC-treated mice confirmed at death. *$P<0.05$ compared with PBS-treated control. **$P<0.005$ compared with PBS-treated control. (**d**) Spleens of DAC-treated mice were decreased in size compared with vehicle-treated mice, but not to a statistically significant extent (organs harvested at different time-points per the Kaplan–Meier plot).

levels of CEBPA, but relatively low levels of the key late differentiation driver CEBPE (submitted manuscript). Similarly, we have noted that the promoter CpG methylation profile of MDS and AML cells is consistent with partial differentiation. It could be the partial differentiation of AML cells at baseline, suggested by surface phenotype, lineage-specifying transcription

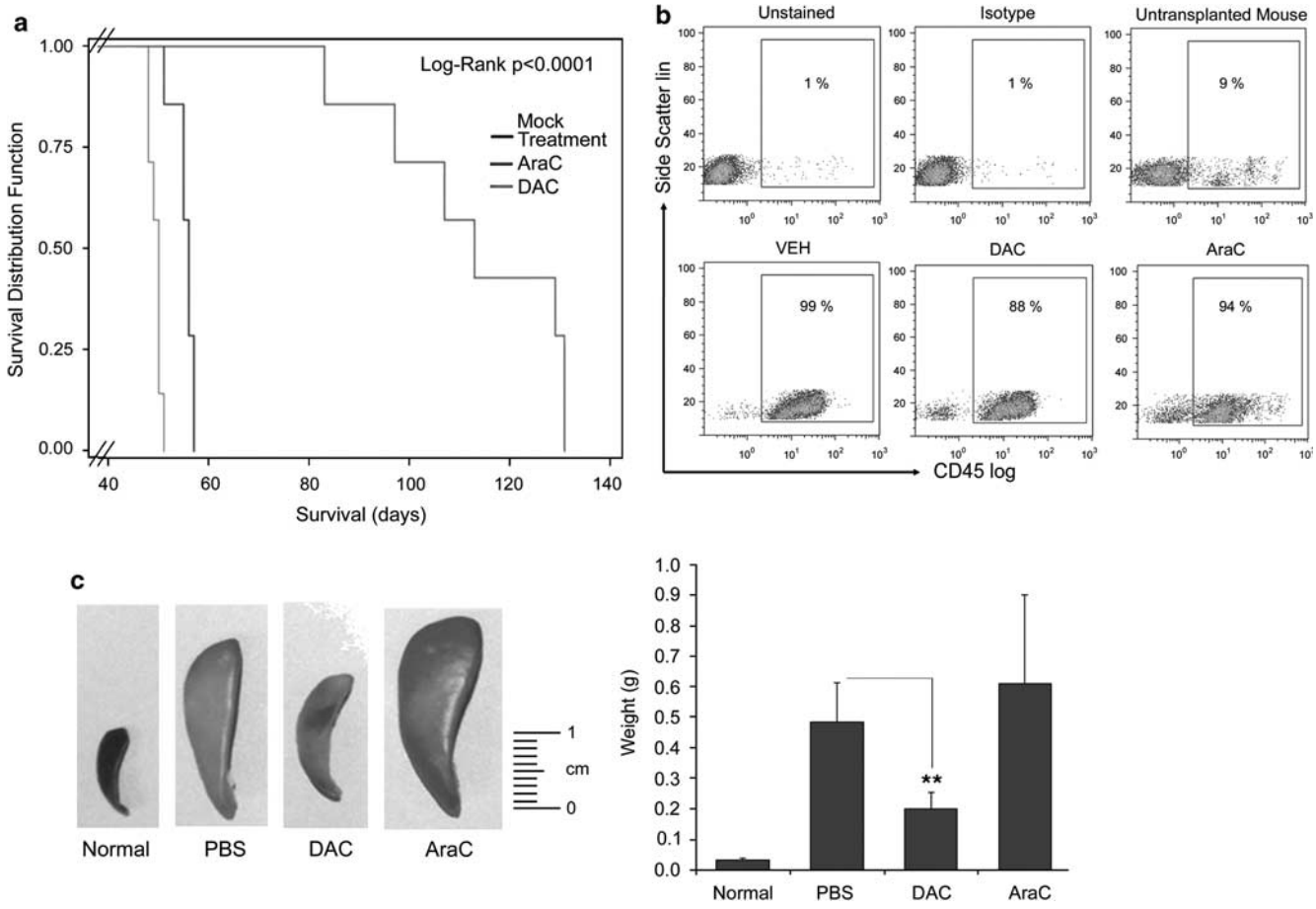


Figure 6 Better survival with non-cytotoxic decitabine (DAC) than cytotoxic cytarabine (AraC) in a murine xenotransplantation model of refractory/relapsed human AML. Fresh AML cells from a patient with relapsed, chemotherapy treatment refractory AML were transplanted by tail vein injection (1×10^6 cells/mouse) into NSG mice. These AML cells contained multiple chromosome abnormalities including t(8;18)(q22:q23) and t(11;13)(q21:q12). Starting day 5 after transplantation, mice were treated with vehicle (control) ($n=7$), AraC 75 mg/kg per day IP for 5 days ($n=7$), or DAC 0.2 mg/kg SC $3\times$/week for 2 weeks then $1\times$/week ($n=7$). (**a**) Better survival in DAC-treated mice. Mice were killed for signs of distress. (**b**) Spleens of DAC-treated mice were significantly decreased in size compared with AraC or vehicle-treated mice. $^{**}P<0.005$ compared with phosphate buffered saline (PBS)-treated control. Normal, non-transplanted, non-treated mouse. Spleens were harvested at later time-points in DAC-treated mice as per the Kaplan–Meier plot. (**c**) Bone marrow replacement by human leukemia cells. Human CD45 staining was used to identify human cells in bone marrow after death at different time-points per the Kaplan–Meier plot.

factor expression and promoter CpG methylation patterns, contributes to the contrasting differentiation responses of normal HSC and AML cells to non-cytotoxic DNMT1 depletion.

Although this treatment can bypass the p53-dependence of conventional cytotoxic treatment, it is still limited by the pharmacologic properties of decitabine. The S phase specific mechanism of action of decitabine was underlined by the correlation of AML cell line sensitivity with KI67 expression (a measure of growth fraction). Therefore, scheduling and duration of exposure is a critical determinant of treatment efficacy. However, decitabine is rapidly metabolized by ubiquitously expressed cytidine deaminase (rapid metabolism by cytidine deaminase, which is highly expressed in the liver, could explain why the liver was a sanctuary site for liver tropic THP1 cells from the effects of cytarabine and decitabine). Hence, the *in vivo* half-life of decitabine after IV push is in the order of minutes, compared with many hours *in vitro*.[35,64] An obvious mechanism for treatment failure therefore is that some or many AML cells may complete S phase while decitabine is absent *in vivo*. As decitabine was originally developed to induce apoptosis in malignant cells,[65] doses to treat AML were escalated to maximum tolerated levels (up to 80 mg/kg infused over 36–44 h), requiring many subsequent weeks without treatment to allow for recovery from cytotoxic side-effects.[64] A role for differentiation in decitabine-induced cell cycle exit of leukemia cells does not exclude a role for activation of apoptosis genes, anti-metabolite effects and apoptotic cell cycle exit: these could be the dominant consequences of treatment in some malignant cells, and certainly with higher dose.[19,50,66–68] However, a decrease in the dose (to 15 mg/m^2 infused over 3 h $3\times$/day on day 1–3, repeated every 6 weeks) led to United States Food and Drug Administration approval of decitabine as a treatment for MDS.[69] A further decrease in the daily dose and administration more frequently (20 mg/m^2 infused over 1 h $1\times$/day on day 1–5, repeated every 28 days, has further improved MDS treatment clinical results.[69,70] The results here provide a biological rationale to continue this trend towards smaller dose and more frequent administration: very low drug levels are sufficient to deplete DNMT1, and the decrease in toxicity can be used to administer treatment 1–3 $\times$/week for months or years, to capture AML cells entering S phase asynchronously and decrease treatment-free intervals that allow unimpeded AML cell division (Supplementary Figure S2). Clinical trials in the hemoglobinopathies have shown that

decitabine 0.2 mg/kg ($\sim$7.5 mg/m^2) administered SC has non-cytotoxic DNA hypomethylating and differentiation modifying effects, and can be administered safely from 1–3 $\times$ /week.[25]

These *in vitro* and *in vivo* results provide a rationale for adjusting *in vivo* dose, schedule and route of administration of decitabine to emphasize a non-cytotoxic, normal HSC sparing, p53-independent mechanism of action. Although pharmacologic barriers to optimal clinical translation remain, these can potentially be addressed through further pre-clinical and clinical investigation.

Conflict of interest

The authors declare no conflict of interest.

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