TITLE: p53 independent epigenetic-differentiation treatment in xenotransplant models of acute myeloid leukemia

Running title: p53 independent treatment for AML

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ABSTRACT

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 cytosine analogue decitabine that deplete the chromatin modifying enzyme DNA methyl-transferase 1 (DNMT1) without phosphorylating p53 or inducing early apoptosis were determined. These decitabine regimens but not equimolar DNA-damaging cytarabine up regulated the key late differentiation factors CEBPε and p27/CDKN1B, induced cellular differentiation, and terminated AML cell-cycle, even in cytarabine-resistant p53- and p16/CDKN2A-null AML cells. Leukemia initiation by xeno-transplanted AML cells was abrogated but normal hematopoietic stem cell (HSC) 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 xeno-transplant models of p53-null and relapsed/refractory AML, the non-cytotoxic regimen significantly extended survival compared to conventional cytotoxic cytarabine. Modifying i*n vivo* dose and schedule to emphasize this pathway of decitabine action can bypass a mechanism of resistance to standard therapy.

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 (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 (5): in blast transformed myeloproliferative disease, which responds only transiently if at all to conventional therapy, 45.5% of cases had either *TP53* defects or gain of *MDM4* (6). In MDS and AML cases with complex cytogenetic abnormalities, another group of patients with very poor treatment outcomes, the rate of *TP53* mutation can exceed 70% (7).

Hence, especially for certain sub-types 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 (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. 5-azacytidine and decitabine are cytosine analogues, and at high concentrations, can cause DNA damage that triggers apoptosis. It is also possible that the chromatin-modifying effects of these drugs renew expression of tumor suppressor genes that mediate apoptosis. Hence, it has not been clear that differentiation-mediated cell cycle exit is the most important therapeutic action of these compounds (reviewed in (19)). There has even been debate about whether inhibition of chromatin-modifying enzymes is the most important effect of these drugs (19). Therefore, if such treatment is to bypass a major limitation of conventional therapy, demonstration of a p53-independent mechanism of action, then rationalization of dose and schedule to emphasize this pathway of action, and demonstration of *in vivo* efficacy, are important translational challenges.

Unlike the cytosine analogues cytarabine or gemcitabine, the sugar back-bone of decitabine is unmodified. Therefore, at low concentrations, decitabine can incorporate into the newly synthesized DNA strand during S-phase without terminating chain elongation (20;21). These non-DNA damaging, non-cytotoxic concentrations

of decitabine can nonetheless deplete DNMT1, both *in vitro* and *in vivo* (12;20-24). Our first objective was to determine if these concentrations of decitabine, which do not kill normal hematopoietic stem cells (HSC) (low concentrations of decitabine have increased normal hematopoietic stem cell self-renewal in a number of studies (25-28)), 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 (29-32). Therefore, our 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. *In vitro* experiments were conducted in p53 wild-type and p53-null MLL-AF9 AML cells. The effects of treatment 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, we examined if a decitabine dose, schedule and route of administration rationalized for a non-cytotoxic mechanism of action *in vivo*, could be efficacious in murine xenotransplantation models of p53-null and relapsed/refractory human AML, to evaluate the translational potential of this alternative, non-cytotoxic treatment approach.

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 was 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) 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 were analyzed – **(i)** p53 wild-type MLL-AF9 cells were generated as described (33). These cells have a gene-expression profile similar to primary human MLL-AF9 leukemia cells (33), and self-renew indefinitely both in vitro and in vivo, initiating invasive AML in transplanted mice (33). **(ii)** p53-null THP1 cells were purchased from ATCC (Manassas, VA). This morphologically monocytoid AML cell line (M5 morphology) contains an MLL-AF9 fusion and is homozygously mutated at the *TP53* and *CDKN2A* loci. THP1 cells used for xeno-transplantation were transfected to express luciferase. **(iii)** Primary (fresh) AML cells from a patient with relapsed/refractory AML. These cells had a myelomonocytic morphology (M4) and by standard metaphase karyotyping 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 10ng/ml of the following human cytokines: stem cell factor (SCF), FLT3 ligand (FLT3), thrombopoietin (TPO), interleukin-3 (IL-3) and interleukin-6 (IL-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 ^{0}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.

Apoptosis detection: Apoptosis was detected by Annexin-V and 7AAD or PI co-staining using the APOAF commercial kit (Sigma).

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 treated and untreated control cultures were plated in decitabine free-semisolid media (MethoCult H4434, Stem Cell Technology, 2000 cells/ml). Colony-forming units (CFU) were identified by morphology and counted under an inverted microscope at 10 days post-plating.

SDS-PAGE and Western Blotting: Approximately 100 μg of cytoplasmic and nuclear protein extracts from cells, together with molecular weight markers, were subjected to SDS-PAGE on 4-12% gradient gels (Invitrogen) followed by transfer to PVDF membranes (Invitrogen). Blots were probed using antibodies for DNMT1 (Abcam ab16632), Phospho-p53 (Cell Signaling #9286), p53 (Sigma P6874), p15 (Cell Signaling #4822), p21 (Cell Signaling #2946), p16 (Santa Cruz Biotech sc-81613 ; Cell Signaling #4824), 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 injection into non-irradiated 6-8 week old NOD/SCID or NSG mice. Mice were anesthetized with isofluorane before transplantation. 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) with isotype-matched immunoglobulin as a control in all experiments. Livers and spleens were weighed and fixed. To anatomically localize THP-1cells in living mice, the substrate D-Luciferin (15 mg/ml D-luciferin in sterile PBS (Promega), was injected IP and mice were imaged after 10 minutes mice using an IVIS-200 CCD camera imaging system (Xenogen, Alameda,CA).

Correlation of KI67 gene expression with GI50: Quality controlled raw data (Affymetrix CEL files, SOFT files) from previously published experiments (GSE5846 (34)) were downloaded from Gene Expression Omnibus (GEO) datasets (www.ncbi.nlm.nih.gov/geo). KI67 gene expression data in 6 leukemia cell lines (CCRF-CEM, HL60, K562, MOLT4, RPMI8226, SR) was 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 plots, Spearman and Pearson correlation coefficients were generated using SAS statistical analysis software.

RESULTS

Equimolar decitabine or cytarabine in p53 wild-type MLL-AF9 cells. Identical concentrations of decitabine and cytarabine (cytosine analogues metabolized through the same nucleotide pathways) were added to p53 wild-type MLL-AF9 AML cells (33). Decitabine 0.5 μM depleted DNMT1 (**figure 1A**) without causing significant apoptosis (**figure 1B**) (annexin staining quantified by flow-cytometry 24 hours after decitabine or cytarabine treatment). In contrast, an equimolar concentration of cytarabine caused substantial apoptosis (**figure 1B**). Both decitabine and cytarabine 0.5 μM added on days 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 expression of the monocyte marker CD14 (measured by flow-cytometry on day 4), although the increase produced by decitabine was greater (median fluorescence intensity 6.02 versus 4.09), quantified by flow-cytometry 96 hours after decitabine or cytarabine treatment) (**figure 1E**). Neither drug increased expression of the granulocyte marker CD11b.

Differential effect on AML leukemia initiating cells and normal hematopoietic stem cells. Engraftment in an immuno-compromised murine host is a functional assay for both normal HSC and leukemia initiating cells (35;36). Normal CD34+ hematopoietic cells and p53 wild-type MLL-AF9 cells were treated with the identical regimen of decitabine 0.5 μM (days 1 and day 5) and decitabine 0.2 μM (days 2 and day 6) (the objective was to maximize *in vitro* exposure to decitabine but without reaching concentrations that produce measurable DNA damage). 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 (**figure 2A, B, figure S1**). Mice receiving the combination of decitabine-treated normal and decitabine-treated MLL-AF9 cells remained healthy and were sacrificed at week 13 (greater than twice the period of survival of the control group) (**figure 2A, figure S1**). These mice demonstrated normal human hematopoietic cell engraftment, comparable to that seen in mice receiving $4x10^6$ normal human CD34+ cells without leukemia cells. Analysis of their BM showed no morphologic or flow-cytometric evidence of leukemia cell engraftment (**figure 2B, figure S1**).

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 (37)). The concentration of decitabine used depleted DNMT1 without causing significant apoptosis (**Figure 3A, 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 (**figure 3C**). 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 3D**). Decitabine treatment markedly increased expression of the granulocyte marker CD11b, with slight effects on CD14 expression. Cytarabine treatment did not affect these differentiation markers (**figure 3E**).

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, and possibly upregulation of cyclin-dependent kinase inhibitor 1A (p21/CDKN1A) and p16/CDKN2A (reviewed in (38)). Myeloid lineage-commitment and differentiation requires lineage-specifying transcription factors such as CEBPα and PU.1 (reviewed in (39)). Late myeloid differentiation and cell cycle exit is associated with upregulation of the key late transcription factor CEBPε (40-43) and p27/CDKN1B (p27) (29-32), and possibly upregulation of p15/CDKN2B. 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 4A**). p16/CDKN2A protein was not detected in these cells using two different antibodies (**figure 4A**), 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 hours after decitabine treatment, and p27/CDKN1B protein, which peaked 96 hours after decitabine treatment (**figure 4A**). Cytarabine had minimal effects on the expression of these key late differentiation proteins (**figure 4A**). Despite use of two separate antibodies from different manufacturers, we were unable to detect p15/CDKN2B protein. However, decitabine treatment did increase CDKN2B mRNA >2-fold measured by QRT-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 4B**). Cytarabine did not produce these effects (**figure 4B**). 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 p15 nor p16 mRNA nor protein were detected in the THP1 cells (**figure 4B**, data not shown).

Sensitivity of leukemia cell lines to decitabine inversely correlates with the proliferative index. The proliferation index of leukemia cells may predict sensitivity to decitabine, since decitabine is S-phase specific in its mechanism of action. In 6 leukemia cell lines, the concentration of decitabine that produced 50% growth

inhibition (GI50) inversely correlated with the expression of KI67 (a proliferation marker expressed only in cycling cells; KI67 expression is widely used in clinical pathology as an index of malignant cell proliferation) (GI50 data from the Developmental Therapeutics Program of the NCI [http://dtp.nci.nih.gov/index.html]; gene expression data from GEO Datasets GSE5846 (34)) (**figure S2**).

Better survival with non-cytotoxic decitabine than with cytotoxic cytarabine in murine xeno-transplant models of p53-null human AML. The correlation between sensitivity of AML cells to decitabine and the proliferation index emphasizes the importance of optimizing scheduling and administration, to increase and distribute the windows of drug exposure and capture AML cells entering S-phase at different points in time (**figure S3**). Since the objective is not high peak levels of drug to cause DNA damage and cytotoxicity, sub-cutaneous (SC) administration could have advantages over intra-peritoneal (IP) or intravenous administration by producing lower peak levels but longer half-life. Since such doses are non-cytotoxic (24), it is feasible to administer drug more frequently, to create more windows of drug exposure (**figure S3**). To confirm that 0.2 mg/kg administered SC weekly depletes DNMT1 without causing cytotoxicity or severe cytopenia *in vivo*, non-transplanted NSG mice (n=4) were treated 1-2X/week for 8 weeks. There was no treatment associated cytopenia (**figure S4A**). DNMT1 was substantially depleted in bone marrow cells analyzed at sacrifice with no measurable increase in apoptosis and a small increase in phospho-H2AX levels (**figure S4B-D**).

Weekly SC decitabine was then used to treat a xeno-transplant model of p53-null human MLL-AF9 AML. Non-irradiated NSG mice were transplanted with $3x10^6$ THP1 cells by tail vein injection. Starting at day 5 after transplant, mice were treated with vehicle (PBS), cytarabine 75 mg/kg/day IP for five consecutive days (to model conventional chemotherapy (44)), or decitabine 0.2 mg/kg SC 3X/week for 2 weeks then 2X/week for 2 weeks then 1X/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 but disease concentrated in the region of the liver in decitabine and cytarabine mice (**figure 5B**). This pattern was recapitulated at sacrifice, when vehicle treated mice demonstrated disseminated tumor masses in the thoracic and abdominal cavities and subcutaneously, but disease was concentrated in the livers of cytarabine and decitabine treated mice, with large numbers of leukocytic nodules (**figure 5C**). Spleen weights and sizes (obtained at different time-points of euthanasia as per the Kaplan-Meir plot) were comparable between the different treatment groups, although decreased to a non-statistically significant extent in decitabine treated mice (**figure 5D**). The liver expresses high levels of cytidine deaminase, the enzyme that rapidly metabolizes cytosine analogues, which could explain liver sanctuary for liver tropic THP1 cells from the effects of cytarabine and decitabine.

Better survival with non-cytotoxic decitabine than with cytotoxic cytarabine in a murine xeno-transplant model of refractory/relapsed human AML. To complement the above experiment in which an

AML cell line was used, a xeno-transplant 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 $1x10^6$ patient cells by tail vein injection. Starting at day 5 after transplant, mice were treated with vehicle (PBS), cytarabine 75 mg/kg/day IP for five consecutive days (44), or decitabine 0.2 mg/kg SC 3X/week for 2 weeks then 2X/week for 2 weeks then 1X/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 corresponding to the Kaplan-Meir plot), the bone marrow of all mice was replaced by human leukemia cells (**figure 6B, figure S5A, B**); spleens of decitabine treated mice were significantly decreased in size compared to cytarabine or vehicle treated mice (**figure 6C, figure S5C**). Unlike the THP1 cells, these AML cells did not demonstrate liver tropism (livers were not increased in size or weight at sacrifice).

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 (25-28;45). However, after the repression of stem cell genes that occurs with lineage-commitment, decitabine can augment expression of late differentiation genes and accelerate differentiation instead (45). Therefore, baseline differentiation stage is a major determinant of the cell fate response to decitabine treatment. Surface phenotype can be used to sort AML cell populations into subsets. These subsets can then be xeno-transplanted into immunocompromised mice for evaluation of leukemia-initiating efficiency. The earliest studies suggested that AML cells with leukemia-initiating capacity had a surface phenotype resembling that of normal HSC (CD34+38-) (36;46). This suggested that AML cell populations might recapitulate the hierarchical structure of normal hematopoiesis, with a small sub-set of AML cells with a stem cell phenotype sustaining the bulk AML cell population (46). Recently, it has been reported that the antibodies used to sort for CD38+ may inhibit proliferation and might have technically influenced the earliest studies (47). Accordingly, in a number of recent studies, AML initiating cells had a surface phenotype suggesting lineage-commitment (CD34+38+, CLL-1+, CD71+, CD90 -, c-Kit -) (47-53). Cross-species barriers are another major influence on the outcome of leukemia or cancer initiating cell assay experiments (54). With use of more immuno-compromised mice, or mice which express human cytokines, AML initiating cell surface-phenotypes are not stem cell restricted (54-56). Differentiation absolutely requires and is driven by lineage-specifying transcription factors such as CEBPA. CEBPA is expressed at high levels with lineage-commitment. AML cells, including CD34+ and CD34+38- subsets express high levels of CEBPA, but relatively low levels of the key late differentiation driver CEBPE (supporting 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 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, 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 also 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 intravenous administration is in the order of minutes, compared to many hours *in vitro* (57;58). An obvious mechanism for treatment failure therefore is that some or many AML cells may complete S-phase while decitabine is absent from the system (most of the time). Decitabine was originally developed for cytotoxic therapy (59). Therefore, doses to treat AML were escalated to maximum tolerated levels (up to 80 mg/kg infused over 36-44 hours) in traditional phase 1 studies (57), followed by many weeks without treatment to allow for recovery from cytotoxic side-effects. A decrease in the dose (to 15 mg/m^2 infused over 3 hours 3X/day on day 1-3, repeated every 6 weeks) led to United States Food and Drug Administration (FDA) approval of decitabine as a treatment for MDS (60). A further decrease in the daily dose and administration more frequently (20 mg/m^2 infused over 1 hour 1X/day on Day 1-5, repeated every 28 days), has further improved MDS treatment clinical results (60;61). The results here provide a biological rationale to continue this clinical trend of decreasing dose and administering drug more frequently: very low drug levels are sufficient for non-cytotoxic DNMT1 depletion, and the decrease in toxicity can be used to administer treatment frequently, to increase windows of drug exposure and capture AML cells entering S-phase at different points in time (**figure S3**). That lower doses of SC decitabine (0.2 mg/kg, ~5 mg/m^2) are clinically active, with non-cytotoxic epigenetic and differentiation modifying effects, and can be administered safely from 1-3 X/week, has been demonstrated in sickle cell disease and β-thalassemia clinical trials (24).

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. Pharmacologic barriers to optimal clinical translation remain. However, these can potentially be addressed through further pre-clinical and clinical investigation.

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Supplementary information is available at Leukemia's website

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FIGURE LEGENDS

Figure 1. Non-cytotoxic concentrations of decitabine (DAC) induce differentiation and terminate proliferation of p53 wild-type MLL-AF9 cells. p53 wild-type cells were treated with DAC or AraC 0.5 μM on day 1 and 4. **A) DAC 0.5 μM depleted DNMT1**. DNMT1 measured by Western blot. **B) DAC 0.5 μM did not cause significant early apoptosis.** Annexin staining measured by flow-cytometry 24 hours after DAC treatment. Positive control AraC treatment. **C) DAC and AraC treatment were anti-proliferative.** Cell quantity estimated by MTT assay at day 2, 4 and 7. DAC or AraC 0.5 μM added to cells on Day 1 and 4. Colony formation in semi-solid media used as an assay of stem/progenitor activity. THP1 cells in liquid culture were treated with 0.5 μM of decitabine or AraC on Day 1 and 4 then plated into methylcellulose on day 5 (2000 viable cells plated per ml of methylcellulose). **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. Giemsa staining of cytospin preparations on day 4. **E) DAC and AraC treatment increased expression of the monocytic marker CD14.** CD14 and CD11b (granulocytic marker) measured by flow-cytometry on day 4.

Figure 2. Low concentration decitabine inhibits AML LIC but spared normal HSC. Engraftment in an immuno-compromised murine host is a functional assay for both normal HSC and LIC (35;36). Normal CD34+ HSC and MLL-AF9 leukemia cells were treated in vitro with the identical decitabine 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. Cells harvested on day 7 were combined (3 x 10^5 each MLL-AF9 + normal cells), then transplanted by tail-vein injection into sub-lethally irradiated NOD/SCID recipient mice (n=6). Additional controls: mice transplanted with PBS-treated normal CD34+ cells alone (4 x 10^6 cells/mouse). **A) Significant survival difference between groups**. Surviving mice were sacrificed for analysis at week 13. **B) >90% AML cell engraftment in bone marrow of mice receiving PBS-treated cells; ~80% normal, multi-lineage, human hematopoietic cell engraftment in mice receiving decitabine treated cells**. Additional engraftment data and controls in **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.

Figure 3. Decitabine (DAC), but not an identical regimen of AraC, terminated proliferation of p53-null MLL-AF9 cells (THP1 cells (37)). **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 hours after DAC or AraC treatment. **C) DAC, but not AraC treatment, was anti-proliferative.** Cell quantity estimated by MTT assay at day 1, 3, 6 and 8. DAC or AraC 0.5 μM added to cells on Day 1 and 4. Colony formation in semi-solid media used as an assay of stem/progenitor activity. THP1 cells in liquid culture were treated with 0.5 μM of decitabine or AraC on Day 1 and 4 then plated into methylcellulose on day 5 (2000 viable cells plated per ml of methylcellulose). **D) DAC induced morphologic changes of monocytic differentiation** (decreased nuclear-cytoplasmic ratio, increased cell size, granulation and vacuolization of the cytoplasm). Arrows = mitotic cells. Giemsa staining of cytospin preparations on day 4. **E) DAC, but not AraC, increased expression of the granulocyte marker CD11b, and to a lesser extent the monocyte marker CD14.** CD11b and CD14 measured by flow-cytometry on day 4.

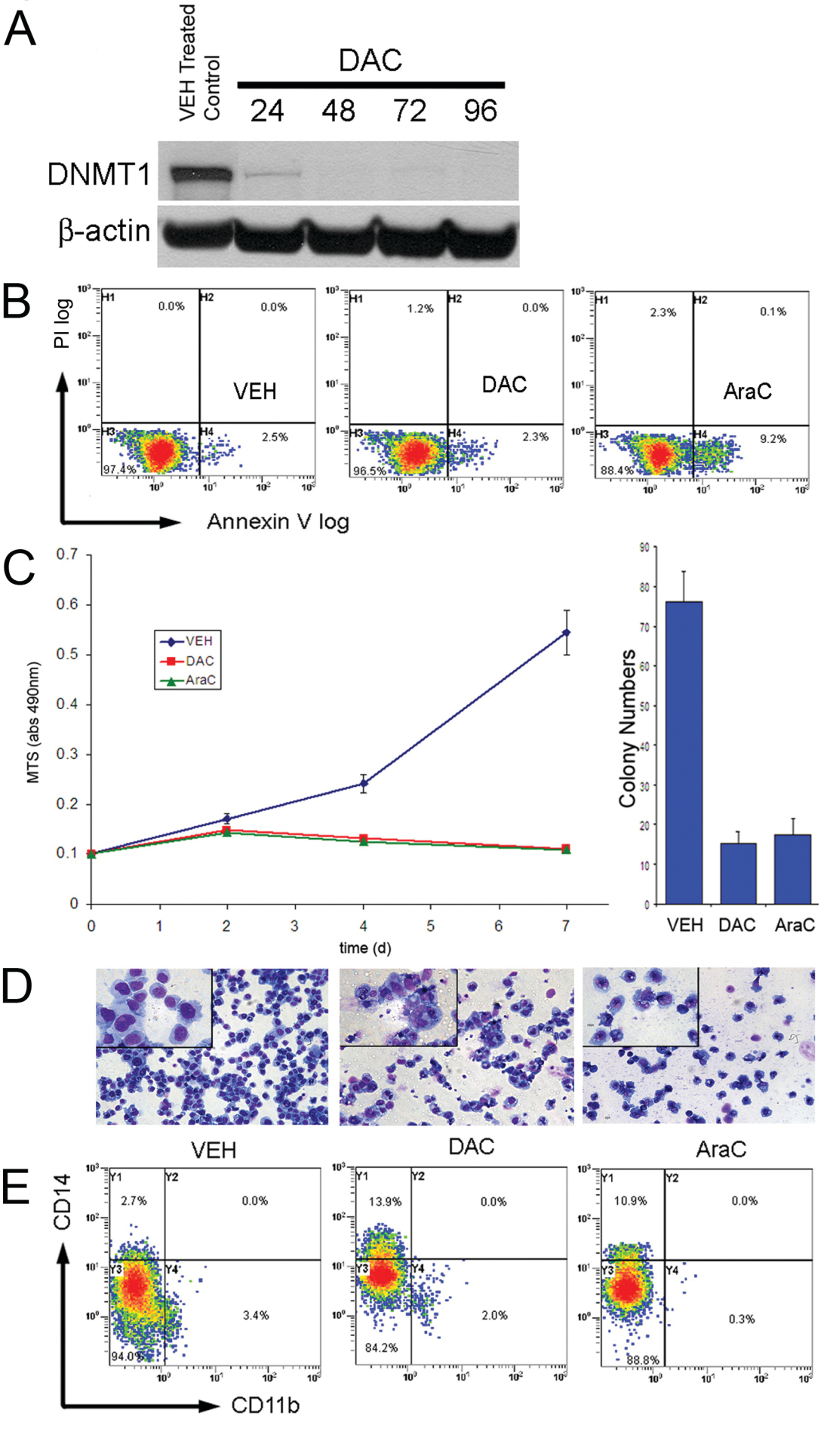
Figure 4. Decitabine (DAC) and AraC differentially regulate apoptosis and differentiation proteins in p53 wild-type and p53-null MLL-AF9 cells. Key events associated with apoptosis and differentiation mediated cell cycle exit were examined in p53 wild-type and p53-null MLL-AF9 cells treated with DAC or AraC 0.5 μM on day 1 and 4. **A) Western blots of p53 wild-type MLL-AF9 cells treated with DAC and AraC**. Numbers = hours after initiation of treatment. Red 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 and AraC**.

Figure 5. Better survival with non-cytotoxic decitabine than with cytotoxic AraC in a murine xeno-transplantation model of p53-null human AML. A) Kaplan-Meier plot of survival distribution function. X-axis: days after xeno-transplant of AML cells. Non-irradiated NSG mice were transplanted with $3x10^6$ THP1 cells (p53-null MLL-AF9 AML cells) by tail vein injection. Starting day 5 after transplantation, mice were treated with vehicle (PBS, n=5), cytarabine (AraC) 75 mg/kg/day IP for five days (n=5) (to model conventional chemotherapy (44)), or decitabine (DAC) 0.2 mg/kg SC 3X/week for 2 weeks then 1X/week (n=5). **B) Bioluminescent imaging to show anatomic localization of engrafted THP1 cells**. Images from dorsal perspective obtained ten minutes after IP administration of luciferin substrate, and on day 28 after 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) Extensive liver disease in a murine xeno-transplantation model of p53-null human AML treated with non-cytotoxic decitabine or cytotoxic AraC.** Non-irradiated NSG mice were transplanted with $3x10^6$ THP1 cells (p53-null MLL-AF9 AML cells) by tail vein injection. Starting day 5 after transplantation, mice were treated with vehicle (mock treated control), AraC 75 mg/kg/day IP for five days (to model conventional chemotherapy (44)), or decitabine 0.2 mg/kg SC 3X/week for 2 weeks then 1X/week. 5 mice per treatment group. * = $p < 0.05$ compared with PBS treated control. ** = $p < 0.005$ compared with PBS treated control. **D) At sacrifice, the spleens of decitabine treated mice were decreased in size compared to vehicle treated mice, but not to a statistically significant extent** (sacrifice was at different time-points as per the Kaplan-Meir curve)**.**

Figure 6. Better survival with non-cytotoxic decitabine than with cytotoxic AraC in a murine xeno-transplantation model of refractory/relapsed human AML. To complement the above xeno-transplantation experiment in which an AML cell line was used, a xeno-transplantation model was generated using fresh AML cells from a patient with relapsed, chemotherapy treatment refractory AML. These AML cells contained multiple chromosome abnormalities including a t(8;18)(q22:q23) and t(11;13)(q21:q12). *TP53* was not mutated by sequencing of genomic DNA and cDNA (data not shown). Non-irradiated NSG mice were transplanted with $1x10^6$ patient cells by tail vein injection. Starting day 5 after transplantation, mice were treated with vehicle (control), AraC 75 mg/kg/day IP for five days (44), or decitabine 0.2 mg/kg SC 3X/week for 2 weeks then 1X/week (7 mice per group). **A) Mice treated with decitabine had significantly longer median survival (>100% increase) than AraC or vehicle treated mice** (median survival 113, 56, and 50 days respectively, Log-Rank $p<0.0001$). **B) At sacrifice, the bone marrow of all mice was replaced by human leukemia cells**. Human CD45 staining used to identify human cells (sacrifice was at different time-points as per the Kaplan-Meir curve). **C) At sacrifice, spleens of decitabine treated mice were significantly decreased in size compared to AraC or vehicle treated mice**. ** = $p < 0.005$ compared to PBS treated control. Normal = non-transplanted, non-treated mouse.

Figure 1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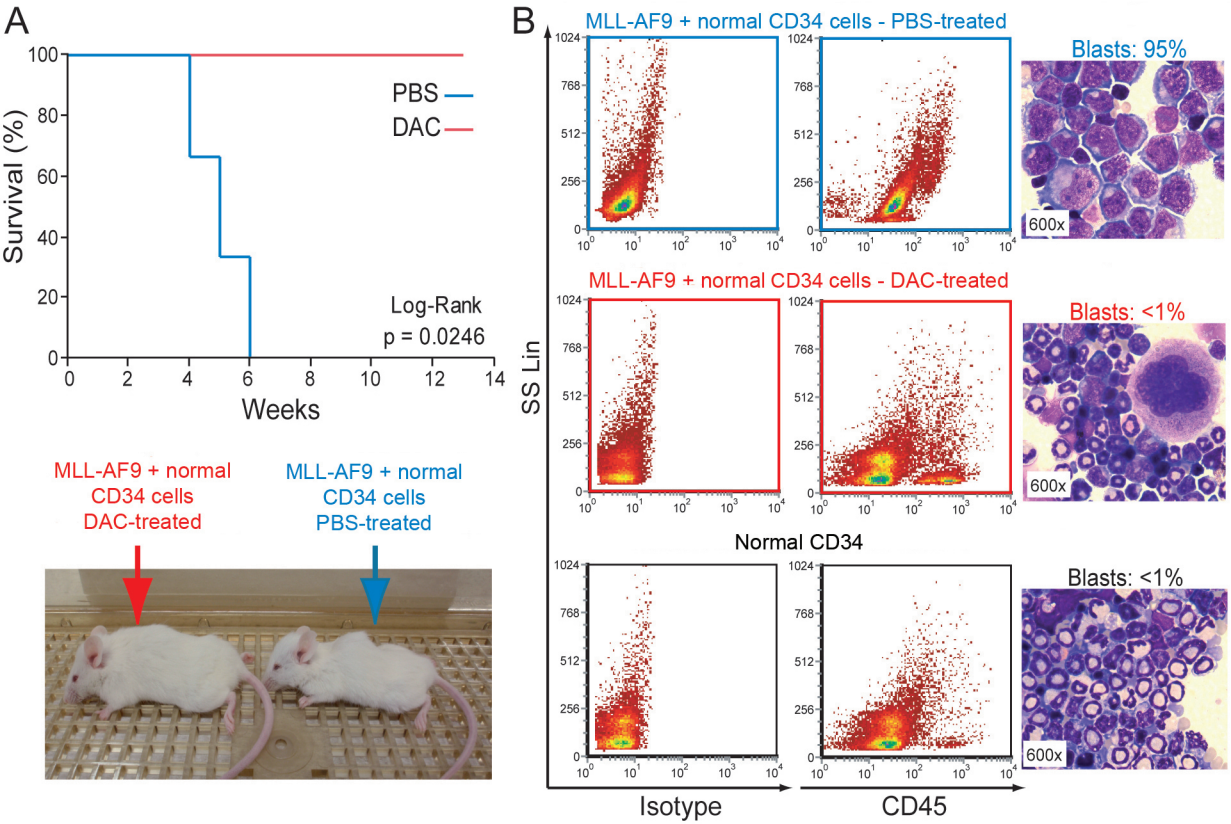
VEH Treated Control
DAC
24
48
72
96
DNMT1
β-actin
B
PI log
Annexin V log
VEH
DAC
AraC
0.0%
0.0%
2.5%
97.4%
1.2%
0.0%
2.3%
96.5%
2.3%
0.1%
9.2%
88.4%
C
MTS (abs 490nm)
time (d)
VEH
DAC
AraC
Colony Numbers
D
VEH
DAC
AraC
E
CD14
CD11b
2.7%
0.0%
3.4%
94.0%
13.9%
0.0%
2.0%
84.2%
10.9%
0.0%
0.3%
88.8%

Figure 2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Survival (%)
100
80
60
40
20
0
PBS
DAC
Log-Rank
p = 0.0246
0
2
4
6
8
10
12
14
Weeks
MLL-AF9 + normal CD34 cells DAC-treated
MLL-AF9 + normal CD34 cells PBS-treated
B
MLL-AF9 + normal CD34 cells - PBS-treated
Blasts: 95%
MLL-AF9 + normal CD34 cells - DAC-treated
Blasts: <1%
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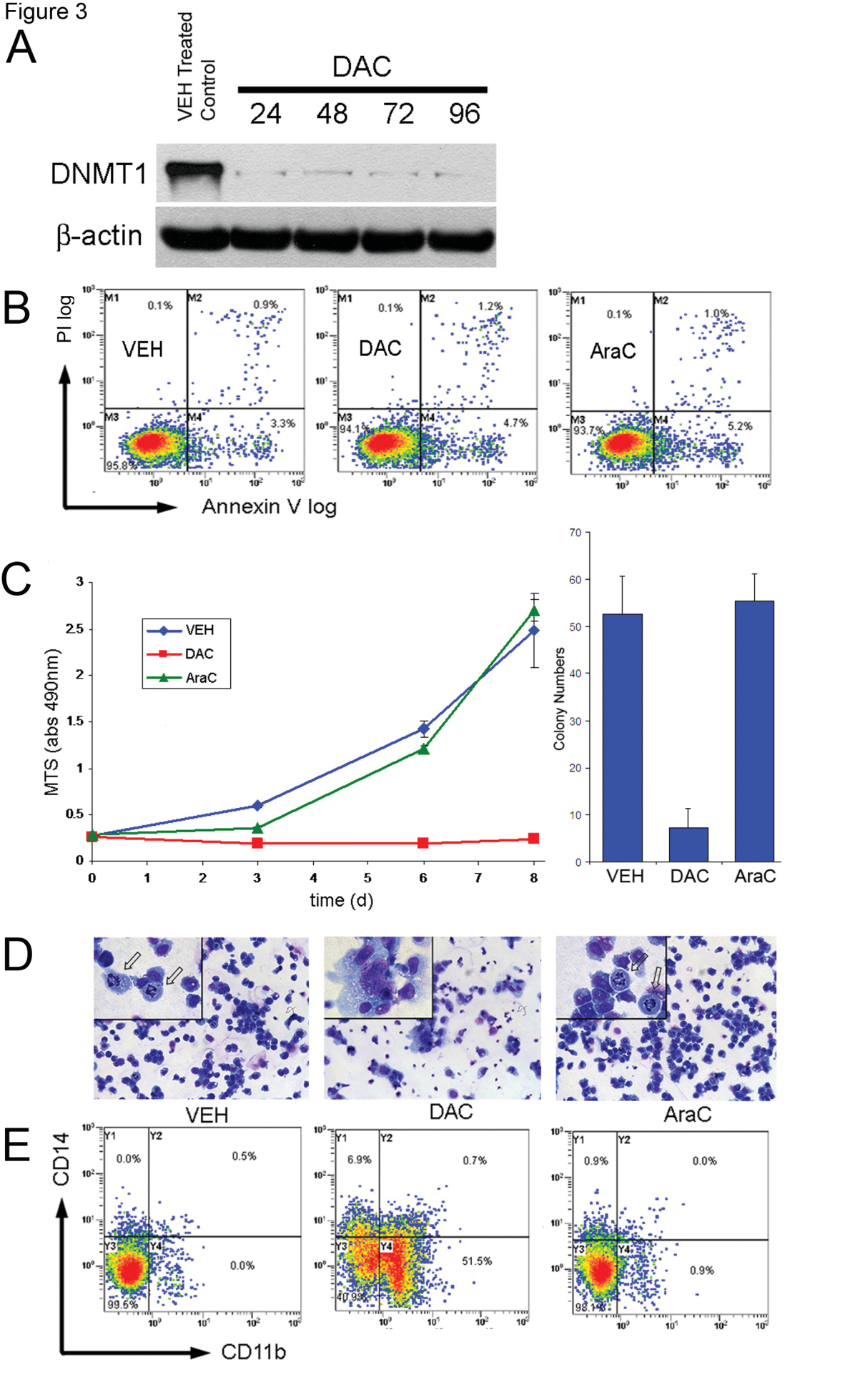
Normal CD34
Blasts: <1%
600x
Isotype
CD45

Figure 3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VEH Treated Control
DAC
24
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72
96
DNMT1
β-actin
B
PI log
VEH
DAC
AraC
M1
M2
M3
M4
0.1%
0.9%
3.3%
95.8%
0.1%
1.2%
4.7%
94.1%
0.1%
1.0%
5.2%
93.7%
Annexin V log
C
MTS (abs 490nm)
VEH
DAC
AraC
time (d)
Colony Numbers
VEH
DAC
AraC
D
VEH
DAC
AraC
E
CD14
CD11b
Y1
Y2
Y3
Y4
0.0%
0.5%
0.0%
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40.9%
0.9%
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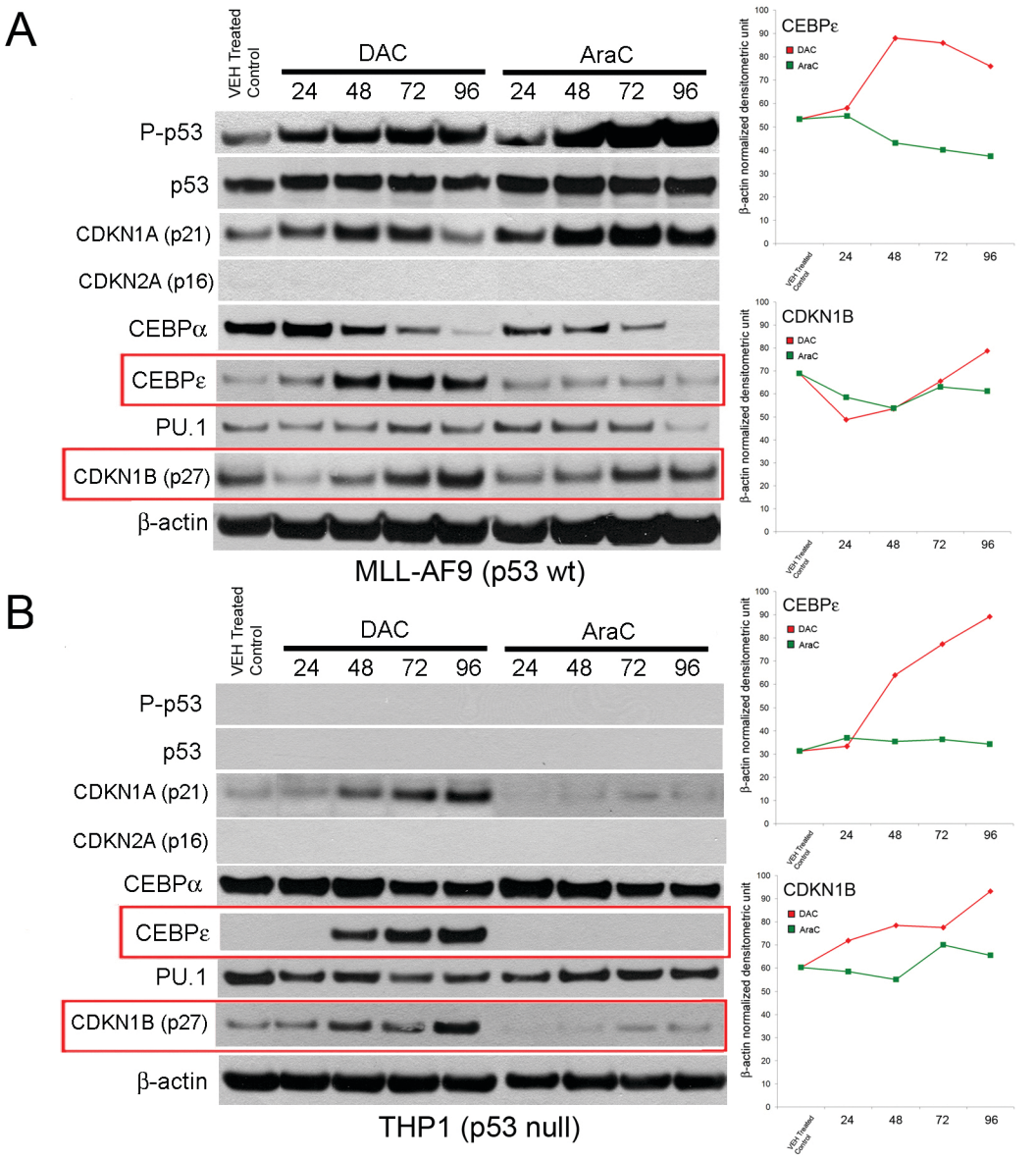
98.1%

Figure 4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VEH Treated Control
DAC
AraC
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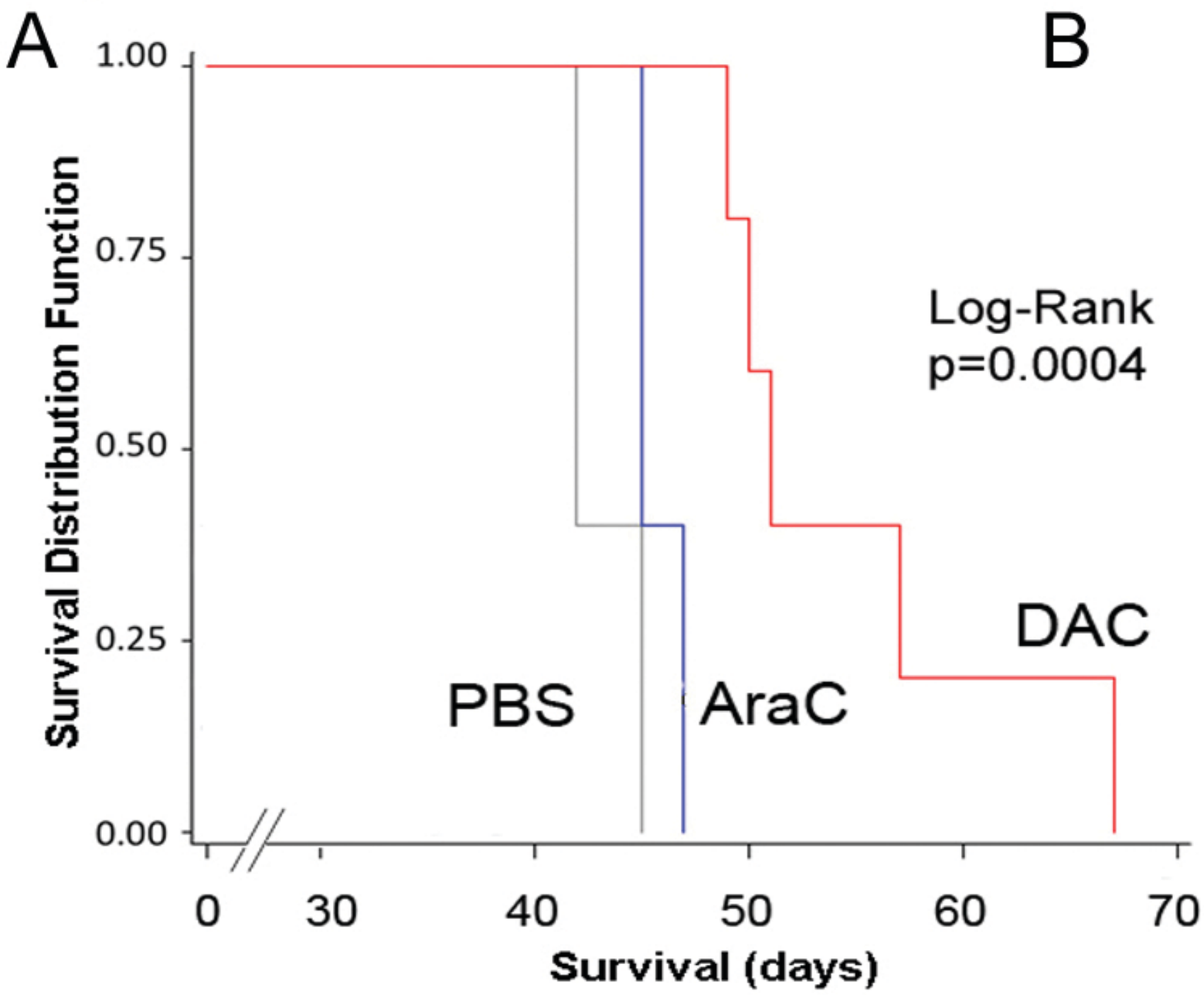
p53
CDKN1A (p21)
CDKN2A (p16)
CEBPα
CEBPε
PU.1
CDKN1B (p27)
β-actin
MLL-AF9 (p53 wt)
CEBPε
DAC
AraC
β-actin normalized densitometric unit
CDKN1B
B
THP1 (p53 null)

Figure 5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1.00
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Survival Distribution Function
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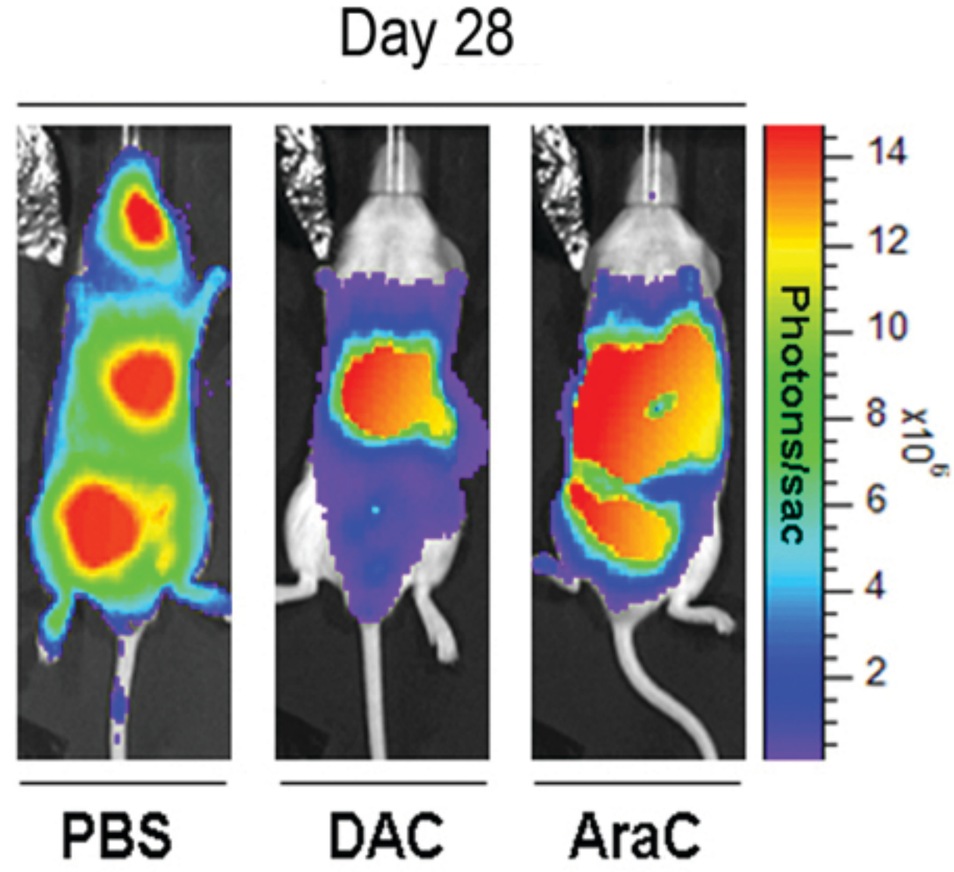
AraC
DAC
0
30
40
50
60
70
Survival (days)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Day 28
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14
12
10
8
6
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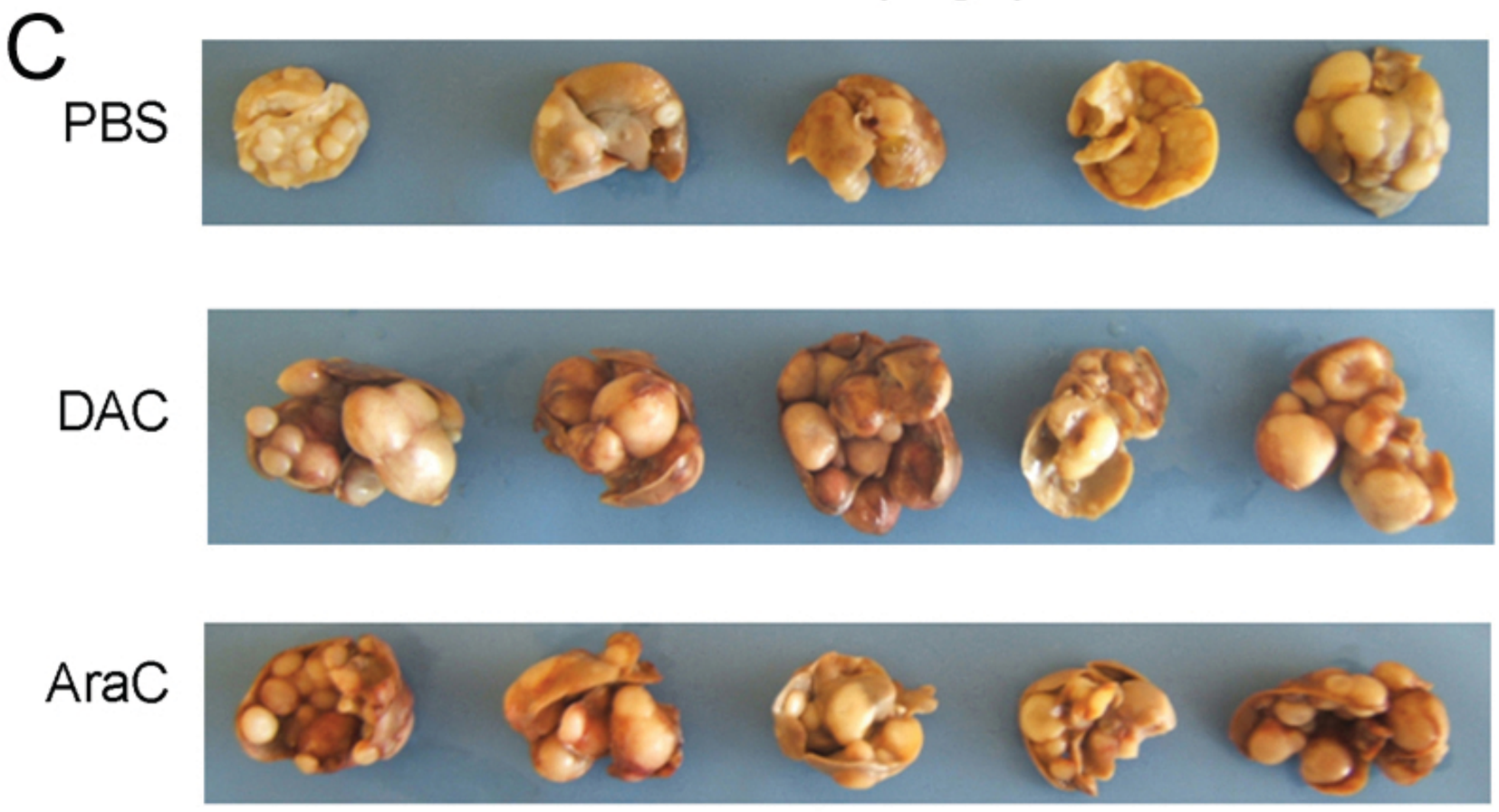
PBS
DAC
AraC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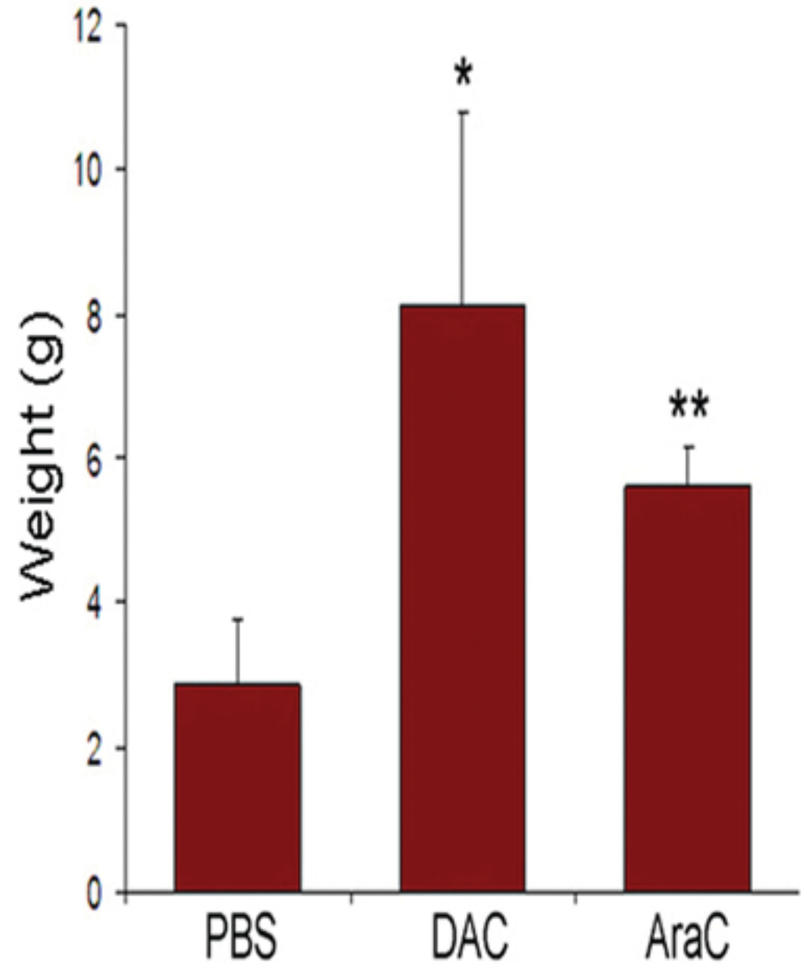
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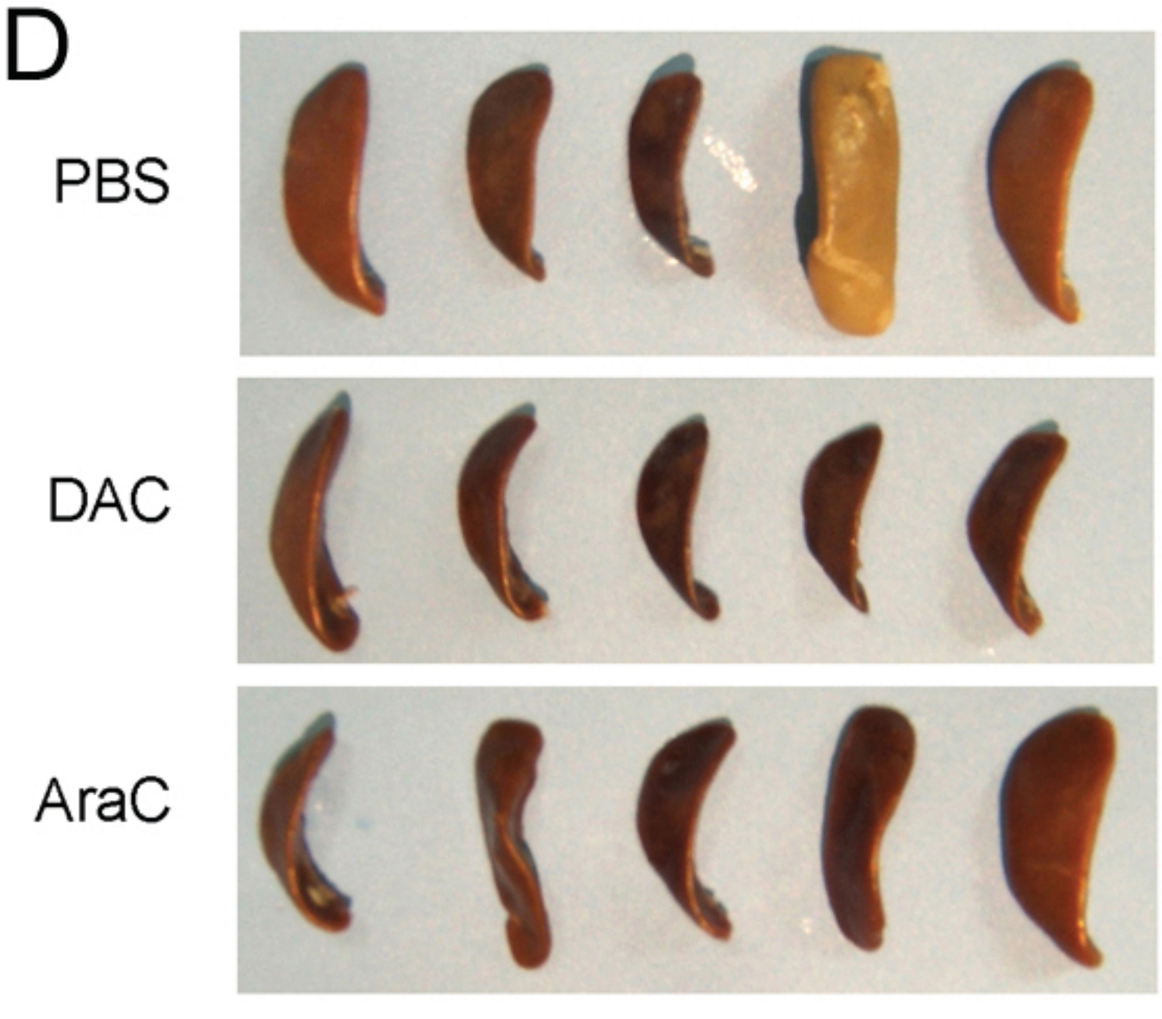
D
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DAC
AraC

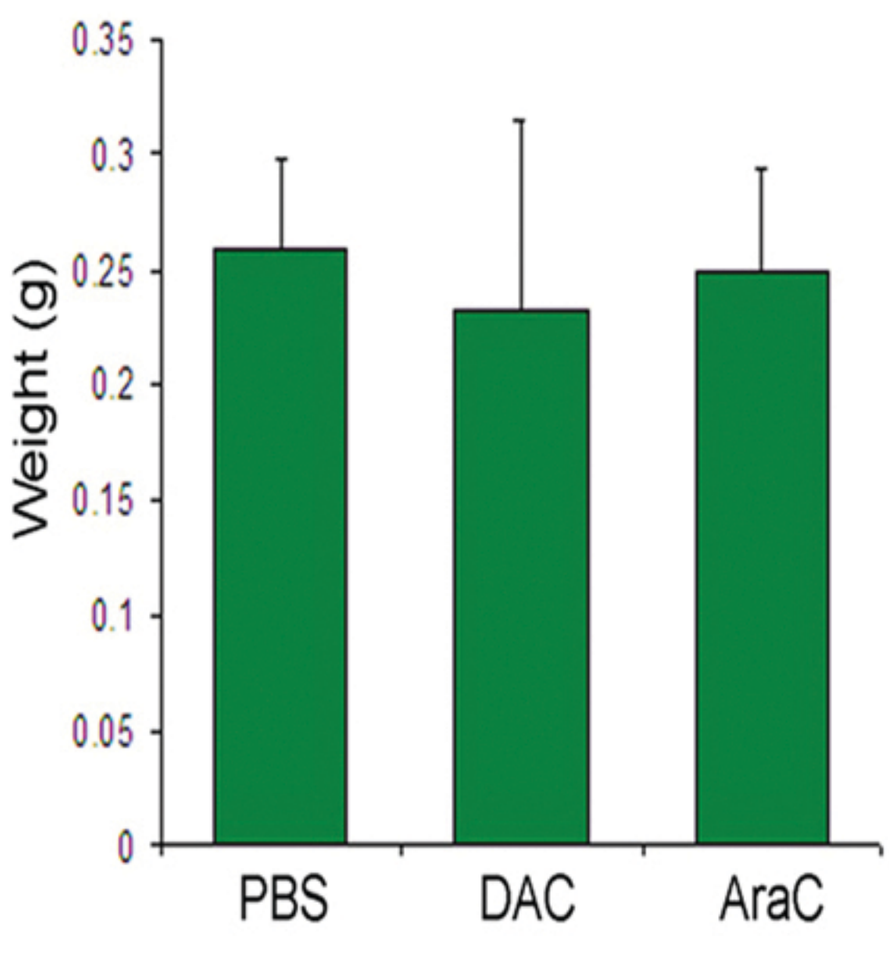
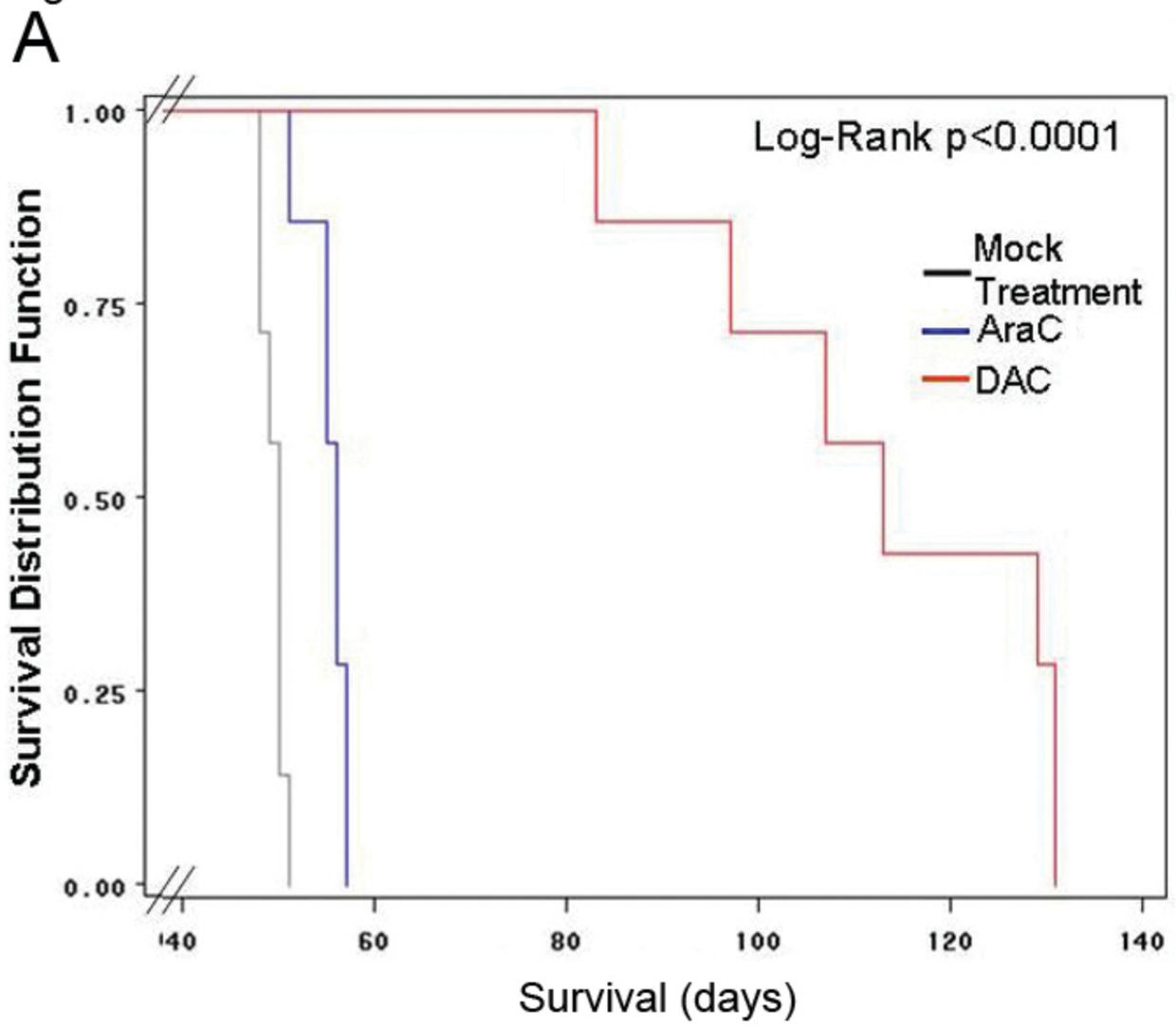
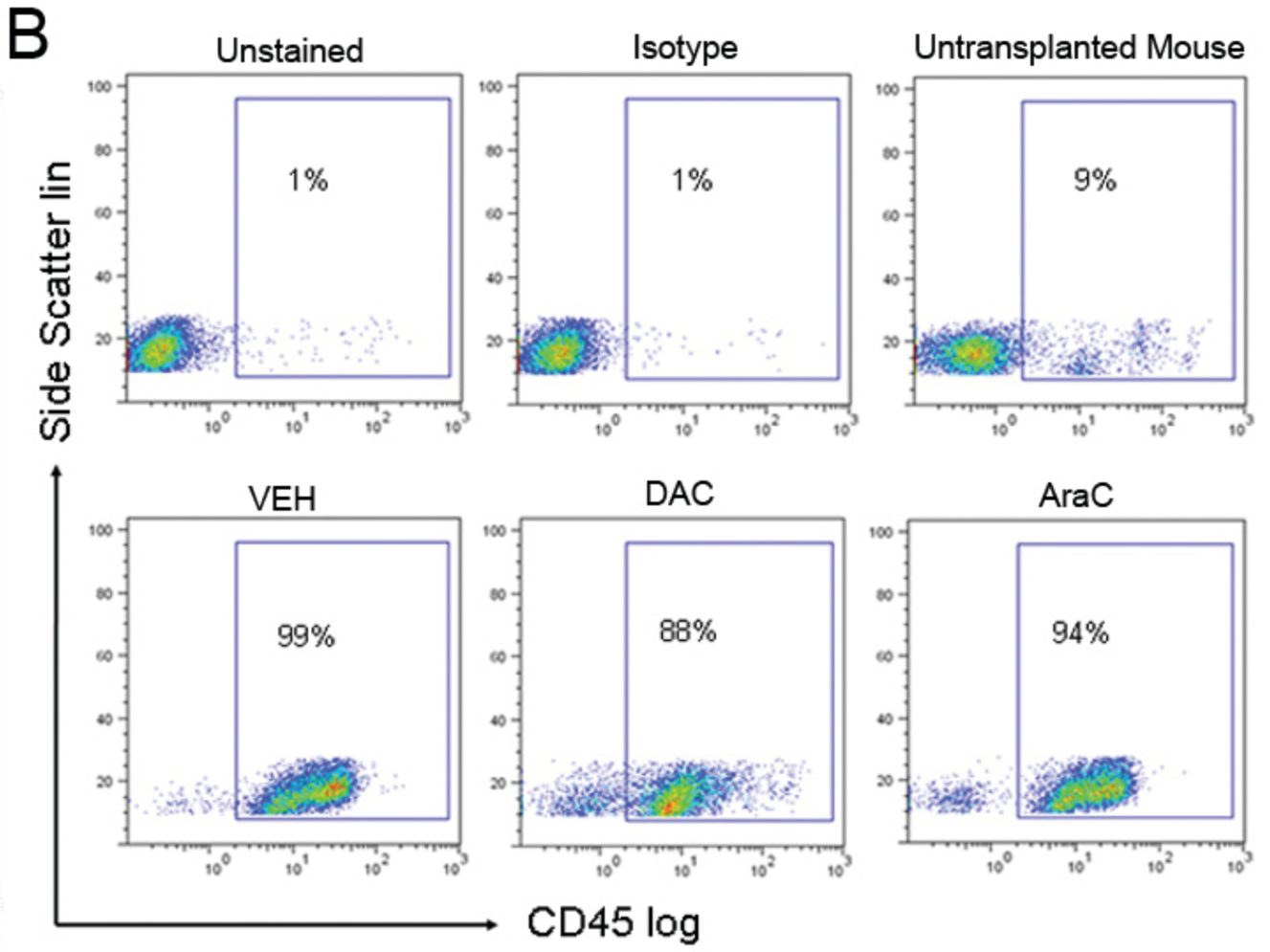
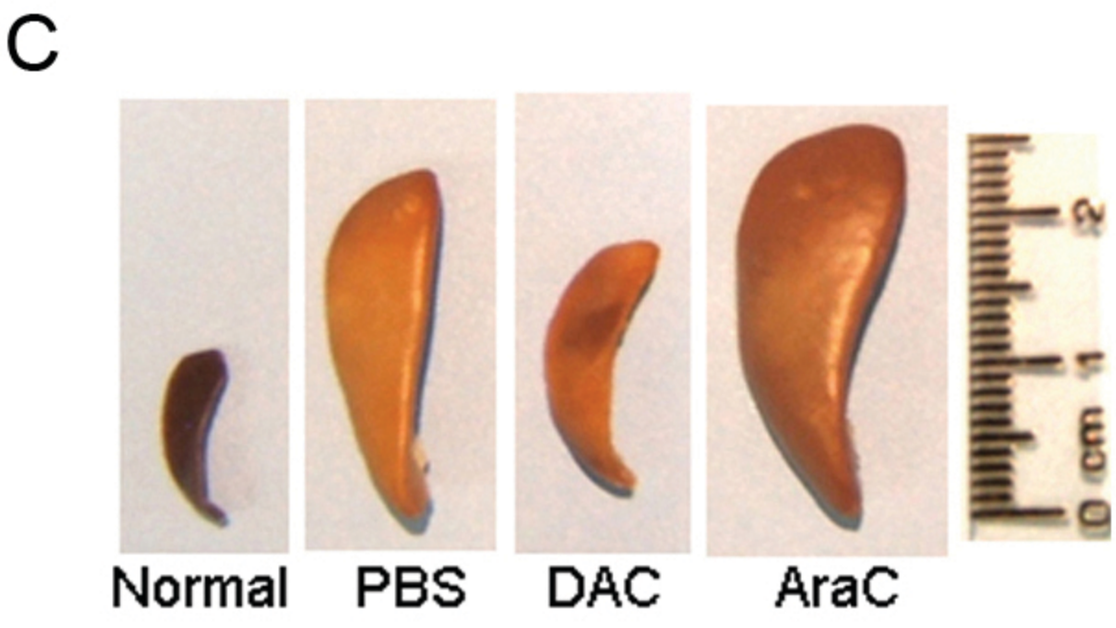
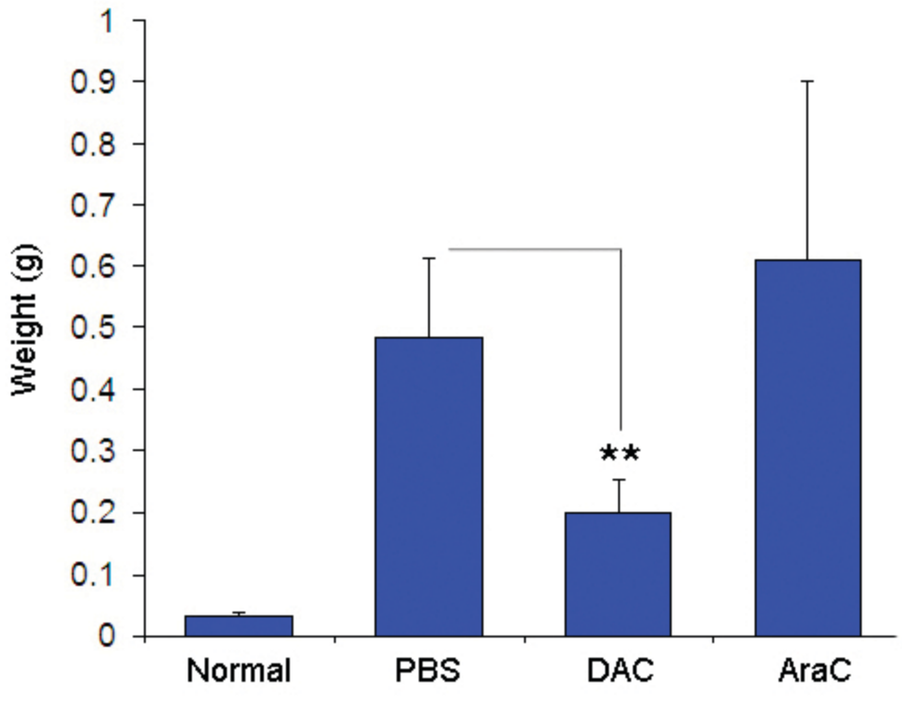

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Weight (g)
PBS
DAC
AraC

Figure 6


A
Log-Rank p<0.0001
Mock Treatment
AraC
DAC
Survival Distribution Function
1.00
0.75
0.50
0.25
0.00
40
60
80
100
120
140
Survival (days)


B
Unstained
Isotype
Untransplanted Mouse
1%
1%
9%
VEH
DAC
AraC
99%
88%
94%
Side Scatter lin
CD45 log


C
Normal
PBS
DAC
AraC


Weight (g)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
Normal
PBS
DAC
AraC