Antitumor synergy with SY-1425, a selective RARα agonist, and hypomethylating agents in retinoic acid receptor pathway activated models of acute myeloid leukemia

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Supplemental Information Methods

Cell lines and Tissue Culture

To investigate synergistic antiproliferative activity between SY-1425 and HMAs, AML cell lines expressing high levels of *RARA* or *IRF8* (OCI-AML3, NOMO-1, SIG-M5, MV-4-11) or low levels of *RARA* (OCI-M1) were studied. Qualification of *RARA* or *IRF8* levels were determined based off work previously published from our group.[1] Cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Brunswick, Germany), except MV-4-11, which was obtained from the American Tissue Culture Collection (ATCC, Manassas, Virginia). Cells were cultured in RPMI1640 +10% FBS media for all experiments described, and treated with the following agents at varying concentrations: vehicle (DMSO), SY-1425 (0.1-100 nM), azacitidine (0.16-16 µM), or decitabine (0.8-8 µM). Cells were cultured for 5 days in varying concentration combinations of SY-1425 and azacitidine or decitabine followed by a cell viability measurement using ATPlite (PerkinElmer), and luminescence read on a PerkinElmer Envision instrument. Cell viability was calculated by normalizing luminescence between drug treated to vehicle (DMSO) treated wells.

Synergy in vitro assays

Using a Biotek EL406, 50 µL of cell media containing 20-60,000 cells/mL was distributed into white 384-well Nunc plates (Thermo Fisher Scientific). The compounds to be tested were dissolved in DMSO and arrayed on 384 well compound storage plates (Greiner). Each compound plate received 1 compound in 5-point dose response approximately centered on the EC_{50} of the given compound for a given cell line.

Compound arrays were distributed to assay plates using a 20 nL 384-well pin transfer manifold on a Janus MDT workstation (PerkinElmer). After addition of compound, cell plates were incubated for 5 days in a 37°C incubator. Cell viability was evaluated using ATPlite (PerkinElmer) following manufacturer protocols. Data was analyzed in CalcuSyn using the median effect principle[2] and visualized using GraphPad Prism Software. Key parameters assessed were combination index and dose reduction index.

Apoptosis measurements

Cells lines (OCI-AML3, MV-4-11, SigM5, OCI-M1 and Kasumi-1) were treated in $10cm^2$ dishes with DMSO, 100 nM azacitidine, 100 nM decitabine, or 10µM RG108 for 24 hours. Cells were then counted, resuspended in fresh media without drug, and plated in a black walled, clear bottom 96-well plate (Corning 9604) @ 10,000 cells/well. Cells were then treated with either DMSO or 50nM of SY-1425 and incubated in 37C CO_2 incubator for 24 Hours. After 24 hours, induction of apoptosis was determined using Promega™ Caspase-Glo® 3/7 Assay Systems (CN: G8093) per manufacture protocol, and luminescence read on a PerkinElmer Envision instrument. Each condition was run in sextuplicate. Relative change in apoptosis was calculated by taking the log2(Experimental Condition/DMSO condition).

Western blots

OCI-AML3, NOMO-1 and OCI-M1 were treated in $10cm^2$ dishes with DMSO, 100 nM azacitidine, 100 nM decitabine, or 10µM RG108 for 24 hours. Cells were than counted, resuspended in drug free media and plated in 6-well dishes at 1e6 cells/mL and treated with either DMSO or 50 nM of SY-1425 for 24 hours. Cells were then collected, and cell lysates were

prepared, run on a 4%-12% Bis-Tris gradient gel (NuPAGE™ 4%-12% Bis-Tris Protein Gels), and transferred to PVDF membranes. Membranes were blocked with Odyssey® Blocking buffer(TBS) (P/N 927-50000) and stained with primary antibody overnight at 4°C. Blots were visualized using Odyssey® CLx imager.

Antibody List:

Manufacturer	Antibody	Catalog #	Molecular Wt	Species	Dilution
Cell Signal	DNMT-1	35032	200kDa	Rabbit	1:500
Cell Signal	PARP	9542L	116 KDa 89KDa Cleaved	Rabbit	1:1000
Cell Signal	Phospo-Histone H2A	9718	15kDa	Rabbit	1:1000
Cell Signal	Beta Tubulin	86298S	55kDa	Mouse	1:1000
Cell Signal	GAPDH	97166	37kDa	Mouse	1:1000
LiCor	Goat Anti Mouse	926-68070	N/A	N/A	1:10000
LiCor	Goat Anti Rabbit	926-32211	N/A	N/A	1:10000

Xenograft mouse model

HuKemia Model AM-5512 female NOD SCID mice (Crown Bioscience, Inc) were used for all *in vivo* experiments. This model was previously established as an SY-1425 sensitive PDX model among a panel of AML PDXs.[1] All animal protocols were approved by the Institutional Animal Care and Use Committee at Crown Bioscience, Inc prior to conduct, and the care and use of animals was conducted in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were inoculated with $2x10^6$ cells from passage 3, and treatment was initiated 20 days later upon detection of hCD45+ cells in the peripheral blood at ~5%.

For initial testing, 4 groups were treated: group 1, vehicle (1% DMSO in pH8 adjusted PBS) twice a day for the duration of the study or until death; group 2, SY-1425 3 mg/kg twice a day for

35 days; group 3, azacitidine 2.5 mg/kg once a day for 7 days; group 4, azacitidine 2.5 mg/kg once a day for 7 days and SY-1425 3 mg/kg twice a day for 35 days. Peripheral tumor burden was determined based on the percentage of circulating human CD45 (Biolegend antibody, clone HI30) cells identified by fluorescence-activated cell sorting (FACS). Bone marrow and spleen sections were stained with H&E stain to determine cell pathology. Percentage of huCD45+ in Bone Marrow and Spleen were determined via human CD45 staining (Biolegend antibody, clone HI30) of cell dispersions identified by fluorescence-activated cell sorting (FACS). These results were derived from an exploratory study, thus were not powered.

To determine the optimal combination treatment regimen, mice were treated in 6 different groups: group 1, vehicle (1% DMSO in PBS) twice a day for 8 weeks; group 2, azacitidine 2 mg/kg once a day for 2 weeks; group 3, SY-1425 3 mg/kg twice a day for 8 weeks; group 4, azacitidine 2 mg/kg once a day for 2 weeks (weeks 2 and 6) and SY-1425 3 mg/kg for 6 weeks (weeks 1,3,4 and 5,7,8); group 5, azacitidine 2 mg/kg once a day for 2 weeks (weeks 1 and 5) and SY-1425 3 mg/kg twice a day for 6 weeks (weeks 2,3,4 and 6,7,8); group 6, azacitidine 2 mg/kg once a day for 2 weeks (weeks 1 and 5) and SY-1425 3 mg/kg twice a day for 8 weeks. Peripheral tumor burden was determined based on the percentage of circulating human CD45 cells identified by FACS. Kaplan-Meier plots were generated in GraphPad Prism version 6.0 with statistical analysis performed by Mantel-Cox method.

RNA-seq sample preparation

Triplicate samples were treated with 1µM azacytidine for 72 hours with SY-1425 50nM added during the final 24 hours for SY-1425 alone or combination. AML samples were homogenized in 1ml Trizol and RNA isolated by RNAeasy (Ambion) according to manufacturer instructions. RNA libraries were prepared and sequenced as described previously.[3]

RNA-seq analysis

RNA-Seq data was quantified using RSEM v1.2.21 software[4] (rsem-calculate-expression, parameters = --samtools-sort-mem 3G --ci-memory 3072 --bowtie-chunkmbs 1024 ---bowtie2 --strand-specific), aligning to the HG19 transcriptome. The RSEM software produces expression for each gene in Expected Counts. These files were then read into R and processed with DESeq2[5] for differential expression analysis. The counts per gene were compared for the samples in each condition to determine the differentially expression genes.

References:

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4. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 2011;12323–323.

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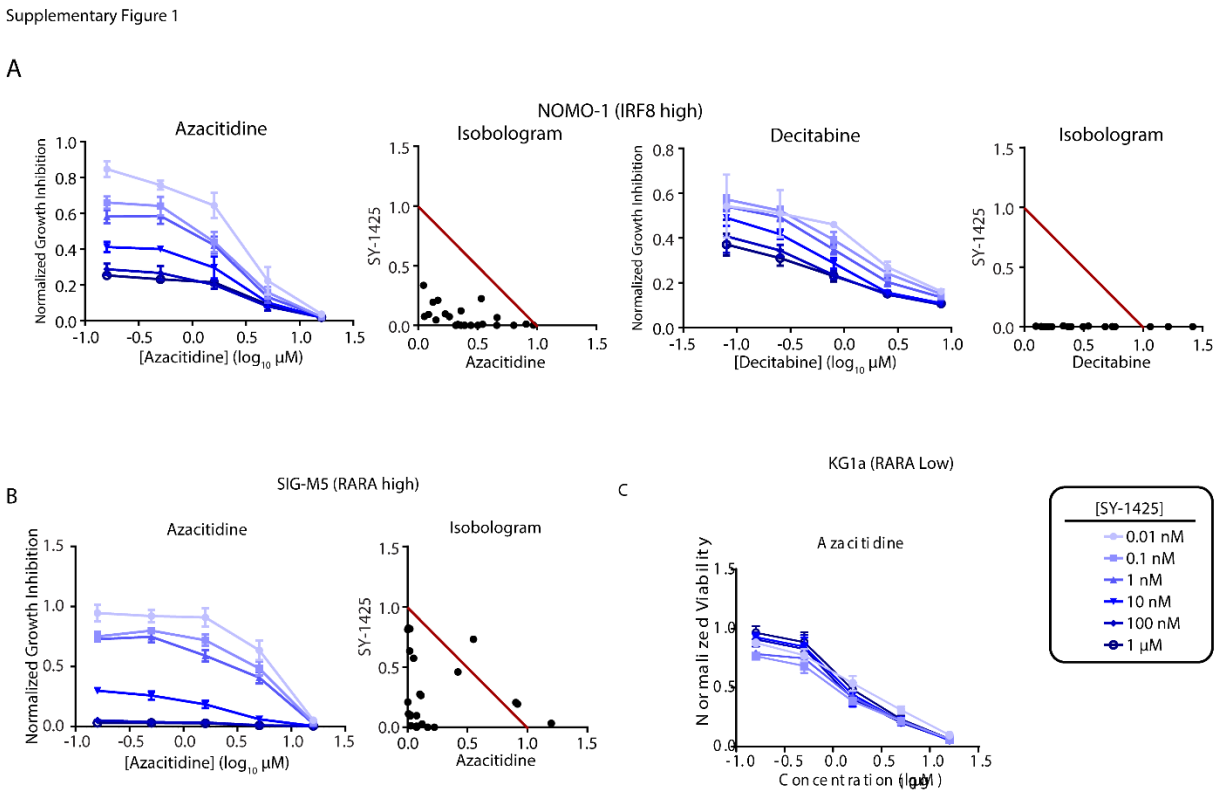
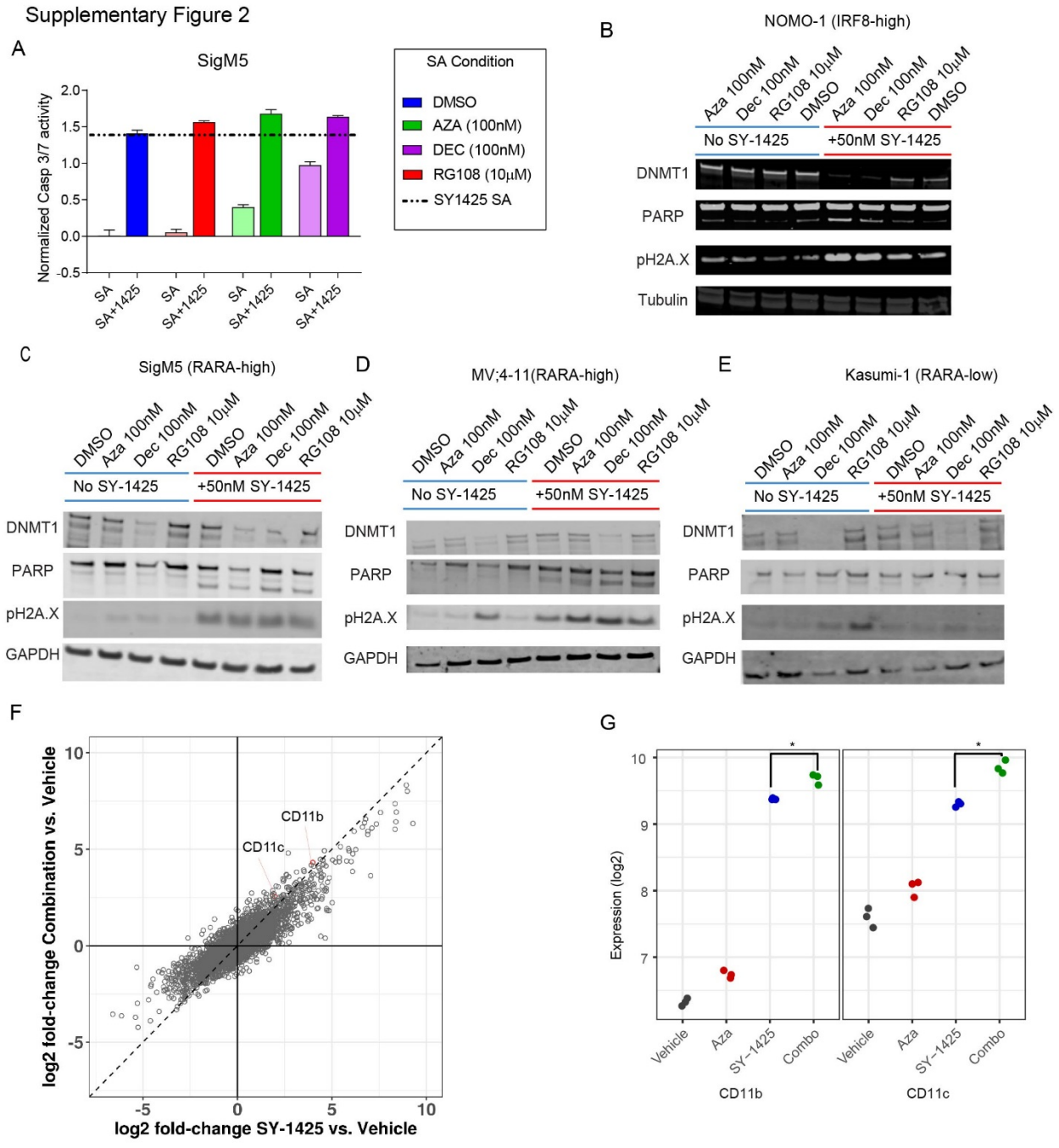
Supplemental Data and Figure legends:



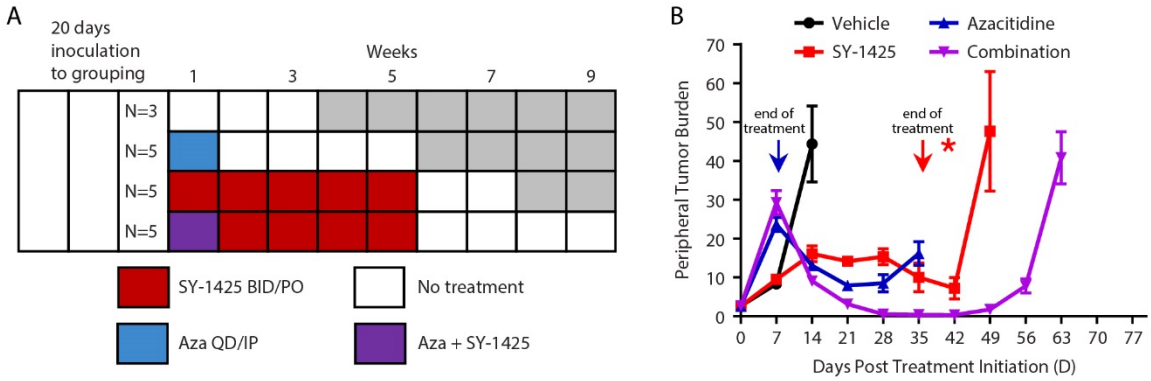
Supplementary Figure 1. SY-1425 was added in increasing concentrations in combination with azacitidine two additional AML cell lines: (A) *IRF8* High NOMO-1 and (B) *RARA* High SIG-M5 and one(C) *RARA* low line (KG1a). Cellular growth inhibition was determined using ATPlite, via normalization of compound treated wells to vehicle treated wells. Isobolograms illustrate the degree of synergy in reducing cell proliferation, where data points below the 1:1 line are synergistic, data points at or near the line are additive, and data points > 1 are antagonistic. Compounds did not display synergy in KG1a thus an isobologram could not be generated.



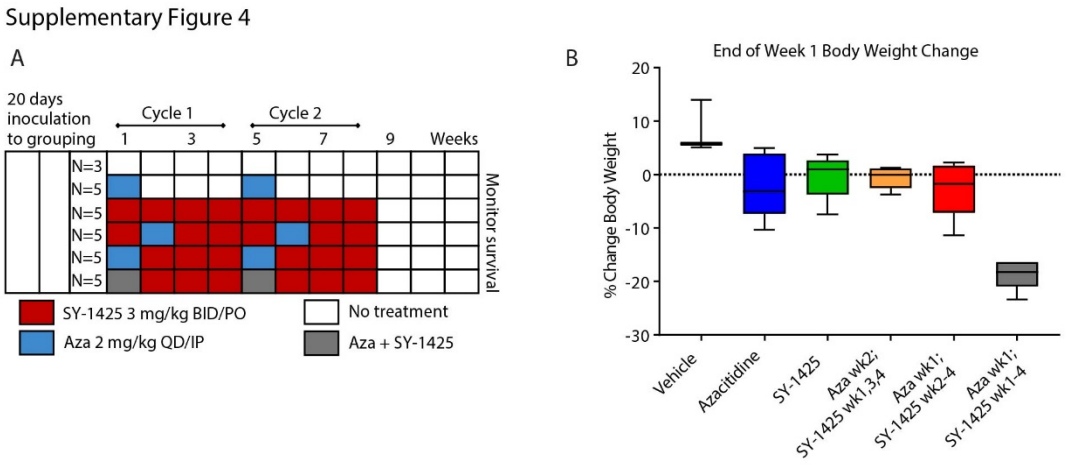
Supplemental Figure 2. Induction of apoptosis was measured via increase in caspase 3/7 activation with Promega™ Caspase-Glo® 3/7 Assay Systems (CN: G8093) per manufacture protocol. (A) In *RARA*-high SIG-M5 cells, cells were pre-treated for 24 hours with HMAs then

treated SY-1425. After 24 hours cells were measured for levels of activated Caspase-3/7. The combination of azacitidine and 50nM SY-1425 showed a significant increase (P<0.001) of Caspase-3/7 positive cells over single agent SY-1425 or HMA in SIG-M5 cells. (B-E) Western blot for DNA damage markers, PARP cleavage and pH2A.X in four cell lines. In all cases, the combination of SY-1425 +Aza or Dec, showed an increase in these markers directly related to the expression level of *RARA* or *IRF8*. (B) *IRF8*-high cells NOMO-1 showed increased PARP cleavage and pH2A.X in the combination only, with minimal effect from any single agents. (C) *RARA*-high SigM5 cells showed significant increase in PARP cleavage in pH2A.X with SY-1425 alone, which was further enhanced in the combination. (D) *RARA*-high MV;4-11 cells showed slight increase in PARP cleavage and pH2A.X with SY-1425, which was significantly enhanced in the combination. (E) *RARA*-low Kasumi-1 cells show little to no change in DNA damage markers, except for the response to single agent HMAs. (F) The log2 fold-change of expression vs. vehicle treatment for SY-1425 and the combination, with ITGAM and ITGAX in red. (G) Expression (log2) by condition. '*' indicates FDR<0.05 (only shown for comparison of SY-1425 vs. combination).

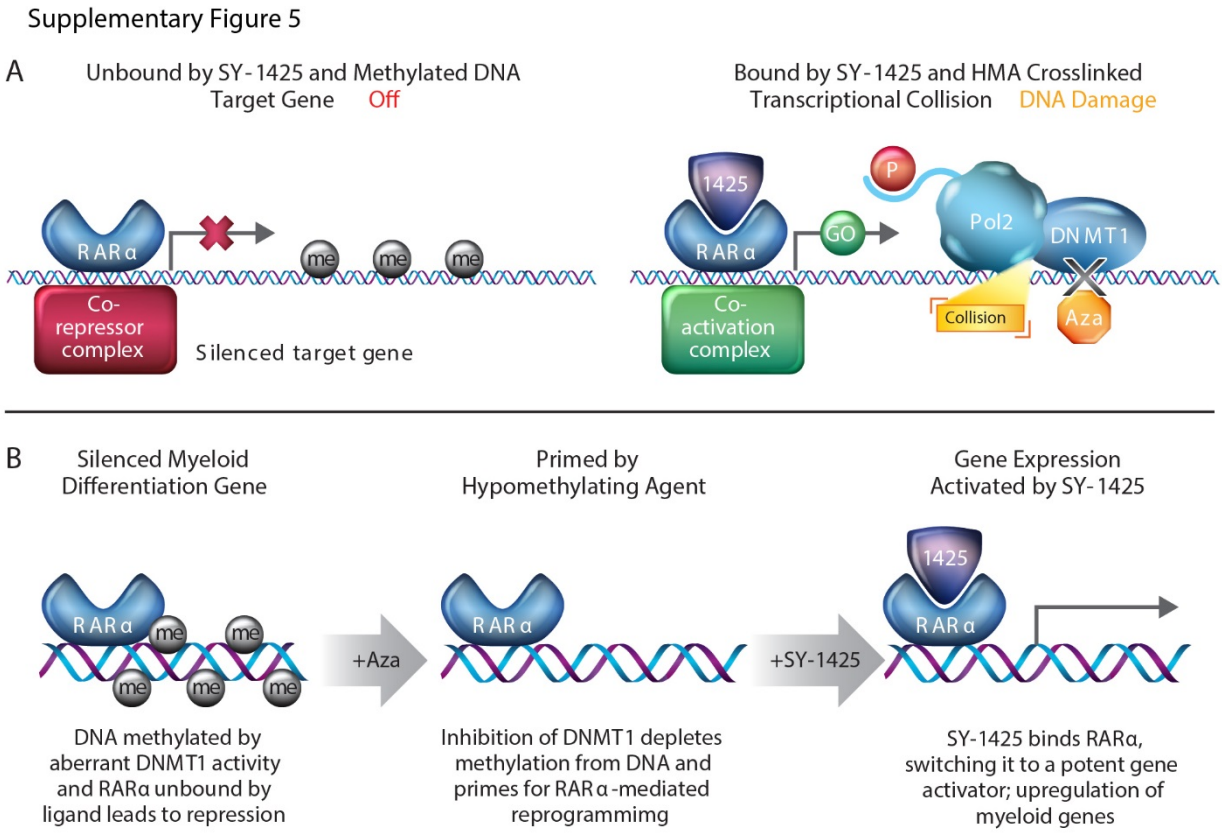
Supplementary Figure 3



Supplementary Figure 3. (A) Treatment schema for the patient-derived AML mouse models. Mice were inoculated with human tumor cells followed by initiation of treatment 20 days later. Treatment groups included vehicle only, azacitidine alone for 1 week, SY-1425 alone for 5 weeks, or azacitidine and SY-1425 for 1 week followed by SY-1425 alone for 4 weeks. SY-1425 was dosed at 3 mg/kg PO BID and azacitidine at 2.5 mg/kg IP QD for the first 7 days. (B) The percentage of huCD45+ cells in peripheral blood was assayed by FACS weekly to determine peripheral tumor burden in each treatment group. Arms were terminated by human endpoints as predefined by IACUC protocol. The blue and red arrowheads indicate the end of treatment for the azacitidine only and SY-1425 only arms, respectively. The asterisk (*) indicates that the combination therapy treatment ended on the same day as the SY-1425 only treatment.



Supplementary Figure 4. SY-1425 and HMA combination regimen study to optimize tolerability and tumor burden reduction. This figure shows the full treatment schema for all 6 groups tested. (A) Treatment schema for the patient-derived AML mouse models. Mice were inoculated with human tumor cells followed by initiation of treatment 20 days later. All groups had 2 cycles of treatment. The following treatment cycles were used for the 6 groups of mice: vehicle only; azacitidine alone for 1 week followed by no treatment for 3 weeks; SY-1425 alone for 4 weeks; SY-1425 alone for 1 week followed by azacitidine alone for 1 week, followed by SY-1425 alone for 2 weeks; azacitidine alone for 1 week followed by SY-1425 alone for 3 weeks; SY-1425 and azacitidine for 1 week followed by SY-1425 alone for 1 week. (B) Change in body weight after the first week of treatment. Percent change in body weight for each group is shown as a box and whisker plot. All treatment groups showed minimal BWL except for the group with concomitant administration of SY-1425 and azacitidine (gray). Aza, azacitidine; BID, twice a day; BM, bone marrow; huCD45+, human CD45+; IP, intraperitoneal; PO, orally; QD, once a day; wk, week.



Supplementary Figure 5. Proposed mechanism of SY-1425 and HMA synergy. (A) The combination of increased DNA methylation due to aberrant DNMT1 activity plus ligand-free RARα bound to gene promoters results in silencing of myeloid differentiation genes in tumor cells. Upon addition of HMAs such as azacitidine and decitabine, DNMT1 remains crosslinked to the DNA, resulting in a road block to replication and transcriptional machinery. Addition of the RARα agonist SY-1425 switches RARα to a potent gene activator and upregulates transcription, which cannot proceed due to the DNMT1-Aza blockade, resulting in DNA damage and cell death. (B) An alternate mechanism, where addition of HMAs inhibits DNMT1 and decreases methylation at gene promoters, priming RARα-mediated reprogramming. Addition of the RARα agonist SY-1425 switches RARα to a potent gene activator and upregulates the transcription of myeloid genes, resulting in cell differentiation that pushes blast cells to a postmitotic state. Aza, azacitidine; DNMT1, DNA methyltransferase 1; P, phosphorylation; Pol2, DNA polymerase II; me, methylation.