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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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Scientific category: MYELOID NEOPLASIA

Acute myeloid leukemia (AML) and biologically related myelodysplastic syndrome (MDS)1 are hematologic malignancies with poor outcomes. While recent approvals of new targeted therapies have increased options for some patients, those unfit for intensive treatment have few options,2–4 with single agent hypomethylating agents (HMAs) remaining as standard of care.1,5,6 The retinoic acid receptor alpha (RARα) transcription factor, encoded by the RARA gene, plays a critical role in myeloid cells and shows dysregulation in a subset of AML and MDS tumors.7 We recently demonstrated that the selective RARα agonist SY-1425 (tamibarotene) had biologic and clinical activity in 43% of evaluable relapsed or refractory AML and higher-risk MDS patients with activation of the RARA pathway.8 In this study, we sought to determine whether HMAs and SY-1425 exerted synergistic antiproliferative effects in AML models of RARA pathway activation in vitro and in vivo. Addition of HMAs and SY-1425 to RARA-high or IRF8-high, but not RARA-low, AML cell lines resulted in synergistic antiproliferative effects supported by evidence of DNA damage and apoptosis to a far greater extent than either agent alone. Studies in a patient-derived xenograft mouse model also demonstrated deeper and more durable responses with the combination than either agent alone. Furthermore, preclinical testing of various regimens determined that treating with azacitidine for 1 week followed by treatment with SY-1425 for 3 weeks maximized tumor suppression and tolerability. These findings directly support the ongoing clinical study of SY-1425 in combination with azacitidine.8

Both AML and MDS arise, in part, due to genetic alterations in transcription factors (ie RUNX1, NPM1) and epigenetic modifying genes (ie MLL, DNMT3A) leading to inactivation of tumor suppressor genes thus enabling proliferation of immature cells.9 Alterations in DNA methyltransferases (DNMTs) specifically result in DNA hypermethylation which contributes to gene silencing through promoter inactivation, and can be targeted by HMAs that mimic native nucleoside residues and incorporate into DNA. Once incorporated, HMAs are recognized by DNMT1 as a cytosine, however this interaction creates an irreversible DNA-DNMT1 adduct that requires DNA damage repair to resolve. This then results in loss of DNMT1, as the DNA-protein adduct is degraded by the DNA damage response pathway.9 After loss of DNMT1, the cell cannot maintain its methylation enabling re-initiation of cellular differentiation pathways and induction of proliferative arrest.5,9,10

We recently demonstrated that super-enhancer (SE) analysis can define novel epigenomic subtypes of non-APL AML. In non-APL AML, RARA pathway activation, detected by the presence of SEs at the RARA or IRF8 gene loci, was found to be predictive of response to SY-1425 in preclinical models, establishing the potential for biomarker-guided clinical studies.7,11
SY-1425 (tamibarotene) is a selective and potent agonist for the nuclear hormone receptor RARα, with improved pharmacological properties over first-generation pan-retinoids such as ATRA. Tamibarone is currently approved in Japan for the treatment of patients with relapsed/refractory APL. Since both SY-1425 and HMAs have demonstrated roles in treating myeloid malignancies, operate on the gene regulatory level, and have non-overlapping toxicities, we hypothesized that their combination could show synergistic therapeutic potential.

To examine whether there is synergistic anti-proliferative activity between HMAs and SY-1425, we explored the combination of either azacitidine or decitabine with SY-1425 in RARA-high, IRF8-high, and RARA-low AML cell lines (Figure 1, Supplemental Figure 1). Increasing concentrations of SY-1425 showed synergistic anti-proliferative effects in combination with either azacitidine or decitabine in RARA-high and IRF8-high AML cell lines as shown by the growth curves and combination isobolograms (Figures 1A, 1B, Supplemental Figure 1A, 1B). Synergy was noted over a wide range of concentrations supportive of potential for pharmacologic interaction across clinically relevant ranges. The RARA/IRF8-low cell lines, OCI-M1, and KG1a, did not show a synergistic interaction despite sensitivity to HMAs alone, as shown in Figure 1C (Figure 1C, Supplemental Figure 1C). This supports the exploration of the combination for enhanced anti-tumor activity in RARA pathway biomarker-positive tumors.

The strong reduction in cell number seen with the combination in vitro supported the potential for induction of cell death. To characterize the combination relationship with cell death, we treated the RARA-high cell lines (OCI-AML3, MV;4-11, and SigM5) with azacitidine or decitabine for 24 hours, followed by SY-1425 for 24 hours (Figure 2A, 2B, Supplemental Figure 2A). In all cases, treatment with single agent alone led to low levels of induction of apoptosis as evidenced by caspase 3/7 activation, but the combination of SY-1425 and azacitidine led to significantly higher levels of apoptosis. However, the degree of response to the combination was dependent on the relative RARA expression and sensitivity to SY-1425. RARA high cells OCI-AML3 and MV;4-11 each had increases in apoptosis of log2(FC)>0.5 in the combination (SY1425+Aza or SY-1425+Dec) over single agents. By comparison, the low cells Kasumi-1 and OCI-M1, both had weaker enhancement of apoptosis in the combination, as measured by log2(FC)<0.5, over the single agents (Figure 2C, 2D, Supplemental Figure 2E). Intriguingly, pretreatment with RG108, a mechanistically distinct DNMT inhibitor that is non-covalent and non-DNA integrating, did not lead to the same degree of apoptosis when combined with SY-1425, indicating that the mechanism for induction of apoptosis may rely on the unique way that azacitidine and decitabine function through DNA incorporation and DNMT1 covalent trapping (Figures 2A-2D).

Since both agents are directed to DNA interacting targets, we hypothesized that the source of cell killing could potentially originate from DNA damage. Indeed, the combination of SY-1425 with azacitidine or decitabine in RARA-high (OCI-AML3, MV;4-11, and SigM5) or IRF8-high (NOMO-1) cells resulted in induction of DNA damage as detected by PARP cleavage and phosphorylation of H2A.X, beyond that seen by HMA treatment alone (Figure 2E, Supplemental Figure 2B-2D). The RARA-low cell lines OCI-M1 and Kasumi-1 showed high levels of pH2A.X with HMA treatment alone, but little enhanced pH2A.X in the combination with SY-1425 (Figure 2F). Additionally, RG108 did not result in enhanced pH2A.X in the combination in any of the cell lines. This further supports that the DNA incorporation and covalent trapping of DNMT1 that occur with azacitidine and decitabine is needed for apoptosis induction (Figure 2E, 2F and Supplemental Figure 2B).
The combination could alternatively boost re-initiation of terminal differentiation through HMA mediated hypomethylation, priming RARα target genes for enhanced SY-1425 mediated activation. To explore this, we examined change gene expression changes in MV;4-11 (RARA high AML cells) to determine if any genes were significantly enhanced in the combination versus the single agents. Overall, there were minor differences in expression changes driven by SY-1425 single agent compared to the combination (Supplemental Figure 2C). However, certain key macrophage lineage genes such as ITGAX (CD11c) and ITGAM (CD11b), showed a slight increase in expression level between SY-1425 single agent and the combination (log2 fold-change 0.59 and 0.32 respectively, FDR<0.01, Supplemental Figure 2D). However, given that these genes already showed a large enhancement with SY-1425 alone, the slight increase in the combination is likely not enough to explain the in vivo response.

To further support the rationale for clinical investigation of the combination, SY-1425 and azacitidine were administered to a disseminated patient-derived xenograft (PDX) mouse model of RARA-high AML. An initial study assessing pharmacodynamics (design schematic Supplemental Figure S3A) found that while single agent administration resulted in stable disease over a five week course of treatment, only the combination resulted in regressions of tumors to undetectable levels, as assessed by quantification of human CD45+ cells in peripheral blood (PB). This deeper response also led to greater duration of response after treatment cessation compared with single agent administration (Supplemental Figure 3B). Tumor burden was further examined in the spleen (SP) and bone marrow (BM) of mice from each treatment group (Figure 3A-B). Based on morphology, there was a marked reduction in poorly differentiated blast cells in tissues following the combination treatment, including morphologic evidence of increased mature myeloid cells at 2 weeks, as compared to either agent alone.

Using the same PDX model, we sought to optimize the regimen in an expanded study by comparing vehicle to single agents, or combinations, varying the first two weeks of a cycle (design schematic Supplemental Figure 4A). Mice treated with either single agent showed stable disease in peripheral blood measurements while on treatment and some survival benefit over vehicle (Figure 3C, 3D). The combination of SY-1425 with azacitidine showed a decrease of peripheral tumor burden over vehicle and sustained reduction post cessation of treatment over single agents (Figure 3C) irrespective of the regimen chosen. Furthermore, there was reduction of disseminated tumor in tissues at end of treatment (Figure 3D) when compared between combination and vehicle (p<0.05 in PB, BM, SP) or single agents (azacitidine: p<0.05 in PB, BM, SP; SY-1425: p<0.05 PB, SP). However, only when azacitidine was given in the first week (either alone for 7 days or concomitantly with SY-1425) did we see a significant (p<0.05) benefit in survival over single agent treatment (Figure 3E). This observation aligns with the proposed mechanistic models wherein hypomethylation and DNMT1 cross linking primes cells before SY-1425 activates transcription through RARα, thus allowing optimal induction of apoptosis seen in the combination. In addition, while concomitant administration resulted in some weight loss, the staggered treatment arm demonstrated stable mouse weight (Supplemental Figure 4B), suggesting reasonable tolerability. Both HMA's (in AML and MDS) and SY-1425 (tamibarotene in APL) are generally well tolerated with non-overlapping side-effect profiles in their approved indications, supporting a potentially useful therapeutic combination strategy.

The results of our in vitro and in vivo studies provide a strong mechanistically-guided rationale for combining SY-1425 and HMA's in the clinical setting in patients with AML and RARA pathway activation (hypothetical model Supplemental Figure 5). Our evidence suggests the
combination may work via DNMT1 inactivation by HMA covalent cross-linking to DNA, causing DNA strain and damage when combined with strong target gene induction by SY-1425 at genes in AML that were previously shown to maintain a hypermethylated state. The importance of the cross-linking mechanism is further supported by the increased DNA damage observed with azacitidine/decitabine compared to the competitive inhibitor RG108. The single agents showed modest growth-arrest-driven effects \textit{in vivo}, while the combination led to a strong reduction in tumor burden to nearly undetectable levels, and < 5% in tissues. This supports the cell-killing effect of the combination that was characterized \textit{in vitro} as DNA damage and apoptosis. The safety and efficacy of SY-1425 in combination with azacitidine, as supported by these data, are currently being explored in a phase 2 trial in genomically defined subsets of patients with AML (NCT02807558).\(^7\)\(^,\)\(^15\)

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**References:**


Figure 1. SY-1425 and hypomethylating agents had synergistic antiproliferative activity in RARA-high and IRF8-high cells. SY-1425 was added in increasing concentrations in combination with either azacitidine or decitabine in (A) RARA-high OCI-AML3, or (B) MV;4-11 (B), or (C) RARA-low OCI-M1. Cellular growth inhibition was determined using ATPlite, as a calculation of fold change in signal in compound treated wells over vehicle (DMSO) 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. An isobologram for the RARA-low cell line (C) could not be calculated since there was no meaningful dose effect of SY-1425 in combination despite HMA activity. A marked decrease in cell viability was seen with increasing amounts of SY-1425 in RARA-high, but not which is not strongly seen in the RARA-low, cell line. Data shown is representative of results of three separate experiments.

Figure 2. Combination of SY-1425 and hypomethylating agents result in apoptosis and DNA damage in RARA-high and IRF8-high cells. Induction of apoptosis was measured via increase in caspase 3/7 with Promega™ Caspase-Glo® 3/7 Assay Systems in RARA-high AML cell lines (A) OCI-AML3 and (B) MV;4-11 as well as (C) RARA-low AML cell OCI-M1 and (D) Kasumi-1 pretreated with either DMSO, azacitidine, decitabine, or RG108 for 24 hours, followed by 24 Hours with SY-1425. There was a statistically significant increase in apoptosis induction (p<0.001 as measured by a two-tailed t-test) in cells treated with combination versus either SY-1425 alone or HMA alone, where the degree of response was related to the RARA expression. Upon addition of SY-1425 to cells treated with HMAs, DNA damage was detected via Western blot based on induction of pH2A.X and PARP cleavage in (E) RARA-high OCI-AML3, and (F) RARA-low OCI-M1 AML cell lines. OCI-AML3 showed synergistic increase in pH2A.X only in the combination of azacitidine or decitabine with SY-1425 but not with RG108. Contrastingly, OCI-M1 show high pH2A.X with treatment of azacitidine or decitabine alone, which was only slightly increased when combined with SY-1425. Data shown is representative of results of three separate experiments. AZA, Azacitidine; DEC, Decitabine; SA, Single Agent

Figure 3. Patient-derived AML xenograft models showed increased sensitivity to SY-1425 and HMA combination. Mice were inoculated with human tumor cells followed by initiation of treatment 20 days later when peripheral huCD45+ cells reached ~5%. Representative images from spleen and bone marrow from initial pharmacodynamic study. (A) Bone marrow (%huCD45 Cells: Vehicle: 29.3%, Aza: 12.8%, SY-1425: 12.7%, Aza+SY-1425:5.3%) and (B) spleen (%huCD45 Cells: Vehicle: 63.1%, Aza: 14.9%, SY-1425: 22.5%, Aza+SY-1425:19.5%) samples were sectioned and stained with H&E to characterize the cellular morphology. Numbers shown represent percentage of huCD45+ cells in respective condition as determined through FACS analysis of cell dispersion. Follow-up regimen study had 2 cycles of treatment for each group. The following treatment cycles were used for the 6 groups of mice (N=5 per group): 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. (C) The percentage of
human CD45+ cells in peripheral blood was assayed by FACS weekly to determine peripheral tumor burden in each treatment group. Arms terminated when all mice on arm had died. (D) The percentage huCD45+ cells in peripheral blood, bone marrow, and spleen was assayed by FACS at time of termination for tissues to determine tumor burden reduction. Data is reflected of tissue harvested at termination as determined by humane endpoint. (E) Survival curves for the 6 treatment groups. Both groups where Aza was given in the first week (red and gray curves) had a significant (p<0.05 Mantel-Cox test) increase in survival over either SY-1425 (red*) or Aza (black*) alone. In (C) & (D), the vertical dashed line indicates the end of treatment for all arms, and the horizontal dashed line indicates a tissue burden decreasing to < 5%. Aza, azacitidine; BID, twice a day; BM, bone marrow; huCD45+, human CD45+; IP, intraperitoneal; PO, orally; QD, once a day; wk, week.
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 \textit{RARA} or \textit{IRF8} (OCI-AML3, NOMO-1, SIG-M5, MV-4-11) or low levels of \textit{RARA} (OCI-M1) were studied. Qualification of \textit{RARA} or \textit{IRF8} levels were determined based off work previously published from our group.\footnote{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 \mu M), or decitabine (0.8-8 \mu M). 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.} 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 \mu M), or decitabine (0.8-8 \mu M). 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 \mu L 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\textsubscript{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\degree C incubator. Cell viability was evaluated using ATPlite (PerkinElmer) following manufacturer protocols. Data was analyzed in CalcuSyn using the median effect principle\footnote{Compounds were arrayed in 5-point dose response. Each compound plate received 1 compound. The 5-point dose response was centered on the EC\textsubscript{50} for the given cell line.} 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\textsuperscript{2} dishes with DMSO, 100 nM azacitidine, 100 nM decitabine, or 10\mu M 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 37\degree C CO\textsubscript{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\textsuperscript{2} dishes with DMSO, 100 nM azacitidine, 100 nM decitabine, or 10\mu M 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:**

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**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. 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 2x10⁶ 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 software4 (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 DESeq25 for differential expression analysis. The counts per gene were compared for the samples in each condition to determine the differentially expression genes.
References:


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.

(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.