Expansion of Primary Human AML by Aryl Hydrocarbon Receptor Antagonism Minimally Affects Leukemic Transcriptional Profiles but Alters Cellular Metabolism

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Abstract: Small molecule-based antagonism of the aryl hydrocarbon receptor (AHR) by StemRegenin1 (SR1) promotes *ex vivo* expansion and maintenance of primary human hematopoietic stem cells (HSCs) as well as acute myeloid leukemia (AML) cells. However, basis and nature of SR1 induced expansion of human AML remains unknown. Here, global expression profiling on 7 clinically diverse human AML patient samples treated *ex vivo* with a synthesized analog of SR1 (aSR1) uncovered that only as few as 750 genes were differentially regulated. Uniquely, aSR1 treatment did not modulate self-renewal associated pathways including HEDGEHOG, NOTCH or WNT across patient samples, but instead resulted in overall upregulation of the oxidative phosphorylation metabolic pathway. Higher oxygen consumption rates, along with increased sensitivity to the chemotherapeutic agent cytarabine (AraC) validated that aSR1-induced transcriptional profiles lead to functional enhancement of oxidative phosphorylation. Our study reveals that aSR1 induces minor alterations to the leukemic transcriptional profile leading to a shift in cellular metabolism. This finding should further instruct use of SR1-mediated expansion for mechanistic studies of leukemic self-renewal and the development of drug screening platforms using patient specific AML samples.

Keywords: Acute myeloid leukemia, aryl hydrocarbon receptor antagonism, cellular metabolism, *ex vivo* expansion, gene expression, oxidative phosphorylation, StemRegenin1.

1. INTRODUCTION

Acute Myeloid Leukemia (AML) is a complex and heterogeneous cancer of the human hematopoietic system [1], thought to be initiated and maintained by primitive, self-renewing leukemic cells [2]. An incomplete mechanistic understanding of the molecular dynamics that govern the leukemic state is largely attributed to the inability to effectively culture and maintain primary AML samples for study [3]. This has contributed to minimal changes to frontline disease treatment and poor rates of AML patient survival over the last 40 years [4]. Multiple approaches have aimed to define culture conditions that would enable mechanistic studies of leukemic self-renewal and development of drug-screening platforms *in vitro*. Maintaining AML *in vitro* using cytokines has proven ineffective [5], while stromal co-cultures that enable leukemic cell expansion require complex, long-term cultures [6]. Furthermore, efficient expansion techniques using genetic modifications [7, 8] or modulation of self-renewal pathways [9, 10] in healthy hematopoietic stem cells (HSCs) have either not been applied to AML or were unsuccessful. However, recent pharmacological screens building on an HSC expansion approach [11] have identified that StemRegenin1 (SR1) supports the expansion of $CD34^+$ AML cells *ex vivo* [12] through antagonism of the aryl hydrocarbon receptor (AHR) [11], an evolutionary conserved PER/ARNT/SIM (PAS)-domain-containing protein that senses and responds to environmental cell conditions [13].

Given the potential implications of this finding to the study of human AML, the effects of SR1-mediated expansion beyond AHR antagonism [11] need to be better defined. Here we use gene expression profiling and functional *in vitro* assays to discern that a synthesized analog of SR1 (aSR1) minimally alters the leukemic transcriptome, but enriches for genes involved in the oxidative phosphorylation (OXPHOS) metabolic pathway leading to a functional shift in cellular metabolism.

2. MATERIALS AND METHODS

2.1. Sample Collection

Primary, diagnostic AML (Table **A.1**) and healthy samples were obtained after informed consent,

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according to McMaster University Research Ethics Board-approved protocols. Mononuclear cells were isolated as previously described [14].

2.2. SR1 Analog Synthesis

Chemical synthesis of SR1 analog was performed based on previous description [15-17], as SR1 was not commercially available during our experimentation. Synthesis methods and characterization data are provided in Appendices.

2.3. Ex vivo Culture

AML cells were cultured in StemSpan serum-free media (StemCell Technologies) supplemented with stem cell factor (100ng/mL), FLT3 ligand (100ng/mL), thrombopoietin (20ng/mL) and vehicle control (DMSO) or aSR1 (1μM).

2.4. Phenotypic and Functional Analyses

Treated cell suspensions were harvested after seven days for phenotypic or clonogenic analysis. CD34 and CD45 antibodies (BD Pharmingen) were used for phenotypic analysis using a LSRII Flow Cytometer (BD). Data were analyzed using FlowJo software (TreeStar Inc., v.9.2). To assess clonogenic progenitor capacity using the colony forming unit (CFU) assay, AML cells (25,000) were plated in Methocult H4434 (StemCell Technologies). Colonies were stained with calcein at Day 7, imaged using an automated microscope (Operetta, Perkin Elmer) and quantified using customized Nearest-Neighbor based scripts implemented in Acapella. Significant differences between treatments were established using Student t-test p-value<0.05 (*) (GraphPad Prism v.4).

2.5. Gene Expression Profiling

As known AHR target genes are affected within 24h of treatment [11], and small molecule-mediated changes in global gene expression are best detected within this time frame [18, 19], AML cells treated with vehicle control or aSR1 were harvested after 24h. RNA was extracted using RNeasy Micro Kit (Qiagen), converted to cDNA and hybridized to a Human Gene 1.0 ST Array (Affymetrix). Raw data was normalized using Robust Multi-array Average (RMA) method. Principal Component Analysis and fold-change values were obtained using Partek Genomics Suite software (Partek Inc.). Functional analysis of gene expression profiling was performed using Gene Set Enrichment Analysis (GSEA) software [20].

2.6. Metabolic Characterization

Treated AML cells were harvested after 24h, and plated in XF96 V3 PS cell-culture microplates (50,000/well) in XF Base Medium (Seahorse Bioscience) supplemented with 0.5mM sodium pyruvate, 1mM L-glutamine and 3.2g/L glucose, adjusted to pH 7.4. Cells were incubated for 1h at 37^{o}C and placed on XFe96 Flux Pak hydrated cartridges for data acquisition using an XFe96 analyzer (Seahorse Bioscience). Oxygen consumption rates and extra-cellular acidification rates were compared across DMSO vs aSR1 treated samples to establish changes in cellular metabolism [21].

2.7. Inhibitory Concentrations of AraC

Dose-response curves of AraC in co-treatments with aSR1 or vehicle were performed for 24h (25,000 cells in 0.2mL). For 50% inhibitory concentrations (IC_{50}) analysis, cell counts of Hoechst positive and propidium iodide negative cells were performed on well images acquired using Operetta and analyzed using FCS Express (De Novo Software). IC_{50} fitting was done using the variable slope, four-parameters, least square fit model in Prism; and statistical significance was analyzed using Two-way ANOVA.

3. RESULTS AND DISCUSSION

3.1. AML Transcriptome is Minimally Affected by aSR1 Treatment, but is Enriched for Genes Involved in Oxidative Phosphorylation

AHR antagonism by SR1 promotes the expansion of primary human AML cells *in vitro* [12]. Toward investigating the transcriptional effects of AHR antagonism we first confirmed that treatment of primary healthy and AML patient samples with a synthesized analog of SR1 (aSR1) led to decreased expression of AHR target genes AHRR and CYP1B1, increased total numbers of $CD34^{+}$ cells, and increased frequencies of progenitor cells with CFU capacity *in vitro* (Figure **1**), like previous findings using SR1 [11, 12]. Having validated the efficacy of aSR1 expansion, we next selected seven primary AML samples that reflected the diversity and heterogeneity of disease observed clinically (Table **A.1**) and performed global gene expression analyses to assess aSR1 effects on their transcriptomes. The untreated AML transcriptional profile was generally conserved following aSR1 treatment (Figure **2A**), with only as few as 750 differentially regulated genes ($\geq$1.5 fold change, $p \leq 0.05$) observed in six of seven samples (Figure **2B**). These results indicate that aSR1 treatment minimally

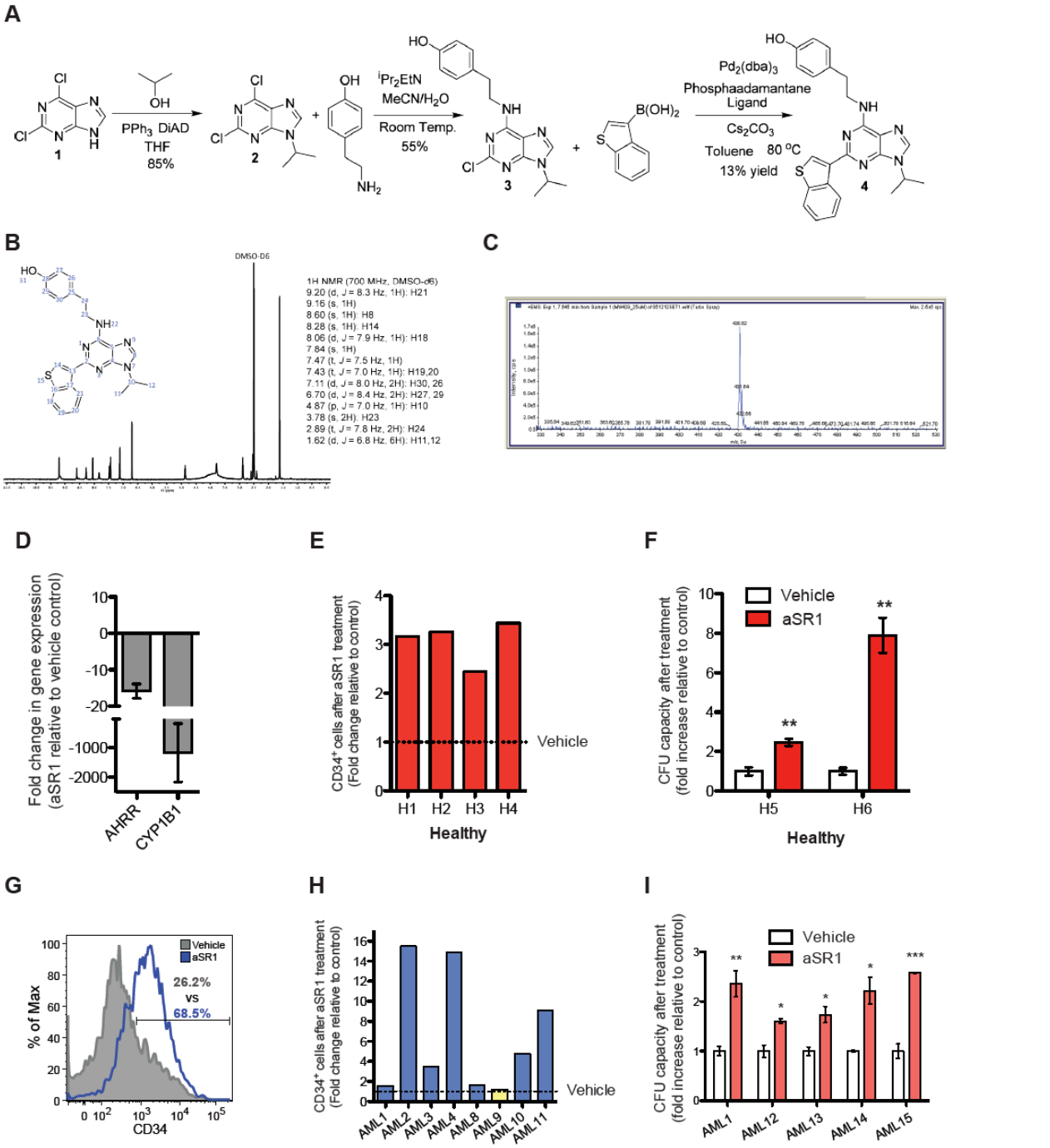

Figure 1: Synthesis and characterization of aSR1. (**A**) A Mitsunobu alkylation reaction converted 2,6-dichloropurine 1 into compound 2 in the presence of triphenylphosphine (PPh3), dry tetrahydrofuran (THF), anhydrous isopropanol, and diisopropyl azodicarboxylate (DiAD) with a 85% yield. Conversion of compound 2 into compound 3 was performed by addition of tyramine and diisopropylethylamine (iPr2EtN). The reduced crude reaction was purified by mass-directed preparative LC/MS with a 55% yield. Synthesis of compound 4 was done via condensation of compound 3 with boronic acid in the presence of phosphaadamantane ligand, cesium carbonate and the palladium catalyst: Pd2(dba)3. Purification of compound 4 was done using preparative LC/MS with a 13% yield. (**B**) Proton Nuclear Magnetic Resonance (1H NMR) spectra of compound 4 was analyzed in DMSO-D6. (**C**) The mass spectra of compound 4 dissolved in DMSO showed the strongest peak intensity at m/z=430.62. (**D**) Change in indicated gene expression in primary AML samples following aSR1 treatment relative to vehicle control. N=2 AML samples, performed in three technical replicates. (**E, F**) Bar graphs depicting the increased fold expansion of primary healthy $CD34^+$ cells and clonogenic CFU capacity following 7 days of culture in aSR1 relative to vehicle control. For clonogenic CFU assays, bars represent mean of three technical replicates $\pm$ Std dev. **p<0.01. (**G**) Representative flow cytometric histogram depicting a higher frequency of $CD34^+$-expressing primary AML cells following 7 days of culture with aSR1 vs vehicle control. N=8 primary AML samples. (**H, I**) Bar graphs depicting $\geq$ 1.5 fold expansion of total primary AML $CD34^+$ cells (7/8 samples) and significant increases in clonogenic CFU capacity after 7 days of culture in aSR1 relative to vehicle control. For clonogenic CFU assays, bars represent mean of three technical replicates per sample $\pm$ Std dev. *<0.05, **<0.01, ***<0.001.

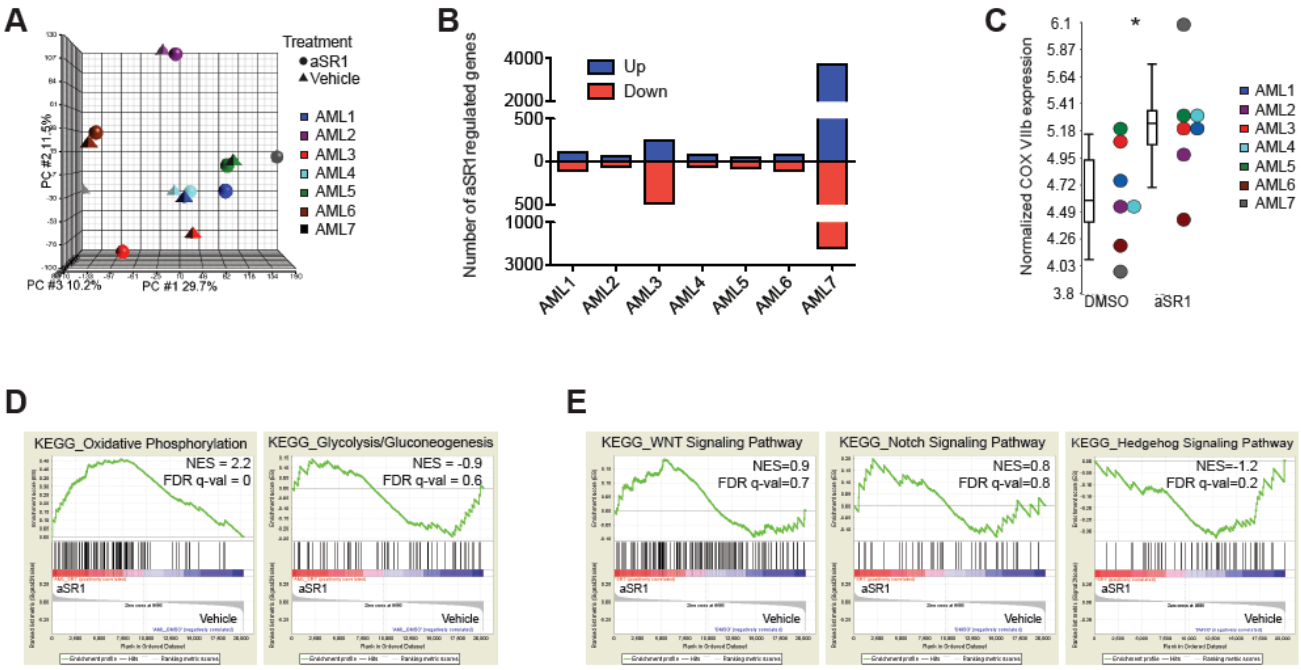

Figure 2: AML transcriptome is minimally affected by aSR1 treatment, but is enriched for genes involved in oxidative phosphorylation. (A) Principal component analysis (PCA) plot of global gene expression reveals that AML transcriptional profiles are conserved in the majority of AML samples treated with aSR1 for 24h; N=7 primary AML samples. (B) Bar graph depicting the number of genes differentially regulated by aSR1 treatment across 7 primary AML samples; fewer than 750 genes are affected by aSR1 in 6/7 samples. (C) Normalized COX -VIIb expression in 7 primary AML samples reveals a significant up-regulation in all samples following aSR1 treatment. One-Way ANOVA, *$p \leq 0.05$, FC$\geq$ 1.5. (D, E) Gene set enrichment analyses of primary AML samples demonstrating that OXPHOS pathway is enriched following aSR1 treatment (comparison of 7 aSR1-treated samples relative to 7 DMSO treated), but not glycolytic or self-renewal pathways. Normalized enrichment score (NES), and false-discovery rate (FDR) q-value are indicated.

affects global transcription signatures in most primary AML samples, in contrast to *in vitro* expansion achieved through modulation of self-renewal pathways where greater than 3500 genes can be differentially regulated (Figure **A1 A-B**) [22]. Further analysis revealed COX-VIIb to be the only gene that was affected (significantly upregulated, $p \leq 0.05$) across all AML samples (Figure **2C**), and one that has been previously demonstrated to increase in response to AHR ligands in mouse liver [23]. Since COX-VIIb is a subunit of the cytochrome-c oxidase complex that contributes to the OXPHOS metabolic pathway [24], we next investigated whether aSR1 caused a transcriptional enrichment in metabolic pathways. Overall, the OXPHOS pathway, but not the glycolytic pathway, was enriched (Figure **2D**). Moreover, WNT, NOTCH and HEDGEHOG pathways commonly associated with self-renewal and stemness were not affected (Figure **2E**) [25], indicating that aSR1 effects are not mediated through modulation of previously established early self-renewal pathways [9, 10]. Together, these results demonstrate that the leukemic transcriptional profile is minimally affected by aSR1 in the majority of AML samples assessed, and uniquely suggests that aSR1 affects cellular metabolism.

3.2. aSR1 Treatment Functionally Enhances Oxidative Phosphorylation in AML *in vitro*

To validate whether transcriptional observations manifested as changes to cellular metabolism, we measured changes in oxygen consumption rate (OCR) and extra-cellular acidification rate (ECAR) following aSR1 treatment as indicators of OXPHOS and glycolysis, respectively. Given the limited number of cells available and the inability to effectively expand the vehicle control-treated AML cells [5], we used a practical cell concentration (50,000 per well) for OCR and ECAR measurements and observed a consistent increase in OCR in six of seven AML samples following aSR1 treatment (Figure **3A**), with minimal changes to ECAR (Figure **3B**). These results suggested that cellular metabolism had shifted toward enhanced OXPHOS following aSR1 treatment. We next aimed to functionally validate these findings *in vitro*. It has been previously shown that increased sensitivity to AraC *in vitro* serves as a functional readout of a shift in cellular metabolism away from glycolysis [26]. Therefore, to functionally address and validate our finding that aSR1 treatment of AML mediates .a shift towards OXPHOS (away from glycolysis) (Figure **3B**), we interrogated

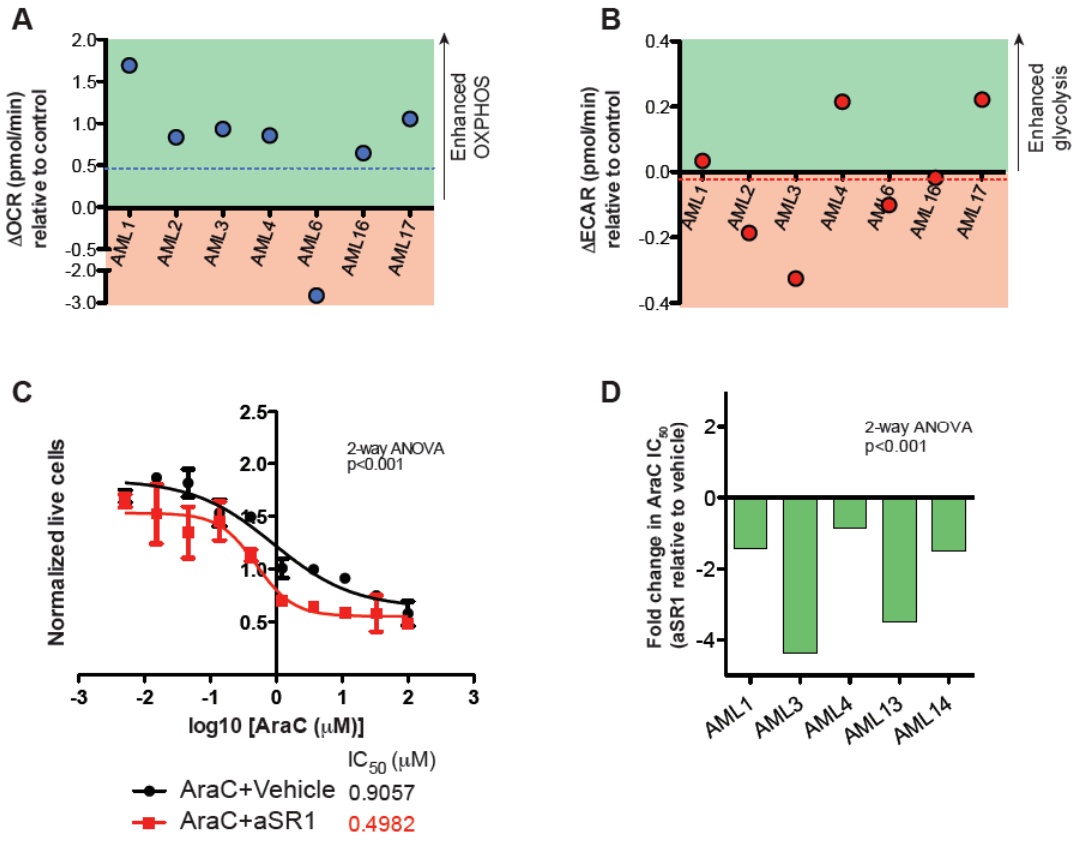

Figure 3: aSR1 treatment enhances oxidative phosphorylation in AML cells *in vitro*. (A) Dot plot depicting an increase in OCR across most AML samples treated with aSR1 relative to vehicle control. Dots represent average (n=3-6 technical replicates) ΔOCR in aSR1 versus vehicle control; dotted line represents average ΔOCR. Positive ΔOCR represents enhanced oxidative phosphorylation. (B) Dot plot depicting no consistent ΔECAR across aSR1 treated AML samples relative to vehicle control. Dots represent average (n=3-6 technical replicates) ΔECAR in aSR1 versus vehicle control; dotted line represents average ΔECAR. Positive ΔECAR represents enhanced glycolysis. (C) Representative dose-response curves demonstrating decreased AraC IC50 in primary AML sample co-treated with aSR1 versus vehicle control. Two-way ANOVA, p<0.001. (D) Bar graph depicting decreased AraC IC50 across five primary AML samples following co-treatment with aSR1 relative to vehicle control. Dose response curves were statistically analyzed using Two-way ANOVA, p<0.001."

whether aSR1 treatment increased the cytotoxic effects of AraC *in vitro.* These analyses indicated an increased sensitivity to AraC in all five AML patient samples tested that were treated with aSR1 (Figure **3C-D**). Together, our findings provide the first evidence that aSR1 alters the cellular metabolism of AML cells toward enhanced OXPHOS.

Unlike previous expansion approaches [7-10, 22], aSR1-mediated expansion does not act through modulation of expected self-renewal pathways and conserved global AML cell transcriptional profiles in the majority of AML patient samples. As such, SR1 treatment represents a viable means of expanding primary AML samples for further characterization of mechanisms that govern the leukemic state, without majorly altering transcriptional programs in a manner that may convolute or affect the results of molecular response studies to drug combinations. We reveal that SR1 shifts cellular metabolism toward enhanced OXPHOS. Future mechanistic studies that manipulate components of this pathway, including COXVIIb, using a systematic knockdown or gene expression-modulation approach may provide further insights into the individual contributions of metabolism-related proteins in mediating aSR1 expansion effects. Currently, we propose that aSR1-mediated metabolic changes may provide a cellular context that promotes AML expansion *ex vivo* in a manner similar to OXPHOS-driven expansion in $CD8^+$ T cells [27]. Accordingly, studies using SR1 expansion for the development of drug screening platforms should be cognizant of the novel effects on leukemic cell metabolism induced by SR1 treatment that may alter their sensitivity to candidate therapeutics. Together our findings provide a deeper and unique mechanistic understanding of SR1's effects on AML cells that should further instruct *in vitro* based studies and drug targeting campaigns using primary AML patient specific samples.

AUTHORSHIP

F.C. conceived and designed the study, acquired analyzed and interpreted data, drafted the manuscript; K.R.S. wrote and revised the manuscript for important intellectual content; Z.S. analyzed and interpreted data; B.G. acquired and interpreted data; T.J.C. interpreted data; M.B. conceived and designed the study, interpreted data, finalized writing of the manuscript, and approved the manuscript for submission.

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ABBREVIATIONS

AHR	Aryl hydrocarbon receptor
AraC	Cytarabine
AML	Acute myeloid leukemia
CFU	Colony forming unit
ECAR	Extracellular acidification rate
HSC	Hematopoietic stem cell
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
SR1	StemRegenin1
aSR1	Synthesized analog of SR1

APPENDIX

MATERIALS AND METHODS

In house synthesis of aSR1 and chemical characterization. The purine-derived analog of AHR antagonist SR1 (MW 430.62) was synthesized (aSR1) as described below and dissolved in DMSO (Sigma) for *in vitro* experiments. Synthesis started when 2,6-dichloropurine (2.64mmol) and triphenylphosphine (PPh3, 4.76mmol) were placed into a vacuum dried 100mL round-bottom flask under argon, followed by 25mL of dry tetrahydrofuran (THF)[16]. Using a syringe, anhydrous isopropanol (3.43mmol) was added while cooling in an ice bath. Diisopropyl axodicarboxylate (DiAD, 3.97mmol) was added drop wise until solution turned from yellow/orange to clear (5 drops). The mixture was stirred for 30 minutes when a white precipitate formed. Then, the mixture was removed from the ice bath and stirred overnight at room temperature under argon. The solvent was reduced and the reaction was purified using a Teledyne Isco Combiflash in 100% hexanes to 40% ethyl acetate. The collected product fractions contained compound 2 (2.24mmol) in 85% yield. Three mL of distilled water were added to a 25mL round-bottom flask containing tyramine (2.16mmol) and 15mL of acetonitrile (MeCN), followed by diisopropylethylamine (iPr2EtN, 4.13mmol). This mixture was added using a syringe to a separate 25mL round-bottom flask containing compound 2(2.05mmol) dissolved in 3mL of MeCN. The reaction was stirred at room temperature for 30 minutes. The crude reaction was reduced and purified by preparative liquid chromatography-mass spectrometry (LC/MS), yielding compound 3 (1.12mmol, 55% yield). ^{1}H NMR (700 MHz, DMSO-*d*6) δ 8.24 (m, 1H), 7.06 (m, 1H), 6.67 (m, 1H), 4.66 (p, J = 7.0 Hz, 1H), 3.58 (m, 2H), 2.78 (t, J = 7.8 Hz, 2H), 1.49 (d, J = 6.8 Hz, 6H). ^{13}C NMR (176 MHz, DMSO-*d*6) δ 155.6, 154.9, 152.9, 149.2, 139.1, 129.5, 129.3, 118.3, 115.1, 46.7, 41.9, 33.9, 22.1. A 25mL round bottom flask containing boronic acid (0.517mmol), phosphaadamantane ligand (0.051mmol)[17], cesium carbonate (1.04mmol) and Pd2(dba)3 (0.026mmol) was purged with argon. Three mL solution of compound 3 (0.259mmol) in 1,4-dioxane was added to the mixture via syringe. The reaction was heated to 80°C under argon for 4 hours, then cooled to room temperature and purified using mass-directed preparative LC/MS to obtain compound 4. ^{13}C NMR (176 MHz, DMSO-d^6) δ 157.9, 157.7, 155.6, 155.5, 154.0, 140.5, 139.0, 137.0, 135.4, 130.5, 129.8, 129.5, 125.5, 124.5, 124.3, 122.9, 115.1, 46.7, 41.9, 34.5, 22.2. aSR1 was dissolved into a stock solution of 10mM in DMSO and stored at-80^{o}C before dilutions were made for *in vitro* studies.

Table A.1: Primary AML Patient Samples

Sample	Disease Status	Source	Cytogenetic Abnormality	Molecular Abnormality
AML1	Progression from MDS§	PB	inv(3)(q21q26.2)	ND†
AML2	New diagnosis	BM	t(11;17)(q13;q21)	ND†
AML3	New diagnosis	PB	Normal	ND†
AML4	New diagnosis	PB	Normal	NPM1
AML5	Progression from MDS§	BM	inv(3)(q21q26.2)	ND†
AML6	New diagnosis	PB	-Y, add(18)(q21.1)	ND†
AML7	New diagnosis	BM	t(9;11); t(11q23)	Normal
AML8	New diagnosis	BM	Normal	FLT3-ITD
AML9	Relapse	PB	Normal	NPM1 and FLT3-ITD
AML10	New diagnosis	PB	Normal	NPM1 and FLT3-ITD
AML11	Progression from MDS§	BM	Normal	ND†
AML12	New diagnosis	PB	+8, +13	FLT3-ITD
AML13	New diagnosis	PB	Normal	NPM1
AML14	New diagnosis	BM	t(8,21), -Y	Normal
AML15	New diagnosis	BM	Normal	NPM1 and FLT3-D835
AML16	New diagnosis	PB	Normal	Normal
AML17	New diagnosis	PB	Normal	Normal

†, ND = Not Determined; §, MDS = Myelodysplastic Syndrome; AML= Acute Myeloid Leukemia.

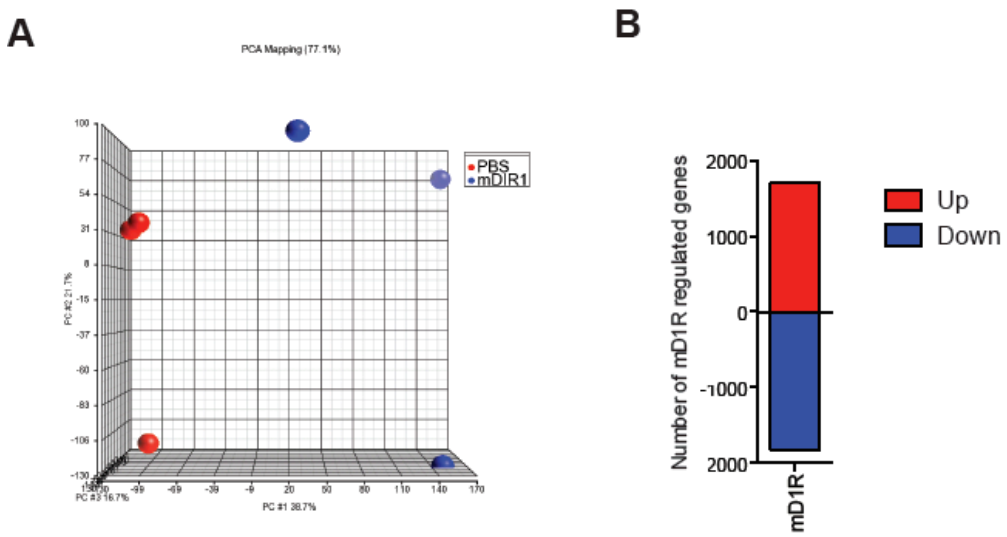

Figure A.1: Effects of Notch-mediated expansion on global transcription. (**A**) PCA plot of mouse bone marrow cells expanded *ex vivo* using mD1R ligand 20. (**B**) Total number of genes differentially regulated (≥1.5 fold, p≤0.05) by mD1R ligand.

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