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The novel Isatin analog KS99 targets stemness markers in acute myeloid leukemia

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ABSTRACT

Leukemic stem cells are multipotent, self-renewing, highly proliferative cells that can withstand drug treatments. Although currently available treatments potentially destroy blast cells, they fail to eradicate leukemic progenitor cells completely. Aldehyde dehydrogenase and STAT3 are frequently up-regulated in pre-leukemic stem cells as well as in acute myeloid leukemia (AML) expressing the $CD34^+CD38^-$ phenotype. The Isatin analog, KS99 has shown anticancer activity against multiple myeloma which may, in part, be mediated by inhibition of Bruton's tyrosine kinase activation. Here we demonstrate that KS99 selectively targets leukemic stem cells with high aldehyde dehydrogenase activity and inhibits phosphorylation of STAT3. KS99 targeted cells co-expressing CD34, CD38, CD123, TIM-3, or CD96 immunophenotypes in AML, alone or in combination with the standard therapeutic agent cytarabine. AML with myelodysplastic-related changes was more sensitive than *de novo* AML with or without *NPM1* mutation. KS99 treatment reduced the clonogenicity of primary human AML cells as compared to normal cord blood mononuclear cells. Downregulation of phosphorylated Bruton's tyrosine kinase, STAT3, and aldehyde dehydrogenase was observed, suggesting interaction with KS99 as predicted through docking. KS99 with or without cytarabine showed *in vivo* preclinical efficacy in human and mouse AML animal models and prolonged survival. KS99 was well tolerated with overall negligible adverse effects. In conclusion, KS99 inhibits aldehyde dehydrogenase and STAT3 activities and causes cell death of leukemic stem cells, but not normal hematopoietic stem and progenitor cells.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease with treatment relying primarily on traditional cytotoxic agents and hematopoietic stem cell transplantation. AML arises from hematopoietic stem and progenitor cells (HSPC) through various alterations in stem cells.[1] During blast transformation, mutant progenitors undergo stepwise genetic, epigenetic and clonal changes, and give rise to pre-leukemia stem cells (pre-LSC) as well as fully transformed leukemia stem cells (LSC).[2] These cells are frequently chemo-resistant, and their division leads to clonally aggressive AML.[1] Thus, effective therapies are warranted to destroy AML stem cells selectively, but not normal HSPC. While LSC were initially defined as cells with $CD34^+CD38^-$ phenotype with ability to engraft in mouse models,[2-5] recent data have demonstrated $CD34^+CD38^+$ AML cells also have an engraftment poten-

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tial in animal models.[4,6-8] After relapse, numbers of LSC increase dramatically and CD34$^-$ cells often acquire engraftment potential.[6,9]

Inclusion of additional AML-specific LSC surface antigens, including CD123, CD96 and TIM-3, can help identify and target resistant leukemic cells.[10-13] It has been suggested that the self-renewal capacity of otherwise quiescent AML-LSC is supported by upregulation of the surface marker T-cell immunoglobulin mucin-3 (TIM-3). TIM-3 is not expressed in normal HSC, suggesting that the TIM-3$^+$ population may contain the great majority of functional LSC in most types of AML.[14] These markers play a role in activating the inactive LSC for the purpose of self-renewal and disease maintenance, thus facilitating relapse with minimal to moderate survival benefit.[12-16]

Stem cells protect themselves by upregulation of aldehyde dehydrogenase (ALDH), a cytosolic enzyme that guards them against the DNA damage induced by reactive oxygen species and reactive aldehydes.[17] A population of CD34$^+$CD38$^-$ leukemic cells with moderate ALDH activity has been shown to contribute to relapse in AML.[18] Targeting intracellular markers including ALDH and signal transducer and activator of transcription 3 (STAT3) in LSC marked by additional surface markers like CD34, CD123, TIM-3 or CD96 may validate therapeutic targets more efficiently. Despite substantial advances in the understanding of LSC markers, so far, no agents have been made available in the clinic to selectively target these progenitors. Cytarabine (Ara-C) and anthracyclines (7+3) are the current standard induction and consolidation therapy for AML, but these regimes only provide moderate therapeutic benefit.[19] The recent approval of novel agents including venetoclax, gilteritinib, and midostaurin has advanced therapy.

In this study, we identify the unexplored anti-LSC activity of the recently published small molecule Isatin analog, KS99. Earlier studies had established KS99 as an anti-microtubule agent with a dual role as Bruton's tyrosine kinase (BTK) inhibitor in multiple myeloma (MM).[20] Since BTK has a role in the maturation and regulation of dendritic cells (DC) *via* interleukin 10 (IL-10) and Signal transducer and activator of transcription 3 (STAT3), blocking BTK carefully modulates the STAT3.[21] Modulation of STAT3 is important in prolonging survival of AML patients, especially considering that upstream mutations result in the activation of STAT3 and the protein per se is not mutated in this condition.[22] STAT3 activity in LSC is associated with a poor prognosis in AML patients, possibly because it contributes to resistance to chemotherapy.[22,23] ALDH has been identified as a potential biomarker and therapeutic target in chemoresistant AML.[24-26] Here, we report that, besides BTK inhibition, KS99 targets stemness markers, STAT3, and ALDH, in putative LSC expressing surface CD34, CD123, TIM-3, and CD96. We demonstrate that KS99 is active against AML as a single agent or in combination with standard of care Ara-C.

Methods

The *Online Supplementary Appendix* contains detailed information on experimental methods and materials.

Cell lines and cell culture

Details of the acute myeloid leukemia cell line culture conditions are provided in the *Online Supplementary Appendix*.

Acute myeloid leukemia patient and healthy donor cells

Bone marrow (BM) aspirates or peripheral blood (PB) samples were obtained from AML patients, and cord blood (CB) samples were obtained from the freshly delivered placenta of healthy donors after informed consent using protocols approved by the Penn State College of Medicine Institutional Review Board (IRB) (#2000-186). Mononuclear cells (MNC) were isolated by density gradient separation (Ficol-Paque, GE Healthcare Life Sciences, Pittsburgh, PA, USA) and frozen for later use. Details are provided in the *Online Supplementary Appendix*.

Cell viability and Annexin V assay

Cell viability and apoptosis were determined using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) and Muse Annexin V & Dead Cell Kit (MCH100105, Millipore, Burlington, MA, USA). Details are provided in the *Online Supplementary Appendix*.

Colony-forming assay

Cryopreserved human AML patient samples and cord blood mononuclear cells were thawed and washed with RPMI 1640 (10% FBS) and used for the colony formation assay. Details are provided in the *Online Supplementary Appendix*.

Western blot analysis

Acute myeloid leukemia cells were treated with indicated concentrations of KS99 or DMSO. Cells were collected, washed with cold PBS, and whole cell lysates were harvested. Further details are provided in the *Online Supplementary Appendix*.

Flow cytometry

To detect apoptosis in LSC, DMSO, KS99 or Ara-C-treated cells were washed and stained with various markers; anti-human CD45 conjugated with APC-Cy7, CD34-FITC, CD38-APC, CD123-APC, TIM-3-PE-Cy7, or CD96-BV711 monoclonal antibodies for 30 minutes on ice, followed by Annexin V-BV421 and 7AAD staining. Further details are provided in the *Online Supplementary Appendix*.

Aldehyde dehydrogenase assay

The enzyme activity of ALDH was measured by using ALDEFLUOR kit (StemCell Technologies, Vancouver, Canada), as described in the manufacturer's protocol. It is a fluorescent-based assay that detects ALDH1A1 isoform, which is highly expressed in stem cells. Further details are provided in the *Online Supplementary Appendix.*

Animal studies

Acute myeloid leukemia cell transplantable models luciferase-expressing human AML cell lines (U937 and MV4-11) and murine AML cell line (C1498) were used to investigate the efficacy of KS99. In addition, the pharmacokinetics of the drug were examined to determine the circulating levels of KS99 in the blood. Further details are provided in the *Online Supplementary Appendix.*

In silico docking of KS99 with ALDH1A1, BTK, and STAT3

Details are provided in the *Online Supplementary Appendix*.

Statistical analysis

The statistical analysis methodology is described in the *Online Supplementary Appendix.*

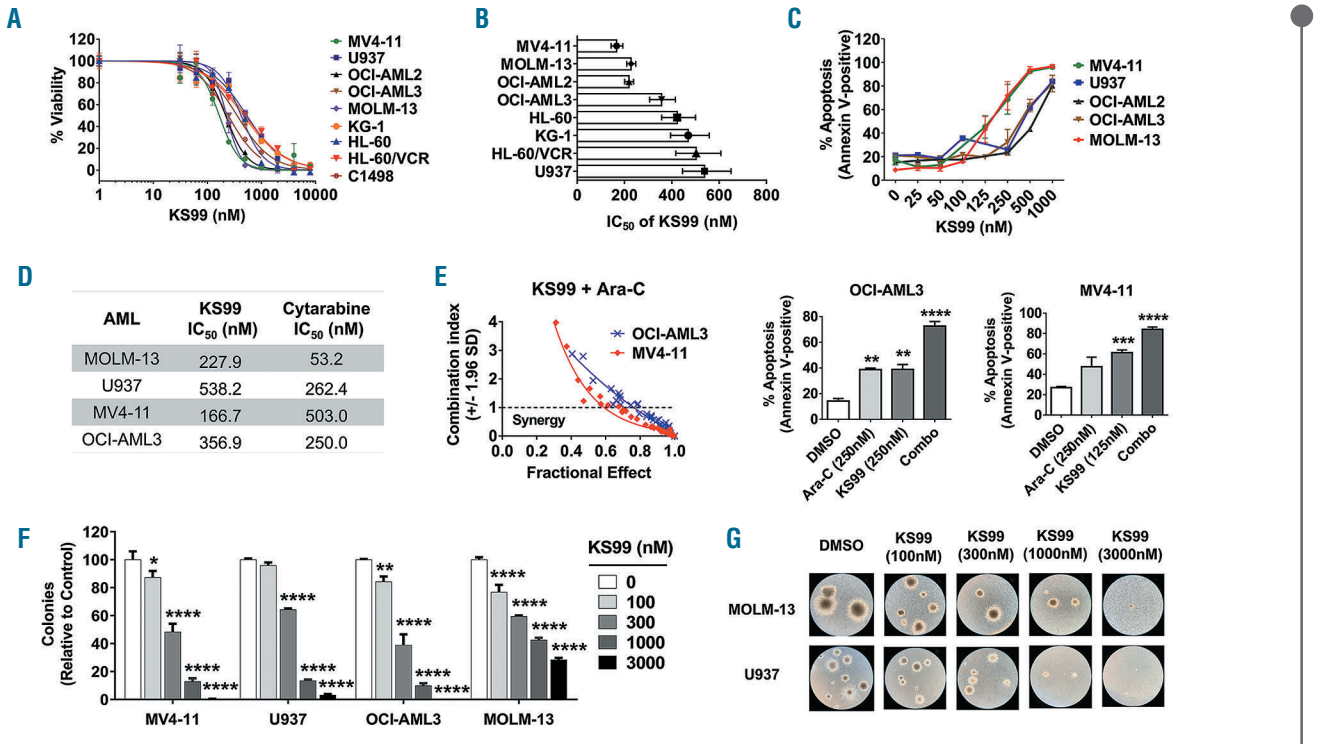

Figure 1. KS99 inhibits cell proliferation and clonogenicity and induces apoptosis in human acute myeloid leukemia (AML) cell lines. (A) Cell viability of AML cell lines after the treatment with KS99. (B) IC_{50} values for AML cell lines were plotted with 95% confidence intervals. (C) Induction of apoptosis with KS99 was determined as the percentage of Annexin V-positive cells. (D) The sensitivity of human AML cell lines to KS99 or Cytarabine (Ara-C) alone. (E) OCI-AML3 and MV4-11 cells were treated with increasing doses of KS99, Ara-C or combination. (E, left) Combination Index (CI) values of KS99 and Ara-C co-treatment were calculated by CalcuSyn. Synergy CI<0.9. (E, right) Apoptosis was determined as the percentage of Annexin V-positive cells. (F and G) KS99 reduced the colony-forming ability of AML cell lines. The representative colony microscopy images (4X) are shown as indicated. Data are the mean±standard error of the mean. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; one-way ANOVA.

Results

KS99 induces apoptosis and reduces cell survival of human acute myeloid leukemia cell lines

To assess the effect of KS99 on AML cells *in vitro*, a panel of human leukemic cell lines [MOLM-13, MV4-11, OCI-AML2, OCI-AML3, HL-60, vincristine resistant HL-60 (HL-60/VCR), U937, and KG-1], and mouse leukemia C1498 cells were selected. Cell viability was measured after treatment with KS99 (10 nM-10 μM) for 48 hours (h). For most of the cell lines, KS99 treatment led to a decrease in the viability in the nanomolar (nM) range (Figure 1A). The half inhibitory concentration (IC_{50}) values for all the human cell lines were between 100 nM and 600 nM. Specifically, MV4-11, MOLM-13, and OCI-AML2 manifested higher sensitivity with lower IC_{50} values of 166 nM, 228 nM, and 218 nM, respectively. The other cell lines showed relatively higher IC_{50} values (300-600 nM) (Figure 1B). Similarly to human AML cells, mouse C1498 cells were sensitive to KS99 with an IC_{50} of 217 nM.

Furthermore, flow cytometry demonstrated apoptosis on selected cell lines. KS99 induced apoptosis within the nanomolar range (100-500 nM) in a dose-dependent manner (Figure 1C). MOLM-13 cells were chosen for time-dependent survival, and the outcome showed an early decrease in cell proliferation at 6-8 h with a prominent decline in viability post 24-48 h of treatment (*Online Supplementary Figure S1A*). Simultaneously, apoptosis was evident as early as 4 h of treatment with maximum effect at 48 h (*Online Supplementary Figure S1B*). These time points were considered when further functional assays were conducted. Chemotherapeutics are clinically often used in combination to achieve complete remission (CR) in AML patients. Hence, we decided to compare and combine KS99 to Ara-C to evaluate an increase in efficacy of Ara-C. The sensitivity of AML cell lines to KS99 and Ara-C is shown in Figure 1D. For the synergy studies, OCI-AML3 and MV4-11 cells were treated with Ara-C (0.062-4 μM) and KS99 (0.03125-2 μM) for 72 h, and the combination index (CI) was calculated. KS99 significantly increased cytotoxic responses, in combination with Ara-C, and showed synergy (CI<0.9) in both cell lines (Figure 1E, left panel). Furthermore, subtoxic KS99 concentrations (lower than IC_{50}) reduced IC_{50} of Ara-C by a median of 2-3-fold (*Online Supplementary Figure S1C*). Similarly, KS99 augmented the pro-apoptotic effect of Ara-C in OCI-AML3 and MV4-11 cells (Figure 1E, right panel). Next, the inhibitory effect of KS99 on colony forming ability of human AML cell lines was determined. The treatment of KS99 led to a decrease in the number and size of colonies across the selected range and cell lines (Figure 1F and G). These results show that KS99 is active in AML and can be combined with Ara-C to enhance anti-leukemic activity.

KS99 exerts a cytotoxic effect in primary human acute myeloid leukemia and favors cases with poor prognosis

The pro-apoptotic activity of KS99 was tested on newly diagnosed and untreated primary human AML patients (n=21). Cells were treated with increasing concentrations of KS99 (0.1-6 μM) for the 48 h. The IC_{50} values were cal-

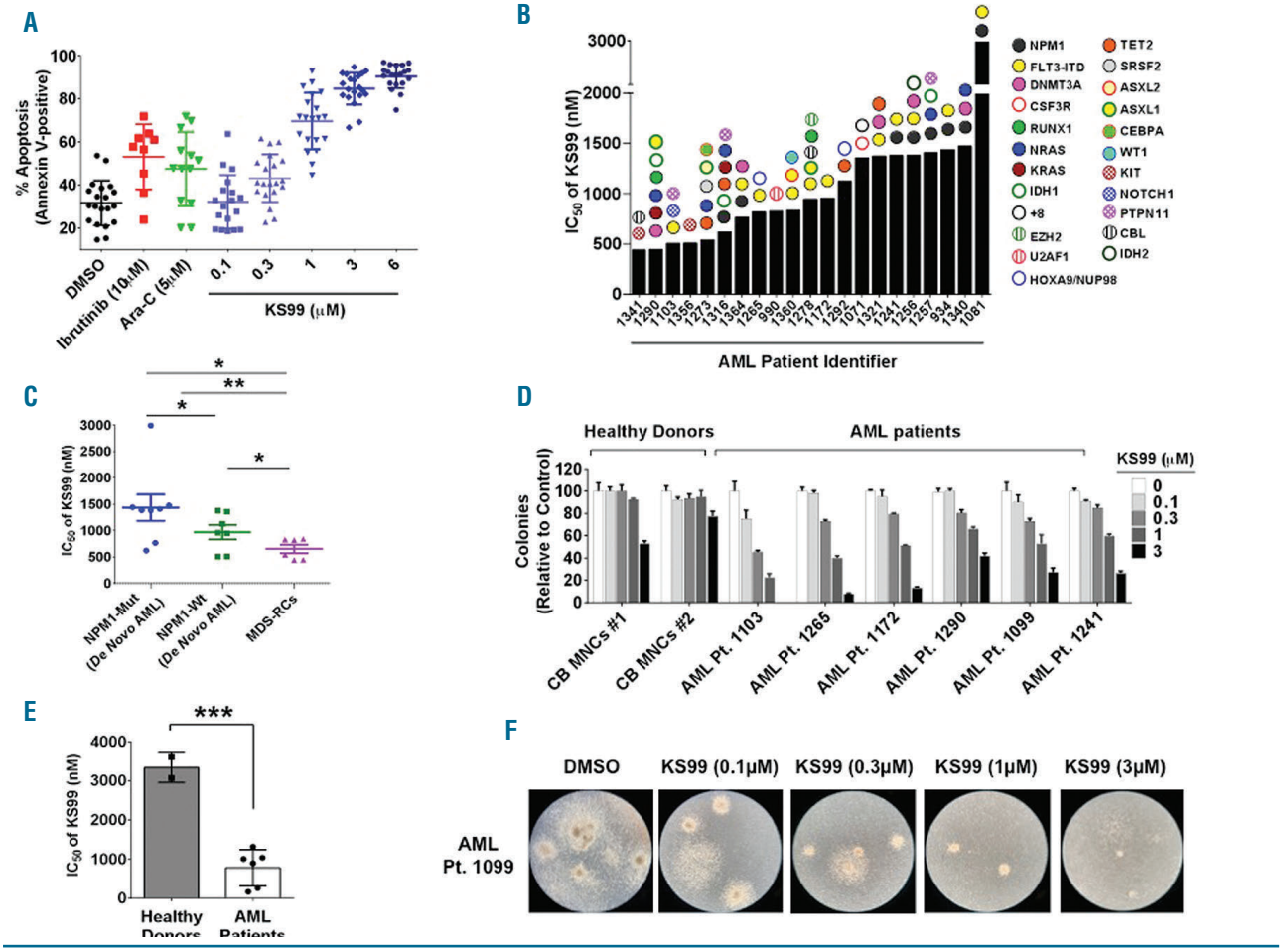

Figure 2. KS99 induces apoptosis and inhibits clonogenicity in primary human acute myeloid (AML) leukemia cells. Sensitivity of primary human AML cells to KS99 (n=21), Cytarabine (Ara-C) (n=13) or ibrutinib (n=9) after 48 hours (h) treatment. (A) Apoptosis was determined as the percentage of Annexin V-positive cells. (B) The IC_{50} values of KS99 for primary human AML cells, based on the number of viable cells (i.e. Annexin $V^-/7AAD^-$). Individual genetic mutations are indicated by colored points. (C) Comparison of IC_{50} values of KS99 for AML subgroups. AML with myelodysplastic-related changes (MDS-RC) was compared to *de novo* AML (*NPM1* mutant *vs.* wild type). (D-F) Primary human AML samples and cord blood mononuclear cells (CB MNC) obtained from healthy donors were treated with indicated concentrations of KS99 and colonies were counted after 10-14 days. Data represent triplicate wells (n=3). (D) Bar graphs show the dose-dependent response of KS99. (E) Bar graph shows a comparison of IC_{50} values of KS99 for AML patient samples and CB MNC in the colony-forming assay. (F) Representative microscopy images (4X) of AML Patient 1099 colonies after KS99 treatment. Data are the mean±standard error of the mean (SEM) **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; unpaired *t*-test.

culated on the basis of Annexin-V/7AAD signals determined by flow cytometry. The sensitivity of primary human AML cells to KS99 was compared to AML standard of care agent, Ara-C. Since KS99 has been reported earlier by our group as BTK inhibitor,[20,27,28] a known BTK inhibitor, ibrutinib[27] was also included in this study for comparative purposes. Interestingly, almost all the primary cells from AML patients were quite sensitive to KS99 at a lower micromolar range (0.3-3 μM) (Figure 2A). The effectiveness of 5 μM Ara-C or 10 μM ibrutinib was equivalent to 0.3-1 μM KS99, defining the potential of relatively low doses of KS99 on patient samples (Figure 2A). We next tested whether sensitivity to KS99 is correlated with the mutational status of primary AML samples. The sensitivity of AML patient samples (n=21) with individual mutation status is shown in Figure 2B. Clinical and genetic data for AML patient samples are shown in *Online Supplementary Table S1*. It is important to note that cases associated with poor prognosis had lower IC_{50} values and thus higher sensitivity to KS99 (left to right of Figure 2B). *NPM1*/FLT-3-ITD status seems to be resistant (found in 4 of 6 with highest IC_{50}). The AML subset, AML with myelodysplastic-related changes (MDS-RC), were more sensitive than *de novo* AML cases (*P*=0.0077) with or without an *NPM1* mutation (*P*=0.012, *P*=0.045, respectively) (Figure 2C). Within the *de novo* AML, *NPM1* wild-type cases were more sensitive than *NPM1* mutant cases (*P*=0.02) (Figure 2C).

KS99 targets leukemic progenitor cells while sparing normal hematopoietic stem and progenitor cells

Primary human AML cases (n=6) with various cytogenetic and molecular status (*Online Supplementary Table S1*) were selected to test the anti-leukemic activity of KS99 in colony-forming assay. Normal cord blood mononuclear cells (CB-MNC) (n=2) were used as HSPC controls to demonstrate the selectivity of KS99 towards LSC. The colony-forming capacity of primary human AML cases was significantly reduced in the presence of KS99, while normal CB-MNC were much less sensitive (Figure 2D). CB-MNC had significantly higher IC50 values (4.2-fold) as compared to primary human AML cases (Figure 2E).

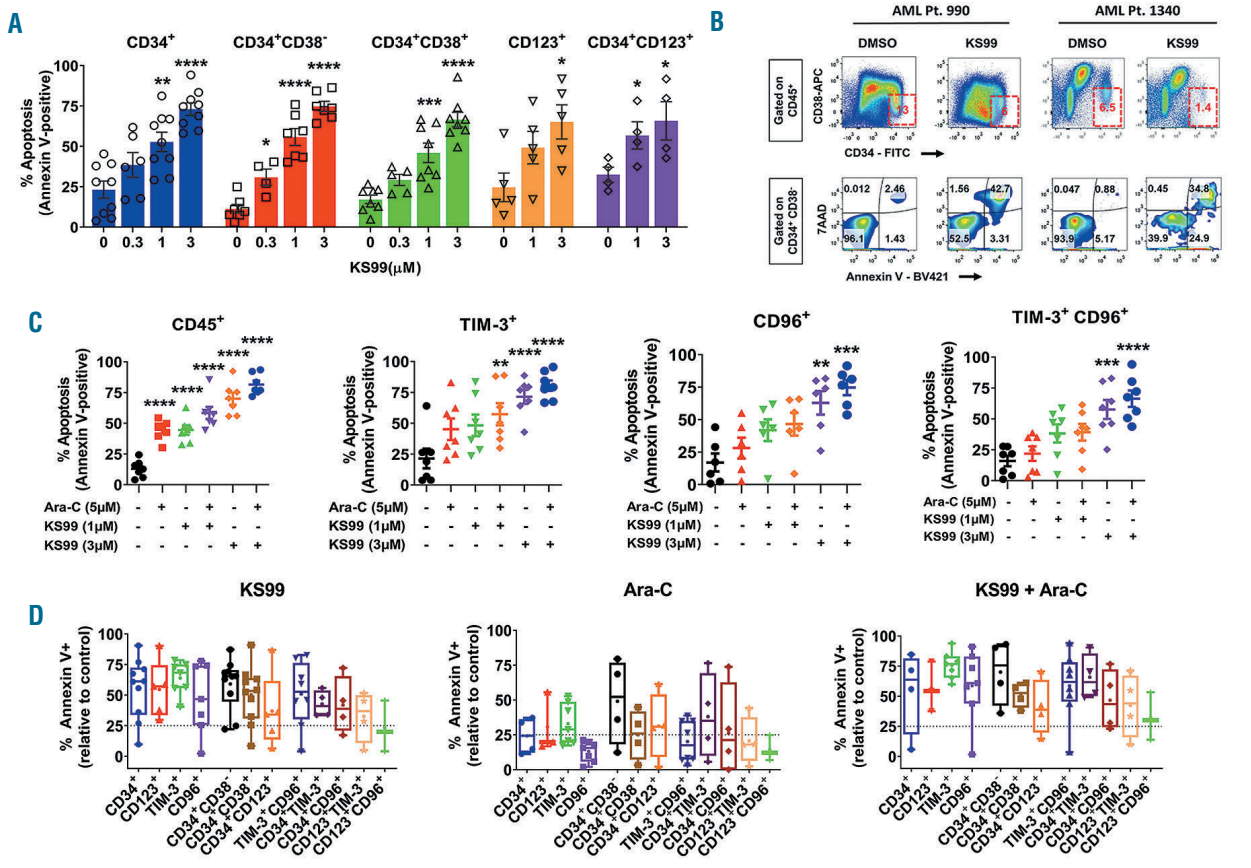

Figure 3. KS99 induces apoptosis in leukemic stem cells (LSC). (A) Dose-dependent apoptotic response of KS99 in primary human leukemic stem cells (LSC) identified as CD34$^+$, CD34$^+$CD38$^-$, CD34$^+$CD38$^+$, CD123$^+$, or CD34$^+$CD123$^+$ cells. Error bars are mean±standard error of the mean (SEM). (B) Representative flow cytometric analysis of cell death in LSC. (C) Apoptosis in CD45$^+$, TIM-3$^+$, CD96$^+$, or TIM-3$^+$CD96$^+$ cells after the treatment with KS99 and Cytarabine (Ara-C). Error bars are mean±SEM. (D) Apoptotic response of KS99, Cytarabine (Ara-C) or combination in primary human AML cells expressing or co-expressing LSC immunophenotypes; CD34, CD38, CD123, TIM-3, and CD96. Data were normalized to DMSO-treated cells. Error bars represent maximum and minimum values. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; unpaired t-test.

KS99-treated cells formed smaller AML blast colonies than the control treatment, as shown in Figure 2F and *Online Supplementary Figure S3*. Overall, these results showed that KS99 targets clonogenicity of leukemic cells as monotherapy while sparing normal HSPC. Since clonogenic activity is an indicator of pre-LSC, LSC and HSPC,[29] these observations were followed by flow cytometric analysis for cell surface markers of LSC.

KS99 induces apoptosis in primary human leukemic stem cells

To validate the anti-LSC activity of KS99, primary human AML cells were treated with increasing concentrations of KS99 (0.3 μM, 1 μM or 3 μM), Ara-C (5 μM), or combinations of KS99 with Ara-C for 24 h under the LSC culture conditions described by Pabst *et al.*[27] Since there is no one perfect LSC marker, we studied multiple well reported markers.[30,31] LSC were phenotypically defined by gating on CD45$^+$ followed by CD34$^+$C-D38$^-$/CD38$^+$, CD123$^+$, TIM-3$^+$, or CD96$^+$. Induction of apoptosis was observed in CD123$^+$ and CD34$^+$CD123$^+$ cells with KS99 treatment in a dose-dependent manner (Figure 3A). CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ cells were analyzed to see whether KS99 has a pro-apoptotic activity in subpopulations of CD34$^+$ cells, and we found that CD34$^+$ cells were sensitive to KS99 regardless of CD38 status (Figure 3A and B). We also observed that KS99 selectively targeted blast-like cells as compared to granulocyte-like or lymphocyte-like cells in causing reduction of CD34$^+$ cells (*Online Supplementary Figure S2*). Next, KS99 was compared and combined with Ara-C in CD45$^+$, TIM-3$^+$, CD96$^+$ or TIM-3$^+$CD96$^+$ human AML cells. Cells showed similar sensitivity to KS99 as observed in CD34$^+$ and CD123$^+$ cells (Figure 3C). When KS99 was added to Ara-C, it increased Ara-C's pro-apoptotic activity, especially in CD96$^+$ or TIM-3$^+$CD96$^+$ cells (Figure 3C). Furthermore, we extended our analysis by evaluating the pro-apoptotic activity of KS99 alone and in combination with Ara-C in LSC co-expressing CD34, CD38, CD123, TIM-3, or CD96 immunophenotypes (Figure 3D). Interestingly, cells coexpressing CD123 and TIM-3 or CD96 immunophenotypes were less sensitive to Ara-C or KS99 compared to other co-expressions. However, their sensitivity was increased with combination treatment (Figure 3D, right panel). Overall, these results show that KS99 induces apoptosis not only in CD45$^+$ or CD34$^+$ human AML cells, but also in TIM-3$^+$, CD96$^+$, or cells co-expressing various LSC phenotypes. In addition, it also has the potential to enhance the activity of Ara-C in AML stem cells, given that most cells of each phenotype are sensitive to the combination.

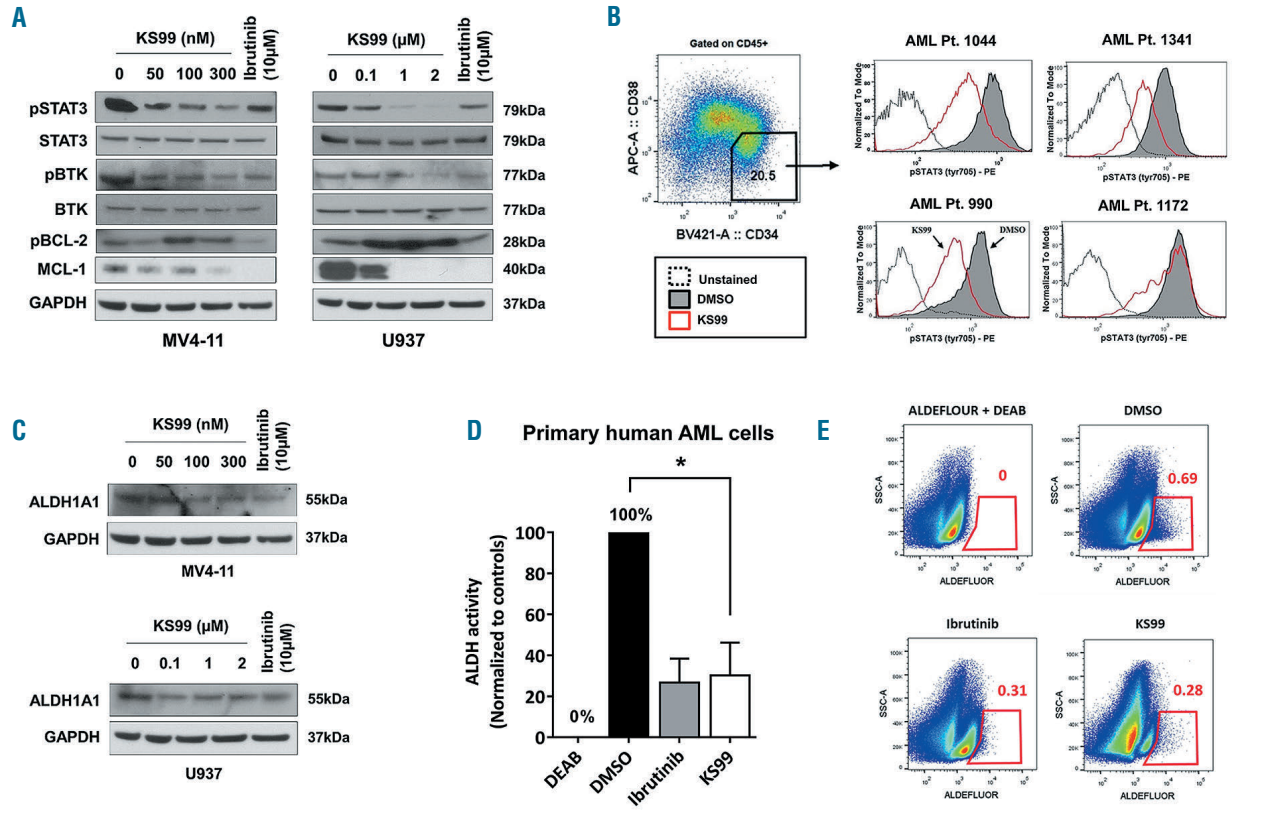

Figure 4. KS99 down-regulates pBTK, pSTAT3 and inhibits aldehyde dehydrogenase (ALDH) activity. (A) Immunoblot analysis of the whole MV4-11 and U937 cell lysates after the treatment either with DMSO, increasing concentrations of KS99 or ibrutinib (10 μM) for 24 hours (h). GAPDH was used as a loading control. (B) Flow cytometric detection of pSTAT3 in LSC of primary human acute myeloid leukemia (AML) samples (n=4). (C) Immunoblotting of whole-cell lysates of KS99 or ibrutinib-treated MV4-11 and U937 cells with ALDH1A1. GAPDH was used as a loading control. (D and E) Primary human AML samples were treated either with DMSO, KS99 (3 μM) or ibrutinib (10 μM) for 48 h. ALDH activity was measured by ALDEFLOUR assay kit *via* flow cytometry. Results are mean±standard error of the mean. n=3. **P*<0.05 was assessed by unpaired *t*-test.

KS99 interacts with BTK, STAT3, and ALDH1A1

A molecular docking approach was used to examine the interaction of KS99 with BTK, STAT3, and ALDH1A1 (isoform) at the atomic level. For ALDH1A1, STAT3 and BTK, the lowest binding energies were -9.65 kcal/mol, -6.76 kcal/mol, and -9.31 kcal/mol, respectively (*Online Supplementary Figure S4*). The inhibition constant values for the aforesaid proteins was 84.19 nM, 11.14 μM, and 150.61 nM, respectively, suggesting that KS99 interacts with them. It should be noted that the lower energy scores correlate with a higher binding affinity,[32] i.e. better for ALDH1A1 and BTK, followed, in this case, by STAT3. KS99 binds poorly with the developed structure of STAT3 with higher binding energy and inhibition constant as compared to ALDH1A1 and BTK. The phosphotyrosine 48 amino acid binding pocket of STAT3 interacted with KS99 at comparatively higher binding energy and inhibition constant than other proteins docked in this study. Further details are provided in *Online Supplementary Table S2*.

KS99 mediated downregulation of pBTK and pSTAT3 with reduced aldehyde dehydrogenase activity in acute myeloid leukemia cell lines and primary human acute myeloid leukemia cells

To extend studies of KS99 from earlier observations, we examined BTK and STAT3 signaling in AML cell lines by western blotting and in primary AML cells by flow cytometry. Western blot results confirmed that KS99 significantly inhibits phosphorylation of BTK, and STAT3 in a dose-dependent manner in MV4-11 and U937 cells (Figure 4A). Moreover, the degree of inhibition of pSTAT3 and pBTK with 10 μM ibrutinib was achieved with low nanomolar concentrations of KS99 in both cell lines. As reported previously for MM cells,[20] KS99 up-regulated BCL-2 phosphorylation and down-regulated MCL-1 in AML cells (Figure 4A). We did not observe BCL-2 phosphorylation with ibrutinib treatment but did see MCL-1 downregulation.

We obtained comparable results in primary human AML samples using flow cytometry. KS99 or vehicle-treated (DMSO) primary human AML cells were initially gated on the 7AAD-negative population (live cells) followed by $CD45^+$ and $CD34^+CD38^-$, respectively. pSTAT3 was reduced by KS99 treatment in 3 out of 4 of the $CD34^+CD38^-$ subpopulation of tested primary human AML cases (Figure 4B). Overall, these results suggest that KS99 down-regulates pBTK and pSTAT3.

Earlier studies have shown that LSC exhibit ALDH activity and it associates with drug resistance.[17] Western blot data showed downregulation of ALDH1A1, an isoform of ALDH with KS99 treatment in a dose-dependent manner in MV4-11 cells, whereas, in U937 cells, all the concentrations showed the same degree of downregulation (Figure 4C). Ibrutinib also down-regulated ALDH1A1, as reported previously for ovarian cancers.[33-35] ALDH activ-

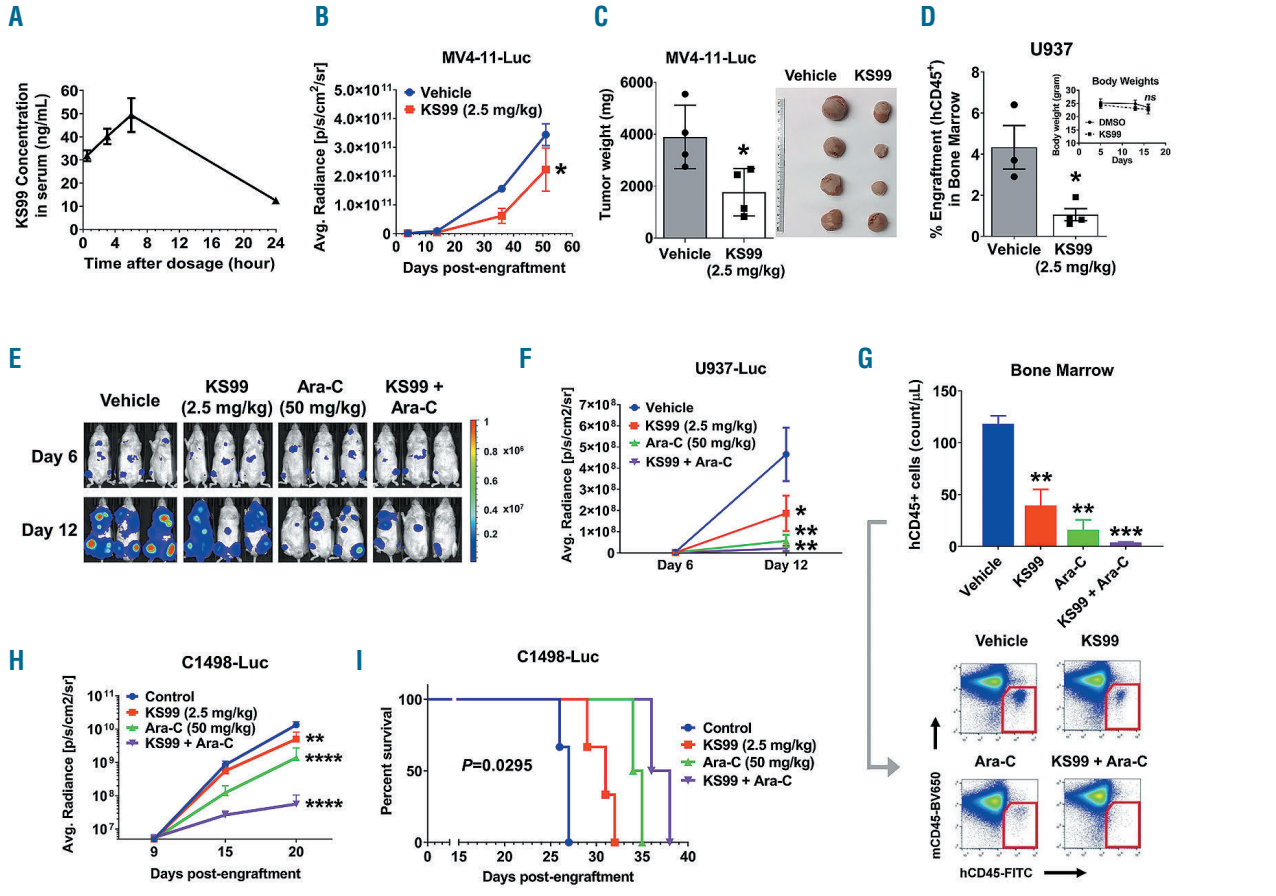

Figure 5. Preclinical efficacy of KS99 in acute myeloid leukemia (AML) mouse models. (A) Pharmacokinetics of KS99 (2.5 mg/kg body weight) concentration in plasma of NSG mice (n=3 per time point) measured after 0.5, 3, 6 and 24 hours (h) of treatment. Results are mean±standard error of the mean (SEM). (B) Mice engrafted with luciferase-expressing MV4-11 (MV4-11-Luc) cells subcutaneously were treated either with vehicle control (DMSO) or KS99 (2.5 mg/kg). Bioluminescence imaging (BLI) and quantification of radiance as a surrogate for tumor of mice (n=4) over the time course of the study were assessed. (C) Tumors were isolated and weighed at the termination of the study. (D) U937-bearing NSG mice (n=3-4) were treated either with vehicle control (DMSO) or KS99 (2.5 mg/kg). Bar graph indicates the percentage of human CD45⁺ cells in the bone marrow of mice. The insert is the body weight of mice throughout the study. Points represent individual mice (mean±SEM). (E-G) Luciferase-expressing U937 (U937-Luc)-bearing NRG mice (n=3) were treated either with vehicle control (DMSO), KS99 (2.5 mg/kg), Cytarabine (Ara-C, 50 mg/kg) or combination of KS99 and Ara-C. (E and F) Bioluminescence imaging signals of mice over the time course of the study. (G) Bar graph depicts a number of human CD45⁺ cells in the bone marrow of mice as detected using flow cytometry (top) and representative flow cytometric analysis of bone marrow cells (bottom). *P<0.05; **P<0.01; ***P<0.001 were assessed by two-way ANOVA analysis. (H and I) Luciferase-expressing C1498 (C1498-Luc)-bearing albino C57BL/6 mice (n=3) were treated either with vehicle control (DMSO), KS99 (2.5 mg/kg), Cytarabine (Ara-C, 50 mg/kg) or a combination. (H) Quantification of BLI signals of mice over the time course of the study. (I) Kaplan-Meier survival analysis of animals. Results are mean±SEM. NS: not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, unpaired t-test.

ity in primary human AML cells was tested by ALDEFLOUR assay as described above. Briefly, cells were treated with DMSO, ibrutinib (10 μM) or KS99 (3 μM) for 48 h, harvested and subjected to flow cytometer. Both KS99 and ibrutinib treatments caused a decrease in the ALDH⁺ subpopulation in all primary human AML cases studied (Figure 4D and E).

KS99 reduces the leukemic burden in preclinical acute myeloid leukemia animal models and improves the survival of animals

The study by Pandey *et al.* has shown the efficacy of KS99 at 1 mg/kg body weight in multiple myeloma.[20] In the current work, we determined the maximum-tolerated dose (MTD) of KS99 in NSG mice to be 2.5 mg/kg *via* an intraperitoneal (i.p.) route. Next, a single dose pharmacokinetic (PK) study was performed in NSG mice. Mice were injected with KS99 (2.5 mg/kg), sacrificed at various time points post-dosing (n=3), and plasma levels were analyzed by LC-MS/MS. PK data showed a maximum of 49 ng/mL (105.1 nM) plasma drug concentration and rapid elimination from the systemic circulation after 8 h (Figure 5A).

To evaluate the preclinical efficacy of KS99 *in vivo*, subcutaneous (s.c.) and disseminated xenograft mouse models were used. For the subcutaneous xenograft model, MV4-11-Luc cells were injected subcutaneously into NSG mice. Once visible tumors had been established, mice were treated with either vehicle control or KS99 (2.5 mg/kg) three times a week (*Online Supplementary Figure S5A*). Data showed a 65% decrease in bioluminescent signals in KS99-treated mice compared to the vehicle. Tumor sizes and weight (approx. 70%) were lower than vehicle-treated animals (Figure 5C).

To extend our findings in a subcutaneously implanted AML xenograft model, the efficacy of KS99 was further evaluated in a disseminated mouse model using human

AML U937 cells. Leukemia-bearing mice were randomized into treatment groups according to body weight and treated as above, followed by the treatment regimen shown in *Online Supplementary Figure S5B*. Analysis of bone marrow (BM) at termination showed an approximate 76% reduction of hCD45$^+$ cells in the KS99-treated group compared to the vehicle-treated group (P<0.05) (Figure 5D). Overall, KS99 treatment at 2.5 mg/kg was well tolerated by animals, as evidenced by no significant changes in body weight, suggesting negligible drug-associated toxicity (Figure 5D, see insert).

Next, KS99 was compared and combined with Ara-C in U937-Luc-bearing NOD.Cg-Rag1tm1Mom Il2rgtm1 Wjl/SzJ (NRG) mice. The treatment was initiated with vehicle control, KS99, Ara-C (50 mg/kg, IP) or combination (*Online Supplementary Figure S5C*). Although single-agent treatments KS99 (approx. 2.5 fold) or Ara-C (approx. 8.2-fold) showed a decrease in the progression of AML, co-treatment (approx. 22.2-fold decrease) was more effective at suppressing AML than either drug alone, as assessed by BLI (Figure 5E and F). Furthermore, efficacy was confirmed by flow cytometric analysis of bone marrow cells at the time of termination (Figure 5G). Mice treated with KS99, or Ara-C monotherapy exhibited a decrease of approximately 66% or approximately 86% in hCD45$^+$ cells, respectively (Figure 5G). Interestingly, almost complete eradication (approx. 96%) of the leukemic burden was noticed in the combined treatment (KS99 + Ara-C) group (Figure 5G).

The above observations in human AML animal models were validated in an immunocompetent syngeneic animal model of AML. As for the above results, treatment with KS99, Ara-C or combination in C1498-Luc-bearing albino C57BL/6 mice showed a reduction in leukemia progression analyzed by BLI (P<0.01, P<0.0001, and P<0.0001, respectively) (Figure 5H). Moreover, KS99 improved overall survival of mice (P=0.0295) (Figure 5I). Mice treated with both agents survived longer than single agent-treated mice (Figure 5I).

Overall these results show that KS99 reduces the leukemic burden in AML xenograft models and improves animal survival. Efficacy can be further improved by combining KS99 with Ara-C or other AML chemotherapeutics.

Discussion

Current first-line cytotoxic chemotherapy for AML shows limited success, with 50% of younger patients and 80% of older patients succumbing to the disease.[36] Unfortunately, these therapies have dose-limiting normal HSPC toxicity, which is one of the major obstacles in the treatment of AML.[37] It is well established that cancer stem cells have a major role in the initiation, progression, and relapse of solid tumors.[38] In AML, the leukemic stem cells have a similar role, and these cells acquire resistance to conventional chemotherapeutic drugs upon the accumulation of molecular mutations post-primary chemotherapy.[39] The LSC within AML are responsible for tumor growth and maintenance.[38] Relapse is frequently observed, and is largely attributed to acquired resistance of LSC to chemotherapeutic agents.[2,3,37] Therefore, eradication of the LSC is likely necessary to increase survival of AML patients.[40] Our studies here focus on small molecule KS99, a novel Isatin derivative which we show has the potential to target LSC. KS99 inhibited cell growth, induced apoptosis, and suppressed survival in both human AML cell lines and primary human AML cells.

Studies have shown that BTK is highly expressed and remains an important target in AML.[41] BTK inhibitors, ibrutinib and CG-806, are proving to be promising AML agents [27,42,43] Our previous studies showed that KS99 is a dual inhibitor of BTK/tubulin in multiple myeloma.[20] However, its role in targeting the LSC which control the poor prognosis of AML have remained undiscovered until now. BTK functions by initiating a cascade of downstream transcriptional factors that increase cell proliferation in cancer and favor cell survival.[20] While ibrutinib blocks the BTK and causes apoptosis of cancer cells, the fact that the LSC are functional may lead to relapse in some cases. Our data show that KS99 inhibits the BTK-driven STAT3 phosphorylation, which is an empirical player of LSC signaling.[44,45] Various reports have clarified the role of STAT3 signaling in pre-LSC and have associated it to poor prognosis in multiple cancers, including AML.[22,46-48] The effect of KS99 in CD34$^+$CD38$^-$ LSC has shown decreased levels of phosphorylated STAT3, which leads to reduced expression of MCL-1 against increased phosphorylated BCL, probably due to BTK inhibition. This observation corroborates with MM and earlier studies.[20,22,46-48] *In silico* analysis shows binding of KS99 to STAT3 and emphatically to the phosphotyrosine-containing SH2 domain of STAT3. This further proves that the interaction between KS99 and BTK has a major role in down-regulating STAT3.

Aldehyde dehydrogenase, a detoxifying enzyme, is expressed in both normal progenitor cells and AML stem cells.[17,25,33-35] In colony-forming assays, KS99 illustrates the reduction in the colony-forming capacity of AML progenitor cells but mostly spares normal HSPC. This finding, with a specific selectivity of KS99, is further supported by a reduction in ALDH activity. Our western blot and flow cytometry data showed a significant decrease in ALDH expression and activity. Furthermore, the data are supported by docking studies which predict the strong binding affinity of KS99 to ALDH1A1 isoform at very low predictive inhibition constant. Collectively, our data suggest that KS99 induces apoptosis by inhibiting the STAT3 and ALDH activation in LSC. *In silico* data described here also support the effective binding of KS99 to ALDH1A1 at an inhibitory constant of 84.19 nM, suggesting that the molecule has a role in directly inhibiting the activity of ALDH and further inhibiting growth and survival of AML cells. The amino acid residues involved in the probable KS99-ALDH hydrophobic interaction likely increase binding affinity, stabilizing the ligand at its binding site and further affecting ALDH activity.

We showed that KS99 is highly active in CD34$^+$, CD34$^+$CD38$^-$ or CD34$^+$CD38$^+$ expressing cells in AML patient samples. However, a number of previous studies have shown that LSC in AML can also reside within CD123$^+$, TIM-3$^+$ or CD96$^+$ cells.[12-16] We, therefore, extended our studies to these cells and were able to show that primary human AML cells expressing or co-expressing CD123, TIM-3 or CD96 were also sensitive to KS99. These results are consistent with colony-forming and ALDH observations. Furthermore, the combination of KS99 helped to augment the pro-apoptotic efficacy of Ara-C in LSC, especially in TIM-3$^+$ cells co-expressing CD34 or

CD123. Altogether, it indicated that KS99 targets LSC in AML.

Study of 21 primary human AML cases with KS99 showed a higher sensitivity of cases with mutations associated with poor prognosis compared to cases with favorable prognosis. NPM1 mutation is linked to better prognosis and responds well to traditional chemotherapy, but is relatively insensitive *in vitro* to KS99. In contrast, AML with poor prognosis MDS-RC[49-54] were killed by KS99 at lower concentrations. AML with MDS-RC represents 25-35% of all AML, and, given its poor prognosis, calls for better therapies. Currently, experimental and investigational agents are available to target different subgroups of AML, but few address AML with MDS-RC. Our study suggests that KS99 not only targets LSC, but has potential activity against AML with MDS-RC.

Finally, our preclinical animal data are clear evidence of the therapeutic potential of KS99 in immunocompromised and immunocompetent AML xenograft models. In addition, KS99 augmented the efficacy of standard of care agent, Ara-C. Although KS99 has shown *in vivo* AML inhibitory potential in this study, the activity has been modest. We believe that these findings are expected given the PK profile of this agent. The plasma concentrations achievable at the MTD were well below the IC_{50} determined from *in vitro* studies and, together with the relatively rapid decay in these levels, provide data explaining less than robust *in vivo* activity. Current efforts are directed at the study of different formulations for drug delivery and structural variants of KS99 which may yield similar *in vitro* activity with improved toxicity and pharmacokinetic characteristics.

In summary, we demonstrate that KS99 inhibits ALDH, pSTAT3, and pBTK in AML and decreases cell proliferation and clonogenicity while increasing apoptosis. BTK phosphorylation is also inhibited, though the evidence presented here suggests that this may not be the primary target of drug inhibition. We believe that *in silico* data suggest that ALDH may be such a primary target. Almost all human AML cases, including those with MDS-RC, have been sensitive to KS99. This agent targets LSC and progenitors with limited toxicity towards normal HSPC. Our study offers a comparative validation of KS99 to the standard agent Ara-C in specific targeting of AML-LSC. This study advances KS99 alone or in combination as a candidate agent for therapeutic development after reformulation or chemical modification of KS99 or its derivatives which may prove active in clinical trials of AML.

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