

# Sunitinib Reduces Acute Myeloid Leukemia Clonogenic Cells *in Vitro* and Has Potent Inhibitory Effect on Sorted AML ALDH<sup>+</sup> Cells

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## Abstract

Sunitinib is an orally administered, multi-target tyrosine kinase inhibitor that has been approved by the FDA for the treatment of renal cell carcinoma and imatinib resistant gastro-intestinal tumors. Anti-leukemic activity of sunitinib has been examined in early clinical trials with limited success. However, recent trials on acute myeloid leukemia (AML) patients carrying FLT3 mutations have shown promising results. Effects of sunitinib on leukemic clonogenic cells and potential leukemic stem cells have not been examined so far. We analyzed the anti-proliferative and apoptotic properties of sunitinib on AML-derived cell lines. We also tested the effect of sunitinib on AML patient derived clonogenic cells (AML-CFC), as well as flow-sorted potential leukemic progenitors. Peripheral blood or bone marrow samples were obtained from newly diagnosed AML patients and flow sorted for CD34<sup>+</sup> CD133<sup>+</sup> or ALDH<sup>+</sup> cells. Umbilical cord blood derived CD34<sup>+</sup> cells were used as normal controls. Sunitinib induced growth arrest and apoptosis in AML derived cell lines. In addition, 7  $\mu$ M sunitinib induced 75% reduction of AML-CFC as compared to DMSO treated control ( $\pm 6.79\%$ ; n = 4). In contrast, 7  $\mu$ M sunitinib treatment of umbilical cord blood derived normal CD34<sup>+</sup> cells showed 29% reduction in AML-CFC ( $\pm 6.77\%$ ; n = 5). Treatment of ALDH<sup>+</sup> cells sorted from 2 AML cases and CD34<sup>+</sup> CD133<sup>+</sup> cells from one patient showed reduction of AML-CFC on treatment with sunitinib. Our study highlighted a potent anti-proliferative and proapoptotic

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**effect of sunitinib on AML cell lines, AML patient derived clonogenic cells and potential leukemic stem cells.**

## **Keywords**

**Acute Myeloid Leukemia, Sunitinib, Tyrosine Kinase Inhibitor, AML-CFC, Leukemic Stem Cells**

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## **1. Introduction**

Acute myeloid leukemia (AML) is a clonal proliferative disorder of the myeloid progenitors that results in an accumulation of immature myeloid cells in the bone marrow and peripheral blood. Substantial improvement in the outcome of AML patients is seen during the recent times, which is mainly due to the development of risk-stratified treatment approaches to patient management [1]. However, treatment of elderly AML patients, patients carrying mutations in FLT3 and those with complex cytogenetic abnormalities and monosomies still remain challenging. In this regard, development of novel targeted therapeutic approaches is crucial for improving the clinical outcome of AML subsets [2].

Sunitinib is a multi-targeted tyrosine kinase inhibitor (TKI) that targets platelet-derived growth factor receptors (PDGFRs), vascular endothelial growth factor receptors (VEGFRs) and other constitutively active kinases like c-KIT. Sunitinib has been approved by the United States Food and Drug Administration (FDA) for the treatment of metastatic renal cell carcinoma (mRCC) and gastrointestinal stromal tumor (GIST) [3]. Sunitinib has also been shown to decrease the phosphorylation of wild type fms-related tyrosine kinase (FLT3) and internal tandem duplications (ITDs) using *in vitro* and *in vivo* AML models [4] [5]. In clinical trials evaluating safety and efficacy in AML patients, sunitinib showed molecular and clinical responses, however, this was transient and associated with significant toxicities [6]. Use of sunitinib in combination with conventional chemotherapy had shown promising results. Combination of sunitinib with AraC or Daunorubicin demonstrated synergistic inhibition of FLT3-ITD positive AML cells [7]. A recent phase I/II study in older AML patients with FLT3 mutations studied addition of sunitinib to standard induction and consolidation chemotherapy, followed by maintenance on sunitinib. In this study, a 25 mg/day dose of sunitinib was well tolerated and complete remission was achieved in 59% AML patients irrespective of the type of FLT3 mutation [8]. In the present study, we have evaluated the activity of sunitinib on AML cell lines and clonogenic cells from primary AML samples and potential AML stem cells subpopulation.

## **2. Material and Methods**

### **2.1. Materials**

A stock solution of 20mM sunitinib malate (Sigma-Aldrich, USA) was prepared by dissolving in the dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and stored at  $-20^{\circ}\text{C}$ . Further dilutions were done in DMSO. Cell-Titer blue viability kit (Promega, UK) was used to study the effect of sunitinib on cell line viability. CD34-PE, Annexin V450 and 7-AAD antibodies were purchased from the BD (BD biosciences, USA). CD34 Microbead kit was obtained from Miltenyi (Miltenyi Biotech, Germany). Propidium iodide and RNase A were purchased from Sigma (Sigma-Aldrich, USA). Lymphoprep and MethoCult™ (H4434 and H4100) were purchased from Stem Cell Technologies (Stem Cell Technologies, Canada).

### **2.2. Sample Collection and Isolation of CD34+ Cells**

Umbilical cord blood (UCB) samples from normal deliveries and primary AML patient bone marrow (BM) or peripheral blood (PB) samples were collected after informed consent. This study has been approved by the institution review board in accordance with the Helsinki declaration. Mononuclear cells (MNCs) were isolated by density gradient centrifugation from the UCB/AML samples using Lymphoprep (Stem Cell Technologies, Canada). UCB was diluted 1:2 with sterile  $1\times$  PBS (Gibco, Life Technologies, USA) and layered on the Lymphoprep. Following centrifugation at 750 g for 30 mins, mononuclear cells were collected from the gradient. CD34<sup>+</sup>

cells were enriched from the MNCs by positive selection using CD34 Microbead kit according to the manufacturer's protocol (Miltenyi Biotec, Germany). Isolated CD34<sup>+</sup> cells were checked for their purity by labelling with anti-CD34-PE on the BD FACS Aria III (BD biosciences, USA) flow cytometer. In this study, we included only samples having purity above 95%. The enriched samples were counted for their viability and cryo preserved in fetal bovine serum with 10% DMSO and stored in liquid nitrogen for next use.

### 2.3. Cell Culture

K562 (erythroleukemia), HL60 (acute promyelocytic leukemia) and NB4 (acute promyelocytic leukemia) cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Gibco, Life Technologies, USA) supplemented with 10% FBS (Lonza, USA) and incubated in humidified 5% CO<sub>2</sub> 37°C incubator. All the cell lines were purchased from Cell Lines Service (CLS GmbH, Germany). AML mononuclear cells and sorted human hematopoietic stem/progenitors cells were cultured in serum free expansion media (Stem cell Technologies, Canada) for drug assays.

### 2.4. Cell Proliferation Assay

Cell proliferation assay was performed using Cell-Titer blue cell viability kit. Briefly,  $1 \times 10^4$  cells were incubated in a quadruplicate with different doses of sunitinib in a 96-well plate with a total volume of 100  $\mu$ L medium. DMSO treated cells served as control. After 48 hours of incubation, 20  $\mu$ L of cell titer blue reagent was added and fluorescence was recorded at 560/590 nm on a microplate reader (Spectramax i3, Molecular Devices, USA) after 2 hr of incubation at 37°C. Percentage cell viability was calculated as fluorescence value of the treated cells/fluorescence value of the control  $\times$  100. At least three independent experiments were performed.

### 2.5. Detection of Apoptosis

Annexin V assay was performed to detect apoptotic cells. Briefly,  $2 \times 10^5$  cells were plated in a 6-well plate and treated with various concentrations of sunitinib including DMSO treated control and incubated for 48hrs. The cells were harvested and stained with Annexin V450 and 7-AAD. The stained samples were analyzed by acquiring 10,000 cells using BD FACS Aria III.

### 2.6. Cell Cycle Analysis

Cells were seeded in a 6-well plate at density of  $5 \times 10^5$  cells per well and treated with various doses of sunitinib. After 48 hrs, cells were collected and fixed with ice-cold ethanol and kept at  $-20^\circ\text{C}$  for 1 hour. Cells were washed and then stained with propidium iodide (PI) staining solution (PI 40  $\mu$ g/ml, RNase 20  $\mu$ g/ml). Flow cytometric analysis was performed by acquiring 20,000 doublet-excluded cells using BD FACS Aria III sorter.

### 2.7. Aldefluor Assay and Flow Cell Sorting

Aldehyde dehydrogenase (ALDH) activity was evaluated in AML patient MNCs as per manufacturer's instruction (Aldefluor, Stem Cell Technologies, Canada). DEAB was added to create a negative control. Co-staining with anti-CD34-VioBlue (Miltenyi Biotech, Germany) was done in some samples. Stained samples were then analyzed and isolated by sorting ALDH<sup>+</sup> cells (BD FACS Aria III).

### 2.8. CFC Assay

Colony forming cell (CFC) assay was performed by plating cells following 24 h sunitinib or DMSO (Control) treatment. AML-CFC assays were performed by plating un-sorted AML cells at 20,000 - 60,000 cells/ml or ALDH<sup>+</sup>/CD34<sup>+</sup> cells at 10,000 cells/ml in Methocult as described [9] [10]. The colonies were evaluated after 10 - 14 days. CFC assay for drug treated normal CD34<sup>+</sup> cells were performed by plating 1000 cells/ml Methocult and evaluated after 12 days.

### 2.9. Statistical Analysis

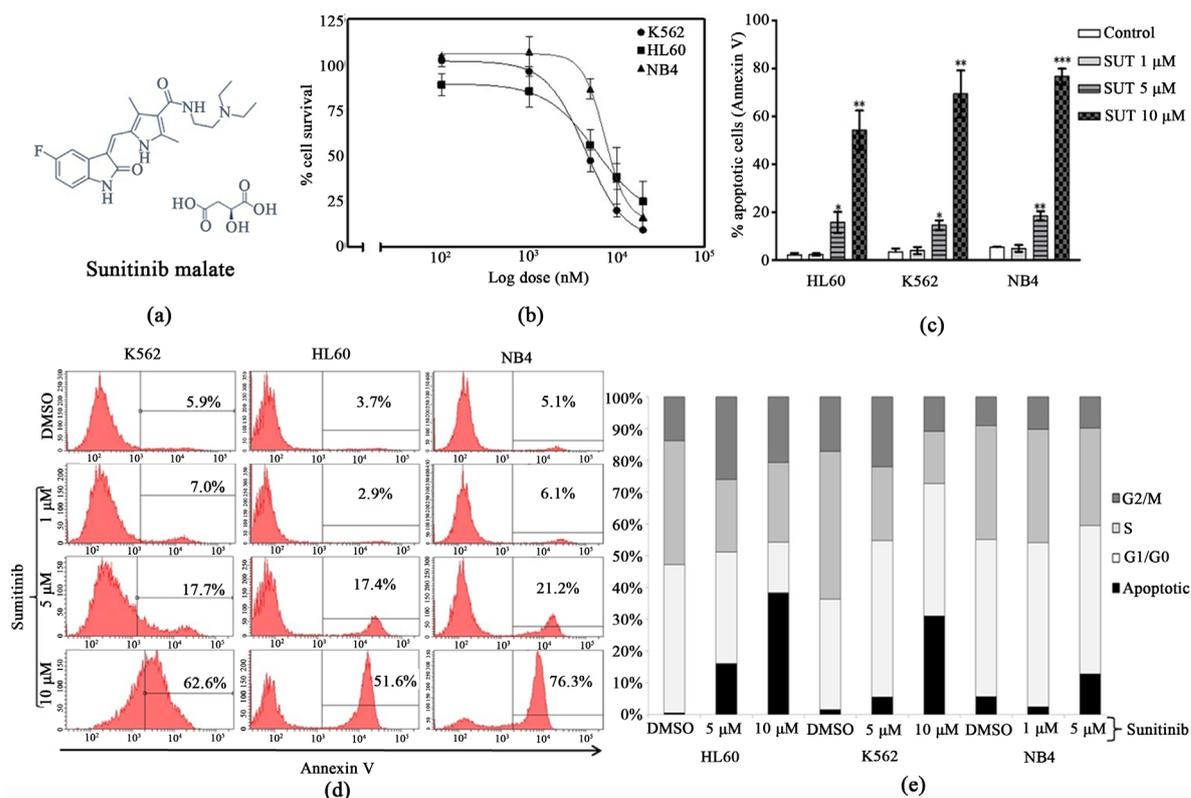
Cell proliferation curves were generated using Graphpad Prism (Graphpad Software Inc, USA). Data was presented as mean  $\pm$  SEM. Unpaired two-tailed Student's *t*-test was used for all *in vitro* cell line experiments and

AML-CFC assays. A  $p < 0.05$  was considered significant. Mann-Whitney U test was used to determine significant differences between two individual AML patient groups.

### 3. Results

#### 3.1. Anti-Proliferative Effect of Sunitinib in Different Leukemia Cell Lines

In order to explore the antiproliferative effects of sunitinib (Figure 1(a)), on AML cells, human HL60, K562 and NB4 cell lines were cultured with increasing concentrations of sunitinib. Cell proliferation and viability was assessed using the Cell-Titre Blue Kit after 48 h treatment. As represented in Figure 1(b), sunitinib inhibited HL60, K562 and NB4 cell proliferation in a dose-dependent manner with  $IC_{50}$  values of 5.7, 4.4 and 7.3  $\mu\text{M}$  respectively. In order to determine the precise mechanism for the anti-proliferative action of sunitinib, annexin V staining and cell cycle distribution profile was studied. As shown in Figure 1(c) & Figure 1(d), dose dependent increase in annexin V positive cells was observed in sunitinib-treated cells, suggesting apoptosis as the reason for anti-proliferative action of sunitinib on AML cell lines. Additionally, as shown in Figure 1(e), exposure of AML cells to sunitinib for 48 h led to an increase in the number of sub-G1 population of cells representing apoptotic cells. Increasing doses of sunitinib results in a concomitant decrease in the proportion of cells in the S phase for all cell lines tested, indicating a decrease in the proliferation of cells.



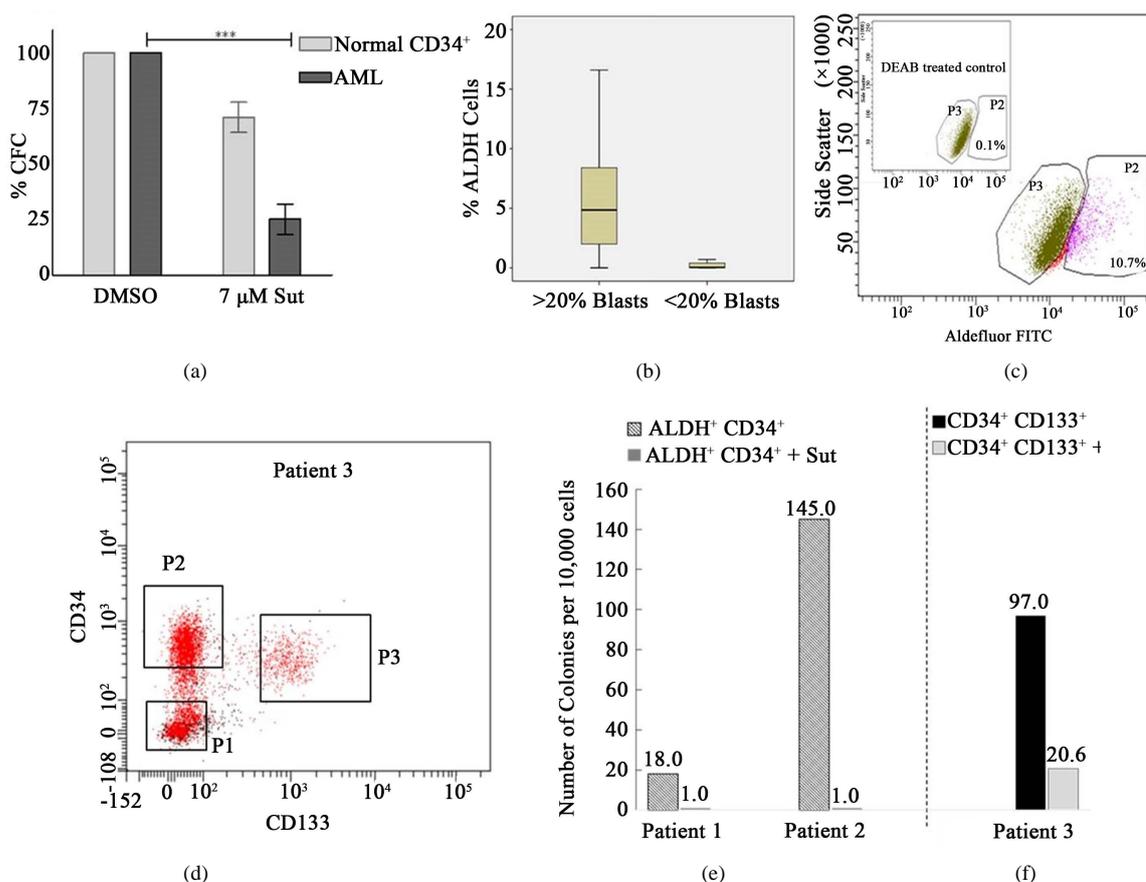
**Figure 1.** Antiproliferative effect of Sunitinib on AML cell lines. (a) Chemical structure of Sunitinib malate; (b) Cell proliferation assay performed using Cell-Titre Blue reagent at 48 h demonstrated a dose-dependent antiproliferative effect on AML cell line K562, NB4 and HL60. The data is represented as mean  $\pm$  SEM of three independent experiments. Curve fitting was performed by non-linear regression using log (inhibitor) vs. response-variable slope (four parameters) using Graphpad Prism. The  $IC_{50}$  for sunitinib in K562 was 4.4  $\mu\text{M}$ , in HL60 was 5.7  $\mu\text{M}$  and in NB4 was 7.3  $\mu\text{M}$ ; (c) Mean  $\pm$  SEM of three independent experiments showing percentage apoptotic cells in different AML cell lines upon treatment with increasing dose of sunitinib. \* represents  $p < 0.05$ , \*\* represents  $p < 0.01$ , \*\*\* represents  $p < 0.001$  compared to the control; (d) Apoptosis assays performed using annexin V staining shows a dose dependent increase in apoptosis with sunitinib in all AML cell lines and 48 h. Analysis was done on BD FACS Aria III flow cytometer; (e) Cell cycle analysis of HL60, K562 and NB4 cells were performed by staining the cells with propidium iodide and analyzed with the flow cytometer.

### 3.2. Effect of Sunitinib on Primary AML Clonogenic Cells

In order to evaluate the effect of sunitinib on primary AML patient derived leukemic cells, AML-BM or PB samples were incubated in the presence of 7 $\mu$ M sunitinib or DMSO for 24 h before performing AML-CFC assay. Normal UCB CD34<sup>+</sup> was used for comparison. As shown in **Figure 2(a)**, treatment with 7  $\mu$ M sunitinib resulted in a 75% reduction in mean AML-CFC as compared to DMSO treated control ( $\pm 6.8\%$ ;  $n = 4$ ). Treatment of normal UCB CD34<sup>+</sup> cells with 7  $\mu$ M sunitinib showed only 29% reduction ( $\pm 6.77\%$ ;  $n = 5$ ). ALDH activity was checked in 20 newly diagnosed AML samples. Patients with blast count >20% showed higher ALDH activity as compared to patients with <20% blasts ( $U = 6.0$ ,  $p = 0.007$ , **Figure 2(b)**). In order to check the effect of sunitinib on potential leukemic stem cells, we sorted ALDH<sup>+</sup> CD34<sup>+</sup> and ALDH<sup>-</sup> cells from two AML patients and plated them in clonogenic assay with or without 7 $\mu$ M sunitinib. ALDH<sup>-</sup> cells did not show any CFC growth, however, colonies were detected only in ALDH<sup>+</sup> sorted cells. In the presence of sunitinib, ALDH<sup>+</sup> AML cells lost the clonogenic potential in both AML samples tested (**Figure 2(d)**, **Figure 2(e)**). From another AML sample showing CD34<sup>+</sup> CD133<sup>+</sup> cells, various subpopulations were sorted out as indicated in **Figure 2(d)**. Only cells expressing CD34 formed colonies. Treatment of CD34<sup>+</sup> CD133<sup>+</sup> sorted cells and CD34<sup>+</sup> CD133<sup>-</sup> cells with sunitinib reduced the number of colonies (**Figure 2(f)**).

### 4. Discussion

Activating mutations in RTKs are common in AML and have adverse clinical outcome. FLT3 mutations that in-



**Figure 2.** Effect of sunitinib on clonogenic cells (a) Sunitinib (Sut) effectively reduces CFC derived from primary AML samples. Treatment of normal CD34<sup>+</sup> cells showed weaker inhibition of CFC; (b) Expression of ALDH using aldefluor staining in AML patients grouped according to blast percentage. Mann-Whitney U test showed patients with >20% blasts have higher ALDH activity ( $p = 0.007$ ); (c) ALDH<sup>+</sup> cells were sorted from 2 different AML samples ( $n = 2$ ) generated colonies in CFC assay(e); (d) Sorting of potential leukemic stem cells from patient 3 demonstrated clonogenic potential only in CD34<sup>+</sup> CD133<sup>+</sup> cells P3 and CD34<sup>+</sup> CD133<sup>-</sup> cells (P2); (f) 7 $\mu$ M sunitinib eradicates these leukemic CFCs.

clude internal tandem duplication (FLT3-ITD) and the tyrosine kinase domain mutation (FLT3-TKD), are found in almost 30% of newly diagnosed AML [11]. These mutations confer an adverse prognosis in AML [12]. Mutations in the KIT receptor gene have been reported in 3% - 15% of AML cases [13] [14] and with a significantly higher proportion in core binding factor AML (CBF-AML) [15]. KIT mutations confer adverse prognosis to CBF-AML [16] [17]. Owing to the high frequency of RTK mutations and poor clinical outcome there has been continuous efforts to develop targeted inhibitors of RTK. Many compounds have entered clinical trials and have demonstrated varying efficacies either as single agents or combination with conventional chemotherapies [8] [18] [19] [20]. Sunitinib is an orally available multi-targeting TKI, mainly targeting VEGFR, PDGFR, CD117 (c-KIT) and FLT3 [21]. Although clinical studies of sunitinib have shown some promising results, the effect of sunitinib on clonogenic leukemic cells and ALDH<sup>+</sup> potential leukemic stem cells has not been evaluated so far. In this paper we are able to demonstrate potent antiproliferative effect of sunitinib on three myeloid leukemia cell lines (K562, HL-60 and NB4) with varying IC<sub>50</sub> values. The slight differences in the IC<sub>50</sub> values from previously published reports in K562 and NB4 cells [22] [23], could be due to different times points for studying viability. We were able to demonstrate that sunitinib induces dose-dependent apoptosis in AML cell lines. Similar findings were reported earlier in other leukemic and non-leukemic cell lines [22] [24].

To study the effect of sunitinib on clonogenic AML cells, we evaluated primary AML patient with >20% blasts in CFC assay. Impairment of clonogenic survival was more pronounced in AML cells as compared to umbilical cord blood derived normal CD34 cells. The main reason for preferential killing of AML cells over normal CD34 cells by Sunitinib could be dependence of AML cells on multiple RTKs for proliferation. A previous study of *in vitro* FLT3 inhibition in AML showed no clear relationship between mRNA levels of FLT3 and response to RTK inhibitors lestaurtinib and PKC412 [25]. This non-concordance of response to FLT3 inhibitors might be due to other RTKs involved in AML cell signaling. In order to look for potential stem cell markers in AML samples, a number of groups have described ALDH<sup>+</sup> cells to be enriched in AML stem cells [26] [27]. Analysis of ALDH<sup>+</sup> cells was possible in 24 AML samples but sufficient number of cells could be sorted from only 2 samples for *in vitro* targeting experiments with sunitinib. Higher expression of ALDH was observed in patients having blast % more than 20. Additionally, ALDH expression correlated with high CD34% (data not shown). In view of the reports of presence of leukemic stem cells in ALDH-bright or ALDH-intermediate blasts [27] [28], our experiments were aimed at targeting these potential LSC using sunitinib. Our results highlight the possibility of elimination of potential LSC by sunitinib. The mechanism of sunitinib action on ALDH positive AML cells could not be elaborated in this limited study. In the third patient that was highly positive for CD34 and co-expressed CD133, only cells that expressed CD34 formed colonies. CD34<sup>+</sup>CD133<sup>+</sup> cells formed colonies with a higher frequency than the CD34<sup>+</sup>CD133<sup>-</sup> cells indicating a more primitive clonogenic feature. Although a functional hierarchy among the CD34<sup>+</sup>CD133<sup>+</sup> and CD34<sup>+</sup>CD133<sup>-</sup> has been demonstrated in UCB derived normal cells, so far no study has demonstrated any such functional hierarchy in AML samples [29] [30]. While our *in vitro* results do indicate a functional hierarchy in the ability to form leukemic colonies, *in vivo* studies are needed to fully characterize this hierarchy.

## 5. Conclusion

Our study highlights the relevance of TKI inhibitors in targeting clonogenic AML cells and potential AML stem cells. Our data supports the evidence of functional hierarchy in ALDH expressing AML cells. Moreover, our data demonstrate strong activity of sunitinib on potential LSC candidates *in vitro*.

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