Sunitinib, an orally available receptor tyrosine kinase inhibitor, induces monocytic differentiation of acute myelogenous leukemia cells that is enhanced by 1,25-dihydroxyvitamin D₃.

To the Editor: Sunitinib, an orally available multitargeted receptor tyrosine kinases (RTK) inhibitor, effectively induced growth arrest and apoptosis of acute myelogenous leukemia (AML) cells with gain of function mutation in fms-like tyrosine kinase 3 (FLT3) (FLT3-ITD) in vitro and in vivo.¹ This study found that sunitinib induced monocytic differentiation of AML cells irrespective of FLT3 gene status. For example, exposure of HL60 cells with the wild-type FLT3 as well as MOLM13 cells expressing FLT3-ITD to sunitinib increased levels of CD11b antigen, a marker of myeloid differentiation, on their cell surface (Figs 1A). Sunitinib also increased levels of CD14 cell surface antigens and stimulated their endocytic capacity, as measured by internalization of FITC-labeled dextran (data not shown). Moreover, sunitinib stimulated production of inflammatory cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in AML cells (Figs 1B,C), indicating monocytic differentiation of AML cells. Sunitinib-mediated monocytic differentiation was further confirmed by morphological change (D). Interestingly, sunitinib-induced differentiation was significantly potentiated in the presence of 1,25(OH)₂D₃, a known inducer of monocytic differentiation in leukemia cells (Figs 1A-C).² Furthermore, 1,25(OH)₂D₃
potentiated the ability of sunitinib to inhibit the clonogenic growth of AML cells (Figs 1E).

CCAAT/enhancer binding protein α (C/EBPα) belongs to a family of nuclear transcription factor and is required for myeloid differentiation. Sunitinib increased levels of C/EBPα in association with downregulation of c-Myc, a negative regulator of differentiation, in MOLM13 and HL60 cells (Fig 2). Sunitinib might induce monocytic differentiation of AML cells via modulation of these transcription factors.

We next examine whether sunitinib affected activity of normal hematopoietic cells in vivo. Treatment of C57BL/6 mice (n=5) with sunitinib (20 mg/kg, 5 days) significantly increased population of CD14⁺ mononuclear cells and CD8⁺ T cells in their spleen as well as bone marrow (Table 1). Also, sunitinib stimulated production of IFN-γ and TNF-α in their spleen mononuclear cells (Fig 3). These observations suggested that sunitinib activated the inflammatory cells in vivo.

Finally, we attempted to verify the molecular mechanisms by which sunitinib induced monocytic differentiation of hematopoietic cells. Notable, when AML cells were pre-incubated with anti-human TNF-α monoclonal antibody infliximab (100 ng/ml), sunitinib-induced expression of CD11b was almost completely blocked (Table 2), suggesting the involvement of TNF-α-mediated signaling in sunitinib-inducing monocytic differentiation of AML cells. This study utilized the very low concentrations
of sunitinib (2.5-20 nM), which was not able to dephosphorylate FLT3 and its downstream signal pathways in MOLM13 and HL60 cells (data not shown). Sunitinib also induced the differentiation of U937 cells which did not express FLT3 on their cell surface (data not shown). Thus, sunitinib-induced differentiation was probably independent of FLT3. Recently, erlotinib as well as gefitinib, an inhibitor of the epidermal growth factor receptor (EGFR), induced growth arrest and differentiation of AML cells in vitro and in vivo, which was independent of EGFR signaling. Erlotinib and gefitinib could produce TNF-α, resulting in differentiation of AML cells.

Taken together, sunitinib induced monocytic differentiation of AML cells irrespective of the FLT3 gene status. Clinical studies with this class of agents should include the individuals with AML expressing the wild-type FLT3.

**Acknowledgements**

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References


Figure Legend

Fig 1. Effects of 1,25(OH)$_2$D$_3$ on sunitinib-induced differentiation and growth arrest of AML cells. (A) MOLM13 and HL60 cells were cultured with either sunitinib (2.5-20 nM) and/or 1,25(OH)$_2$D$_3$ (0.005-0.5 μM). After 2 days, CD11b expressing population was measured by FACS. Results represent the mean ± SD of three experiments performed twice in duplicate plate. The statistical significance of difference was determined by one-way ANOVA followed by Boneferroni’s multiple comparison tests. *, p< 0.01, with respect to control cells treated with either sunitinib or 1,25(OH)$_2$D$_3$ alone. VitD, 1,25(OH)$_2$D$_3$. Effects of 1,25(OH)$_2$D$_3$ and/or sunitinib on macrophage-related cytokines. (B, C) MOLM13 and HL60 cells were cultured with either sunitinib (2.5-20 nM) and/or 1,25(OH)$_2$D$_3$ (0.01-0.5μM). After 2 days, RNA was extracted and cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of (B) IFN-γ and (C) TNF-α. Results represent mean ± SD of triplicate cultures. The statistical significance of difference between cytokine production induced by either 1,25(OH)$_2$D$_3$ and/or sunitinib was determined by one-way ANOVA followed by Boneferroni’s multiple comparison tests. *, p<0.01. VitD, 1,25(OH)$_2$D$_3$. (D) May-Giemsa stain. MOLM13 cells were culture by replacing culture media containing sunitinib (2.5 μM) or control diluent (DMSO) every another day. After 8 days, cells were harvested and cytospin preparations were stained with May-Giemsa. Their

morphology was assessed under a light microscope. Original magnifications were x 400. **1,25(OH)\textsubscript{2}D\textsubscript{3} potentiated anti-proliferative effect of sunitinib.** (E) Colony forming assay. MOLM13 and HL60 cells were cloned in soft agar and cultured with sunitinib (2.5-20 nM) or 1,25(OH)\textsubscript{2}D\textsubscript{3} (1.25-50 nM) either alone or in combination. After 10 days, colonies were counted. Results are expressed as a mean percentage of control plates containing diluent. The statistical significance of difference between growth inhibition produced by either sunitinib or 1,25(OH)\textsubscript{2}D\textsubscript{3} alone and those resulting from the combination of both was determined by one-way ANOVA followed by Bonferroni’s multiple comparison tests. Results represent the mean ± SD of 3 experiments performed in triplicate. *, \( p < 0.01 \). VitD, 1,25(OH)\textsubscript{2}D\textsubscript{3}.

**Fig 2. Effects of sunitinib on differentiation-related molecules. Western blot analysis.** MOLM13 and HL60 cells were cultured with sunitinib (5-20 nM). After 2 days, cells were harvested and subjected to Western blot analysis to monitor the levels of C/EBP\(\alpha\) and c-Myc. Each lane was loaded with 30 \( \mu \text{g} \) of nuclear proteins. Levels of GAPDH were measured as a loading control.

**Fig 3. Effects of sunitinib on macrophage-related cytokines in vivo.** Either sunitinib (n=5, 20 mg/kg) or control diluent (n=5) was administered to C57BL/6 mice by gavage for 5 days. At the end of the experiments, spleen was removed and RNA was extracted. cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of
IFN-γ and TNF-α. Results represent mean ± SD of triplicate cultures. *, $p<0.05$; **, $p<0.01$. 
Fig 1

A

MOLM13

Population of CD11b expressing cells (%)

HL60

Population of CD11b expressing cells (%)

B

MOLM13

Relative expression of IFN-γ (fold of control)

HL60

Relative expression of IFN-γ (fold of control)
**Figure Legend:**

The figure illustrates the effect of vitamin D (VitD) and sunitinib on the relative expression of TNF-α in MOLM13 and HL60 cell lines. The x-axis represents different concentrations of VitD and sunitinib, while the y-axis shows the relative expression of TNF-α (fold of control).

**C**

- **Control**
- **Sunitinib**

The control and sunitinib treatment groups are depicted in the images below, showing the cellular morphology and changes in TNF-α expression.
E

**MOLM13**

![Bar chart showing colony numbers (% of control) for MOLM13 cells treated with different concentrations of Vitamin D (VitD) and sunitinib.](image)

**HL60**

![Bar chart showing colony numbers (% of control) for HL60 cells treated with different concentrations of Vitamin D (VitD) and sunitinib.](image)
Fig 2

<table>
<thead>
<tr>
<th>MOLM13</th>
<th>HL60</th>
</tr>
</thead>
<tbody>
<tr>
<td>sunitinib (nM)</td>
<td>-</td>
</tr>
</tbody>
</table>

![Western blot images for MOLM13 and HL60 cells with sunitinib treatment.](image)

- c-Myc
- C/EBPα
- GAPDH

Fig 3

**p<0.05**

![Bar charts showing relative expression of IFN-γ and TNF-α with and without sunitinib treatment.](image)
Table 1. CD14 and CD11b expression.

<table>
<thead>
<tr>
<th></th>
<th>CD14</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>control spleen</td>
<td>15.5±1.9%</td>
<td>35.5±2.9%</td>
</tr>
<tr>
<td>sunitinib spleen</td>
<td>22.3±6.1%**</td>
<td>39.6±2.6%**</td>
</tr>
<tr>
<td>control BM</td>
<td>42.3±1.1%</td>
<td>10.6±3.8%</td>
</tr>
<tr>
<td>sunitinib BM</td>
<td>47.0±3.6%**</td>
<td>22.4±2.3%*</td>
</tr>
</tbody>
</table>

Mice were treated with either sunitinib (20 mg/kg/day, for 5 days) or control diluent by gavage. At the end of experiments, mice were sacrificed, and spleen and BM were removed. Population of CD14- or CD8-expressing cells was quantified by FACS.

* p<0.01, ** p<0.05. BM; bone marrow
Table 2. Expression of CD11b.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>infliximab 100 ng/ml</th>
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</thead>
<tbody>
<tr>
<td>MOLM13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>4.3 ± 1.9%</td>
<td>3.4 ± 0.5%</td>
</tr>
<tr>
<td>sunitinib 2.5nM</td>
<td>25.6 ± 2.0%</td>
<td>3.5 ± 0.3%</td>
</tr>
<tr>
<td>sunitinib 5 nM</td>
<td>39.8 ± 3.3%</td>
<td>4.5 ± 0.6%</td>
</tr>
<tr>
<td>HL60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>4.1 ± 1.7%</td>
<td>0.8 ± 0.04%</td>
</tr>
<tr>
<td>sunitinib 10 nM</td>
<td>29.2 ± 6.5%</td>
<td>3.4 ± 0.3%</td>
</tr>
<tr>
<td>sunitinib 20 nM</td>
<td>42.3 ± 3.2%</td>
<td>5.8 ± 0.2%</td>
</tr>
</tbody>
</table>

AML cells were pre-incubated with anti-human TNF-α monoclonal antibody infliximab (100 ng/ml) for 1 hr, and then exposed to various concentrations of sunitinib (2.5-20 nM). After 2 days, cells were harvested, and CD11b expressing population was analyzed by flow cytometry.