Ortho-topolin riboside induced differentiation through inhibition of STAT3 signaling in Acute Myeloid Leukemia HL-60 Cells

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Abstract

We previously demonstrated that ortho-topolin riboside (oTR), a naturally occurring cytokinin, exhibits potential anticancer effects via the mitochondrial apoptotic pathway and endoplasmic reticulum stress pathway. In the present study, we revealed that oTR induced the differentiation of acute myeloid leukemia (AML) HL-60 cells, which represent the M2 sub-type of AML. We found that oTR arrested the cell cycle at S phase, upregulated the expression of myeloid surface marker CD11b, reduced the nuclear cytoplasmic ratio, and altered the horseshoe shape of nuclei, as evidenced by
Wright-Giemsa staining. Further, we found that oTR reduced the protein level of phosphorylated STAT3 and activated the protein level of phosphorylated STAT1, but not total STAT1 and STAT3. Moreover, we found time-dependent inhibition of the expression of both phosphorylated STAT3 and its upstream kinase, Janus kinase 2, when cells were treated with oTR. Additionally, the levels of phosphorylated SHP-1 were increased while phosphorylated SHP-2 was decreased. Collectively, our data indicate a differentiation-induced mechanism underlying the inhibition of STAT3 signaling upon treatment with oTR. Therefore, oTR may constitute a novel differentiation-induced therapeutic for use in clinical treatment of AML.

**Keywords**  Ortho-topolin riboside; differentiation ; STAT3 signal ; HL-60 cells
Introduction

Leukemia comprises a group of malignant blood diseases characterized by uncontrolled overproduction of hematopoietic progenitors or terminally differentiated leukocytes [1]. It remains particularly difficult to treat acute myeloid leukemia (AML) [2]. Thus far, cytotoxic drugs targeting proliferating cells have shown limited efficacy in the treatment of AML; notably, such drugs also exhibit significant toxicity. All-trans retinoic acid (ATRA) and arsenic trioxide (ATO) provide new options for differentiation therapy, but have been limited to treatments of AML-M3, and are not suitable for other subtypes of AML [3]. Therefore, development of new chemotherapy drugs that can effectively promote differentiation and eliminate AML is urgently needed.

During a variety of cellular processes (e.g., growth, differentiation, metabolism, migration, and survival), the level of tyrosine phosphorylation must be balanced; this is maintained by protein tyrosine kinases and protein tyrosine phosphatases (PTPs) [4]. SHP-1 and SHP-2 (SH2 domain-containing phosphatases 1 and 2) comprise two PTPs that play important roles in lymphocytes and other hematopoietic cells [5]. Signal transducer and activator of transcription (STAT) proteins play important roles in the regulation of cell proliferation, survival, differentiation, and immune response [6]. Aberrant STAT signaling has often been observed in hematopoietic malignancies, including AML [7-9]. Notably, some natural products have been reported to inhibit STAT3 activity through the regulation of SHP-1 and/or SHP-2 in cancer cells [10].

Cytokinins are important purine derivatives that act as hormones in plants, where they control many processes in the regulation of plant growth and differentiation [11]. Moreover, cytokinins have been shown to exhibit anticancer activity in vitro and in vivo in mammals [12, 13]. Cytokinin ribosides (N6-substituted adenosines) have been found to exhibit anticancer activity through the induction of apoptosis and blockage of cell cycling in various cancer cell lines, as well as in several xenografts and a small clinical trial [14].

Ortho-topolin riboside (oTR; also known as 6-(2-hydroxybenzylamino)-9-D-
ribofuranosylpurine, Fig. 1) is a naturally occurring riboside form of cytokinins that is present at micromolar concentrations in poplar leaves after sunrise [15]. oTR has shown exceptional cytotoxic activity against NCI60 cell lines, compared with the activity of other cytokinin ribosides [14]. However, a detailed molecular mechanism underlying the effect of oTR on differentiation has not been elucidated with respect to AML cells. We previously reported that oTR exhibited antitumor activity by inducing differentiation in the U937 human leukemia cell line, but the differentiation-inducing properties of oTR remain undefined in HL-60 human myeloid leukemia cells. In the present study, we investigated the anti-tumor effect of oTR on HL-60 cells.

Materials and Methods

Materials
Ortho-topolin riboside (oTR, purity >99%) was purchased from olchemm GmbH (Czech Republic). RPMI 1640 and fetal bovine serum were purchased from Gibico. Anti-STAT3, anti-phospho-STAT3Y705, anti-JAK2, anti-phospho-JAK2Y1007/1008, Anti-phospho-SHP1Tyr564, Anti-phospho-SHP2Tyr542, anti-SHP1, anti-SHP2 and β-actin were purchased from Cell Signaling (Danvers, M). Wright-Giemsa staining solution was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Penicillin-streptomycin, Cell Counting Kit-8 (CCK-8), Phosphatase Inhibitor complex, Anti-Human CD11b-PE was purchased from eBioscience (San Diego, CA, USA).

Cell culture
HL-60 cells were purchased from the Cell Bank of the Chinese Academy of Sciences
(Shanghai, China). Cells were incubated in complete RPMI 1640 (RPMI 1640 with 10% (v/v) FBS and 100 U/mL of penicillin and streptomycin) at 37°C in a humidified atmosphere containing 5% CO2.

**Cell viability assay**

Cell viability was assayed in 96-well flat-bottomed plastic microplates. Cells were seeded at a density of 1×10⁴ cells per well, then treated with increasing concentrations of oTR (0.1, 1, 10, 50 and 100 μM) for 24 h. Cell viability was analysed using the Cell Counting Kit-8 (CCK-8), 10 μL of CCK-8 solution was added to each well before incubation for a further 3 h at 37°C. After incubation, the absorbance was measured at 450 nm using a microplate reader (3001, Thermo Scientific, Finland). The cell viability was expressed as: sample absorbance/control absorbance ×100%.

**Wright-Giemsa staining**

After treating the cells in the 96-well plate with the compound for different hours, the cells were collected and washed with PBS. Cells were mounted on glass slides by bench-top low speed centrifuge (L2-4K, Hunan, China), and morphological evaluation of differentiation was assessed using a Wright-Giemsa staining kit, dried overnight at room temperature and observed using a microscope (Leica, DMI 4000B and Germany).

**CD11b protein expression analysis**

The treated cells were collected and analyzed for expression of the cell surface differentiation marker CD11b by using a FACS Calibur flow cytometer (FACS Calibur, Becton Dickinson, USA). After treatment, cells were harvested and washed with PBS, and the cells were incubated with the blocking antibody Anti-Mouse CD16/CD32 for 15 minutes at room temperature, and then incubated with Anti-Human CD11b-PE antibody for 30 minutes at room temperature in the dark, then analyzed by flow cytometry.

**Western blot analysis**

When cells were treated with oTR for time indicated, the cells were washed twice in PBS and resuspended in RIPA buffer (50 mM Tris–HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM PMSF, 1 mg/mL aprotinin, 1 mg/mL
leupeptin, 1 mM Na$_3$VO$_4$, 1 mM NaF and a protease inhibitor cocktail), incubated on ice for 20 min, and centrifuged at 12,000 rpm for 20 min at 4°C. The protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, CA, USA).

The protein extracts (30μg) were boiled in Laemmli buffer, resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% dry milk in Tris-buffered saline Tween-20 (TBST) at room temperature for 1 h. The membranes were incubated overnight using primary specific antibodies for STAT3, phospho-STAT3, JAK2, phospho-JAK2, SHP1, SHP2, phospho-SHP1, phospho-SHP2 and β-actin. The membranes were washed three times with TBST for 10 min each, followed by incubation with a secondary antibody for 1 h. Immunoreactive proteins were detected by an enhanced chemiluminescence blotting detection system (FluorChem HD2, Alpha Innotech).

RT-PCR analysis

The oTR-treated HL-60 cells were collected, washed three times with PBS, and the total RNA in the cells was extracted with Trizol according to the reagent instructions, and quantified by NanoDrop. According to the manufacturer's description, the genomic DNA was removed by adding gDNA Eraser Buffer, gDNA Eraser, total RNA, RNase Free dH$_2$O using the PrimeScript™ RT kit. Then the above DNA-removing reaction solution which mixed with RNase Free dH$_2$O, PrimeScript Buffer, RT Prime Mix and PrimeScript RT Enzyme Mix were reverse transcribed using a Thermal Cycler Dice instrument (TaKaRa). The PCR reaction mixture consisted of TB GreenTM Premix Ex TaqTM II (Tli RNaseH Plus), PCR Primer, DNA template, sterilized ddH$_2$O according to the kit TaKaRa (NO. RR820A) TB GreenTM Premix Ex TaqTM II (Tli RNaseH Plus). The CT value of each gene mRNA was detected by Real time PCR using LightCycler® 96, and analyzed by $2^{-\Delta\Delta CT}$ method. The PCR primer sequences are as follows:

<table>
<thead>
<tr>
<th>Table 1: The sequences of primers for real-time PCR</th>
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<tr>
<td><strong>PU.1</strong> forward: 5’-GCCCTATGACACGGATCTAT-3’</td>
</tr>
<tr>
<td>reverse: 5’-AAGTCCAGTAATGGCGCTAT-3’</td>
</tr>
</tbody>
</table>
C/EBPα forward: 5’-GACAAGAACAGCAACGAGTAC-3’
reverse: 5’-TCATTGTCACTGGTCAGCTC-3’

C/EBPβ forward: 5’-CATCGACTCAGCCCCGTAC-3’
reverse: 5’-GAGAAGAGGTCGGAGAGGAAG-3’

GAPDH forward: 5’-TGGTGAAGCAGGCATCTGAG-3’
reverse: 5’-CTCCTGCAGACTTCAACAGCA-3’

Statistical analysis

Results are expressed as the mean ± SD. The student's t-test and one-way ANOVA were used to compare the test and control values. *p<0.05, **p<0.01 were considered statistically significant compared to the control.

Results

oTR inhibited cell proliferation in HL-60 cells

HL-60 cells were treated with increasing concentrations of oTR (0.1, 1, 10, 50 and 100 μM) for 24 h. We tested the cell viability on cell proliferation by using the CCK-8 assay. The results showed that the rate of cell viability was inhibited significantly from 91.1% to 11.3% when treated with increased concentrations of oTR. The half maximal inhibitory concentration IC50 value is 3.4 μM (Fig. 2).

![Cell viability graph](image)

**Fig 2.** Effects of cell viability of oTR on HL-60 cells. Cell viability was analyzed by the CCK-8 assay after 24 h of treatment with increasing concentrations of oTR. The values are expressed as the mean ± SD from three individual experiments. **p < 0.001 versus control.

oTR induced the differentiation of HL-60 cells
To detect the antiproliferative activity in HL-60 cells, we examined the change of cell-cycle arrest when treated with oTR. We found oTR arrested the cell cycle at S in HL-60 cells. (Figure 3A). Because cell differentiation is tightly coupled with cell growth arrest in cancer cell, we used CD11b as a mature granulocytes marker to test the differentiation of HL-60 cells. We found the levels of myeloid (CD11b) marker was increased in a dose dependent manner after incubation with 1μM oTR for 24 h(Fig. 3B). We also confirmed the inducing differentiation by morphologic analysis using Wright Giemsa staining. We found untreated HL-60 cells were round with large and round nuclei and a sparse cytoplasm. Treatment with oTR reduced the nuclear cytoplasmic ratio and altered the horseshoe morphology of nuclei (Fig. 3C). All these indicated that oTR induced the differentiation of HL-60 cells into mature granulocytes.
Fig 3. Differentiation-inducing activity of oTR in HL-60 cells. (A) The HL-60 cells were treated with oTR (1 μM) for 24 h, and cell cycle analysis was performed using flow cytometry. (B) The percentage of CD11b expressed in HL-60 cells. *P < 0.05, **P < 0.01 versus the control group without any treatment. (C) Effects of oTR on the morphology of HL-60 cells. Cells were treated with 1 μM oTR or vehicle (0.1% DMSO) as a positive control for 24 h, and morphological changes were observed by phase contrast microscopy. Black arrows: Differentiated cells.

**oTR suppressed STAT3 activation in oTR-induced AML differentiation while induced the activation of STAT1**

It has been reported that signal transducer and activator of transcription (STAT) proteins as latent cytoplasmic transcription factors played an important role in cellular processes including cell proliferation, differentiation and apoptosis[16] [17]. Here we found oTR obviously decreased the protein level of p-JAK2\(^{\text{Y1007}}\) and p-STAT3\(^{\text{Y705}}\) but not the total protein of JAK2 and STAT3 in HL-60 cells (Fig.4). We also determined whether the activation of STAT1 phosphorylation is involved in the differentiation. Here we found oTR induced the differentiation in HL-60 cells by acting through the activation of p-STAT1.

Fig 4. Effects of oTR on protein levels of phosphorylated STAT1, STAT3 and JAK in HL-60 cells. HL60 cells were treated with oTR (1 μM) for 24h. The protein levels were detected by Western blot analysis. The experiments were repeated three times and the data show the representative results.

**oTR changed the effects of oTR on mRNA levels of C/EBPα, C/EBPβ and PU.1**

To further examine the ability of myeloid differentiation after treatment with oTR in
HL-60 cells, we detected the mRNA expression on the monocytic transcription factor C/EBPα, C/EBPβ and PU.1. Consistent with monocytic differentiation, we found C/EBPα, C/EBPβ and PU were upregulated compared with untreated cells at 48 h after treatment with oTR (Fig. 5).

Fig 5. Cells were treated with 1 μM oTR for 48 h. The mRNA expression of C/EBPα, C/EBPβ and PU.1 were detected by qRT-PCR. Data presented are the mean±SD of three independent experiments. *** for P < 0.001, ** for P < 0.01, * for P < 0.05.

oTR inhibited the phosphorylation of SHP2, while increased the phosphorylation of SHP1

It has been report that SHP-1 and SHP-2 have an important role in haematopoietic cells [18, 19]. The expression of SHP-1 in HL-60 cells were examined. We found oTR increased phosphorylated SHP-1 protein expression (Figure 6). Since SHP-2 participates in the JAK/STAT signalling, and positively contributes to cell differentiation and cell cycle maintenance [20-22]. The effect of oTR on the expression of phosphorylated SHP-2 also was detected. Here we found oTR inhibited the phosphorylation of SHP-2.

Fig 6. Effects of oTR on protein levels of phosphorylated SHP1, and SHP2 in HL-60 cells. HL60 cells were treated with oTR (1 μM) for 24h. The protein levels were detected by Western blot analysis. The experiments were repeated three times and the data show the representative results.
Discussion

Differentiation therapies involve conversion of malignant tumors to curable tumors or terminally differentiated cells that undergo no further proliferation [23].

AML has been classified into eight subtypes: M0 to M7 according to the French, American, British (FAB) group, which used morphology and cytochemistry to characterize AML [24, 25]. APL is AML-M3 subtype characterized by the t(15;17) translocation, which fuses the promyelocytic leukemia (PML) gene to the retinoic acid receptor α (RARα) gene, and leads to the production of the PML/RARα fusion protein[26]. The ATRA and ATO are therapeutics specifically designed for this molecular feature[27]. Although the use of these two drugs has greatly improved prognosis for patients with APL, they are not suitable for other subtypes of AML. Therefore, new differentiation-induced agents are needed for AML.

HL60 cell line is the M2 sub-type of AML[28]. In the present study, we found that cytokinin oTR was effective for inducing granulocytic differentiation of HL-60 AML cells. oTR increased the phosphorylation of SHP-1 while inhibiting the phosphorylation of SHP-2. We also found that oTR reduced the expression of phosphorylated STAT3 and the upstream kinase, Janus kinase 2, in a time-dependent manner. Our findings indicate that oTR can exert antitumor activity in HL-60 cells by inducing differentiation through the STAT3 signaling pathway.

Cytokinins are a class of naturally occurring plant hormones and purine derivatives that play key roles in the regulation of plant growth and differentiation; moreover, they exhibit anticancer activity in vitro and in vivo in mammals [12, 14]. Thus, cytokinins can affect growth and differentiation in animals, and may be useful in treating human diseases that involve dysregulated cell proliferation and/or differentiation [12].

oTR is a naturally occurring nucleoside that can be extracted from plants. In this study, we showed that oTR significantly inhibited the proliferation of HL-60 AML cells, as indicated by reduced viability of HL-60 cells upon treatment with oTR. Furthermore, we found that oTR induced the differentiation of HL-60 cells into
mature granulocytes; oTR arrested the cell cycle at S phase and upregulated the expression of CD11b. We confirmed the induction of differentiation by morphologic analysis. We found that treatment with oTR reduced the nuclear cytoplasmic ratio and altered the horseshoe morphology of nuclei. Taken together, these findings indicated that oTR induced the differentiation of HL-60 cells into mature granulocytes.

STAT1 and STAT3 activation is important for the terminal differentiation of immature leukemia cells [29]. The activation of STAT1 has been confirmed in the differentiation of various drug-induced leukemia cells [30-32]. Activation of STAT1 is important in ATRA and other various drug-induced differentiation therapies for myeloid cells in APL and other subtypes of acute leukemia. Phosphorylated STAT1 can transactivate downstream target genes, such as PU.1, C/EBPa, C/EBPβ, CXCL-10, RIG-G, and IRF-I, in order to induce cell differentiation.

Abnormal STAT3 activation is often detected in many human cancer cells, including leukemia, lymphoma, and solid tumors [33]. Homodimerization of STAT3 can lead to nuclear translocation, DNA binding, and subsequent gene transcription involved in the activation of STAT3 [33]. During activation, STAT3 phosphorylation is performed through activation of its upstream Janus kinases [34]. Agents that suppress the activation of STAT3 reportedly have potential for cancer prevention and treatment [6]. Here, we found that oTR suppressed STAT3 activation in oTR-induced AML differentiation therapy, while it induced the activation of STAT1; moreover, oTR induced the regulation of transcription factors C/EBPa, C/EBPβ, and PU.1, all of which are important during myeloid differentiation.

SHP-1 is encoded by the PTPN6 gene and expressed widely in the hematopoietic system; it exhibits various impacts on cell signaling pathways [6, 35]. SHP-1 has been reported to negatively regulate the phosphorylation of STAT3 during tumor development, including in the formation of leukemias, as well as in gastric and breast cancers [20, 36]. SHP-2 is encoded by the PTPN11 gene; its overexpression has been observed at both protein and RNA levels in several human AML cell lines and primary tumor samples [37]. Notably, SHP-2 can inhibit apoptosis in cancer stem cells and enhance the growth of leukemia stem cells [20, 37, 38]. Although SHP-2 is
traditionally regarded as a PTP, such that it should inactivate kinases and serve as a negative regulator of cell function, SHP-2 has been reported to promote cell growth and function through both upregulation of positive signaling pathways and downregulation of negative signaling pathways [39-41]. Here, we found that oTR inhibited the phosphorylation of SHP-2, whereas it increased the phosphorylation of SHP-1 in HL-60 cells.

In conclusion, our results demonstrated that oTR induced the differentiation of HL-60 human AML cells by suppression of the STAT3 signaling pathway and induction of STAT1 activation. We also found that oTR reduced the level of phosphorylated SHP-2, while it increased the level of phosphorylated SHP-1 in HL-60 cells. Our data suggest that oTR might be applicable in treatment of AML patients with M2 subtype by inducing cell differentiation, but not all subtypes of AML. Therefore, future researches on the antitumor effect of oTR are needed.

**Conflict of Interest**

All authors declare that they have no conflict of interest.

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