Enzasataurin Enhances ATRA-induced Differentiation of Acute Myeloid Leukemia Cells

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Research

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Abstract

Background

All-trans retinoic acid (ATRA) is considered to be the sole clinically useful differentiating agent in the treatment of acute myeloid leukemia (AML). However, it has been effective only in acute promyelocytic leukemia (APL) but not other subtypes of AML. Therefore, finding strategies to sensitize cells to ATRA may develop ATRA-based therapy in the treatment of non-APL AML patients.

Methods

Cell proliferation was assessed by cell growth. Cell death was evaluated by cell viability and Annexin-V assay. Cell differentiation was analyzed by CD11b expression and morphology. To explore the underlying mechanisms, we studied the role of PKCβ, MEK, ERK, AKT, PU.1, C/EBPβ and C/EBPε by Western-blotting analysis.

Results

In this study, a clinically achievable concentration of enzastaurin enhanced ATRA-induced differentiation of AML cell lines, HL-60 and U937 as well as non-APL AML primary cells, while it also restored ATRA sensitivity in ATRA-resistant cell line, HL-60Res. Mechanistically, in all these cell lines, enzastaurin-ATRA (enz-ATRA) enhanced the protein levels of PU.1, CCAAT/enhancer binding protein β (C/EBPβ) and C/EBPε. The activity of protein kinase C β (PKCβ) was suppressed by enz-ATRA treatment in HL-60 and HL-60Res cells. However, another PKCβ-selective inhibitor mimicked the cellular and molecular effects of enzastaurin only in HL-60 cells. Only in U937 cells, enz-ATRA activated MEK and ERK, and a MEK specific inhibitor suppressed enz-ATRA-triggered differentiation and reduced the protein levels of PU.1, C/EBPβ and C/EBPε. Enz-ATRA activated Akt in HL-60 and HL-60Res cells. However, an Akt inhibitor blocked enz-ATRA-triggered differentiation and restored the protein levels of PU.1, C/EBPβ and C/EBPε only in HL-60Res cells. Therefore, PKCβ inhibition, MEK/ERK and Akt activation are involved in enz-ATRA-induced differentiation in HL-60, U937 and HL-60Res cells, respectively by modulation of the protein levels of C/EBPβ, C/EBPε and PU.1.

Conclusions

Enzastaurin, at the clinically achievable concentration, enhances ATRA-induced differentiation of AML cells by PKCβ inhibition, MEK/ERK and Akt activation. This study may provide a potential therapeutic strategy for AML patients.

Background

Acute myeloid leukemia (AML) accounts for 80% of adult acute leukemia [1]. The median age at diagnosis is about 70 years with 5-year survival rate of only 10% for the patients above the age of 60 years while 40% for younger patients (18–60 years) [1]. Due to the improvement of lifespan of the general population, AML is predicted to increase 38% in elder patients by 2031. Over the last four decades, cytarabine/anthracycline-based chemotherapy has been the main therapeutic strategy for AML patients resulting in the remission rate of 60–85% for patients younger than 60 years of age and 40–60% for elder patients [1]. However, most patients relapse and become resistant to the treatment. As mentioned above, survival is worse for elder patients who neither can tolerate intensive treatment nor are suitable for stem cell transplantation. Therefore, there is a pressing need to develop new therapeutic strategy for AML patients. Over the last couple of years, some new genetic driver mutations in AML have been identified and several mutation-targeted agents with promising results in clinical trials have been developed [2]. However, only a small portion of AML patients can benefit from these novel treatment strategies. Thus, the development of other effective anti-AML therapies is still required.

Differentiation therapy, which clears tumor bulk by terminal maturation with relatively less severe side effects, may be an alternative to chemotherapy in this circumstance. All-trans retinoic acid (ATRA), the active metabolite of vitamin A, has been successfully applied in the treatment of acute promyelocytic leukemia (APL) by differentiation induction [3]. However, due to the complicated physiopathology of non-APL AML, the clinical trial of ATRA in AML had disappointing results [4]. Since ATRA is a master regulator of myeloid cell differentiation, research strategies to extend the efficacy of ATRA-based therapy to non-APL AML is ongoing. *Nucleophosmin1* (NPM1) mutation without FLT3 internal tandem duplications (FLT3-ITD), *isocitrate dehydrogenase 1 (IDH1)* R132H mutation or over expression of *ecotropic viral integration site 1* (EVI-1) have been demonstrated to increase the response of non-APL AML cells to ATRA [5–7], suggesting that AML patients with certain genetic alteration might benefit from ATRA-based therapy. Combination of ATRA with chemotherapy, epigenetic modifiers or arsenic trioxide may be a rational approach to some AML patients [8]. Alternative strategies to increase the expression or the activity of retinoic acid receptor α (RARα) or inhibit its degradation have shown to restore the sensitivity of AML cells to ATRA in *vitro* or *in vivo* [8].

MEK/ERK pathway is required for myeloid differentiation induced by certain cytokine and ATRA-triggered differentiation in HL-60 and APL cells [9–12]. Except for limited studies demonstrating that Src inhibitors can promote ATRA-induced differentiation in AML cells by MEK/ERK, the MEK/ERK pathway is rarely used to improve ATRA sensitivity in AML cells [13, 14]. Since MEK/ERK is an important cytoplasmic pathway for myeloid differentiation, it may serve as a target for enhancing ATRA sensitivity in AML cells. Enzastaurin, a derivative of protein kinase C (PKC) pan-inhibitor.
staurosporine, has been designed to suppress the activation of PKCβ [15]. It has been proven to be safe and well tolerated in multiple clinical trials, and has shown promising anti-cancer activity [15]. Moreover, it can also reverse ATRA resistance and synergize with ATRA to induce differentiation in ATRA-resistant APL cells via MEK/ERK [16]. However, whether enzastaurin can promote ATRA-induced differentiation in AML cells has not yet been investigated.

In this study, non-APL AML cell lines HL-60, U937 and ATRA-resistant HL-60 cell line HL-60Res were used as in vitro models. To be pointed out, these cell lines are all without NPM1 mutation which is associated with increased responsiveness to ATRA [17, 18]. Clinical achievable concentration of enzastaurin enhanced ATRA-induced differentiation in HL-60, U937 and non-APL AML primary cells while it also reversed ATRA resistance in HL-60Res cells. Mechanistically, different pathways were involved in. PKCβ inhibition, MEK/ERK and AKT regulated the combination of enzastaurin and ATRA (enz-ATRA) induced differentiation in HL-60, U937 and HL-60Res cells, respectively. PU.1, CCAAT/enhancer binding protein β (C/EBPβ) and C/EBPε were the downstream molecules of these signaling pathways.

Methods

Reagents

ATRA was obtained from Sigma-Aldrich (St Louis, MO, USA). Enzastaurin, trametinib and Ly294002 were purchased from Selleckchem Chemicals (Houston, TX, USA). A PKCβ inhibitor was obtained from Merck (Darmstadt, Germany). All reagents were dissolved in dimethyl sulfoxide (DMSO).

Primary cells and cell culture

Bone marrow samples were collected at the time of diagnosis at the Department of Hematology of Ruijin Hospital. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki, and the study was approved by the Medical Science Ethic committee of Shanghai Jiao Tong University School of Medicine. Mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque Plus (GE healthcare bio-sciences, Uppsala, Sweden) and maintained in Iscove’s Modified Dulbecco’s Medium(IMDM)(GE healthcare bio-sciences) supplemented with 20% fetal bovine serum (GE healthcare bio-sciences), 10 ng/mL recombinant human interleukin-3 (rhIL-3), 10 ng/mL rhIL-6 and 50 ng/mL recombinant human stem cell factor (rh SCF) (PeproTech Inc China, Suzhou, Jiangsu, China). HL-60 and HL-60Res cells were cultured in IMDM, supplemented with 20% fetal bovine serum while U937 cells were cultured in RPMI-1640 medium (GE healthcare bio-sciences), supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO2 at 37 ºC.

Annexin-V analysis

According to instructions provided in the Annexin V-7AAD Apoptosis Detection Kit (BD Biosciences Pharmingen, San Diego, CA, USA), 5 × 10⁵ cells were harvested and washed with binding buffer. Subsequently, cells were incubated with 5 µL 7-Amino-Actinomycin and 5 µL annexin-V in the dark at room temperature for 15 min. Fluorescent intensities were evaluated by flow cytometry (EPICS XL, Coulter, Hialeah, FL, USA).

Cell differentiation assays

Cell maturation was determined by cellular morphology and the content of cell surface differentiation-related antigen CD11b. Morphology was evaluated with May-Grunwald-Giemsa's staining and observed at 1000x magnification. The expression of cell surface differentiation-related antigen CD11b (Coulter, Marseilles, France) was determined by flow cytometry (EPICS XL).

Western-blotting analysis

After lysed with RIPA buffer (Sigma-Aldrich) and centrifuged at 13,000 rpm for 10 min at 4 ºC, supernants were collected and quantified by Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA, USA). 20 or 50 µg protein extracts were loaded onto 8% SDS–polyacrylamide gel, subjected to electrophoresis, and transferred to polyvinylidene difluoride membranes (GE Healthcare UK Ltd, Buckinghamshire, UK). Blocking with 5% nonfat milk or BSA in PBS, the membranes were incubated with the following primary antibodies: C/EBPβ, C/EBPε, PU.1 from Santa Cruz Biotech (Santa Cruz, CA, USA); phospho-p44/42 Erk1/2 (Thr202/Tyr204), phospho-MEK1/2 (Ser218/222), Phospho-PKC (pan) (βII Ser660), Phospho-PKCa/β II (Thr638/641), Phospho-Akt (Ser473), Phospho-Akt (Thr308) from Cell Signaling Technology (Beverly, MA, USA); GAPDH from Proteintech (Rosemont, IL, USA). Then membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare UK Ltd). Immunocomplexes were visualized with chemiluminescence kit (GE Healthcare UK Ltd). To detect Erk1/2, MEK1/2, PKCβ and AKT, the same membrane incubated with accordingly phosphorylated antibody was stripped with stripping buffer (2% SDS, 100 mM beta-mercaptoethanol, 50 mM Tris, pH6.8), followed by blocking and probing respectively with anti-Erk1/2 (Cell Signaling Technology), anti-MEK1/2 (Cell Signaling Technology), anti-PKCβ (Santa Cruz Biotech) or anti-AKT (Cell Signaling Technology).

Statistical analysis

For cell growth, cell survival, Annexin-V assay and histogram of CD11b, values are expressed as mean ± SD, p values are mentioned in the corresponding figure legends. Chi-square test (n = 20,000) was used to analyze the flow-cytometric analysis of CD11b, P < 0.05 was taken to indicate statistical significance.
Results

Enzastaurin enhances ATRA-induced differentiation in HL-60, U937 cells and patient-derived AML blasts while it also reverses the ATRA resistance in HL-60Res cells.

Since 2 µM has been demonstrated to be the clinical achievable concentration of enzastaurin [19], such concentration was used as the maximum concentration of enzastaurin in all the cell lines. Only 2 µM enzastaurin alone or in combination with ATRA inhibited cell growth in HL-60 and U937 cells (Additional file1: Figure S1a and c) while the proliferation was not affected with any treatment in HL-60Res cells (Additional file1: Figure S1b). The cell viability was maintained above 95% with any treatment in all the cell lines (Additional file1: Figure S1d-f). Meanwhile, the content of Annexin V+ cells only increased slightly with some treatments in all the cell lines (Additional file1: Figure S1g-i).

Morphologically, as illustrated in Fig. 1a, all the cell lines presented a characteristic morphology of primitive cells such as round nucleus and large nuclear/cytoplasm ratio. With ATRA treatment for 3 days in HL-60 and U937 cells, some cells displayed decreased nuclear/cytoplasm ratio with kidney-shape nuclei. However, there was no obvious change in HL-60Res cells with ATRA treatment for 10 days. More matured cells were presented in all the cell lines with the combination of any concentration of enzastaurin and ATRA, especially with co-treatment of ATRA and 2 µM enzastaurin (Fig. 1a). Consistent with the morphology, a synergistic effect of enzastaurin and ATRA on the content of CD11b+ cells was also observed in a dose-dependent manner in all the cell lines (Fig. 1b-e). Therefore, enzastaurin enhanced ATRA-induced differentiation of HL-60 and U937 cells while restored ATRA sensitivity in HL-60Res cells. For non-APL AML primary cells, 5 out of 9 samples were AML-M4 and AML-M5 (Table 1). In 4 out of these 5 samples, enzastaurin enhanced ATRA-induced differentiation as assayed by morphology and the content of CD11b+ cells (Fig. 2a and b). In samples diagnosed as AML-M1 (No.2), AML-M2 (No.8 and No.9), MDS transforming to AML (No.7) and AML-M5 with AML-ETO fusion gene and c-kit mutation (No.6), such effect was not observed (Table 1). One difference between these two groups was that 4 cases that were effective to enz-ATRA had partial response to ATRA, while 5 cases that were invalid to enz-ATRA had no response to ATRA (Table 1). Thus, enzastaurin enhanced ATRA-induced differentiation in some AML primary cells.

Table 1
Patients data and response to enzastaurin and/or ATRA

| No. | sex | age | karyotype | Gene mutation/fusion | Blast(%) | WBC(x10^9/L) | FAB classification | CD11b+cells(%) | DMSO | RA | EN | EN + RA |
|-----|-----|-----|-----------|----------------------|----------|--------------|--------------------|----------------|------|----|-----|---------|
| 1   | M   | 65  | 46,XY(t(6;11) (q27;q23) MLL-AF6 | 85.5     | 9.17        | AML-M4           | 9.53           | 33.4         | 33.9 | 56.7 |
| 2   | M   | 55  | 46,XY     | C/EBPa G141C mutation, C/EBPa P192_H193 inserts PP, C/EBPa A303_K313 duplication, C/EBPa Q305_R306 inserts HNVETQQKAKQ | 88       | 288         | AML-M1            | 0.6           | 3.8  | 0.2 | 5.5    |
| 3   | M   | 57  | 46,XY     | FLT3Y599_D600 inserts GSTGSSDNEYFYVDFREY | 50       | 97.73       | AML-M5            | 19.4          | 20.8 | 26.9 | 37.5 |
| 4   | F   | 44  | 48~49,XX,t(10;11),p12;q23,+21,+3 | MLL-AF10, N-RAS mutation | 83       | 7.8         | AML-M5a           | 3.33          | 24   | 9.87 | 33.4 |
| 5   | M   | 76  | 45~47,XY,7,+M1~M6 NRAS G12S mutation | 73       | 74.71       | AML-M4b          | 10.4           | 53  | 20.4 | 63.5 |
| 6   | F   | 55  | 45,X,-X,t(8;21;12),q22;q22p13 | AML1-ETO,C-KIT T380 duplication | 86       | 14          | AML-M5            | 3.2           | 3.9  | 9.3  | 9      |
| 7   | M   | 51  | 46,XY     | DNMT3A N-terminal catalytic domain mutation ND | 3.79     | ND          | MDS transforming to AML | 3.8           | 4.9  | 5.8  |
| 8   | F   | 56  | 46,XX     | C/EBPa K313 duplication | 54       | 8.45        | AML-M2a           | 2.2           | 1.9  | 7.2  | 8      |
| 9   | F   | 38  | 46,XX     | C/EBPa Q305P mutation | 36.5     | 6.12        | AML-M2            | 0.8           | 0.8  | 1.8  | 2.6    |

Mononuclear cells were isolated by density gradient centrifugation and maintained in IMDM supplemented with 20% fetal bovine serum, 10 ng/mL rhIL-3, 10 ng/mL rhIL-6 and 50 ng/mL rhSCF. The cells were treated with 2 µM enzastaurin (EN) and/or 1 µM ATRA (RA) for 4 days and CD11b-positive cells were calculated by flow cytometry. ND indicates not done.

Enzastaurin enhances ATRA-induced differentiation in HL-60 cells by inhibition of PKCβ.
To investigate the mechanisms of enz-ATRA treatment-triggered differentiation in these three cell lines, we used 2 μM enzastaurin in the following studies. Since enzastaurin has been designed to suppress the activation of PKCβ [15], we first studied the role of PKCβ in enz-ATRA-induced differentiation. Phosphorylation of Ser660 or Thr641 is essential for activation of PKCβ [20]. As shown in Fig. 3a, comparing with ATRA treatment, with enz-ATRA treatment, the phosphorylation of PKCβ Ser660 was decreased in HL-60 cells while the phosphorylation of PKCβ T641 was reduced in HL-60Res cells. However, in U937 cells, neither the phosphorylation of PKCβ Ser660 nor that of PKCβ T641 was reduced with enz-ATRA treatment comparing with ATRA treatment. Thus, enzastaurin inhibited PKCβ in HL-60 and HL-60Res cells. To confirm the role of PKCβ, another PKCβ inhibitor was combined with ATRA to examine whether it could mimic the effect of enzastaurin to augment ATRA-induced differentiation. 500 nM, 200 nM and 100 nM PKCβ inhibitor was used in HL-60, HL-60Res and U937 cells, respectively, with no obvious effects on survival. To note, such concentration of PKCβ is 5–25 fold higher than the IC50 to inhibit PKCβI and PKCβII as indicated in the instructions. Like enz-ATRA treatment, fully differentiated cells with lobed nuclei accompanied by markedly decreased nuclear/cytoplasm ratio were presented in HL-60 cells with PKCβ inhibitor-ATRA treatment for 4 days (Fig. 3b).

Moreover, comparing with enz-ATRA treatment, the content of CD11b+ cells was increased to the similar level in HL-60 cells with PKCβ inhibitor-ATRA treatment (Fig. 3c and d). Similar to enz-ATRA treatment, comparing with ATRA treatment, the phosphorylation of PKCβ Ser660 was reduced with PKCβ inhibitor in HL-60 cells (Fig. 3e). Thus, by suppression of PKCβ, PKCβ inhibitor could enhance ATRA-triggered differentiation in HL-60 cells just like enzastaurin. These results suggested that PKCβ inhibition might regulate enzastaurin-enhanced ATRA-triggered differentiation in HL-60 cells. However, PKCβ inhibitor could neither elevate ATRA-triggered differentiation in U937 cells nor restore ATRA sensitivity in HL-60Res cells as evaluated by morphology (Fig. 3b) and the content of CD11b+ cells (Fig. 3c and d). Therefore, PKCβ may not be involved in enz-ATRA treatment-triggered differentiation in U937 and HL-60Res cells.

PKCβ inhibition and MEK/ERK activation are involved in enz-ATRA-induced differentiation in HL-60 and U937 cells, respectively by upregulation of the protein levels of C/EBPβ, C/EBPε and PU.1.

To further survey the mechanisms of enz-ATRA treatment-triggered differentiation, we studied several proteins and signal pathways involved in ATRA-induced differentiation in HL-60 cells or granulocytes. As mentioned above, MEK/ERK signal pathway regulates certain cytokine-induced myeloid differentiation and ATRA-triggered granulocytic differentiation in APL cells and HL-60 cells [9–12]. C/EBPβ, C/EBPε and PU.1 are required for the maturation of the myeloid lineages, as well as ATRA-induced differentiation in APL cells [21–24]. Moreover, by MEK/ERK modulating the protein levels of C/EBPβ, C/EBPε and PU.1, some medicines including enzastaurin synergize with ATRA to induce differentiation in ATRA-resistant APL cells [16, 25–27]. As Fig. 4 shown, comparing with ATRA treatment, the protein levels of C/EBPβ, C/EBPε and PU.1 were increased remarkably by enz-ATRA treatment in all the cell lines. Meanwhile, comparing with ATRA treatment, PKCβ inhibitor-ATRA treatment also augmented the protein levels of C/EBPβ, C/EBPε and PU.1 in HL-60 cells. It was suggested that enzastaurin promoted ATRA up-regulated protein levels of C/EBPβ, C/EBPε and PU.1 by PKCβ inhibition to enhance ATRA induced-differentiation in HL-60 cells. However, comparing with ATRA, the phosphorylation levels of MEK and ERK were enhanced only in U937 cells with enz-ATRA treatment. In HL-60 and HL-60Res cells, ATRA phosphorlated MEK and ERK, while enz-ATRA did not elevate their phosphorylation levels. Trametinib, a highly specific and potent MEK1/2 inhibitor [28] did attenuate MEK activity in all the cell lines, as determined by Western-blotting of phosphorylated ERK1/2 (Fig. 5a). With trametinib pretreatment, fully differentiated cells with lobed nuclei and a decreased nuclear/cytoplasm ratio were replaced by primitive cells with round nuclei and a large nuclear/cytoplasm ratio in U937 cells (Fig. 5b). The content of CD11b+ cells was also significant suppressed by trametinib in U937 cells (Fig. 5c and d). Moreover, in the presence of trametinib, enz-ATRA treatment-enhanced protein levels of C/EBPβ, C/EBPε and PU.1 were remarkably decreased in U937 cells (Fig. 5e). Thus, enz-ATRA treatment-induced differentiation in U937 cells via MEK/ERK modulation of the protein levels of C/EBPβ, C/EBPε and PU.1. Trametinib slightly inhibited enz-ATRA treatment-triggered differentiation in HL-60 cells while unexpectedly augmented enz-ATRA treatment-induced differentiation in HL-60Res cells as evaluated by morphology (Fig. 5b) and the content of CD11b+ cells (Fig. 5c and d). Therefore, MEK/ERK signal pathway may not regulate enz-ATRA treatment-triggered differentiation in HL-60 and HL-60Res cells.

Akt activation positively regulates enz-ATRA-induced differentiation in HL-60Res cells by modulation of the protein levels of C/EBPβ, C/EBPε and PU.1.

Besides MEK/ERK, PI3K/AKT is another signal pathway demonstrated to be essential for ATRA-induced differentiation in HL-60 cells [29]. Phosphorylation of Ser473 or Thr308 is essential for activation of Akt [30]. In HL-60 cells, ATRA treatment for 48 h phosphorylated Akt at Ser473 and Thr308 while enz-ATRA treatment enhanced ATRA-promoted phosphorylation of both sites (Fig. 6a). In HL-60Res cells, ATRA did not enhance the phosphorylation of Akt, but enz-ATRA treatment for 72 h increased the phosphorylation of Akt at Ser473 (Fig. 6a). LY294002, the inhibitor of PI3K, did attenuate the activation of Akt in both cell lines (Fig. 6b). However, it suppressed enz-ATRA-induced differentiation in HL-60Res cells but not in HL-60 cells as determined by morphology (Fig. 6c) and the content of CD11b+ cells (Fig. 6d and e). Moreover, with LY294002 pretreatment, the protein levels of C/EBPβ, C/EBPε and PU.1 enhanced by enz-ATRA in HL-60Res cells were significantly reduced (Fig. 6f). Therefore, Akt was not involved in enz-ATRA treatment-induced differentiation in HL-60 cells. However, enz-ATRA treatment induced differentiation in HL-60Res cells via Akt modulation of the protein levels of C/EBPβ, C/EBPε and PU.1.

**Discussion**

In this study, we demonstrated that enzastaurin enhanced ATRA-induced differentiation in HL-60, U937 and non-APL AML primary cells as well as reversed ATRA-resistance in HL-60Res cells. As mentioned above, these three cell lines are all without NPM1 mutation, the marker of ATRA sensitivity [17, 18]. As the ATRA resistant cells, HL-60Res cells may better reflect the clinical effect of ATRA and enz-ATRA in non-APL AML patients. Moreover, the concentration of enzastaurin used in this study is clinically achievable [19]. Taken together, it indicates that such combination may provide a potential
therapy strategy for some AML patients. For patient-derived AML blasts, in 4 out of 5 AML-M4 and AML-M5 samples, enzastaurin enhanced ATRA-induced differentiation. It seemed that such combination might be effective in AML-M4 and AML-M5 patients. However, we also observed that the effectiveness of enz-ATRA in AML primary cells might be associated with ATRA sensitivity. Therefore, the association of sensitivity to enz-ATRA treatment with age, gender, chromosomal and genetic changes has not been investigated.

PKC is a family of serine/threonine kinases, consisting of 13 isoforms that play a crucial role in regulation of proliferation, differentiation, apoptosis, cell migration and gene expression. The role of different PKC isoforms in granulocytic differentiation is quite controversial. For ATRA-induced granulocytic differentiation in HL-60 cells, PKCa and PKCβII are activated and positively regulate granulocytic differentiation [31]. However, Zauli G et al reported that only PKCa and PKCγ but neither PKCβII nor PKCδ showed significant modification upon ATRA treatment in HL-60 cells [32]. Kambhampati S et al showed that only PKCδ was activated with ATRA treatment in HL-60 cells [33]. For myeloid differentiation, PKCα is suggested not to be required for granulocytic differentiation because its mRNA and protein are decreased with ATRA treatment in HL-60 and undetectable in peripheral blood neutrophils [34]. However, PLCγ2 and PKC are demonstrated to be crucial upstream signals that modulate myelopoiesis by G-CSF [35]. In this study, the inhibition of PKCβ by either enzastaurin or another PKCβ inhibitor did enhance ATRA-induced differentiation in HL-60 cells. It indicates that PKCβ may negatively regulate ATRA-induced differentiation in HL-60 cells. Further study showed that enzastaurin and PKCβ inhibitor both increased ATRA-enhanced protein levels of C/EBPβ, C/EBPε and PU.1 in HL-60 cells. It suggests that PKCβ inhibition may control enzastaurin-enhanced differentiation in HL-60 cells by modulation of the protein levels of C/EBPβ, C/EBPε and PU.1. PKC positively or negatively regulates the transcriptional activity of C/EBPβ by phosphorylation on different site [36, 37]. In addition, C/EBPβ can trigger the expressions of C/EBPε and PU.1 in ATRA-triggered differentiation in APL cells [22, 23]. PU.1 can also directly activate the transcription of C/EBPβ [38]. Therefore, there might be PKCβ inhibition-C/EBPβ-PU.1-C/EBPε cascade in enz-ATRA-induced differentiation in HL-60 cells. PKCβ was inhibited by enz-ATRA in HL-60Res cells whereas it was not suppressed in U937 cells. Moreover, another PKCβ inhibitor could not mimic the effect of enzastaurin to enhance ATRA-induced differentiation in both cell lines. Thus, PKCβ might not be involved in enz-ATRA-induced differentiation in these two cell lines. In fact, PKCβ-independent effect of enzastaurin is not rare [16, 39–41].

Comparing with ATRA, the phosphorylation levels of MEK and ERK were enhanced with enz-ATRA treatment only in U937 cells. Moreover, the specific inhibitor of MEK suppressed enz-ATRA-induced differentiation and the protein levels of C/EBPβ, C/EBPε and PU.1 only in U937 cells. Therefore, MEK/ERK-modulated the protein levels of C/EBPβ, C/EBPε and PU.1 in enz-ATRA-induced differentiation only in U937 cells, but not HL-60 cells or HL-60Res cells. Consistent with this study, staurosporine, the parent compound of enzastaurin, enhances ATRA-induced differentiation in U937 cells also via MEK/ERK-mediated modulation of C/EBPs [42]. MEK and ERK have been demonstrated to promote the expression of C/EBPβ and regulate the activity of C/EBPβ and PU.1 [43–45]. Since there is interaction of C/EBPβ, C/EBPε and PU.1 as mentioned above [22, 23, 38], there might be MEK-ERK-C/EBPβ-PU.1-C/EBPε cascade in enz-ATRA-induced differentiation in U937 cells.

Akt can positively or negatively control differentiation of leukemia cells depending on cell type and differentiation inducer [31, 46]. In HL-60 cells, Akt activation is required for ATRA-induced differentiation [29]. In the present study, though Akt was activated by enz-ATRA treatment, the inhibition of Akt did not suppress enz-ATRA-triggered differentiation in HL-60 cells. Thus, the involvement of Akt in enz-ATRA-triggered differentiation in HL-60 cells was excluded. In HL-60Res cells, Akt was activated by enz-ATRA treatment and Akt inhibitor suppressed differentiation and the protein levels of C/EBPβ, C/EBPε and PU.1. Therefore, Akt activation was involved in enz-ATRA-induced differentiation in HL-60Res cells by modulation of the protein levels of C/EBPβ, C/EBPε and PU.1. Akt induces transcriptional activity and expression of C/EBPβ and PU.1 [47–50]. In addition, C/EBPβ can trigger the expressions of C/EBPε and PU.1 [22, 23]. Collectively, there might be Akt-C/EBPβ-PU.1-C/EBPε cascade in enz-ATRA-induced differentiation in HL-

Conclusions

A clinically achievable concentration of enzastaurin enhances ATRA-induced differentiation of HL-60, U937 and non-APL AML primary cells as well as reverses ATRA-resistance in HL-60Res cells. PKCβ inhibition, MEK/ERK and Akt activation are involved in enz-ATRA-induced differentiation of HL-60, U937 and HL-60Res cells, respectively by modulating the protein levels of C/EBPβ, C/EBPε and PU.1. This study may provide a potential therapeutic strategy for some AML patients.

Abbreviations

AML acute myeloid leukemia; APL:acute promyelocytic leukemia; ATRA:all-trans retinoic acid; C/EBPβ:CCAAT/enhancer binding protein β; DMSO:dimethyl sulfoxide; IDH1:isocitrate dehydrogenase 1; EVI-1:ecotropic viral integration site 1; FLT3-ITD:FLT3 internal tandem duplications; NPM1:Nucleophosmin1; PKC:protein kinase C; RARα:retinoic acid receptor α; rhIL-3:recombinant human interleukin-3; rh SCF:recombinant human stem cell factor

Declarations

Ethics approval and consent to participate
This study involved bone marrow samples from AML patients. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki, and the study was approved by the Medical Science Ethic committee of Shanghai Jiao Tong University School of Medicine.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its additional information files].

Competing interests

The authors declare that they have no competing interests.

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Author contributions

ZY L, L C, M D, XQ W, Y S, J W and H L carried out the experiments. ZY L prepared all the figures. X C designed the study and wrote the manuscript. All authors read and approved the final manuscript.

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**Figures**
Figure 1

The effect of enz-ATRA treatment on cell differentiation in HL-60, HL-60Res and U937 cells. (a) HL-60 cells were treated with 1/2 μM enzastaurin (1EN/2EN) and/or 0.1 μM ATRA (RA) for 3 days. HL-60Res and U937 cells were treated with 1/2 μM enzastaurin (1EN/2EN) and/or 1 μM ATRA (RA) for 10 days and 3 days, respectively. One representative morphology of HL-60 (upper panel), HL-60Res (middle panel) and U937 (lower panel) cells are shown. Magnification is 1,000. Similar results were obtained in three independent experiments. Differentiation was also evaluated by flow-cytometric analysis of CD11b expression in HL-60 (b), HL-60Res (c) and U937 cells (d) with the indicated treatment for 4 days, 6 days and 3 days, respectively.
Each value represents the mean ± SD of three independent measurements. **P<0.001, versus DMSO treated cells. ### P<0.001, versus ATRA treated cells. &&& P<0.001, as compared with 1EN+RA in U937 and HL-60Res cells or 1EN+0.1RA in HL-60 cells. (e) The representative histogram of flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4 days, 6 days and 3 days, respectively. The percentages of CD11b+ cells are shown in the corresponding panels.
Enzastaurin enhances ATRA-induced differentiation in some primary blasts from non-APL AML patients. Primary blasts from patients were treated with 2 μM enzastaurin (EN) and/or 1 μM ATRA (RA) for 4 days. The morphology (a) and the histogram of flow-cytometric analysis of CD11b expression (b) are shown. Magnification of morphology is 1,000. The percentages of CD11b+ cells are shown in the corresponding panels.
PKCβ-inhibition mediates enzastaurin-enhanced ATRA-triggered differentiation of HL-60 cells. (a) HL-60 cells were treated with 2 μM enzastaurin (EN) and/or 0.1 μM ATRA (RA) for 1 h and 3 h. HL-60Res and U937 cells were treated with 2 μM enzastaurin (EN) and/or 1 μM ATRA (RA) for 3 h. The activation of PKCβ was evaluated by Western-blotting analysis of phosphorylated PKCβ at serine 660 and threonine 641. The same membrane incubated with anti-phospho-PKCβ was stripped and followed by detection of PKCβ. Different time points for protein collections have corresponding GAPDH expression as internal control. (b) HL-60 cells were treated with 500 nM PKCβ inhibitor and/or 0.1 μM ATRA (RA) for 3 days. HL-60Res cells
were treated with 200 nM PKCβ inhibitor and/or 1 μM ATRA (RA) for 10 days. U937 cells were treated with 100 nM PKCβ inhibitor and/or 1 μM ATRA (RA) for 3 days. The representative morphology of HL-60, HL-60Res and U937 cells are shown. Magnification is 1,000. (c) Differentiation was also evaluated by flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4 days, 6 days and 3 days, respectively. Each value represents the mean ± SD of three independent measurements. ***P<0.001, versus DMSO treated cells. ### P<0.001, versus ATRA treated cells. (d) The representative histogram of flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4 days, 6 days and 3 days, respectively. The percentages of CD11b+ cells are shown in the corresponding panels. (e) HL-60 cells were treated with 500 nM PKCβ inhibitor and/or 0.1 μM ATRA (RA) for 2 h and 3 h. Phosphorylation of PKCβ was measured by Western-blotting analysis. The same membrane incubated with anti-phospho-PKCβ was stripped and followed by detection of PKCβ. Different time points for protein collections have corresponding GAPDH expression as internal control.
The effect of Enz-ATRA and PKCβ inhibitor-ATRA on C/EBPs, PU.1 and MEK/ERK. HL-60 cells (upper left) were treated with 2 μM enzastaurin (EN) and/or 0.1 μM ATRA (RA) for 24 h. For PKCβ inhibitor, 500 nM was used in HL-60 cells for 24 h (lower right). U937 cells (lower left) were treated with 2 μM enzastaurin (EN) and/or 1 μM ATRA (RA) for 6 h, 12 h and 24 h. HL-60Res (upper right) cells were treated with 2 μM enzastaurin (EN) and/or 1 μM ATRA (RA) for 48 h and 72 h. The same membrane incubated with the antibodies to phosphorylated Erk1/2 or MEK1/2 was stripped and followed by

Figure 4
detection of MEK and ERK1/2. Since diverse time points for collecting protein were used, each has the expression of GAPDH as internal control. Similar results were obtained in three independent experiments.
Figure 5

MEK/ERK-inhibition suppresses enz-ATRA-triggered differentiation of U937 cells by restoring the expression of C/EBPs and PU.1. HL-60, HL-60Res and U937 cells were pretreated with 0.01 μM, 0.1 μM and 5 μM trametinib for 2 h, respectively. (a) The attenuation of MEK activation by trametinib (T) was detected by Western-blotting analysis of phosphorylated ERK1/2 in HL-60, HL-60Res and U937 cells with indicated treatments for 24 h, 48 h and 4 h, respectively. The same membrane incubated with the antibodies to phosphorylated Erk1/2 was stripped and followed by detection of ERK1/2. The expression of GAPDH was evaluated as internal control. Similar results were obtained in three independent experiments. (b) Effect of trametinib on
morphology in HL-60, HL-60Res and U937 cells incubated with the indicated drugs for 4 days, 10 days and 3 days, respectively. The magnification is 1,000. One representative experiment among three independent assays is shown. Similar results were obtained in three independent experiments. (c) Differentiation was also evaluated by flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4 days, 6 days and 3 days, respectively. Each value represents the mean ± SD of three independent measurements. ### P<0.001, as compared with 2EN+RA in U937 and HL-60Res cells or 2EN+0.1RA in HL-60 cells. (d) The representative histogram of flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4 days, 6 days and 3 days, respectively. The percentages of CD11b+ cells are shown in the corresponding panels. (e) The protein levels of C/EBPα, C/EBPδ and PU.1 in U937 cells with the indicated drugs for 24 h was assayed by Western-blotting. The expression of GAPDH was evaluated as internal control. Similar results were obtained in three independent experiments.
AKT controls enz-ATRA-induced differentiation of HL-60Res cells by enhancing the expression of C/EBPs and PU.1. (a) HL-60 cells were treated with 2 μM enzastaurin (EN) and/or 0.1 μM ATRA (RA) for 48 h. HL-60Res cells were treated with 2 μM enzastaurin (EN) and/or 1 μM ATRA (RA) for 72 h. The activation of AKT was evaluated by Western-blotting analysis of phosphorylated AKT at serine 473 and threonine 308. The same membrane incubated with anti-phospho-AKT was stripped and followed by detection of AKT. The expression of GAPDH was evaluated as internal control. (b) The attenuation of AKT activation by LY294002 (LY) was detected by Western-blotting analysis of phosphorylated AKT in HL-60 and HL-60Res cells with indicated
treatments for 48 h and 72 h, respectively. The same membrane incubated with the anti-phospho-AKT was stripped and followed by detection of AKT. The expression of GAPDH was evaluated as internal control. (c) The effect of LY294002 on morphology in HL-60 and HL-60Res cells incubated with the indicated drugs for 4 days and 10 days, respectively. The magnification is 1,000. One representative experiment among three independent assays is shown. (d) Differentiation was also evaluated by flow-cytometric analysis of CD11b expression in HL-60 and HL-60Res cells with the indicated treatment for 4 days and 6 days, respectively. Each value represents the mean ± SD of three independent measurements. ### P<0.001, as compared with 2EN+RA in HL-60Res cells. (e) The representative histogram of flow-cytometric analysis of CD11b expression in HL-60 and HL-60Res cells with the indicated treatment for 4 days and 6 days, respectively. The percentages of CD11b+ cells are shown in the corresponding panels. (f) The protein levels of C/EBPα, C/EBPβ, and PU.1 in HL-60Res cells with the indicated drugs for 72 h was assayed by Western-blotting. The expression of GAPDH was evaluated as internal control.

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