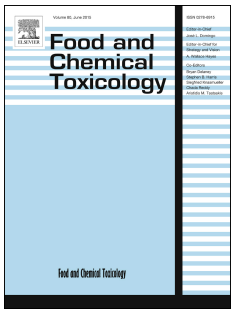
Accepted Manuscript



Dasatinib synergizes with ATRA to trigger granulocytic differentiation in ATRA resistant acute promyelocytic leukemia cell lines via Lyn inhibition-mediated activation of RAF-1/MEK/ERK

Ming Ding, Xiang-qin Weng, Yan Sheng, Jing Wu, Cui Liang, Xun Cai

PII: S0278-6915(17)30651-8

DOI: 10.1016/j.fct.2017.10.053

Reference: FCT 9378

To appear in: *Food and Chemical Toxicology*

Received Date: 27 September 2017

Revised Date: 27 October 2017

Accepted Date: 28 October 2017

Please cite this article as: Ding, M., Weng, X.-q., Sheng, Y., Wu, J., Liang, C., Cai, X., Dasatinib synergizes with ATRA to trigger granulocytic differentiation in ATRA resistant acute promyelocytic leukemia cell lines via Lyn inhibition-mediated activation of RAF-1/MEK/ERK, *Food and Chemical Toxicology* (2017), doi: 10.1016/j.fct.2017.10.053.

Dasatinib synergizes with ATRA to trigger granulocytic differentiation in ATRA resistant acute promyelocytic leukemia cell lines via Lyn inhibition-mediated activation of RAF-1/MEK/ERK

Ming Ding, Xiang-qin Weng, Yan Sheng, Jing Wu, Cui Liang, Xun Cai*

Shanghai Institute of Hematology and State Key Laboratory of Medical Genomics, Rui-jin Hospital, Shanghai Jiao Tong University School of Medicine,

No.197 Rui-jin Road II, Shanghai 200025, China

*Corresponding author at:

Shanghai Institute of Hematology and State Key Laboratory of Medical Genomics, Rui-jin Hospital, Shanghai Jiao Tong University School of Medicine

No.197 Rui-jin Road II, Shanghai, 200025, China

Tel: 86-21-64370045 extension 610709

Fax: 86-21-64743206

E-mail address: xuncai@yahoo.com[1]

[1] **Abbreviations**

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; APL, acute promyelocytic leukemia; ATO, arsenic trioxide; ATRA, all-trans retinoic acid; C/EBP, CCAAT/enhancer□binding protein; CML, chronic myeloid leukemia; DMSO, dimethylsilfoxide; FTS, salirasib; GO, gemtuzumab ozogamicin; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; RBD, Ras-binding-domain; SFK, Src family kinase; SDS, sodium dodecyl sulfate

Abstract

All-trans retinoic acid (ATRA) resistance has been a critical problem in acute promyelocytic leukemia (APL) relapsed patients. In this study, dasatinib synergized with ATRA to trigger differentiation in ATRA-resistant APL cell lines. The combined treatment activated RAF-1, MEK and ERK as well as enhanced ATRA-promoted up-regulation of the protein level of PU.1, C/EBPβ and C/EBPε. U0126 (MEK specific inhibitor) and sorafenib tosylate (RAF-1 specific inhibitor) suppressed the combined treatment-induced differentiation, ERK phosphorylation and the up-regulation of C/EBPs and PU.1. Sorafenib tosylate also attenuated the MEK activity. However, the combined treatment did not enhance Ras activity and Ras inhibitor neither blocked MEK activation nor inhibited differentiation. Therefore, the combined treatment induced differentiation via Ras independent RAF-1/MEK/ERK. Earlier than RAF-1 activation, dasatinib suppressed Lyn activity, the predominant activated Src family kinase (SFK) and dephosphorylated RAF-1 at S259. Furthermore, SFK inhibitor, PP2 did suppress Lyn activity and mimicked the effect of dasatinib on ATRA-induced differentiation as well as decreased phosphorylation of RAF-1 at S259. Thus, it was suggested that Lyn inhibition might activate RAF-1 by the dephosphorylation of RAF at S259 and lead to differentiation. In conclusion, the combination of dasatinib and ATRA could overcome ATRA resistance through Lyn inhibition-mediated activation of RAF-1/MEK/ERK.

Keywords: acute promyelocytic leukemia; dasatinib; all-trans retinoic acid; Lyn; RAF; differentiation

1. Introduction

Since the introduction of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) in the conventional chemotherapy of acute promyelocytic leukemia (APL), APL has transformed from a highly fatal cancer to a highly curable disease(Shen et al., 2004). However, there are subsets of patients that eventually relapse and/or develop resistance to ATRA. Increased ATRA metabolism and the up-regulation of cellular RA-binding protein were once suggested to be the mechanisms of ATRA resistance in early studies. However, ATRA-induced mutations in the ligand binding domain of the *RARα* moiety of *PML-RARα* fusion gene resulting in deficient ATRA binding were the major cause of ATRA resistance (Tomita et al., 2013). Besides defects in *PML-RARα*, mutations in *FLT3-ITD* or *TP53*, overexpression of *BP1* or co-expression of *AML-ETO* were associated with ATRA resistance (Abreu E Lima et al., 2005; Awwad et al., 2008; Gallagher et al., 2012; Welch et al., 2012). Furthermore, altered ligand-induced co-repressor release, co-activator recruitment might also contribute to ATRA resistance (Farris et al., 2012). Overexpression of topoisomerase II β by PKCδ and suppressed transcription of certain ATRA-targeted genes by BRG1 and nucleophosmin were both demonstrated to be the mediators of ATRA resistance (McNamara et al., 2010; Nichol et al., 2016).

Over the years, extensive efforts were made to identify other medicines for the treatment of refractory APL. Though *in vitro* studies showed that some agents, such as granulocyte colony-stimulating factor, tumor necrosis factor, oridonin, staurosporine, matrine and interferon-γ could cooperate with ATRA to induce differentiation in

ATRA resistant APL cells, further *in vivo* studies and clinical trials were urgently needed to verify their clinical efficacy (Gao et al., 2010; Ge et al., 2014; He et al., 2012; Witcher et al., 2004; Wu et al., 2014). Gemtuzumab ozogamicin (GO)，an anti-CD33 monoclonal antibody conjugated to the toxin calicheamicin, exhibited significant activity in APL (Takeshita et al., 2005). GO has been used successfully in combination with ATRA and ATO for newly diagnosed APL patients as well as a single agent for patients with molecular relapsed APL or patients who are unfit for conventional chemotherapy (Aribi et al., 2007; Lo-Coco et al., 2004; Ravandi et al., 2009). However, no large randomized study of GO for APL treatment has been performed and only one long-term follow-up of GO combined with ATRA and ATO in APL treatment was reported (Abaza et al., 2017). Allogeneic bone marrow transplantation was another therapy choice, but only applicable to small amount of patients. Till now, ATO was the widely proven effective therapy other than ATRA and chemotherapy in the treatment of APL, especially for the ATRA-resistant relapsed APL patients (McCulloch et al., 2017). Unfortunately, ATO resistance has also been observed recently (Goto et al., 2011; Lou et al., 2015). Therefore, the development of alternative therapies to avoid or reverse drug resistance continues to be a goal in the treatment of APL.

Dasatinb, one of the second generation of tyrosine kinase inhibitors, was administered for chronic, blastic, or accelerated phase chronic myeloid leukemia (CML) patients who are resistant or intolerant to imatinib, even as the frontline treatment of CML-chronic phase in some countries (Wei et al., 2010). Its capability to

inhibit ABL also allowed its use in the treatment of Philadelphia chromosome–positive acute lymphoblastic leukemia (ALL) with the combination of other therapies (Foà et al., 2011; Rousselot et al., 2016; Sasaki et al., 2016).

In addition to blocking BCR-ABL kinase activity, dasatinib also inhibits other Src family kinases (SFKs), such as LCK, HCK, FYN, YES, FGR, BLK, LYN, and FRK (Chen and Chen, 2015). SFKs are commonly overexpressed and/or hyperactivated in acute myeloid leukemia (AML) cell lines and primary cells (Johnson, 2008). Inhibition of SFKs by its selective inhibitor PP2 enhanced ATRA-induced myeloid differentiation(Jensen et al., 2015; Miranda et al., 2007). Furthermore, as a SFK inhibitor, dasatinib promoted ATRA-triggered differentiation in some AML cell lines, such as APL cell line NB4, acute monocytic leukemia cell line U937 and acute myeloblastic leukemia with maturation cell line HL-60 (Congleton et al., 2012; Kropf et al., 2010). Among these cell lines, NB4 and HL-60 cell lines are ATRA-sensitive while U937 is ATRA low-responsive cell line. Therefore, it was speculated that dasatinib might overcome ATRA-resistance in some cell lines. However, whether dasatinib could restore differentiation response of ATRA-resistant APL cells to ATRA has not yet been reported.

In the present study, we investigated the effect of the combination of ATRA and dasatinib in ATRA-resistant APL cell lines, NB4-R1 and NB4-R2. The combined treatment synergized to trigger terminal granulocytic differentiation accompanied by G0/G1 phase arrest in both cell lines. Furthermore, it was showed that the combined treatment overcome differentiation block via RAF-1/MEK/ERK-mediated modulation

of the protein level of CCAAT/enhancer-binding protein β (C/EBPβ), C/EBPε and PU.1. Meanwhile, before the activation of RAF-1, dasatinib suppressed the activation of Lyn, the predominant activated SFK in NB4-R1 and NB4-R2 cell lines. Further study indicated that the linkage between the inhibition of Lyn activity and RAF-1 activation was the dephosphorylation of RAF-1 at S259.

2. Material and Methods

2.1 Reagents

ATRA and okadaic acid were purchased from Sigma-Aldrich (St Louis, MO). U0126 were obtained from EMD Chemicals (San Diego, CA). Dasatinib, sorafenib tosylate, PP2 and salirasib (farnesylthiosalicylic acid，FTS) were from Selleckchem Chemicals (Houston, TX). They were all dissolved by dimethylsilfoxide (DMSO).

2.2 Cell culture and cell proliferation

The ATRA resistant cell lines, NB4-R1 and NB4-R2 cells were obtained from Dr Michel Lanotte (Hopital Saint Louis, Paris, France) (Nason-Burchenal et al., 1997)and were cultured in RPMI-1640, supplemented with 10% fetal calf serum (Thermo Fisher Scientific Inc, Waltham, MA) in a humidified atmosphere of 95% air/5% CO2 at 37℃. To avoid possible effects of cell density on cell growth and survival, cells were maintained at less than 5×10^5 cells/ml. Actual viable cell numbers were calculated by multiplying diluted times with counted viable cell numbers.

2.3 Cell differentiation assays

Cell maturation was evaluated by cellular morphology, nitroblue tetrazolium

(NBT) reduction assay and the content of cell surface differentiation-related antigen CD11b. Morphology was determined with May-Grunwald-Giemsa's staining of cells centrifuged onto slides by cytospin (Shandon, Runcon, UK; 500 r.p.m., 5 min) and viewed at x1000 magnification. For NBT reduction, 1×10^6 cells were collected and incubated with 1mg/ml NBT (Sigma-Aldrich) solution containing 10μM phorbol 12-myristate 13-acetate (Sigma-Aldrich) at 37℃ for 1 hour. Cells were lysed by 10% sodium dodecyl sulfate (SDS) and 0.04N hydrochloric acid. The absorbance at O.D 570nm was detected by spectrophotometer (Beckman Coulter, Brea, CA). The expression of cell surface differentiation-related antigen CD11b (Coulter, Marseilles, France) was determined on flow cytometry (EPICS XL, Coulter, Hialeah, FL).

2.4 Cell cycle analysis

Cells were collected, rinsed and fixed overnight in 70% cold ethanol. Then, cells were rinsed with phosphate-buffered saline (PBS), treated with Tris-HCl buffer (pH 7.4) supplemented with 1% RNase (Sigma-Aldrich) and stained with 50 mg/ml propidium iodide (Sigma-Aldrich). Cell cycle distribution was determined by flow cytometry (EPICS XL, Coulter). Data were collected, stored and analyzed by Modfit software (BD Biosciences, San Jose, CA).

2.5 Western blot analysis

Cells were washed with cold PBS and lysed with RIPA buffer (Sigma-Aldrich) containing protease inhibitor and phosphatase inhibitor cocktail (Selleckchem Chemicals). Cell lysates were centrifuged at 13000 rpm for 10 minutes at 4℃. Supernants were collected and quantified by Bio-Rad Dc protein assay (Bio-Rad

Laboratories, Hercules, CA). 50 or 100μg protein extracts were loaded on 8% SDS-polyacrylamide gel, subjected to electrophoresis, and transferred to polyvinylidene difluoride membranes (GE Healthcare UK Ltd, Buckinghamshire, UK). After blocking with 5% nonfat milk or BSA in PBS, the membranes were probed with the following primary antibodies: RARα, C/EBPβ, C/EBPε, PU.1, p-RAF-1(Tyr340/341) from Santa Cruz Biotech (Santa Cruz, CA); phospho–c-RAF(Ser259), phospho-p44/42 Erk1/2 (Thr202/Try 204), phospho-MEK1/2 (Ser217/Try 221)， p-RAF-1(Ser338) and Phospho-Src family (Tyr416) from Cell Signaling Technology (Beverly, MA); Phospho-PP2A (Y307) catalytic subunit from R&D systems (Minneapolis, MN); Ras from EMD Millipore Corporation (Temecula, CA); β-actin from Sigma-Aldrich. Subsequently, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare UK Ltd). Immunocomplexes were visualized by chemiluminescence kit (GE Healthcare UK Ltd) according to the manufacturer's instruction. To detect PP2A, RAF-1, Lyn, Erk1/2 and MEK1/2, the same membrane incubated with the phosphorylated PP2A, RAF-1, Src family, Erk1/2 or MEK1/2 was stripped with stripping buffer (2% SDS, 100mM beta-mercaptoethanol, 50 mM Tris, pH6.8) followed by blocking and probing with anti-PP2A C subunit (Cell Signaling Technology), anti-RAF-1(Cell Signaling Technology), anti-Lyn (Santa Cruz Biotech), anti-Erk1/2 (Cell Signaling Technology), or anti-MEK1/2 (Cell Signaling Technology).

2.6 Ras pull down assay

Cells were lysed in ice-cold Mg2+ lysis/wash buffer (EMD Millipore Corporation) supplemented with protease inhibitor and phosphatase inhibitor cocktail. Lysates were centrifuged at 13000 rpm for 10 minutes at 4°C. Aliquots of lysates were set aside to allow quantitation of total Ras by immunoblotting. The remainder of the lysates was incubated with 10 µl of beads coated with a fusion protein (GST-RAF-1-RBD) (EMD Millipore Corporation) at 4°C for 1 hour. Beads were washed three times with lysis/wash buffer, boiled for 10 minutes in 2×Laemmli reducing buffer, and analyzed by immunoblotting for Ras.

2.7 Immunoprecipitations

Cells were lysed in lysis buffer (20mM HEPES, (pH7.4), 137mM sodium chloride, 1.5mM magnesium chloride, 1mM EGTA, 10%(v/v) glycerol, 1% Triton X-100, protease inhibitor and phosphatase inhibitor cocktail). Lysates were centrifuged at 13000 rpm for 10 minutes at 4°C. Aliquots of lysates were set aside to allow quantitation by immunoblotting. The remainder of the lysates was incubated respectively with 2µg of Ig G or anti-lyn antibody overnight at 4°C, followed by protein G plus agarose (Santa Cruz Biotech) incubation for 2h at 4°C. Immunocomplexes were collected by centrifugation and washed three times with lysis buffer. Bound proteins were eluted by boiling in 2×Laemmli reducing buffer for 10 minutes and analyzed by immunoblotting. To avoid the interference of Ig G heavy chain, the membrane probed with anti-lyn or anti- Phospho-Src family (Tyr416) was incubated with mouse anti-rabbit IgG (light-chain specific) (Cell Signaling Technology) following by HRP-conjugated secondary antibody.

2.8 Statistical analysis

For NBT reduction, two-tailed unpaired Student's t test was used, n value was 3. The flow cytometric analysis of CD11b was analyzed by chi-square test, n value was 20,000.

3. Results

3.1 Dasatinib synergized with ATRA to induce differentiation accompanied by G0/G1 phase arrest in NB4-R1 and NB4-R2 cells.

To investigate the effect of combined treatment of dasatinib and ATRA on NB4-R1 and NB4-R2 cells, we first tested the maximum concentration of dasatinib studied in both cell lines. DMSO treatment was regarded as solvent control since both ATRA and dasatinib were dissolved in it. 1μM and 2μM dasatinib was determined to be the maximum concentration used in NB4-R1 and NB4-R2 cells respectively with no obvious effects on survival (data not shown). Comparing with DMSO treated cells, 0.5μM and 1μM dasatinib alone slightly inhibited the proliferation (Figure 1a) without marked alteration of cell cycle distribution in NB4-R1 cells (Figure 1c), while 1μM and 2μM dasatinib treatment suppressed the proliferation (Figure 1b) accompanied with G0/G1 phase arrest in NB4-R2 cells (Figure 1d). Comparing with dasatinib treatment, the combination of dasatinib and ATRA showed greater growth inhibition in both cell lines in a dose and time-dependent manner (Figure 1a and 1b) paralleled by more obvious G0/G1 phase arrest (Figure 1c and 1d). The cell viability was maintained above 90% with any treatment in both cell lines (data not shown).

To study the effect of combined treatment on triggering differentiation, both cell

lines were treated with 1μM ATRA and the corresponding concentrations of dasatinib for 72 hours. As shown in Figure 1e and 1f, both cell lines had a characteristic morphology of APL blast such as irregular nucleus and large nuclear/cytoplasm ratio. After incubated with lower concentration of dasatinib alone for 72 hours, no significantly morphological change was observed in both cell lines. However, some cells treated with higher concentration of dasatinib or 1μM ATRA presented decreased nuclear/cytoplasm ratio in these two cell lines (Figure 1e and 1f). In both cell lines, any concentration of dasatinib and ATRA co-treated cells displayed more matured appearances, such as kidney-shape or lobed nuclei accompanied by markedly decreased nuclear/cytoplasm ratio (Figure 1e and 1f). Moreover, almost all the cells treated with the combination of higher concentration of dasatinib and ATRA presented fully differentiation (Figure 1e and 1f). Consistent with the morphology, 1μM ATRA or high concentration of dasatinib alone for 72 hours modestly increased NBT reduction activity in both cell lines (Figure 1g and 1h). More remarkably enhanced NBT reduction activity was observed in both cell lines with dasatinib/ATRA co-treatment (Figure 1g and 1h). Accordingly, a synergistic effect of dasatinib and ATRA on the content of CD 11b positive cells was observed in a dose-dependent manner in both cell lines (Figure 1i-1l). Taken together, these results demonstrated that co-treatment of dasatinib and ATRA induced granulocytic differentiation and indicated that the addition of dasatinib could reverse ATRA resistance in ATRA-resistant APL cells.

3.2 The combination of dasatinib and ATRA activated MEK/ERK signal pathway,

modulated the protein level of C/EBPs and PU.1.

To survey the molecular mechanisms of combined treatment-induced differentiation, high concentrations of dasatinib were used in the subsequent studies. Since dasatinib could reverse ATRA resistance in these two cell lines and RA signaling pathway is involved in the regulation of myeloid differentiation, we focused on certain proteins and signaling pathways involving in ATRA-induced granulocytic differentiation.

The degradation of PML-RARα, the pathogenic protein of APL, was deemed to be the key mechanism of ATRA-induced granulocytic differentiation of APL cells(Ohnishi, 2007). As illustrated in Figure 2, ATRA treatment alone or dasatinib/ATRA co-treatment degraded RARα. However, PML-RARα fusion protein was not destructed by any treatment in both cell lines even at 72 hours when the cells had already been induced differentiation (Figure 2). ATRA and the combined treatment decreased the protein level of PML-RARα fusion protein at the similar level in NB4-R1 cells while the addition of dasatinib partially inhibited ATRA-mediated reduction of PML-RARα in NB4-R2 cells (Figure 2). Therefore, the cooperation differentiation effect of ATRA and dasatinib might be independent of the regulation of PML-RARα.

The induction of C/EBPβ and C/EBPε expression is implicated in the later stage of granulocytic differentiation(Lekstrom-Himes, 2001). The transcription factor PU.1 is a member of the Ets transcription factor family and plays a vital role in commitment and maturation of the myeloid lineages. Moreover, it was reported that

their expression could be induced by ATRA and they were all required for ATRA-induced differentiation in APL cells(Duprez et al., 2003; Mueller et al., 2006; Park et al., 1999). As shown in Figure 2, in both cell lines, ATRA or dasatinib treatment alone for 24 hours slightly elevated the protein level of C/EBPε and C/EBPβ but not PU.1. However, the combination of dasatinib with ATRA remarkably enhanced the protein level of C/EBPβ, C/EBPε and PU.1 in both cell lines (Figure 2).

Activation of MEK/ERK was demonstrated to be required for some cytokine-induced myeloid differentiation as well as ATRA-triggered granulocytic differentiation in APL cells(Miranda et al., 2005, 2002; Weng et al., 2016). To explore whether MEK/ERK signaling pathway was activated, the phosphorylated MEK and ERK1/2 were assessed by Western blot analysis in cells treated with ATRA or/and dasatinib for 24 hours. As illustrated in Figure 2, the combined treatment increased the amount of phosphorylation of MEK and ERK1/2 more significantly than that with any single treatment in both cell lines. The total amount of MEK and ERK1/2 in both cell lines remained almost unaltered after any treatment.

Therefore, it indicated that rather than PML-RARα fusion protein, the activation of MEK/ERK cascade and the up-regulation of C/EBPs and PU.1 might be involved in the combined treatment-induced differentiation.

3.3 The combination-induced differentiation and the up-regulation of C/EBPs as well as PU.1 were dependent on MEK/ERK activation.

Having validated the activation of MEK/ERK by dasatinib/ATRA co-treatment, we next studied whether MEK/ERK signaling pathway was required for the combined

treatment-induced granulocytic differentiation. Cells were pretreated with 1μM U0126, a specific inhibitor of MEK at the maximum concentration with no effect on cell viability when added to the combined treatment. The effectiveness of U0126 was evaluated by ERK1/2 phosphorylation with the indicated treatment for 24 hours. Such concentration of U0126 did block ERK1/2 activation in NB4-R1 cells while attenuate ERK1/2 activity in NB4-R2 cells (Figure 3a and 3b). With U0126 pretreatment, matured granulocytes presented in dasatinib/ATRA co-treatment were replaced by primary cells with round nuclei and large nuclear/cytoplasm ratio in NB4-R1 cells while partial differentiated cells with kidney-shaped nuclei in NB4-R2 cells (Figure 3c and 3d). Accordingly, 1μM U0126 pretreatment suppressed combined treatment-enhanced NBT reduction assay (Figure 3e) and the content of CD11b positive cells (Figure 3f-3h) more significantly in NB4-R1 cells than that in NB4-R2 cells. These results were consistent with the difference of MEK inhibition by U0126 in these two cell lines. Meanwhile, in the presence of U0126, the combined treatment-enhanced the protein level of C/EBPβ, C/EBPε and PU.1 was remarkably decreased in both cell lines (Figure 3i and 3j). These results highlighted a major role of MEK/ERK signal pathway in the differentiation-inducing effect of ATRA and dasatinib in ATRA resistant-APL cells. It was also suggested that MEK/ERK signal pathway positively regulated dasatinib/ATRA-induced differentiation by modulating the protein level of PU.1, C/EBPβ and C/EBPε.

3.4 RAF-1 was an upstream positive modulator of MEK/ERK signaling pathway and required for the combination-induced differentiation.

Having demonstrated the critical role of MEK/ERK pathway in the combination-induced differentiation and some of its downstream targets, the upstream molecules of MEK/ERK signal pathway was to be surveyed. RAF-1 is a classical upstream regulator of MEK/ERK signal pathway. The activation of RAF-1 was analyzed by Western blot analysis of p-RAF-1(Ser338) and p-RAF-1(Tyr340/341). The phosphorylated RAF-1 at Ser 338 was only detected in NB4-R1 cells with ATRA treatment for 6 hours (Figure 4a). However, in both cell lines, phosphorylated RAF-1 at Tyr340/341 was presented more remarkably with the combined treatment for 6 hours, 18 hours earlier than MEK activation (Figure 4a and 4b).

To further investigate whether RAF-1 activation was required for MEK/ERK signal pathway, cells were pretreated for 1 hour with sorafenib tosylate, a specific inhibitor of RAF-1. 5μM and 2μM sorafenib tosylate was determined to be used in NB4-R1 and NB4-R2 cells respectively with no obvious effects on survival when added to the combined treatment (data not shown). As illustrated in Figure 4c and 4d, sorafenib tosylate pretreatment suppressed the combination-enhanced phosphorylation of MEK and ERK in both cell lines. Thus, the activation of MEK/ERK by the combined treatment was dependent on RAF-1. Moreover, sorafenib tosylate also attenuated the combination-triggered differentiation in both cell lines as assessed by morphology (Figure 4e and 4f), NBT reduction assay (Figure 4g) and CD11b expression (Figure 4h-4j). Consistent with the difference of the concentration of sorafenib tosylate used in these two cell lines, the inhibitory effect of sorafenib tosylate on differentiation was more distinct in NB4-R1 cells than that in NB4-R2

cells. In addition, the combination-enhanced protein level of C/EBPβ, C/EBPε and PU.1 was significantly decreased by sorafenib tosylate pretreatment in both cell lines (Figure 4k and 4l). These data demonstrated that RAF-1 was an upstream positive modulator of MEK/ERK signaling pathway and required for the combination-induced differentiation.

3.5 Ras might not be involved in the combined treatment-induced differentiation and the activation of MEK.

To further explore the upstream molecules of RAF-1, we first determined the role of Ras, a well-known upstream regulator of RAF-1, in the combination-induced differentiation. It has been well documented that Ras, in its active-GTP-bound state, binds to RAF-1 through Ras-binding-domain (RBD). Thus, Ras activation was measured by the level of Ras-GTP in both cell lines with dasatinib or/and ATRA treatment for 3 hours, a few hours earlier than RAF-1 activation. As shown in Figure 5a, there was baseline activation of Ras in both cell lines and the combined treatment hardly altered the Ras activity. Therefore, the combined treatment did not enhance the Ras activity prior to RAF-1 activation and it indicated that the combination-induced RAF-1 activation might not depend on Ras. To confirm the role of Ras, both cell lines were pretreated with 50μM salirasib (FTS) for 1 hour and then exposed to other treatments for 24 hours. FTS is an S-farnesyl cysteine analog that affects docking of active GTP-Ras in the membrane by competing with Ras for its membrane anchorage sites, resulting in hydrolyzing GTP-Ras to inactive GDP-Ras(Marom et al., 1995). FTS did remarkably suppress the baseline Ras activity in both cell lines while slightly

inhibited Ras activation with the combined treatment in NB4-R1 and NB4-R2 cells (Figure 5b). Unexpectedly, FTS pretreatment elevated the combined treatment-induced phosphorylation of MEK in both cell lines (Fig 5c and 5d). Meanwhile, FTS did not affect the combination-triggered differentiation in both cell lines as assessed by morphology (Figure 5e and 5f), NBT reduction assay (Figure 5g) and CD11b expression (Figure 5h-5j). Taken together, FTS, the inhibitor of Ras activation, did suppress Ras activity in both cell lines while it neither inhibit MEK activation nor affect differentiation. It was concluded that Ras might not be involved in the combined treatment-induced differentiation and the activation of MEK.

3.6 Dasatinib inhibited the activity of Lyn, the predominant activated SFK in NB4-R1 and NB4-R2 cells.

SFKs are the key targets of dasatinib and SFK inhibition could enhance ATRA-induced myeloid differentiation (Congleton et al., 2012; Jensen et al., 2015; Kropf et al., 2010; Miranda et al., 2007). To investigate whether dasatinib affect the activity of SFKs, the whole cell lysates of both cell lines treated with dasatinib or/and ATRA for 3 hours were immunoblotted with the antibody to phospho-Src Y416. This antibody recognized a conserved phosphotyrosine corresponding with activated form of different SFK members. The molecular weights of the SFK members are between 50-kDa to 70-kDa. As shown in Figure 6a left, some of SFKs with the molecular weight around 55-kDa were phosphorylated with DMSO or ATRA treatment. Meanwhile, the phosphorylation was decreased by dasatinib and the combined treatment at the same level in both cell lines (Figure 6a left). Lyn, with the molecular

weight of 53 to 56-kDa, is the major activated SFK in NB4 cells (Kropf et al., 2010), the original cell line of NB4-R1 and NB4-R2. Therefore, the membrane immunoblotted with the antibody to phospho-Src Y416 was stripped and reprobed with the antibody to Lyn. The bands of Lyn were in the similar pattern of phospho-Src Y416 in both cell lines (Figure 6a right). Thus, it indicated that Lyn might be the predominant activated SFK which was inhibited by dasatinib in NB4-R1 and NB4-R2 cells.

To further confirm whether Lyn was the major activated SFK suppressed by dasatinib, immunodepletion study of Lyn was performed with dasatinib or/and ATRA treatment for 3 hours, followed by immunoblotting with the antibody to phospho-Src Y416. The same experiment with the cell lysate from untreated NB4 cells was used as positive control. Theoretically, after immunoprecipitation of Lyn, the immunoprecipitate pellets contained the immunocomplex of Lyn while proteins other than the Lyn complex presented in the immunodepleted supernant. As illustrated in Figure 6b middle and lower, like NB4 cells, phosphorylated SFKs were not detected in the immunodepleted supernant but only in the immunoprecipitate pellets with DMSO or ATRA treatment in both cell lines. Furthermore, Lyn was only presented in the immunoprecipitate pellets but not the immunodepleted supernant in these cell lines (Figure 6b middle and lower). Therefore, it was demonstated that only Lyn was the activated SFK member in NB4-R1 and NB4-R2 cells and dasatinib suppressed the activity of Lyn.

3.7 The inhibition of Lyn activity by the combined treatment initiated RAF-1

activation by reducing the phosphorylation of RAF-1 at S259.

Our above results showed that dasatinib synergized with ATRA to trigger terminal granulocytic differentiation by Ras independent RAF-1/MEK/ERK pathway, accompanied by Lyn inhibition. Since Lyn inhibition was 3 hours earlier than the activation of RAF-1 and Lyn has been implicated as a negative regulator of ERK(Lannutti and Drachman, 2004), it prompted us to investigate the link between Lyn inhibition and RAF-1 activation.

Dephosphorylation of RAF-1 phospho-S259, one of the inhibitory sites of RAF-1, initiates the activation of RAF-1(Lavoie and Therrien, 2015). Interestingly, PP2A is one of the phosphotases responsible for dephosphorylation of RAF-1 at S259 and Lyn can inhibit PP2A through phosphorylating of Y307 in its catalytic subunit(Zonta et al., 2015). Thus, we hypothesized that dasatinib-induced suppression of Lyn might release the inhibition of PP2A and subsequently initiate RAF activation by dephosphorylation of RAF-1 at S259. As Figure 7a and 7b shown, 3 hours before RAF-1 activation, the same time point of Lyn inhibition, the phosphorylation of RAF-1 at S259 was dramatically decreased by the combined treatment in both cell lines. However, the phosphorylation of PP2A at Y307 kept the similar level by any treatment (Figure 7a and 7b). Moreover, PP2A selective inhibitor, okadaic acid could not affect the combined treatment-induced differentiation as assayed by morphology (Figure 7c and 7d) and CD11b expression (Figure 7e and 7f).

To further confirm the correlation between Lyn inhibition and dephosphorylation of RAF-1 at S259, we evaluated whether the SFK specific inhibitor PP2 could mimic

the effect of dasatinib. Since Lyn was verified to be the predominant activated SFK in both cell lines, it meant that the main target of SFK specific inhibitor PP2 was Lyn. Similar to the co-treatment of dasatinib and ATRA, the level of phosphorylated/activated Lyn and the phosphorylation of RAF-1 at S259 were significantly reduced with the combination of 1μM ATRA and 10 mM PP2 for 3 hours (Figure 8a and 8b). Moreover, co-treatment of PP2 and ATRA also induced terminal granulocytic differentiation in both cell lines as assessed by morphology (Figure 8c and 8d), NBT reduction assay (Figure 8e) and CD11b expression (Figure 8f-8h). Thus, it was suggested that the inhibition of Lyn could dephosphorylate RAF-1 at S259 and finally lead to differentiation.

4. Discussion

In this work, we demonstrated that dasatinib, a tyrosine kinase inhibitor currently used in the treatment for CML and Philadelphia chromosome–positive ALL, could restore ATRA-sensitivity in two retinoid resistant APL cell lines. However, the effect of the combination of dasatinib and ATRA on primary cells from ATRA-resistant APL patients needs further investigation.

PML-RARα fusion protein is believed to contribute to the pathogenesis of APL in a dominant negative fashion. Moreover, the destruction of PML-RARα was widely accepted as the key mechanism of ATRA-induced granulocytic differentiation of APL cells. However, in this work, PML-RARα fusion protein was not destructed by the combined treatment even at 72 hours when the cells had already been induced differentiation. Thus, it indicated that the combination could induce differentiation

independent of PML-RARα degradation. Consistent with our observation, other agents such as oridonin, G-CSF, TNF and staurosporine could synergize with ATRA to induce differentiation in ATRA-resistant APL cell lines without any effect on PML-RARα(Gao et al., 2010; Ge et al., 2014; Higuchi et al., 2004; Witcher et al., 2004). Since dasatinib could also promote ATRA-induced differentiation in some non-APL AML cells which is PML-RARα fusion protein negative, suggesting that PML-RARα degradation was not required for dasatinib enhancement of ATRA-triggered differentiation.

To survey the molecular mechanism of the combined treatment-induced granulocytic differentiation, we focused on some known transcription factors and pathway involving in ATRA-triggered differentiation in APL cells. As mentioned above, the expression of C/EBPβ, C/EBPε and PU.1 were all required for ATRA-induced differentiation in APL cells(Duprez et al., 2003; Mueller et al., 2006; Park et al., 1999). Among them, *C/EBPε* was identified to be a downstream ATRA target gene in APL treatment while PU.1-mediated transactivation was repressed by PML-RARα and restored by the treatment of ATRA(Park et al., 1999; Qian et al., 2013). A majority of PU.1 target genes are directly involved in myeloid differentiation and function, such as CD11b, CD45, the granulocyte/macrophage colonystimulating factor receptor, myeloperoxidase, lysozyme, neutrophil elastase, microtubule-associated protein 1S and glycolytic enzyme hexokinase 3 (Federzoni et al., 2012; Haimovici et al., 2014; Kastner and Chan, 2008). In this study, the combined treatment activated MEK/ERK and significantly enhanced protein level of

C/EBPβ, C/EBPε and PU.1. Attenuation of the MEK activation blocked not only the differentiation but also the increased protein level of C/EBPs and PU.1. It meant that MEK/ERK signal pathway positively regulated the combined treatment-induced differentiation by modulating the protein level of PU.1, C/EBPβ and C/EBPε. Furthermore, RAF-1 was activated much earlier than MEK phosphorylation and inhibition of RAF-1 activation suppressed the MEK/ERK activation, differentiation as well as the enhanced protein level of C/EBPs and PU.1. Thus, the combination-induced differentiation was via RAF-1/MEK/ERK-mediated modulation of the protein level of C/EBPβ, C/EBPε and PU.1. The exact mechanisms of how RAF-1/MEK/ERK regulated their expression level remains to be elucidated. Since activated ERK translocates from cytoplasm to nucleus, it might modulate the expression or the activity of several transcription factors by phosphorylation. Indeed, accumulating evidence showed that MEK/ERK signaling pathway could promote C/EBPβ expression and modify the activity of C/EBPβ and PU.1 (Lee et al., 2010; Lu et al., 2013; Murakami et al., 2011). Moreover, in ATRA-treated APL cells, C/EBPβ could induce the expression of C/EBPε and PU.1(Duprez et al., 2003; Mueller et al., 2006). PU.1 could also directly activate the transcription of C/EBPε (Yoshida et al., 2007). Hence, there might be MEK-C/EBPβ-C/EBPε, MEK-PU.1-C/EBPε or MEK-C/EBPβ-PU.1-C/EBPε cascade in the combination-induced differentiation.

In further study, NB4-R1 and NB4-R2 cells were shown to have basal activity of Ras and dasatinib or/and ATRA treatment did not enhance its activity. Attenuation of the Ras activity by FTS neither suppressed the activation of MEK nor inhibited

differentiation. Therefore, Ras might not be involved in the combined treatment induced differentiation and the activation of MEK. In other words, the combined treatment induced differentiation was via Ras-independent RAF-1/MEK/ERK pathway. Similar to dasatinib, another SFK inhibitor PP2 was demonstrated to enhance phorbol myristate acetate and H_2O_2-induced activation of RAF-1 in Ras independent manner (Lee et al., 2004).

Dasatinib, a tyrosine kinase inhibitor which also targets to SFK, did suppress SFK activity in NB4-R1 and NB4-R2 cells 3 hours earlier than RAF-1 activation. Further study proved that Lyn was the prominent activated SFK which was inhibited by dasatinib in these two cell lines. Lyn was demonstrated to function as a negative regulator of myelopoiesis (Harder et al., 2004). *Lyn*$^{-/-}$ granulocyte precursors showed obvious hyperresponsiveness to G-CSF via increased ERK activation(Mermel et al., 2006). It is similar to our observation that dasatinib overcome ATRA resistance through RAF-1/MEK/ERK activation accompanied by the inhibition of Lyn activity. It indicated that the inhibition of Lyn activity might link to the activation of RAF-1, that is, Lyn might negatively regulate RAF activation. However, there was no detailed mechanism of the negative role of Lyn in G-CSF-activated ERK in *Lyn*$^{-/-}$ granulocyte precursors. Then, we first focused on certain negative regulators of RAF-1 which are activated by SFK. RAF-1 kinase inhibitor protein, 14-3-3, Sprouty 2 and Sprouty 4 are by far the known negative modifiers in RAF-1 complex (Lavoie and Therrien, 2015). Among them, only Sprouty 2 is activated through tyrosine phosphorylation by a Src-like kinase and leads to consequent suppression of RAF-1 activation through

sequestering RAF-1 or Grb2 (Lavoie and Therrien, 2015). However, we did not detect tyrosine phosphorylation of Sprouty 2 with any treatment in both cell lines (data not shown). Hence, Sprouty 2 was not activated and might not be involved in the regulation of RAF-1 activity by Lyn in our model. Next, inhibitory sites of RAF-1 which are positively regulated by Lyn were taken into consideration. Phosphorylation of S43, S233, S259, S289, S296 and S301 mediate the repression of RAF-1 activity (Lavoie and Therrien, 2015). Interestingly, PP2A, one of the phosphotases responsible of S259 dephosphorylation resulting in initiating the RAF-1 activation, can be inhibited by Lyn through phosphorylation of Y307 in the catalytic subunit (Zonta et al., 2015). Therefore, suppression of Lyn might release PP2A inhibition by dephosphorylation, lead to dephosphorylation of RAF at S259 and initiation of RAF-1 activation. Our further study showed that the combined treatment really decreased the phosphorylation of RAF-1 at S259 but not Y307 in the catalytic subunit of PP2A. Okadaic acid, the antagonist of PP2A could not suppress the combined treatment-induced differentiation. Moreover, SFK inhibitor, PP2 really suppressed Lyn activity and mimicked the effect of dasatinib on ATRA-induced differentiation as well as dephosphorylation of RAF-1 at S259. Thus, it was suggested that Lyn inhibition might trigger the dephosphorylation of RAF at S259 so that lead to the activation of RAF-1 and differentiation. It also indicated that Lyn inhibition mediated dephosphorylation of RAF S259 was not by PP2A. The detailed mechanism of Lyn inhibition resulting in dephosphorylation of RAF at S259 remains to be further investigated. Consistent with our results, Lyn knockdown was confirmed to decrease

phosphorylation of RAF-1 at S259 in HL-60 cells, while dasatinib also promoted ATRA-triggered differentiation in this cell line (Congleton et al., 2012).

5. Conclusions

Dasatinib, the medicine currently used for CML and Ph+ ALL therapy could synergize with ATRA to trigger terminal granulocytic differentiation in ATRA resistant APL cell lines. The detailed mechanisms were illustrated in Figure 9. Briefly, Lyn might inhibit RAF-1 activity by Ser 259 phosphorylation. Dasatinib could release RAF-1 inhibition by suppressing Lyn. With the addition of ATRA, the combination dephosphorylated RAF-1 at Ser259 and activated RAF-1 by Tyr 340/341 phosphorylation. Subsequently, RAF-1 downstream targets MEK and ERK were activated. Finally, the enhancing protein levels of C/EBPs and PU.1 modulated by ERK resulted in differentiation. These findings might provide potential therapy approach for ATRA resistant APL patients and also suggest Lyn as the target to overcome ATRA resistance.

Funding source

This work is supported by the Natural Science Foundation of Shanghai (17ZR1417100).

Conflict of interest

The authors declare that they have no competing interests.

References

Abaza, Y., Kantarjian, H.M., Garcia-manero, G., Estey, E., Borthakur, G., Faderl, S., Brien, S.O., Wierda, W., Pierce, S., Brandt, M., Luthra, R., Patel, K., Kornblau,

S., Kadia, T., Daver, N., 2017. Long-term Outcome of Acute Promyelocytic Leukemia Treated with All-Trans- Retinoic Acid , Arsenic Trioxide , and Gemtuzumab. Blood 129, 1275–1283. doi:10.1182/blood-2016-09-736686

Abreu E Lima, R.S., Rozera Baruffi, M., Gouveia De Lima, A.S., De Oliveira, F.M., De Figueiredo-Pontes, L.L., Gonzaga Tone, L., Rogatto, S.R., Passetto Falcão, R., Ferrari Chauffaille, M.D.L.L., Rego, E.M., 2005. The co-expression of PML/RARα and AML1/ETO fusion genes is associated with ATRA resistance. Br. J. Haematol. 128, 407-409. doi:10.1111/j.1365-2141.2004.05343.x

Aribi, A., Kantarjian, H.M., Estey, E.H., Koller, C.A., Thomas, D.A., Kornblau, S.M., Faderl, S.H., Laddie, N.M., Garcia-Manero, G., Cortes, J.E., 2007. Combination therapy with arsenic trioxide, all-trans retinoic acid, and gemtuzumab ozogamicin in recurrent acute promyelocytic leukemia. Cancer 109, 1355–1359. doi:10.1002/cncr.22524

Awwad, R.T., Do, K., Stevenson, H., Fu, S.W., Lo-Coco, F., Costello, M., Campbell, C.L., Berg, P.E., 2008. Overexpression of BP1, a homeobox gene, is associated with resistance to all-trans retinoic acid in acute promyelocytic leukemia cells. Ann. Hematol. 87, 195–203. doi:10.1007/s00277-007-0402-7

Chen, R., Chen, B., 2015. The role of dasatinib in the management of chronic myeloid leukemia. Drug Des. Devel. Ther. 9, 773-779. doi:10.2147/DDDT.S80207

Congleton, J., MacDonald, R., Yen, A., 2012. Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Raf (S259) and enhance MAPK-dependent differentiation of myeloid leukemia cells. Leukemia 26,

1180–1188. doi:10.1038/leu.2011.390

Duprez, E., Wagner, K., Koch, H., Tenen, D.G., 2003. C/EBPβ: A major PML-RARA-responsive gene in retinoic acid-induced differentiation of APL cells. EMBO J. 22, 5806–5816. doi:10.1093/emboj/cdg556

Farris, M., Lague, A., Manuelyan, Z., Statnekov, J., Francklyn, C., 2012. Altered nuclear cofactor switching in retinoic-resistant variants of the PML-RARα oncoprotein of acute promyelocytic leukemia. Proteins Struct. Funct. Bioinforma. 80, 1095–1109. doi:10.1002/prot.24010

Federzoni, E.A., Valk, P.J.M., Torbett, B.E., Haferlach, T., Löwenberg, B., Fey, M.F., Tschan, M.P., 2012. PU.1 is linking the glycolytic enzyme HK3 in neutrophil differentiation and survival of APL cells. Blood 119, 4963–4970. doi:10.1182/blood-2011-09-378117

Foà, R., Vitale, A., Vignetti, M., Meloni, G., Guarini, A., De Propris, M.S., Elia, L., Paoloni, F., Fazi, P., Cimino, G., Nobile, F., Ferrara, F., Castagnola, C., Sica, S., Leoni, P., Zuffa, E., Fozza, C., Luppi, M., Candoni, A., Iacobucci, I., Soverini, S., Mandelli, F., Martinelli, G., Baccarani, M., 2011. Dasatinib as first-line treatment for adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. Blood 118, 6521–6528. doi:10.1182/blood-2011-05-351403

Gallagher, R.E., Moser, B.K., Racevskis, J., Poiré, X., Bloomfield, C.D., Carroll, A.J., Ketterling, R.P., Roulston, D., Schachter-Tokarz, E., Zhou, D.C., Chen, I.M.L., Harvey, R., Koval, G., Sher, D.A., Feusner, J.H., Tallman, M.S., Larson, R.A.,

Powell, B.L., Appelbaum, F.R., Paietta, E., Willman, C.L., Stock, W., 2012. Treatment-influenced associations of PML-RARα mutations, FLT3 mutations, and additional chromosome abnormalities in relapsed acute promyelocytic leukemia. Blood 120, 2098–2108. doi:10.1182/blood-2012-01-407601

Gao, F., Tang, Q., Yang, P., Fang, Y., Li, W., Wu, Y., 2010. Apoptosis inducing and differentiation enhancement effect of oridonin on the all-trans-retinoic acid-sensitive and -resistant acute promyelocytic leukemia cells. Int. J. Lab. Hematol. 32, e114-122. doi:10.1111/j.1751-553X.2009.01147.x

Ge, D.-Z., Sheng, Y., Cai, X., 2014. Combined staurosporine and retinoic acid induces differentiation in retinoic acid resistant acute promyelocytic leukemia cell lines. Sci. Rep. 4, 4821. doi:10.1038/srep04821

Goto, E., Tomita, A., Hayakawa, F., Atsumi, A., Kiyoi, H., Naoe, T., 2011. Missense mutations in PML-RARA are critical for the lack of responsiveness to arsenic trioxide treatment. Blood 118, 1600–1609. doi:10.1182/blood-2011-01-329433

Haimovici, A., Brigger, D., Torbett, B.E., Fey, M.F., Tschan, M.P., 2014. Induction of the autophagy-associated gene MAP1S via PU.1 supports APL differentiation. Leuk. Res. 38, 1041–1047. doi:10.1016/j.leukres.2014.06.010

Harder, K.W., Quilici, C., Naik, E., Inglese, M., Kountouri, N., Turner, A., Zlatic, K., Tarlinton, D.M., Hibbs, M.L., 2004. Perturbed myelo/erythropoiesis in Lyn-deficient mice is similar to that in mice lacking the inhibitory phosphatases SHP-1 and SHIP-1. Blood 104, 3901–3910. doi:10.1182/blood-2003-12-4396

He, P., Liu, Y., Zhang, M., Wang, X., Xi, J., Wu, D., Li, J., Cao, Y., 2012.

Interferon-gamma enhances promyelocytic leukemia protein expression in acute promyelocytic cells and cooperates with all-trans-retinoic acid to induce maturation of NB4 and NB4-R1 cells. Exp. Ther. Med. 3, 776–780. doi:10.3892/etm.2012.488

Higuchi, T., Kizaki, M., Omine, M., 2004. Induction of differentiation of retinoic acid-resistant acute promyelocytic leukemia cells by the combination of all-trans retinoic acid and granulocyte colony-stimulating factor. Leuk. Res. 28, 525–532. doi:10.1016/j.leukres.2003.09.014

Jensen, H.A., Bunaciu, R.P., Varner, J.D., Yen, A., 2015. GW5074 and PP2 kinase inhibitors implicate nontraditional c-Raf and Lyn function as drivers of retinoic acid-induced maturation. Cell. Signal. 27, 1666–1675. doi:10.1016/j.cellsig.2015.03.014

Johnson, D.E., 2008. Src family kinases and the MEK/ERK pathway in the regulation of myeloid differentiation and myeloid leukemogenesis. Adv. Enzyme Regul. 48, 98–112. doi:10.1016/j.advenzreg.2007.11.002

Kastner, P., Chan, S., 2008. PU.1: A crucial and versatile player in hematopoiesis and leukemia. Int. J. Biochem. Cell Biol. 40, 22-27. doi:10.1016/j.biocel.2007.01.026

Kropf, P.L., Wang, L., Zang, Y., Redner, R.L., Johnson, D.E., 2010. Dasatinib promotes ATRA-induced differentiation of AML cells. Leukemia 24, 663–665. doi:10.1038/leu.2009.267

Lannutti, B.J., Drachman, J.G., 2004. Lyn tyrosine kinase regulates thrombopoietin-induced proliferation of hematopoietic cell lines and primary

megakaryocytic progenitors. Blood 103, 3736–3743. doi:10.1182/blood-2003-10-3566

Lavoie, H., Therrien, M., 2015. Regulation of RAF protein kinases in ERK signalling. Nat. Rev. Mol. Cell Biol. 16, 281–298. doi:10.1038/nrm3979

Lee, M., Kim, J.Y., Anderson, W.B., 2004. Src tyrosine kinase inhibitor PP2 markedly enhances Ras-independent activation of Raf-1 protein kinase by phorbol myristate acetate and H 2O2. J. Biol. Chem. 279, 48692–48701. doi:10.1074/jbc.M403132200

Lee, S., Shuman, J.D., Guszczynski, T., Sakchaisri, K., Sebastian, T., Copeland, T.D., Miller, M., Cohen, M.S., Taunton, J., Smart, R.C., Xiao, Z., Yu, L.-R., Veenstra, T.D., Johnson, P.F., 2010. RSK-mediated phosphorylation in the C/EBP{beta} leucine zipper regulates DNA binding, dimerization, and growth arrest activity. Mol Cell Biol 30, 2621–2635. doi:10.1128/MCB.00782-09

Lekstrom-Himes, J. a, 2001. The role of C/EBP(epsilon) in the terminal stages of granulocyte differentiation. Stem Cells 19, 125–133. doi:10.1634/stemcells.19-2-125

Lo-Coco, F., Cimino, G., Breccia, M., Noguera, N.I., Diverio, D., Finolezzi, E., Pogliani, E.M., Di Bona, E., Micalizzi, C., Kropp, M., Venditti, A., Tafuri, A., Mandelli, F., 2004. Gemtuzumab ozogamicin (Mylotarg) as a single agent for molecularly relapsed acute promyelocytic leukemia. Blood 104, 1995–1999. doi:10.1182/blood-2004-04-1550

Lou, Y., Ma, Y., Sun, J., Ye, X., Pan, H., Wang, Y., Qian, W., Meng, H., Mai, W., He,

J.S., Tong, H., Jin, J., 2015. Evaluating frequency of PML-RARA mutations and conferring resistance to arsenic trioxide-based therapy in relapsed acute promyelocytic leukemia patients. Ann. Hematol. 94, 1829–1837. doi:10.1007/s00277-015-2477-x

Lu, J., Wu, D., Zheng, Y., Hu, B., Cheng, W., Zhang, Z., Li, M., 2013. Troxerutin Counteracts Domoic Acid-Induced Memory Deficits in Mice by Inhibiting CCAAT/Enhancer Binding Protein beta-Mediated Inflammatory Response and Oxidative Stress. J. Immunol. 190, 3466–3479. doi:10.4049/jimmunol.1202862

Marom, M., Haklai, R., Ben-Baruch, G., Marciano, D., Egozi, Y., Kloog, Y., 1995. Selective inhibition of ras-dependent cell growth by farnesylthiosalisylic acid. J. Biol. Chem. 270, 22263–22270. doi:10.1074/jbc.270.38.22263

McCulloch, D., Brown, C., Iland, H., 2017. Retinoic acid and arsenic trioxide in the treatment of acute promyelocytic leukemia: Current perspectives. Onco. Targets. Ther. 10, 1585-1601. doi:10.2147/OTT.S100513

McNamara, S., Nichol, J., Wang, H., Miller, W.J., 2010. Targeting PKC delta mediated topoisomerase ii beta overexpression subverts the differentiation block in a retinoic. Leukemia 24, 729-739. doi:10.1038/leu.2010.27

Mermel, C.H., McLemore, M.L., Liu, F., Pereira, S., Woloszynek, J., Lowell, C.A., Link, D.C., 2006. Src family kinases are important negative regulators of G-CSF-dependent granulopoiesis. Blood 108, 2562–2568. doi:10.1182/blood-2006-05-024307

Miranda, M.B., McGuire, T.F., Johnson, D.E., 2002. Importance of MEK-1/-2

signaling in monocytic and granulocytic differentiation of myeloid cell lines. Leukemia 16, 683–92. doi:10.1038/sj.leu.2402400

Miranda, M.B., Redner, R.L., Johnson, D.E., 2007. Inhibition of Src family kinases enhances retinoic acid induced gene expression and myeloid differentiation. Mol. Cancer Ther. 6, 3081–3090. doi:10.1158/1535-7163.MCT-07-0514

Miranda, M.B., Xu, H., Torchia, J.A., Johnson, D.E., 2005. Cytokine-induced myeloid differentiation is dependent on activation of the MEK/ERK pathway. Leuk. Res. 29, 1293–1306. doi:10.1016/j.leukres.2005.03.016

Mueller, B.U., Pabst, T., Fos, J., Petkovic, V., Fey, M.F., Asou, N., Buergi, U., Tenen, D.G., 2006. ATRA resolves the differentiation block in t(15;17) acute myeloid leukemia by restoring PU.1 expression. Blood 107, 3330–3338. doi:10.1182/blood-2005-07-3068

Murakami, M., Ito, H., Hagiwara, K., Kobayashi, M., Hoshikawa, A., Takagi, A., Kojima, T., Tamiya-Koizumi, K., Sobue, S., Ichihara, M., Suzuki, M., Banno, Y., Nozawa, Y., Murate, T., 2011. Sphingosine kinase 1/S1P pathway involvement in the GDNF-induced GAP43 transcription. J. Cell. Biochem. 112, 3449–3458. doi:10.1002/jcb.23275

Nason-Burchenal, K., Maerz, W., Albanell, J., Allopenna, J., Martin, P., Moore, M.A.S., Dmitrovsky, E., 1997. Common defects of different retinoic acid resistant promyelocytic leukemia cells are persistent telomerase activity and nuclear body disorganization. Differentiation 61, 321–331. doi:10.1046/j.1432-0436.1997.6150321.x

Nichol, J.N., Galbraith, M.D., Kleinman, C.L., Espinosa, J.M., Miller, W.H., 2016. NPM and BRG1 Mediate Transcriptional Resistance to Retinoic Acid in Acute Promyelocytic Leukemia. Cell Rep. 14, 2938–2949. doi:10.1016/j.celrep.2016.02.074

Ohnishi, K., 2007. PML-RARα inhibitors (ATRA, tamibaroten, arsenic troxide) for acute promyelocytic leukemia. Int. J. Clin. Oncol.12, 313-317. doi:10.1007/s10147-007-0694-6

Park, D.J., Chumakov, A. M., Vuong, P.T., Chih, D.Y., Gombart, a F., Miller, W.H., Koeffler, H.P., 1999. CCAAT/enhancer binding protein epsilon is a potential retinoid target gene in acute promyelocytic leukemia treatment. J. Clin. Invest. 103, 1399–408. doi:10.1172/JCI2887

Qian, M., Jin, W., Zhu, X., Jia, X., Yang, X., Du, Y., Wang, K., Zhang, J., 2013. Structurally differentiated cis-elements that interact with PU.1 are functionally distinguishable in acute promyelocytic leukemia. J. Hematol. Oncol. 6, 25. doi:10.1186/1756-8722-6-25

Ravandi, F., Estey, E., Jones, D., Faderl, S., O'Brien, S., Fiorentino, J., Pierce, S., Blamble, D., Estrov, Z., Wierda, W., Ferrajoli, A., Verstovsek, S., Garcia-Manero, G., Cortes, J., Kantarjian, H., 2009. Effective treatment of acute promyelocytic leukemia with all-trans-retinoic acid, arsenic trioxide, and gemtuzumab ozogamicin. J. Clin. Oncol. 27, 504–510. doi:10.1200/JCO.2008.18.6130

Rousselot, P., Coudé, M.M., Gokbuget, N., Passerini, C.G., Hayette, S., Cayuela, J.M.,

Huguet, F., Leguay, T., Chevallier, P., Salanoubat, C., Bonmati, C., Alexis, M., Hunault, M., Glaisner, S., Agape, P., Berthou, C., Jourdan, E., Fernandes, J., Sutton, L., Banos, A., Reman, O., Lioure, B., Thomas, X., Ifrah, N., Lafage-Pochitaloff, M., Bornand, A., Morisset, L., Robin, V., Pfeifer, H., Delannoy, A., Ribera, J., Bassan, R., Delord, M., Hoelzer, D., Dombret, H., Ottmann, O.G., 2016. Dasatinib and low-intensity chemotherapy in elderly patients with Philadelphia chromosome-positive ALL. Blood 128, 774–782. doi:10.1182/blood-2016-02-700153

Sasaki, K., Jabbour, E.J., Ravandi, F., Short, N.J., Thomas, D.A., Garcia-Manero, G., Daver, N.G., Kadia, T.M., Konopleva, M.Y., Jain, N., Issa, G.C., Jeanis, V., Moore, H.G., Garris, R.S., Pemmaraju, N., Cortes, J.E., O'Brien, S.M., Kantarjian, H.M., 2016. Hyper-CVAD plus ponatinib versus hyper-CVAD plus dasatinib as frontline therapy for patients with Philadelphia chromosome-positive acute lymphoblastic leukemia: A propensity score analysis. Cancer 122, 3650–3656. doi:10.1002/cncr.30231

Shen, Z.-X., Shi, Z.-Z., Fang, J., Gu, B.-W., Li, J.-M., Zhu, Y.-M., Shi, J.-Y., Zheng, P.-Z., Yan, H., Liu, Y.-F., Chen, Y., Shen, Y., Wu, W., Tang, W., Waxman, S., de The, H., Wang, Z.-Y., Chen, S.-J., Chen, Z., 2004. All-trans retinoic acid/As2O3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. Proc. Natl. Acad. Sci. 101, 5328–5335. doi:10.1073/pnas.0400053101

Takeshita, A., Shinjo, K., Naito, K., Matsui, H., Sahara, N., Shigeno, K., Horii, T.,

Shirai, N., Maekawa, M., Ohnishi, K., Naoe, T., Ohno, R., 2005. Efficacy of gemtuzumab ozogamicin on ATRA- and arsenic-resistant acute promyelocytic leukemia (APL) cells. Leukemia 19, 1306–1311. doi:10.1038/sj.leu.2403807

Tomita, A., Kiyoi, H., Naoe, T., 2013. Mechanisms of action and resistance to all-trans retinoic acid (ATRA) and arsenic trioxide (As2O 3) in acute promyelocytic leukemia. Int. J. Hematol. 97, 717–25. doi:10.1007/s12185-013-1354-4

Wei, G., Rafiyath, S., Liu, D., 2010. First-line treatment for chronic myeloid leukemia: dasatinib, nilotinib, or imatinib. J. Hematol. Oncol. 3, 47. doi:10.1186/1756-8722-3-47

Welch, J.S., Ley, T.J., Link, D.C., Miller, C.A., Larson, D.E., Koboldt, D.C., Wartman, L.D., Lamprecht, T.L., Liu, F., Xia, J., Kandoth, C., Fulton, R.S., McLellan, M.D., Dooling, D.J., Wallis, J.W., Chen, K., Harris, C.C., Schmidt, H.K., Kalicki-Veizer, J.M., Lu, C., Zhang, Q., Lin, L., O'Laughlin, M.D., McMichael, J.F., Delehaunty, K.D., Fulton, L.A., Magrini, V.J., McGrath, S.D., Demeter, R.T., Vickery, T.L., Hundal, J., Cook, L.L., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T.N., Walker, J.R., Watson, M.A., Heath, S.E., Shannon, W.D., Varghese, N., Nagarajan, R., Payton, J.E., Baty, J.D., Kulkarni, S., Klco, J.M., Tomasson, M.H., Westervelt, P., Walter, M.J., Graubert, T.A., Dipersio, J.F., Ding, L., Mardis, E.R., Wilson, R.K., 2012. The origin and evolution of mutations in acute myeloid leukemia. Cell 150, 264–278. doi:10.1016/j.cell.2012.06.023

Weng, X. qin, Sheng, Y., Ge, D. zheng, Wu, J., Shi, L., Cai, X., 2016. RAF-1/MEK/ERK pathway regulates ATRA-induced differentiation in acute promyelocytic leukemia cells through C/EBPβ, C/EBPε and PU.1. Leuk. Res. 45, 68–74. doi:10.1016/j.leukres.2016.03.008

Witcher, M., Hoi, Y.S., Guo, Q., Miller, W.H., 2004. Combination of retinoic acid and tumor necrosis factor overcomes the maturation block in a variety of retinoic acid-resistant acute promyelocytic leukemia cells. Blood 104, 3335–3342. doi:10.1182/blood-2004-01-0023

Wu, D., Shao, K., Sun, J., Zhu, F., Ye, B., Liu, T., Shen, Y., Huang, H., Zhou, Y., 2014. Matrine cooperates with all-trans retinoic acid on differentiation induction of all-trans retinoic acid-resistant acute promyelocytic leukemia cells (NB4-LR1): Possible mechanisms. Planta Med. 80, 399–408. doi:10.1055/s-0034-1368183

Yoshida, H., Ichikawa, H., Tagata, Y., Katsumoto, T., Ohnishi, K., Akao, Y., Naoe, T., Pandolfi, P.P., Kitabayashi, I., 2007. PML-retinoic acid receptor alpha inhibits PML IV enhancement of PU.1-induced C/EBPepsilon expression in myeloid differentiation. Mol. Cell. Biol. 27, 5819–34. doi:10.1128/MCB.02422-06

Zonta, F., Pagano, M.A., Trentin, L., Tibaldi, E., Frezzato, F., Trimarco, V., Facco, M., Zagotto, G., Pavan, V., Ribaudo, G., Bordin, L., Semenzato, G., Brunati, A.M., 2015. Lyn sustains oncogenic signaling in chronic lymphocytic leukemia by strengthening SET-mediated inhibition of PP2A. Blood 125, 3747–55. doi:10.1182/blood-2014-12-619155

Figure Legends

Figure 1 Effects of combined treatment on cell growth, cell cycle distribution and differentiation. Cell growth of NB4-R1 (a) and NB4-R2 (b) was calculated as mentioned in methods. NB4-R1 cells were treated with 0.5μM (0.5D), 1μM dasatinib (1D), 1μM ATRA (RA) and in combination for 4 days while NB4-R2 cells were treated with 1μM (1D), 2μM dasatinib (2D), 1μM ATRA (RA) and in combination for 3 days. One representative experiment was shown. Each value represented the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. Cell cycle distribution of NB4-R1 (c) and NB4-R2 cells (d) treated with dasatinib or/and ATRA for 48 hours. The percentages of G0/G1 phase cells were shown in the corresponding panels. Results were representative among three independent experiments. Representative morphologic analysis of NB4-R1 (e) and NB4-R2 cells (f) treated with the indicated drugs for 72 hours. Similar results were obtained in three independent experiments. Differentiation was also evaluated by NBT reduction assay (g, h) and flow cytometric analysis of CD11b expression (i, j) in NB4-R1 (g, i) and NB4-R2 cells (h, j) with the indicated treatment for 72 hours. For NBT reduction assay, one representative experiment was shown. Each value represented the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. For flow cytometric analysis of CD11b expression, each value represented the mean ± SD of three independent measurements. **P*<0.05, ***P*<0.01, ****P*<0.001 versus DMSO treated cells. ### *P*<0.001, versus ATRA

treated cells. &&& $P<0.001$, as compared with 0.5D+RA in NB4-R1 cells or 1D+RA in NB4-R2 cells. The representative histogram of flow cytometric analysis of CD11b expression in NB4-R1 (k) and NB4-R2 cells (l) with the indicated treatment for 72 hours were also shown. The percentages of CD11b positive cells were shown in the corresponding panels.

Figure 2 The combined treatment modulated the protein level of C/EBPβ, C/EBPε, PU.1 and activated MEK/ERK cascade. Except the Western blot analysis of PML-RARα, cells were treated with dasatinib (D), ATRA (RA) alone and in combination (D+RA) for 24 hours. For Western blot analysis of PML-RARα**,** cells were treated with the indicated drugs for 72 hours. The same membrane incubated with the phosphorylated Erk1/2 or MEK1/2 was stripped and followed by detection of MEK and ERK1/2. The expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments.

Figure 3 MEK inhibition blocked differentiation and restored the protein levels of C/EBPs as well as PU.1. Cells were exposed to 1μM U0126 for 1 hour prior to other treatment. The attenuation of MEK activation by U0126 (U) was measured by Western blot analysis of phosphorylated ERK1/2 in NB4-R1 (a) and NB4-R2 cells (b) with indicated treatments for 24 hours. The same membrane incubated with the phosphorylated Erk1/2 was stripped and followed by detection of ERK1/2. Similar results were obtained in three independent experiments. Inhibitory effect of U0126 on morphologic changes in NB4-R1 (c) and NB4-R2 cells (d) incubated with the indicated drugs for 72 hours. One representative experiment among three independent

assays was shown. The inhibitory effect of U0126 on differentiation was also confirmed by NBT reduction assay (e) and flow cytometric analysis of CD11b expression (f) with the indicated drugs for 72 hours. For NBT reduction assay, one representative experiment was shown. Each value represented the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. For flow cytometric analysis of CD11b expression, each value represented the mean ± SD of three independent measurements. ### $P<0.001$ versus D+RA. The representative histograms of flow cytometric analysis of CD11b expression in NB4-R1 (g) and NB4-R2 cells (h) with the indicated drugs for 72 hours were also shown. The percentages of CD11b positive cells were shown in the corresponding panels. The protein level of C/EBPβ, C/EBPε and PU.1 in NB4-R1 (i) and NB4-R2 (j) cells with the indicated drugs for 24 hours was determined by Western blot analysis. Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments.

Figure 4 The activation of MEK/ERK signaling pathway and the combined treatment-induced differentiation were dependent on RAF-1. RAF-1 activation was evaluated by the phosphorylation of RAF-1 at Ser338, Tyr340 and 341 with the indicated treatments for 6 hours in NB4-R1 (a) and NB4-R2 cells (b). Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments. NB4-R1 and NB4-R2 cells were pretreated with 5μM and 2μM sorafenib tosylate (SORA) for 1 hour respectively. The attenuation of RAF-1 activation by SORA was measured by phosphorylated MEK and ERK1/2 with the

indicated treatments for 24 hours in NB4-R1 (c) and NB4-R2 cells (d). Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments. The effect of SORA on the combined treatment-induced differentiation for 72 hours was observed by morphologic changes in NB4-R1 (e) and NB4-R2 cells (f). One representative experiment among three independent assays was shown. The inhibitory effect of SORA on differentiation was also confirmed by NBT reduction assay (g) and flow cytometric analysis of CD11b expression (h). For NBT reduction assay, one representative experiment was shown. Each value represented the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. For flow cytometric analysis of CD11b expression, each value represented the mean ± SD of three independent measurements. ### $P<0.001$ versus D+RA. The representative histograms of flow cytometric analysis of CD11b expression in NB4-R1 (i) and NB4-R2 cells (j) with the indicated drugs for 72 hours were also shown. The percentages of CD11b positive cells were shown in the corresponding panels. The effect of SORA on the combined treatment modulated protein level of C/EBPs and PU.1 was measured by Western blot analysis with the indicated treatments for 24 hours in NB4-R1 (k) and NB4-R2 cells (l). Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments.

Figure 5 Ras was not required for the combined treatment-induced differentiation and the activation of MEK/ERK cascade. Ras activation was evaluated with the indicated treatment for 3 hours. Active GTP-bound Ras was pulled

down by a GST fusion protein containing the human Ras binding domain of RAF-1(GST-RAF-1-RBD) and immunoblotted with anti-Ras antibody. Equal amount of Ras in the extracts were verified by immunoblotting an aliquot of the total cell lysate taken before pull down (a). Similar results were obtained in three independent experiments. Pretreated with 50μM salirasib (FTS) for 1 hour, the inhibitory effect of FTS on Ras activation was measured in both cell lines with the indicated treatment for 24 hours (b). The effect of FTS on the activation of MEK was evaluated by Western blot analysis of phosphorylated MEK with the indicated treatments for 24 hours in NB4-R1 (c) and NB4-R2 cells (d). Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments. Pretreated with FTS for 1 hour, the effect of FTS on the combined treatment-induced differentiation for 72 hours was observed by morphologic changes in NB4-R1 (e) and NB4-R2 cells (f). One representative experiment among three independent assays was shown. The effect of FTS on differentiation was also confirmed by NBT reduction assay (g) and flow cytometric analysis of CD11b expression (h). For NBT reduction assay, one representative experiment was shown. Each value represented the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. For flow cytometric analysis of CD11b expression, each value represented the mean ± SD of three independent measurements. The representative histograms of flow cytometric analysis of CD11b expression in NB4-R1 (i) and NB4-R2 cells (j) with the indicated drugs for 72 hours were also shown. The percentages of CD11b positive cells were shown in the corresponding panels.

Figure 6 Dasatinib inhibited the activity of Lyn, the major activated SFK in NB4-R1 and NB4-R2 cells. (a) The activation of SFK was assessed by Western blot analysis of phospho-Src Y416 in the lysates from NB4-R1 and NB4-R2 **c**ells with the indicated treatments for 3 hours (left panel). The same membrane incubated with the phospho-Src Y416 was stripped and followed by detection of Lyn (right panel). Molecular weights in kDa were shown in the middle. Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments. (b) Aliquot of cell lysates (input) from NB4-R1 and NB4-R2 cells with the indicated treatment for 3 hours as well as the cell lysates from NB4 untreated cells were immunoblotted with anti-p-Src Y416. The same membrane incubated with the phospho-Src Y416 was stripped and followed by detection of Lyn. Expression of β-actin was assessed as internal control (upper panel). The remainder of cell lysates was immunoprecipitated with Lyn antibody. ATRA-treated cell lysates from NB4-R1 and NB4-R2 cells were also subjected to immunoprecipitation with control IgG antibody. After immunoprecipitation of Lyn, the immunoprecipitate pellets and the immunodepleted supernant were subjected to immunoblotting with anti-p-Src Y416. The same membrane incubated with the phospho-Src Y416 was stripped and followed by detection of Lyn (middle and lower panel). Similar results were obtained in three independent experiments.

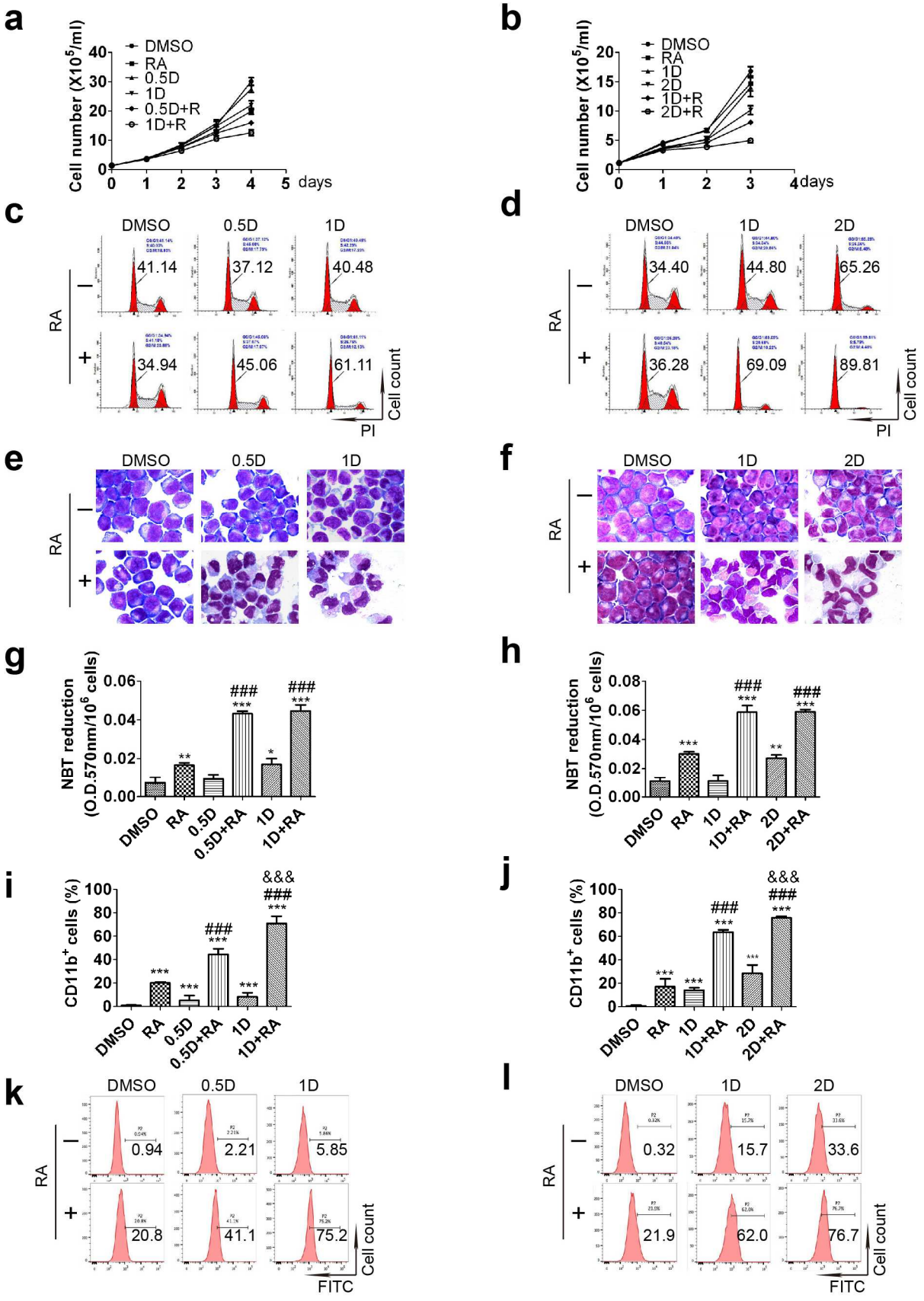
Figure 7 The combined treatment dephosphorylated RAF-1 S259 but not PP2A. The phosphorylation of RAF-1 S259 and PP2A Y307 were assessed by Western blot in the lysates from NB4-R1 (a) and NB4-R2 (b) **c**ells with the indicated treatments for

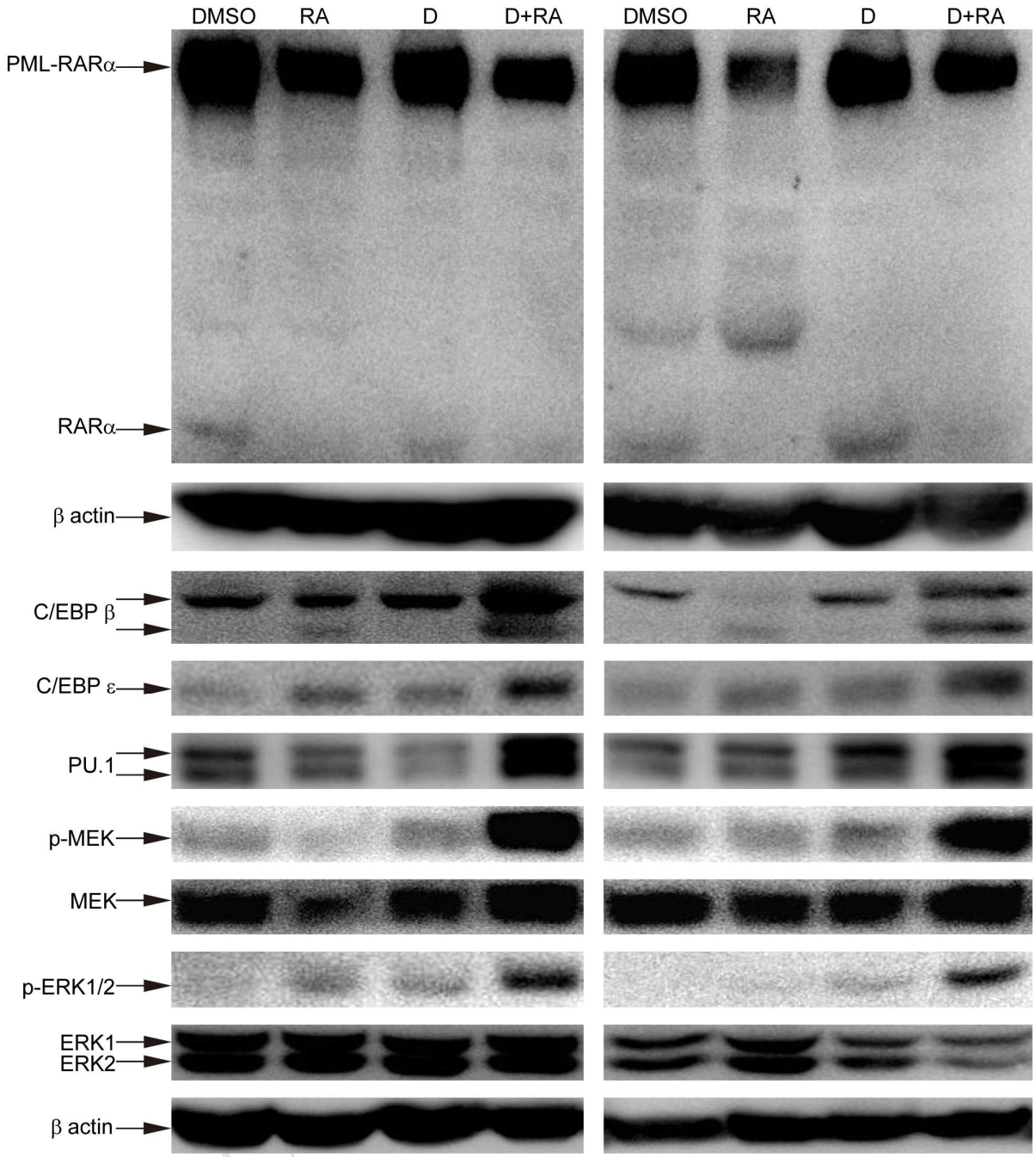
3 hours. The same membrane incubated with the p-RAF(Ser259) or p-PP2A(Y307) was stripped and followed by detection of RAF-1 or PP2A catalytic unit. Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments. Pretreated with 5nM okadaic acid (OA) for 1 hour, the effect of okadaic acid on the combined treatment-induced differentiation for 72 hours was observed by morphologic changes in NB4-R1 (c) and NB4-R2 cells (d). One representative experiment among three independent assays was shown. The effect of okadaic acid on differentiation was also confirmed by flow cytometric analysis of CD11b expression. The representative histograms of flow cytometric analysis of CD11b expression in NB4-R1 (e) and NB4-R2 cells (f) with the indicated drugs for 72 hours were also shown. The percentages of CD11b positive cells were shown in the corresponding panels.

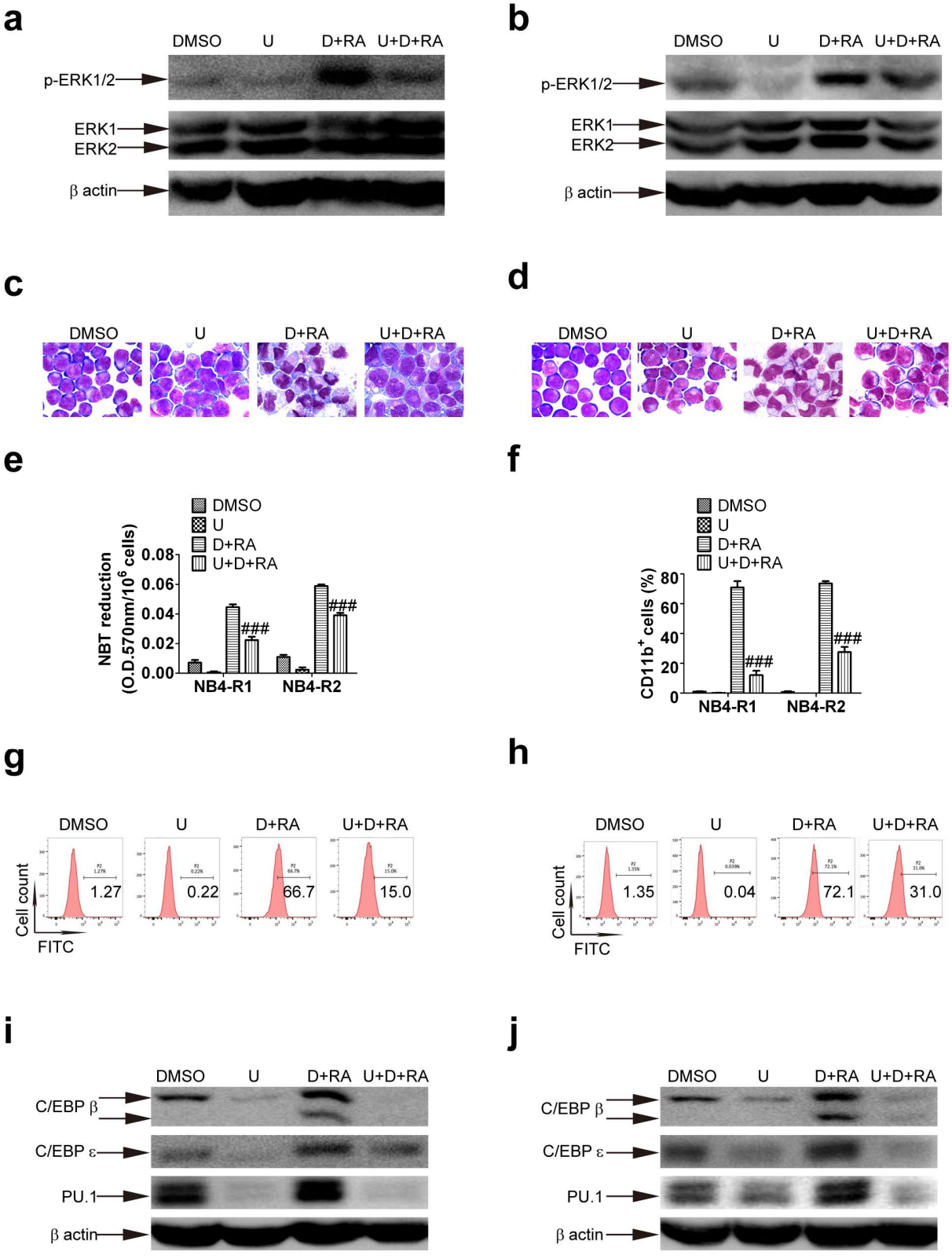
Figure 8 SFK inhibitor, PP2 mimicked the effect of dasatinib. The phosphorylation of RAF-1 S259 and Src family Y416 were assessed by Western blot in the lysates from NB4-R1 (a) and NB4-R2 (b) **c**ells with 1μM ATRA and/or 10 mM PP2 for 3 hours. The same membrane incubated with the p-Src family (Y416) or p-RAF(Ser259) was stripped and followed by detection of Lyn or RAF-1. Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments. Cells were treated with 1μM ATRA and/or 10 mM PP2 for 72 hours. Representative morphologic analysis of NB4-R1 (c) and NB4-R2 cells (d) was shown. Differentiation was also evaluated by NBT reduction assay (e) and flow cytometric analysis of CD11b expression (f) with the indicated treatment for 72 hours.

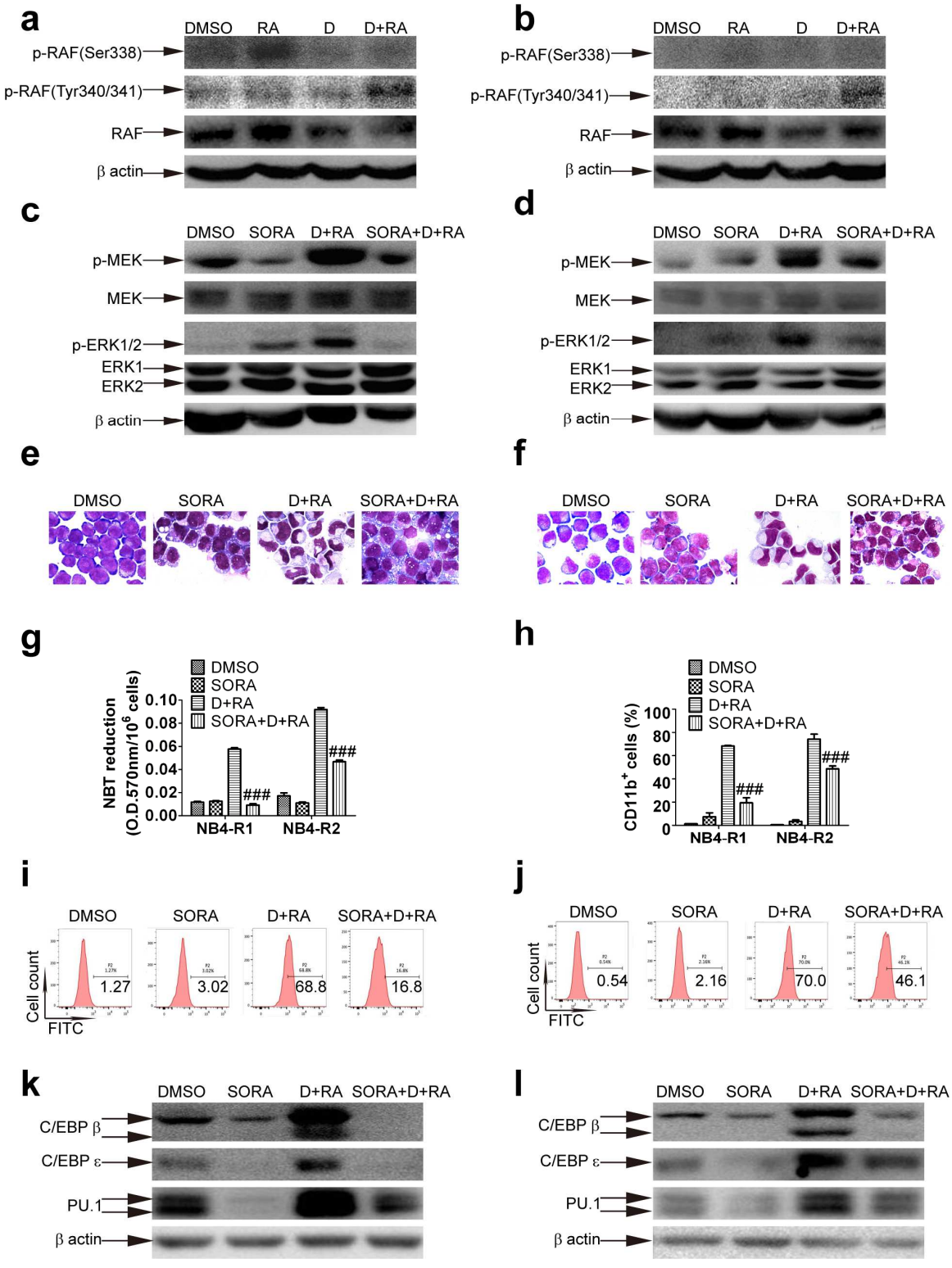
For NBT reduction assay, one representative experiment was shown. Each value represented the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. For flow cytometric analysis of CD11b expression, each value represented the mean ± SD of three independent measurements. ***P<0.001 versus DMSO treated cells. ### P<0.001, versus ATRA treated cells. The representative histogram of flow cytometric analysis of CD11b expression in NB4-R1 (g) and NB4-R2 cells (h) with the indicated treatment for 72 hours were also shown. The percentages of CD11b positive cells were shown in the corresponding panels.

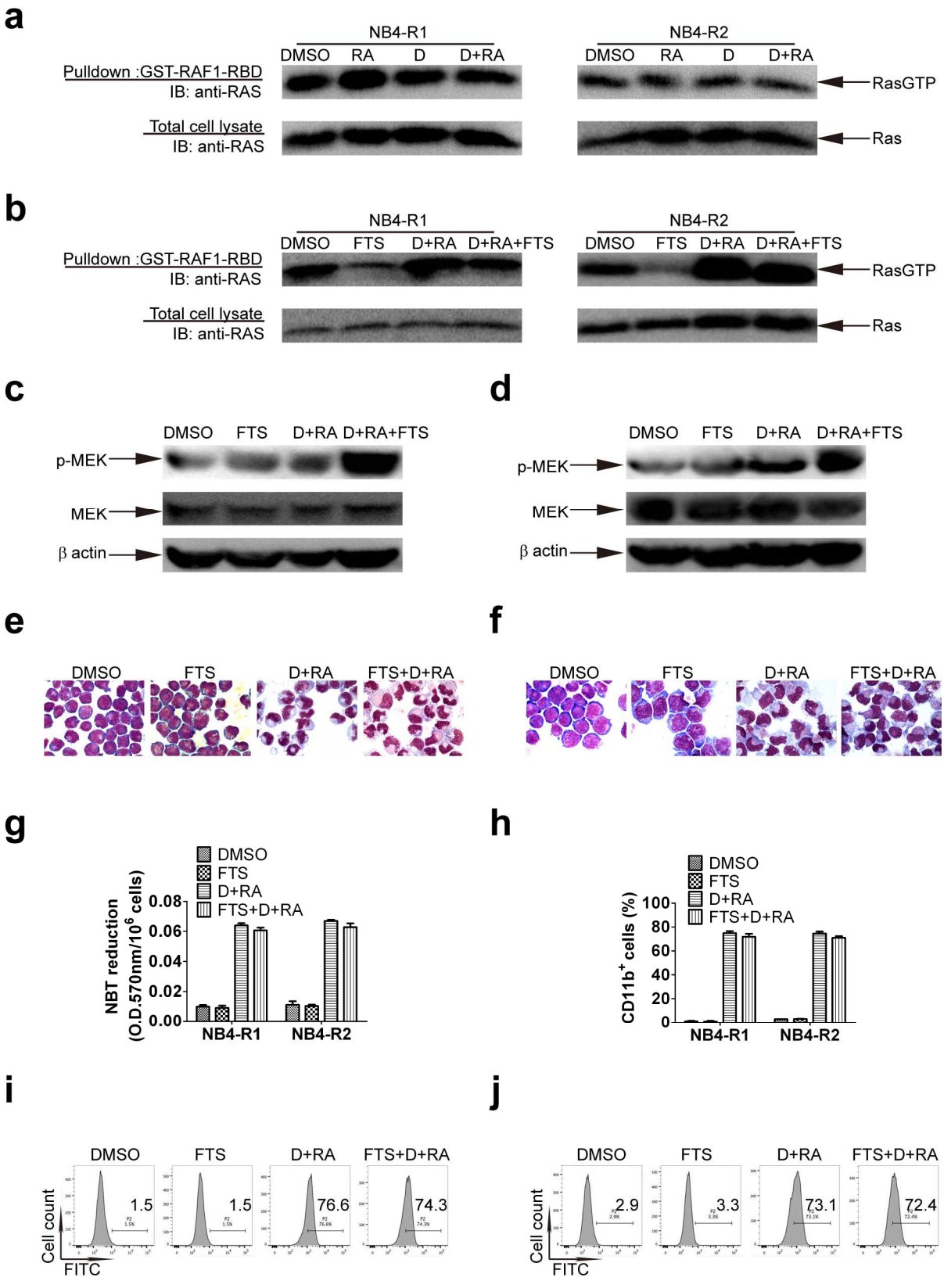
Figure 9 Schematic diagram of the mechanisms of the combined treatment induced differentiation. Dasatinib initiated RAF-1 activation by inhibition of Lyn, the kinase related to the phosphorylation of RAF-1 at Ser 259. With the addition of ATRA, RAF-1 Ser259 was dephosphorylated and activated by Tyr340/341 phosphorylation resulting in downstream MEK/ERK activation. Activated ERK enhanced the protein levels of C/EBPs and PU.1 and led to differentiation.

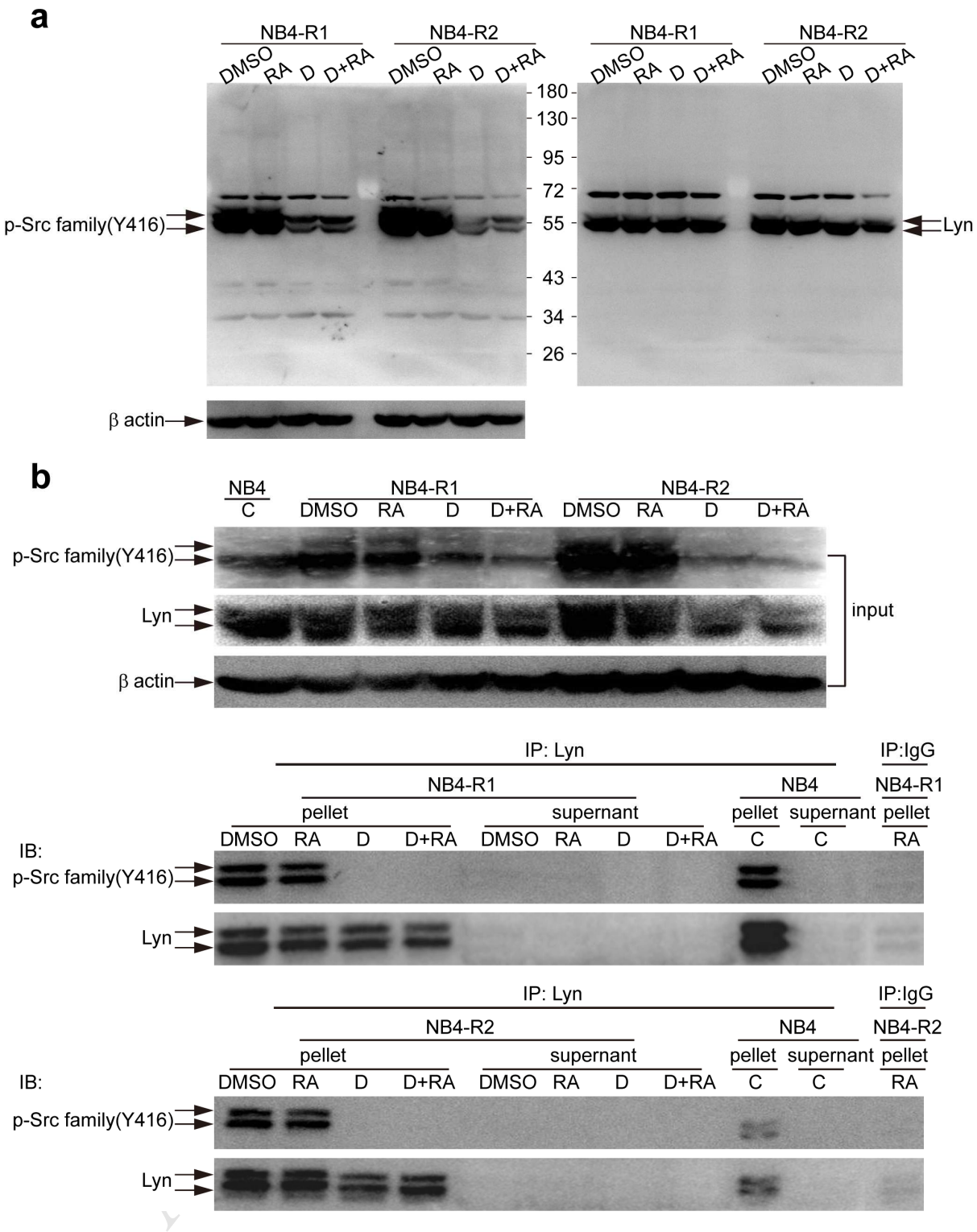

a
Cell number (X10^5/ml)
DMSO
RA
0.5D
1D
0.5D+R
1D+R
days
b
Cell number (X10^5/ml)
DMSO
RA
1D
2D
1D+R
2D+R
days
c
DMSO
0.5D
1D
RA
41.14
37.12
40.48
34.94
45.06
61.11
Cell count
PI
d
DMSO
1D
2D
RA
34.40
44.80
65.26
36.28
69.09
89.81
Cell count
PI
e
DMSO
0.5D
1D
RA
f
DMSO
1D
2D
RA
g
NBT reduction (O.D.570nm/10^6 cells)
DMSO
RA
0.5D
0.5D+RA
1D
1D+RA
h
NBT reduction (O.D.570nm/10^6 cells)
DMSO
RA
1D
1D+RA
2D
2D+RA
i
CD11b+ cells (%)
DMSO
RA
0.5D
0.5D+RA
1D
1D+RA
j
CD11b+ cells (%)
DMSO
RA
1D
1D+RA
2D
2D+RA
k
DMSO
0.5D
1D
RA
0.94
2.21
5.85
20.8
41.1
75.2
Cell count
FITC
l
DMSO
1D
2D
RA
0.32
15.7
33.6
21.9
62.0
76.7
Cell count
FITC

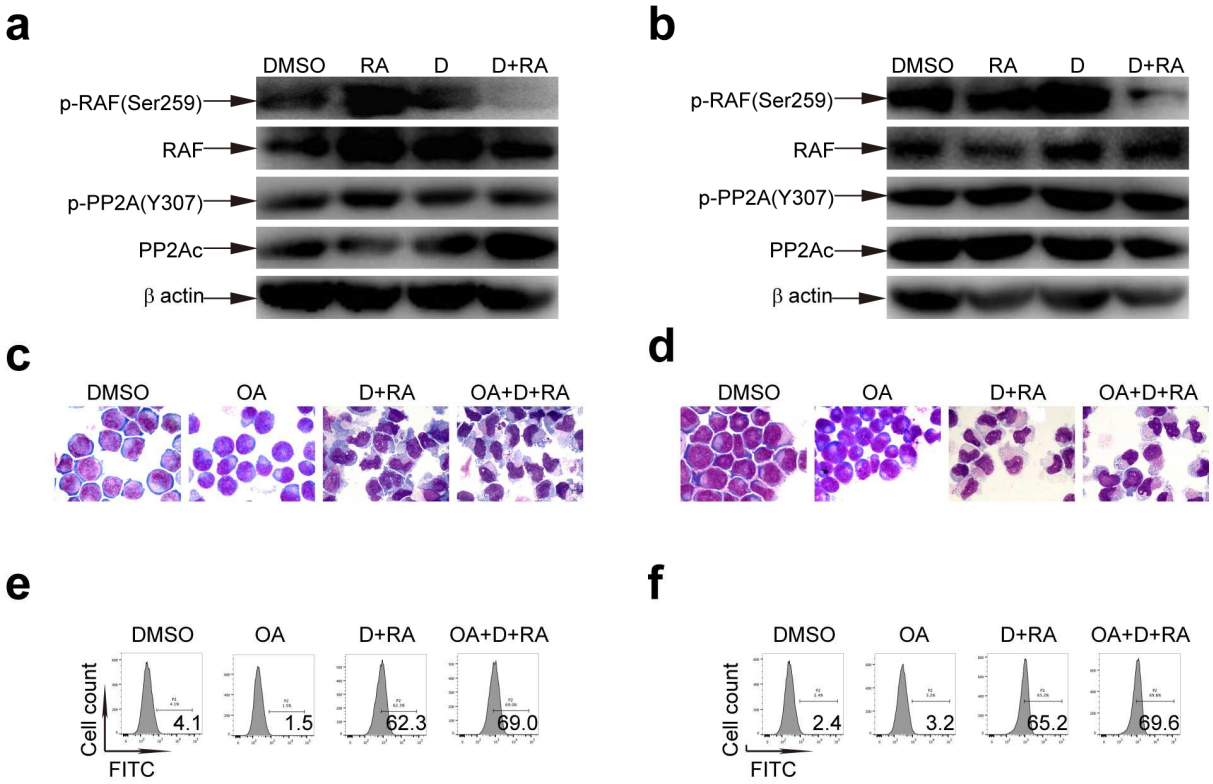


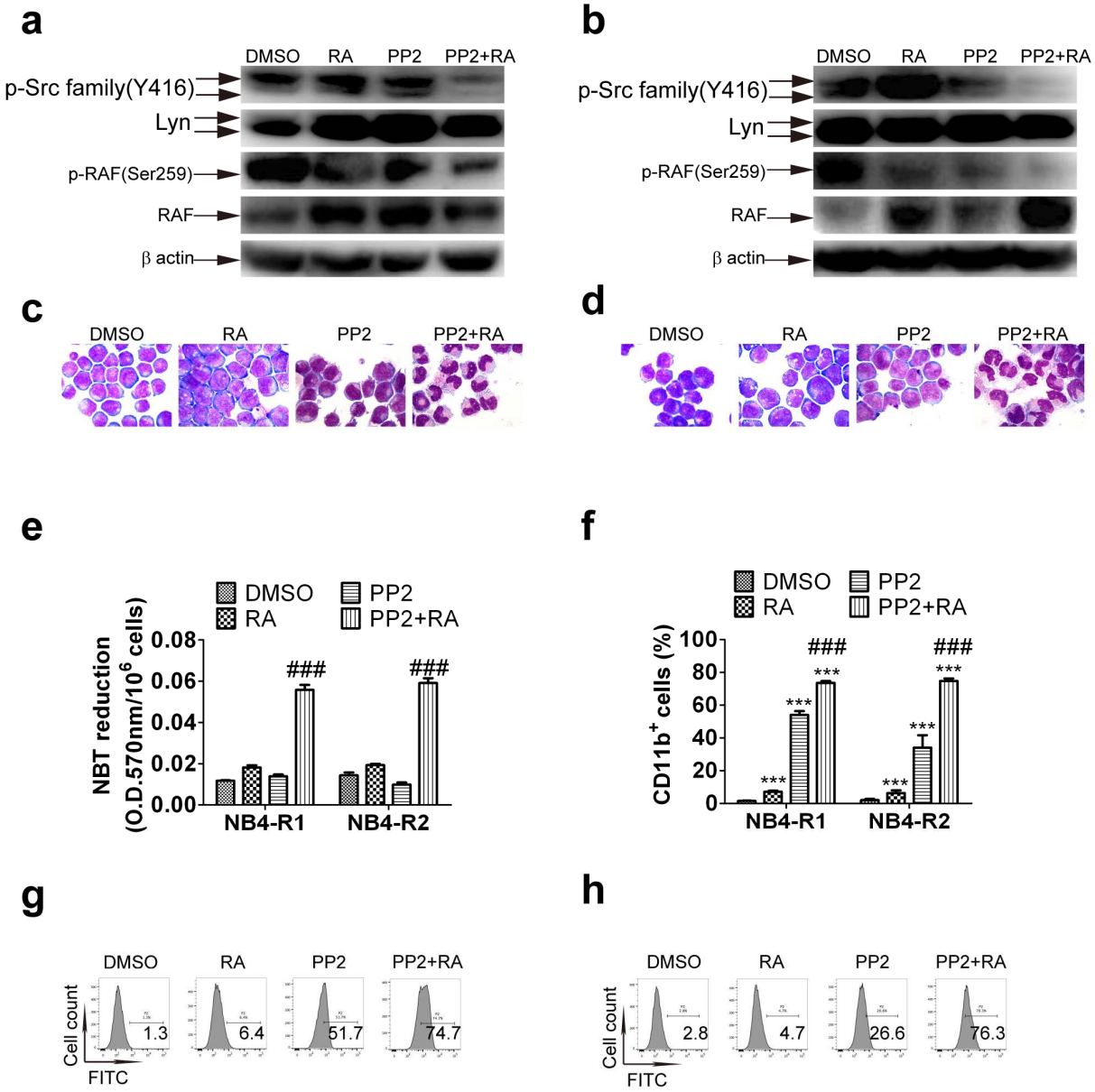

a
DMSO
U
D+RA
U+D+RA
p-ERK1/2
ERK1
ERK2
β actin
b
DMSO
U
D+RA
U+D+RA
p-ERK1/2
ERK1
ERK2
β actin
c
DMSO
U
D+RA
U+D+RA
d
DMSO
U
D+RA
U+D+RA
e
DMSO
U
D+RA
U+D+RA
NBT reduction (O.D.570nm/10^6 cells)
0.08
0.06
0.04
0.02
0.00
###
###
NB4-R1
NB4-R2
f
DMSO
U
D+RA
U+D+RA
CD11b+ cells (%)
80
60
40
20
0
###
###
NB4-R1
NB4-R2
g
DMSO
U
D+RA
U+D+RA
Cell count
1.27
0.22
66.7
15.0
FITC
h
DMSO
U
D+RA
U+D+RA
Cell count
1.35
0.04
72.1
31.0
FITC
i
DMSO
U
D+RA
U+D+RA
C/EBP β
C/EBP ε
PU.1
β actin
j
DMSO
U
D+RA
U+D+RA
C/EBP β
C/EBP ε
PU.1
β actin

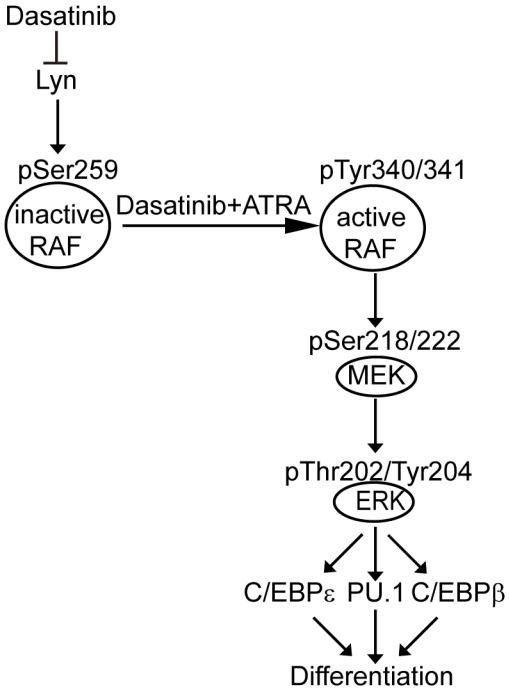

a
DMSO
RA
D
D+RA
p-RAF(Ser338)
p-RAF(Tyr340/341)
RAF
β actin
b
DMSO
RA
D
D+RA
p-RAF(Ser338)
p-RAF(Tyr340/341)
RAF
β actin
c
DMSO
SORA
D+RA
SORA+D+RA
p-MEK
MEK
p-ERK1/2
ERK1
ERK2
β actin
d
DMSO
SORA
D+RA
SORA+D+RA
p-MEK
MEK
p-ERK1/2
ERK1
ERK2
β actin
e
DMSO
SORA
D+RA
SORA+D+RA
f
DMSO
SORA
D+RA
SORA+D+RA
g
DMSO
SORA
D+RA
SORA+D+RA
NBT reduction (O.D.570nm/10^6 cells)
0.10
0.08
0.06
0.04
0.02
0.00
###
###
NB4-R1
NB4-R2
h
DMSO
SORA
D+RA
SORA+D+RA
CD11b+ cells (%)
100
80
60
40
20
0
###
###
NB4-R1
NB4-R2
i
DMSO
SORA
D+RA
SORA+D+RA
Cell count
FITC
1.27
3.02
68.8
16.8
j
DMSO
SORA
D+RA
SORA+D+RA
Cell count
FITC
0.54
2.16
70.0
46.1
k
DMSO
SORA
D+RA
SORA+D+RA
C/EBP β
C/EBP ε
PU.1
β actin
l
DMSO
SORA
D+RA
SORA+D+RA
C/EBP β
C/EBP ε
PU.1
β actin


a
NB4-R1
DMSO RA D D+RA
NB4-R2
DMSO RA D D+RA
Pulldown :GST-RAF1-RBD
IB: anti-RAS
RasGTP
Total cell lysate
IB: anti-RAS
Ras
b
NB4-R1
DMSO FTS D+RA D+RA+FTS
NB4-R2
DMSO FTS D+RA D+RA+FTS
Pulldown :GST-RAF1-RBD
IB: anti-RAS
RasGTP
Total cell lysate
IB: anti-RAS
Ras
c
DMSO FTS D+RA D+RA+FTS
p-MEK
MEK
β actin
d
DMSO FTS D+RA D+RA+FTS
p-MEK
MEK
β actin
e
DMSO FTS D+RA FTS+D+RA
f
DMSO FTS D+RA FTS+D+RA
g
DMSO
FTS
D+RA
FTS+D+RA
NBT reduction (O.D.570nm/10^6 cells)
0.08
0.06
0.04
0.02
0.00
NB4-R1 NB4-R2
h
DMSO
FTS
D+RA
FTS+D+RA
CD11b+ cells (%)
100
80
60
40
20
0
NB4-R1 NB4-R2
i
DMSO FTS D+RA FTS+D+RA
Cell count
1.5 1.5 76.6 74.3
FITC
j
DMSO FTS D+RA FTS+D+RA
Cell count
2.9 3.3 73.1 72.4
FITC


a
NB4-R1
NB4-R2
NB4-R1
NB4-R2
DMSO RA D D+RA DMSO RA D D+RA DMSO RA D D+RA DMSO RA D D+RA
180
130
95
72
55
43
34
26
p-Src family(Y416)
Lyn
β actin
b
NB4
C
NB4-R1
NB4-R2
DMSO RA D D+RA DMSO RA D D+RA
p-Src family(Y416)
Lyn
β actin
input
IP: Lyn
IP:IgG
NB4-R1
NB4
NB4-R1
pellet
supernant
pellet supernant pellet
DMSO RA D D+RA DMSO RA D D+RA C C RA
IB:
p-Src family(Y416)
Lyn
IP: Lyn
IP:IgG
NB4-R2
NB4
NB4-R2
pellet
supernant
pellet supernant pellet
IB:
DMSO RA D D+RA DMSO RA D D+RA C C RA
p-Src family(Y416)
Lyn




a
DMSO
RA
PP2
PP2+RA
p-Src family(Y416)
Lyn
p-RAF(Ser259)
RAF
β actin
b
DMSO
RA
PP2
PP2+RA
p-Src family(Y416)
Lyn
p-RAF(Ser259)
RAF
β actin
c
DMSO
RA
PP2
PP2+RA
d
DMSO
RA
PP2
PP2+RA
e
DMSO
RA
PP2
PP2+RA
NBT reduction (O.D.570nm/10^6 cells)
0.00
0.02
0.04
0.06
0.08
###
###
NB4-R1
NB4-R2
f
DMSO
RA
PP2
PP2+RA
CD11b+ cells (%)
0
20
40
60
80
100
***
***
***
***
###
###
NB4-R1
NB4-R2
g
DMSO
RA
PP2
PP2+RA
Cell count
1.3
6.4
51.7
74.7
FITC
h
DMSO
RA
PP2
PP2+RA
Cell count
2.8
4.7
26.6
76.3
FITC



- Dasatinib and ATRA triggered differentiation in ATRA resistant APL cell lines.
- The combination induced differentiation via Ras independent RAF-1/MEK/ERK.
- Dasatinib suppressed Lyn activity, the predominant activated SFK in both cell lines.
- RAF-1 was activated by Lyn inhibition-mediated dephosphorylation of S259.