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The combination of UCN-01 and ATRA triggers differentiation in ATRA resistant acute promyelocytic leukemia cell lines via RAF-1 independent activation of MEK/ERK

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

[a] 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

[b] Department of Hematology Oncology, Central Hospital of Minhang District, No. 170 Xin Song Road, Shanghai, 201199, China

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ABSTRACT

With the introduction of arsenic trioxide and all-trans retinoic acid, the prognosis of acute promyelocytic leukemia has greatly improved. However, all-trans retinoic acid resistance is still unresolved in acute promyelocytic leukemia relapsed patients. In this study, the clinical achievable concentration of 7-hydroxystaurosporine synergized with all-trans retinoic acid to induce terminal differentiation in all-trans retinoic acid resistant acute promyelocytic leukemia cell lines. Though 7-hydroxystaurosporine is a PKC inhibitor, PKC might not be involved in the combination-induced differentiation since other PKC selective inhibitors, Gö 6976 and rottlerin failed to cooperate with all-trans retinoic acid to trigger differentiation. The combination significantly enhanced the protein level of CCAAT/enhancer binding protein β and/or PU.1 as well as activated MEK/ERK. U0126 (MEK specific inhibitor) not only suppressed the combination-induced differentiation but also restored the protein level of CCAAT/enhancer binding protein β and/or PU.1. However, RAF-1 inhibitor had no inhibitory effect on MEK activation and the combination-induced differentiation. Therefore, the combination overcame differentiation block via RAF-1 independent MEK/ERK modulation of the protein level of CCAAT/enhancer binding protein β and/or PU.1. These findings may provide a preclinical rationale for the potential role of this combination in the treatment of all-trans retinoic acid resistant acute promyelocytic leukemia patients.

1. Introduction

Acute promyelocytic leukemia (APL) is characterized by the characteristic chromosomal translocation t(15; 17) (q22; q21), resulting in the fusion gene consisting of *promyelocytic leukemia (PML)* and *retinoic acid receptor α* (*RARα*)(Shen et al., 2004). With the introduction of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) in the conventional chemotherapy of APL, the prognosis of APL has greatly improved and made it as a highly curable disease(Shen et al., 2004). However, 5–10% patients achieved complete remission still eventually relapsed and/or developed resistance to ATRA(Wang et al., 2016). For decades, finding novel therapy approaches to overcome ATRA resistance has been an important goal in the treatment of APL. Though some agents could induce differentiation in ATRA resistant APL cells in combination with ATRA, the clinical applicability of these therapies remains to be determined(Gao et al., 2010; Ge et al., 2015; Gianni' et al., 2001; Guillemin et al., 2002; He et al., 2012; Higuchi et al., 2004; Witcher et al., 2004; Wu et al., 2014). Gemtuzumab ozogamicin (GO), an anti-CD33 monoclonal antibody conjugated to the toxin calicheamicin, has been used successfully as a single agent for patients with molecular relapsed APL as well as in combination with ATRA and ATO for newly diagnosed APL patients(Abaza et al., 2017; Aribi et al., 2007; Godwin et al., 2017; Lo-coco et al., 2004; Ravandi et al., 2009). Large randomized study of GO for APL treatment remains to be performed. As a single agent, LG-362B overcame the ATRA resistance on cellular differentiation and transplantable APL mice with potential target of PML-RARα(Wang et al., 2016). Clinical trials were urgently needed to verify its clinical efficacy. Till now, ATO was the widely proven effective

Abbreviations: ATRA, all-trans retinoic acid; ATO, arsenic trioxide; APL, acute promyelocytic leukemia; C/EBP, CCAAT/enhancer-binding protein; GO, gemtuzumab ozogamicin; DMSO, dimethyl sulfoxide; MEKK, MEK kinase; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-3 kinase; PKA, protein kinase A; PKC, protein kinase C; PML, promyelocytic leukemia; RARα, retinoic acid receptor α; SDS, sodium dodecyl sulfate; UCN-01, 7-hydroxystaurosporine

* Corresponding author.
E-mail address: Cx10901@rjh.com.cn (X. Cai).

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therapy in the treatment of APL, especially for the ATRA-resistant relapsed APL patients(McCulloch et al., 2017). Though adverse effects of ATO are relatively rare, it can still lead to serious adverse effects. Moreover, acquired ATO resistance is still inevitable(Goto et al., 2011; Lou et al., 2015; Zhu et al., 2014). Therefore, alternative approach to avoid or reverse ATRA resistance needs to be sought to improve the outcome of APL patients.

Signaling pathway and effectors involved in myeloid differentiation or ATRA-induced granulocytic differentiation might provide new therapeutic targets to release the differentiation block. 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). Furthermore, enhanced activation of MEK/ERK restored ATRA sensitivity in ATRA-resistant APL cell lines(Ge et al., 2015). Therefore, MEK/ERK signal pathway might be the potential target to overcome ATRA resistance. 7-hydroxystaurosporine (UCN-01), a derivative of staurosporine, was original developed as a PKC inhibitor but was also subsequently identified as an inhibitor of Chk1 and PDK1/AKT(Dai et al., 2005). Staurosporine is an indolo[2,3-a] carbazole alkaloid from microorganism. Though staurosporine is one of the most powerful PKC inhibitors, its intolerable toxicity due to its poor kinase selectivity hampered its clinical use(da Rocha et al., 2002). Unlike staurosporine, UCN-01 exhibits *in vivo* antitumor activity in animals and its safety was demonstrated in multiple clinical trials(Akinaga et al., 1991; Chalovich and Eisenberg, 2013; Fracasso et al., 2011; Gojo et al., 2014). It is interesting that UCN-01 activates MEK/ERK signaling pathway in several myeloid leukemia and myeloma cells(Dai et al., 2002, 2001). However, its ability to stimulate MEK/ERK was never used for the development of therapeutic approach. On the contrary, due to its capacity to function as a checkpoint abrogator, a variety of agents which interrupt MEK/ERK were investigated to combine with UCN-01 to exert antitumor activity via apoptosis(Dai et al., 2007, 2005; Hahn et al., 2005; Jia et al., 2003).

Since UCN-01 is capable to activate MEK/ERK, we studied whether UCN-01 could restore ATRA sensitivity in ATRA resistant APL cell lines via MEK/ERK signaling pathway. The clinical achievable concentrations of UCN-01 were used. The combination of UCN-01 and ATRA mainly induced terminal granulocytic differentiation in dose-dependent manner in ATRA resistant APL cell lines NB4-R1 and NB4-R2. PKC and PML-RARα might not be involved in the combined treatment-triggered differentiation. Further study demonstrated that the combination overcame differentiation block via RAF-1 independent MEK/ERK modulation of the protein level of C/EBPβ and/or PU.1.

2. Material and Methods

2.1. Reagents

ATRA was obtained from Sigma-Aldrich (St Louis, MO, USA). Gö 6976, rottlerin and U0126 were purchased from EMD Millipore (Billerica, MA, USA). UCN-01 and sorafenib tosylate were from Selleckchem Chemicals (Houston, TX, USA). All these reagents were dissolved by dimethyl sulfoxide (DMSO).

2.2. Cell culture, cell viability and cell proliferation

The ATRA resistant cell lines, NB4-R1 and NB4-R2 were provided by 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, USA) in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Cells were maintained at less than 5×10^5 cells/mL to avoid possible effects of cell density on cell growth and survival. Cell viability was measured by trypan-blue exclusion. Actual viable cell numbers were calculated by multiplying diluted times with counted viable cell numbers.

2.3. Cell differentiation assays

Cell differentiation was determined by morphology, nitroblue tetrazolium (NBT) reduction assay and the content of cell surface differentiation-related antigen CD11b. Morphology was evaluated with May-Grunwald-Giemsa's staining of cells centrifuged onto slides by cytospin (Shandon, Runcon, UK; 800 rpm., 5 min) and observed at x1000 magnification. For NBT reduction, 1×10^6 cells were harvested and incubated with 1 mg/mL NBT (Sigma-Aldrich) solution containing 10 μM phorbol 12-myristate 13-acetate (Sigma-Aldrich) at 37 °C for 1 h. Then, cells were lysed by 10% sodium dodecyl sulfate (SDS) and 0.04 M hydrochloric acid. The absorbance at O.D 570 nm was measured by spectrophotometer (Beckman Coulter, Brea, CA, USA). The expression of CD11b (Coulter, Marseilles, France) was examined by flow cytometry (EPICS XL, Coulter, Hialeah, FL, USA). Data were collected and analyzed by FlowJo software (FlowJo, LLC,Ashland, Oregon, USA).

2.4. Annexin-V analysis

Cells (5×10^5) were collected and washed with binding buffer provided in the Annexin V-PI Apoptosis Detection Kit (BD Biosciences Pharmingen, San Diego, CA, USA). Then, 5 μL annexin-V and 5 μL 7-Amino-Actinomycin (7-AAD) were added to the cells and incubated at room temperature in the dark for 15 min. Fluorescent intensities was determined on flow cytometry (Coulter).

2.5. Western blot analysis

Whole cell lysates were prepared, and quantified by Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA, USA). 50 or 100 μg protein extracts were resolved by denaturing electrophoresis on 8% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes (GE Healthcare UK Ltd, Buckinghamshire, UK). After blocking with 5% nonfat milk or BSA, the membranes were immune-stained with the following primary antibodies: RARα, C/EBPβ, C/EBPε, PU.1 from Santa Cruz Biotech (Santa Cruz, CA, USA); phospho-p44/42 Erk1/2 (Thr202/Try204), phospho-MEK1/2 (Ser217/Try221) from Cell Signaling Technology (Beverly, MA, USA); β-actin from Sigma-Aldrich. Immunoreactivity was detected by the horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare UK Ltd) and visualized by chemiluminescence kit (GE Healthcare UK Ltd) according to the manufacturer's instruction. To examine Erk1/2 and MEK1/2, the same membrane immune-stained with the phosphorylated Erk1/2 or MEK1/2 was stripped with stripping buffer (2% SDS, 100 mM beta-mercaptoethanol, 50 mM Tris, pH6.8), and then probed with anti-Erk1/2 (Cell Signaling Technology) or anti-MEK1/2 (Cell Signaling Technology).

2.6. Statistical analysis

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

3. Results

3.1. UCN-01 synergizes with ATRA to trigger granulocytic differentiation in NB4-R1 and NB4-R2 cells

To investigate the effect of the combination of UCN-01 and ATRA on NB4-R1 and NB4-R2 cells, we first determined the concentration of UCN-01 studied in both cell lines. One Phase I trial of UCN-01 by 72 h continuous infusion showed that a maximum tolerated dose of 42.5 mg/m^2/day for 3 days achieved the free drug concentration of 111 nM, measured by salivary concentration to reflect plasma levels(Sausville et al., 2001). Another Phase I investigation using 24 mg/m^2/day for 3 days indicated the free UCN-01 in plasm to exceed 200 nM(Chalovich

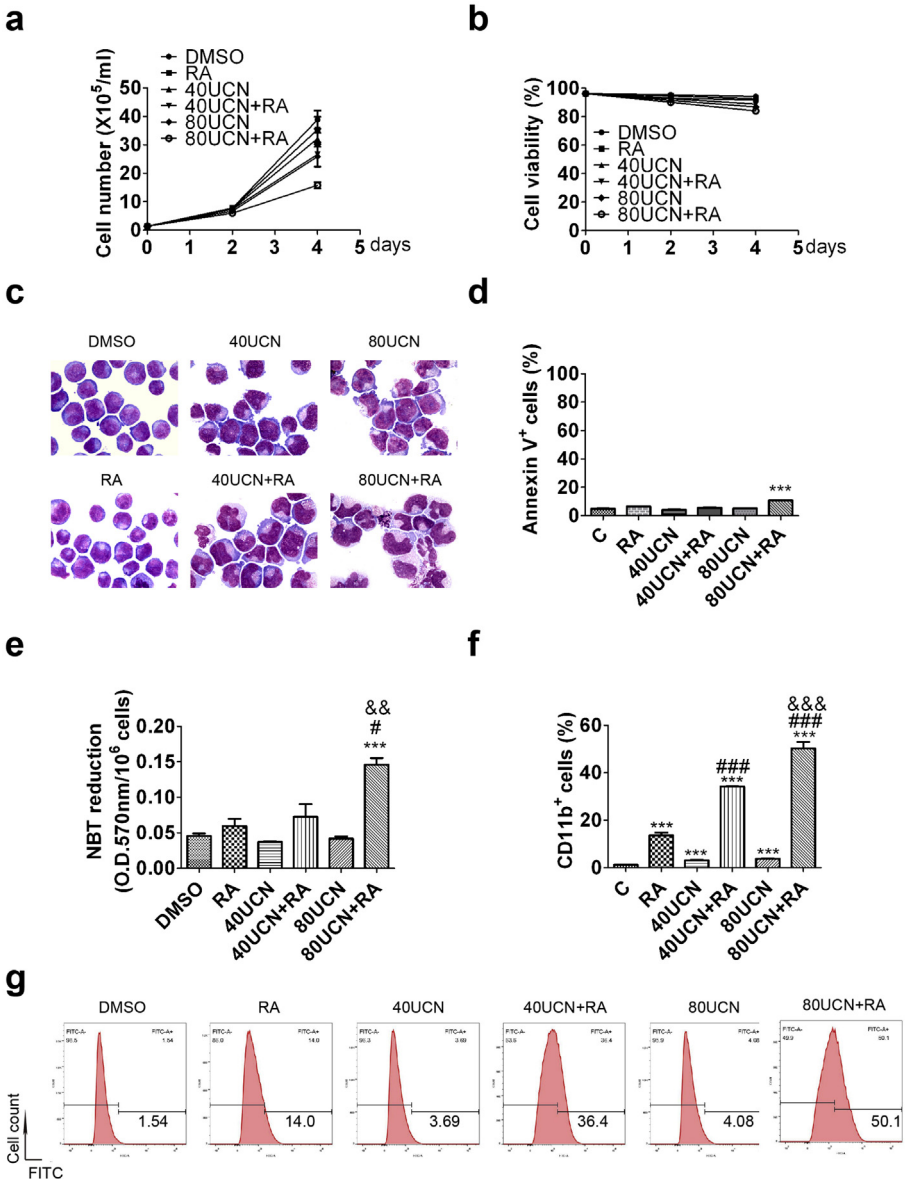

Fig. 1. Effects of combined treatment on the growth, survival and differentiation in NB4-R1 cells. NB4-R1 cells were treated with 40 nM, 80 nM UCN-01 (UCN), 1 μM ATRA (RA) and in combination for 4 days. Cell growth (a) and cell viability (b) was calculated as mentioned in Material and Methods. One representative experiment was shown. Each value represented the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. Representative morphologic analysis of NB4-R1 cells treated with the indicated drugs for 4 days (c). Similar results were obtained in three independent experiments. Annexin-V assay of NB4-R1 cells treated with UCN-01 or/and ATRA for 4 days (d). Each value represented the mean ± SD of three independent measurements. ***$P < 0.001$ versus DMSO treated cells. Differentiation was also evaluated by NBT reduction assay (e) and flow cytometric analysis of CD11b expression (f) in NB4-R1 cells with the indicated treatment for 4 days. 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. #$P < 0.05$, ###$P < 0.001$, versus ATRA treated cells. && $P < 0.01$, &&& $P < 0.001$, as compared with 40UCN-01 + RA in NB4-R1 cells. The representative histogram of flow cytometric analysis of CD11b expression in NB4-R1 cells with the indicated treatment for 4 days were also shown (g). The percentages of CD11b positive cells were shown in the corresponding panels.

and Eisenberg, 2013). Clinically achievable UCN-01 concentration 80 nM and 100 nM were determined to be the maximum concentrations used in NB4-R1 and NB4-R2 cell lines respectively with no effect on cell survival (Fig. 1b and Fig. 2b). DMSO treatment was regarded as control since both ATRA and UCN-01 were dissolved in it. Low concentration of UCN-01 slightly inhibited cell growth while high concentration of UCN-01 and the combination of low concentration of UCN-01 and ATRA suppressed proliferation moderately in both cell lines (Figs. 1a and 2a). However, the combination of high concentration of UCN-01 and ATRA showed significantly growth inhibition in both cell lines (Figs. 1a and 2a). With the co-treatment of high concentration of UCN-01 and ATRA for 4 days, the cell viability decreased mildly in both cell lines (UCN-01 + RA compared with DMSO, 84.0 ± 0.8% *vs* 94.2 ± 1.5% in NB4-R1 cells and 79 ± 1.7% *vs* 95.4 ± 0.7% in NB4-R2 cells, Figs. 1b and 2b). Thus, the combined treatment inhibited proliferation and modestly induced cell death in both cell lines.

As shown in Figs. 1c and 2c, both cell lines presented a characteristic morphology of APL blast such as round nucleus and large nuclear/cytoplasm ratio. No typical differentiated or apoptosis cells were observed in both cell lines treated with ATRA or UCN-01 alone (Figs. 1c and 2c). In both cell lines, any concentration of UCN-01 and ATRA co-treated cells displayed more matured appearances, such as kidney-shape or lobed nuclei accompanied by markedly decreased nuclear/cytoplasm ratio (Figs. 1c and 2c). Moreover, almost all the cells treated with the combination of high concentration of UCN-01 and ATRA presented fully differentiation (Figs. 1c and 2c). Consistent with cell viability, a few typical apoptotic cells could only be observed in both cell lines with co-treatment of ATRA and high concentration of UCN-01 (Figs. 1c and 2c). Annexin-V assay showed that Annexin-V^+ cells were slightly increased with the co-treatment of high concentration of UCN-01 and ATRA in both cell lines (UCN-01 + RA compared with DMSO, 10.8 ± 0.1% *vs* 4.9 ± 0.9% in NB4-R1 cells and 13.0 ± 1.2% *vs* 6.4 ± 0.7% in NB4-R2 cells, Figs. 1d and 2d). Therefore, the pattern of cell death induced by the combined treatment of high concentration of UCN-01 and ATRA in both cell lines was apoptosis. Consistent with morphological change, high concentration of UCN-01 combined with ATRA enhanced NBT reduction remarkably in both cell lines (Figs. 1e and 2e). Accordingly, a synergistic effect of UCN-01 and ATRA on the content of $CD11b^+$ cells was observed in a dose-dependent manner in both cell lines (Fig. 1f, 1g, 2f and 2g). Taken together, these results demonstrated that co-treatment of UCN-01 and ATRA mainly induced granulocytic differentiation accompanied by slightly apoptosis in these

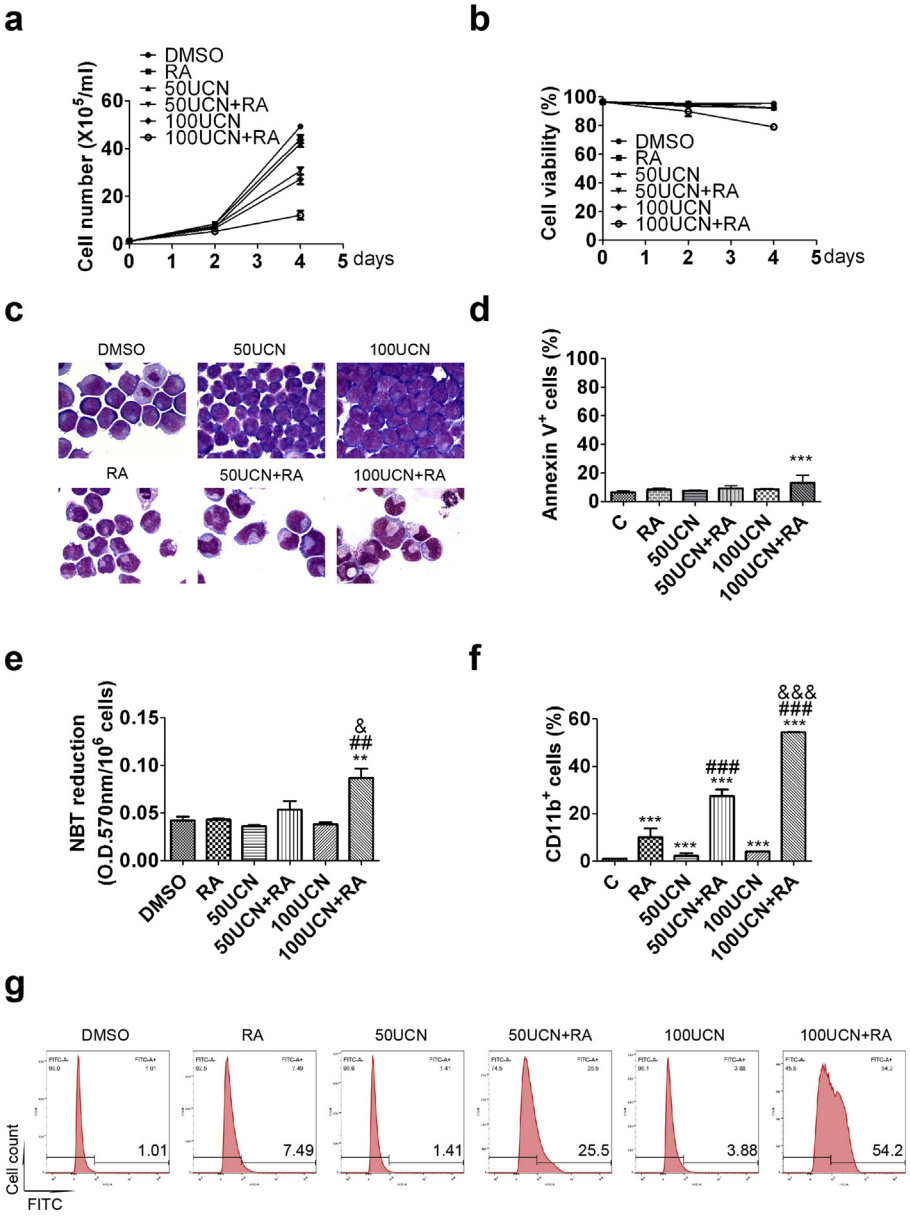

Fig. 2. Effects of combined treatment on the growth, survival and differentiation in NB4-R2 cells. NB4-R2 cells were treated with 50 nM, 100 nM UCN-01 (UCN), 1 μM ATRA (RA) and in combination for 4 days. Cell growth (a) and cell viability (b) was calculated as mentioned in Material and Methods. One representative experiment was shown. Each value represented the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. Representative morphologic analysis of NB4-R2 cells treated with the indicated drugs for 4 days (c). Similar results were obtained in three independent experiments. Annexin-V assay of NB4-R2 cells treated with UCN-01 or/and ATRA for 4 days (d). Each value represented the mean ± SD of three independent measurements. $^{***}P < 0.001$ versus DMSO treated cells. Differentiation was also evaluated by NBT reduction assay (e) and flow cytometric analysis of CD11b expression (f) in NB4-R2 cells with the indicated treatment for 4 days. 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. $^{**}P < 0.01$, $^{***}P < 0.001$ versus DMSO treated cells. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, versus ATRA treated cells, & $P < 0.05$, &&& $P < 0.001$, as compared with 50UCN-01 + RA in NB4-R2 cells. The representative histogram of flow cytometric analysis of CD11b expression in NB4-R2 cells with the indicated treatment for 4 days were also shown (g). The percentages of CD11b positive cells were shown in the corresponding panels.

two cell lines.

3.2. The combined treatment induced differentiation is independent of PKC

Since the combined treatment mostly induced differentiation, we focused on the mechanisms of combined treatment-induced differentiation. High concentration of UCN-01 was used in the subsequent studies. As UCN-01 is the derivative of staurosporine, a potent PKC inhibitor, we first determined whether PKC was required for the combination-induced differentiation. UCN-01 can inhibit PKC-α, -β, -γ, -δ, and -ε isoforms. To further confirm the role of PKC, we evaluated whether the combination of ATRA with several selective PKC inhibitors could mimic the effect of UCN-01 on ATRA-induced differentiation. Two PKC inhibitors, Gö 6976 (an inhibitor of PKC-α, -βI and –μ isoforms) and rottlerin (an inhibitor of PKC-δ, -α, -β, -γ, -ε and -ζ isoforms), whose inhibition spectrum covered all the UCN-01 inhibited PKC isoforms were used in the following experiment. No typical fully differentiated cells could be observed in both cell lines with the combined treatment of ATRA and any of the above selective PKC inhibitors (Fig. 3a and b). Moreover, compared with ATRA treatment alone, $CD11b^+$ cells were only slightly increased by the co-treatment of ATRA and Gö 6976 or ATRA and rottlerin in both cell lines, much less than the combination of ATRA and UCN-01 (Fig. 3c and d). Therefore, these two PKC selective inhibitors could not synergize with ATRA to induce terminal differentiation in NB4-R1 and NB4-R2 cells. It was suggested that the combined treatment induced differentiation might be independent of PKC.

3.3. The combination of UCN-01 and ATRA activates MEK/ERK signal pathway, enhances the protein level of C/EBPβ and/or PU.1

The degradation of PML-RARα, the pathogenic protein of APL, has been regarded as the crucial mechanism of ATRA-induced granulocytic differentiation of APL cells(Ohnishi, 2007). ATRA treatment alone for 96 h decreased the protein level of PML-RARα and RARα in both cell lines (Fig. 4). However, addition of UCN-01 inhibited ATRA-mediated reduction of PML-RARα in NB4-R1 cells, whereas the combined treatment in NB4-R2 cells hardly affected ATRA-reduced protein level of PML-RARα (Fig. 4). It was suggested that the combined treatment induced differentiation might be independent of the regulation of PML-

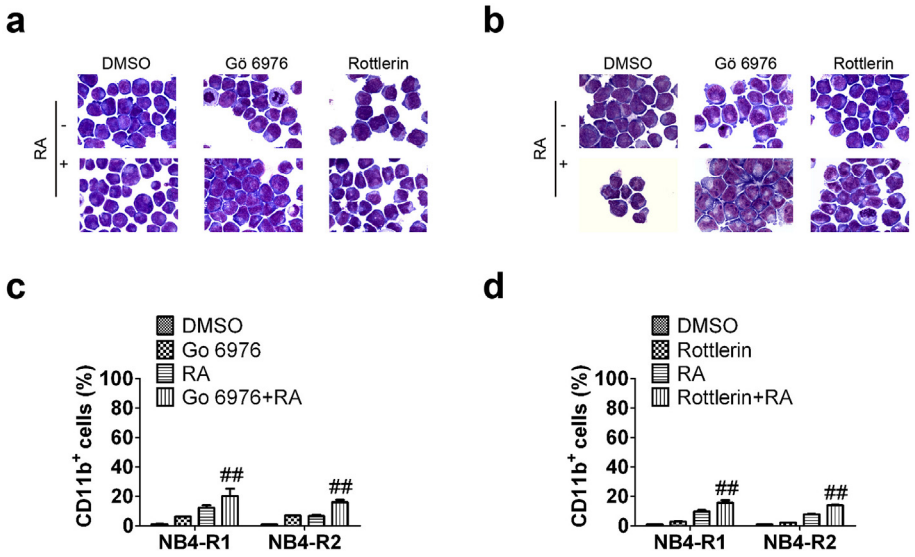

Fig. 3. The effect of the combination of PKC selective inhibitors and ATRA on the differentiation. NB4-R1 (left panel) and NB4-R2 (right panel) cells were treated with 200 nM Gö 6976, 2 μM rottlerin, 1 μM ATRA (RA) and in combination for 4 days. The representative morphologic analysis of NB4-R1 (a) and NB4-R2 (b) cells were shown. Similar results were obtained in three independent experiments. Differentiation was also evaluated by flow cytometric analysis of CD11b expression with the co-treatment of Gö 6976 and ATRA (c) or rottlerin and ATRA for 4 days (d). Each value represented the mean ± SD of three independent measurements. $##P < 0.01$, versus ATRA treated cells.

RARα.

Proceeding from the promyelocyte stage to granulocyte requires several transcription factors. CCAAT/enhancer binding protein β (C/EBPβ), C/EBPε and PU.1 (a member of the ets family) are critical to terminal granulocyte differentiation(Lekstrom-Himes, 2001). In addition, their expression could be induced by ATRA and they were all required for ATRA-mediated differentiation in APL cells(Duprez et al., 2003; Mueller et al., 2006; Park et al., 1999). As shown in Fig. 4, in both cell lines, the co-treatment for 48 h enhanced the protein level of PU.1 but not C/EBPε. Though ATRA treatment for 48 h elevated the protein level of C/EBPβ, mainly the short form in both cell lines, the combined treatment increased it more remarkably only in NB4-R1 cells (Fig. 4). Therefore, the protein level of PU.1 and C/EBPβ were increased with the combined treatment in NB4-R1 cells while only PU.1 was augmented with the co-treatment in NB4-R2 cells.

As mentioned above, UCN-01 was reported to activate MEK/ERK signaling pathway in several leukemia and myeloma cells(Dai et al., 2002, 2001). Moreover, MEK/ERK signaling pathway was essential to myeloid differentiation as well as ATRA-triggered granulocytic differentiation in APL cells(Miranda et al., 2005, 2002; Weng et al., 2016). The phosphorylated MEK and ERK1/2 were assessed by Western blot analysis to examine whether MEK/ERK signaling pathway was activated. The amount of phosphorylation of MEK and ERK1/2 was increased with the combined treatment as well as UCN-01 for 12 h in NB4-R1 cells (Fig. 4). In NB4-R2 cells, only the combined treatment for 36 h enhanced the phosphorylated MEK while the phosphorylated ERK was elevated with UCN-01/RA co-treatment for 40 h (Fig. 4). Therefore, the activation of MEK/ERK was earlier than the modulation of C/EBPβ and PU.1. Taken together, these results indicated that the up-regulation of C/EBPβ and/or PU.1 as well as the activation of MEK/ERK might mediate the combined treatment-induced differentiation.

3.4. The combined treatment induces differentiation via MEK/ERK modulated protein level of C/EBPβ and/or PU.1

To explore the role of MEK/ERK in the combined treatment-induced differentiation, both cell lines were pretreated with 0.5 μM U0126, a specific inhibitor of MEK. The effectiveness of U0126 was evaluated by ERK1/2 phosphorylation. U0126 did attenuate ERK1/2 activation in these two cell lines (Fig. 5a and b). U0126 suppressed cell differentiation triggered by the co-treatment in both cell lines as assessed by morphology (Fig. 5c) and CD11b expression (Fig. 5d–f). Moreover, in the presence of U0126, the combined treatment-enhanced the protein level of C/EBPβ and PU.1 in NB4-R1 cells as well as PU.1 in NB4-R2 cells was remarkably decreased (Fig. 5g and h). These results confirmed the critical role of MEK/ERK in the combined treatment-induced differentiation and also suggested PU.1 and C/EBPβ as the downstream targets of MEK/ERK.

3.5. RAF-1 is not required for the combination-activated MEK/ERK and differentiation

RAF-1 is a classical upstream regulator of MEK/ERK signal pathway. To further determine whether RAF-1 was required for the combined treatment-induced MEK/ERK activation and differentiation, cells were pretreated with 0.5 μM sorafenib tosylate, a specific inhibitor of RAF-1. To note, 0.5 μM was the maximum concentration of sorafenib tosylate with no obvious effects on survival when added to the combined treatment (data not shown). Unexpectedly, sorafenib tosylate alone activated MEK in both cell lines (Fig. 6a and b). Moreover, sorafenib tosylate could not reduce the combined treatment-induced phosphorylation of MEK in both cell lines (Fig. 6a and b). Meanwhile, as determined by morphology (Fig. 6c) and CD11b expression (Fig. 6d–f), the combined treatment-induced differentiation could not be suppressed by sorafenib tosylate in both cell lines. Therefore, the combined treatment activated MEK/ERK and induced differentiation might not depend on RAF-1.

4. Discussion

In this study, UCN-01 was demonstrated to synergize with ATRA to induce differentiation in ATRA-resistant APL cell lines to restore ATRA sensitivity. To the best of our knowledge, this is the first study to report the differentiation-enhancing effect of UCN-01. However, the efficacy of the combination on the primary cells from APL relapsed patients needs to be further confirmed.

UCN-01 is a derivative of staurosporine, the potent PKC inhibitor. The IC_{50} of UCN-01 to inhibit PKCα, -β, -γ, - δ, -ε is 29 nM, 34 nM, 30 nM, 590 nM and 530 nM. Thus, the high concentration used in this study might suppress PKCα, PKCβ and PKCγ. In further study, the other two selective PKC inhibitors, Gö 6976 and rottlerin, whose inhibition spectrum covered these PKC isoforms were used. However, neither could mimic the effect of UCN-01 on differentiation induction with the combination of ATRA. Therefore, PKC might not involve in the combination-induced differentiation. Since the IC_{50} value of UCN-01 was tested in purified enzyme, it was possible that much higher concentration were required to suppress PKC activity in the whole cell system. Moreover, the role of different PKC isoforms in granulocyte

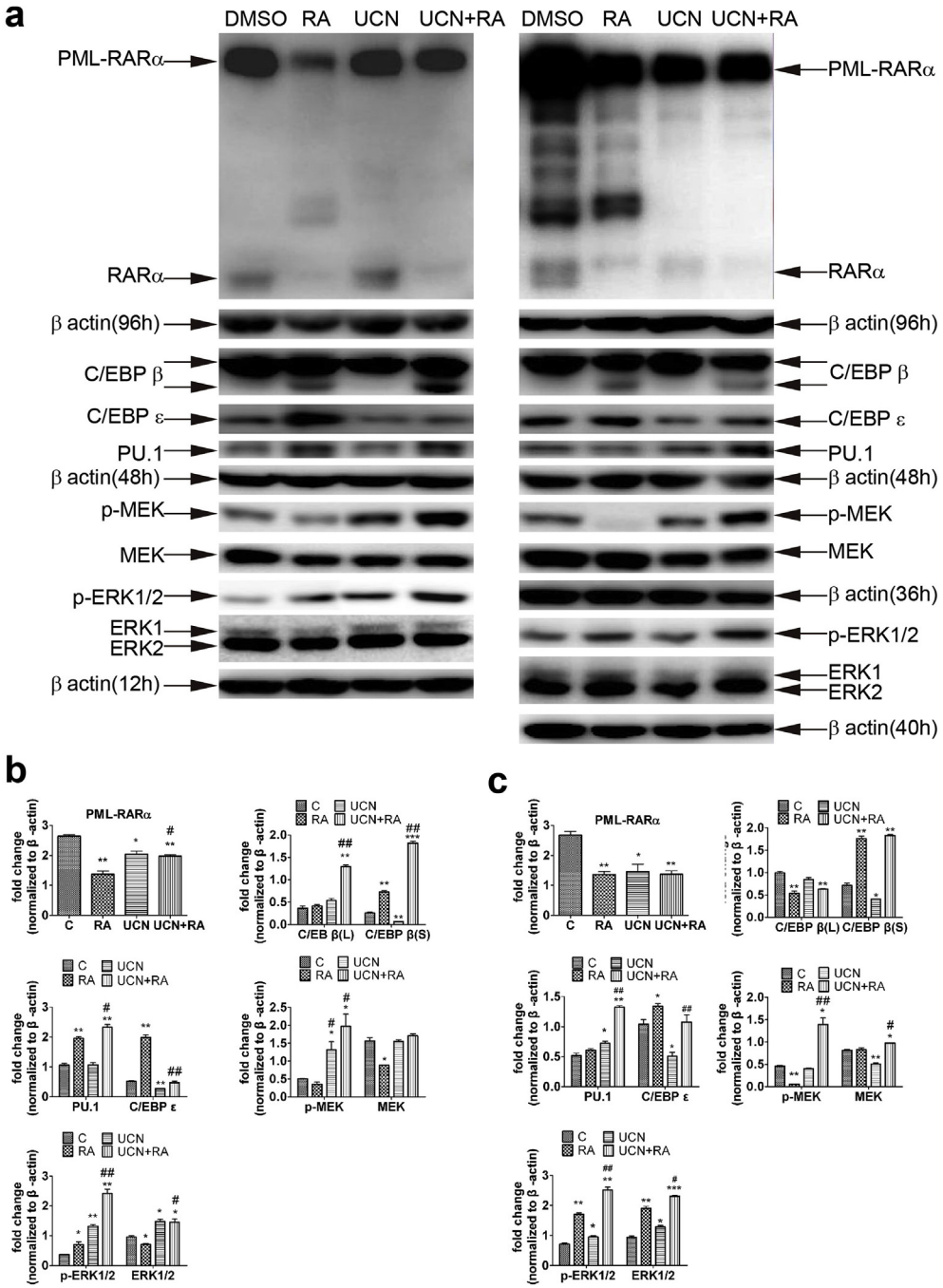

Fig. 4. The combined treatment enhanced the protein level of C/EBPβ and/or PU.1 and activated MEK/ERK. (a) NB4-R1 (left panel) and NB4-R2 (right panel) cells were treated with UCN-01 (UCN), ATRA (RA) alone and in combination (UCN + RA) for 12, 36, 40, 48 and 96 h. The same membrane incubated with the phosphorylated Erk1/2 or MEK1/2 was stripped and followed by detection of MEK and ERK1/2. Due to different time points for collecting protein, each time point has the corresponding expression of β-actin as internal control. Similar results were obtained in three independent experiments. Quantitated analysis of proteins in NB4-R1 cells (b) and NB4-R2 cells (c) with the indicated treatment. Values were expressed as protein/β-actin. The data were expressed as mean ± SD of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus DMSO treated cells. $\#P < 0.05$, $\#\#P < 0.01$, versus ATRA treated cells. C/EBP β(L) indicated the long form of C/EBP β while C/EBP β(S) indicated its short form.

differentiation is inconclusive. PKCα was suggested to negatively regulate neutrophil differentiation while whether activated PKCδ positively modulated ATRA-induced differentiation in APL cells was quite controversial(Devalia et al., 1992; Kambhampati et al., 2003; McNamara et al., 2010). Besides PKC isoform mentioned above, UCN-01 can also inhibit Chk1, Cdc25C-associated protein kinase 1 and Cdk1 with the IC_{50} lower than 100 nM. The role of these kinases in the combined treatment-induced differentiation remained to be surveyed.

PML-RARα is demonstrated to contribute to the pathogenesis of APL and both ATRA and ATO are successful PML-RARα targeted therapies for APL(Shen et al., 2004). However, in this work, ATRA treatment alone could not induce differentiation in both cell lines even the protein level of PML-RARα was decreased. Meanwhile, though the addition of UCN-01 inhibited or had no effect on ATRA-mediated reduction of PML-RARα, the combination still induced terminal differentiation. It indicated that the degradation of PML-RARα was not sufficient to promote differentiation and the combined treatment-triggered differentiation might be independent of PML-RARα destruction. Besides our study, PML-RARα degradation was also confirmed to be not required for differentiation induction by other agents combined with ATRA(Gao et al., 2010; Ge et al., 2015; Gianni' et al., 2001; Higuchi et al., 2004; Witcher et al., 2004).

To further investigate the mechanisms of the combined treatment-triggered differentiation, several proteins and signal pathway involved in ATRA-induced differentiation in APL cells were surveyed. MEK/ERK was activated and the protein level of PU.1 was augmented remarkably with the combined treatment in both cell lines. Besides PU.1, the combined treatment also significantly enhanced the protein level of C/EBPβ in NB4-R1 cells. Moreover, MEK specific inhibitor U0126 could suppress the combined treatment-induced differentiation and restore

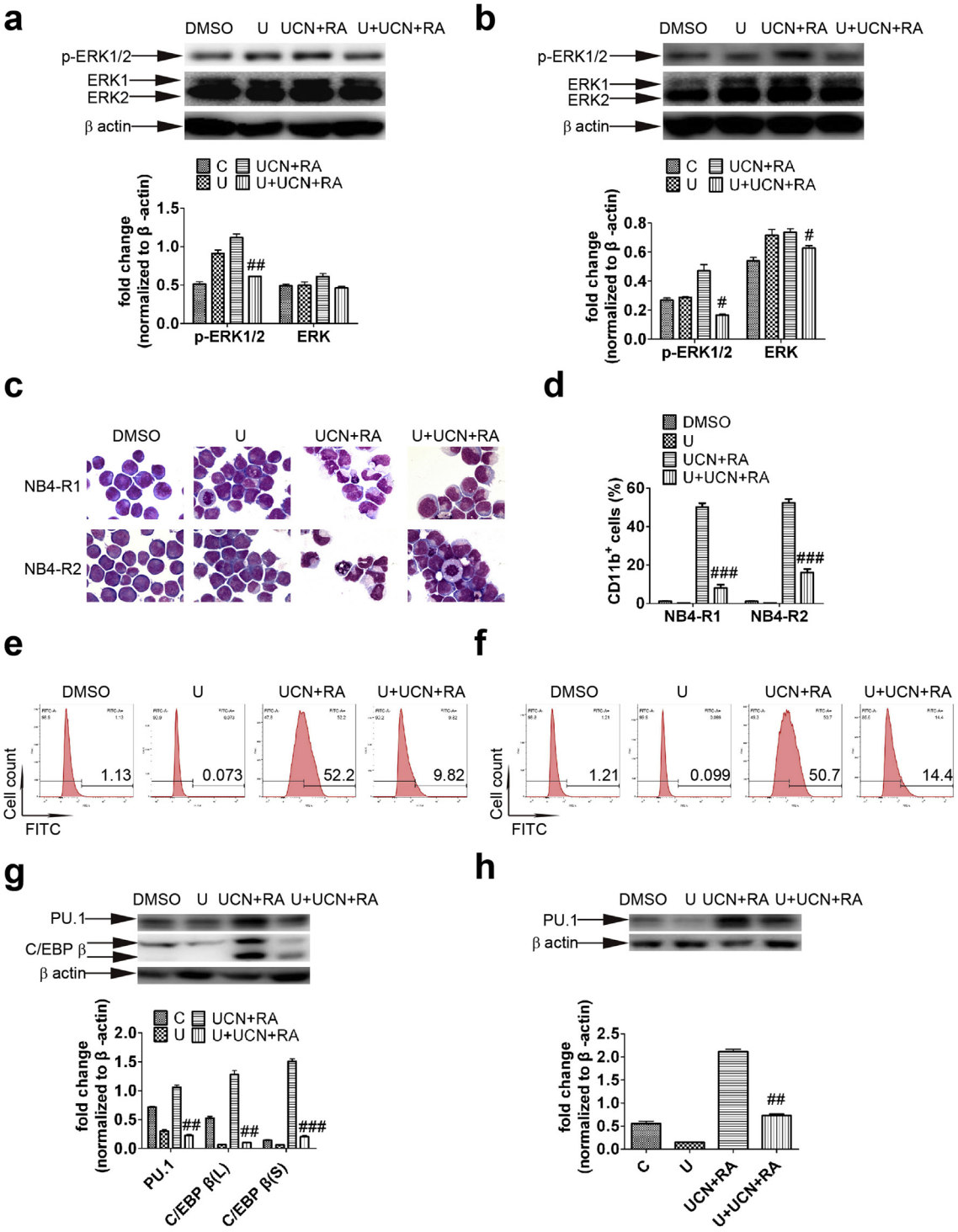

Fig. 5. Inhibition of MEK suppressed differentiation and restored the protein levels of C/EBPβ and/or PU.1. Cells were exposed to 0.5 μM U0126 for 1 h prior to other treatments. The attenuation of MEK activation by U0126 (U) was detected by Western blot analysis of phosphorylated ERK1/2 in NB4-R1 (a, upper panel) and NB4-R2 cells (b, upper panel) with the indicated treatments for 12 h and 40 h respectively. 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. Quantitated analysis of proteins in NB4-R1 cells (a, lower panel) and NB4-R2 cells (b, lower panel) with the indicated treatment. Values were expressed as protein/β-actin. The data were expressed as mean ± SD of three experiments. $\#P < 0.05$, $\#\#P < 0.01$, versus UCN-01 + ATRA treated cells. Inhibitory effect of U0126 on morphologic changes in NB4-R1 and NB4-R2 cells (c) incubated with the indicated drugs for 96 h. One representative experiment among three independent assays was shown. The inhibitory effect of U0126 on differentiation was also confirmed by flow cytometric analysis of CD11b expression in NB4-R1 and NB4-R2 cells (d) with the indicated drugs for 96 h. Each value represented the mean ± SD of three independent measurements. $\#\#\#P < 0.001$ versus UCN-01 + RA. The representative histograms of flow cytometric analysis of CD11b expression in NB4-R1 (e) and NB4-R2 cells (f) with the indicated drugs for 96 h were also shown. The percentages of CD11b positive cells were shown in the corresponding panels. The protein level of C/EBPβ and PU.1 in NB4-R1 (g, upper panel) as well as PU.1 in NB4-R2 (h, upper panel) cells with the indicated drugs for 48 h was determined by Western blot analysis. Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments. Quantitated analysis of proteins in NB4-R1 cells (g, lower panel) and NB4-R2 cells (h, lower panel) with the indicated treatment. Values were expressed as protein/β-actin. The data were expressed as mean ± SD of three experiments. $\#\#P < 0.01$, $\#\#\#P < 0.001$, versus UCN-01 + ATRA treated cells. C/EBP β(L) indicated the long form of C/EBP β while C/EBP β(S) indicated its short form.

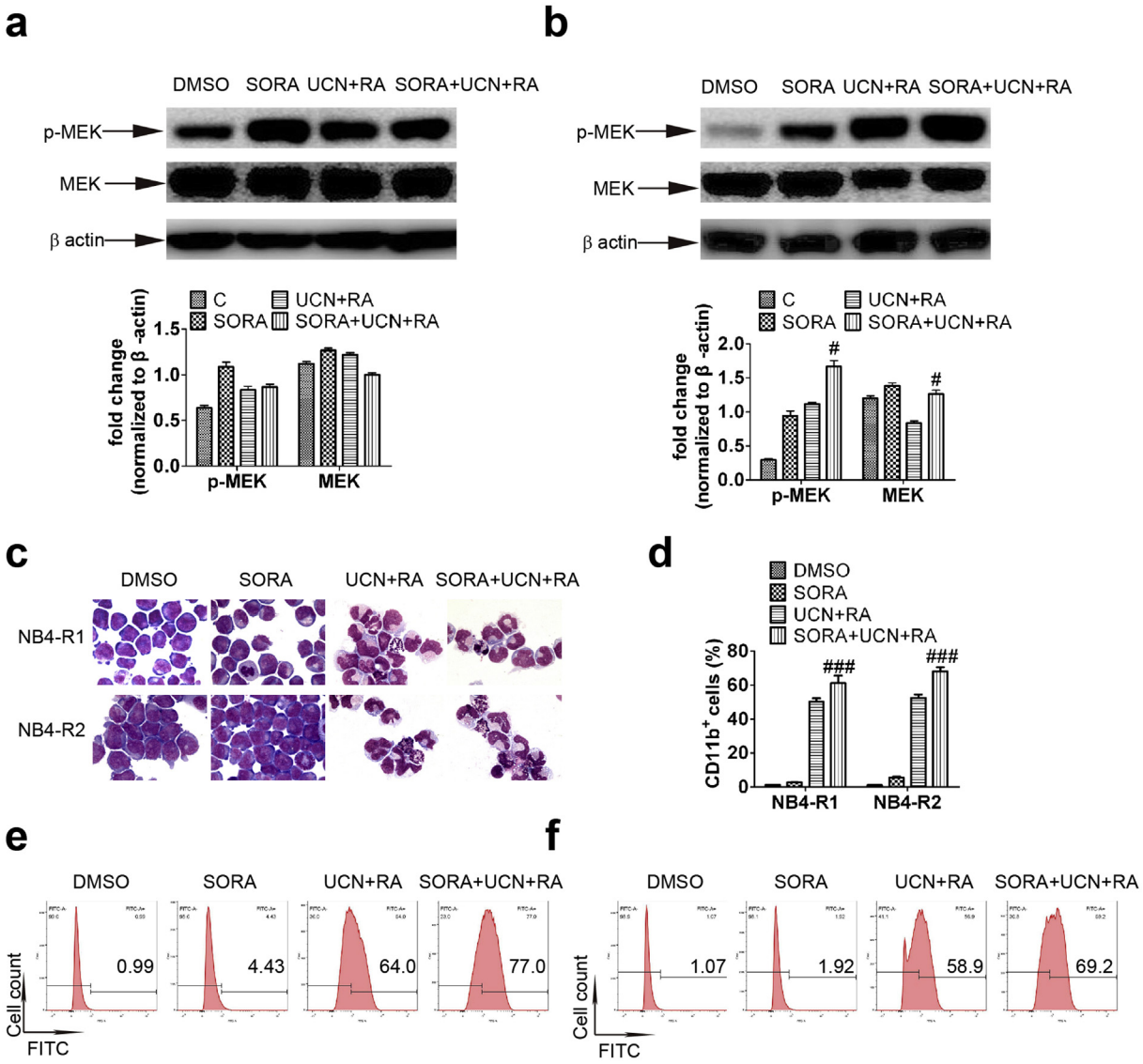

Fig. 6. The combined treatment-activated MEK/ERK signaling pathway and differentiation was independent of RAF-1. NB4-R1 and NB4-R2 cells were pretreated with 0.5 μM sorafenib tosylate (SORA) for 1 h. The effect of SORA on MEK activation was measured by phosphorylated MEK with the indicated treatments for 12 h and 36 h respectively in NB4-R1 (a, upper panel) and NB4-R2 cells (b, upper panel). Expression of β-actin was assessed as internal control. Similar results were obtained in three independent experiments. Quantitated analysis of proteins in NB4-R1 cells (a, lower panel) and NB4-R2 cells (b, lower panel) with the indicated treatment. Values were expressed as protein/β-actin. The data were expressed as mean ± SD of three experiments. $\#P < 0.05$ versus UCN-01 + ATRA treated cells. The effect of SORA on the combined treatment-induced differentiation for 96 h was observed by morphologic changes in NB4-R1 and NB4-R2 cells (c). One representative experiment among three independent assays was shown. The effect of SORA on the combined treatment-induced differentiation was also confirmed by flow cytometric analysis of CD11b expression in NB4-R1 and NB4-R2 cells (d). Each value represented the mean ± SD of three independent measurements. $\#\#\#P < 0.001$ versus UCN-01 + RA. The representative histograms of flow cytometric analysis of CD11b expression in NB4-R1 (e) and NB4-R2 cells (f) with the indicated drugs for 96 h were also shown. The percentages of CD11b positive cells were shown in the corresponding panels.

the protein level of C/EBPβ and/or PU.1. Thus, it indicated that the combined treatment-induced differentiation was via MEK/ERK modulated the protein level of C/EBPβ and/or PU.1. Though the mechanisms of how MEK/ERK regulated the expression of C/EBPβ and PU.1 are remained to be elucidated, MEK/ERK-induced expression of C/EBPβ or PU.1 was also reported in a few studies(Belmonte et al., 2001; Hu et al., 2001; Kusuyama et al., 2016; Lu et al., 2013; Salmenpera et al., 2002; Zhang et al., 2011). As a transcription factor, many of PU.1 targeted genes, such as *CD11b, the microtubule-associated protein 1S, glycolytic enzyme hexokinase 3, the granulocyte/macrophage colonystimulating factor receptor, myeloperoxidase, lysozyme* and *neutrophil elastase* directly modify myeloid differentiation(Federzoni et al., 2012; Haimovici et al., 2014; Kastner and Chan, 2008). In this study, PU.1 was enhanced in both cell lines also highlights its important role in granulocyte differentiation. Further study excluded RAF-1 as the upstream molecule of MEK/ERK since RAF-1 inhibitor failed to suppress the activation of MEK and differentiation. Thus, the combined treatment induced terminal granulocytic differentiation by RAF-1 independent MEK/ERK activation. The activation of MEK/ERK via RAF-1 independent manner is not rare(Cerioni et al., 2003; Faure and Bourne, 1995; Kartha et al., 1999; Lin et al., 2001). Besides RAF-1, protein kinase A (PKA), PKC, phosphatidylinositol-3 kinase (PI3K) and MEK kinase (MEKK) are known upstream kinases to modulate MEK activity(Dajas-Bailador et al., 2002; Winston et al., 1995). The exact upstream regulator of MEK in the combined treatment remains to be identified.

5. Conclusions

UCN-01, at clinical achievable concentrations, synergizes with ATRA to induce terminal granulocytic differentiation in ATRA-resistant APL cell lines. RAF-1 independent MEK/ERK cascade mediates the protein level of C/EBPβ and/or PU.1 and leads to the differentiation. These findings may provide a preclinical rationale for the potential role of this combination in the treatment of ATRA-resistant APL patients.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.02.033.

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