Potentiation of Acute Promyelocytic Leukemia Cell Differentiation and Prevention of Leukemia Development in Mice by Oleanolic Acid

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Abstract. *Although differentiation therapy with all-trans retinoic acid (ATRA) induces complete remission in most acute promyelocytic leukemia (APL) patients, it is associated with organ toxicity. The present study focused on investigating the effects of the natural compounds oleanolic acid (OA) and ursolic acid (UA) on proliferation and differentiation of human APL HL-60 cells in vitro and murine APL WEHI-3 cells in vivo. Results demonstrated that OA and UA significantly inhibited cellular proliferation of HL-60 in a concentration- and time-dependent manner. Non-cytotoxic concentration of OA exhibited a marked differentiation-inducing effect on HL-60 and enhanced ATRA-induced HL-60 differentiation. In contrast, UA showed only a moderate effect. Activation of MAPK/NF-κB signaling pathway was likely found to be involved in the mechanism. Moreover, OA increased survival duration of WEHI-3 transplanted BALB/c mice, and decreased leukemia cells infiltration in the liver and spleen. Thus, these results may provide new insight for developing alternative therapy in APL patients.*

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Acute promyelocytic leukemia (APL) is a sub-type of acute myeloid leukemia, a human cancer of white blood cells in which malignant proliferative leukemic cells fail to terminally differentiate and accumulate in blood and bone marrow. It is indicated by a t(15;17)(q22;q12) chromosomal translocation, leading to generation of PML-RARα fusion protein. APL remains a significant health risk in the general population with rising incidence in aging adults with overal early death rate of 24.2 % (1). Recent approaches in the treatment of APL include the induction of terminal differentiation, as cells subjected to differentiation inducers obtain phenotypic characteristics of end-stage adult cell forms with unreplicative ability and eventually enter pathways of programmed cell death. A variety of agents to induce terminal differentiation of leukemic cells has been effectively used and significantly improved prognosis for patients with APL, indicating that the malignant state is reversible (2).

In the presence of 1,25-dihydroxyvitamin D_3 or all-*trans* retinoic acid (ATRA), HL-60 are differentiated into cells of monocytic lineage or granulocytic lineage, respectively (3, 4). ATRA has been used as an effective therapeutic agent for APL and was reported to induce complete remission in almost all APL patients (5). ATRA induced cell differentiation by activating MAPK pathway and increased levels of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K) (6, 7). At the level of gene transcription, ATRA induced degradation of the PML-RARA oncoprotein, that enables dissociation of the co-repressor complex (CoR) and association of co-activators complex (CoA). As a result,

the chromatin structure became relaxed, allowing for transcriptional repression and terminal differentiation of APL cells into granulocytes (8). Despite its effectiveness, continuous treatment with ATRA causes significant treatment-related toxicity, hypercalcemia (5), and progressive reduction in plasma drug concentration, leading to ATRA resistance (9). To overcome these side-effects, combination treatment of ATRA with chemotherapy has been suggested.

Bioactive triterpenoids have been widely discussed regarding their anticancer potential in both *in vitro* and *in vivo* models (10). Oleanolic acid (OA, 3β-hydroxy-olean-12-en-28-oic-acid) and its isomer ursolic acid (UA, 3β-hydroxy-urs-12-en-28-oic-acid) are bioactive pentacyclic triterpenoids, widely distributed in medicinal herbs and plants used in the human diet. UA and OA are effective in inhibiting angiogenesis, invasion of tumor cells, and metastases (11, 12); however, its usefulness in APL treatment has only been recently suggested.

The present study was designed to investigate the anti-proliferation, differentiation, induction and differentiation-enhancing potential of OA and UA on HL-60. Furthermore, we evaluated the leukemia-preventive effects of OA in BALB/c mice.

Materials and Methods

Chemicals. OA, UA, ATRA and nitroblue tetrazolium (NBT) were obtained from Sigma Chemical (St. Louis, MO, USA). RPMI-1640 Medium, Iscove's Modified Dulbecco's Medium (IMDM), L-glutamine were obtained from GIBCO (Carlsbad, CA, USA). Stock solutions of OA, UA and ATRA were prepared in DMSO with at least 1,000-fold dilution in medium to ensure that the final concentration of DMSO had no effect on cells. The inhibitors PD98059, SB203580, SP600125 and Bay11-7082 were obtained from Cell Signaling Technologies (Beverly, MA, USA).

Cell culture and cell proliferation analysis. Human acute promyeloid leukemia HL-60 and WEHI-3 promyelocytic mice leukemia cells were cultured in RPMI-1640 medium and IMDM, respectively. Both media were supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. Cell proliferation and viability were assessed by using a hemocytometer following exclusion assays by trypan blue. The blue-stained cells were considered as non-viable.

NBT cell differentiation assay. NBT differentiation assay of leukemic cells was assessed using the NBT reduction test. Briefly, cells were cultured in 24-well culture plate containing 2×10^5/ml cells with different concentrations of compounds for 24 to 72 h. 1% NBT dissolved in PBS containing 200 ng/ml phorbol 12-myristate 13-acetate (PMA) followed by incubation for 1 h. Cell differentiation was observed by preparing cytospin slides an examining blue-black intracellular formazan deposits, indicative of a PMA-stimulated respiratory burst (NBT positive cells).

Effect of specific inhibitors on cell differentiation and western blot analysis. HL-60 were pre-treated with 20 μM specific MAPK

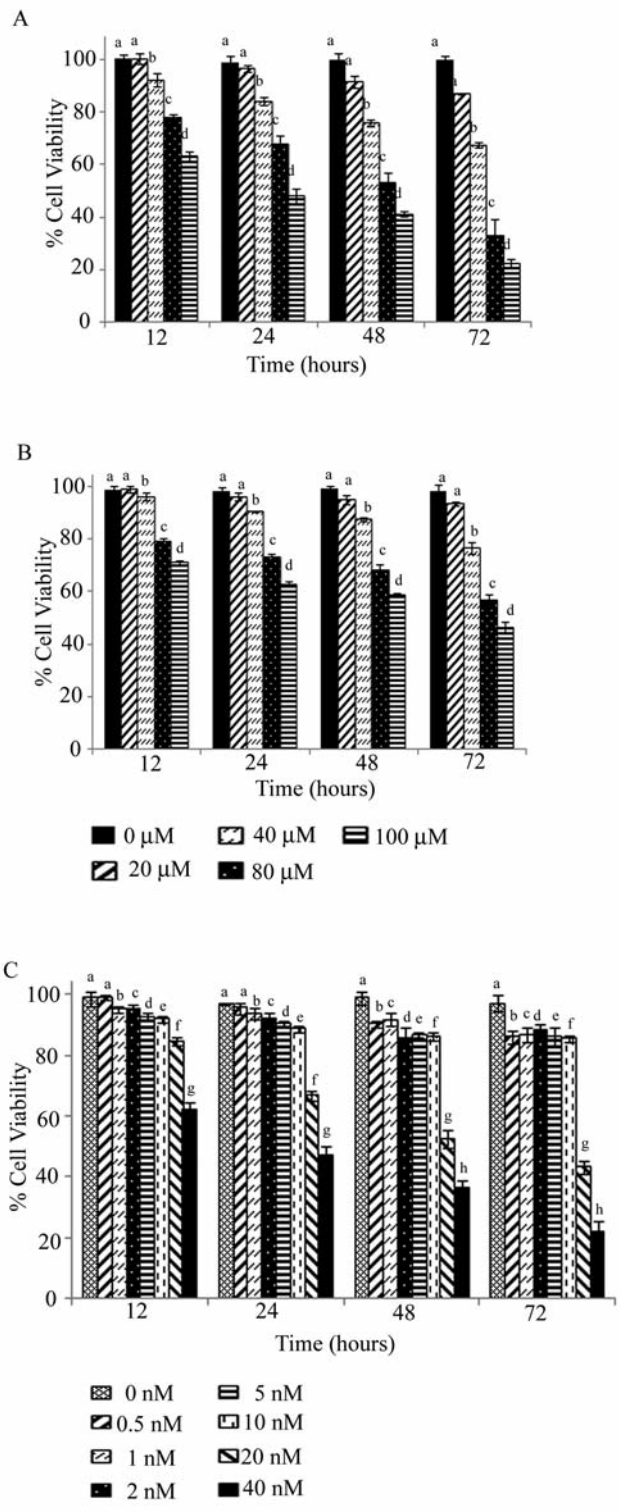

Figure 1. *Effects of OA, UA, and ATRA on HL-60 cell proliferation. (A) % Cell viability in the absence and presence of different concentrations of OA treated for 0-72 h; (B) UA treated for 0-72 h and (C) ATRA treated for 0-72 h. Viable cells were counted using trypan blue staining. Data represent mean±SD from three independent experiments.* $^{a\text{-}h}p<0.05$ *significantly different compared to 0 μM of each exposure time.*

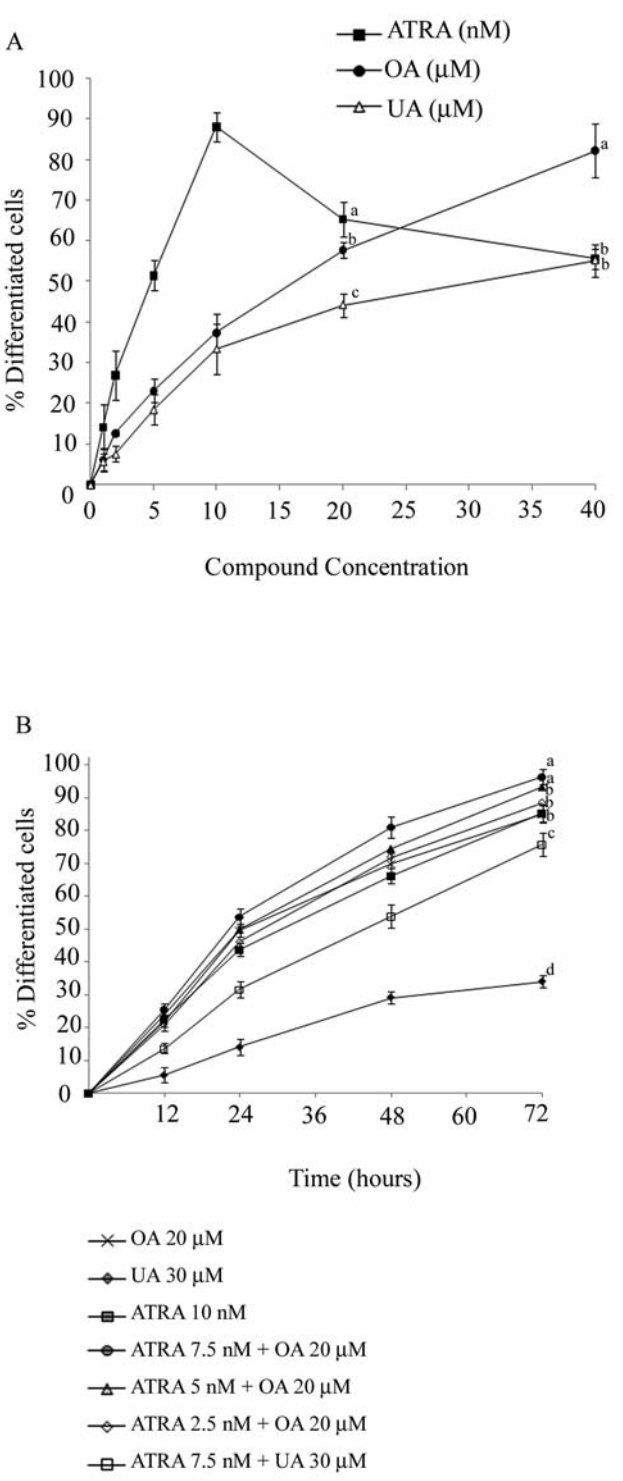

Figure 2. *Differentiation-inducing effect of OA and UA on HL-60. (A) Cells were treated for 72 h with increasing concentrations of OA and UA and ATRA for comparison. (B) Cells were treated with compounds and 10 nM ATRA alone or in combination with decreasing concentrations of ATRA for various time periods and performed NBT reduction assay. Each value represents mean±s.d. of three independent experiments.* $^{a\text{-}e}p<0.05$*, relative to group treated with ATRA alone.*

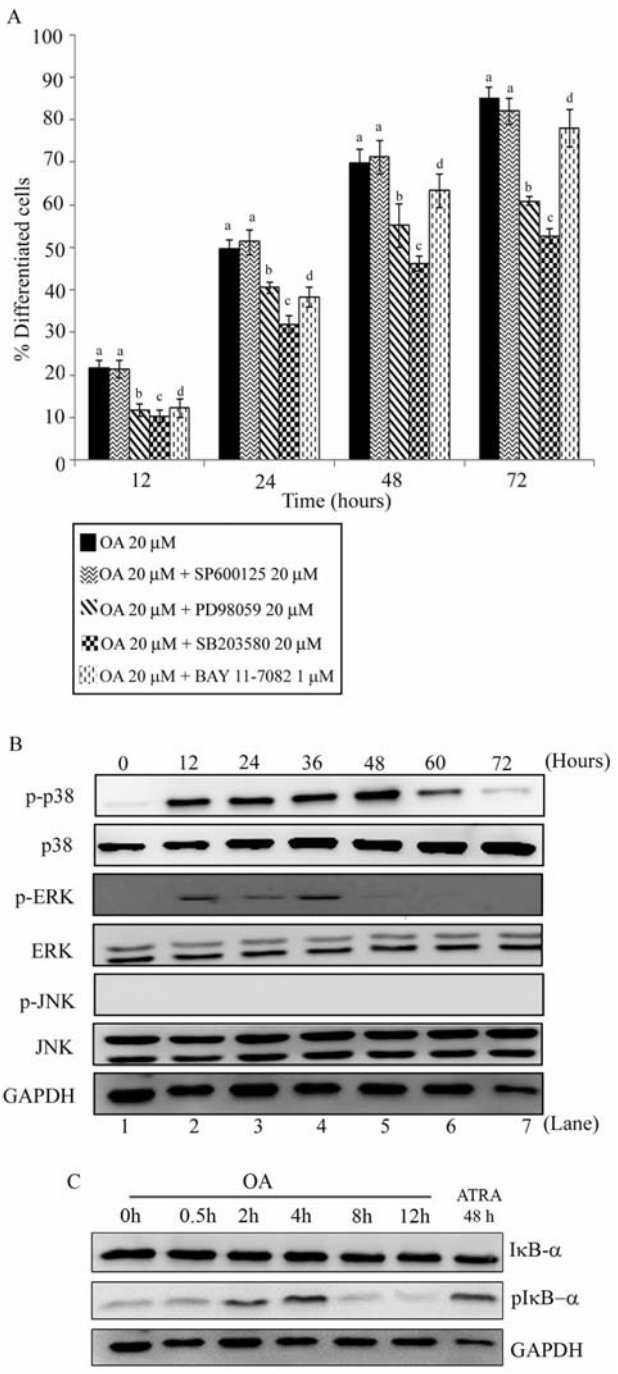

Figure 3. *OA affects MAPK/NF-κB signaling in HL-60. (A) Effects of specific inhibitors on OA-induced HL-60 cell differentiation. Cells were pre-cultured with indicated inhibitors, following treatment with OA for 72 hand NBT assay. Data represent mean±SD derived from three independent experiments.* $^{a\text{-}d}p<0.05$ *compared to group treated with OA alone. (B) Time-dependent analysis of JNK, p-38 and ERK phosphorylation were determined by western blot at various timing after the addition of 20 μM OA (C) Time dependent analysis of IκB-α and IκB-α phosphorylation by western blot after addition of 20 μM OA, ATRA were used as a positive control. Experiments were repeated at least three times producing similar results.*

Table I. *Symptoms and survival duration observed in WEHI-3 leukemic BALB/c mice treated with OA 50 mg/kg b.w.*

Treatment	Dose	(D/T)	Survival duration	Symptoms
Corn Oil	(Compound Vehicle)	0/5	30 days	None
OA	50 (mg/kg b.w)	0/5	30 days	Sedation, piloerection, convulsion
WEHI-3	1×10^6 cells/ 0.2 ml	5/5	5-7 days	Sedation, piloerection, convulsion, drastically decrease in food and water intake
WEHI-3 + OA and 50 (mg/kg b.w)	1×10^6 cells/ 0.2 ml	2/5	12-13 days	Sedation, piloerection, convulsion, drastically decrease in food and water intake

D/T: Number of dead mice/ number of treated mice.

inhibitors for 6 h or 1 μM NF-κB inhibitor BAY 11-7082 for 2 h followed by incubation with 20 μM OA for 24 h to 72 h. Afterwards, the degree of cell differentiation was assessed by the NBT reduction assay. Cell lysates for western blot analysis was prepared by treating HL-60 with OA (20 μM) for 12-72 h prior to harvesting. The procedures of western blotting was described in the literature (13).

Measurement of in vivo anti-leukemic activity in BALB/c mice. All animal experiments were performed according to the approved Institutional Animal Care and Use committee. Twenty 6-week-old male and female BALB/c mice (BioLASCO Laboratories Co., Ltd, Taipei, Taiwan) were randomized into 4 groups. Group I was the control and received intraperitoneal injection (*i.p.*) of corn oil (0.1 ml, compound vehicle) on alternate days for 30 days; Group II received 50 mg/kg b.w. OA in 0.1 ml corn oil on alternate days for 30 days; Group III received WEHI-3 only, WEHI-3 were transplanted to BALB/c mice intravenously (*i.v.*) with 1×10^6 cells/ 0.2 ml PBS on day 15 of the experiment; Group IV was received *i.p.* of 50 mg/kg b.w, based on our previous publication (14). OA in 0.1 ml corn oil every second day for two weeks prior WEHI-3 transplantation. OA treatments were continued on alternate days after transplantation, up to day 30 of the experiment. At the end of the experiment healthy and moribund mice were euthanized.

Observation of general behavior and mortality rate. Within 24 h, mice were assessed to any alteration of general behavior including convulsions, disorientation, weakness, hyperventilation, and salivation. Mortality rate was continuously monitored three times every day until 30 days after inoculation was completed. The external appearance of mortal mice and appearance of the viscera, heart, lungs, stomach, intestine, kidney, testis, and spleen were carefully monitored and any apparent deviation in features were recorded. Further, body weights and food intake were recorded on alternating days to monitor significant changes.

Histopathological study. At the end of the study, all mice were dissected to obtain their spleens, livers, heart and kidneys. Organs were than separately weighed and fixed in 10% buffered formalin. Pathological examination was done by haematoxylin and eosin with spleen and liver. All histopathological evaluations were carried-out in a double-blind manner by a professional pathologist from the Animal Disease Diagnostic Center, National Pingtung University of Science and Technology.

Statistical analysis. Results are presented as means±SEM and one-way ANOVA in SPSS v.14.0 (SPSS Inc., Chicago, IL, USA) was applied to the variables that could contribute to find significant effects on HL-60 growth and differentiation. Statistical significance at $p\leq0.05$ was considered as the level that would be significant. Tukey–HSD post-hoc analysis was conducted following ANOVA with $p\leq0.05$.

Results

Inhibition of HL-60 cell proliferation by OA and UA. On most occasions, cell differentiation is accompanied with withdrawal from the cell cycle, resulting in inhibition of proliferation (15, 16). Therefore, in the present study we initially determined the cell proliferation and viability of human HL-60 after treatment with OA, UA (0-100 μM), and ATRA (0-40 nM) for 0-72 h incubation period. Data clearly showed that OA and UA significantly inhibited HL-60 proliferation in a concentration- and time-dependent manner within 0-72 h after treatment (Figure 1A and B). Higher numbers of non-viable cells were noted in cells cultured at high OA and UA concentrations with doses ranging from 80-100 μM. As shown in Figure 1C, the exposure of 20 to 40 nM ATRA to HL-60 also resulted in a dose- and time-dependent increase in numbers of non-viable cells. Calculation of the IC_{50} using the linear regression equation, resulted in 62 μM, 93 μM, and 23 nM for OA, UA, and ATRA respectively (72-h treatment). However, lower concentrations of OA (20 μM), UA (30 μM), and ATRA (10 nM), corresponding to >90% cell survival, were chosen for treatment in further investigations.

Induction of cell differentiation in HL-60 by OA and UA at non-toxic concentration. Cells were exposed to increasing concentrations of compounds from 0 to 72 h and NBT cell differentiation assays were performed. Results demonstrated that OA (0-40 μM) showed a significant ($p<0.05$) dose-dependent differentiation-inducing effect on HL-60 cell compared to UA (0-40 μM) (Figure 2A). Increasing concentrations of ATRA (0-40 nM) were also investigated for comparison. Results indicated that 10 nM ATRA induced highest % differentiated cells (88%±4%). On the contrary, higher concentration of ATRA ranging from 20 nM to 40 nM, lowered the percentage of differentiated cells. This

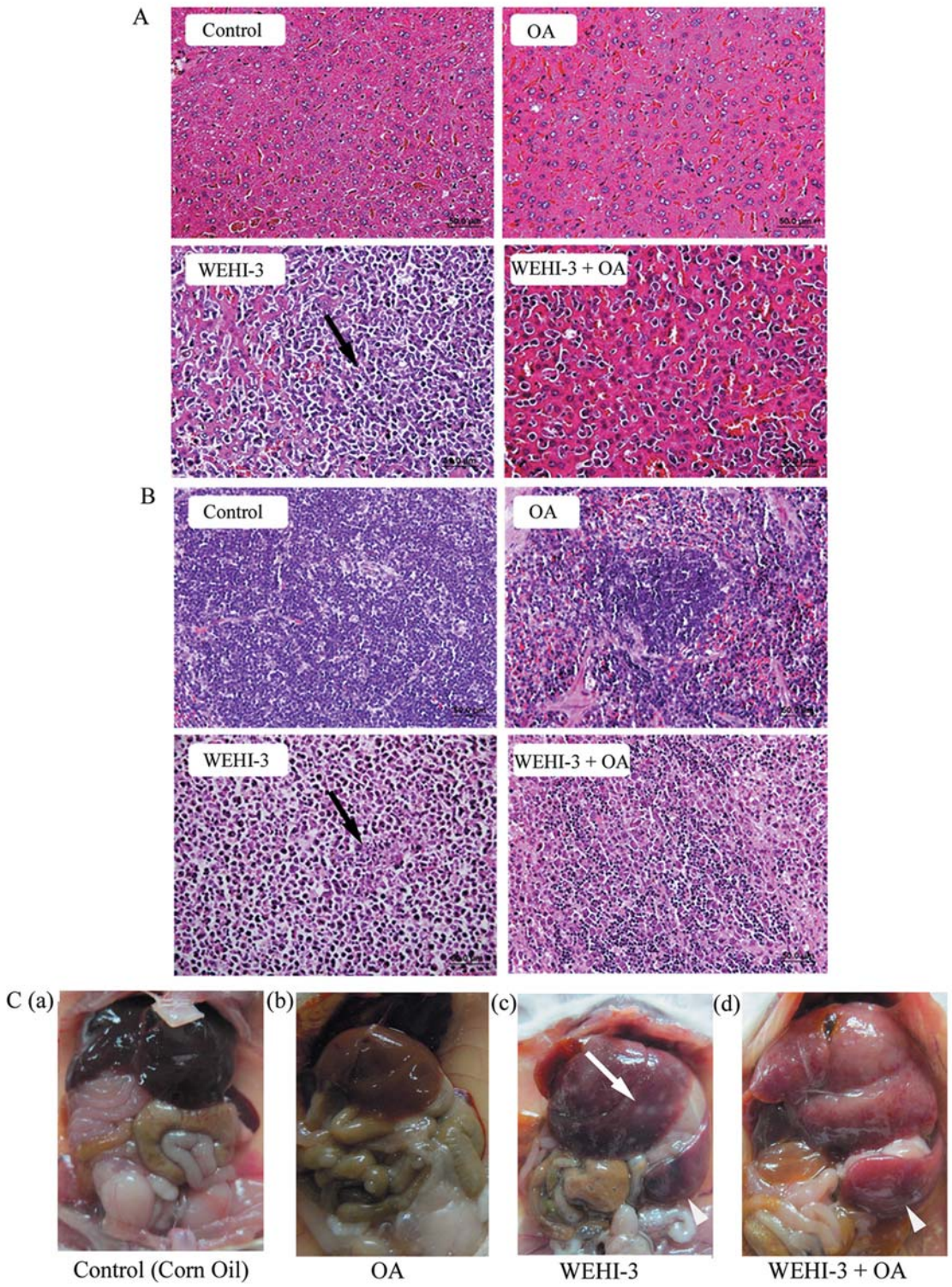

Figure 4. *Histopathological and organ examination. (A) Histopathology of liver and (B) spleen tissues on OA-treated leukemic BALB/c mice. Animals were initially treated i.p with OA on alternate days for 8 times, then injected i.v with WEHI-3 cells in PBS. Dissected leukemic mice and hematoxylin-eosin stain for the paraffin sections of spleens and liver from OA-treated and un-treated leukemic mice, as described in Materials and Methods. Arrow indicates infiltration of immature myeloblastic cellsinto red pulp of the spleen. (C) Demonstration of BALB/c mice organ condition after treatment with (a) corn oil control, dissected in the end of experiment (b) injected i.p with 50 mg/kg b.w. OA in 0.1 ml corn oil on alternate days, dissected in the end of experiment, (c) i.v injected with WEHI-3 cells dissected after 5 survival days, and (d) injected with OA and WEHI-3 cell dissected after 12 days. Arrow indicates splenomegaly and white dot leukemia cells aggregates.*

is most likely due to the decrease of number of viable cells when HL-60 were treated with higher concentrations of ATRA. Given the fact that 20 μM OA remarkably induced the differentiation of HL-60 without cytotoxicity, our attention turned to investigating the enhancing effect of OA on ATRA-induced cell differentiation. To achieve this, HL-60 were treated with 20 μM OA in combination with increasing non-toxic concentration of ATRA (0-10 nM). Interestingly, our results suggested that the addition of OA to cultures exposed to increasing concentrations of ATRA resulted in marked time-dependent percentage of NBT-positive cells. As shown in Figure 2B, the addition of 20 μM OA to 2.5 nM, 5 nM, and 7.5 nM ATRA resulted in a significant ($p<0.05$) enhancement of differentiated HL-60 (89%, 93%, and 96% differentiated cells, respectively) compared to cultures exposed to a sub-optimal concentration of ATRA (10 nM), which by itself caused a relatively lower level of cell differentiation (85% differentiated cells) after 72 h treatment. In contrast, addition of 30 μM UA to 7.5 nM ATRA had no enhancing effect on HL-60 cell differentiation (72% differentiated cells).

Involvement of specific MAPKs and NF-κB in OA-induced HL-60 cell differentiation. Mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal protein kinase (JNK), and p38 kinase, played an important role in the regulation of cell proliferation, differentiation and survival (17). Thus, investigating the constituents of this pathway may be beneficial when considering chemotherapeutic options for treating various neoplasias (18). First of all, specific MAPK inhibitors, including p38 MAPK inhibitor SB203580, ERK inhibitor PD98059, and JNK inhibitor SP600125 were employed. HL-60 was pre-treated with non-toxic concentrations of individual inhibitor, then the degree of cell differentiation was assessed. We found that the presence of SB203580 and PD98059 resulted in time-dependent reduction of OA-induced cell differentiation. In contrast, addition of the JNK-specific inhibitor SP600125, did not significantly affect OA-induced HL-60 cell differentiation (Figure 3A). In addition, one of the well-studied downstream components of the MAPK signaling pathway is the nuclear transcription factor-kappa B (NF-κB) (19). To define the function of NF-κB in HL-60 cell differentiation induced by OA, cells were treated with 1 μM BAY 11-7082, a pharmacological inhibitor of IκBα phosphorylation and NF-κB, 2 h before cell differentiation, and maintained up to 72 h after OA treatment. Our results demonstrated that treatment with BAY 11-7082 resulted in a significant time-dependent reduction of OA-induced HL-60 cell differentiation (Figure 3A, $p<0.05$), indicating that enhancement of OA-induced HL-60 differentiation is closely related to activation of NF-κB. However, the differentiation inhibition pattern of BAY 11-7082 differs from that of MAPK inhibitors. As shown in Figure 3A, BAY 11-7082 plays a role at the early stage of cell differentiation (0-24 h), whereas MAPK maintained its activity up to later times (0-72 h).

Activation of p38, ERK and NF-κB upon OA treatment. To further confirm the involvement of p38, ERK and NF-κB in mediating OA-induced HL-60 cell differentiation, western blot analysis was performed to visualize phosphorylation levels. Figure 3B demonstrated that OA promoted p38 and ERK phosphorylation in a time-dependent manner, suggesting that activation of this signaling cascade is essential for the anti-leukemic effects of OA. Conversely, JNK was not phosphorylated between 12 h and 72 h of treatment with OA. Moreover, we investigated the expression of IκB-α and of its phosphorylated form at different times after addition of 20 μM OA. Phosphorylation of IκB-α is required for NF-κB dimers to translocate to the nucleus and regulate transcription (20). As a positive control, HL-60 were also treated with 20 nM ATRA for 48 h. ATRA has been reported to be closely linked to activation of NF-κB in HL-60 (21). Consistent with previous observations on the involvement of NF-κB inhibitor BAY-7082, our results demonstrated that OA induced IκB-α phosphorylation (Figure 3C). Importantly, we found that ATRA induced phosphorylation of IκB-α at a later time than OA (48 h). This highlights that the differentiation-inducing potential of OA on HL-60 and NF-κB activation is essential for its activity. Several reports have demonstrated the interference of NF-κB activation as a common feature of compounds that enhance differentiation in HL-60 (22).

Leukemia prevention by OA in a mouse model. Since *in vitro* experiments showed that OA remarkably inhibits HL-60 cell proliferation and induces differentiation, it is necessary to further demonstrate its activity in an *in vivo* model. As shown on Table I, control groups given corn oil and treated with OA remained alive until the end of the experiment. In contrast, mice transplanted with WEHI-3 resulted in 40% mortality as soon as 5 days after injection, and remaining mice followed 7 days after *i.v.* injection leading to 100% mortality of the group. Whereas treatment with 50 mg/kg OA increased mice survival rate up to 12 to 13 days. Histopathological data of spleen and liver are shown on in Figure 4A and B. The arrow indicates in the spleen tissue a marked infiltration of immature myeloblastic cells. Expansion in red pulp and white pulp showed significant change. Neoplastic cells contained large irregular nuclei accompanied with clumped chromatin and prominent nucleoli and abundant clear and light eosinophilic cytoplasm. In addition to microscopical examination, we also examined pathological abnormalities during dissection. Enlarged spleen, lung and liver of WEHI-3 transplanted BALB/c mice were notable characteristics of leukemic mice. The mentioned organs were weighted and presented in Table II. Figure 4C shows

Table II. *Body and organ weight of WEHI-3 transplanted BALB/c mice after treatment with OA for up to 30 days.*

Parameters	Groups			
	Corn oil	OA	WEHI-3	WEHI-3 + OA
Weight (g)				
Body initial	20.18±2.87	19.78±2.12	22.24±2.32	21.93±1.98
Body final	22.95±2.13	22.24±1.89	19.11±1.59	19.89±1.23
Change (%)	+13.73	+12.29	–14.07	–9.30
Liver	1.09±0.18	1.35±0.14	1.83±0.23	1.58±0.06*
Spleen	0.09±0.03	0.11±0.03	0.42±0.08	0.41±0.07
Lung	0.14±0.03	0.24±0.03	0.46±0.05	0.34±0.03*
Heart	0.20±0.01	0.18±0.01	0.19±0.01	0.20±0.01
Kidney	0.39±0.01	0.33±0.03	0.31±0.02	0.30±0.02
Food (g/days)	3.3±0.46	3.26±0.48	2.53±0.53	2.21±0.33
Water (ml/days)	4.2±1.2	5.3±1.9	4.1±1.1	4.2±1.9

Each point is mean±S.D. (n=5). *$p<0.05$ indicates significant difference by Tukey's HSD test between the WEHI-3 leukemic mice and OA-treated experimental group.

representative results of organs of each mice group, as described in the experimental section. Arrow indicates liver damage and splenomegaly as soon as 5 days post-injection.

Discussion

OA as well as UA are well-known regarding their inhibition of tumor promotion. In recent studies, several lines of evidence have demonstrated that OA, UA and its derivatives have significant anti-tumor effects and marked cytotoxic activity towards many cancer cells both *in vitro* and *in vivo* (23, 24). We demonstrated that both OA and UA were able to inhibit leukemic cell proliferation, although the effectiveness of OA is greater than that of UA. Despite the fact that greater effectiveness of the modified synthetics derivatives has been reported, our study demonstrated that the natural form of OA also shows a significant inhibitory effect on cancer cell proliferation, which is in accordance with previous findings (25). However, the mechanism of inhibition of triterpenoids against cancer cells might be complicated and not easily understood. It was shown that OA acid and its derivatives contribute to anticancer effects by apoptosis induction and modulating the tumor environment by anti-inflammatory activities (26). NF-κB, a key transcription factor involved in inflammation is commonly overexpressed in cancer cells thereby suppressing apoptosis of tumor cells and maintaining a chronically inflamed microenvironment beneficial for cancer (27). Furthermore, OA and its derivatives target cancer cells by inhibition of DNA topoisomerase I (28), multidrug resistance-associated protein (MRP1) activities (23), and angiogenesis (29), but it does not provide explanation for involvement of cell differentiation.

Herein, we report for the first time, the ability of OA and UA to induce differentiation and enhance the differentiation signal exerted by a low concentration of ATRA. HL-60 were markedly differentiated when treated with OA, and showed a moderate-inducing effect when treated with UA. In combination with low dose of ATRA, OA cooperatively enhanced the percentage of differentiated HL-60 cells, in contrasts to UA that did not significantly affect the differentiation level exerted by ATRA. On the basis of our findings, we suggest that OA may be used as part of initial screening for potential synergistic anti-leukemic agents with ATRA in an attempt to decrease the side-effects related to the use of this well-established differential-therapy agent.

The induction of HL-60 cell differentiation requires activation of a variety of signal transduction systems such as MAPK pathways (7). Previous studies demonstrated that the MEK/ERK MAP kinase pathways are involved in PMA-, ATRA- and G-CSF-induced myeloid differentiation (30). Treatment with sesquiterpene lactones in combination 1,25-$(OH)_2D_3$ were also reported to induce monocytic differentiation of HL-60 through PKC and ERK pathways (31). In the present study, we illustrate that OA possesses significant inducing and enhancing effects on HL-60 cell differentiation through activation of ERK and p38 phosphorylation, with a time-dependent increase in protein levels. Although defining the mechanism of action requires further in-depth analyses, OA markedly induced HL-60 cell differentiation by activating MAPK pathway and enhanced the percentage of differentiated cells in ATRA-induced HL-60 cell differentiation. However, the precise role of MAPK signaling in this enhancement should be further investigated.

By employing the above *in vivo* model, it was shown that OA increased the survival duration and decreased the number of leukemia cells that infiltrated into the liver and spleen in leukemic mice. In conclusion, our results provide further evidence that OA might be a promising candidate or adjunct in the treatment of APL. When used in combination with ATRA, OA may exert a greater therapeutic response than ATRA alone and possibly produce less ATRA treatment-related side-effects.

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