Retraction Notice

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This article was published online on April 26, 2016 in *Integrative Cancer Therapies*, and its DOI is 10.1177/1534735416635275. The authors wish to retract this article because the third author made mistakes in calculation of the concentration of the compounds used in experiments shown in Tables 1 and 2. This mistake affected the accuracy of data shown in these tables, the reproducibility of data, and weakened the reported conclusion. Therefore, all authors agreed to retract this article. This request has been approved by the editorial staff of *Integrative Cancer Therapies*. 
RETRACTED: Apoptotic Effects of Rotundic Acid on Human Esophagus and Lung Cancer Cells

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Abstract
Rotundic acid (RA) is a pentacyclic triterpenic acid. Apoptotic effects of RA at 4, 8, or 16 µM in OE33 and A549 cell lines, an esophageal squamous cancer cell line and a non–small-cell lung cancer cell line, were examined. RA at 4 to 16 µM inhibited the survival of both cell lines. RA at 8 and 16 µM decreased Bcl-2 expression and at 4 to 16 µM upregulated Bax and cleaved caspase-3 expression. RA treatments decreased the ratio of Bcl-2/Bax in those cells. RA at 4 to 16 µM lowered Na+–K+·ATPase activity and reduced mitochondrial membrane potential in OE33 and A549 cells. RA at 8 and 16 µM enhanced DNA fragmentation and caspase-3 and caspase-9 expression in OE33 cells. In A549 cells, RA increased caspase-3 expression in a concentration-dependent manner; but only at 8 and 16 µM, it upregulated caspase-9 expression. RA treatments at 4 to 16 µM decreased protein kinase c activity, suppressed the expression of cytochrome c and apoptosis-inducing factor, and lowered the production of reactive oxygen species, vascular endothelial growth factor, transforming growth factor-β1, and tumor necrosis factor-α in both cell lines. RA at 8 and 16 µM downregulated hypoxia-inducible factor-1α expression. These findings suggest that RA could penetrate into OE33 and A549 cells and execute cytotoxic activities.

Keywords
rotundic acid, esophagus cancer, lung cancer, apoptosis

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Introduction
Esophagus and lung cancers are thoracic cancers. Human lung cancers are classified into 2 major types: small-cell lung cancer (SCLC) and non–small-cell lung cancer (NSCLC); the latter is the most common type.1 Lung cancer remains the most lethal malignancy. The incidence of esophageal cancer is relatively low when compared with that of lung cancer; however, its survival rate is poor.2 Thus, the development of agents with inhibitory effect against esophagus cancer cells warrants attention.

Rotundic acid (RA; Figure 1) is a pentacyclic triterpenic acid naturally occurring in many medicinal plants such as Mussaenda macrophylla, olive (Olea europaea), and Glochidion obliquum.3-5 It is reported that this triterpenic acid could exhibit cytotoxic activities toward human hepatocellular carcinoma (HepG2), malignant melanoma (A375), SCLC (NCI-H446), breast cancer (MCF-7), and colon cancer (HT-29) cell lines.6,7 These studies implied that this triterpenic acid was a potent anticancer agent. However, it is unknown if RA could inhibit the growth of esophageus cancer and/or NSCLC cells. OE33, a human esophageal squamous cell line, and A549, a NSCLC cell line, have been widely used to investigate the apoptotic effects and possible mechanism of certain compounds against esophageus cancer and NSCLC.8,9 In the present study, both cancer cell lines were also used to examine the cytotoxic potential and action mode of RA at various concentrations.

Cancer cell death could be induced by activating the mitochondrial apoptotic pathway. This pathway is mainly mediated by BCL family of proteins, in which the expression of apoptotic factors such as Bax is increased and/or the expression of antiapoptotic factors such as Bcl-2 is decreased.10 The changes in these proteins trigger permeabilization of the outer mitochondrial membrane of cancer cells, which causes the loss of mitochondrial membrane potential (MMP),11 enhances the release of cytochrome c...
and apoptosis-inducing factor (AIF), and activates the mitochondria-derived caspase cascade, such as caspase-3 and caspase-9. These events eventually lead to cancer cell death. In addition, Na⁺-K⁺-ATPase, a key enzyme in the tricarboxylic acid cycle, is a transmembrane protein responsible for exchanging intracellular Na⁺ for extracellular K⁺. Abnormality in this enzyme disturbs ion homeostasis and impairs mitochondrial functions. So far, little information is available regarding the influence of RA on the variation of Bcl-2, Bax, MMP, caspases, cytochrome c, and Na⁺-K⁺-ATPase activity in OE33 and A549 cells.

Malignant tumor growth creates a hypoxic environment because of insufficient blood supply, which stimulates reactive oxygen species (ROS) production and hypoxia-inducible factor (HIF)-1α expression. Increased HIF-1α in turn activates protein kinase c (PKC) and promotes tumor proliferation and differentiation. Furthermore, greater HIF-1α facilitates the transcription of several genes encoding for angiogenic and autocrine growth factors, including vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β1, and tumor necrosis factor (TNF)-α, which consequently benefits the progression of cancer cell angiogenesis and metastasis. Thus, if RA could decrease the production of these factors, it might provide more efficient anticancer activities.

Materials and Methods

Chemicals

RA (98.5%) was obtained from Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China. Medium, chemicals, plates, and antibiotics used for cell culture were purchased from Difco Laboratory (Detroit, MI).

Cell Culture

Normal human esophagus cell (Het-1A), normal human bronchial epithelial cell (BEAS-2B), and OE33 and 549 cell lines were bought from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium, which contained 10% fetal bovine serum, 100 U/mL of penicillin, and 100 U/mL of streptomycin (pH 7.4) at 37°C in 5% CO₂. The culture medium was refreshed every 3 days, and cells were subcultured every week. Phosphate-buffered saline (PBS, pH 7.2) was added to adjust the cell number to 10⁵/mL for experiments and analyses.

Experimental Design

Stock solution of RA was prepared in dimethyl sulfoxide (DMSO) and diluted with medium. The final DMSO concentration in medium was 0.1%. DMSO at this concentration did not affect cell viability and other measurements (data not shown). Test cells (10⁵/mL) were treated with RA at 0 (control), 0.5, 1, 2, 4, 8, or 16 µM for 48 hours at 37°C, which resulted in 95.9% ± 1.5% incorporation of RA.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

MTT assay was done to examine cell viability. After RA treatment, cells were incubated with 0.25 mg MTT/mL for 3 hours at 37°C. The amount of MTT formazan product was determined by measuring the absorbance at 570 nm using a Bio-Rad microplate reader (Hercules, CA), with 630 nm as a reference. Cell viability was expressed as a percentage of control groups.

Measurement of MMP

MMP was assayed by flow cytometry (Beckman-FC500, Beckman Coulter, Fullerton, CA), and the fluorescent dye rhodamine 123 (Rh123) was purchased from Sigma Chemical Co (St Louis, MO). Cells were suspended in RPMI 1640 medium. Rh123 (100 µg/L) was added into cell medium and followed by incubation for 45 minutes at 37°C. After washing twice with PBS, the mean fluorescence intensity was analyzed.

Preparation of Mitochondrial Fractions

Control or treated cells were lysed in 5 mL cold lysis buffer containing 10 mM HEPES (pH 7.9), 0.1 mM EDTA (ethylenediaminetetraacetic acid), 1.5 mM MgCl₂, 10 mM KCl, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT (dithiothreitol), and 0.6% Nonidet P-40 for 30 minutes. After centrifuging at 200 g for 10 minutes at 4°C, the supernatant was collected and further centrifuged at 10000 g for 20 minutes at 4°C to obtain mitochondria pellet. This pellet was then resuspended in PBS. Protein concentration of the mitochondrial fraction was determined by an assay kit (Pierce Biotechnology Inc, Rockford, IL).

Na⁺-K⁺-ATPase Activity Assay

Na⁺-K⁺-ATPase activity was assayed by measuring the amount of inorganic phosphate (Pi) released from ATP. The reaction mixture contained 100 mmol/L NaCl, 20 mmol/L KCl, 2 mmol/L ATP, 30 mmol/L Tris-HCl buffer (pH 7.4), and the freshly isolated cellular mitochondria. This assay was initiated by adding ATP and terminated by adding 15% trichloroacetic acid after 15 minutes of incubation at 37°C. The released Pi was quantified by measuring the absorbance at 640 nm. The value was expressed as a percentage of control groups.
Determination of DNA Fragmentation

A commercial ELISA plus kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to measure DNA fragmentation. After incubation with RA at various concentrations, cells were lysed for 30 minutes at room temperature and followed by centrifugation at 200g for 10 minutes. Then, 20 µL supernatant was transferred onto the streptavidin-coated plate, and 80 µL freshly prepared immunoreagent was added into each well and followed by incubation for 2 hours at room temperature. After washing with PBS, the substrate solution was added and then incubated for 15 minutes. The absorbance at 405 nm (reference wavelength 490 nm) was measured using a microplate reader. DNA fragmentation was expressed as the enrichment factor using the following equation: enrichment factor = (Absorbance of RA treated cells)/(Absorbance of controls).

Measurement of ROS, TNF-α, TGF-β1, and VEGF

ROS level was measured by 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Cell homogenate at 100 µL was reacted with 100 µL DCFH-DA (2 mg/mL) for 30 minutes at 37°C. Fluorescence values were determined by a fluorescence plate reader with 488 nm excitation and 525 nm emission. The result was expressed as relative fluorescence unit (RFU)/mg protein. The levels of TNF-α, TGF-β1, and VEGF in cell culture supernatant were measured using ELISA kits (R&D Systems, Minneapolis, MN).

PKC Activity Assay

PKC activity was determined by a peptide pseudosubstrate, which could be phosphorylated by PKC. In brief, cell pellets were washed twice in ice-cold PBS and suspended in 1 mL cold buffer (50 mM Tris-Cl, 5 mM EDTA, 50 mM β-mercaptoethanol, 10 mM EGTA [ethyleneglycoltetraacetic acid], 10 mM benzamidine, and 1 mM PMSF; pH 7.5). After centrifugation, protein concentration was determined. PKC activity was assayed by a protein kinase assay kit (EMD Biosciences, Darmstadt, Germany). Color intensity was measured at 492 nm, and the relative kinase activity was calculated.

Western Blot Analyses

Cells were suspended in lysis buffer, and protein content was determined. Sample at 40 µg protein was applied to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Bedford, MA) for 1 hour. After blocking with a solution containing 5% nonfat milk for 1 hour, the membrane was incubated with mouse anticleaved caspase-3, anti-Bcl-2, anti-Bax (1:2000), anti–caspase-3, anti–caspase-9, anticytochrome c, anti-AIF (1:1000), and anti-HIF-1α (1:500) monoclonal antibody (Boehringer-Mannheim, Indianapolis, IN, USA) at 4°C overnight and followed by reaction with horseradish peroxidase conjugated antibody for 2 hours. The detected bands were quantified by Scion Image analysis software (Scion Corp, Frederick, MD), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The blot was imaged by autoradiography and normalized to GAPDH.

Statistical Analysis

The effect of each treatment was analyzed from 10 different preparations (n = 10). Data were reported as means ± SD and subjected to analysis of variance. Differences among means were determined by the least significance difference test, with significance defined at P < .05.

Results

RA Decreased Cancer Cell Viability

RA treatments at 0.5 to 16 µM did not affect the viability of Het-1A and BEAS-2B cells and normal human esophagus and bronchial epithelial cell lines (Figure 2, P > .05). RA treatments at 0.5, 1, and 2 µM did not affect the viability of OE33 and A549 cells and esophagus and lung cancer cell lines. However, RA at 4 to 16 µM inhibited the survival of both cell lines (P < .05) in a concentration-dependent manner. After calculation, the values of IC50 of RA for OE33 and A549 cells were 6.7 and 8.3 µM, respectively. In OE33 cells, RA at 4 to 16 µM decreased Bcl-2 expression and increased the expression of Bax and cleaved caspase-3 (Figure 3, P < .05), and Bax expression was enhanced in a
Figure 3. Effects of RA at 0 (control), 4, 8, or 16 µM on Bcl-2, Bax, and cleaved caspase-3 expression and ratio of Bcl-2/Bax in human OE33 and A549 cells. After 48 hours of treatment with RA at various concentrations, cells were further processed by Western blot analyses. Data are mean ± SD (n = 10).

Abbreviation: RA, rotundic acid.

*Means among bars without a common letter differ; P < .05.
concentration-dependent manner ($P < .05$). In A549 cells, RA at 8 and 16 µM suppressed Bcl-2 expression ($P < .05$) and, at 4 to 16 µM, upregulated Bax and cleaved caspase-3 expression ($P < .05$). RA at 4 to 16 µM decreased the ratio of Bcl-2/Bax in both cell lines ($P < .05$).

**RA Impaired Mitochondrial and DNA Stabilities**

As shown in Table 1, RA at 4 to 16 µM reduced MMP in OE33 and A549 cells ($P < .05$) in a concentration-dependent manner. RA at these concentrations, without a concentration-dependent effect, also lowered Na$^+$-K$^+$-ATPase activity in both cell lines ($P < .05$). RA only at 8 and 16 µM raised DNA fragmentation in OE33 and A549 cells ($P < .05$). As shown in Figure 4, RA at 8 and 16 µM upregulated caspase-3 and caspase-9 expression in OE33 cells ($P < .05$). In A549 cells, RA enhanced caspase-3 expression in a concentration-dependent manner but increased caspase-9 expression only at 8 and 16 µM ($P < .05$). RA treatments at 4 to 16 µM decreased PKC activity in both cell lines, and a concentration-dependent effect appeared in A549 cells (Figure 5, $P < .05$).

**RA Suppressed Cytochrome c, AIF, and HIF-1α Expression**

As shown in Figure 6, RA at 4 to 16 µM suppressed cytochrome c and AIF expression in both cell lines ($P < .05$) and a concentration-dependent effect was observed in decreasing AIF expression in OE33 cells ($P < .05$). RA at 8 and 16 µM downregulated HIF-1α expression in both cell lines ($P < .05$). RA at 4 to 16 µM decreased PKC activity in OE33 cells (Figure 4, $P < .05$), in which a concentration-dependent effect was observed in A549 cells. These novel findings prove that RA was a potent agent against esophagus cancer and NSCLC. Our data revealed that RA suppressed protein expression of Bcl-2 and HIF-1α, lowered MMP, increased DNA fragmentation, promoted caspase activation, and decreased VEGF and TGF-β1 production in both cancer cell lines, which consequently led to cell apoptosis. These novel findings prove that RA was a potent agent against esophagus cancer and NSCLC. In addition, RA at test concentrations did not affect the survival of normal human esophagus and bronchial, Het-1A, and BEAS-2B cells. Thus, the application of RA for esophagus or lung cancer prevention or therapy might be safe.

### Discussion

The cytotoxic effects of RA against liver, breast, and colon cancer cells have been reported. Our present study extended the inhibitory activity of this triterpenic acid toward OE33 and A549 cells, esophagus cancer, and NSCLC cells. Our data revealed that RA suppressed protein expression of Bcl-2 and HIF-1α, lowered MMP, increased DNA fragmentation, promoted caspase activation, and decreased VEGF and TGF-β1 production in both cancer cell lines, which consequently led to cell apoptosis. These novel findings prove that RA was a potent agent against esophagus cancer and NSCLC. In addition, RA at test concentrations did not affect the survival of normal human esophagus and bronchial, Het-1A, and BEAS-2B cells. Thus, the application of RA for esophagus or lung cancer prevention or therapy might be safe.
PKC activation stimulates tumor cell signaling pathways, such as the Ras kinase pathway, and favors tumor proliferation and progression. It is reported that PKC inhibitors benefited the induction of lung and esophageus cancer cell apoptosis. In our study, PKC activity in OE33 and A549 cells was substantially diminished by RA treatments at 4 to 16 µM. The inhibitory effects of RA on PKC activation in those cells might contribute to retarding esophagus cancer and NSCLC progression via decreasing associated pathways. Both cytochrome c and AIF play important roles in the mitochondrial respiratory chain and cell survival. It is reported that increased AIF enhanced tumor differentiation in esophageal squamous cell carcinoma. Thus, the lower protein expression of cytochrome c and AIF after RA treatments that we observed implied that the mitochondrial respiratory chain of OE33 and A549 cells was damaged. These data once again prove that RA could cause mitochondrial malfunctions in those cells. HIF-1α regulates the essential adaptive process for cancer cells to the hypoxic microenvironment, the major pathophysiological condition for cancer progression. HIF-1α overexpression was a predictive factor for poor prognosis or worse survival rate for NSCLC and esophagus cancer patients. Thus,
Figure 6. Effects of RA at 0 (control), 4, 8, or 16 μM on cytochrome c, AIF, and HIF-1α expression in human OE33 and A549 cells. After 48 hours of treatment with RA at various concentrations, cells were further processed by Western blot analyses. Data are mean ± SD (n = 10).

Abbreviations: RA, rotundic acid; AIF, apoptosis-inducing factor; HIF, hypoxia-inducible factor.

Table 2. Effects of RA at 0 (Control), 4, 8, or 16 μM on ROS, TNF-α, and VEGF Levels (pg/mg protein) and TGF-β1 Level (ng/mg protein) in Human OE33 and A549 Cells.  

|          | ROS       | TNF-α     | VEGF      | TGF-β1     |
|----------|-----------|-----------|-----------|------------|
| OE33 cells |           |           |           |            |
| Control  | 2.13 ± 0.15b | 192 ± 10c | 207 ± 13b | 188 ± 6b   |
| RA, 4    | 1.57 ± 0.18c | 143 ± 9d  | 173 ± 7c  | 150 ± 8c   |
| RA, 8    | 1.06 ± 0.13d | 135 ± 12d | 128 ± 9d  | 116 ± 10d  |
| RA, 16   | 0.77 ± 0.09d | 97 ± 5a   | 76 ± 5a   | 72 ± 6a    |
| A549 cells |           |           |           |            |
| Control  | 1.87 ± 0.17c | 201 ± 11c | 215 ± 14c | 197 ± 9c   |
| RA, 4    | 1.38 ± 0.12d | 164 ± 13d | 170 ± 6d  | 158 ± 10d  |
| RA, 8    | 0.76 ± 0.08c | 107 ± 8c  | 121 ± 8c  | 145 ± 13d  |
| RA, 16   | 0.63 ± 0.11c | 98 ± 9a   | 104 ± 10a | 96 ± 5a    |

Abbreviations: RA, rotundic acid; ROS, reactive oxygen species; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; TGF, transforming growth factor.

Cells were exposed to RA for 48 hours at 37°C. Data are mean ± SD (n = 10).

Means in a column without a common letter differ; P < .05.
the suppressive effects of RA on HIF-1α expression in both OE33 and A549 cells shows that this compound could weaken the adaptability of these cancer cells to hypoxic conditions, which in turn decreased the survival and metastasis of these cells. Furthermore, HIF-1α is a transcriptional factor for VEGF expression.33 Because RA treatments already downregulated HIF-1α expression in both cancer cell lines, the lower VEGF generation in these cells could be explained.

ROS is an oxidant, and TNF-α is an inflammatory mediator. Both VEGF and TGF-β1 are angiogenic factors. Increased ROS, TNF-α, VEGF, and TGF-β1 production enhance oxidative and inflammatory stress, which benefits microvascular permeability and cancer metastasis. Saji et al34 indicated that TGF-β1, via its immunosuppressive effect, promoted pulmonary metastasis in NSCLC patients. von Rahden et al35 reported that TGF-β1 overexpression was highly associated with poor prognosis in patients with esophagus cancer. Thus, the lower production of ROS, TNF-α, VEGF, and TGF-β1 in RA-treated OE33 and A549 cells suggested that angiogenic and metastatic actions in these cancer cells were diminished. These findings imply that RA might provide antiangiogenic and antimetastatic activities in these cancer cells by decreasing oxidative, inflammatory, and angiogenic stress. RA is a natural compound present in many edible plant foods or herbs such as G obliquum, O europaea, Ilex integra, and Randia fo massa.43 Its natural property could enhance its use for disease prevention or therapy. However, the information regarding the in vivo activity and bioavailability of this compound is limited. Thus, further in vivo studies are necessary to verify and ensure the safety and efficiency of RA.

In summary, RA treatments at 4 to 10 μM induced apoptosis in OE33 and A549 cells. This triterpenoid increased DNA fragmentation, decreased MMP and Na(+)-K(+)ATPase activity, and lowered VEGF and TGF-β1 production in these cancer cells. This agent also effectively suppressed protein expression of Bcl-2, HIF-1α, and cytochrome c. These findings suggest that RA is a potent agent against esophagus and lung cancer.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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