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Pro-oxidant and cytotoxic activities of atractylenolide I in human promyeloleukemic HL-60 cells

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Abstract

The dried rhizome of Bai Zhu (*Atractylodes ovata*) is widely used as a Chinese herbal medicine. Two sesquiterpenolides of similar structures (atractylenolide I, AT-I; atractylenolide III, AT-III) were isolated from dried rhizome of *Atractylodes ovata*. Incubation of AT-I with recombinant human Cu,Zn-superoxide dismutase (rhCu,Zn-SOD) resulted in rhCu,Zn-SOD fragmentations and Zn releases. However, these were not observed in the AT-III reaction. The AT-1 showed dose-dependent cytotoxic activities (7.5, 15, and 30 μg/ml) on the human promyeloleukemic HL-60 cells while AT-III did not, and the IC_{50} of the former being 10.6 μg/ml (corresponding to 46 μM) on 12 h-treated cells. The results of DNA ladder and DNA contents in sub-G1 type revealed that AT-I induced apoptosis in human promyeloleukemic HL-60 cells. The cytotoxic and pharmacological mechanisms of AT-I against human promyeloleukemic HL-60 cells was investigated. The AT-I appeared to exhibit both pro-oxidant and antioxidant properties after an ESR spectrometer was used to detect hydroxyl radical productions in vitro and flow cytometry to detect intracellular ROS productions in AT-I treated cells. The AT-1 also showed dose-dependent Cu,Zn-SOD inhibitory activity in HL-60 cells treated for 12 h, confirmed by activity and immune stainings. However, catalase, Mn-SOD, and glutathione peroxidase did not apparently change activities under the same treatments. The addition of commercial rhCu,Zn-SOD (25–100 U/mL) to the AT-I-treated HL-60 cells (15 μg/ml) resulted in significant differences ($p < 0.01$) and could reduce the AT-I cytotoxicity from 78% to 28% on HL-60 cells. It was proposed that the AT-I might work via Cu,Zn-SOD inhibition in HL-60 cells to induce apoptosis and bring about cytotoxicity.
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Keywords: Apoptosis; *Atractylodes ovata*; Atractylenolide I; Cu,Zn-superoxide dismutase; Flow cytometry; Human promyeloleukemic HL-60 cells; Sesquiterpenoides

1. Introduction

Bai Zhu is the dried rhizome of *Atractylodes ovata* (Compositae), a popular traditional Chinese herb used as a tonic for spleen and stomach ailments in Asia. The extracts of the herb have been reported to have several pharmacological activities, such as anti-inflammatory (Endo et al., 1979) and anti-ulcer properties (Matsuda et al., 1991; Nogami et al., 1986; Kubo et al., 1983), lipid peroxidation inhibition (Kiso et al., 1983; Kiso et al., 1985), and inhibitory activities against *tert*-butyl

Abbreviations: AT-I, atractylenolide I; rhCu,Zn-SOD, recombinant human Cu,Zn-superoxide dismutase; DCFH/DA, dichlorodihydrofluorescein diacetate; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; ESR, electron spin-resonance; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; Zincon, (2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene).

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hydroperoxide-induced cytotoxicity in primary culture of rat hepatocytes (Satoh et al., 1996; Bakurai et al., 1993).

Copper-zinc superoxide dismutase (Cu,Zn-SOD) is a first-line cytosolic enzyme for protecting cells from superoxide radical injury (Valentine et al., 2005), and a supplement of Cu,Zn-SOD could increase neuroprotective effects against ischemic neuronal damage in the gerbil hippocampus (Hwang et al., 2005). However, hydrogen peroxide (Choi et al., 1999) or peroxynitrite (Alvarez et al., 2004) were reported to inactivate Cu,Zn-SOD, and nitric oxide (Niketić et al., 1999) was also reported to inactivate Mn-SOD and Fe-SOD, and fragmented SOD was found in PAGE gels. Huang et al. (2000) pointed out that 2-methoxyoestradiol and its structural derivatives could selectively kill human leukemia cells through inhibitions of Cu,Zn-SOD, and the inhibition of SOD caused accumulation of cellular superoxide radicals, finally leading to apoptosis.

In a previous study, four structure-related sesquiterpenes (atractylon and atractylenolides (AT)-I, AT-II and AT-III) were isolated from *A. ovata*. Both atractylon and AT-I showed dose-dependent cytotoxicities against HL-60 and P-388 cell lines, and the atractylon-treated HL-60 cells were further investigated in the apoptotic parameters of DNA ladder, sub-G_1 DNA contents, and PARP cleavages (Wang et al., 2002). In this report, the cytotoxic mechanism of AT-I against human promyeloleukemic HL-60 cells was investigated in comparison with structure-related AT-III. We proposed the cytotoxicity of AT-I against human promyeloleukemic HL-60 cells relative to its pro-oxidant activity and the inhibition against Cu,Zn-SOD activity.

2. Materials and methods

2.1. Isolation of AT-I and AT-III from A. ovata

The isolation and structure identification of AT-I and AT-III (Fig. 1A) from *A. ovata* were according the previous report (Wang et al., 2002). The molecular mass of AT-I and AT-III was 230.1 Da and 248.1 Da, respectively. The difference between AT-I and AT-III was that the former had a double bond between the C-8 and C-9 positions while the latter had a hydroxyl group in the C-8 position.

2.2. Cell cultures

Human promyeloleukemic HL-60 cells were obtained from American Type Cell Culture (ATCC) (Rockville, MD, USA). The human promyeloleukemic HL-60 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 100 mg/l streptomycin and were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Cell viability

A stock solution of AT-I and AT-III (20 mg/ml) was prepared by dissolving them in DMSO and then storing at −20 °C until use. Serial dilutions of these stock solutions were prepared in the culture medium in 24-well microtiter plates. AT-I or AT-III at different concentrations (7.5, 15, and 30 μg/ml, respectively corresponding to 32.6, 65.2, and 130.4 μM for AT-I; and 30.2, 60.5, and 120.9 μM for AT-III) was added to cell cultures for 12 h without renewal of the medium. The cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining (Allen et al., 1997). To prevent AT-I toxicity (15 μg/mL) to HL-60 cells, the rhCu,Zn-SOD (25, 50, and 100 U/ml, 574595, Calbiochem Co.) was pre-treated for 1 h and then co-cultured with AT-I for 12 h and assayed for cell viability.

2.4. DNA isolation and electrophoresis

The human promyeloleukemic HL-60 cells (1×10^6 cells/well) were treated with AT-I at different concentrations (7.5, 15, and 30 μg/ml) for 12 h. Cells were collected, washed with PBS twice and then lysed in 100 ml of lytic buffer (50 mM Tris, pH 8.0; 10 mM EDTA; 0.5% sodium sarkosinate, and 1 mg/mL proteinase K) for 3 h at 56 °C. DNA was then extracted with phenol/chloroform/isoamyl alcohol (V/V/V, 25/24/1) before loading. The extent of DNA fragmentation was assessed by 1.5% agarose gel electrophoresis (Allen et al., 1997).

2.5. Flow cytometry analysis of sub-G_1 DNA contents

The AT-I-treated (7.5, 15, and 30 μg/ml) HL-60 cells (5×10^5 cells/well) were harvested by centrifugation and washed with PBS. They were then fixed with ice-cold 80% ethanol for at least 30 min and then stained with propidium iodide (Sigma). Total fluorescence intensities were quantified by a FACScan flow cytometer (Becton Dickinson, CA, USA). The results of sub-G_1 DNA contents were calculated as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence (Allen et al., 1997).

2.6. Measurement of intracellular peroxide levels by flow cytometry

The intracellular peroxide levels were assessed by flow cytometry using dichlorodihydrofluorescein diacetate (DCFH/DA, Molecular Probes) as a probe (Gorman et al., 1997). The DCFH/DA penetrated into cells and was hydrolyzed by cellular esterase to DCFH, which was further oxidized by intracellular peroxide into a strong fluorescent compound, dichlorofluorescein. The AT-I (0.125, 0.25, and 0.5 μg/ml), or 0.015% H_2O_2 (positive controls) were used to treat human promyeloleukemic HL-60 cells (1×10^5 cells/well) in the culture medium for 30 min, and then cells were cultured for another 30 min after the addition of 5 μM DCFH/DA (15 mM in DMSO as a stock solution). The cells were washed twice with PBS and quantified by a FACScan flow cytometer (Becton Dickinson, CA, USA) with excitation and emission settings of 488 and 530 nm, respectively. The peroxide levels in cells were plotted as one-parameter histograms with cell count on the *y*-axis and the fluorescence on the *x*-axis. The calculated area of fluorescent distributions in the fixed cell counts were expressed as the fluorescent intensity.

2.7. Western blots and activity staining

Crude extracts (30 μg proteins) of AT-I-treated or AT-III-treated HL-60 cells were harvested and separated on 10% native PAGE gels and then were stained either for catalase (Woodbury et al., 1971) or SOD (Beauchamp and Fridovich, 1971) activity or transferred onto Hybond-P PVDF membranes (Amersham Pharmacia Biotech Co.) for Cu,Zn-SOD, Mn-SOD, or glutathione peroxidase immune staining, which was detected by anti-Cu/Zn SOD (574597, Calbiochem Co.), anti-Mn SOD (574596, Calbiochem Co.), or anti-glutathione peroxidase (CR2144SP, Cortex Biochem., CA, USA) antibodies, respectively.

2.8. SOD inhibition and released Zn determination

Each of the 30 and 60 μg of AT-I or AT-III were pre-mixed with 10 U of commercial recombinant human Cu,Zn-SOD (574595, Calbiochem Co.) for 12 h and were separated on 10% native PAGE gels. The gel was stained for SOD activity (clear bands against purple backgrounds, (Beauchamp and Fridovich, 1971)) or transferred onto Hybond-P PVDF

membranes for Cu,Zn-SOD immune staining to check the SOD inhibition and fragmentation. For released Zn determinations, the 50 μg AT-I was pre-mixed with 50, 100, and 150 U of rhCu,Zn-SOD in the total 35 μl mixture for 12 h, and the released Zn from rhCu,Zn-SOD was determined by Zincon reagent (2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene) (Pattanaik et al., 1992) to form Zn–Zincon complex. The absorbance at 620 nm was determined, and the only rhCu,Zn-SOD was used as a blank. The $ZnCl_2$ was used to plot the standard curve.

2.9. Pro-oxidant and antioxidant properties of AT-1 in Fenton reaction detected by ESR spectrometer

The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (1991). The total 500 μL mixture included 0.02, 0.025, 0.0375, and 0.05 μg/ml of AT-I, 5 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and 0.05 mM ferrous sulfate. After mixing, the solution was transferred to an ESR quartz cell and placed at the cavity of the ESR spectrometer, and then hydrogen peroxide was added to a final concentration of 0.25 mM. Deionized water was used instead of sample solution for blank experiments. After forty seconds, the intensity of the signal of DMPO-OH spin-adduct ($ISA_{DMPO\text{-}OH}$) was measured. All ESR spectra were recorded at the ambient temperature (298 K) on a Bruker EMX-6/1 ESR spectrometer equipped with WIN-ESR SimFonia software, Version 1.2. The conditions of ESR spectrometry were as follows: center field, 345.4 ± 5.0 mT; microwave power, 8 mW (9.416 GHz); modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 0.6 s; scan time, 1.5 min. The intensities of DMPO-OH spin signal in ESR spectrometry were used to evaluate the pro-oxidant or antioxidant properties of AT-I and were calculated as the peak height which was standardized by WIN-ESR SimFonia software. The calculated DMPO-OH adducts in the control was assumed as 100%.

2.10. Statistics

Means of triplicates were measured. Student's *t*-test was used for comparison between two treatments. A difference between the control and each treatment was considered statistically significant when $P < 0.05$ (*) or $P < 0.01$ (**).

3. Results

3.1. Induction of apoptosis of HL-60 cells by AT-1

Though the difference between AT-I and AT-III structures is minor (Fig. 1A), the AT-1 showed dose-dependent cytotoxic activities (7.5, 15, and 30 μg/ml, Fig. 1B) on the human promyeloleukemic HL-60 cells while AT-III did not, and the IC_{50} of the former was 10.6 μg/ml (corresponding to the 46 μM) on 12 h-treated cells. The apoptotic parameters, such as DNA ladders (Fig. 1C) and sub-G_1

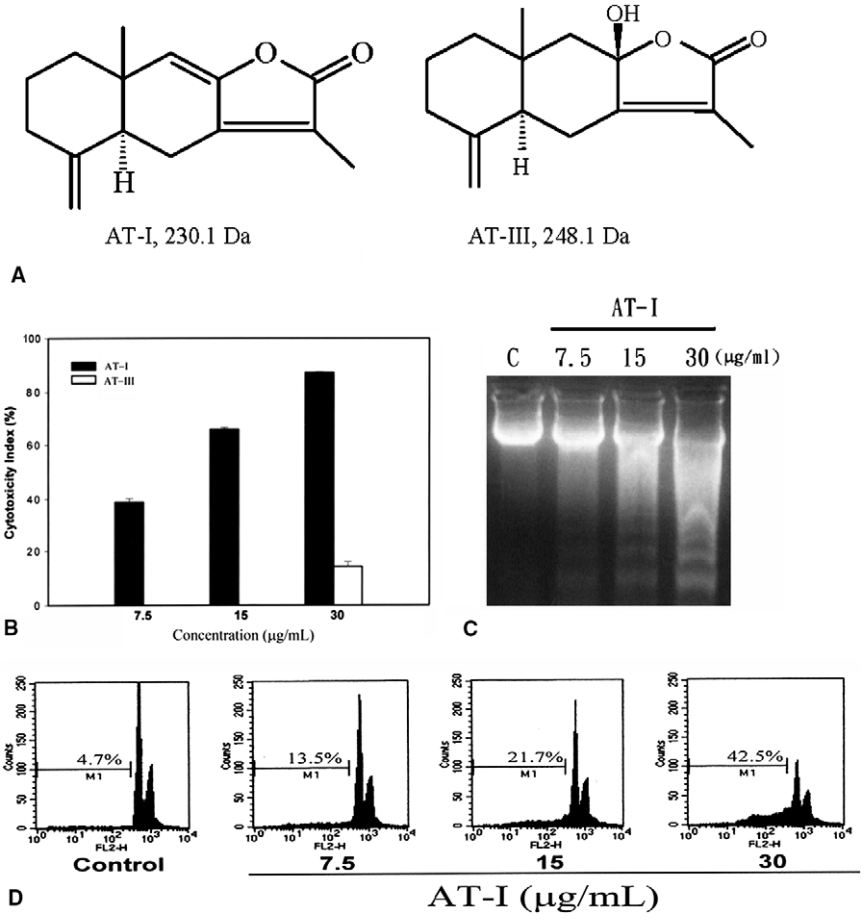

Fig. 1. The cytotoxic activity of AT-I and AT-III on the HL-60 cells. (A) The structures of AT-I and AT-III, (B) the cytotoxicity index of AT-I and AT-III in the concentrations of 7.5, 15, and 30 μg/ml on 12 h-cultured HL-60 cells, (C) the apoptotic parameter of DNA ladder of AT-I-treated HL-60 cells for 12 h in the concentrations of 7.5, 15, and 30 μg/ml, (D) the apoptotic parameter of sub-G_1 DNA contents (M1) of AT-I-treated HL-60 cells for 12 h in the concentrations of 7.5, 15, and 30 μg/ml. The M1 indicated the number of cells versus the amount of DNA as indicated by the intensity of fluorescence.

DNA contents (Fig. 1D), showed that AT-1 induced apoptosis and resulted in cytotoxicity to human promyeloleukemic HL-60 cells. The values most below the G_1 peak in the DNA contents (M1) were 4.7%, 13.5%, 21.7%, and 42.5%, respectively, for controls, 7.5, 15, and 30 μg/ml of AT-I-treated HL-60 cells.

3.2. SOD inhibition and released Zn determination

Recombinant human Cu,Zn-SOD was inhibited by AT-1, as observed by activity staining (faint clear bands, Fig. 2A) and immune staining (Fig. 2B). Each of the 30 μg (lanes 1–3) and 60 μg (lanes 2–4) of AT-I (lanes 1 and 2) or AT-III (lanes 3 and 4) were pre-mixed with 10 U of commercial rhCu,Zn-SOD for 12 h and were separated on 10% native PAGE gels. Compared to the control (Cu,Zn-SOD only, lane C), it was found that AT-1, but not AT-III, could inactivate rhCu,Zn-SOD (faint clear bands, lanes 1 and 2, Fig. 2A) and resulted in SOD fragmented patterns (lanes 1 and 2, Fig. 2A and B). The 60 μg of AT-I (lane 2, Fig. 2A and B) made more serious SOD fragmentations than 30 μg did (lane 1, Fig. 2A and B). The released Zn contents from Cu,Zn-SOD were determined using a Zn–Zincon complex standard curve (Fig. 2C). It was found that the released Zn contents increased (Fig. 2D) with increased rhCu,Zn-SOD (50, 100, and 150 U) at a fixed amount of AT-I (50 μg). The released Zn from rhCu,Zn-SOD was 3.92, 5.86, and 15.59 μg/ml, respectively, for 50, 100, and 150 U of rhCu, Zn-SOD.

3.3. The changes of antioxidant enzymes in AT-I-treated or AT-III-treated HL-60 cells

The human promyeloleukemic HL-60 cells were treated with AT-I (lanes 1–3 were 7.5, 15, and 30 μg/ml, respectively) or AT-III (lanes 4–6 were 7.5, 15, and 30 μg/ml, respectively) for 12 h. Each cell extract (30 μg protein) was separated on 10% native PAGE gels for Cu,Zn-SOD activity staining (clear band against the purple backgrounds, Fig. 3A) or immune stainings of Cu,Zn-SOD (Fig. 3B) and Mn-SOD (Fig. 3C). Compared to the control lane (lane C, without AT-I or AT-III treatments), Cu,Zn-SOD dose-dependent inhibitions were found in AT-I-treated cells (lanes 1–3, Fig. 3A, activity staining; lanes 1–3,

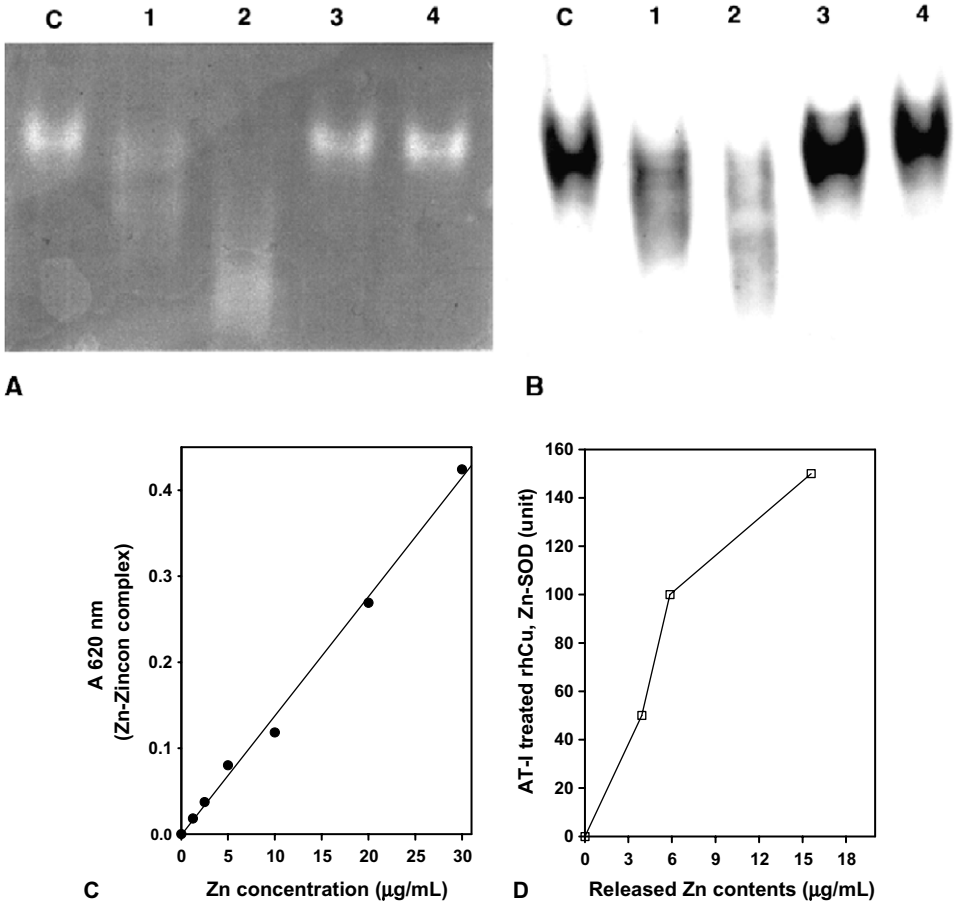

Fig. 2. The effects of AT-I on recombinant human Cu,Zn-SOD activity detected by (A) activity staining (clear bands against purple background) or (B) immune staining. The 30 μg (lanes 1–3) and 60 μg (lanes 2–4) of AT-I (lanes 1 and 2) or AT-III (lanes 3 and 4) were pre-mixed with 10 U of commercial rhCu,Zn-SOD for 12 h and were separated on 10% native PAGE gels. (C) The $ZnCl_2$ was used to plot the standard curve as detected by Zincon reagent to form Zn–Zincon complex (absorbance at 620 nm). (D) The released Zn from rhCu,Zn-SOD (50, 100 and 150 U) after AT-I treatments was calculated from the Zn standard curve, and the only rhCu,Zn-SOD was used as a blank.

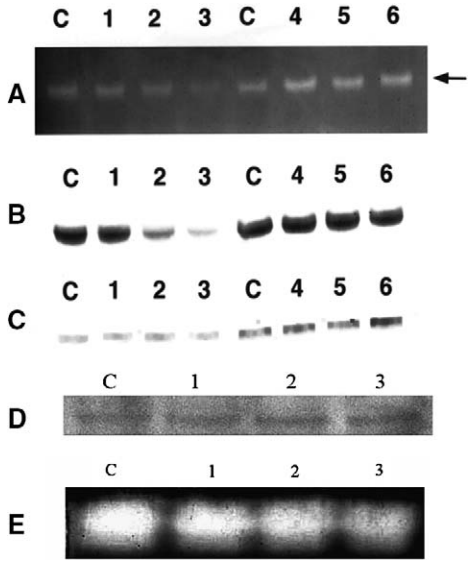

Fig. 3. The changes of antioxidant enzyme activity in AT-I-treated or AT-III-treated HL-60 cells for 12 h. The activity stainings (clear bands against the dark background) of Cu,Zn-SOD (A) and catalase (E) or immune stainings of Cu,Zn-SOD (B), Mn-SOD (C), and glutathione peroxidase (D). The HL-60 cells were treated with AT-I (lanes 1–3 were 7.5, 15, and 30 μg/ml, respectively) or AT-III (lanes 4–6 were 7.5, 15, and 30 μg/ml, respectively) for 12 h. Each cell extracts (30 μg protein) was separated on 10% native PAGE gels for activity stainings or transfer onto PVDF membrane for immune stainings.

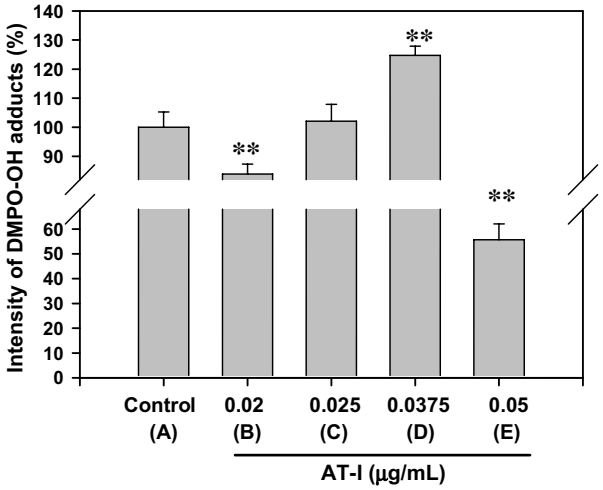

Fig. 4. Pro-oxidant and antioxidant properties of AT-1 in Fenton reaction detected by ESR spectrometer in the magnetic field from 3426 to 3526 Gauss. The intensity of DMPO-OH adduct was measured without (as the control, A) or with AT-I additions in the concentrations of 0.02 (B), 0.025 (C), 0.0375 (D), and 0.05 μg/ml (E). The intensities of DMPO-OH spin signal in ESR spectrometry were used to evaluate the pro-oxidant or antioxidant properties of AT-I and were calculated as the peak height which was standardized by WIN-ESR SimFonia software. The calculated DMPO-OH adducts in the control was assumed as 100%, the relative intensities were 83.92%, 102.07%, 124.73%, and 55.68%, respectively, for 0.02, 0.025, 0.0375, and 0.05 μg/ml. A difference between the control and each treatment was considered statistically significant when $p < 0.05$ (*) or $p < 0.01$ (**).

Fig. 3B, immune staining). AT-III, however, did not show any apparent inhibitory activities on Cu,Zn-SOD. Neither AT-I nor AT-III treatments inhibited the expression of Mn-SOD activity in HL-60 cells (Fig. 3C, immune staining). AT-I treatments also did not have any apparent impact on glutathione peroxidase (lanes 1–3, Fig. 3D, immune staining) or catalase (lanes 1–3, Fig. 3E, activity staining, yellow band against the deep blue backgrounds[1]) activities.

3.4. Pro-oxidant and antioxidant properties of AT-1 in Fenton reaction detected by ESR spectrometer

The intensities of DMPO-OH adducts in the magnetic field from 3426 to 3526 Gauss were used to detect the hydroxyl radicals generated by the Fenton reaction. The intensities of DMPO-OH spin signal in ESR spectrometry were used to evaluate the pro-oxidant or antioxidant properties of AT-I and were calculated as the peak height which was standardized by WIN-ESR SimFonia software. The calculated DMPO-OH adducts in the control was assumed as 100%. AT-I was added in the Fenton reaction system to evaluate the promotion (pro-oxidant) or inhibition (antioxidant) properties on hydroxyl radical generations in vitro. The relative intensity of calculated DMPO-OH adducts (Fig. 4) in the control was assumed to be 100%, and the added AT-I of 0.02 (B), 0.025 (C), 0.0375 (D), and 0.05 μg/ml (E) showed 83.92% ($p < 0.01$), 102.07%, 124.73% ($p < 0.01$), and 55.68% ($p < 0.01$) relative intensities, respectively. The AT-I showed pro-oxidant effects on hydroxyl radical productions in the concentrations of 0.025–0.0375 μg/ml, and antioxidant effects in the concentrations higher than 0.05 μg/ml. From the intensity data in the ESR spectra it can be seen that the AT-I exhibited pro-oxidant and antioxidant properties on hydroxyl radical generations in the Fenton reaction system.

3.5. Measurement of intracellular peroxide levels in AT-I treated HL-60 cells

The DCF fluorescent intensity was subjected to flow cytometric analysis to assess the intracellular peroxide levels in each set of AT-I treated HL-60 cells. The peroxide levels in cells were plotted as one-parameter histograms with cell count on the *y*-axis and the fluorescence on the *x*-axis. The calculated area of fluorescent distributions in the fixed cell counts were expressed as the fluorescent intensity (Fig. 5). Peroxide levels in the untreated control was 187.69 (A), and the fluorescent intensities of AT-I-treated cells in the concentrations of 0.125 (B), 0.25 (C), and 0.5 μg/ml (D) were 410.47 ($p < 0.01$), 319.08 ($p < 0.01$), and 224.68 ($p < 0.05$), respectively. All of the tested concentrations increased the peroxide levels in HL-60 cells. Hydrogen peroxide (E, positive control, 0.015%) dramatically

[1] For interpretation of color in this figure, the reader is referred to the web version of this article.

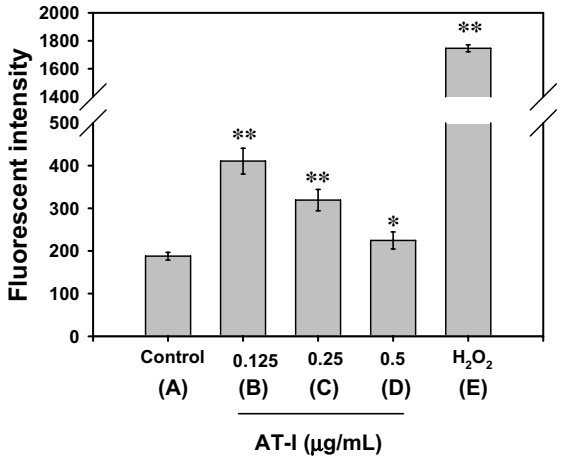

Fig. 5. Measurement of intracellular peroxide levels in AT-I treated HL-60 cells by flow cytometric analysis using DCFH/DA fluorescent probes. The intracellular peroxide levels without (A) or with AT-I treated HL-60 cells in the concentrations of 0.5 (B), 0.25 (C), and 0.125 μg/ml (D) for 30 min, and the 0.015% of hydrogen peroxide (E) was used as a positive control. The peroxide levels in cells were transferred as one-parameter histograms with cell count on the *y*-axis and the fluorescence on the *x*-axis. The calculated area of fluorescent distributions in the fixed cell counts were expressed as the fluorescent intensity. A difference between the control and each treatment was considered statistically significant when $p < 0.05$ (*) or $p < 0.01$ (**).

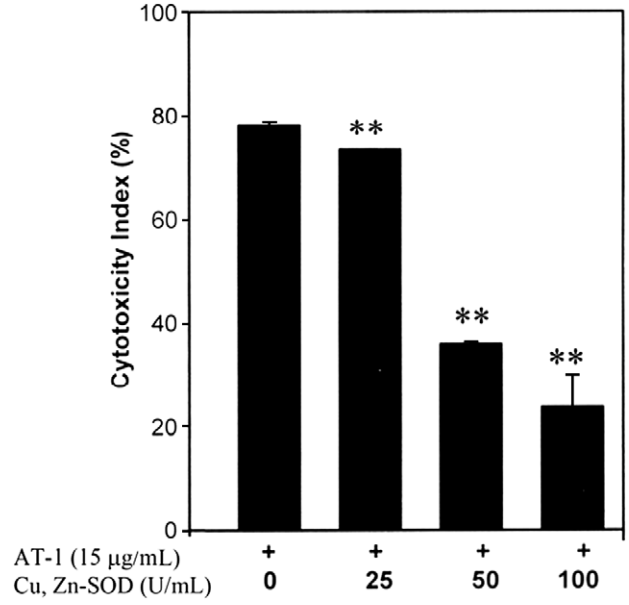

Fig. 6. The effects of added rhCu,Zn-SOD on cytotoxicity of AT-I-treated HL-60 cells. Pre-treatment of rhCu,Zn-SOD (25, 50, 100 U/ml) 1 h before AT-I-treated HL-60 cells (15 μg/ml) decrease the cytotoxicity from 78% to 28% (100 U/ml). A difference between the control and each treatment was considered statistically significant when $p < 0.05$ (*) or $p < 0.01$ (**).

increased the intracellular fluorescent levels. We found the AT-I treatments to exhibit reverse effects on the intracellular peroxide levels, with the lower concentration (0.125 μg/ml) of AT-I increasing them. However, the higher concentration of AT-I (0.5 μg/ml) decreased them in line with the untreated ones.

3.6. Prevention of cytotoxicity of AT-I-treated HL-60 cells by adding Cu,Zn-SOD

The AT-I (15 μg/ml) treatment resulted in the death of 78% of the human promyeloleukemic HL-60 cells. However, the pre-treatment of rhCu,Zn-SOD (25, 50, 100 U/ml) for 1 h had a significant impact ($p < 0.01$) and decreased the cytotoxicity to 28% (100 U/ml) (Fig. 6).

4. Discussion

Bai Zhu is the dried rhizome of *A. ovata* (Compositae), a popular traditional Chinese herb used as a tonic for spleen and stomach ailments in Asia. Although several pharmacological activities were reported (Endo et al., 1979; Matsuda et al., 1991; Nogami et al., 1986; Kubo et al., 1983; Kiso et al., 1983; Kiso et al., 1985; Satoh et al., 1996; Bakurai et al., 1993), however, few reports were concerned for anticancer treatments. In the previous report (Wang et al., 2002), the sesquiterpenes (atractylenolides (AT)-I, and AT-III) were isolated from *A. ovata*. The molecular mass of AT-I and AT-III was 230.1 Da and 248.1 Da, respectively. The difference between AT-I and AT-III was that the former had a double bond between the C-8 and C-9 positions while the latter had a hydroxyl group in the C-8 position. The AT-I was reported to have anticancer activity (Wang et al., 2002), therefore, in the present study, the cytotoxic mechanism of AT-I against human promyeloleukemic HL-60 cells was investigated. We proposed the AT-I against HL-60 cells relative to pro-oxidant and inhibition against Cu,Zn-SOD activity.

Though AT-I and AT-III had similar structures, the former exhibited apparent cytotoxicity to HL-60 cells under the same concentrations. AT-I showed dose-dependent cytotoxicities to HL-60 cells (IC_{50}, 10.6 μg/ml corresponding 46 μM for 12 h-treated cells), and the DNA ladder and increased sub-G_1 DNA contents revealed that AT-I induced apoptosis (Fig. 1). The IC_{50} (46 μM) of AT-I in cells treated for 12 h was lower than that of myricetin, apigenin, and close to that of baicalein and fisetin (Lee et al., 2002). The IC_{50} of taraxinic acid (a hydrolysate sesquiterpene lactone glycoside) was 34.5 μM for the 48-h treated HL-60 (Choi et al., 2002) which was toxic than that of the AT-I, however, the treatment time (48 h) was longer than the present report (12 h). Several sesquiterpenoids isolated from *Pulicaria canariensis* had IC_{50} values higher than that of AT-I in HL-60 cells treated for 12 h (Triana et al., 2005). Citrinin had cytotoxic activity toward HL-60 cells, and the IC_{50} value was close to 50 μM in 24 h-treated cells (Yu et al., 2006). From above data, AT-I had potent cytotoxicity toward HL-60 cells. Investigations into the AT-I-induced cytotoxic mechanism and into the effects of AT-I on antioxidant enzymes, on ROS productions in the treated cell system, and on hydroxyl radical productions in the cell-free system continue.

Cu,Zn-SOD is a first-line cytosolic enzyme for protecting cells from superoxide radical injury (Valentine et al., 2005), and the Cu,Zn-SOD supplements could increase neuroprotective effects against ischemic neuronal damage in the gerbil hippocampus (Hwang et al., 2005). However, hydrogen peroxide (Choi et al., 1999) or peroxynitrite (Alvarez et al., 2004) were reported to inactivate Cu,Zn-SOD and fragmented SOD was found in PAGE gels. Choi et al. proposed that hydroxyl radicals resulted in SOD fragmentations and could be recovered by carnosine, homocarnosine, and anserine (Choi et al., 1999). Therefore, different amounts of AT-I were pre-mixed with rhCu, Zn-SOD and separated on native PAGE gels. It was found that AT-I inhibited rhCu,Zn-SOD activity and resulted in SOD fragmentations detected by activity (faint clear bands) and immune stainings (Fig. 2A and B). The released Zn increased with more rhCu, Zn-SOD (50–150 U) added at the fixed AT-I concentration (Fig. 2D). Pattanaik et al. (1992) used the dichlorodiammineplatinum (II) to react with Zn-metallothionein, and the released Zn was determined by the Zincon reagents. The present results (Fig. 2) suggested that the released Zn might be from the fragmented rhCu, Zn-SOD by AT-I treatments.

In the AT-I-treated cell system, several antioxidant enzymes—such as Cu,Zn-SOD, Mn-SOD, glutathione peroxidase, and catalase (Fig. 3)—were detected either by activity staining or immune staining to allow us to observe the enzyme changes. It was found that only Cu,Zn-SOD (Fig. 3A, activity stainings; Fig. 3B, immune stainings) was apparently inactivated in cells treated for 12 h. Huang et al. (2000) pointed that 2-methoxyoestradiol and its structural derivatives could selectively kill human leukemia cells through inhibitions of Cu,Zn-SOD, and the inhibition of SOD caused accumulation of cellular superoxide radicals, finally leading to apoptosis.

Ueda et al. (2001) reported that baicalin acted as a pro-oxidant and induced apoptosis of Jurkat cells. The procyanidin B2 showed dual antioxidant and pro-oxidant effects on metal-mediated DNA damage in HL-60 cells (Sakano et al., 2005). The hydroxyl radical productions with or without AT-I additions in the cell-free Fenton reaction system detected by ESR spectrometer (Fig. 4) or changes of intracellular peroxide levels in the AT-I-treated HL-60 cell system detected by flow cytometric analysis (Fig. 5) were investigated. Both cell-free (Fig. 4) and cell-treated (Fig. 5) systems revealed that AT-I acted as a pro-oxidant in lower concentrations and as an antioxidant in higher concentrations, which was similar to the behavior of uric acid (Abuja, 1999; Filipe et al., 2002).

On the other hand, the cytotoxicities of AT-I-treated cells could be recovered by adding rhCu,Zn-SOD (Fig. 6). Choi et al. (1999) proposed that hydroxyl radicals resulted in SOD fragmentations and could be recovered by carnosine, homocarnosine, and anserine. In the present results, AT-I was able to directly inhibit rhCu,Zn-SOD and cause SOD fragmentations (30 and 60 μg). The AT-I behaved as an pro-oxidant in the Fenton reaction system in the ranges of 0.025–0.0375 μg/ml; the AT-I elevated intracellular peroxide levels after 30 min treatment in the ranges of 0.125–0.25 μg/ml; the dose-dependent cytotoxicity of AT-I (the actual amounts in cultured medium were 7.5, 15, and 30 μg) in cells treated 12 h accompanying Cu,Zn-SOD inhibitions. The sesquiterpene-related compounds were hydrophobic properties in nature. If AT-I can diffuse from medium into cells, then it can inhibit the Cu,Zn-SOD and result in ROS accumulations and finally the apoptosis of HL-60 cell. If it can not diffuse into HL-60 cells, the pro-oxidant effects in hydroxyl radical productions of AT-I in the cultured medium may attack the membranes and elevate the intracellular peroxide levels (may be via the inhibition of Cu,Zn-SOD), finally resulting in the apoptosis of HL-60 cell.

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