

## Research Article

# The Critical Role of Redox Homeostasis in Shikonin-Induced HL-60 Cell Differentiation via Unique Modulation of the Nrf2/ARE Pathway

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Among various cancer cell lines, the leukemia cell line HL-60 was most sensitive to Shikonin, with evidence showing both the prooxidative activities and proapoptotic effects of micromolar concentrations of Shikonin. However, the mechanism involved in the cytotoxicity of Shikonin in the submicromolar range has not been fully characterized. Using biochemical and free radical biological experiments *in vitro*, we identified the prodifferentiated profiles of Shikonin and evaluated the redox homeostasis during HL-60 differentiation. The data showed a strong dose-response relationship between Shikonin exposure and the characteristics of HL-60 differentiation in terms of morphology changes, nitroblue tetrazolium (NBT) reductive activity, and the expression level of surface antigens CD11b/CD14. During drug exposure, intercellular redox homeostasis changes towards oxidation are necessary to support Shikonin-induced differentiation, which was proven by additional enzymatic and non-enzymatic redox modulators. A statistically significant and dose-dependent increase ( $P < 0.05$ ) was recorded with regard to the unique expression levels of the Nrf2/ARE downstream target genes in HL-60 cells undergoing late differentiation, which were restored with further antioxidants employed with the Shikonin treatment. Our research demonstrated that Shikonin is a differentiation-inducing agent, and its mechanisms involve the Nrf2/ARE pathway to modulate the intercellular redox homeostasis, thus facilitating differentiation.

## 1. Introduction

Acute myeloid leukemia (AML) is characterized by the uncontrolled proliferation of undifferentiated or poorly differentiated myeloid blasts. Differentiation therapy is an alternative AML treatment based on the induction of leukemic blasts to mature beyond the differentiation block, which can restore the normal cellular phenotype and cell cycle arrest [1]. The studies were performed in HL-60 cells, a cell line that was originally isolated from an acute promyelocytic leukemia patient and is a well-characterized model for studying terminal differentiation events. A number of compounds are known to induce differentiation in HL-60 cells, including cytokines, polar planar compounds, purine and pyrimidine analogs, and chemotherapeutic agents. These

agents exert their differentiation effects through various mechanisms, which include the following: remodeling chromatin, hypomethylating DNA, inhibiting histone deacetylase, inhibiting topoisomerase, interfering with DNA and RNA synthesis, and disrupting signal transduction [2].

In the context of AML, increased oxidative stress in myeloid leukemias may indeed represent a therapeutic target, and early encouraging results from *in vitro* studies and clinical trials suggest that reactive oxygen species (ROS) modulation therapy in myeloid leukemia patients warrants further investigation, especially in signal transduction [3]. However, observations in this area are controversial, as either prooxidant or antioxidant approaches appear to be beneficial. For example, arsenic trioxide and realgar were approved for treatment of relapsed acute promyelocytic leukemia

(APL) due to their prooxidant properties [4, 5]. Our previous work has shown that the antioxidant isoliquiritigenin is able to induce the monocytic differentiation in leukemia cells [6], though the specific mechanisms involved remain unclear. The intracellular thiol redox environment is thought to affect many cell processes including differentiation. The reduced glutathione GSH is the most abundant intracellular thiol and is therefore the major regulator of intracellular redox homeostasis [7]. There are many redox-sensitive transcription factors, including Nrf2, AP-1, c-Jun, Bach1, NF $\kappa$ B, IKK $\beta$  subunit, interferon regulatory factor 3, p53, and Pax-8, each of which may contribute to the effects of redox homeostasis on cell differentiation. There is growing evidence that Keap1-Nrf2 activation can alter differentiation outcome; however, the effects vary from the stimulation to inhibition of differentiation depending on the cell type as well as the chemical properties and dose of the stimulus used to modulate Nrf2 [8].

In the quest for new AML treatments, natural products derived from traditional Chinese medicine (TCM) are attractive clinical candidates due to the long-proven use of their traditional therapy effects in China, that is, Pishuang (arsenic trioxide) and Xionghuang (realgar). *Lithospermum erythrorhizon* Sieb. et Zucc., referred to as “Zicao” in Chinese and “Shikon” in Japanese, is a plant that grows in the western Xin Jiang region of China and is used as a “heat clearing and blood cooling” medicine. Zicao root extracts have been used in Chinese traditional medicine for many years as a cancer treatment [9]. Its use for this purpose, however, is missing from several current pharmacopoeias of TCM and may be less common than the applications already described. Shikonin, the main naphthoquinone compound of Zicao root, was recently reported as having prooxidative activities and proapoptotic effects on various cancer cells [10]. In reviewing the literature in AML treatments, no data were found on the association between Shikonin and the prodifferentiated effect. To better understand the chemotherapeutic effects of Shikonin, the present study aims to determine whether Shikonin induces differentiation in HL-60 cells. We also studied the role of cellular redox homeostasis in association with Shikonin treatments.

## 2. Materials and Methods

**2.1. Reagents.** Shikonin was purchased from Chengdu Biopurify Phytochemicals Ltd. (purity 98%, Chengdu, China). Fetal bovine serum (FBS) was purchased from Sijiqing (Hangzhou Sijiqing Co., China). Nitroblue tetrazolium (NBT), methyl thiazolyl tetrazolium (MTT), Hoechst 33258 and 33342, phorbol-12-myristate-13-acetate (PMA), 2,7-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA), L-S, R-buthionine sulfoximine (BSO), catalase (CAT), N-acetyl-L-cysteine (NAC), superoxide dismutase (SOD), Tiron (4,5-dihydroxybenzene-1,3-disulfonate), glutathione diethyl ester (GSH-EE), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5-chloromethylfluorescein diacetate (CMF-DA) was purchased from Molecular Probes (Eugene, OR, USA), and gentamicin was

obtained from Shandong Sunrise Pharmaceutical Co., Ltd. (Zibo, China). All other chemicals were of analytical grade and were commercially available.

**2.2. Cell Culture and Treatments.** HL-60 cells were purchased from the ATCC (Manassas, VA, USA) and cultured in Iscove's modified Dulbecco's medium (ATCC) with L-glutamine, 10% FBS and 10  $\mu$ g/mL gentamicin at 37°C in 5% CO<sub>2</sub>. For all experiments, HL-60 cells were seeded at  $0.3 \times 10^6$  cells/mL. Cells were treated with Shikonin at the indicated concentration for 72 h either alone or in combination with a redox modulator for 4 h pretreatment prior to Shikonin treatment. After pretreatments, medium was replaced to avoid the additive or synergistic interaction between redox modulators and Shikonin. The concentrations used for the redox modulators are as follows: CAT (25 U/mL), NAC (100 mM), Tiron (200  $\mu$ M), GSH-EE (2 mM), and BSO (5 mM).

**2.3. Cell Viability Assay.** The cytotoxic activity of Shikonin was evaluated using the MTT assay. HL-60 cells were harvested during the exponential phase of growth and plated in 96-well plates. Shikonin was prepared in DMSO and subsequently diluted with medium prior to use. The final concentration of DMSO was less than 0.1%. The vehicle control group received the same amount of DMSO. The cells were exposed to Shikonin for 72 h, and then 10  $\mu$ L of 5 mg/mL of MTT was added to each well for 4 h. The reaction was halted by the addition of 150  $\mu$ L of DMSO, and the absorbance (A) at 550 nm was determined by a spectrophotometry (Varioskan Flash 3001, Thermo, USA). The cell inhibition rate was calculated as  $100\% \times (\text{control group A values} - \text{experimental group A values}) / \text{control group A values}$ .

**2.4. Determination of Cell Differentiation.** Two morphological assays for cell differentiation were used in this study. HL-60 cells were cultured in 0, 50, 75, and 100 ng/mL of Shikonin for 72 h. After the treatment, single-cell suspensions were prepared and loaded into a centrifuge tube followed by centrifugation at  $1000 \times g$  in a cytospin centrifuge. The slides were fixed with methanol, air-dried, and stained for 10 min with Giemsa staining solution. After staining, the slides were rinsed in deionized water and air-dried again prior to optical analysis. To observe the morphological changes of cell nuclei, Shikonin-treated cells were washed twice with cold PBS, fixed with 4% paraformaldehyde, and stained with Hoechst 33258 (2.5 mg/mL). Cells were assessed with regard to size, regularity of the cell margin, and morphological characteristics of the nuclei [11, 12]. The samples were observed using a fluorescence microscope (Axio Observer, Zeiss, Germany). The degree of differentiation was assayed by the ability of the cells to reduce nitroblue tetrazolium (NBT) into insoluble blue-black formazan upon stimulation with PMA. Each cell suspension (100  $\mu$ L) was mixed with an equal volume of 2 mg/mL NBT dissolved in PBS containing 1  $\mu$ g/mL PMA and incubated at 37°C for 30 min. The reaction was halted with the addition of 0.4 mL of cold 2 M HCl. The formazan product was obtained by

centrifugation of the sample at  $700 \times g$  for 10 min. The supernatant was discarded, and the formazan was dissolved in  $600 \mu\text{L}$  of DMSO. The percentage of NBT-positive cells with formazan deposits in the cytoplasm was determined by a spectrophotometry at 572 nm. The data are expressed as the percentage of the control value.

**2.5. Determination of Intracellular ROS, GSH, and GSSG/GSH.** Cells were treated with the indicated chemical(s) for 4 h, washed with PBS and treated with  $20 \mu\text{M}$  H<sub>2</sub>DCFDA (Ex/Em = 488 nm/525 nm) or  $5 \mu\text{M}$  CMFDA (Ex/Em = 492 nm/517 nm) at 37°C for 30 min. The cells were washed twice with PBS, and the relative fluorescence intensity was read by spectrophotometry. The ROS and GSH levels were calculated as the mean fluorescence intensity (MFI) per 1,000 nonnecrotic cells, and measurement of both probes in a single cell was performed using a fluorescence microscope. The cell numbers were calculated by staining with Hoechst and propidium-iodide (Pi) [13, 14]. Standard curves were made by a series of cell numbers in 96-well plates ranging from  $5 \times 10^6$  to  $5 \times 10^3$ . Cells were collected, sonicated, and mixed with either  $1.15 \mu\text{g/mL}$  of Hoechst-33342 or  $20 \mu\text{g/mL}$  of Pi dye at a 1:9 ratio and maintained at 37°C for 30 min. DNA-associated Hoechst and Pi fluorescence was measured at 460 nm and 620 nm, respectively. Subtracting necrotic cells (Pi stained cells) from the total number of cells (Hoechst stained cells) gave the number of nonnecrotic cells in each treatment. As for the assay of GSSG/GSH, Shikonin-treated cells was collected by centrifugation at  $500 \times g$  10 min and washed twice with PBS. The harvested cells were resuspended in protein removal reagent and vortexed vigorously. The samples were frozen rapidly and thawed twice with liquid nitrogen at 37°C and then at 4°C for 5 min. The supernatant was collected by centrifugation at  $10,000 \times g$  for 10 min. The GSH/GSSG ratio was determined using a GSH and GSSG assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions.

**2.6. RNA Extraction and Real-Time RT-PCR.** To analyze the expression levels of CD11b/CD14 (after 72 h Shikonin treatment) and the Nrf2 downstream target genes (after 4 h Shikonin treatment), cells were treated as indicated, washed with PBS, and collected. The primer sequences are described in Table 1 [12, 15–18]. RNA was extracted from cells using the EZ-10 Spin Column Total RNA Minipreps Super Kit (Bio Basic Inc.) according to the manufacturer's instructions. Two micrograms of RNA per sample was converted to cDNA using the PrimeScript RT Reagent Kit (TaKaRa). Relative gene expression was quantified using real-time PCR (Rotor Gene Q, QIAGEN, USA) with a SYBR-Green Kit (QIAGEN), enabling real-time detection of PCR products according to the manufacturer's protocol. The cDNA was submitted to real-time PCR using the primer pairs as given below. The PCR primers' (synthesized by Shanghai Sangon Biotechnology Co., Ltd.) cycling conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, Tm (°C) for 30 s, and 72°C for 30 s. The relative amount of target mRNA was calculated by the  $2^{-\Delta\Delta\text{Ct}}$  method.

Each sample was analyzed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous reference gene for mRNA normalization.

**2.7. Statistical Analysis.** There are 3 replicates at least performed in each experiment. Data obtained from different experiments are presented as the mean  $\pm$  standard deviation of at least three independent experiments and evaluated by ANOVA. Student's *t*-test for multiple comparisons was used to identify differences among groups. Values were considered to be statistically significant at  $P < 0.05$ .

### 3. Results

**3.1. Shikonin Inhibits the Proliferation of HL-60 Cells.** Previous studies have determined that the optimal proapoptotic dose range of Shikonin is 1.25 to  $10 \mu\text{mol/L}$  (360 to 2883 ng/mL) [19]. The experiments in this study used a lower concentration range of Shikonin (25 to 1600 ng/mL) to study its inhibition on HL-60 proliferation.

The optimal time point for harvesting cells was set at 72 h based on previous observations in HL-60 cells [6]. HL-60 cell proliferation was inhibited in a dose-dependent manner compared to that of the control group after 72 h of treatment, whereas the inhibition rates showed a significant "S" shape curve (Figure 1(a)). As the concentration of Shikonin increased from 50 to 200 ng/mL, there was a rapid increase in the inhibition rates. At 100 ng/mL, Shikonin had an inhibition rate of 48.1%, which was almost equivalent to the IC<sub>50</sub> value. The lower concentrations of Shikonin (<100 ng/mL) inhibited HL-60 cells proliferation, but no necrotic cells were observed and there were few apoptotic cells (Figure 1(b)). These results indicated that the inhibitory effect of Shikonin on HL-60 cells proliferation was not due to directly killing the HL-60 cells. Therefore, we selected a relatively lower concentration range from 0 to 100 ng/mL to study the mechanism involved in the nonkilling effects for the following experiments.

**3.2. Shikonin Induces the Morphology Changes of HL-60 Cells.** Cells grown under the influence of Shikonin quickly exhibited morphological changes that were characteristic of more mature cells in the monocytic pathway. As shown in Figure 1(b), the Giemsa and Hoechst stained undifferentiated control HL-60 cells were found to be predominantly myelocytes with round, regular cell margins and large nuclei, which indicated that the cells were highly malignant with regard to DNA synthesis and that they proliferated at a rapid rate.

The treatment with Shikonin at the indicated concentrations resulted in an increase in cell size and the reduction of the nuclear-cytoplasmic ratio, suggesting that these cells were less active with regard to DNA synthesis. There was no horseshoe morphology observed in the nuclei of the untreated cells, but such morphology was clearly observed in the nuclei of cells treated with Shikonin, which suggests monocytic differentiation. Additionally, the population of horseshoe-shaped nuclei increased significantly after Shikonin treatment in a dose-dependent manner (Figure 1(b)).

TABLE 1: Primer sequences used.

Gene	Primers	Tm (°C)
CD14 [15]	sense: 5'-AAAGGACTGCCAGCCAAGCT-3' anti-sense: 5'-GATTCCCGTCCAGTGTTCAGGT-3'	58
CD11b [12]	sense: 5'-CAGAGCGTGGTCCAGCTTCA-3' anti-sense: 3'-CCTTCATCCGCCGAAAGTCA-5'	58
NQO1 [16]	sense: 5'-GAGGACCTCCTTCAACTATGCC-3' anti-sense: 5'-CCTTTGTCATACATGGCAGCG-3'	59
GCLC [17]	sense: 5'-ATGATGCCAACGAGTCTGAC-3' anti-sense: 5'-CGCCTTTGCAGATGTCTTTC-3'	57
GCLM [17]	sense: 5'-AGGAGCTTCGGGACTGTATT-3' anti-sense: 5'-TGGGCTTCAATGTCAGGGAT-3'	57
GST [18]	sense: 5'-ATGGGCCTAGAGCTGTTTCT-3' anti-sense: 5'-AGCCCAGGTACTCATGAACA-3'	57
GPX [18]	sense: 5'-GGGGCCTGGTGGTCTCGGCT-3' anti-sense: 5'-CAATGGTCTGGAAGCGGCGGC-3'	65
CAT [17]	sense: 5'-AACTGGGATCTTGTGGGAA-3' anti-sense: 5'-GACAGTTCACAGGTATCTG-3'	55
GR [18]	sense: 5'-GAAAAGGCTGTAATTTTATTTCAA-3' anti-sense: 5'-ATCAAAAGTCCCTTCCTCTGC-3'	55
GAPDH	sense: 5'-CAAGGTCATCCATGACAACCTTG-3' anti-sense: 5'-GTCCACCACCCTGTTGCTGTAG-3'	57

**3.3. Shikonin Increases NBT Reductive Activity in HL-60 Cells.** HL-60 cells can differentiate into phagocytic cells, which, when activated, generate superoxide anions as a method to eliminate phagocytized pathogens. Measurement of superoxide anion production by using the NBT assay provides information about the differentiation status [20]. Accordingly, this assay was performed to show the induction of mature differentiation by Shikonin. In Shikonin-treated cells, the NBT reductive activity increased in a dose-dependent manner after 72 h of treatment, indicating that Shikonin can induce HL-60 cell differentiation (Figure 1(c)).

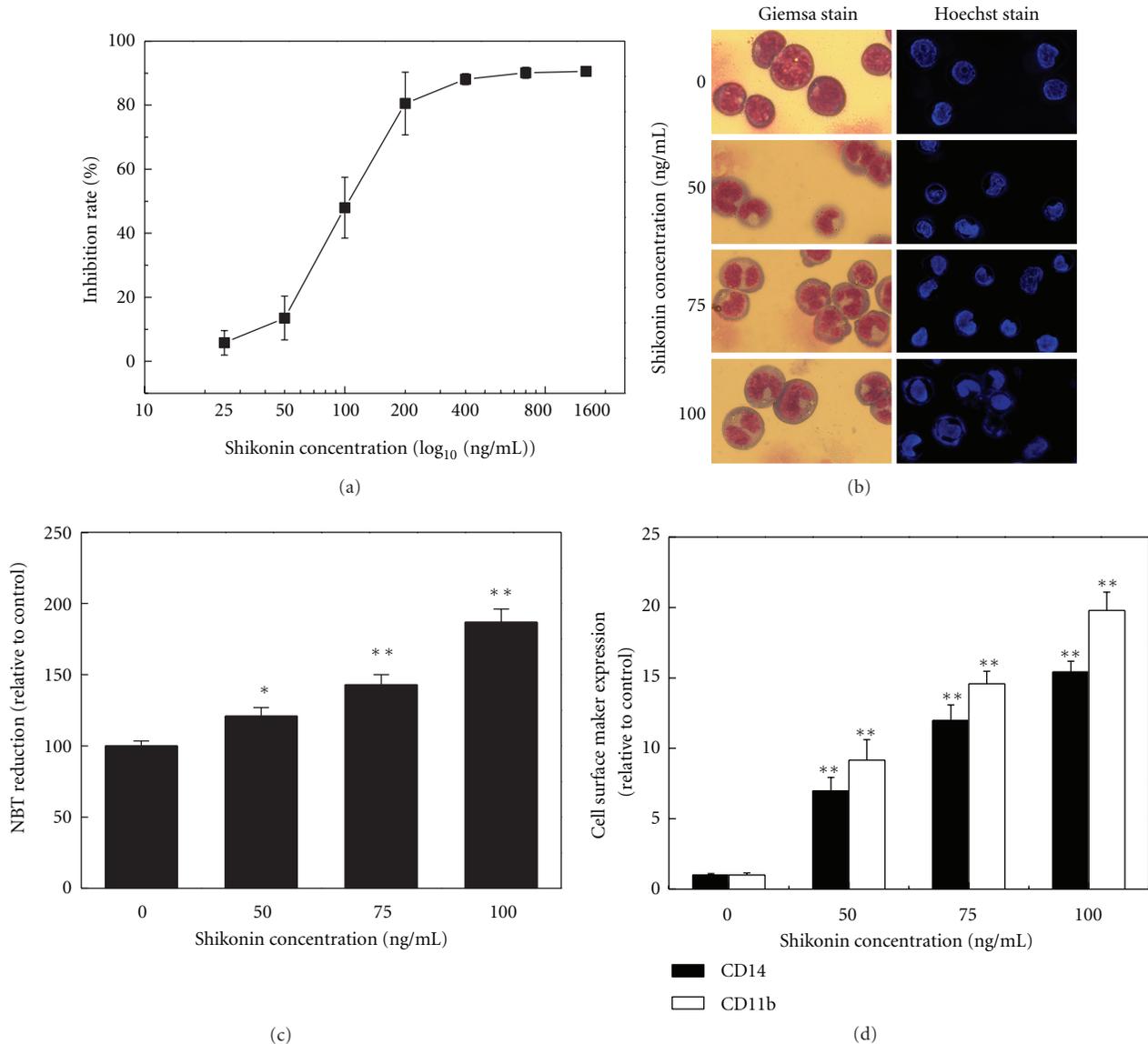
**3.4. Shikonin Increases the mRNA Expression of CD11b and CD14 in HL-60 Cells.** The expression of the monocytic and myelomonocytic surface markers CD14 and CD11b is one of the important characteristics of differentiation in HL-60 cells [12, 15, 21, 22]. To confirm the differentiation in Shikonin-treated HL-60 cells, the gene expression levels of CD11b and CD14 were determined. The results show that the mRNA expression levels of CD11b and CD14 were elevated by Shikonin treatment in a dose-dependent manner (Figure 1(d)).

**3.5. The Intercellular Redox Homeostasis Shifts Towards Oxidation in the Shikonin-Induced HL-60 Cell Differentiation.** The fluorescence probes H<sub>2</sub>DCFDA and CMF-DA were used to evaluate the level of the intracellular redox products ROS and GSH in HL-60 cells (Figure 2(a)). The time of exposure to Shikonin was limited to 4 h, the time frame in which ROS events are initiated prior to drug-induced differentiation, as described in previous works [3]. The

relative DCF fluorescence density (mean ROS production) significantly increased with Shikonin treatment ranging from 0 to 100 ng/mL, whereas the relative CMF fluorescence density (mean GSH production) decreased inversely to the Shikonin concentration (Figures 2(b) and 2(c)). To define the precise changes of intercellular redox homeostasis, the ratio of the total reduced glutathione GSH to its oxidized product GSSG was analyzed after Shikonin treatment. The results show that treatment with Shikonin consistently reduced the GSH/GSSG ratio (Figure 2(d)).

**3.6. The Intercellular Redox Homeostasis Is Closely Connected with Shikonin-Induced HL-60 Cell Differentiation.** To understand whether the changes in intracellular redox homeostasis were important determinants for Shikonin-induced cell differentiation, it is necessary to correlate the level of produced ROS and/or GSH with the extent of the event. The addition of the prooxidant BSO, as described in the methods, significantly enhanced ROS by reducing GSH in Shikonin-treated cells (Figures 3(a) and 3(b)). The Shikonin-induced cell differentiation was further facilitated in terms of the enhanced superoxide anion production and the increased mRNA expression levels of CD11b and CD14 (Figures 3(c) and 3(d)).

In this study, both enzymatic and nonenzymatic antioxidative agents were used to identify the key redox events involved in Shikonin-induced differentiation. When the GSH precursors NAC and GSH-EE were added, GSH production was increased and concurrent with the alleviated ROS production (Figures 3(a) and 3(b)). In contrast to the BSO pretreatment, both GSH precursors suppressed Shikonin-induced cell differentiation significantly in terms of reduced



**FIGURE 1: Shikonin inhibits proliferation and induces differentiation in HL-60 cells.** (a) The effects of Shikonin on HL-60 cell proliferation. The inhibition ratio of cell proliferation was determined by the MTT assay after a 72 h incubation with Shikonin as indicated concentrations. The effects of Shikonin on the morphologic changes (b), NBT-reducing activity (c), and CD11b and CD14 mRNA expression (d) of HL-60 cells treated with 0, 50, 75, and 100 ng/mL Shikonin for 72 h. The vehicle control group (0 ng/mL) received 0.01% DMSO. The differentiation of the HL-60 cells was defined as an increase in NBT absorbance at 572 nm. The cells were collected by cytopsin centrifugation, stained with either Giemsa (left column) or Hoechst 33258 (right column), and then observed by microscopy (400x). Transcript levels were monitored by real-time PCR analysis and normalized to levels of GAPDH. All data are presented as the mean  $\pm$  SEM of the samples from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  compared to the control group.

NBT reductive activity and the decreased mRNA expression levels of CD11b and CD14 (Figures 3(c) and 3(d)). Tiron, another nonenzymatic antioxidant, also inhibited Shikonin-induced cell differentiation and showed a remarkable recovery effect on the Shikonin-induced decrease in GSH (Figures 3(b), 3(c), and 3(d)).

After the enzymatic antioxidative agents SOD and CAT, which function as ROS scavengers, were added as pretreatments prior to Shikonin treatment, a similar trend was

observed in the suppression of ROS production but also in the restoration of GSH (Figures 3(a) and 3(b)). The kinetics of NBT reductive activity and the mRNA expression levels of CD11b and CD14 in differentiating HL-60 cells in the presence of the SOD and CAT are shown in Figures 3(c) and 3(d). The individual addition of both enzymes suppressed Shikonin-induced HL-60 cell differentiation, and SOD showed a stronger inhibition compared to CAT. Additionally, all redox modulators in present work proved no distinct

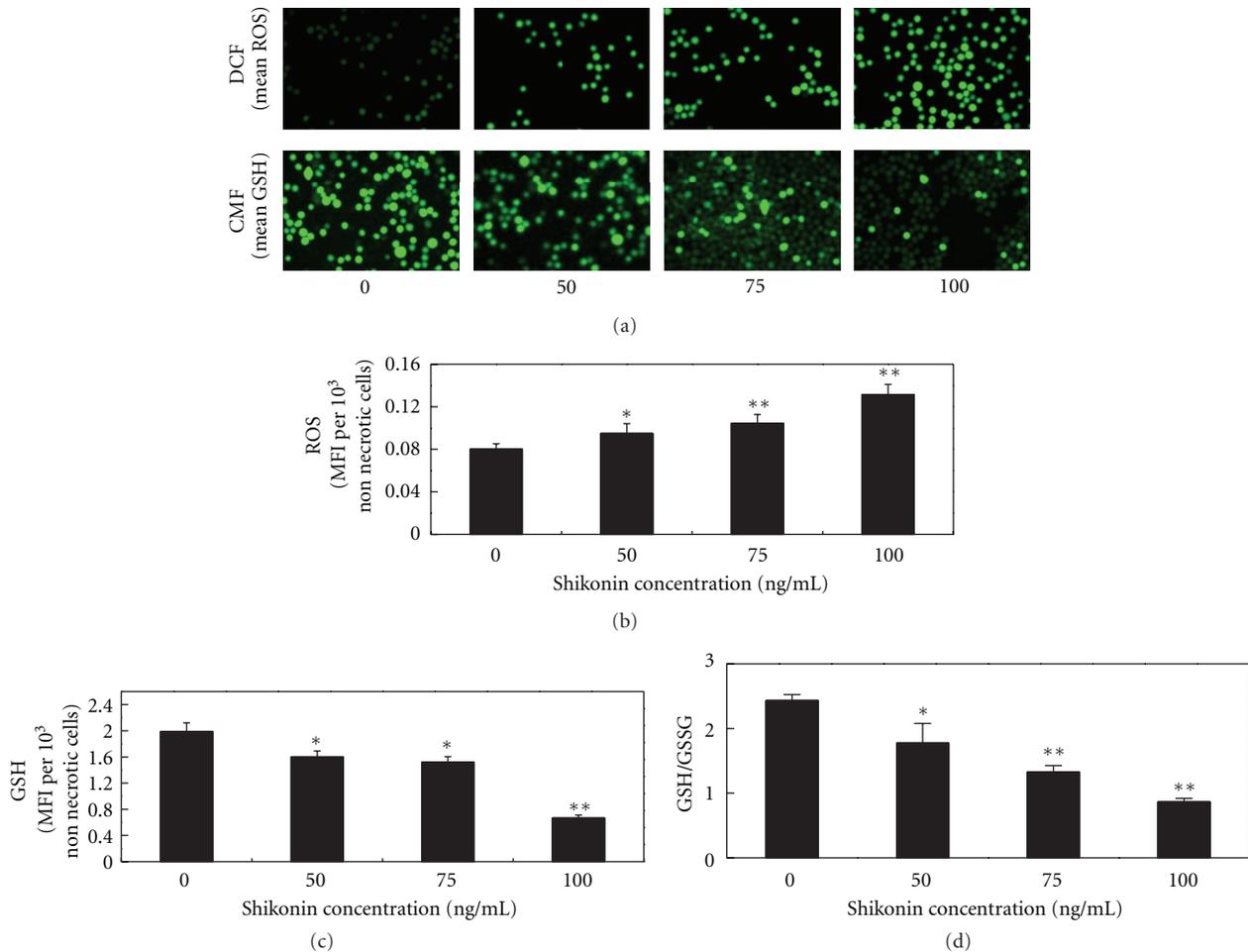


FIGURE 2: The changes of intercellular redox homeostasis in HL-60 cells prior to Shikonin-induced differentiation. (a) The fluorescence probes DCF and CMF were used to evaluate the level of the intracellular redox products ROS and GSH in HL-60 cells by microscopy (40x). The changes of intracellular ROS (b) and GSH (c) levels in HL-60 cells treated with 0, 50, 75, and 100 ng/mL Shikonin for 4 h. The ROS and GSH levels were calculated as the mean fluorescence intensity (MFI) per 1,000 nonnecrotic cells. (d) The changes of the intracellular GSH/GSSG ratios in HL-60 cells treated with 0, 50, 75, and 100 ng/mL Shikonin for 4 h.

effect on HL-60 differentiation and basal levels of NBT reduction and CD11b/CD14 expression (data not shown).

**3.7. The Nrf-2/ARE Signaling Pathway Is Involved in Response to Shikonin Treatment.** Major Nrf2/ARE-regulated gene products are NADP(H):quinone oxidoreductase-1 (NQO1), superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR), glutamate cysteine ligase (GCL), and catalase (CAT) [3, 7]. To determine whether the differentiation-inducing effect of Shikonin is associated with its ability to activate the Nrf2/ARE pathway, these enzymes were analyzed in terms of their mRNA expression levels. As shown in Figures 4(a) and 4(e), the mRNA expression levels of GPX and CAT were markedly upregulated by Shikonin in a dose-dependent manner. At a concentration of 100 ng/mL, the expression level of CAT was 6 times higher compared to the control. However, other ARE genes' expression levels including GR, GST, NQO1, and GCL were suppressed in unison

with increasing Shikonin concentrations up to 100 ng/mL (Figures 4(a), 4(c), and 4(e)).

Based on these observations, we can better understand the kinetics of the Nrf2/ARE signaling pathway in response to Shikonin treatment in HL-60 cells. However, the enhanced or suppressed cell differentiation mediated by those redox modulators from the Nrf2/ARE signaling pathway still requires further detailed explanation. As shown in Figure 4(b), BSO pretreatment facilitated cell differentiation and displayed the highest expression levels of the GPX gene and the lowest expression levels of the GR gene with all Shikonin treatments. In other groups proven to inhibit Shikonin-induced cell differentiation, the GPX expression levels were decreased, but the GR expression levels were increased in all of the groups except for the Tiron-treated group. Interestingly, both enzymatic and nonenzymatic agents reduced CAT gene expression levels more efficiently compared Shikonin treatment alone (Figure 4(f)). The effect of Shikonin on the inhibition of GR, GST, NQO1, and GCL gene expression

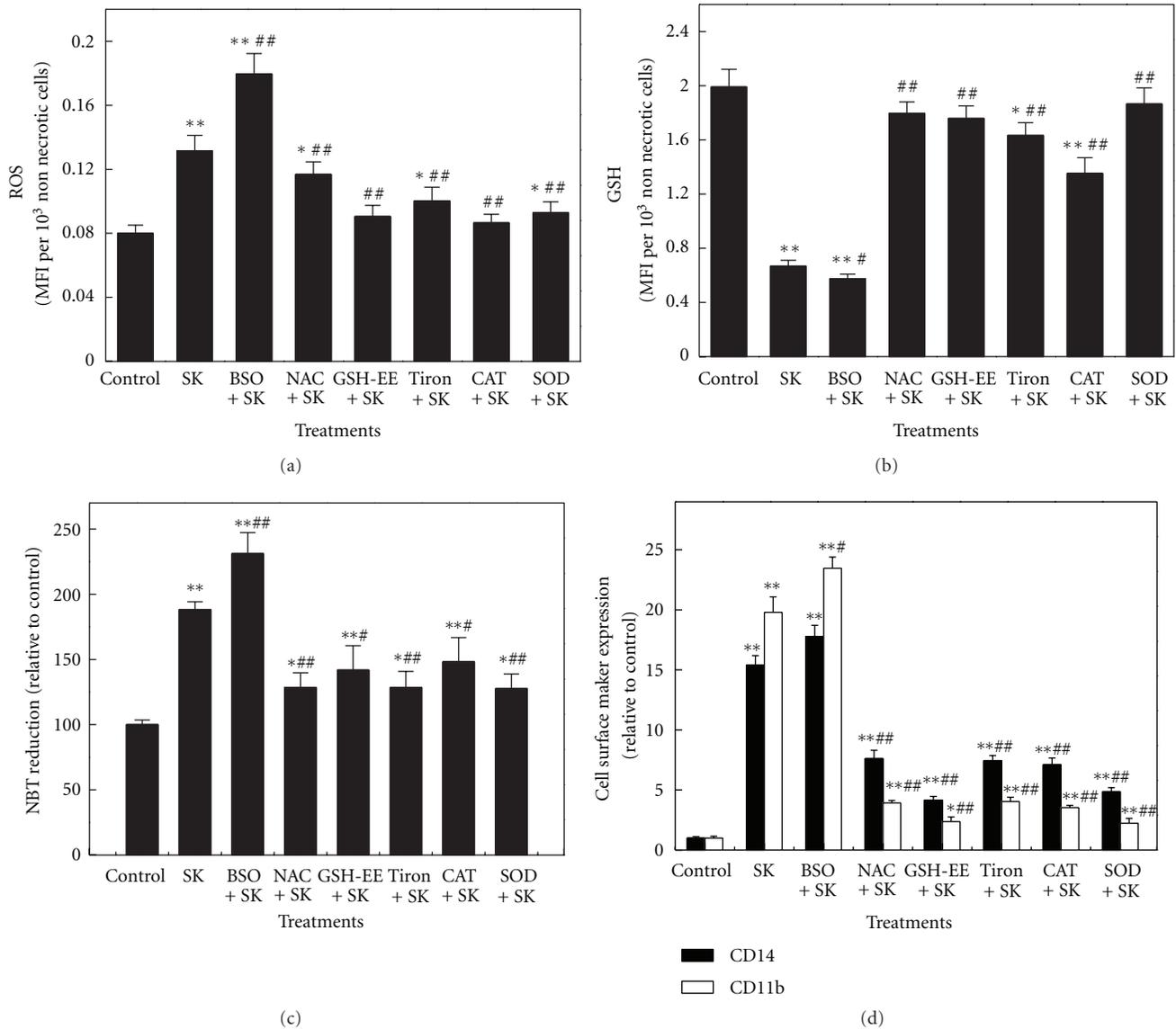


FIGURE 3: The role of intercellular redox homeostasis in Shikonin-induced HL-60 cell differentiation. The effects of the GSH modulators on intracellular ROS (a) and GSH (b) levels in HL-60 cells after 4 h of Shikonin treatment. The effects of the GSH modulators SOD, Tiron, BSO, NAC, CAT, and GSH-EE on NBT-reducing activity (c) and the mRNA expression levels of CD11b and CD14 (d) in HL-60 cells after 72 h of Shikonin treatment. SK mean Shikonin treatment. \* $P < 0.05$ , \*\* $P < 0.01$  compared to the Shikonin-treated (100 ng/mL) group.

levels was blocked at varying degrees with pretreatment with all of anti-oxidants except BSO (Figures 4(b), 4(d), and 4(f)).

#### 4. Discussion

Zicao has been broadly applied as a TCM for thousands of years in China for its traditional therapeutic effects of “heat clearing and blood cooling.” As a major component of Zicao, Shikonin was originally purified by the Japanese chemists Majima and Kuroda, and its pharmacological activities were characterized, such as accelerating tissue granulation proliferation and exerting antibacterial and anti-inflammatory

and antitumor effects [23]. Shikonin has been reported to exert cytotoxicity against various tumor cell lines [23]. Our present study found that the  $IC_{50}$  value of Shikonin on HL-60 cells was approximately 100 ng/mL ( $3.47 \times 10^{-7}$  M), which is similar to an NCI report (HL-60 (TB),  $\log_{10}IC_{50} = -7.5$ ) (<http://dtp.nci.nih.gov/>) (see Supplementary Material available online at doi:10.1155/2012/781516). Previously reported data suggest that leukemia cell lines are the most sensitive cancer cell lines to Shikonin. The killing activities of Shikonin were observed in a range of 360 to 2883 ng/mL in terms of cell apoptosis and/or necrosis [19]. These cytotoxic events were also observed in the present work when

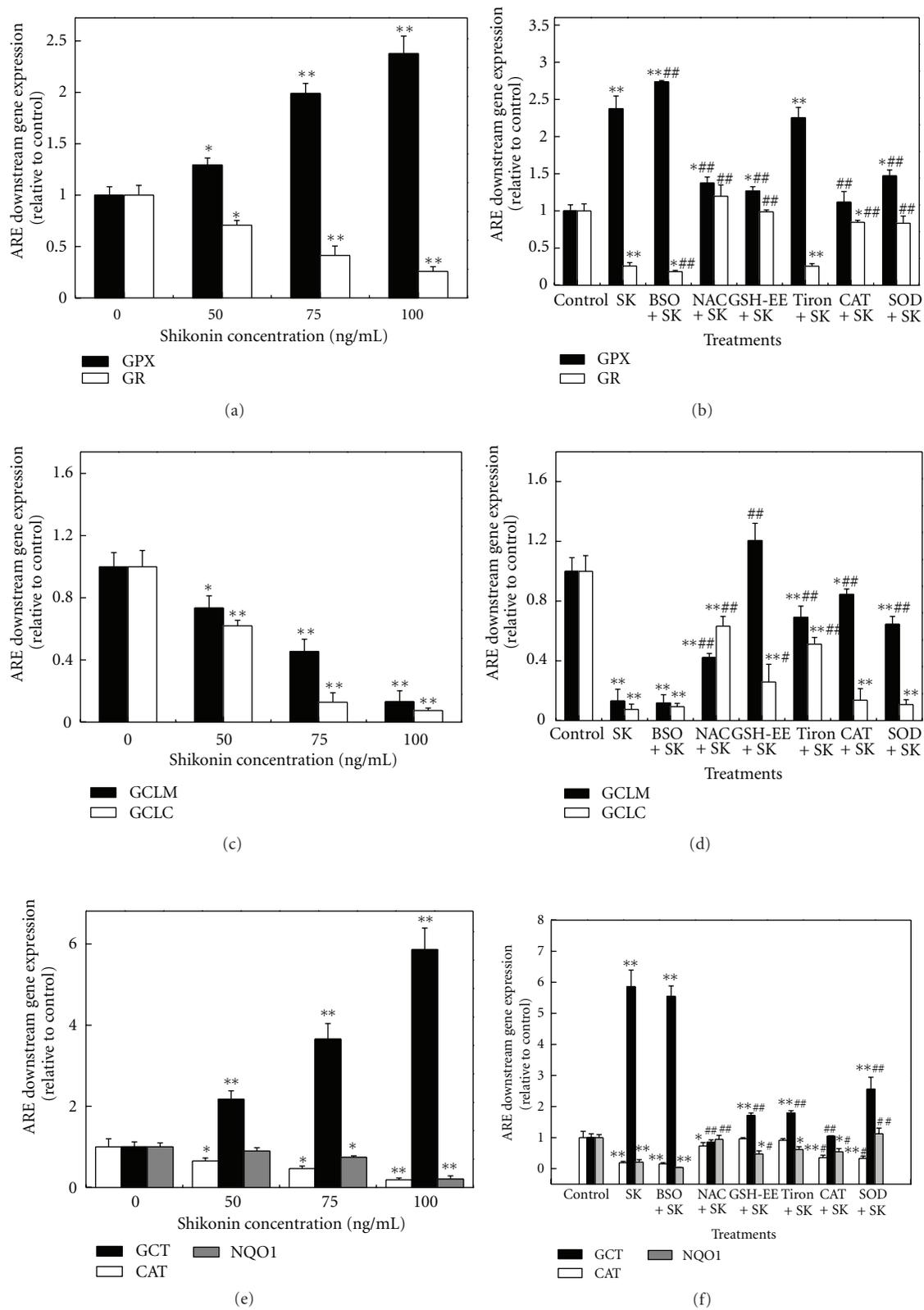


FIGURE 4: The modulation of the Nrf2/ARE pathway downstream target genes in Shikonin-induced HL-60 cell differentiation. The mRNA expression levels of GPX and GR genes (a), GCLM and GCLC genes (c), and GST, CAT, and NQO1 genes (e) in HL-60 cells after 4 h of treatment with 0, 50, 75, or 100 ng/mL of Shikonin. The effects of the GSH modulators on the mRNA expression levels of GPX and GR genes (b), GCLM and GCLC genes (d), and GST, CAT, and NQO1 genes (f) in HL-60 cells treated with Shikonin for 4 h.

the Shikonin concentration was higher than 200 ng/mL (data not shown). The effects of the cellular inhibition unrelated to cell death not only were attributed to the applied lower doses of Shikonin ( $\leq 100$  ng/mL), but also highlight the sensitivity of leukemia cells to Shikonin.

Leukemia cells exhibit immature features, such as immature development and scattered, large, and irregular nuclei as well as other biochemical function errors [1, 3, 24]. Cell morphology, NBT-reducing ability, and the presence of cell surface antigens CD11b/CD14 were considered to be specific markers of differentiation in HL-60 cells [20–22]. These identifiers for differentiation were widely used when modern pharmacological efforts were made for seeking new AML chemotherapeutic agents in TCM. The present study shows that the Shikonin-treated cells exhibited characteristics of mature monocytic differentiation in terms of relatively small and deformed nuclei as well as the enhanced production of superoxide anions and the increased gene expression of the cell surface antigens CD11b/CD14. Shikonin at 50 ng/mL ( $0.174 \mu\text{M}$ ) showed a significant prodifferentiation activity after 72 h treatment. In a study of Jian et al.,  $0.1 \mu\text{M}$  ATRA could induce a remarkable HL-60 cell differentiation with the same duration [25]. Although there are quite some differences in the parameters of those two studies, Shikonin was proved to be an attractive leading compound in searching new AML chemotherapeutics. The proapoptotic activities were nearly proven prior to the prodifferentiation activities in other TCM such as arsenic trioxide and realgar [4, 5, 26]. Those studies report that the extent of cell growth limitation was closely correlated to the doses of additional agents. It was proposed that an agent exhibits its cytotoxicity initially on differentiation, followed by apoptosis and necrosis with increasing doses [7]. This phenomenon is similar to the observed prodifferentiation effects on HL-60 cell treated with lower concentrations of Shikonin (50 to 100 ng/mL).

Unique ROS metabolism in myeloid leukemia may represent a therapeutic target [3]. The variety of ROS levels in Shikonin treatment was a primary focus in this study. We observed a rapid enhancement in ROS production and GSH depletion as a preliminary effect of Shikonin-induced HL-60 cell differentiation, which is in agreement with previously published proapoptotic reports [19, 27]. Shikonin has also been reported to generate ROS and electrophilic molecules [28]. Nevertheless, other literatures revealed that Shikonin shows highly efficient antioxidative activities against several types of ROS, such as singlet oxygen ( $^1\text{O}_2$ ), superoxide anion radicals ( $\text{O}_2^-$ ), hydroxyl radicals ( $\bullet\text{OH}$ ), and *tert*-butyl peroxy radical ( $\text{BuOO}\bullet$ ) as well as iron-dependent microsomal lipid peroxidation [29]. In this context, a simple explanation for Shikonin-induced differentiation was difficult given the production of multiple types of ROS. The three most important redox systems within the cells are NADPH/NADP<sup>+</sup>, thioredoxin ( $\text{TRX}_{\text{red}}/\text{TRX}_{\text{ox}}$ ), and glutathione (GSH/GSSG). Among these systems, GSH/GSSG is the most important because glutathione concentrations are approximately 500–1000-fold higher than NADPH and TRX; thus, changes in reduced/oxidized glutathione buffer directly reflect intracellular redox alterations coupled to various cell processes [7]. Redox homeostasis is thought to play an important

role in HL-60 apoptosis and differentiation [1, 3, 8]. The redox homeostasis of GSH/GSSG in Shikonin-treated cells was shifted towards oxidation in a dose-dependent manner, suggesting higher levels of oxidative stress prior to the induced differentiation.

To determine the decisive role of redox homeostasis in differentiation rather than death, more evidence was needed to test this idea via the activity of both enzymatic and nonenzymatic antioxidants as well as prooxidants in Shikonin treatment. Pretreatments with the redox modulators separately prior to drug treatment were proved to be a reasonable approach according to literatures [30, 31]. In the study of Krance et al.,  $\text{VD}_3$ -treated cells incubated with diethyl maleate (a GSH depletor) followed by BSO had higher expression of differentiation marker CD11b than unexposed cells [8]. In addition, treatment with BSO alone had no effect on control cells. In Krance's GSH depletion system, BSO was seemed as a weak GSH depletor. In order to avoid self recovery of GSH in HL-60 cells,  $100 \mu\text{M}$  BSO was employed in whole treatment to sustained GSH depletion effects by other reagents. In our experiment, BSO and other GSH modulators were used in pretreatment for only 4 h, when higher concentration of BSO (5 mM) was selected to keep a GSH depletion effect (Figure 3(b)). In such context, it can be acceptable that BSO and other redox modulators have no significant effect on basal levels of cell differentiation markers. Further, HL-60 cell differentiation was enhanced with specific depletion of GSH when pretreated with BSO, a selective and irreversible inhibitor of GCL, which coordinates GSH synthetase to perform GSH synthesis in the cytoplasm [7]. However, HL-60 cell differentiation was suppressed with enhanced GSH production when pretreated with the GSH precursors NAC and GSH-EE. SOD is a superoxide anion scavenger, which catalyzes the reaction of  $2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2$ , and CAT clears a variety of peroxides. This study shows that SOD exerts a stronger inhibition on Shikonin-induced HL-60 cell differentiation compared to CAT. All of the specific modulators of ROS and GSH were sufficient to interrupt redox homeostasis in unique ways. However, Tiron, a vitamin E analog, showed nonspecific ROS scavenging and exhibited inhibitory effects on Shikonin-induced cell differentiation. Though neither the direct production of ROS by Shikonin nor the involvement of the superoxide anion release from NADPH oxidase in HL-60 cells can be ruled out, the data presented here suggest a mechanism involved in Shikonin-induced differentiation. Enhanced ROS production in addition to GSH depletion by a submicromolar concentration of Shikonin induced mild stress in the HL-60 cells, which resulted in the fine modulation of the intercellular redox homeostasis to facilitate differentiation [3, 4, 7, 23].

Based on the above discussion, we elaborated on a hypothesis explaining the possible reactions involved in Shikonin-induced HL-60 cell differentiation. Among various signaling pathways, the Nrf2/ARE pathway and redox homeostasis are tightly connected in cells [1]. Evidence indicates a regulatory role of the Nrf2/ARE pathway to supply GSH and other antioxidants to buffer ROS and other electrophilic molecules [1, 8]. In the study of Ahmed et al., their results show that Shikonin was able to induce Hsp70 at  $0.1 \mu\text{M}$

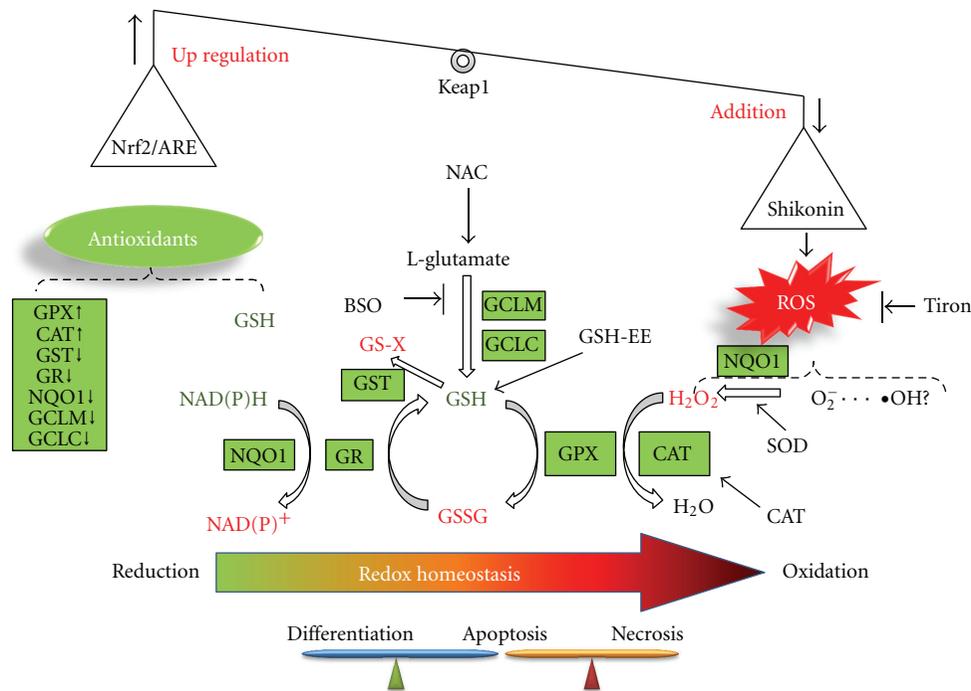


FIGURE 5: Schematic representation of the role of redox homeostasis in Shikonin-treated acute promyelocytic leukemia HL-60 cells. Low doses (<100 ng/mL) of Shikonin exert very mild oxidative stress via unique modulation of the Nrf2/ARE pathway by shifting the cells from proliferation to differentiation. However, higher concentrations of Shikonin result in the predominance of cell death because the oxidative stress is more severe and overcome the antioxidative capacity of Nrf2/ARE pathway, resulting in cell death.

(28.8 ng/mL) by a 3 h treatment, which also show a significant increase in the Nrf2-mediated oxidative stress response in a myeloid cell line U937, a line that is closely related to HL-60's [32]. Therefore, we sought to determine whether the application of Shikonin caused differential expression of the Nrf2 downstream target genes involved in the redox changes in HL-60 cells. In the construction of the GSH system mentioned above, GCL, a key enzyme to perform GSH synthesis as GR, is a heterodimer that is composed of a catalytic subunit (GCLC) and a modulatory subunit (GCLM) [17]. GPX was responsible for GSH oxidation to GSSG by  $H_2O_2$  [7]. It was suggested that Shikonin suppressed gene expression of the enzymes (GR, GCLC, GCLM) but enhanced GPX gene expression levels to compensate for the GSH depletion in the present study. GST is also responsible for the transformation and consumption of reducing matter (GSH) [33]. The primary metabolic function of NQO1 is the reduction of quinones to hydroquinones by consuming a reducing matter (NADPH). The embedded  $O_2^-$  scavenging activity of NQO1 is attractive because it could play a potential role in minimizing the deleterious consequences of the generation of redox active hydroquinones, which also raises the issue of the relevance of this effect in cells where there are more efficient systems for superoxide removal such as SOD [34]. We observed decreases in the GST and NQO1 expression levels by Shikonin, which were markedly restored by pretreatment with all of the tested antioxidants

except for BSO. These results suggested that the production of hydroquinones and glutathione-S-conjugates is reduced. When coupled with the oxidative stress induced by Shikonin treatment, these findings emphasize the sensitivity of GST in response to GSH depletion. A higher dose of Shikonin causes apoptosis in human glioma cells by interrupting intracellular redox homeostasis, which included CAT downregulation and SOD-1 upregulation as well as decreased Bcl-2 and increased Bax expression levels [28]. However, our study shows that the CAT gene expression levels were upregulated significantly in conjunction with GPX to scavenge the  $H_2O_2$  produced by Shikonin treatment. We noticed that both enzymatic and nonenzymatic antioxidative agents reduced the CAT and GPX gene expression levels more efficiently compared to Shikonin treatment alone. Furthermore, treatment with BSO caused GSH depletion in addition to the highest expression levels of the GPX gene. This evidence suggests that both enzymes play an important role in response to Shikonin-induced oxidative stress coupled with GSH depletion as well as  $H_2O_2$  production. Altogether, as shown in Figure 5, these changes in the major Nrf2/ARE-regulated gene products displayed a unique method of Shikonin-induced HL-60 differentiation.

Collectively, our study is the first investigation of the prodifferentiation effect of Shikonin on HL-60 cells, specifically the role of redox homeostasis in regulating HL-60 cell differentiation. Further studies are needed to explain

the exact mechanisms that activate Nrf2 signal pathway and the involvement of other C/EBP family of transcription factors in Shikonin treatment.

## Abbreviations

AML:	Acute myeloid leukemia
ARE:	Antioxidant responsive element
BSO:	L-S,R-buthionine sulfoximine
CAT:	Catalase
CMF-DA:	5-chloromethylfluorescein diacetate
DMSO:	Dimethyl sulfoxide
FBS:	Fetal bovine serum
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GCL:	Glutamate cysteine ligase
GCLC:	Catalytic subunit of glutamate cysteine ligase
GCLM:	Modulatory subunit of glutamate cysteine ligase
GPX:	Glutathione peroxidase
GR:	Glutathione reductase
GSH:	Reduced glutathione
GSH-EE:	Glutathione diethyl ester
GSSG:	Oxidized glutathione
GST:	Glutathione S-transferase
H <sub>2</sub> DCFDA:	2,7-dichlorodihydrofluorescein diacetate
MTT:	Methyl thiazolyl tetrazolium
NAC:	N-acetyl-L-cysteine
NBT:	Nitroblue tetrazolium
NQO1:	NADP(H):quinine oxidoreductase-1
Nrf2:	NF-E2-related factor 2
PMA:	Phorbol-12-myristate-13-acetate
ROS:	Reactive oxygen species
SK:	Shikonin
SOD:	Superoxide dismutase
TCM:	Traditional Chinese medicine
Tiron:	4,5-dihydroxybenzene-1,3-disulfonate.

## Authors' Contribution

These authors contributed equally in this paper.

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## References

[1] I. Bobilev, V. Novik, I. Levi et al., "The Nrf2 transcription factor is a positive regulator of myeloid differentiation of acute

myeloid leukemia cells," *Cancer Biology and Therapy*, vol. 11, no. 3, pp. 317–329, 2011.

- [2] A. S. Tsiptsoglou, I. S. Pappas, and I. S. Vizirianakis, "Mechanisms involved in the induced differentiation of leukemia cells," *Pharmacology and Therapeutics*, vol. 100, no. 3, pp. 257–290, 2003.
- [3] P. S. Hole, R. L. Darley, and A. Tonks, "Do reactive oxygen species play a role in myeloid leukemias?" *Blood*, vol. 117, no. 22, pp. 5816–5826, 2011.
- [4] D. Sumi, Y. Shinkai, and Y. Kumagai, "Signal transduction pathways and transcription factors triggered by arsenic trioxide in leukemia cells," *Toxicology and Applied Pharmacology*, vol. 244, no. 3, pp. 385–392, 2010.
- [5] L. W. Wang, Y. L. Shi, N. Wang, B. D. Gou, T. L. Zhang, and K. Wang, "Association of oxidative stress with realgar-induced differentiation in human leukemia HL-60 cells," *Chemotherapy*, vol. 55, no. 6, pp. 460–467, 2009.
- [6] D. F. Li, Z. H. Wang, H. M. Chen et al., "Isoliquiritigenin induces monocytic differentiation of HL-60 cells," *Free Radical Biology and Medicine*, vol. 46, no. 6, pp. 731–736, 2009.
- [7] F. Q. Schafer and G. R. Buettner, "Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple," *Free Radical Biology and Medicine*, vol. 30, no. 11, pp. 1191–1212, 2001.
- [8] S. M. Krance, P. C. Keng, J. Palis, and N. Ballatori, "Transient glutathione depletion determines terminal differentiation in HL-60 cells," *Oxidative Medicine and Cellular Longevity*, vol. 3, no. 1, pp. 53–60, 2010.
- [9] V. P. Papageorgiou, A. N. Assimopoulou, E. A. Couladouros, D. Hepworth, and K. C. Nicolaou, "The chemistry and biology of alkannin, shikonin, and related naphthazarin natural products," *Angewandte Chemie*, vol. 38, pp. 270–301, 1999.
- [10] X. Mao, C. R. Yu, W. H. Li, and W. X. Li, "Induction of apoptosis by shikonin through a ROS/JNK-mediated process in Bcr/Abl-positive chronic myelogenous leukemia (CML) cells," *Cell Research*, vol. 18, no. 8, pp. 879–888, 2008.
- [11] D. S. Kim, S. H. Kim, J. H. Song, Y. T. Chang, S. Y. Hwang, and T. S. Kim, "Enhancing effects of ceramide derivatives on 1,25-dihydroxyvitamin D<sub>3</sub>-induced differentiation of human HL-60 leukemia cells," *Life Sciences*, vol. 81, no. 25–26, pp. 1638–1644, 2007.
- [12] N. Wang, L. W. Wang, B. D. Gou, T. L. Zhang, and K. Wang, "Realgar-induced differentiation is associated with MAPK pathways in HL-60 cells," *Cell Biology International*, vol. 32, no. 12, pp. 1497–1505, 2008.
- [13] A. M. Tonary and J. P. Pezacki, "Simultaneous quantitative measurement of luciferase reporter activity and cell number in two- and three-dimensional cultures of hepatitis C virus replicons," *Analytical Biochemistry*, vol. 350, no. 2, pp. 239–248, 2006.
- [14] K. Wrobel, E. Claudio, F. Segade, S. Ramos, and P. S. Lazo, "Measurement of cytotoxicity by propidium iodide staining of target cell DNA application to the quantification of murine TNF- $\alpha$ ," *Journal of Immunological Methods*, vol. 189, no. 2, pp. 243–249, 1996.
- [15] S. Caprodossi, M. Pedinotti, C. Amantini et al., "Differentiation response of acute promyelocytic leukemia cells and PML/RAR $\alpha$  leukemogenic activity studies by real-time RT-PCR," *Molecular Biotechnology*, vol. 30, no. 3, pp. 231–238, 2005.
- [16] T. W. Fischer, M. A. Zmijewski, B. Zbytek et al., "Oncostatic effects of the indole melatonin and expression of its cytosolic and nuclear receptors in cultured human melanoma cell lines," *International Journal of Oncology*, vol. 29, no. 3, pp. 665–672, 2006.

- [17] H. R. Lee, J. M. Cho, D. H. Shin et al., "Adaptive response to GSH depletion and resistance to L-buthionine-(S,R)-sulfoximine: involvement of Nrf2 activation," *Molecular and Cellular Biochemistry*, vol. 318, no. 1-2, pp. 23–31, 2008.
- [18] M. G. Baigi, L. Brault, A. Néguesque et al., "Apoptosis/necrosis switch in two different cancer cell lines: influence of benzoquinone- and hydrogen peroxide-induced oxidative stress intensity, and glutathione," *Toxicology in Vitro*, vol. 22, no. 6, pp. 1547–1554, 2008.
- [19] W. Han, J. Xie, L. Li, Z. Liu, and X. Hu, "Necrostatin-1 reverts shikonin-induced necroptosis to apoptosis," *Apoptosis*, vol. 14, no. 5, pp. 674–686, 2009.
- [20] E. L. Batista, M. Warbington, J. A. Badwey, and T. E. van Dyke, "Differentiation of HL-60 cells to granulocytes involves regulation of select diacylglycerol kinases (DGKs)," *Journal of Cellular Biochemistry*, vol. 94, no. 4, pp. 774–793, 2005.
- [21] A. E. Barton, C. M. Bunce, R. A. Stockley, P. Harrison, and G. Brown, "1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> promotes monocytopoiesis and suppresses granulocytopoiesis in cultures of normal human myeloid blast cells," *Journal of Leukocyte Biology*, vol. 56, no. 2, pp. 124–132, 1994.
- [22] D. Brackman, F. Lund-Johansen, and D. Aarskog, "Expression of leukocyte differentiation antigens during the differentiation of HL-60 cells induced by 1,25-dihydroxyvitamin D<sub>3</sub>: comparison with the maturation of normal monocytic and granulocytic bone marrow cells," *Journal of Leukocyte Biology*, vol. 58, no. 5, pp. 547–555, 1995.
- [23] T. Efferth, H. Miyachi, and H. Bartsch, "Pharmacogenomics of a traditional Japanese herbal medicine (Kampo) for cancer therapy," *Cancer Genomics and Proteomics*, vol. 4, no. 2, pp. 81–91, 2007.
- [24] J. Schwarz, P. Stöckbauer, J. Souček, and K. Smetana, "Features of immaturity in cells derived from granulocytic differentiation inducer treated human myeloid leukaemia (ML-1) cells," *Leukemia Research*, vol. 11, no. 10, pp. 869–876, 1987.
- [25] P. Jian, Z. W. Li, T. Y. Fang et al., "Retinoic acid induces HL-60 cell differentiation via the upregulation of miR-663," *Journal of Hematology and Oncology*, vol. 4, article 20, 2011.
- [26] C. Yedjou, P. Tchounwou, J. Jenkins, and R. McMurray, "Basic mechanisms of arsenic trioxide (ATO)-induced apoptosis in human leukemia (HL-60) cells," *Journal of Hematology and Oncology*, vol. 3, article 28, 2010.
- [27] W. Han, J. Xie, Y. Fang, and Z. Wang, "Pan H. Nec-1 enhances shikonin-induced apoptosis in leukemia cells by inhibition of RIP-1 and ERK1/2," *International Journal of Molecular Sciences*, vol. 13, pp. 7212–7225, 2012.
- [28] C. H. Chen, M. L. Lin, P. L. Ong, and J. T. Yang, "Novel multiple apoptotic mechanism of Shikonin in human glioma cells," *Annals of Surgical Oncology*, vol. 19, no. 9, pp. 3097–3106, 2012.
- [29] D. Gao, M. Kakuma, S. Oka, K. Sugino, and H. Sakurai, "Reaction of  $\beta$ -alkannin (shikonin) with reactive oxygen species: detection of  $\beta$ -alkannin free radicals," *Bioorganic and Medicinal Chemistry*, vol. 8, no. 11, pp. 2561–2569, 2000.
- [30] P. Bondza-Kibangou, C. Millot, V. E. Khoury, and J. M. Millot, "Antioxidants and doxorubicin supplementation to modulate CD14 expression and oxidative stress induced by vitamin D<sub>3</sub> and seocalcitol in HL60 cells," *Oncology Reports*, vol. 18, no. 6, pp. 1513–1519, 2007.
- [31] J. Savickiene, G. Treigyte, A. Gineitis, and R. Navakauskiene, "A critical role of redox state in determining HL-60 cell granulocytic differentiation and apoptosis via involvement of PKC and NF- $\kappa$ B," *In Vitro Cellular and Developmental Biology—Animal*, vol. 46, no. 6, pp. 547–559, 2010.
- [32] K. Ahmed, Y. Furusawa, Y. Tabuchi et al., "Chemical inducers of heat shock proteins derived from medicinal plants and cytoprotective genes response," *International Journal of Hyperthermia*, vol. 28, no. 1, pp. 1–8, 2012.
- [33] D. M. Townsend and K. D. Tew, "The role of glutathione-S-transferase in anti-cancer drug resistance," *Oncogene*, vol. 22, no. 47, pp. 7369–7375, 2003.
- [34] A. T. Dinkova-Kostova and P. Talalay, "NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector," *Archives of Biochemistry and Biophysics*, vol. 501, no. 1, pp. 116–123, 2010.



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