Research Article

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

Bo Zhang, 1 Na Chen, 1 Hongmei Chen, 1 Zhenhua Wang, 1 and Qiusheng Zheng 1, 2

1 Key Laboratory of Xinjiang Endemic Phytomedicine Resources of Ministry of Education, School of Pharmacy, Shihezi University, Shihezi 832002, China
2 Life Sciences School, Yantai University, Yantai 264005, China

Correspondence should be addressed to Qiusheng Zheng, zqsyt@sohu.com

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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 isoiquiritigenin 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, NFκB, IKKβ 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 and “Shikon” in Japanese, is a plant that grows in the

2. Materials and Methods

2.1. Reagents. Shikonin was purchased from Chengdu Biopurify Pharmaceutical Co., Ltd. (purity 98%, Chengdu, China). Fetal bovine serum (FBS) was purchased from Sijiqing (Hangzhou Sijiqing Co., China), nitroblue tetrazolium (NBT), phorbol-12-myristate-13-acetate (PMA), 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA), L-S,R-buthionine sulfoximine (BSO), catale (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 μg/mL gentamicin at 37°C in 5% CO2. For all experiments, HL-60 cells were seeded at 0.3 × 106 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 μM), 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 μL of 5 mg/mL of MTT was added to each well for 4 h. The reaction was halted by the addition of 150 μL 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% × (control group A values − experimental group A values)/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 × g in a cytopsin 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 μL) was mixed with an equal volume of 2 mg/mL NBT dissolved in PBS containing 1 μg/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 × g for 10 min. The supernatant was discarded, and the formazan was dissolved in 600 μL of DMSO. The percentage of NBT-positive cells with formazan deposits in the cytoplasm was determined by a spectrophotometry at 572 nm.

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 ± 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 μmol/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 IC50 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 monocyte 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)).
The fluorescence probes H$_2$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)).
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 cytospin 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 ± 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
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):quinine 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
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 IC50 value of Shikonin on HL-60 cells was approximately 100 ng/mL (3.47 × 10−7 M), which is similar to an NCI report (HL-60 (TB), log10IC50 = −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 (≤ 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 μM) showed a significant prodifferentiation activity after 72 h treatment. In a study of Jian et al., 0.1 μM 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 (¹O₂), superoxide anion radicals (O₂•−), hydroxyl radicals (•OH), and tert-butyl per-oxyl radical (BuOO•) 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⁺, thioredoxin (TRXₓ/gl/TRXₓ), 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/oxygen 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., VD₃-treated cells incubated with diethyl maleate (a GSH depleter) 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 depleter. In order to avoid self recovery of GSH in HL-60 cells, 100 μM 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 2O₂•− + 2H⁺ → H₂O₂ 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 μM
(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 H2O2 [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 O2− scavenging activity of NQO1 is attractive because it could play a potential role in minimizing the deleterious consequences of the generation of reducto 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 H2O2 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 H2O2 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  
H2DCFDA: 2,7-dichlorodihydrofluorescein diacetate  
MTT: Methyl thiazolyl tetrazolium  
NAC: N-acetyl-L-cysteine  
NBT: Nitroblue tetrazolium  
NQO1: NADP(H):quinone 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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