Original Paper

Anti-Leukemic Activity of Shikonin: Role of ERP57 in Shikonin Induced Apoptosis in Acute Myeloid Leukemia

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Key Words

Acute Myeloid Leukemia • HL-60 • Shikonin • ERP57 • Apoptosis • ER-Stress

Abstract

Background/Aims: ER-Stress and activation of unfolded protein response belong to the major factors involved in chemoresistance in cancer cells. In this study we investigated the effect of shikonin on the survival of acute myeloid leukemia cells and the role of ER-stress protein ERP57, a protein disulfide isomerase, in improvement of chemotherapy. **Methods:** Using MTT assay we studied cytotoxic effects of shikonin on HL-60 cells. The flow cytometry was adopted to examine the shikonin induced mode of cell death in HL-60 cells. The overall protein expression alteration resulting from shikonin treatment was investigated using proteomics methods. Western blotting was performed to quantify the alteration in protein expression in HL-60 after shikonin treatment. Silencing and overexpression studies were carried out to highlight the therapeutic role of ERP57 in shikonin effect on AML cells. **Results:** Shikonin induces apoptosis in HL-60 cells without significant effect on Primary cells from healthy volunteers. The apoptotic effect was dose and time dependent and was accompanied by strong alteration in cell proteome. Among the proteins targeted by shikonin, ERP57 was significantly downregulated in HL-60 after treatment. Compared to healthy control ERP57 was found to be highly expressed in AML cell line HL60 and was downregulated after shikonin treatment. Overexpression of ERP57 protected HL-60 from shikonin induced apoptosis, whereas knockdown of ERP57 expression resulted in increase in shikonin induced apoptosis. **Conclusions:** Our results demonstrate that ERP57 plays a crucial role in resistance towards shikonin induced apoptosis in AML cells. Targeting of ERP57 might offer a new therapeutic option for the treatment of acute myeloid leukemia.

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Introduction

Acute myeloid leukemia (AML) is heterogeneous group of malignant disorder characterized at cellular level by an imbalance between cell proliferation and differentiation

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[1, 2]. AML is still an incurable disease and innovative treatment strategies are urgently needed [3]. Unfolded protein response (UPR) is one of the factors playing an important role in chemoresistance in cancer cells [4, 5]. UPR is an evolutionary conserved mechanism and is activated to compensate the adverse effects of protein accumulation in the endoplasmic reticulum (ER), following the induction of ER stress [6]. UPR induced activation of both proapoptotic and survival pathway allow eukaryotic cells to acclimatize to ER stress [7]. Protein folding is facilitated by different ER resident chaperones like the glucose regulated protein GRP78, calreticulin, calnexin and protein disulfide isomerases (PDI) [8]. ERP57 (PDIA3) is a member of protein disulfide isomerase and is also a glucose-regulated protein [9, 10]. It plays a major role as a folding enzyme in quality control in the secretory pathway. In mammalian cells, the UPR consists of three transmembrane ER stress sensor proteins-protein kinase RNA-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1), and the activating transcription factor 6 (ATF6) [11]. These three molecules function as individual activators of transcriptional (IRE1, ATF6) and translational (PERK) programs. The UPR initiates transient attenuation of protein translation, degradation of misfolded proteins, and induction of molecular chaperones like calreticulin and GRP78 [12]. UPR is therefore cytoprotective and allow the cells to adapt to the environmental and developmental conditions that invade ER protein folding [13]. However, severe and prolonged ER stress may leads to apoptosis rather than being cytoprotective [14]. The collective activities of PERK, IRE1 and ATF6 leads to increased expression of effector stress response (XBP-1, ATF4 and ATF6) and proapoptotic transcription factor (CHOP) additionally enhancing the expression of ER resident chaperones, such as GRP78 and GRP94 [4, 15].

Shikonin (C₁₆H₁₆O₅) is a naturally occurring naphthoquinone present in the root tissues of herb *Lithospermum erythrorhizon*. Shikonin shows pleiotropic effects including anti-tumor effect, accelerating tissue granulation proliferation, anti-ischemic, anti-inflammatory, and anti-bacterial [16, 17]. The antitumor activities of shikonin are mediated through its ability to down-regulate antiapoptotic proteins like Bcl-2 and Bcl-xL, oxidative stress and caspase activation [18-20]. Shikonin has also been reported to inhibit EGFR phosphorylation and cell cycle arrest through increased p53 expression [21, 22]. Shikonin also activates MAP kinases such as ERK2, JNK, and p38, which are implicated in the upstream regulation of apoptosis [23-25]. Due to its effective anticancer properties and circumventing drug resistance in cancer cells [26], we selected this compound for this study.

We demonstrate here that HL-60 and AML primary cells exhibit high level of ERP57, which was significantly downregulated after shikonin treatment. Shikonin also leads to downregulation of other ER stress protein markers from AML cell line to primary AML cells. Our results uncover the role of ERP57 in protection against shikonin induced apoptosis.

Materials and Methods

Reagents

RPMI-1640 was purchased from Gibco. L-glutamine, urea and DTT were from Sigma. Culture flasks were from Falcon, CHAPS [(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate] was from MERCK. Precision plus protein marker and Bio-LyteH were from Bio-Rad. BSA was from Roche. Colloidal Coomassie blue staining (Roti-Blue) was from Carl-Roth. Annexin V-fluorescein isothiocyanate and propidium iodide were from Invitrogen. Ficoll, Tunicamycin and Bacitracin were purchased from Sigma-Aldrich.

Cell culture and treatments

The human myeloid cell line HL-60 was obtained from ATCC and culture were maintained in RPMI-1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen) and 50U/mL penicillin. The peripheral blood samples were obtained from normal healthy donors at UMG, Goettingen, Germany, after written informed consent in compliance with the Declaration of Helsinki 2002. Peripheral blood mononuclear cells (PBMCs) were separated by ficoll-hypaque density gradient (1 g/ml) centrifugation method. Subsequently, the isolated cells (10⁶/ml) were cultured in complete RPMI 1640

medium supplemented with 10% FBS.

Shikonin was obtained from Calbiochem. 10 mM stock solution was prepared in DMSO. For sequential treatments, cells were treated for 24 h, 48 h and 72 h with increasing doses of shikonin. To study the ER-stress induced apoptosis, cells were pretreated with 10 µg/ml tunicamycin for 6 h before 2.5 µM shikonin treatment for further 24 h. Further, to study the role of ERP57 in protection of shikonin induced apoptosis, HL-60 cells were pretreated with 300 µM Bacitracin for 6 h before 2.5 µM shikonin treatments for next 24 h.

Measurement of cell viability

6 x 10³ cells/well was seeded in 96-well plated and treated with different doses of shikonin for 24 h, 48 h and 72 h. Cell viability was assessed using cell Proliferation Kit I (MTT), a colorimetric assay for the non-radioactive quantification of cell proliferation and viability (Roche Applied Bioscience, Mannheim, Germany).

Analysis of Apoptosis by flow cytometry

To determine apoptosis rate, cells were stained with Annexin/PI after 24 h of shikonin treatment. Samples were incubated at 37°C for 15 minutes and analysed on a Becton Dickinson FACS Calibur.

Western Blot analysis

Whole cell protein extracts were obtained from HL-60 cells. 50 µg cell extracts were run on 12% SDS PAGE after denaturation with Laemmli buffer subsequently western blot analysis was performed as described previously [27]. Antibodies against mouse ERP57 (Enzo lifesciences), rabbit GRP78 (sigma), goat calreticulin (Abcam), rabbit IRE1A(Cell Signalling Technology), rabbit GRP94 (Sigma), mouse cleaved ATF6 (Acris Antibodies) and mouse beta actin (Sigma) were used as primary antibodies, diluted in blocking buffer, then added to the membrane and incubated at 4°C for overnight. Molecular probes AlexaFlour 647 goat anti-mouse IgG antibody or AlexaFlour 647 goat anti-rabbit IgG antibody or AlexaFlour 647 donkey anti-goat IgG antibody were used as secondary antibody. Before imaging, the blots were dried in the dark. The blot membranes were scanned at a resolution of 50 µm on a Fuji FLA-5100 scanner (Fuji Photo, Kanagawa, Japan) with single laser-emitting excitation light at 635 nm and 670 nm, respectively.

2-D gel electrophoresis

2-D gel electrophoresis was performed as described previously [28]. For protein identification, 2-DE gels were additionally stained with colloidal Coomassie blue, Roti-Blue (Roth, Karlsruhe, Germany) overnight. Cells were washed with PBS and the resulting pellet was solubilized in lysis buffer (9.5 M urea, 2% w/v CHAPS, 2% v/v ampholytes, and 1% w/v DTT) at 4°C for 30 min, centrifuged (5 min, 13,000 g), and the protein concentration of the supernatant was determined as described above. The Fluorescent images were captured in 16-bit TIFF files format. Spot matching across gels and normalization based on the internal standard was performed using Delta2D software (Decodon, Greifswald, Germany). For protein identification, 2-DE gels were post-stained with colloidal Coomassie blue (Roti-Blue) overnight. Differentially regulated proteins were excised and processed for identification by MS.

Protein identification from 2-DE gels

In-gel digestion and peptide extraction were carried out as described previously [28]. Subsequently the extracted peptides were subjected to peptide sequence analysis. The samples were dissolved in 0.1% formic acid and processed as described previously [28]. Processed data were searched against MSDB and Swissprot databases through the Mascot search engine using a peptide mass tolerance of 50 ppm (parts per million) and fragment tolerance of 100 mmu (millimass unit). Protein identifications with at least two peptides sequenced were considered significant.

Transfection

For siRNA-mediated gene knockdown, 4 x 10⁶ cells were transfected with siRNA oligonucleotide specific for the knockdown of ERP57 expression (Sense Strand: 5' ACC TCG TCC TTC ACA TCT CAC TAA CAT CAA GAG TGT TAG TGA GAT GTG AAG GACTT 3'), (Antisense strand: 3' CAA AAA GTC CTT CAC ATC TCA CTA ACA CTC TTG ATG TTA GTG AGA TGT GAA GGACG-5') were designed in our laboratory and synthesized by Eurofins MWG Operon (Germany) as control for knock-down efficiency Non Specific Controls from Eurofins

MWG were used. HL-60 cells with 70% confluence were transfected with the siRNA ERP57 using transfection reagent, Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfection reagent was removed after 6 h replaced with normal complete culture medium.

For the overexpression of ERP57, 70% confluent HL-60 cells were transfected either with pcDNA3.1-ERP57 (a gift from Dr. Neil Bulleid University of Glasgow) or empty pcDNA3.1 using transfection reagent, LTX reagent with PLUS reagent (Invitrogen) according to the manufacturer's protocol. After 6 h of transfection media was replaced with normal complete media supplemented with 0.5 mg/ml G-418 (Invitrogen) as a selection factor for stable transfection.

Both overexpression and knockdown of ERP57 was confirmed by performing western blotting.

Statistical Analysis

All the values are represented as mean $\pm$ SEM from at least three independent experiments. Data was analyzed using One-way ANOVA followed by Newman Keuls comparison test. Values with * $P < 0.05$ was considered to be significant. For 2-DE the digitalized images were analyzed; spot matching across gels and normalization were performed using Delta2D 3.4 (Decodon, Braunschweig, Germany). Delta2D computes a 'spot quality' value for every spot detected. This value shows how closely a spot represents the 'ideal' 3D Gaussian bell shape. On the basis of the average spot volume ratio, spots whose relative expression is changed at least two fold (increase or decrease) between the compared samples were considered to be significant. To analyze the significance of protein regulation, Student's t-test was performed, and statistical significance was assumed for * $P < 0.05$. All blots were quantified using the ImageJ software. For comparison between two measures (in the same group) the paired t-test was used.

Results

Shikonin displays potent antileukemic activity

To test the antiproliferative/cytotoxic effect of shikonin (Fig. 1A) against HL-60 (Fig. 1B) and blood cells derived from normal volunteer (Fig. 1C) were treated with increasing doses of shikonin (0.5 - 10 μ M) for 24 h, 48 h and 72 h, and the cell viability was analysed by MTT assay. Shikonin showed significant antileukemic activity in HL-60 cells, with IC₅₀ values 3.83 μ M at 48 h. Shikonin showed no cytotoxic effect against primary cells isolated from healthy donors. Our MTT results clearly showed that shikonin has efficient antileukemic activity without significant effect on normal cells.

Shikonin induces apoptosis in AML cells

To address the question of mode of cell death induced by shikonin, we first examined the morphological features of HL-60 during shikonin treatment using phase contrast light microscopy

Fig. 1. Shikonin inhibits the proliferation of HL-60 cells and Primary AML cells. (A) Structure of shikonin. HL-60 cells and Primary cells from healthy volunteers were treated with indicated doses of shikonin for 24, 48 and 72 h (B-C). Cell viability was measured using Cell Proliferation Kit I (MTT) (Roche Applied Bioscience, Mannheim, Germany).

Fig. 2. Shikonin induces apoptosis in HL-60 cells. (A) Shikonin induced morphological changes were observed in HL-60 cells. HL-60 cells were treated with indicated doses of shikonin for 24 h and morphological changes were observed under the phase contrast microscope. (B) HL-60 cells were treated with increasing doses of shikonin for 24 h and Annexin-V/PI staining was performed to examine the apoptotic cells. (C) Percentage of apoptotic cells after treatment with indicated doses of shikonin for 24 h.

Table 1. List of proteins found to be regulated under shikonin treatment in HL-60 cells. Listed are the gene and proteins names of non-redundant proteins, nominal mass of the proteins, calculated isoelectric point, and the MS/MS Score

Protein name	Gene name	Swiss-prot	Nominal mass	CPI	MS/MS-Score
Superoxide Dismutase Cu-Zn	SOD1	SODC_HUMAN	16154	5.7	58
Tropomycin alpha-3	TPM3	TMP3_HUMAN	32987	4.68	99
Alpha Enolase	ENO1	ENOA_HUMAN	47481	7.01	118
Vinculin	VCL	VINC_HUMAN	124292	5.5	84
GRP78	HSPA5	GRP78_HUMAN	72402	5.07	79
Protein disulfide -isomerase	P4HB	PDIA1_HUMAN	57480	4.76	194
Tubulin beta chain	TUBB	TBB5_HUMAN	50095	4.78	78
Serine/Arginin-rich splicing factor 1	SRSF1	SRSF1_HUMAN	27842	10.37	97
EF-Hand domain-containing protein D2	EFHD2	EFHD2_HUMAN	267.95	5.15	80
Endoplasmic reticulum resident protein 29	ERP29	ERP29_HUMAN	29032	6.77	84
Tropomycin alpha-3	TPM3	TMP3_HUMAN	32987	4.68	61
Protein disulfide -isomerase A3	PDIA3	PDIA3_HUMAN	57146	5.98	203
Calreticulin	CALR	CALR_HUMAN	48255	4.29	95
Prohibitin	PHB	PHB_HUMAN	29843	5.57	98
Eukaryotic translation initiation factor 4E type 1B	EIF4E1B	I4E1B_Human	27750	6.68	167
Collagen alpha-1 chain	COL23A1	CONA1_HUMAN	52254	6.88	230
Myelin basic protein	MBP	MBP_HUMAN	33097	9.79	135
Protein RUFY3	RUFY3	RUFY3_HUMAN	53216	5.36	237
Glycin receptor subunit alpha-2	GLRA2	GLRA2_HUMAN	52425	9.06	142
Transgelin	TAGLN	TAGLN_HUMAN	22653	8.87	124
Elongation factor 1-beta	EF1B2	EF1B_HUMAN	24919	4.5	227
Zinc-finger protein 442	ZNF442	ZN44_HUMAN2	75325	9.03	312
Tropomycin beta chain	TPM2	TMP2_HUMAN	32987	4.68	174
GRIP1-associated protein 1	GRAP1	GRAP1_HUMAN	96273	5.08	140
Ras-related protein Rab-37	RAB37	RAB_HUMAN	25028	5.97	230
Putative inactive carbonic anhydrase 5B-like protein	CA5BP1	CA5BL_HUMAN	22779	10.76	89
RNA-binding motif protein,x chromosome	RBMX	RBMX_HUMAN	42306	10.06	79
Adrenodoxin,mitochondrial	FDX1	ADX_HUMAN	19666	4.9	106
T-complex protein 1 subunit epsilon	CCT5	TCPE_HUMAN	60089	5.45	87
Mettallothionein-1E	MT1	MT1E_HUMAN	7150	8.38	94
Dexamethasone-induced ras related protein 1	RASD1	RASD1_HUMAN	32021	9.15	97
Tubulin beta chain	TUBB	TBB5_HUMAN	50095	4.78	72
Heterogenous nuclear ribonucleoprotein	HNRNPH1	HNRH1_HUMAN	49484	5.89	150
Peptidyl - prolyl cis-trans isomerase A	PPIA	PPIA_HUMAN	18229	7.68	140
Alpha-internexin	INA	AINX_HUMAN	55528	5.34	75
Putative inactive carbonic anhydrase 5B-like protein	CA5BP1	CA5BL_HUMAN	22779	10.76	86
Ribosome biogenesis regulatory protein homolog	RRS1	RRS1_HUMAN	41225	10.69	92

(Fig. 2A). HL-60 cells were treated with various doses of shikonin for 24 h and apoptosis was detected by Annexin V/PI staining. In HL-60 cells, a dose dependent increase in the population of apoptotic cells was observed (Fig. 2B, C). Similar effect was also observed in U937 cell line treated with shikonin (data not shown).

Investigation of the effect of shikonin treatment on HL-60 proteome

To assess global protein expression changes and targeted protein pathways upon shikonin treatment, we applied 2-DE-based proteomics approach. Protein lysates from shikonin-treated and -untreated cells were separated first by IEF followed by SDS-PAGE separation. Protein expression patterns from the two cell extracts were compared with each other using the Delta2D 4.3 software (Decodon GmbH). Two-dimensional protein maps derived from the cell extracts were analyzed with

Table 2. List of proteins which were significantly downregulated after shikonin treatment

Gene Name	Spot volume Ratio Treated/Control
TPM3	0.279
VCL	0.137
P4HB	0.399
SRSF1	0.508
ERP29	0.158
PDIA3	0.085
EIF4E1B	0.36
MBP	0.332
TMP2	0.279
CA5BP1	0.168
RBMX	0.452
HNRNPH1	0.226
PPIA	0.550
RRS1	0.550
CALR	0.213

conventional 2- DE in the pH ranges 4-7 and in total, 37 protein spots were found to be regulated when comparing the spot volumes. The proteins were successfully identified with highly significant MOWSE score (Table 1). Among these proteins 15 were identified as significantly downregulated (Table 2), whereas 7 were found to be significantly upregulated (Table 3). The identified proteins were found to be associated with different biological functions and a large number of them could be classified into four functional categories (Fig. 3B) (Table 4): the first category grouped the proteins that are known to be involved in ER stress and protein folding (CALR, CCT5, ERP29, PPIA, PDIA3, HSPA5, and P4HB), confirming role of protein folding proteins in AML cell survival. The other three categories involved regulation of apoptosis (CALR, HSPA5, PHB PDIA3, SOD1 and TUBB), cytoskeleton organization (CALR, SOD1, INA and TUBB), and cellular homeostasis (CALR, MBP, P4HB, PDIA3 and SOD1). Similar to Mitoxantrone effect [29], ERP57 downregulation was also observed in HL-60 after shikonin treatment. As shown in proteomic data, Fig. 3A, shikonin treatment induces significant downregulation of ERP57 and calreticulin (CALR) after 24h of incubation.

Table 3. List of proteins which were significantly upregulated after shikonin treatment

Gene name	Spot volume Ratio Treated/Control
ENO1	5.834
RUFY3	5.805
GLRA2	11.027
TAGLN	2.693
EF1B2	1.952
INA	1.960
CA5BP1	1.979

Table 4. Gene ontology functional annotation of proteins, which were regulated in this experiment according to their involvement in different biological processes

Biological process	Proteins	Shk>C	C>Shk
Protein folding	7	CCT5	CALR ERP29 PPIA PDIA3 P4HB HSPA5
Regulation of apoptosis	6	PHB SOD1 TUBB	CALR HSPA5 PDIA3
Cytoskeleton organisation	4	SOD1 TUBB INA	CALR
Cellular homeostasis	5	SOD1	CALR P4HB PDIA3 MBP

Shikonin treatment reduces the ER-stress in AML

To validate the downregulation of ER-stress marker proteins result obtained from proteomics data, we performed western blot analysis in untreated and shikonin treated HL-60 cells. We found that ER-stress marker proteins were downregulated after shikonin treatment. Interestingly ERP57 and CALR were significantly downregulated in HL-60 cells after shikonin treatment both in dose (Fig. 4A) and time dependent (Fig. 4B) manner confirming the result obtained from proteomic data.

To further study the role of ERP57 in AML development, we performed western blotting in blood cells derived from healthy donors. The data showed clearly that healthy donors express very low amount of ERp57 and CALR (Fig. 4C). Further validation of overexpression of ERp57 in AML patients is required to confirm this result with AML primary cells.

ER-stress inducer Tunicamycin diminishes the shikonin induced apoptosis

To further study the role of ER-stress in AML cell progression, we pretreated HL-60 cells with Tunicamycin prior to shikonin treatment. The data clearly demonstrate that Tunicamycin reduced the shikonin induced apoptosis (Fig. 5A). Thus, we conclude that ER-stress is a key factor in developing resistance in AML cells against shikonin. As previous study has shown that inhibition of protein disulfide isomerase (PDI) activity increases apoptosis in response to ER-stress inducers [30], we further investigated the effect of PDIs inhibitor bacitracin on shikonin induced apoptosis. And we found that bacitracin increases shikonin induced

Fig. 4. Shikonin reduces ER-stress in HL-60 cells. (A) HL-60 cells were treated with indicated doses of shikonin for 24h and then analyzed by western blotting. (B) HL-60 cells were treated for different time with shikonin and the expression regulation of ER-stress proteins and CHOP were monitored using western blot (C) Cell lysate were prepared from PBMCs derived from healthy volunteers to study the expression of ERP57 and CALR in healthy donors. Bar charts representing the quantification of the blots. Results are given as the means $\pm$ SD from four independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001.

apoptosis confirming the role of PDIs in protection against shikonin induced apoptosis (Fig. 5A).

As the inhibition of PDIs clearly improve the effect of shikonin, and our proteomic data showed that among the PDIs, ERP57 was targeted by shikonin; we investigated the change

Fig. 5. Tunicamycin diminishes Shikonin induced apoptosis in HL-60 cells. (A) HL-60 cells were pretreated with Tunicamycin or Bacitracin alone 1 h before shikonin treatment and Annexin-V/PI staining was performed to study the role of ER-stress and ERP57 in shikonin induced apoptosis. Percentage of apoptotic cells after pretreatment with Tunicamycin and Bacitracin for 1 h before shikonin treatment. (B) HL-60 cells were pretreated with Tunicamycin or Bacitracin alone 1 h before shikonin treatment and cell lysate were prepared to perform the western blotting.

in expression of ERP57 after combination treatment of shikonin and tunicamycin and our results clearly showed that tunicamycin increased expression of ERP57, was significantly inhibited by shikonin treatment (Fig. 5B). On another way, combination of shikonin and bacitracin resulted in significant down regulation of ERP57 in HL-60 cells (Fig. 5C).

Thus, our results demonstrates that shikonin induced apoptosis is accompanied by downregulation of ERP57. Furthermore tunicamycin mediated up-regulation of ERP57 was inhibited by shikonin. Taken this together the data suggest that ERP57 is a key protein in response to shikonin induced apoptosis in AML cells.

ERp57 is a key player in AML cell response to shikonin treatment

To further study the crucial role of ERP57 in shikonin induced apoptosis; HL-60 cells were transfected prior to shikonin treatment with ERP57 siRNA and pcDNA3.1-ERP57 for knockdown and overexpression of ERP57 respectively (Fig. 6A). Furthermore, knockdown of ERP57 expression resulted in increase in apoptosis in HL-60 (Fig. 6B) cells and in U937 cells (data not shown) treated with shikonin, whereas overexpression of ERP57 resulted in decrease in apoptosis (Fig. 6B). Hence our results suggest that ERP57 is the key player in AML cells response to shikonin treatment.

Discussion

Acute Myeloid Leukemia (AML) is a disastrous haematological malignancy characterized by the rapid growth of abnormal white blood cells [31]. In spite of recent improvement in

Fig. 6. ERP57 downregulation is the key mediator of Shikonin induced apoptosis. (A) HL-60 cells were transfected with pcDNA3.1-ERP57 and ERP57 siRNA and treated with shikonin for 24 h. Cell lysates were prepared for the western blot analysis. (B) Non-transfected, pcDNA3.1-ERP57 and ERP57 siRNA transfected cells were treated with 2.5 μ M shikonin for 24 h and Annexin-V/PI staining was performed to measure apoptotic cells. (C) Percentage of apoptotic cells after treating non-transfected and transfected cells with shikonin for 24 h.

therapeutic strategies, AML remains an incurable disease for majority of patients [30, 32, 33]. Predominantly, development of chemoresistance is a major bottleneck limiting the therapeutic efficacy in AML therapy [34]. Natural products are important source of anti-cancer lead molecule and have shown a promising anticancerous activity against various cancers [35, 36]. Shikonin is a natural product and it targets cancer cells through various mechanisms. For instance, shikonin inhibits cancer cell proliferation by targeting tumor specific pyruvate kinase-M2 (PKM2) [5]. Other mechanisms involve shikonin induced proteasome inhibition, NF κ B inhibition, Thioredoxin reductase (TrxR1) inhibition [37-39]. In addition shikonin also sensitizes cancer cells towards chemotherapeutic drugs used for cancer therapy [40, 41]. Our results showed that shikonin reduced the expression of ER-stress proteins in AML cells which suggests that ER-stress promote AML cell survival.

Disturbance in the expression of ER-stress proteins has an important role in some human diseases, especially those involving tissues dedicated to extracellular protein synthesis [42].

Rapidly proliferating cancer cells need increased ER activity to assist the folding, assembly and transport of membrane and secretory proteins, and are thereby subjected to ER stress [43]. Because of insufficient vascularisation and rapid growth, tumor cells run into growth-limiting conditions such as hypoxia and nutrient deprivation [44]. An inadequate deliver of glucose affects protein glycosylation and the production of ATP, both of which could lead to the accumulation of unfolded proteins in the ER, resulting in ER stress [44]. Thus, in response to ER stress, activation of the UPR has been observed in various tumors [12]. In the case of leukemic cells, retroviral infection, the pathological expression of fusion protein PML-RAR α and the accumulation of reactive oxygen species (ROS) can all induce

ER stress [45-47]. There is strong evidence for the role of UPR in the pathogenesis of many tumor types in humans particularly in rapidly growing solid tumors in which the demand for oxygen and nutrients can exceed the supply until new tumor-initiated blood vessels are formed [48]. In contrast, the role of the UPR during leukemogenesis remains largely unknown. As previous reports suggest that UPR is activated in a substantial subset of AML patients indicating a role of the UPR in the pathogenesis of these leukemias [15, 49]. In our first step, we performed functional proteomics to screen potential mechanism and pathways involved in AML cell progression. Our results clearly showed that treatment of HL-60 cells with shikonin leads to sharp change in the proteins involved in proper folding of newly synthesized polypeptides (CALR, CCT5, ERP29, PPIA, PDIA3 (ERP57), HSPA5, and P4HB). Among all these proteins, ERP57 and Calreticulin were significantly downregulated by shikonin treatment.

ERP57 is a key member of the PDI family of proteins and it is mainly situated in the endoplasmic reticulum. It forms disulfide bonds and acts as a chaperone in nascent proteins [9, 10, 50]. However, reports on non-ER localizations of PDI family members identified PDI also in the cytosol and extracellular area suggesting additional roles for PDI. An interacting partner of ERP57, Calreticulin is another ER stress protein which has been reported to be expressed on leukemic blast cell surface in AML patients and its overexpression suppresses CEBPA protein translation thus blocking AML cell differentiation [51]. Recently, ERP57 has been reported to be a potential therapeutic target in renal fibrosis [27]. Our findings indicate that HL-60 and primary cells are found to overexpress ERP57 which was significantly downregulated by shikonin. We also showed that overexpression of ERP57 significantly reduces shikonin induced apoptosis in HL-60 while knocking down the expression of ERP57 leads to significant increase in shikonin induced apoptosis in HL-60 cells.

In conclusion, to the best of our knowledge, our study for the first time provided evidence that ERP57 is overexpressed in AML cells and is downregulated by shikonin. Shikonin may represent a potential therapeutic drug with promising antileukemic activity. A combination of shikonin with ERP57 inhibitor may further enhance its antileukemic effect.

Disclosure Statement

The authors declare that they have no competing interests.

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