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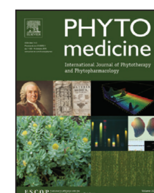

Cryptotanshinone deregulates unfolded protein response and eukaryotic initiation factor signaling in acute lymphoblastic leukemia cells

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A B S T R A C T

Background: Unfolded protein responses (UPR) determine cell fate and are recognized as anticancer targets. In a previous research, we reported that cryptotanshinone (CPT) exerted cytotoxic effects toward acute lymphoblastic leukemia cells through mitochondria-mediated apoptosis.
Purpose: In the present study, we further investigated the role of UPR in CPT-induced cytotoxicity on acute lymphoblastic leukemia cells by applying tools of pharmacogenomics and bioinformatics.
Methods: Gene expression profiling was performed by mRNA microarray hybridization. Potential transcription factor binding motifs were identified in the promoter regions of the deregulated genes by Cistrome software. Molecular docking on eIF-4A and PI3K was performed to investigate the inhibitory activity of CPT on translation initiation.
Results: CPT regulated genes related to UPR and eIF2 signaling pathways. The DNA-Damage-Inducible Transcript 3 (*DDIT3*) gene, which is activated as consequence of UPR malfunction during apoptosis, was induced and validated by *in vitro* experiments. Transcription factor binding motif analysis of the microarray-retrieved deregulated genes in the promoter region emphasized the relevance of transcription factors, such as *ATF2, ATF4* and *XBP1*, regulating UPR and cell apoptosis. Molecular docking suggested inhibitory effects of CPT by binding to eIF-4A and PI3K providing evidence for a role of CPT's in the disruption of protein synthesis.
Conclusion: CPT triggered UPR and inhibited protein synthesis via eIF-mediated translation initiation, potentially supporting CPT-induced cytotoxic effects toward acute leukemia cells.
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Introduction

Proteins have to be folded into specific conformations to properly execute their cellular functions. Besides being a major site for calcium storage and lipid biosynthesis, the endoplasmic reticulum (ER) is an essential organelle for post-translational modifications, structure maturation and correct folding of transmembrane and secretory proteins. These processes require molecular chaperones and enzymes residing in the ER such as oxidoreductases (Braakman and Hebert 2013). Acting as quality control system, the ER exports correctly-folded proteins to the sites of actions and eliminates misfolded proteins through the ER-associated degradation (ERAD) pathway, which disposes misfolded proteins from the ER to the cytosol for ubiquitin-mediated proteolytic degradation (Ruggiano et al. 2014).

Accumulation of misfolded proteins, which is considered to be harmful to cells threatening their survival, results from numerous physiological or pathophysiological factors, such as hypoxia, nutrient deprivation, loss of calcium homeostasis, and elevated uncompleted folding forms of proteins due to mutations and a failure in degradation (Braakman and Hebert 2013). In response to such a cellular condition referred to as ER stress, cells have evolved an adaptive mechanism, termed unfolded protein response (UPR) to maintain cellular homeostasis. UPR is triggered by three major ER-resident transducers: inositol-requiring enzyme-1 (IRE1),

Abbreviations: ALL, acute lymphoblastic leukemia; ATF4, activating transcription factor-4; ATF6, activating transcription factor-6; CHOP, C/EBP homologous protein; CPT, cryptotanshinone; *DDIT3*, DNA damage-inducible transcript 3; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; ERAD, ER-associated degradation pathway; IPA, Ingenuity Pathway Analysis; IRE1, inositol-requiring enzyme-1; mTOR, mammalian target of rapamycin; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PI3K, phosphoinositide-3-kinase; ROS, reactive oxygen species; UPR, unfolded protein responses; XBP1, X-box protein 1.

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protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF6). IRE1 represents a strong homeostatic transcription factor. Upon stimulation, it multimerizes and trans-autophosphorylates, leading to cleavage of X-box protein 1 (*XBP1*) mRNA into a spliced mature form (*XBP1s*). *XBP1s* translocates to the nucleus and induces the transcription of ERAD component genes and genes related to ER chaperones and biogenesis (Plongthongkum et al. 2007). Activated PERK phosphorylates eukaryotic initiation factor 2 (eIF2α) on Ser51 thereby blocking global protein synthesis by decreasing cap-dependent translation from most mRNAs. This in turn alleviates the heavy load of new peptides that require modification and folding in the ER compartment (Yan et al. 2002). Nevertheless, the downstream molecule of PERK, mRNAs encoding *ATF4*, paradoxically sustain translational efficiency, which induces transcription of target genes encoding enzymes involved in amino acid metabolism, enzymes required for protein folding and degradation, GADD34 phosphatase, and the transcription factor C/EBP homologous protein (CHOP/*DDIT*) (Han et al. 2013). ATF6 translocates to the Golgi apparatus for cleavage by site-1 and site-2 proteases. Together with XBP1, activated ATF6 subsequently translocates into the nucleus and regulates transcription of target genes to restore ER function (Li et al. 2000). Taken together, UPR activation serves as adaptive system against ER stress and promotes cell survival. Nevertheless, if prolonged ER stress occurs and UPR fails to restore protein folding homeostasis, PERK and IRE1 stimulate pro-apoptotic signaling and increase CHOP expression. CHOP, a key molecule involved in ER-stress-driven apoptosis, is associated with repression of BCL-2, which in turn translocates BCL-2-associated protein X (BAX) to mitochondria, ultimately leading to release of cytochrome c. This suggests that ER stress modulates intrinsic apoptosis via disruption of mitochondrial membrane potential and a series of caspase activation (Gorman et al. 2012; Weston and Puthalakath 2010). CHOP strongly correlates with ER stress-driven apoptosis and CHOP-deficient cell lines are resistant to ER stress-induced apoptosis (Oyadomari and Mori 2004). In light of the previous facts, sustained UPR activation, which leads to programmed cell death, might be a potential strategy in cancer therapy.

Regulation of gene expression at the level of protein synthesis is a unique mechanism, by which cells rapidly respond to extra- and intracellular stresses. In the case of cancer progression, synthesis of specific proteins required to initiate and maintain the transformed phenotype is hyperactivated by post-transcription via translation initiation. This process utilizes existing mRNA species to produce target proteins and skips transcription steps, favoring cancer cell development (Grzmil and Hemmings 2012). Therefore, the susceptibility of translation initiation to protein synthesis may be a determinant factor in cancer development.

Cryptotanshinone (CPT) is a main lipophilic diterpene quinone isolated from a valuable traditional Chinese herb called *danshen* (*Salvia miltiorrhiza*). Its mechanisms of inhibition of cancer cell growth have been widely investigated (Chen et al. 2013). A previous study revealed that CPT induced UPR-mediated apoptosis in cancer cell lines (Park et al. 2012). We recently observed that CPT stimulated the reactive oxygen species (ROS)-mediated, caspases-dependent pathway resulting in apoptosis in acute lymphoblastic leukemia (ALL) cells (Wu et al., 2015). In the present study, we use pharmacogenomics and bioinformatics to explore, whether UPR is involved in ALL cell death upon CPT treatment.

Materials and methods

Chemicals

CPT (Fig. 1) was purchased from Sigma-Aldrich (Munich, Germany).

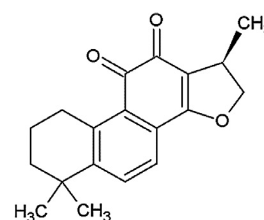

Fig. 1. Chemical structure of CPT.

Culture of cell lines

CCRF-CEM leukemia cells were kept in an incubator containing 5% CO_2 at 37 °C and were cultured in RPMI medium (Invitrogen, Darmstadt, Germany) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin (Invitrogen).

mRNA microarray

Total RNA was isolated using InviTrap Spin Universal RNA Mini kit (Stratec Molecular, Berlin). The procedure of microarray expression profiling including quality check of total RNA, probe labeling, hybridization, scanning and data analysis was performed by the Genomics and Proteomics Core Facility at the German Cancer Research Center (DKFZ) in Heidelberg, Germany (Eberwine et al. 1992).

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated by InviTrap Spin Universal RNA Mini kit (Stratec Molecular) according to the manufacturer's instruction. One microgram RNA was converted to cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, MS, USA). The mRNA levels were analyzed with the use of 5× Hot Start Tag EvaGreen qPCR Mix (no ROX) (Axon Labortechnik, Kaiserslautern, Germany) by CFX384™ Real-Time PCR Detection System (Bio-Rad, Munich, Germany). The running protocol of qPCR was set as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles including denaturation at 95 °C for 15 s, annealing at 58.1 °C (*DDIT3, RPS13*) for 1 min and extension at 72 °C for 1 min following 95 °C for 1 min. *RPS13* gene expression was used for normalization.

Primer sequences were as follows: *RPS13*, forward: GGTTGAAGTTGACATCTGACGA, reverse: CTTGTGCAACACATGTGAAT. *DDIT3*, forward: GGAAACGGAAACAGAGTGGT, reverse: TGTTCTTTCTCCTTCATGCG.

Motif analysis

Transcription factor binding site analysis was performed by the Cistrome analysis software (Liu et al. 2011). Briefly, regulated genes were input and BED formats, a tab-delimited text file which defines data lines displayed in an annotation track, were retrieved with an upstream setting (promoter region) at 2 or 3 kb through the following link: http://genome.ucsc.edu/cgi-bin/hgTables. SeqPos motif analysis was used to screen for enriched motifs in given regions (http://cistrome.org). SeqPos scans all the motifs not only in Transfac, JASPAR, UniPROBE (pbm), hPDI database, but also tries to find *de novo* motifs using MDscan algorithm. The output of genes was ranked by –log10 (*p*-value).

Molecular docking

Preparation of docking files was carried out with AutodockTools-1.5.6rc3 and molecular docking was performed by Autodock4 using lamarckian algorithm (Morris et al. 2009). The three-dimensional CPT structure was prepared in protein data bank (PDB) format from PubChem website. The X-ray crystallography-based structure of eIF-4A and phosphoinositide-3-kinase (PI3K) were obtained from the PDB

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