Curcumin Sensitizes Acute Promyelocytic Leukemia Cells to Unfolded Protein Response-induced Apoptosis by Blocking the Loss of Misfolded N-CoR Protein

Angela Ping Ping Ng¹, Wee Joo Chng¹, ², ⁴ and Matiullah Khan¹, ², #

¹Cancer Science Institute of Singapore, Departments of ²Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, ³NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore, ⁴Department of Haematology-Oncology, National Cancer Institute of Singapore, National University Health System, Singapore

Running title: Curcumin Sensitizes APL cells to UPR-Induced Apoptosis

# Request for Reprints: (M.K.)
Matiullah Khan
Cancer Science Institute (CSI) of Singapore
Yong Loo Lin School of Medicine
National University of Singapore
Center for Life Sciences, Block MD11
10 Medical Drive
Singapore 117597
Phone: (65)-6515-7283
Fax: (65)-6873-9664
Email: csimmk@nus.edu.sg

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Abstract

APL (acute promyelocytic leukemia) is characterized by accumulation of apoptosis-resistant immature promyelocytic cells in the bone marrow and peripheral blood. We have shown that endoplasmic reticulum-associated degradation (ERAD) and protease-mediated degradation of misfolded nuclear receptor co-repressor (N-CoR) confer resistance to unfolded protein response (UPR)-induced apoptosis in APL. These findings suggest that therapeutic inhibition of N-CoR misfolding or degradation may promote growth arrest in APL cells by sensitizing them to UPR-induced apoptosis. Based on this hypothesis, we tested the effects of several known protein conformation modifying agents on the growth and survival of APL cells and identified curcumin, a natural component of turmeric, as a potent growth inhibitor of APL cells. Curcumin selectively inhibited the growth and promoted apoptosis in both primary and secondary leukemic cells derived from APL. The curcumin-induced apoptosis of APL cells was triggered by an amplification of endoplasmic reticulum (ER) stress, possibly from the accumulation of misfolded N-CoR protein in the ER. Curcumin promoted this net accumulation of aberrantly phosphorylated misfolded N-CoR protein by blocking its ERAD and protease-mediated degradation, which then led to the activation of UPR-induced apoptosis in APL cells. The activation of UPR by curcumin was manifested by phosphorylation of PERK and eIF2α, and up-regulation of CHOP and GADD34, the principal mediators of pro-apoptotic UPR. These findings identify the therapeutic potential of curcumin in APL and further establish the rationale of misfolded N-CoR protein as an attractive molecular target in APL.
Introduction

Acute promyelocytic leukemia (APL), designated as AML-M3 (acute myeloid leukemia) in FAB (French American and British) classification, is caused by a fusion protein PML-RARα formed by a reciprocal translocation between \textit{PML} and \textit{RAR}α genes located on chromosome 15 and 17 respectively. APL represents around 10% of all AML in adults with lower incidence reported in children (1, 2). At cellular level, APL is characterized by accumulation of blast cells in the bone marrow and peripheral blood which display features of differentiation arrest and resistance to programmed cell death. Although there is no dispute on the principal role of PML-RARα in the transformation of APL cells, the molecular mechanisms through which PML-RARα promotes transformation is still a matter of intense debate. We have shown that endoplasmic reticulum-associated degradation (ERAD) and protease-mediated degradation of misfolded nuclear receptor co-repressor (N-CoR) plays a significant role in APL cells’ resistance to unfolded protein response (UPR)-induced apoptosis (3, 4). N-CoR, a key component of the generic co-repressor protein complex involved in transcriptional control mediated by various transcription factors, was first identified as a co-repressor of un-ligated nuclear hormone receptors (5, 6). N-CoR was later shown to be essential for the transcriptional repression mediated by the tumor suppressor Mad and other sequence-specific transcription factors (7, 8). We identified N-CoR as a Ski-interacting protein in yeast two hybrid assay (9), and demonstrated the essential role of N-CoR-mediated transcriptional repression in the function of tumor suppressor Mad and Rb (10, 11).
UPR constitutes a coordinated transcriptional activation of a set of genes that encode for endoplasmic reticulum (ER) chaperones and certain death signals, and is activated when net accumulation of misfolded proteins in the ER threatens the survival of cell (12, 13). Functionally, the UPR comprises of two branches, the cytoprotective UPR and the cytotoxic UPR, although distinction between these two branches is not very clear. The cytoprotective UPR protects cells from the harmful effects of misfolded proteins by promoting chaperone-mediated refolding of misfolded proteins and ERAD of misfolded proteins that could not be refolded (12). In many protein conformation disorders such as Alzheimer’s and Parkinson diseases, these corrective measures are often impaired leading to the accumulation of potentially toxic misfolded proteins in the ER and cytosol. This in turn activates cytotoxic UPR and eliminates cells harboring the damaged proteins through programmed cell death (12). The cytotoxic UPR is mediated through the activation of c-Jun N-terminal kinase (JNK) by apoptosis-signal-regulating kinase (ASK1), and up-regulation of C/EBP homologous protein (CHOP; 14) by PERK (protein kinase RNA-like endoplasmic reticulum kinase). The final steps of UPR-induced apoptosis involve the activation and processing of caspases 3 and 9 (15).

We have previously shown that cytoprotective UPR can protect APL cells from the cytotoxic effects of misfolded PML-RAR/N-CoR proteins by inducing their degradation (4). This finding not only identified an important role of cytoprotective UPR in the pathogenesis of APL but also suggested that strategic conversion of cytoprotective UPR into cytotoxic UPR to be an attractive therapeutic strategy for APL. Based on this hypothesis, we screened several known protein conformation modifying agents to identify suitable compounds that could modulate APL cells’ response to UPR and promote growth arrest in APL cells through targeting the degradation of misfolded N-CoR protein. In particular, our goal was to identify agents that would promote selective growth arrest in APL cells either by rescuing the native conformation and function of
N-CoR, or by aggravating ER stress through inducing further accumulation of misfolded N-CoR in the ER. In our initial screening of selected compounds on APL-derived cells, we identified genistein and curcumin as potent inhibitor of growth of multiple APL derived cells. The therapeutic effect of genistein and its underlying molecular mechanism of action has been documented previously (16). Here, we report that curcumin, a natural component of turmeric that has previously been shown to rescue the native conformation of several misfolded proteins such as the amyloid β (Aβ), mutated cystic fibrosis transmembrane conductance regulator (CFTR) and truncated myelin protein zero (MPZ) (17-19), selectively sensitizes APL cells to UPR-induced apoptosis by promoting the net accumulation of misfolded N-CoR protein in the ER.

**Material and Methods**

**Cell lines, human primary leukemia cells and reagents**

The RA (retinoic acid)-sensitive APL cell line NB4 and its RA resistant variant NB4-R1 and UF-1 were from Dr. Homma (Japan), Dr. Lanotte (France) and Dr. Matsushita (Japan) respectively. Other leukemic cell lines were purchased from ATCC (Manassas, VA, USA) and Japan health sciences foundation (Osaka, Japan) and were grown in RPMI, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin and 10 μg/mL streptomycin, with the exception of UF-1 cells which were grown with 15% FBS. The APL cells were validated by their distinct morphology and processing of N-CoR protein. The primary human leukemia samples were collected after informed consent in accordance with the Helsinki Declaration. This study was approved by the Institutional Review Board (IRB) of National University of Singapore and National University Hospital, Singapore.
Cell proliferation assay

The cell proliferation assay was carried out using the cell proliferation kit I (MTT, Roche, Basel, Switzerland) or CellTiter 96® AQueousNon-Radioactive CellProliferation Assay (Promega, Wisconsin) as described by the manufacturers. Cells were seeded into 96-well plates at 5x10^3/well in 100 μL culture medium containing curcumin at various concentrations at 37°C for the durations stated. MTT or MTS labeling reagent was added and incubated further at 37°C for 5 hours. For MTT, this was followed by suspension in solubilisation buffer overnight at 37°C. The spectrophotometrical absorbance of the samples was measured using a microplate reader (Ultramark, Bio-Rad, Hercules, CA) at wavelength 595 nm with a reference wavelength of 655 nm. Alternatively for MTS, the spectrophotometrical absorbance of the samples was measured immediately at wavelength 495nm. Proliferation index of the cells were calculated as (OD_{595} at 20 μM at a particular time-point) / (OD_{595} at 0 μM at the same time point).

Detection of apoptosis through flow cytometry

Bone marrow or peripheral blood from APL patients were collected and processed with Ficoll-Paque PLUS (GE Healthcare) for isolation of mononuclear cells (MNCs) as described by the manufacturer. The MNCs were cultured with IMDM medium supplemented with 10% FBS, 20 ng/ml FLT3 ligand, 20 ng/ml SCF, 20 ng/ml IL-3, 50 ng/ml G-CSF, TPO 50 ng/ml and 50 U/10 μg/mL penicillin/ streptomycin. Cells were seeded at a density of 10 x 10^4 cells/ml and were treated with either DMSO or 20 μM of curcumin for 72 hours. They were then harvested, washed and tested for apoptosis using the Annexin V Apoptosis detection kit (BD Pharmigen).
Data was analyzed using the FlowJo program. Apoptosis assay of APL derived cell lines were performed similarly.

**Giemsa-Wright staining**

Suspension cells were cytopun onto glass slides, stained in May-Gruwald’s eosin methylene blue solution (Merck, Whitehouse Station, NJ) for 5 minutes, followed by staining in Giemsa’s azur eosin methylene blue solution (Merck, Whitehouse Station, NJ) diluted at 1:20 in Giemsa buffer (33 mM of KH₂PO₄, 33 mM Na₂HPO₄, pH 6.81) for 30 minutes with agitation. Slides were mounted with cover slips and visualized in Nikon eclipse TE 2000-S microscope.

**Immunostaining and fluorescence microscopy**

Suspension cells were cytopun onto glass slides while the adherent cells were grown on coverslips in culture medium. Cells were then fixed with 4% freshly prepared paraformaldehyde in PBS for 20 min at 37ºC and permeabilized with 0.2% Triton X-100 in PBS at 4ºC for 5 minutes. They were incubated with the respective primary antibodies at appropriate dilutions in 2.5% BSA in PBS for 2 hours at room temperature or overnight at 4ºC. Alexa Fluor secondary antibodies (Molecular Probes, Eugene, OR) at a dilution of 1:200 in 2.5% BSA in PBS, were incubated with the cells for 1 hours at room temperature. The cells were counter-stained with 150 nM of 4,6-diamidino-2-phenylindole (DAPI).

**N-CoR solubility assay**

293T cells were seeded at 200 x 10⁴ cells per 10-cm plate and transfected with 3 μg pACT-N-CoR-FLAG and 3 μg pACT-PML-RARα-HA expression plasmids. 48 hours after transfection, cells were harvested with NET buffer (20 mM Tris-HCl (pH8), 300 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM NaF, 1 mM Na₃VO₄, 20 mM β-glycerolphosphate, 1.5 mg/mL IAA, protease inhibitor cocktail (Roche) at 4ºC with rotation for 1 hour. The lysates were then centrifuged at 15 000 rpm
at 4°C for 10 minutes. The supernatant was collected as the soluble fraction and the pellet, collected as the insoluble fraction, was suspended in SDS sample buffer and sonicated. The samples were then denatured at 50°C for 10 minutes, separated on 6% SDS-PAGE, transferred to PVDF membrane and blotted with M2 anti-FLAG antibody (Sigma, St. Louis, MO). For solubility assay of endogenous N-CoR protein, around 8x10⁷ vehicle or curcumin treated cells were suspended in 500 μL of NET buffer, centrifuged to separate soluble and insoluble fractions, loaded to 6% SDS-PAGE after brief sonication, and stained with anti-N-CoR antibody (Santa Cruz, CA, USA) after transfer to PVDF membrane.

Immunoprecipitation and Phosphorylation Assays

293T cells transfected with N-CoR-Flag and PML-RARα-HA expression plasmids were treated with vehicle, curcumin or genistein. After incubation for 48 hours, whole cells extracts were prepared in phosphorylation buffer (20 mM Tris HCl pH 7.4, 1 mM EDTA, 10 mM NaCl, 0.1% NP40, 1 mM NaF, 1 mM Na₃VO₄, 20 mM β-glycerolphosphate, 1.5 mg/ml IAA, protease inhibitor cocktail) by mild sonication. Lysates were centrifuged at 15000 rpm for 15 minutes at 4°C and the supernatants were collected. Protein concentration in the supernatant was determined using the Bio-Rad Protein Assay. N-CoR from the supernatant containing 1 mg of protein was immunoprecipitated with Flag M2 antibody (Sigma, St Louis, MO) and level of phosphorylated N-CoR protein was determined in western blotting with generic Phospho antibody. The blot was then stripped and reprobed with FLAG antibody to quantify the amount of immunoprecipitated N-CoR protein.

In-vitro cleavage assay

N-CoR substrate was prepared from 293T cells transfected with pACT-N-CoR-FLAG and pACT-PML-RARα-HA expression plasmids. Cell extracts from NB4, HL60 or K562 were
prepared by pelleting 1x10^7 cells, lysing them on ice in 200 μl of RSB buffer (10 mmol/L Tris (pH 8.0), 10 mmol/L NaCl, 3 mmol/L MgCl2, 0.1% NP40) buffer for 10 minutes. Nuclei were removed from the crude lysates by centrifugation at 3000 rpm at 4°C for 5 minutes. Cleavage assay was then carried out by mixing 25 μl of N-CoR substrate with 25 μl of cell extracts from leukemic cells or purified fractions through size-exclusion chromatography and incubated at 25°C or 37°C for 60 minutes. The cleavage reaction was stopped by adding 16.7 μl of 4 X SDS sample buffer and heating reaction mixture at 50°C for 10 minutes. Proteins were resolved with 6% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes for Western blotting. N-CoR was detected with Flag antibody.

**Analysis of ER expansion**

NB4 cells seeded at 10 x 10^4 cells/mL were treated with 0 μM or 5 μM of curcumin for 72 hours, Cells were incubated with 0.2 μg/mL of brefeldin A-BODIPY for 45 minutes at 37°C in 5% CO2 An aliquot of the stained cells was analyzed by Dako Cytomation Cyan LX flow cytometer using the SUMMIT software. Another aliquot of stained cells were cytospun onto glass slide, fixed with paraformaldehyde and stained with rabbit polyclonal calnexin antibody (SPA-865, Stress Gene, Ann Arbor, Michigan) and rabbit Alexa Fluor 594 antibody.

**Statistical analysis**

The statistical significance of differences in values of data was determined using the unpaired Student's t test. A P value of <0.05 was considered statistically significant.
Results

Selective inhibition of growth of APL cells by curcumin

To investigate whether therapeutic modulation of N-CoR misfolding could sensitize APL cells to UPR-induced apoptosis, we first tested the effect of curcumin on the growth of three APL derived cell lines: the RA-sensitive cell NB4, and the RA-resistant variants NB4-R1 and UF-1. Curcumin, at concentrations ranging from 2.5 to 20 µM, inhibited the growth and viability of all three APL derived cells when analyzed by MTT assay (Fig. 1A) and Wright-Giemsa staining (Supplementary Fig. S1). The effect of curcumin appears to be selective for APL cells as growth and viability of six non-APL cells namely K562, U937, HL60, Jurkat, THP-1 and MOLT-4 were not significantly affected after 72 hours treatment (Fig. 1B & C). Although several non-APL cells also showed varying degree of growth inhibition when exposed to curcumin for 96 hours (Fig. 1B), their viability was not affected as much as observed with NB4 cells (Fig. 1C). The curcumin-induced growth inhibition observed in these non-APL cells at 96 hour could be due to its non-apoptotic growth inhibitory effect that has been documented against various cancer cells. All three curcumin-treated APL derived cells displayed identical features of extensive cell death when analyzed by gross morphological analysis (Supplementary Fig. S1) or FACS based qualitative apoptosis assay (Fig. 2A). As observed in APL derived cell lines, curcumin also triggered apoptosis in two APL derived human primary leukemic cells (Fig. 2B).

Since caspases are known to be principal mediators of apoptosis, we decided to identify the caspase(s) selectively involved in curcumin-induced apoptosis of APL cells. A fluorometric caspase assay was employed to identify the caspases and to measure their degree of activation by curcumin in APL cells. In this assay, caspase activation was quantified by measuring the amount of free AFC (7-amino-4-trifluoromethylcoumarin), which is released when AFC-linked synthetic
substrates are cleaved by activated caspase. AFC-linked substrates of all known mammalian caspases were used to measure the effect of curcumin on the activity of individual caspase in APL cells. As demonstrated in Figure 2C, a selective activation of caspases 2, 3/7 and 9 was observed in NB4 cells treated with curcumin for 72 hours at 10 and 20 µM concentrations. In agreement with this finding, curcumin, when used at 10 µM concentration, induced the cleavage of caspases 9 and 3, which was followed by the activation of PARP (Fig. 2D). Comparable levels of caspases 3, 9 and PARP cleavage by curcumin was also observed in RA resistant APL cells NB4-R1 and UF-1 (Supplementary Fig. S2A and S2B). Apart from promoting apoptosis, curcumin also induced cell cycle arrest at G2/M (M3) phase after 24 hours of treatment (Supplementary Fig. S3), along with a decrease in the percentage of cells at G1 phase (M1). The number of cells at S phase was not significantly affected.

**Curcumin blocks protease-mediated degradation of misfolded N-CoR protein**

We previously reported how selective cleavage or degradation of misfolded N-CoR protein was linked to APL cells’ resistance to UPR-induced apoptosis (4). To investigate if curcumin-induced apoptosis of APL cells was mediated through an inhibition of N-CoR cleavage or degradation, effect of curcumin on the cleavage of N-CoR protein was analyzed. Treatment of NB4 cells with curcumin in a dose-dependent manner led to the stabilization of full length N-CoR protein along with a corresponding reduction in the level of cleaved N-CoR fragment (Fig. 3A). To test if the observed stabilization of N-CoR protein was a result of the inhibition of proteases activity that cleaved N-CoR protein in NB4 cells, an *in vitro* N-CoR cleavage assay was performed. In this assay, Flag-tagged N-CoR protein ectopically expressed in 293T cells was incubated with the extracts of NB4 cells treated with vehicle or curcumin, as well as with AEBSF, a broad spectrum protease inhibitor that abrogated N-CoR cleavage in NB4 cells (4).
Extract of curcumin treated NB4 cells did not cleave Flag-tagged N-CoR protein as efficiently as extract of vehicle treated NB4 cells. This suggests that potency of N-CoR cleaving proteases was significantly reduced after curcumin treatment and the reduction was comparable to that achieved by AEBSF (Fig. 3B). Apparently, the stabilization of N-CoR protein by curcumin was mediated by an inhibition of N-CoR degradation rather than an inhibition of N-CoR misfolding as observed previously with genistein (16). Consistent with this idea, relative level of detergent-insoluble N-CoR protein in NB4 cells or 293T cells over-expressing N-CoR was increased after curcumin treatment while there was hardly any change in the level of detergent-soluble N-CoR protein (Fig. 3C & D). The misfolded N-CoR protein was characterized by aberrant phosphorylation (Fig. 3E), and the level of phosphorylated N-CoR protein was significantly increased after curcumin treatment (Fig. 3F, left panel). On the other hand, genistein treatment resulted in a noticeable reduction in phosphorylated N-CoR level (Fig. 3F right panel). These findings collectively suggested that curcumin-induced N-CoR stabilization mainly resulted from an inhibition of degradation of misfolded N-CoR protein rather than an inhibition of N-CoR misfolding as observed previously with genistein.

**Curcumin blocks proteasomal degradation of misfolded N-CoR protein**

In addition to proteolytic processing, misfolded N-CoR was also subjected to ubiquitin-proteasome mediated degradation specifically stimulated by the E3 ligase activity of PML-RAR protein (3). As curcumin is also known to be a potent inhibitor of proteasomal activity (20-22), we tested whether curcumin-induced stabilization of N-CoR protein was a result from an inhibition of cellular proteasomal activity. Effect of curcumin on the proteasomal activity of 293T cells was tested using the proteasome sensor, pZsProSensor-1, which consists of the mouse
ornithine decarboxylase degradation domain (MODC d410) fused to green florescence protein ZsGreen. The intensity of the green signal emitted from the pZsProSensor-1 is hence inversely proportional to the level of cellular proteasomal activity in cultured cells. As expected, intensity of the green fluorescence signals in 293T cells transfected with pZsProSensor-1 vector increased with increasing concentrations of curcumin, confirming that curcumin could robustly inhibit the cellular proteasomal activity of 293T cells (Supplementary Fig. S4). Next, the effect of curcumin on the degradation of misfolded N-CoR protein in 293T cells was determined using GFP fused N-CoR as a possible substrate of proteasome. When expressed alone, native GFP-N-CoR was confined to the nucleus of 293T cells (Fig. 4A, topmost panel) while misfolded GFP-N-CoR co-expressed with PML-RAR was largely found in the cytosol (Fig. 4A, second panel from top). The intensity of GFP signal from the cytosolic N-CoR was significantly enhanced after treatment with curcumin in a dose-dependent manner, suggesting that curcumin may have blocked the proteasomal degradation of GFP-N-CoR protein (Fig. 4A, lower panels). Furthermore, the level of cytosolic N-CoR protein in NB4 cells was also increased after treatment with curcumin in a dose-dependent manner, confirming that curcumin could trigger cytosolic accumulation of misfolded N-CoR protein in APL cells (Fig. 4B). The proteasome, which is a multi-subunit and multi-catalytic proteinase, possesses multiple trypsin, chymotrypsin and peptidylglutamyl-like hydrolysing activity (23). To find out which of these three catalytic activities was inhibited by curcumin, a flurometric proteasomal activity assay was carried out using synthetic substrates selectively hydrolysed by each activity. In this assay, crude cellular extract of curcumin-treated NB4 cells was incubated separately with peptides, Boc-Leu-Arg-Arg-AMC, Z-Gly-Gly-Leu-AMC or Boc-Leu-Leu-Glu-AMC, which are the synthetic substrates of trypsin, chymotrypsin and caspases-like activities respectively. Curcumin consistently inhibited the activities of all
three catalytic subunits, though the inhibitory effect was most prominent on both the chymotrypsin and caspases-like peptidase activities (Fig. 4C).

**Curcumin activates UPR-induced apoptosis in APL cells**

We have previously shown how degradation of misfolded N-CoR protein led to the attenuation of ER stress and protected APL cells from UPR-induced apoptosis (4). Based on this finding, we hypothesized that amplification of ER stress due to curcumin-induced accumulation of misfolded N-CoR protein in the ER will eventually re-sensitize APL cells to UPR-induced apoptosis. To test this hypothesis, effect of curcumin on the level of ER stress in APL cells was first determined by measuring the relative levels of HMW GRP78 and PDI proteins which are known to be bona-fide ER stress markers in mammalian cells. Curcumin induced the formation of HMW detergent-resistant GRP78 and PDI proteins in NB4 and NB4-R1 cells (Fig. 5A) in a dose-dependent manner, suggesting an amplification of ER stress in APL cells treated with curcumin. In mammalian cells, ER stress amplification is also manifested by physical expansion of ER which could be quantified by selective staining of ER by Brefeldin A-BODIPY (24). As shown in Figure 5B, Brefeldin A-BODIPY signal displayed a shift towards the right after curcumin treatment, suggesting an expansion of ER-GA (golgi apparatus) in NB4 cells after treatment. Staining of Brefeldin A-BODIPY is specific to ER as observed from the colocalisation of Brefeldin A-BODIPY signal with that of calnexin, an ER resident protein (Fig. 5C). An apparent expansion of ER compartment was also evident from the diffused signal of calnexin in curcumin treated NB4 cells (Fig. 5C). The ER expansion by curcumin was a likely outcome of increased accumulation of misfolded N-CoR protein in the ER as suggested by increased co-localization of N-CoR protein with PDI after curcumin treatment (Fig. 5D).
Next, to show that curcumin-induced apoptosis of APL cells was mediated though the activation of cytotoxic UPR, we studied the effect of curcumin on the activation of mediators of cytotoxic UPR. The initial phase of UPR is mediated by the activation of PERK and IRE through phosphorylation (12, 13) while the final outcome of UPR could be either cytoprotective or cytotoxic depending on how efficiently the cytotoxic effect of misfolded proteins accumulated in the ER is neutralized by cells. The cytotoxic UPR is triggered by apoptosis-signal-regulating kinase (ASK1)-induced activation of c-Jun N-terminal kinase (JNK) and by PERK-induced up-regulation of C/EBP homologous protein (CHOP; 14). To assess the effect of curcumin on the activation of UPR in APL cells, we first determined the phosphorylation status of PERK and IRE in NB4 cells. As shown in Figure 6A, a basal level of PERK phosphorylation was already present in untreated NB4 cells, indicating that PERK was already activated in NB4 cells, probably due to a sustained level of chronic ER stress in APL cells. However, level of PERK phosphorylation was augmented significantly after 16 hours of curcumin treatment, which was followed by phosphorylation of eIF2α and up-regulation of pro-apoptotic proteins CHOP and GADD34 (Fig. 6A). Moreover, ASK phosphorylation was induced within 4 hour of curcumin treatment, which peaked at 16 hour and was followed by JNK phosphorylation at 24 hours and BCL2 phosphorylation at 4-24 hours (Fig. 6B). These finding demonstrated a sequential escalation of pro-apoptotic cascade of UPR after curcumin treatment (Fig. 6B). Consistent with our finding, a recent report also demonstrated misfolded PolyQ-induced JNK phosphorylation at 24 hours; although JNK phosphorylation by chemical ER stress inducer thapsigargin (Tg) was evident within 4 hour of treatment (25). Together, these findings provided compelling evidence that curcumin induced-apoptosis of APL cells was mediated through the activation of cytotoxic branch of UPR.
Discussion

Curcumin was selected in our initial screening assay against APL derived cells for its potent inhibitory effect on the misfolding of amyloid β (Aβ), CFTR and MPZ proteins (17-19). Although curcumin was as effective as genistein in stabilizing the full length N-CoR protein, the N-CoR stabilization by curcumin did not result from an inhibition of N-CoR misfolding as observed with genistein. It is likely that molecular mechanism underlying the misfolding of N-CoR protein is substantially different from that involved in the misfolding of amyloid β (Aβ) and CFTR proteins. For instance, misfolding of N-CoR is triggered primarily by aberrant post-translational modification which could be effectively blocked by genistein, a bona fide inhibitor of tyrosine kinase but not by curcumin, which is not known to possess any anti-kinase activity.

Curcumin is the major yellow pigment found in turmeric derived from the roots of the plant Curcuma longa. It possesses strong antioxidant activity and inhibits angiogenesis at pharmacological concentrations (26-28). The principal anti-carcinogenic properties of curcumin, such as inhibition of cellular proliferation, activation of apoptosis, suppression of inflammation and sensitization of tumor cells to chemotherapy have been well documented (29-32). Many of these anti-carcinogenic effects of curcumin have been attributed to the inhibition of various survival pathways, including the PI3K/Akt1 and NF-κB, and up-regulation of genes associated with growth arrest and apoptosis. As demonstrated by us in this report, the major anti-carcinogenic effect of curcumin in APL cells was mediated through its potent inhibitory effect on the protein quality control mechanism, although involvement of other pathways could not be ruled out. Perhaps a more thorough understanding of the mechanisms underlying curcumin-induced apoptosis of APL cells could be gained from the findings in proteasome inhibitor treated
multiple myeloma cells. Multiple myeloma cells exhibit a lower threshold for proteasome inhibitor-induced UPR as they are constitutively under ER stress due to their role as secretory cells. Bortezomib, a proteasome inhibitor, rapidly induced apoptosis or cytotoxic UPR in multiple myeloma cells through the induction of ATF4 and CHOP (33). Likewise, APL cells too experienced a relatively low level of chronic ER stress possibly due to constant production of misfolded N-CoR protein.

Current therapeutic options available for APL in the form of RA, ATO and chemotherapy have proved to be very effective in inducing clinical remission (CR) in majority of APL patients. However, serious systemic toxicity of RA and a relapse after initial remission followed by resistance to RA limits its scope of therapeutic application in clinical practice. Clinical application of ATO is also fraught with complications such as leukocyte activation syndrome and arrhythmia. These obstacles underline the need of newer approaches in the treatment and management of APL. Curcumin, being a natural component of food product, could become an adjuvant to currently available therapeutic agents for APL if its therapeutic utility and usefulness could be established through further preclinical and clinical studies.

References


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Disclosure of Potential Conflicts of Interest

There is no conflict of interest to disclose.

Figure legends

FIGURE 1. Selective inhibition of growth of APL cells by curcumin. A, Proliferation of APL cells is inhibited by curcumin. NB4, NB4-R1 and UF-1 cells were treated in a dose-dependent manner with curcumin for the durations as mentioned and their growth was measured by MTS assay (left and centre panels) or MTT (right panel). Asterisks represent statistical significance using Student’s T test, as compared to the same cell lines grown in 0 µM curcumin at a particular time point, p <0.05. Error bars indicate means ± SD. B & C, Curcumin selectively inhibited the growth of APL cells. Effect of curcumin on the growth and viability of APL and non-APL cells
were determined through (B) MTT assay and (C) Wright-Giemsa staining. NB4 cells were most sensitive to growth inhibition by curcumin while K562 cells were most resistant among the lots.

**FIGURE 2. Curcumin-induced apoptosis of APL cells is mediated by the activation of caspases.** A & B, The percentage of annexin V positive cells in NB4 (A) and two human primary APL samples (B) treated with curcumin for 72 hours in a dose-dependent manner was determined through annexin V assay. C, Curcumin treatment led to the activation of caspases 2, 3/7 and 9. Activity of all the caspases in NB4 cells were treated with 0-20 µM curcumin for 72 hours were calculated based on the fluorescence intensity of their substrate in arbitrary units per µg of protein. Activation of caspases was represented as fold increase of specific activity of each caspase in cells treated with the respective curcumin concentrations over the specific activity of the same caspase in cells treated with DMSO only. D, Activation of caspases by curcumin in APL cells. NB4 cells were treated with various concentrations of curcumin as stated for 72 hours. Cells were then harvested and protein directly extracted with SDS sample buffer. Western blot analysis of the crude lysates were carried out using caspase 9, pro-caspase 3, cleaved caspases 3 and PARP antibodies.

**FIGURE 3. Curcumin promoted the accumulation of misfolded N-CoR protein by blocking its degradation.** A, Curcumin stabilized full length N-CoR protein in NB4 cells. Level of N-CoR protein in NB4 cells treated with curcumin in dose-dependent manner for 48 hours was determined with N-CoR antibody. B, Curcumin inhibited N-CoR cleaving activity in NB4 cells. Relative potency of N-CoR cleaving activity in NB4 cells treated for 48 hours with vehicle, curcumin (10 µM) or AEBSF (100 µM) was determined by in-vitro cleavage assay. Flag-tagged N-CoR protein ectopically expressed in 293T cells was used as substrate. C, Curcumin promoted accumulation of insoluble N-CoR in NB4 cells. Level of N-CoR protein in detergent-soluble (S)
and detergent-insoluble (I) fractions of whole-cell extracts of NB4 cells treated with 0-10 µM curcumin for 48 hours was determined by protein solubility assay. Level of total protein in each sample was quantified by Coomassie staining. D, Curcumin promoted accumulation of insoluble N-CoR in 293T cells transfected with N-CoR and PML-RARα. Level of Flag-tagged N-CoR protein in detergent-soluble (S) and detergent-insoluble (I) fractions of whole-cell extracts of 293T cells transfected with N-CoR and PML-RARα was determined by western blotting using Flag antibody. Level of total protein in each sample was quantified by Coomassie staining. E and F, Curcumin enhanced the aberrant N-CoR phosphorylation. Relative level of phosphorylated N-CoR protein in 293T cells transfected with Flag-tagged N-CoR and PML-RAR plasmids (E) and treated with curcumin or genistein (F) was determined by immunoprecipitation with Flag antibody followed by western blotting with generic phospho antibody. To quantify the level of immunoprecipitated N-CoR protein, the blot were stripped and reprobed with Flag antibody.

FIGURE 4. Curcumin blocked the proteasomal degradation of misfolded N-CoR protein. A, Curcumin inhibited the degradation of the GFP-N-CoR in 293T cells transfected with GFP-N-CoR and PML-RARα, in a dose-dependent manner. 0-10 µM of curcumin was added to transfected 293T cells 2 hours after transfection. GFP-N-CoR was visualized 48 hours after curcumin treatment. GFP-N-CoR was localized in the nucleus in the absence of PML-RARα, but localized mostly in the cytoplasm in the presence of PML-RARα. B, Curcumin inhibited the degradation of endogenous N-CoR in NB4 cells. NB4 cells were treated with 0-10 µM of curcumin for 48 hours. Cells were cytospun onto glass-slides, fixed and stained with N-CoR antibody (red). DNA was stained with DAPI (blue). C, Curcumin inhibited the trypsin-like, chymotrypsin-and caspases-like peptidase activities of the proteasome. Crude extracts of NB4
cells treated with 0-10 μM of curcumin for 48 hours was used to determine the cleavage of proteasome substrates.

**FIGURE 5. Curcumin promoted ER stress and ER expansion in APL cells.** A, The formation of HMW detergent-resistant GRP78 and PDI protein bands in NB4 and NB4-R1 cells treated with 0-20 μM of curcumin for 72 hours was determined by western blotting assay using GRP78 and PDI antibodies. B, Curcumin induced ER-GA expansion in NB4 cells. NB4 cells were treated with DMSO or 5 μM of curcumin for 72 hours. Cells were stained with Brefeldin A-BODIPY (BFA-BODIPY) and analyzed by FACS. C, Curcumin increased the level of endogenous calnexin. NB4 cells were treated with DMSO or 5 μM curcumin for 72 hours and stained with Brefeldin A-BODIPY (green) before cytopspining onto glass slides and then stained with Calnexin antibody (red). DNA was stained with DAPI (blue). D, The level of N-CoR (red) and PDI (green) in NB4 cells treated with vehicle or curcumin was determined by indirect immunoflorescence assay and confocal microscopy. DNA was stained with DAPI (blue).

**FIGURE 6. Curcumin activated UPR-induced apoptosis in APL cells.** A, Curcumin activated the PERK pathway in NB4 cells. NB4 cells were treated with 10 μM of curcumin for 0-72 hours. Western analysis was carried out using phospho-PERK, PERK, phospho-eIF2α, eIF2α, CHOP and GADD34 antibodies. B, Curcumin activated the IRE pathway in NB4 cells. NB4 cells were treated with 10 μM of curcumin for 0-72 hours. Western analysis was carried out using phospho-ASK, ASK, phospho-JNK, JNK, phospho-Bcl2 and Bcl2 antibodies.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

[Diagram showing the effects of curcumin treatment on various proteins over time (A) and (B).]

- pPERK
- PERK
- pelF2α
- eIF2α
- CHOP
- GADD34
- β-actin

Duration of curcumin treatment in hours
0, 0.5, 1, 2, 4, 8, 16, 24, 48, 72

- pASK
- ASK
- pJNK
- p54
- JNK
- p46
- GADD34
- β-actin

Duration of curcumin treatment in hours
0, 0.5, 1, 2, 4, 8, 16, 24, 48, 72

NB4

BCl2

NB4
Molecular Cancer Research

Curcumin Sensitizes Acute Promyelocytic Leukemia Cells to Unfolded Protein Response-induced Apoptosis by Blocking the Loss of Misfolded N-CoR Protein

Angela Ping Ping Ng, Wee J Chng and Matiullah Khan

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