

Two Novel Curcumin Analogues Induced Reactive Oxygen Species Generation and Mitochondrial-Related Apoptosis in Human Breast Cancer MCF - 7 Cells

Shuyue Luo¹, Qingyong Li^{1,2*}, Jian Chen¹ and Wengchao Wang²

¹Key Laboratory of Forest Plant Ecology (Northeast Forestry University), Ministry of Education, 332# No. 26 Hexing Road, Harbin City, Heilongjiang Province, 150040, China

²College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou, 310014, China

*Corresponding author: Qingyong Li, 332# No. 26 Hexing Road, Harbin City 150040, Heilongjiang Province, China, Tel: +86-571-88320984; Fax: +86-571-88320984; E-mail: Liqy@zjut.edu.cn

Received date: February 17, 2016; Accepted date: March 04, 2016; Published date: March 10, 2016

Copyright: © 2016 Luo S, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Objective: Curcumin has been shown to have significant protective effects against cancer and induction of apoptosis is a crucial strategy for cancer therapy, so we have now evaluated the mechanisms involved in two novel asymmetric curcumin analogues induced cell death in MCF - 7 cells.

Methods: The cytotoxicity of two curcumin analogues towards tumor cells was investigated by MTT assays. The morphological analysis using a laser scanning confocal microscope. Further cell cycle analysis, reactive oxygen species (ROS), mitochondrial transmembrane potentials ($\Delta\psi_m$), intracellular Ca^{2+} levels analysis and apoptosis assays via a flow cytometry (FCM). We used western blot assays to determine the expressions of apoptosis-related factors and p38MAPK at protein level.

Results: MCF - 7 cells showed a significant loss of viability, reduced mitochondrial membrane potential ($\Delta\psi_m$), increased intracellular Ca^{2+} levels, and increased production of ROS, which activated the pro-apoptotic p38 mitogen-activated protein kinase. Pretreatment with the antioxidant, N-acetylcysteine, inhibited both two curcumin analogues mediated ROS production and cytotoxicity. Western blotting revealed that the loss of $\Delta\psi_m$ inhibited Bcl-2, and induced Bax and Bak expression; this promoted release of cytochrome c and apoptosis inducing factor from the mitochondria to the cytosol, activation of caspase-9 and caspase-3 in the cytosol, and induction of apoptosis.

Conclusion: The two curcumin analogues displays strong antitumor effect through ROS-dependent mitochondria apoptosis pathway in MCF - 7 cells, and has promising potential to be developed as antitumor compounds.

Keywords: Apoptosis; Curcumin analogues; MCF - 7 cells; Mitochondrial; Reactive oxygen species

Introduction

Breast cancer is a leading cause of death in women. It is responsible for nearly 500,000 deaths every year worldwide [1]. In recent years, curcumin has been shown to have significant protective effects against a range of cancers, including breast cancer [2]. Curcumin inhibits tumor cells by influencing a variety of biological pathways involved in apoptosis, cell cycle regulation, and tumorigenesis [3]. Curcumin and its analogues have attracted attention because of these potent medicinal properties, combined with its low molecular weight and minimal toxicity [4,5]. However, the mechanisms underlying these beneficial effects are not fully understood [6].

Our previous studies also demonstrated that the asymmetric curcumin analogues, (1E,4E)-1-(4-hydroxy-3,5-dimethoxyphenyl)-5-(3,4,5-trimethoxyphenyl)penta-1,4-dien-3-one (F3, Supplementary Figure 1a) and (1E,4E)-1-(3,4-dimethoxyphenyl)-5-(2-hydroxy-4-methoxyphenyl)penta-1,4-dien-3-one (LE, Supplementary Figure 1b), showed greater cytotoxicity and growth suppression in MCF - 7 cells than did curcumin [7].

Apoptosis is a fundamental and complex biological process in which cells play an active role in their own deaths. Dysregulation of apoptosis is the hallmark of all cancer cells and agents that activate programmed cell death can provide valuable anticancer therapeutics [8]. Reactive oxygen species (ROS) are a by-product of normal cellular oxidative processes and have been suggested to be involved in regulating the initiation of apoptotic signaling. Increased levels of ROS have been demonstrated to induce depolarization of the mitochondrial membrane, which eventually produces increased levels of other pro-apoptotic molecules in cells [9].

Curcumin targets have recently been discovered in the mitochondrial pathway. Indeed, curcumin up-regulates pro-apoptotic proteins of the Bcl-2 family and down-regulates two anti-apoptotic proteins, Bcl-2 and Bcl-xL, in tumor cells. Curcumin induces apoptosis by down-regulating Bcl-xL and inhibitor of apoptosis (IAP) proteins, releasing cytochrome c, activating caspase-3, and stimulating uptake of intracellular Ca^{2+} into mitochondria via the uniporter pathway in U937 cells [10].

Both F3 and LE reduced MCF - 7 cell viability by inducing apoptosis. The current study aimed to determine whether the pathway involved in F3- or LE-induced apoptosis was mitochondrial-related

and mediated by ROS. Intracellular ROS generation and mitochondrial membrane potential were measured and found to strongly associate with the mitochondrial-related apoptotic pathway. Our findings suggested that F3 and LE were promising candidates for clinical use in human breast cancer chemotherapy.

Materials and Methods

Chemicals and reagents

F3 and LE (Supplementary Figure 1) were synthesized in our laboratory, as described previously. The purity of each compound was $\geq 98\%$ (as confirmed by high-performance liquid chromatography). F3, LE, and curcumin were dissolved in dimethylsulfoxide (DMSO), and then diluted with 2% of the relevant medium to the indicated concentrations prior to analysis.

The final concentration of DMSO did not exceed 1%. RPMI-1640 medium and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from HyClone (USA). Fetal bovine serum (FBS), penicillin, streptomycin, and trypsinase were purchased from Hao Yang Biological Manufacture Co., Ltd (China). 2,4-Dinitro-phenol, 5-diphenyltetrazolium bromide (MTT), acridine orange (AO), rhodamine123 (Rho123), fluo-3/AM, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), and DMSO were purchased from Sigma-Aldrich Inc. (USA). Phosphate-buffered saline (PBS, pH 7.2 – 7.6) was purchased from Wuhan Boster Biological Technology Ltd. (China).

The Annexin V-FITC Apoptosis Detection Kit, BCA Protein Assay Kit, BCIP / NBT Alkaline Phosphatase Color Development Kit, Cell Mitochondria Isolation Kit, N-acetyl-L-cysteine (NAC), the p38 mitogen-activated protein kinase (p38MAPK) inhibitor (SB203580), the antibodies against cytochrome c, phospho-p38, p38, phospho-JNK, JNK, phospho-ERK, ERK, and all of the apoptosis-related primary and secondary antibodies used in the study were purchased from the Beyotime Institute of Biotechnology (China).

Cell proliferation assay

Cell proliferation was measured by the MTT assay using DMSO to dissolve formazan as mentioned in supporting information.

Morphological analysis, cell cycle analysis and apoptosis assays

MCF - 7 cells were cultured in 6-well plates for 24 h, and then incubated with F3 or LE (10 μM or 20 μM) for 48 h. Subsequently, the cells were washed twice with PBS and incubated with 200 μL AO (10 mg/mL) for 10 min in the dark. The cells were then washed three times with PBS, and the plates were viewed using a laser scanning confocal microscope (Nikon Eclipse TE2000 - E, Tokyo, Japan). The samples were using standard procedures and reagents and then analyzed by flow cytometry (FCM) as mentioned in supporting information.

Measurement of intracellular ROS, mitochondrial membrane potential ($\Delta\psi\text{m}$) and intracellular Ca^{2+} levels

Rho123 was used to analyze $\Delta\psi\text{m}$. The intracellular Ca^{2+} concentration was measured by incubating cells with fluo-3 / AM (10 μL). Intracellular ROS levels were measured using the Reactive Oxygen Species Assay Kit, according to the manufacturer's instructions. The samples were analyzed by FCM.

Inhibitor treatment

We used NAC to inhibit intracellular alteration of redox state, which is a common quencher of ROS, in order to confirm the role of intracellular ROS in both F3- and LE-induced apoptosis [11].

Protein extraction and western blot analysis

The experimental procedures used in this section are listed in supporting information.

Statistical Analysis

The results were expressed as mean \pm standard deviation for triplicate experiments. Statistical significance was determined by one-way analysis of variance, followed by the Bonferroni post hoc test (for multiple comparisons) or the two-tailed Student's t-test. P-values less than 0.05 were considered statistically significant.

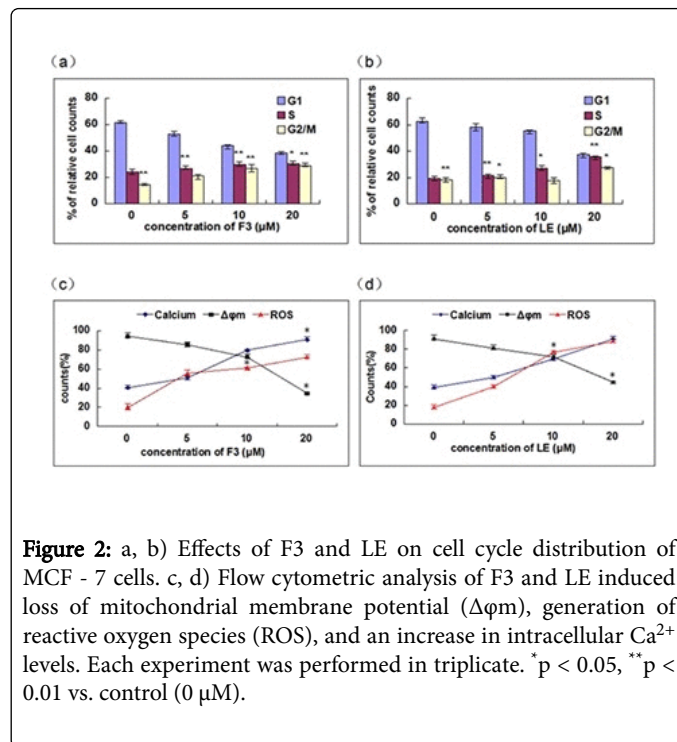
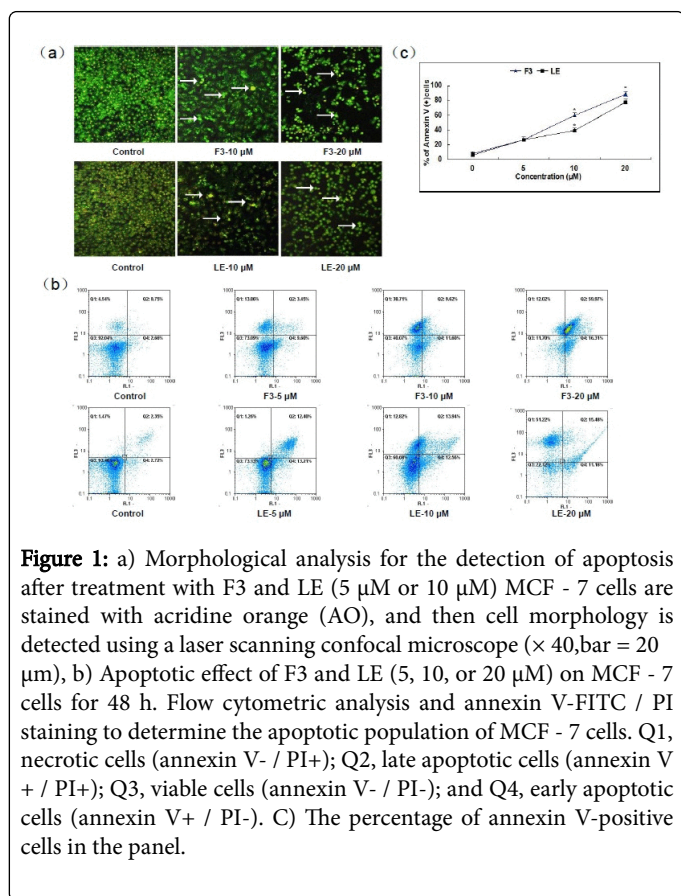
Results and Discussion

F3 and LE selectively inhibited MCF - 7 cell proliferation in a time-dependent manner

Three different tumor cell lines (SMMC-7721, PC-3, and MCF-7) were used to evaluate the effects of F3 and LE. The data are shown in Supplementary Figure 2. These compounds dose-dependently inhibited proliferation of all cell lines and F3 and LE produced greater inhibitory effects than curcumin. Notably, F3 and LE were more cytotoxic towards MCF - 7 cells than they were towards SMMC-7721 or PC-3 cells.

F3 and LE induced apoptosis in MCF - 7 cells

The control cell membranes were orbicular and their nuclei were sharp and regular, while cells treated with F3 or LE displayed typical apoptotic features such as nuclear and cytoplasmic condensation, chromatin fragmentation, marginalization of the fragmented nuclei towards the membrane, and apoptotic bodies (Figure 1a). All of these changes confirmed the cytotoxic effects of F3 and LE. To further investigate apoptotic changes in these cells, annexin V-FITC (to detect exposed phospholipid membrane components) and PI (to detect non-viable cells) double staining FCM analysis was performed. The percentage of apoptotic cells (annexin V+) increased significantly with increasing concentrations of both F3 and LE (from 7.95% to 88.3%, and from 6.54% to 77.88%, respectively) (Figures 1b and 1c). Taken together, these findings indicated that F3 and LE reduced MCF - 7 cell viability by inducing apoptosis.



F3 and LE affected the levels of ROS, intracellular Ca^{2+} , and $\Delta\phi_m$ in MCF - 7 cells

High ROS levels induce cell growth arrest and apoptosis. A number of studies have implicated ROS generated in the mitochondria as key players in the induction of apoptosis [12]. ROS generation increased markedly (by 72.12% and 88.15%) in MCF - 7 cells treated with F3 and LE, as compared with control cells (Figures 2c and 2d). Excessive levels of ROS are reported to lead to an increase in intracellular Ca^{2+} and a loss of $\Delta\phi_m$, leading to mitochondrial dysfunction [13].

To investigate the involvement of the mitochondrial pathway in F3- or LE-induced apoptosis, we assessed $\Delta\phi_m$ in cells exposed to F3 or LE using FCM. We found that both F3 and LE treatments decreased the $\Delta\phi_m$ implying that these analogues caused a rapid dose-dependent dissipation of $\Delta\phi_m$. Both F3 and LE elicited significant increases in the level of intracellular Ca^{2+} , an important messenger molecule in apoptosis (from 40.39% to 90.65% and from 38.65% to 90.88%, respectively) (Figures 2c and 2d).

To confirm this effect on intracellular ROS, MCF - 7 cells were treated with F3 or LE (20 μM) for 48 h in the presence or absence of NAC, a nonspecific ROS scavenger. The treatment F3- and LE-induced increases in ROS generation were blocked by pretreatment with 5 μM NAC (Figures 3a and 3b).

MTT assays showed that cell viability increased significantly (Figures 3c and 3d) in the presence of NAC. This suggested that NAC also protected the MCF - 7 cells from F3- and LE-induced cytotoxicity. These results indicated that F3 and LE caused apoptosis by inducing ROS production and activating the mitochondrial apoptosis pathway.

F3 and LE triggered S and G2 / M arrest in MCF - 7 cells

Cell cycle arrest usually results in apoptosis. FCM detected alterations in the cell cycle in cells treated with F3 or LE (0, 5, 10, and 20 μM) for 48 h. As shown in Figure 2a, the proportion of cells at S phase increased slightly (from 23.93% to 30.00%) and those in G0 / G1 phase decreased in a dose-dependent manner in MCF - 7 cells exposed to F3. An apparent increase in the proportion in G2 / M phase was also observed (from 14.38% to 28.8%).

As shown in Figure 2b, treatment of MCF - 7 cells with LE also significantly increased the proportion of cells in S phase (from 19.28% to 35.58%), reduced the number in G0 / G1 phase in a dose-dependent manner, and slightly increased the proportion in G2 / M phase (from 17.97% to 27.22%). These results suggested that the anti-proliferative effects of both F3 and LE may be related to the accumulation of cells in S and G2 / M phases.

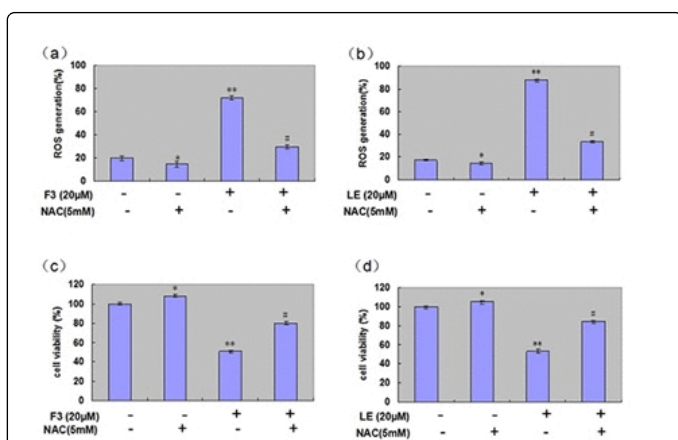


Figure 3: Effects of antioxidant pretreatment on F3-mediated and LE-mediated ROS generation in MCF - 7 cells. Cells were either untreated or treated with F3 or LE (20 µM) in the absence or presence of N-acetylcysteine (NAC; 5 mM) for 48 h. a, b) Production of reactive oxygen species (ROS) was measured by flow cytometry. b) The data represent the percentages of cells within the specified fluorescence intensity range, determined using WinMDI 2.8 software. c, d) Cell viability examined using the MTT assay. *p < 0.05 and **p < 0.01 vs. control (0 µM), # p < 0.05 vs. F3-treated and LE-treated cells (20 µM).

Excessive levels of ROS are reported to lead to an increase in intracellular Ca^{2+} and a loss of $\Delta\psi_m$, leading to mitochondrial dysfunction [13]. To investigate the involvement of the mitochondrial pathway in F3- or LE-induced apoptosis, we assessed $\Delta\psi_m$ in cells exposed to F3 or LE using FCM. We found that both F3 and LE treatments decreased the $\Delta\psi_m$ implying that these analogues caused a rapid dose-dependent dissipation of $\Delta\psi_m$. Both F3 and LE elicited significant increases in the level of intracellular Ca^{2+} , an important messenger molecule in apoptosis (from 40.39% to 90.65% and from 38.65% to 90.88%, respectively) (Figures 2c and 2d).

p38MAPK signaling contributed to both F3-induced and LE-induced apoptosis in MCF - 7 cells

The MAPKs, ERK, JNK, and p38MAPK, are known to be activated in response to oxidative stress [14]. Numerous studies have reported that ROS rapidly activate p38MAPK and promote neuronal cell death [15]. Since F3 and LE both induced ROS generation, we examined the involvement of these kinases in F3- and LE-induced apoptosis. Treatment with either F3 or LE increased the amount of phosphorylated p38 in a concentration dependent manner, without altering the total p38 protein level (Figures 4a and 4b).

In contrast, F3 and LE did not affect the levels of phospho-ERK or phospho-JNK. We further investigated the role of p38MAPK by employing a pharmacological inhibitor of p38MAPK (SB203580). This inhibitor attenuated both F3-induced and LE-induced apoptosis (Figures 4c and 4d).

In addition, SB203580 blocked non-treated cells (Figures 4c and 4d). These data indicated that ROS generation modulated the activities of p38MAPK in F3 - and LE - induced apoptosis. The role of mitochondrial p38MAPK may relate to mitochondrial function and / or the mitochondrial apoptotic pathway.

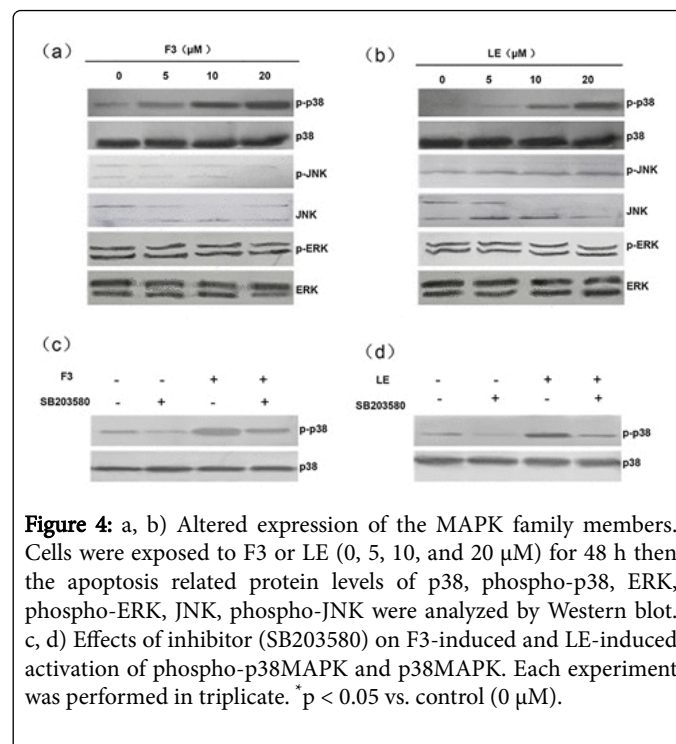


Figure 4: a, b) Altered expression of the MAPK family members. Cells were exposed to F3 or LE (0, 5, 10, and 20 µM) for 48 h then the apoptosis related protein levels of p38, phospho-p38, ERK, phospho-ERK, JNK, phospho-JNK were analyzed by Western blot. c, d) Effects of inhibitor (SB203580) on F3-induced and LE-induced activation of phospho-p38MAPK and p38MAPK. Each experiment was performed in triplicate. *p < 0.05 vs. control (0 µM).

F3- and LE-induced apoptosis in MCF - 7 cells involved the mitochondrial pathway

Natural products or cytotoxic chemicals often induce apoptosis through a mitochondrial pathway, where cytochrome c is released from mitochondria into the cytosol, triggering apoptosome formation and caspase activation [16].

To determine whether F3- and LE-induced apoptosis was mitochondrial-mediated, caspase activation was analyzed. The levels of procaspases - 3 (35 kDa) and - 9 (47 kDa) were reduced and levels of the cleaved caspase - 3 substrate, poly ADP - ribose polymerase (PARP) increased following exposure to F3 and LE, in a dose-dependent manner (Figure 5a, b).

F3 and LE also caused dose-dependent decreases in the level of the X-lined IAP (XIAP) protein, a member of the IAP family that selectively binds and inhibits caspases - 3 and - 9 (Figures 5a and 5b). These data showed that F3 and LE both induced mitochondrial-mediated apoptosis.

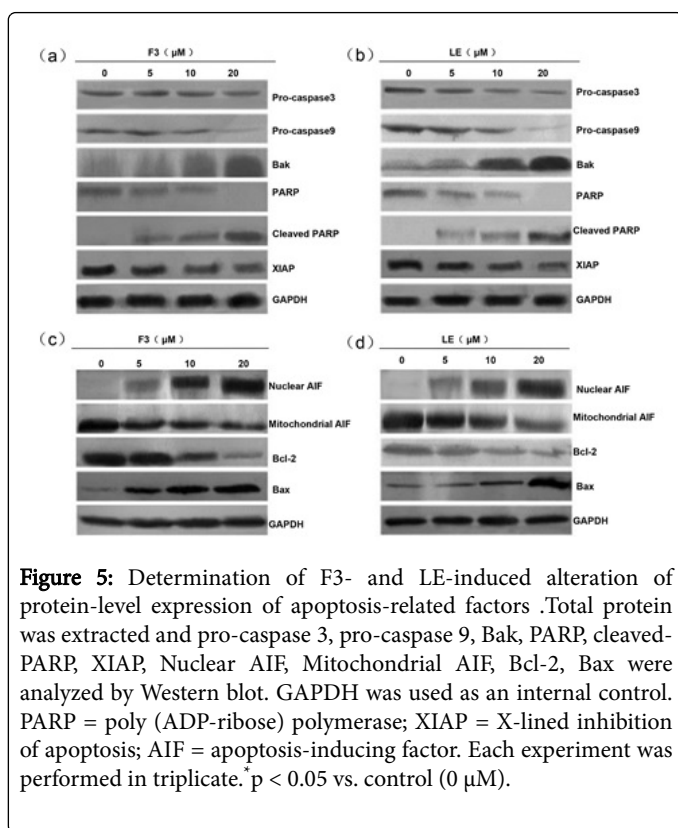


Figure 5: Determination of F3- and LE-induced alteration of protein-level expression of apoptosis-related factors. Total protein was extracted and pro-caspase 3, pro-caspase 9, Bak, PARP, cleaved-PARP, XIAP, Nuclear AIF, Mitochondrial AIF, Bcl-2, Bax were analyzed by Western blot. GAPDH was used as an internal control. PARP = poly (ADP-ribose) polymerase; XIAP = X-lined inhibition of apoptosis; AIF = apoptosis-inducing factor. Each experiment was performed in triplicate. * $p < 0.05$ vs. control (0 μM).

Additionally, AIF that translocates to the nucleus and triggers DNA fragmentation has been identified as an apoptogenic mitochondrial intermembrane protein [17].

AIF precursor protein (67 kDa) was down-regulated, while the mature protein (57 kDa) was up-regulated markedly, indicating that F3 and LE promoted AIF translocation from the mitochondria to the nucleus in a dose-dependent manner (Figures 5c and 5d). Bcl - 2 protein protects cells from mitochondrial apoptosis and the present study found that ectopic expression of Bcl - 2 prevented both F3- and LE-induced apoptosis in MCF - 7 cells (Figures 5c and 5d).

Moreover, both F3 and LE up-regulated the expression of Bax, leading to increases in the Bax / Bcl - 2 ratio, an important marker of apoptosis in cancer cells. Our findings indicated that Bak, an upstream apoptotic factor, was up-regulated by both F3 and LE (Figures 5a and 5b).

Cytosolic cytochrome c levels increased in MCF - 7 cells following exposure to F3 or LE, whereas mitochondrial levels decreased, indicating the release of mitochondrial cytochrome c (Figures 6a and 6b).

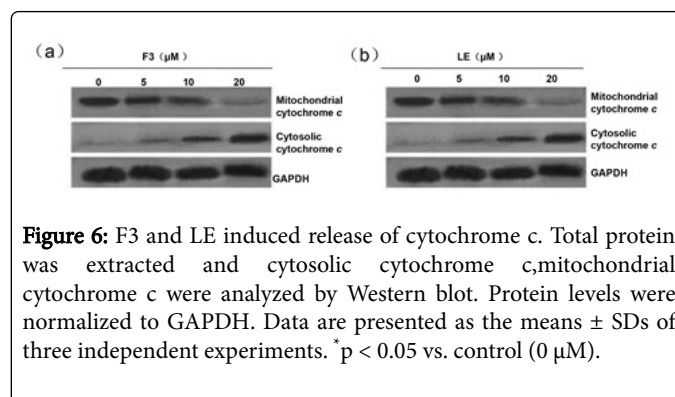


Figure 6: F3 and LE induced release of cytochrome c. Total protein was extracted and cytosolic cytochrome c, mitochondrial cytochrome c were analyzed by Western blot. Protein levels were normalized to GAPDH. Data are presented as the means \pm SDs of three independent experiments. * $p < 0.05$ vs. control (0 μM).

In summary, the results of the present study indicated that both F3 and LE induced apoptosis in MCF - 7 cells. Based on these findings, we propose a model whereby F3 and LE increased ROS generation, leading to MCF - 7 cell apoptosis. Supplementary Figure 3 illustrates that both F3 and LE acted on mitochondria, induced ROS generation, and activated the pro-apoptotic stress kinase p38MAPK, resulting in a loss of $\Delta\psi_m$ and increased intracellular Ca^{2+} . The loss of $\Delta\psi_m$ increased the release of cytochrome c and AIF from mitochondria, and eventually triggered both caspase-dependent and caspase-independent apoptotic pathways. This study may provide an insight into the potential of curcumin analogues for breast cancer therapy.

Acknowledgement

The authors gratefully acknowledge the financial support of the National Natural Science Foundation of China (41312303).

References

- Lin Lu, Fang Ju, Hui Zhao, Xuezen Ma (2015) Antitumor activity of ginseng saponins, 25-OH-PPD and 25-OCH3-PPD, on gastric cancer cells. *Biotechnol Lett* 37: 2461-2466.
- Nagaraju GP, Aliya S, Zafar SF, Basha R, Diaz R, et al. (2012) The impact of curcumin on breast cancer. *Integr Biol (Camb)* 4: 996-1007.
- Cai XZ, Huang WY, Qiao Y, Du SY, Chen Y, et al. (2013) Inhibitory effects of curcumin on gastric cancer cells: a proteomic study of molecular targets. *Phytomedicine* 20: 495-505.
- Liu YL, Yang HP, Gong L, Tang CL, Wang HJ (2011) Hypomethylation effects of curcumin, demethoxycurcumin and bisdemethoxycurcumin on WIF-1 promoter in non-small cell lung cancer cell lines. *Mol Med Rep* 4: 675-679.
- Park BS, Kim JG, Kim MR, Lee SE, Takeoka GR, et al. (2005) Curcuma longa L. constituents inhibit sortase A and *Staphylococcus aureus* cell adhesion to fibronectin. *J Agric Food Chem* 53: 9005-9009.
- Faião-Flores F, Suarez JA, Pardi PC, Maria DA (2012) DM-1, sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3-oxo-penta-1,4-dienyl]-2-methoxyphenolate: a curcumin analog with a synergic effect in combination with paclitaxel in breast cancer treatment. *Tumour Biol* 33: 775-785.
- Li Q, Chen J, Luo S, Xu J, Huang Q, et al. (2015) Synthesis and assessment of the antioxidant and antitumor properties of asymmetric curcumin analogues. *Eur J Med Chem* 93: 461-469.
- Hu W, Kavanagh JJ (2003) Anticancer therapy targeting the apoptotic pathway. *Lancet Oncol* 4: 721-729.
- Pan D, Li W, Miao H, Yao J, Li Z, et al. (2014) LW-214, a newly synthesized flavonoid, induces intrinsic apoptosis pathway by down-regulating Trx-1 in MCF - 7 human breast cells. *Biochem Pharmacol* 87: 598-610.
- Reuter S, Eifes S, Dicato M, Aggarwal BB, Diederich M (2008) Modulation of anti-apoptotic and survival pathways by curcumin as a

-
- strategy to induce apoptosis in cancer cells. *Biochem Pharmacol* 76: 1340-1351.
11. Görsdorf S, Appel KE, Obe G, Hildebrandt AG (1987) Inhibition of bleomycin-induced DNA strand breaks in V 79 Chinese hamster cells by the antioxidant propylgallate. *Toxicol Lett* 38: 135-143.
 12. Kim JY, Yu SJ, Oh HJ, Lee JY, Kim Y, et al. (2011) Panaxydol induces apoptosis through an increased intracellular calcium level, activation of JNK and p38 MAPK and NADPH oxidase-dependent generation of reactive oxygen species. *Apoptosis* 16: 347-358.
 13. Li Q, Zhang L, Zu Y, Liu T, Zhang B, et al. (2013) Generation of reactive oxygen species by a novel berberine-bile acid analog mediates apoptosis in hepatocarcinoma SMMC-7721 cells. *Biochem Biophys Res Commun* 433: 432-437.
 14. Matsuzawa A, Ichijo H (2008) Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochim Biophys Acta* 1780: 1325-1336.
 15. Lan AP, Xiao LC, Yang ZL, Yang CT, Wang XY, et al. (2012) Interaction between ROS and p38MAPK contributes to chemical hypoxia-induced injuries in PC12 cells. *Mol Med Rep* 5: 250-255.
 16. Los M, Wesselborg S, Schulze-Osthoff K (1999) The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. *Immunity* 10: 629-639.
 17. Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, et al. (2000) Two distinct pathways leading to nuclear apoptosis. *J Exp Med* 192: 571-580.