

ORIGINAL ARTICLE

Capsaicin induced apoptosis and gene expression dysregulation of human acute lymphoblastic leukemia CCRF-CEM cells

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Summary

Purpose: Capsaicin, an ingredient of red chili pepper, has possible tumorigenicity/genotoxicity properties. We aimed to determine the effects of capsaicin on the proliferation and gene expression profiles of acute lymphoblastic leukemia (ALL) CCRF-CEM cell line.

Methods: Cell viability and IC₅₀ dose was determined by WST cytotoxicity assay. qRT-PCR, immunohistochemical staining and western blot methods were used to determine target genes' expression levels. Apoptosis was evaluated by measuring the caspase-3 activity.

Results: Capsaicin inhibited the proliferation of CCRF-

CEM cells in a dose-dependent manner. Increased mRNA expressions of caspase gene family members, activated caspase-3 and decreased mRNA and protein expression of BCL-2 gene indicated apoptotic response to capsaicin. Moreover capsaicin treatment suppressed significantly the expression of the key cell signaling pathways of KRAS, AKT, GAB2, PTPN11, BRAF, INPP5D, MAPK7.

Conclusion: Capsaicin induces apoptosis in CCRF-CEM cells and this response is associated with downregulation of cell signaling pathways.

Key words: acute lymphoblastic leukemia, apoptosis, capsaicin, cell signaling

Introduction

Childhood leukemia represents about 35% of all childhood malignancies. ALL is one of the most common forms of childhood cancer, representing 80-85% of all leukemias [1]. As of today, ALL of childhood has become a curable disease in most (80%) of the patients [2]. However, there is still a significant proportion of patients with ALL for whom neither chemotherapy nor allogeneic transplantation offer cure.

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a component of red chili pepper and a widely consumed essential ingredient in numerous cuisines. The analgesic activity of capsaicin allows its usage for the treatment of neuropathic pain [3]. Topical capsaicin treatment has been widely used in different diseases such as arthritis, neuralgias, diabetic neuropathy and cancer [4].

However, there are controversial reports about effectiveness and safety of capsaicin. Antiproliferative and proapoptotic effects of capsaicin have been reported in pancreatic cancer [5], urothelial cancer [6], glioma [7] and leukemia [8] cell lines. On the other hand, mutagenic and proliferative effects have also been reported [9,10]. Therefore, capsaicin is defined as a "double-edged sword" agent for having possible tumorigenicity and genotoxicity properties.

Capsaicin binds to Transient Receptor Potential channels type V1 (TRPV1), which is a ligand gated cationic channel and can be activated by capsaicin. It was reported that TRPV1 is a potential target for pharmacological inhibition of cancer pain in bone metastases, pancreatic cancer and other cancers [11]. TRPV1-mediated apoptosis involves both the mitochondrial and extrinsic apoptotic pathways [6]. Cysteine proteases (caspases)

and BCL-2 are major players of the mitochondrial apoptosis. While caspases are activated during apoptosis, BCL-2 can inhibit their activation and acts as a negative regulator of apoptosis [12].

The fate of the cell is controlled by signal transduction systems that link growth factors and receptors for several cellular functions, and are mostly activated in human cancers. RAS-RAF-MAPK, JAK-STAT, PI3K-AKT are some major signaling pathways and enzymes such as GTPases, protein phosphatases, protein kinases, protein tyrosine phosphatases are key regulatory components in these pathways which are important in the control of cell growth, proliferation, differentiation and transformation. Involvement of the phosphoinositide 3-kinase/AKT pathway in apoptosis induced by capsaicin was reported in a pancreatic cancer cell line [13]. However, the effects of capsaicin in acute ALL have not been studied from a molecular biological viewpoint.

In this study we aimed to investigate the molecular targets of capsaicin in ALL CCRF-CEM cell line and find possible pathways involved in either growth or inhibitory effects. Differences in the gene expression profiles, including apoptosis, cell proliferation and cell cycle pathways, were investigated and capsaicin-induced apoptosis was assessed in the capsaicin ALL treated cells.

Methods

Cell lines

Human ALL cell line CCRF-CEM was obtained from American Type Culture Collection (ATCC, Manassas, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Bio Ind. Kibbutz Beit Haemek, Israel), 100 U/ml penicillin and 100 mg/ml streptomycin (Bio Ind.). Cells were incubated at 37 °C with 5% CO₂. Cells with 95% survival rates and 80% confluency were used in experimental analyses.

Capsaicin

Powder form of pure capsaicin (MW=305.41 g/mol; Sigma, Missouri, USA) was dissolved in DMSO and serial dilutions were prepared using RPMI 1640 medium. Experimental set up was generated with 80 μM capsaicin treatment with IC₅₀ concentration of CCRF-CEM cells. After 72 h of capsaicin exposure, 1x10⁶ and 5x10⁶ cells were collected for apoptosis and gene expression analyses, respectively.

Cytotoxicity assay

Cell viability and proliferation of untreated controls and capsaicin-treated cells were determined by

using the WST-1 assay (Roche Applied Science, Mannheim, Germany). CCRF-CEM cell suspension was placed into 96-well plate at a density of 10x10⁴ cells in 100 μl per well. Then, cells were treated with increasing doses of capsaicin for 72 h. After having performed the proliferation assay, absorbance of each sample was measured spectrophotometrically at 450 nm with an ELISA reader (Thermo, Vantaa, Finland). All experiments were performed in triplicate. The obtained data was evaluated with the GraphPad Prism 5.01, cell proliferation curves were generated and IC₅₀ concentration was calculated for CCRF-CEM cells.

Determination of differentially expressed genes by real-time ready array

Total RNA was isolated using the MagNA Pure LC RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) from untreated and capsaicin-treated CCRF-CEM cells. 10 μg of total RNA were reverse-transcribed with the transcriptor high fidelity cDNA synthesis kit (Roche Applied Science). A real-time ready custom array panel was designed for the quantification of differently expressed gene expressions by real-time PCR using the LightCycler 480 instrument. Relative quantification of each sample with G6PDH (glucose-6-phosphate dehydrogenase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and Beta-actin housekeeping genes was achieved by using the instruments software.

Caspase-3 activity assay

Caspase-3 activity was measured in 1x10⁶ cells by using the Caspase-3 Colorimetric Assay Kit (BioVision Research Products, Mountain View, CA, USA). Briefly, cells were collected and resuspended in chilled lysis buffer. After centrifugation the supernatant was aliquoted and the protein concentrations were measured. For reaching equal amounts of protein (50 μg/ml) samples were diluted with cell lysis buffer. Before samples were incubated at 37 °C for 2 h, reaction buffer and substrate were added. Finally, absorbance was measured spectrophotometrically at 405 nm with an ELISA reader (Thermo, Vantaa, Finland).

Immunohistochemical analyses

Control and capsaicin treated CCRF-CEM cells embedded in paraffin blocks were cut into 2μ sections with microtome (Leica MR 2145) and prepared for immunohistochemical staining as described previously [14]. Primary antibodies of BCL-2 and caspase-3 were obtained from Santa Cruz and diluted at 1/300. In brief, the deparaffinization procedure was accomplished in xylene for 1 h. Rehydration was done in sequential 100, 95, 80 and 70% alcohol series for 2 min each. After leaving in distilled water for 5 min, the tissue samples were delineated on the object slide, washed in phosphate buffered saline (PBS) for 10 min, and then left

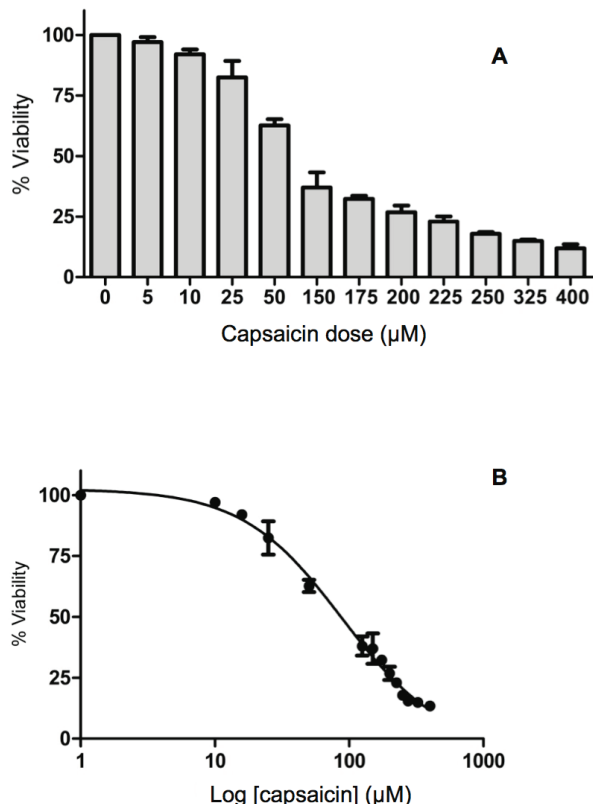


Figure 1. WST cytotoxicity assay results of capsaicin-treated CCRF-CEM cells. **A:** Dose-dependent inhibition of viability in capsaicin-treated CCRF-CEM cells; **B:** Viability inhibition curve.

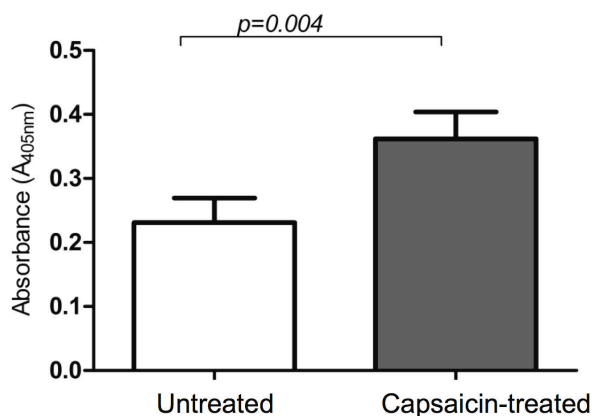


Figure 2. Caspase-3 activity in untreated and capsaicin-treated CCRF-CEM cells.

in trypsin for 15 min. The primary antibody was then applied in an incubator at 57 °C and washed with PBS. Afterwards the biotinylated secondary antibody was applied and washed with PBS before incubating with the enzyme conjugate and 3,3-diaminobenzidine tetrahydrochloride (DAB). Subsequently, sections were

stained with Mayer's hematoxylin (Zymed Laboratories, San Francisco, CA, USA) and mounted with entellan. All sections were examined and photographed with Olympus C-5050 digital camera at Olympus BX51 microscope.

Western blot analyses

Cells were lysed in complete Lysis-M buffer containing Protease Inhibitor Cocktail Tablets (Roche Applied Science, Mannheim, Germany). Protein concentrations were measured with the Bradford method by using bovine serum albumin (BSA) standards in concentrations between 0.25 and 2 mg/ml. 40 µg of protein extract per sample were loaded and run on 8% SDS-PAGE gels and then transferred to PVDF membranes by using the I-Blot System (Invitrogen Corporation, Carlsbad, CA, USA). Primary antibodies used were polyclonal Bcl-2 (1:500 diluted; Biovision, Mountain View, CA, USA) and β -Actin (1:2500 diluted; Cell Signaling Technology, Danvers, MA, USA). WesternBreeze Chromogenic Immunodetection Kit (Anti Rabbit) was used for the chromogenic detection of the proteins (Invitrogen Corporation Carlsbad, CA, USA).

Statistics

Statistical analyses of gene expressions were calculated by CLC Main Workbench software. After normalization to the housekeeping genes, log₂ transformation was performed to the expression values and fold change; FDR (False Discovery Rate) corrected p values were calculated. IC₅₀ cisplatin concentrations were calculated with the GraphPad Prism Software 5.01. The Student's t-test was used to test the probability of significant differences between samples. A p value < 0.05 was considered as statistically significant.

Results

In order to study the effects of capsaicin, CCRF-CEM cells were treated with different concentrations of capsaicin (5, 10, 25, 50, 150, 175, 200, 225, 250, 325 and 400 µM). After 72 h, viability was evaluated by WST cytotoxicity assay. Cell viability was taken as 100% in the control cells and 97, 62, 36, 17 and 11% viabilities were detected at 5, 50, 150, 250 and 400 µM of capsaicin-treated cells, respectively (Figure 1A). These results revealed that cell growth was inhibited by capsaicin in a dose-dependent manner. According to the capsaicin inhibition curve, IC₅₀ dose was calculated as 80 µM for CCRF-CEM cells (Figure 1B).

To examine the apoptotic effects of capsaicin, we analyzed caspase-3 activity and found that its activity was increased to 56% in capsaicin-treated CCRF-CEM cells ($p=0.004$, Figure 2). Data obtained from gene expression and immunohistochemical

Table 1. List of genes that significantly decreased and increased in capsaicin-treated CCRF-CEM cells compared to control

Gene	Full name	Fold change*	FDR p-value**	Control CCRF-CEM Mean	Capsaicin treated CCRF-CEM Mean
KRAS	Kirsten rat sarcoma viral oncogene homolog	-52.89	2.72E-05	0.050	0.010
GAB2	Grb2-associated binding protein	-39.91	0.002	0.090	0.002
BCL2	B cell leukemia/lymphoma-2	-31.22	0.002	0.020	0.001
AKT2	v-akt murine thymoma viral oncogene homolog 2	-23.54	0.002	0.140	0.006
PTPN11	Protein tyrosine phosphatase, non-receptor type-11	-12.7	0.020	0.050	0.004
BRAF	v-raf murine sarcoma viral oncogene homolog-B1	-8.53	0.020	0.050	0.006
INPP5D	Inositol polyphosphate-5-phosphatase	-8.24	0.006	0.130	0.020
MAPK7	Mitogen-activated protein kinase 7	-5.42	0.020	0.005	0.001
AKT1	v-akt murine thymoma viral oncogene homolog 1	-3.63	0.050	0.020	0.006
CHEK2	CHK2 checkpoint homolog (S. pombe)	1.12	0.004	0.003	0.003
CASP7	Caspase-7, apoptosis-related cysteine peptidase	1.14	0.030	0.001	0.002
CASP3	Caspase-3, apoptosis-related cysteine peptidase	1.48	0.006	0.004	0.006
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.00	0.020	0.010	0.020
CDC25C	Cell division cycle 25 homolog C (S. pombe)	2.17	0.020	0.002	0.004
CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18)	2.56	0.007	0.008	0.020
CASP6	Caspase-6, apoptosis-related cysteine peptidase	2.58	0.002	0.0008	0.002
CCNB1	Cyclin B1	3.13	0.020	0.006	0.020
CASP1	Caspase-1, apoptosis-related cysteine peptidase	2.83	0.007	0.001	0.003
CCNB2	Cyclin B2	4.00	0.004	0.030	0.120
CDK1	Cyclin-dependent kinase 1	15.00	0.002	0.010	0.150

*Values represent fold-changes of increased or decreased mRNA expressions. All values were normalized to three housekeeping genes and then log₂ transformation performed. **FDR corrected p-value

analyses also demonstrated increased caspase-3 mRNA and protein expression levels (Figure 3 and 4, Table 1). Furthermore, caspase-1, -3, -6, and -7 mRNA expressions increased significantly in capsaicin-treated CCRF-CEM cells (Table 1). On the other hand, mRNA and protein expression levels of BCL2 gene, which is a negative regulator of apoptosis, decreased significantly in capsaicin-treated CCRF-CEM cells (Figures 3-5, Table 1).

mRNA expressions of cell cycle regulator genes were found dysregulated after capsaicin treatment. For instance CHEK2, CDKN1A, CDC25C, CDKN2C, CCNB1, CCNB2 and CDK1 expressions increased significantly in the capsaicin-treated cells (p=0.004, 0.02, 0.02, 0.007, 0.02, 0.004 and 0.002, respectively).

However, mRNA expressions of adapter proteins (act for transmitting various signals in response to stimuli through cytokine and growth factor receptors, and T- and B-cell antigen receptors) decreased after capsaicin treatment. KRAS was determined as the most expressional reduced gene after capsaicin treatment (-52.89 fold, p<0.001). GAB2, a docking adaptor protein, also exhibited -39.91 fold mRNA expressional downregulation in capsaicin-treated cells (p=0.002). Other genes with

significantly decreased mRNA expression were AKT2 (-23.54 fold, p=0.002), PTPN11 (-12.7 fold, p=0.02), BRAF (-8.53 fold, p=0.02), INPP5D (-8.24 fold, p=0.006) and AKT1 (-3.78 fold, p=0.04), as shown in Table 1.

Discussion

Preventing cancer and causing cancer effects of capsaicin have been reported in several cancers and this commotion leads to discussions over whether its consumption or topical use is entirely safe [4]. It should be considered that hot pepper consumption is not equivalent to the use of pure capsaicin [4], and well-controlled studies are needed to evaluate the safety and efficacy of capsaicin use. Commercially available pure capsaicin was used in this study and our results showed that capsaicin inhibits cell growth and induces apoptosis in CCRF-CEM cells.

Capsaicin-induced apoptosis has been reported in breast [15], bladder [16], colon [17], esophagus [18], prostate [19] and pancreas cancers [20]. However, the reasons of capsaicin-induced leukemic cell apoptosis are still being investigated. Our results suggest some potential mechanisms related



Figure 3. Hierarchical clustering of the gene expression profiles: Gene expression patterns of untreated and capsaicin-treated CCRF-CEM cell groups. Each sample was performed in triplicate.

with induced apoptosis and indicate capsaicin as an anticancer agent.

The RAS-RAF-MEK-ERK-MAPK signaling pathway regulates cell responses to the environmental factors and plays an important role in human cancers. Since KRAS is one of the three RAS, BRAF is also one of the three RAF and exhibit increased expressions in various types of cancer. However, we detected -52.89 and -8.53 fold decrease in KRAS and BRAF expressions respectively

following capsaicin treatment. KRAS is a guanine triphosphatase (GTPase) and its overexpression recruits and activates proteins necessary for the propagation of growth factors and other molecular signals, including phosphoinositide-3 kinase (PI3K), which is involved in numerous signal transduction pathways [6-9]. BRAF is an oncogene and its mutations associate with aberrant activation of the RAS-RAF-MEK-ERK-MAPK signaling pathway in several cancers [21]. In this study, expression-

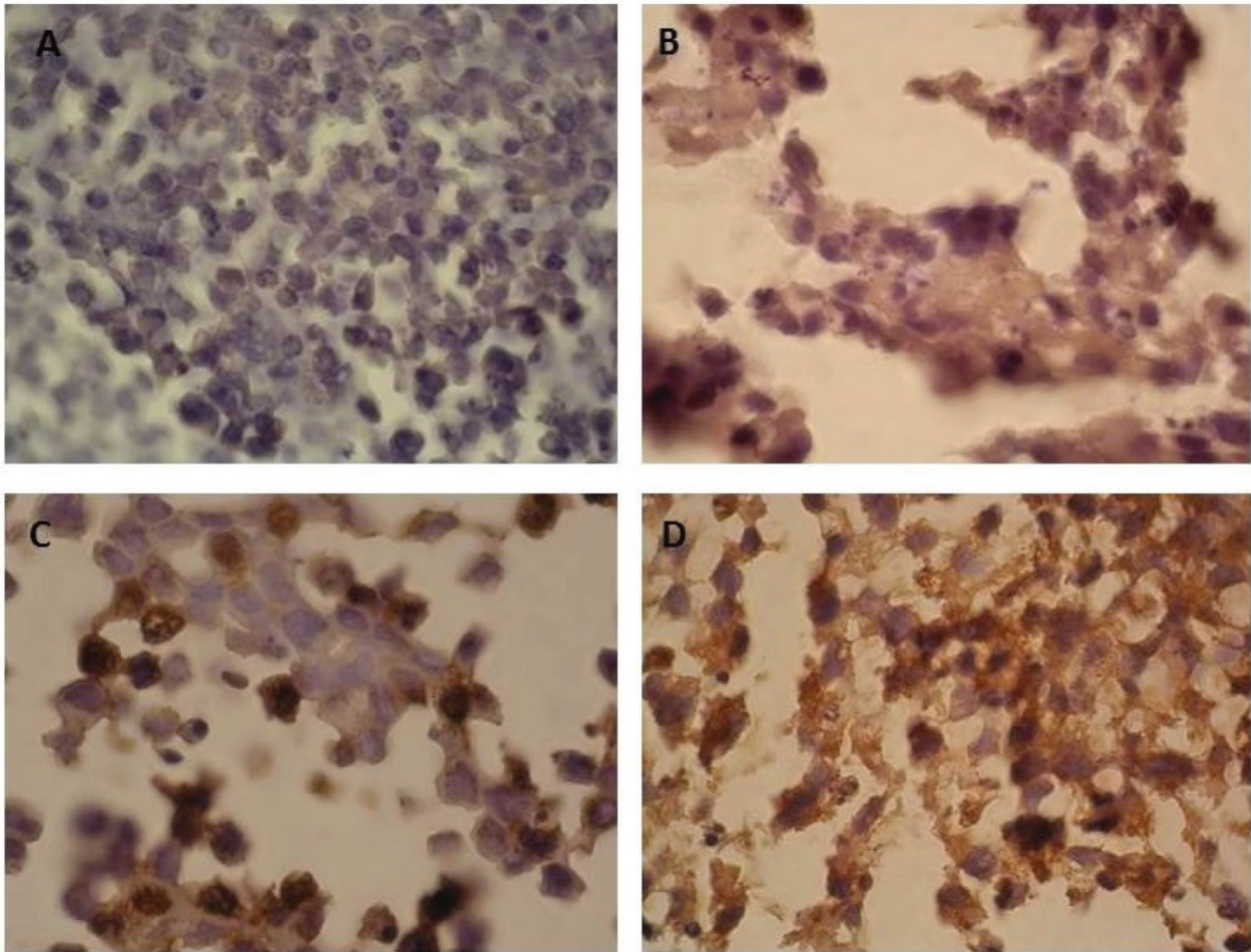


Figure 4. Immunohistochemical expressions of BCL2 and caspase-3 in CCRF-CEM cells with and without capsaicin treatment **A:** Immunoexpression of BCL2 in controls; **B:** capsaicin-treated cells; **C:** Immunoexpression of caspase-3 in controls; **D:** capsaicin-treated cells.

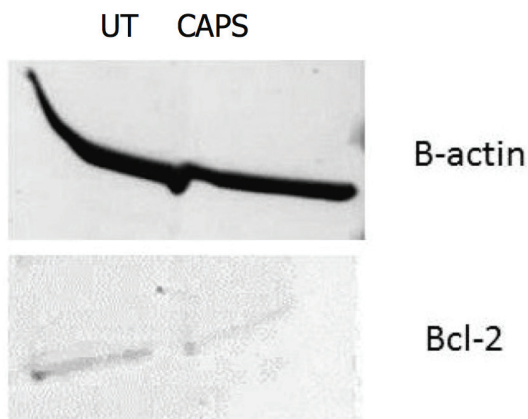


Figure 5. Western blot results of the B-actin and Bcl-2 proteins. UT: untreated CCRF-CEM cells; CAPS: capsaicin-treated CCRF-CEM cells.

al inhibition of KRAS and BRAF showed signaling pathway inhibition at the beginning of the cascade, thus triggering molecules' expressional down-regulation. Furthermore, decreased expression of MAPK7 in -5.42 fold supports this hypothesis with the blockage of signaling at the last part of the

cascade. In conclusion, our data pointed out that RAS-RAF-MEK-ERK-MAPK pathway was strongly inhibited in response to capsaicin treatment.

The GAB proteins, a family of scaffolding or docking adaptor proteins, are recruited to activated receptors [22]. They contain several functional motifs that mediate interactions with multiple signal relay molecules and can assemble signaling complexes. GAB2 undergoes tyrosine phosphorylation, creating a number of docking sites to mediate interactions with SH2 domain-containing proteins such as the tyrosine phosphatase SHP2 and PI3K [23]. The interaction of GAB2 with SHP2 activates RAS-ERK signaling, whereas its association with the p85 subunit of PI3K is crucial in mediating the PI3K-AKT signaling [24]. Suppressed GAB2, AKT1 and AKT2 expressions via capsaicin treatment showed inhibition of PI3K-AKT signaling in our study. Recently, downregulation of the PI3K/AKT signaling pathway for the capsaicin-induced apoptosis was also reported in the PANC-1 human pancreatic cancer cell line [13]. Because PI3K-AKT pathway is leading to growth and survival of can-

cer cells, inhibitors targeting this pathway are more important and capsaicin molecule is a good candidate in this regard.

The inositol 5-phosphatase (INPP5D or SHIP1) is a negative regulator of signaling processes in hematopoietic cells via termination of PI3-K mediated signaling [25]. It was reported that inactivation of INPP5D could play a central role in the deregulation of AKT pathway and tumorigenesis [26]. Similarly, our results revealed dysregulation of AKT1, AKT2 and INPP5D gene expressions together with -3.78, -23.54 and -8.24 fold expressional downregulations, respectively.

PTPN11 encodes the protein tyrosine phosphatase SHP2 and positively regulates physiologic hematopoiesis [27]. Protein tyrosine phosphatases (PTPs) play critical roles in this regulation by controlling the functions of key receptors and intracellular signaling molecules in lymphocytes. Although negatively regulated PTPN11 expression and increased apoptosis have been defined [27], the effect of capsaicin on the PTPN11 expression has not been reported anywhere. We found -12.7 fold decrease in the PTPN11 expression after capsaicin treatment in CCRF-CEM cells.

Capsaicin induced cell cycle arrest in bladder cancer and epithelial carcinoma cells [28, 29]. However, our results demonstrated that capsaicin treatment induced the expression of cell cycle regulator genes (CHEK2, CDKN1A, CDC25C, CDKN2C,

CCNB1, CCNB2 and CDK1). Contrary to our results, inhibition of CDK1 expression after capsaicin treatment was reported in the FaDu pharyngeal carcinoma [30] and leukemia HL-60 cells [31]. However, inhibition of CDK1 started from 100 μ M and 200 μ M high doses of capsaicin for FaDu and HL-60 cells, respectively [30,31], and 75 μ M capsaicin had no effect on CDK1 expression [31]. Since we used 80 μ M capsaicin due to our calculated IC₅₀ dose for CCRF-CEM cells, this lower dose might not be enough to inhibit cell cycle regulators. Increased CDK1 activity and cell apoptosis in the G1 phase was reported in X-irradiated lymphocytic leukemia cells and it was suggested that CDK1 is activated by accumulation of Cyclin B1 [32]. Moreover, CDK1 activation at the wrong time and in the wrong phase, was associated with directly or indirectly triggering BCL2-dependent signaling pathway leading to apoptotic cell death [32]. However, we could not analyze the cell cycle phases and this was a limitation of our study.

In conclusion, our results demonstrated that capsaicin has antiproliferative and apoptotic effects in the CCRF-CEM cell line and could be a new treatment approach for ALL. Activation of the caspase gene family and inhibition of the BCL2 were main players for the capsaicin-induced apoptosis. In addition, capsaicin treatment suppressed significantly the key cell signaling pathway members expression.

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