Original Article:

In vitro antitumoral effects of the combination of Curcuma longa and prednisolone on inhibition of the NF-κB signaling pathway

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common cancer among children. Despite recent treatment advances, about 80 percent of patients indicate resistance to treatment and the relapse remains a significant clinical problem. Apoptotic-resistant leukemia cells exhibit an unusual response to the NF-κB pathway; therefore, inhibiting this pathway can sensitize resistant cells to treatment. IKK-NF-kappaB signaling is an important factor in carciogenesis and a potential target for cancer treatment. Therefore, in this study, the mRNA expression of targets of NF-κB signaling including IKKalpha, IKKbeta and also NF-κB were analyzed, using the real time-PCR in the face of the combined effect of Curcuma longa and prednisolone in the ALL cell line of NALM-6. Results of the study showed significant downregulation of three genes after combination Curcuma longa (5 µg/ml) and prednisolone (1 µM) in comparison with the single agents alone. These findings unveiled the synergistic effect of Curcuma longa and prednisolone on IKKα and IKKβ downregulation and NF-κB inhibition that can be considered as a new approach in the ALL treatment of resistance to chemotherapy.

Keywords: Acute lymphoblastic leukemia; Curcuma longa; prednisolone; NF-κB signaling

INTRODUCTION

Different types of leukemia are the most common cancer in children, and among them, acute lymphoblastic leukemia (ALL) is the most common subtype, with about 25% of childhood tumors [1]. The malignancy is associated with uncontrolled proliferation of lymphoid progenitors in the bone marrow as well as with the accumulation of malignant lymphoblasts in bone marrow and peripheral blood. Modern chemotherapy regimens can effectively treat more than 80% of ALL cases [1]. The vast majority of recurrence occurs due to the failure of therapeutic medications to the complete elimination of the major leukemic clone and subsequently the spread of the disease [2]. Investigating the resistance of cancer cells to chemotherapy drugs has opened a new research topic. Efforts to understand the mechanisms of resistance to treatment have led to significant

improvements over our understanding of signaling pathways involved in the survival of cancer cells. Now that many survival pathways

have been identified, one of the challenges ahead is the discovery and development of molecular factors that have the potential to increase the sensitivity of tumor cells to chemotherapy. Among the signaling pathways that influence on the survival of the tumor, the NF-κB pathway has received considerable attention [3]. When cells are exposed to a suitable stimulus, a triple IKK complex consisting of IKKα, IKKβ, and IKKγ causes phosphorylation of IκB (NF-κB inhibitor protein), which leads to the release of NF-κB and subsequently activate genes expression [4,5]. IKKβ knockout mice have indicated fetal death and an incomplete response to inflammatory cytokines [6], and IKKα knockout mice have showed incomplete

reproduction and morphogenesis [7]. Additionally, IKKα is involved in a variety of biological functions, including apoptosis, tumor suppression, immune system, cell proliferation. Totally, both IKKα and IKKβ are critical to the expression of the genes through NF-κB. Accordingly, in this study, the effect of Curcuma longa extract (previously examined for cytotoxic effects) was evaluated alone as an inhibitor of IKKα/β and in combination with prednisolone as a sensitizer to the drug in ALL cells.

MATERIALS AND METHODS
Chemicals

Prednisolone was purchased from Sigma-Aldrich (Sigma-P6004) and dissolved in sterile-filtered Dimethyl sulfoxide (DMSO) and aliquoted and stored at -20 °C. RPMI 1640 medium (Gibco), FBS (Gibco), RNA extraction kit (GeneAll, Korea), cDNA synthesis kit (Thermo Scientific, USA), and sybr master mix (Takara, Japan) were also purchased.

Plant extract

The extract was obtained from the Curcuma longa roots which was prepared at the Traditional Medicine and Materia Medical Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Total extract was obtained through maceration for 24 h with continuous shaking at room temperature. The filtrate was evaporated to dryness and used for evaluation. A stock solution was prepared with a concentration of 100 mg/ml in DMSO and was stored at -20 °C.

Cell culture

NALM-6 cells as acute lymphoblastic leukemia cells were purchased from the Pasteur Institute of Iran, Tehran. Cells were cultured in RPMI 1640 medium, containing 10% FBS and 1% penicillin at 37 °C with 5% CO2.

RNA isolation and quantitative real-time PCR

The Cells (3×10^5 cells/ml) were treated with different concentrations of prednisolone and Curcuma longa extract and were incubated for 48 h, and total RNA was extracted from each well using the Hybrid-R RNA purification kit (GeneAll, Korea) according to the manufacturer's instructions. The quantity of the RNA samples was analyzed by UV-spectroscopy (NanoDrop TM 2000 Spectrophotometer, Thermo Scientific, USA) at 260 and 280 nm and the quality of extracted RNA was tested by the 1% agarose gel electrophoresis. RNA was reverse transcribed to first-strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA), following the manufacturer's instructions. All the sequence of the primers are shown in Table 1. Real-time PCR analysis was performed using SYBR Premix Ex Taq (Tli RNase H Plus) kit (Takara Biomedical Technology, Japan) in Applied Rotor-Gene Q Real Time PCR System (Qiagen, Valencia, CA). The expression of ABL gene was utilized as an endogenous control to normalize the expression of the target genes. Melting curve analysis of IKKα, IKKβ, NF-κB and ABL showed a single peak. The mean Ct of the target genes was calculated from triplicate measurements and then normalized with the mean Ct of ABL gene. Data extraction was executed using the Rotor-Gene Q series software v. 2.0.2, and the Pfaffl method was used to calculate the relative expression of each gene [8].

Table 1. Quantitative real-time polymerase chain reaction primers

| Name	Sequence
ABL-Forward	5′-CTTCTTGGTGCGTGAGAGTGAG-3′
ABL-Reverse	5′-GACGTAGAGCTTGCCATCAGAAG-3′
IKKα-Forward	5′-CTCCGAAAGCTGCTCAACAAAC-3′
IKKα-Reverse	5′-GCAAATATCGAATCCCAGACCCTA-3′
IKKβ-Forward	5′-TTGCTTGTAGCAAGGTCCGT-3′
IKKβ-Reverse	5′-TGGCTCAGGTAAGCTGTTGG-3′
NF-κB-Forward	5′-AACCAGCCTCTGTGTTTGTC-3′
NF-κB-Reverse	5′-TCCTCTGCACTTCTTCTTTATCTTT-3′

Statistical analysis

The SPSS Statistics version 23 software was used for data analysis. The one-way ANOVA and post hoc Tukey multiple comparison tests were used in the comparison between more than 2 groups. Results are presented as means ± SD. $P < 0.05$ was considered significant.

RESULTS

Effect of Curcuma longa extract and prednisolone combination on the IKKα expression in NALM-6 cells

In this study, doses of 1, 5, 10, and 15 μg/ml of the extract with 1 μM prednisolone alone and in combination with the drug were evaluated over 48 hours of incubation to determine the expression changes of the IKKα gene. Our results indicated that Curcuma longa and prednisolone can reduce the expression of IKKα gene in individual doses and synergy. Concurrent use of Curcuma longa extract and prednisolone in doses of 1, 5 and 10 μg/ml of extract and 1 μM prednisolone significantly reduced the expression of IKKα gene compared to the use of each drug alone. The concentration of 1 μg/ml extract and 1 μM drug resulted in a 1.58-fold decrease in IKKα gene expression compared to control. Additionally, synergy of 5 and 10 μg/ml extract and 1 μM drug reduced the IKKα gene expression by 1.72-fold compared to control (Figure 1).
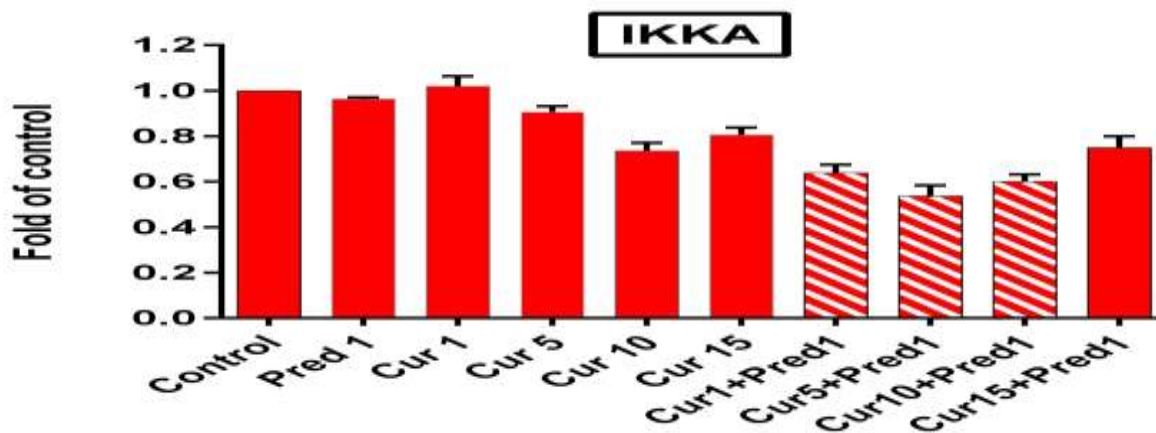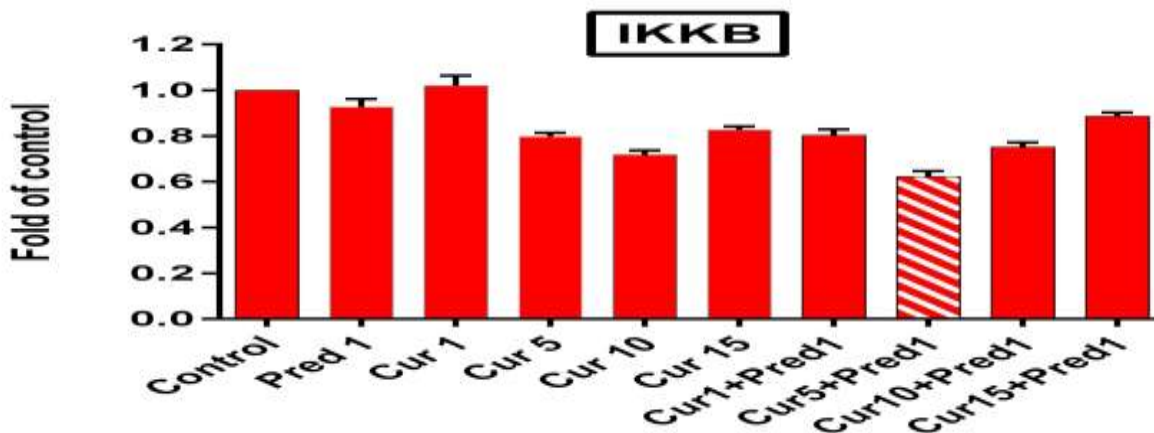

Figure 1. Effect of various concentrations of *Curcuma longa* (1, 5, 10, 15 μg/ml) combined with Prednisolone (1 μM) on IKKα mRNA expression levels in NALM-6 cells. Each bar represents the mean±SD (n=3). P<0.05 (one way ANOVA and post hoc Tukey multiple comparison test).

Effect of Curcuma longa extract and prednisolone combination on the IKKβ expression in NALM-6 cells

In this study, doses of 1, 5, 10, and 15 μg/ml of the extract with 1 μM prednisolone alone and in combination with the drug were evaluated over 48 hours of incubation to determine the expression changes of the IKKβ gene. This study's results indicated that Curcuma longa and prednisolone can reduce the expression of IKKβ gene in individual doses and synergy. Concurrent use of Curcuma longa extract and prednisolone in doses of 1, 5 and 10 μg/ml of extract and 1 μM prednisolone significantly reduced the expression of IKKβ gene compared to the use of each drug alone. The concentration of 5 μg/ml extract and 1 μM drug resulted in a 1.58-fold decrease in IKKβ gene expression compared to control (Figure 2).
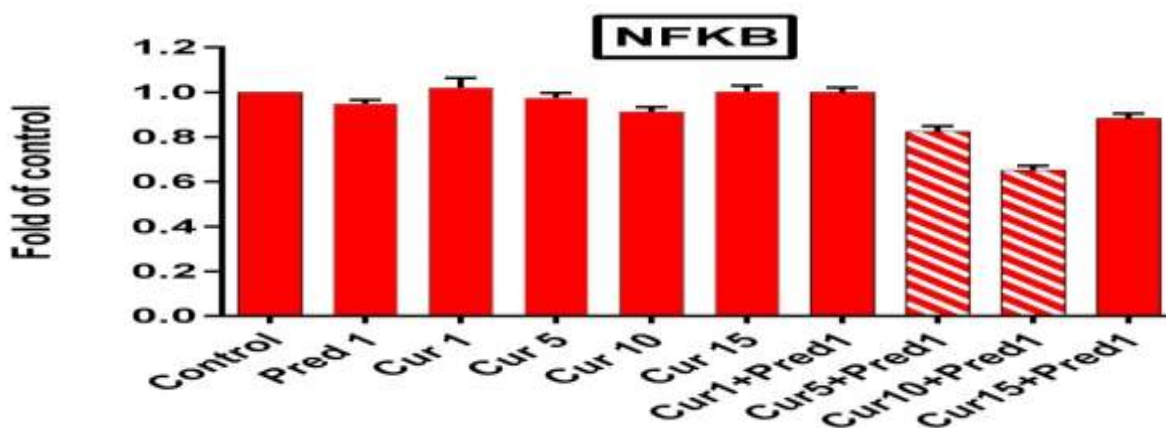
Figure 2. Effect of various concentrations of *Curcuma longa* (1, 5, 10, 15 µg/ml) combined with Prednisolone (1 µM) on IKKβ mRNA expression levels in NALM-6 cells. Each bar represents the mean±SD (n=3). P<0.05 (one way ANOVA and post hoc Tukey multiple comparison test).

Effect of Curcuma longa extract and prednisolone combination on the NF-κB expression in NALM-6 cells

In this study, doses of 1, 5, 10 and 15 µg/ml extract with 1 µM prednisolone alone and also in combination with the drug were evaluated for 48 hours incubation to determine the rate of expression of NF-κB gene expression. The results indicated that Curcuma longa and prednisolone can reduce the expression of NF-κB gene in individual doses and synergy.

Concurrent use of Curcuma longa extract and prednisolone in doses of 5 and 10 µg/ml of extract and 1 µM prednisolone significantly reduced the expression of NF-κB gene compared to the use of each drug alone. The concentration of 5 µg/ml extract and 1 µM drug resulted in a 1.25-fold decrease in NF-κB gene expression compared to control. Also, synergy of 10 µg/ml extract and 1 µM drug reduced the NF-κB gene expression by 1.6-fold compared to control (Figure 3).

Figure 3. Effect of various concentrations of *Curcuma longa* (1, 5, 10, 15 µg/ml) combined with Prednisolone (1 µM) on NF-κB mRNA expression levels in NALM-6 cells. Each bar represents the mean±SD (n=3). P<0.05 (one way ANOVA and post hoc Tukey multiple comparison test).

DISCUSSION

The present study investigated expression changes of IKKα, IKKβ and NF-κB genes on the NALM-6 and REH cells after treatment with

Curcuma longa extract and prednisolone in individual doses and synergy. The results of the synergy of the extract and drug on the REH cell line indicated no significant alteration of these genes; therefore, the results of this cell line were

not included in this report. The investigation of the synergistic effect of the extract and drug on the NALM-6 cell line was significant in some concentrations, especially 5 μg/ml extract and 1 μM drug. Since, apoptotic-resistant leukemia cells exhibit an unusual response to the NF-kB pathway, inhibiting this pathway can sensitize resistant cells to treatment. Totally, the IKK-NF-kappaB signaling pathway is considered as an important factor in carcinogenesis and a potential target for cancer treatment. Inhibition of this pathway in conjunction with inhibition of blast proliferation will have a double effect on the control of this malignancy. Over the past decade, genetic manipulation of NF-κB signaling and the development of IKK inhibitors have improved the treatment of inflammatory and autoimmune diseases [9]. Clinical studies have suggested IKKα/β as a therapeutic target for inhibiting NF-κB activity in a variety of cancers [10], but translating this target into clinical treatment is much more difficult than expected by researchers. Although the specific inhibitor of IKKα/b has not been introduced yet, the ideal inhibitor for the elimination of nuclear IKK function in a particular disease should be designed to minimize systemic toxicity, and may offer more effective therapeutic treatment for cancer treatment [11]. Herbal compounds, such as Curcuma longa, can reduce the side effects of the medication in combination with lower doses of chemotherapy drugs while these combinations can also inhibit the various signaling pathways.

CONCLUSION

In summary, the present study analyzed the expression of IKKα, IKKβ and NF-κB genes in the face of the combined effect of Curcuma longa extract with prednisolone, and the results showed a positive effect of this synergy. Further investigations are required on other ALL cell lines and on the level of protein expression.

REFERENCES
1. Pui CH, Evans WE. Acute lymphoblastic leukemia. N Engl J Med 1998; 339:605-15.
2.Chessells JM. Relapsed lymphoblastic leukaemia in children: a continuing challenge. Br J Haematol.1998;102:423-439.
3.Tatjana stankovic, eliot marston. Molecular mechanisms involved in chemoresistance in paediatric acute lymphoblastic leukaemia. Biblid: 0370-8179, 136(2008) 3-4, p. 187-192.
4.Yang JY, Hung MC, A new fork for clinical application: targeting forkhead transcription factors in cancer. Clin Cancer Res 2009, 15(3):752–757.
5.Solt LA, May MJ, The IkappaB kinase complex: master regulator of NFkappaB signaling. Immunol Res 2008, 42(1–3):3–18.
6.Tanaka M, Fuentes ME, Yamaguchi K, Durnin MH, Dalrymple SA, Hardy KL,Goeddel DV, Embryonic lethality, liver degeneration, and impaired NFkappa B activation in IKK-beta-deficient mice. Immunity 1999, 10(4):421–429.
7.Sil AK, Maeda S, Sano Y, Roop DR, Karin M, IkappaB kinase-alpha acts in the epidermis to control skeletal and craniofacial morphogenesis. Nature 2004, 428(6983):660–664.
8. Pfaffl M W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29: e45.
9.Karin M, Yamamoto Y, Wang QM. The IKK NF-kappa B system: a treasure trove for drug development. Nat Rev Drug Discov 2004, 3(1):17–26.
10.Niederberger E, Geisslinger G. The IKK-NF-kappaB pathway: a source for novel molecular drug targets in pain therapy? FASEB J 2008, 22(10): 3432–3442.
11.Wei-Chien Huang and Mien-Chie Hung.Beyond NF-κB activation: nuclear functions of IκB kinase α. Huang and Hung Journal of Biomedical Science 2013, 20:3.