Tropical Journal of Pharmaceutical Research November 2019; 18 (11): 2293-2298
ISSN: 1596-5996 (print); 1596-9827 (electronic)
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Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i11.9

Original Research Article

Curcumin suppresses leukemia cell proliferation by down-regulation of P13K/AKT/mTOR signalling pathway

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Sent for review: 24 May 2019 *Revised accepted: 20 October 2019*

Abstract

Purpose: *To investigate the effect of curcumin ester on the proliferation of leukemia cell lines in vitro.*
Methods: *Changes in WEHI-3 and THP 1 cell viabilities were measured using Cell Counting Kit 8 (CCK 8). Analysis of cell cycle and determination of apoptosis were carried out using propidium iodide and Annexin V fluorescein isothiocyanate staining. Transmission electron microscopy was used for observing the presence of apoptotic features in cells.*
Results: *Treatment with curcumin ester for 72 h caused significant reduction in the proliferation of WEHI-3 and THP 1 cells. Curcumin ester, at a dose of 50 µM, decreased the proliferations of WEHI-3 and THP 1 cells to 28 and 32 %, respectively. On exposure to curcumin ester for 72 h, cell cycle in WEHI-3 cells was arrested in G1/G0 phase. Curcumin ester at doses of 25, 30 and 50 µM enhanced apoptosis in WEHI-3 cells to 46, 58 and 64 %, respectively. Curcumin ester suppressed the levels of phosphoinositide 3 kinase (PI3K), protein kinase B (AKT) and mechanistic target of rapamycin (mTOR) protein and mRNA in WEHI-3 cells. In curcumin ester-treated WEHI-3 cells, the presence of apop¬totic bodies increased significantly and concentration-dependently.*
Conclusion: *These results demonstrate that curcumin ester inhibits leukemia cell proliferation by inducing apoptosis and arresting cell cycle in G1/G0 phase, probably via suppression of PI3K, AKT and mTOR, and promotion of PTEN. Thus, curcumin ester has potentials for use in the development of an effective treatment strategy for leukemia.*

Keywords: *Leukemia, Curcumin ester, Differentiation, Apoptosis, Phosphorylation, Rapamycin*

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Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Leukemia is one of the malignant cancers characterised by markedly high growth rate and inability of hematopoietic cells to undergo differentiation [1,2]. Worldwide, acute leukemia is diagnosed in approximately 150,000 people every year [3]. The most commonly detected type of acute leukemia in aged people is acute myeloid leukemia which is associated with very high mortality rate [4]. Among all types of malignant hematological diseases, acute myeloid leukemia is most difficult to treat, which accounts for the very poor survival of the affected patients [5]. Therefore, the mechanism involved in the pathogenesis of leukemia needs to be

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investigated in detail so as to develop an effective treatment strategy for the disease.

Therapies used presently for leukemia are chemotherapy, radiotherapy and bone marrow transplantation [6]. These therapies have been found to be unsatisfactory. Therefore, the need for discovery of novel molecules to treat leukemia remains a major clinical challenge.

Apoptosis is considered an important physiological process responsible for elimination of carcinoma cells [7]. Carcinoma cells possess the potential to escape apoptosis. The activation of signaling pathways in carcinoma cells by various natural and synthetic molecules leads to inhibition of tumor growth [8]. An intracellular signalling pathway known as phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) plays important role in the development of several types of cancers including acute leukemia [9]. There is need for development of novel and efficient treatment strategies with limited side effects for leukemia treatment [10].

Curcumin, a major constituent of turmeric and curry powder, possesses a wide range of biological effects. These include anti-oxidant, anti-microbial and anti-inflammatory properties [11,12]. Curcumin has been reported to exert antitumor effects against several kinds of cancer cell lines through induction of apoptosis [11,12]. In the form of combination therapy, curcumin is currently in use in clinical trials for breast and prostate carcinoma treatment. Studies have shown that in malignant pleural mesothelioma cells, curcumin exposure causes cell death through apoptosis, pyroptosis and autophagy [13,14]. The present study investigated the effect of curcumin ester (Figure 1) on the viabilities of WEHI-3 and THP-1 leukemia cell lines *in vitro*.

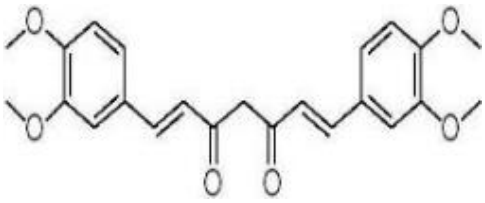

Figure 1: Chemical structure of curcumin ester

EXPERIMENTAL

Cell lines and culture

The WEHI-3 and THP-1 cell lines were supplied by the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10 % fetal bovine serum (FBS) and antibiotics [penicillin (100 IU/mL) and streptomycin (100 µg/mL)] in an incubator in a humidified atmosphere at 37 ˚C and 5 % CO_2.

Cell viability determination

The effect of curcumin ester on the proliferations of WEHI-3 and THP-1 cells was determined using Cell Counting Kit-8 (CCK-8) assay. The WEHI-3 and THP-1 cells were seeded in 96-well plates, each at a density of 3 x 10^5 cells/well, and were grown in RPMI-1640 medium for 24 h. Thereafter, the medium was replaced with a fresh medium containing 5, 10, 15, 20, 25, 30 or 50 µM of curcumin ester. After 72 h of incubation at 37 ˚C, CCK-8 solution (10 µL) was added to each well. Following 3 h incubation, the absorbance of each well was measured at 465 nm using a microplate reader (EL-x 800; BioTek Instruments, Inc., Winooski, VT, USA).

Determination of apoptosis

Apoptosis in WEHI-3 cells exposed to curcumin ester was determined with flow cytometry. The cells were grown for 24 h at a density of 2.5 x 10^5 cells per well in 6-well plates. They were thereafter incubated separately with 25, 30 and 50 µM of curcumin ester for 72 h. Then, the cells were washed with PBS and treated with 300 µL of binding buffer, followed by staining with Annexin V-fluorescein isothiocyanate (FITC, 5 µL) and propidium iodide. The cell staining was carried out for 25 min in the dark at room temperature. Fluorescence measurements were made using an argon laser-linked flow cytometer (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) at 478 nm. The percentage of apoptotic cells was calculated using the FACScan software (version 6.0; BD Biosciences).

Effect of curcumin ester on the cell cycle

The effect of curcumin on distribution of cell cycle in WEHI-3 cells was analysed using flow cytometry. Cells grown for 24 h at a density of 2.5 x 10^5 cells per well in 6-well plates were incubated separately with 25, 30 and 50 µM of curcumin ester for 72 h. After washing with cold PBS, the cells were fixed overnight in ethyl alcohol (70 %) at 4 ˚C. Then, RNase A (1 %) in Tris-hydrochloric acid buffer, pH 7.6 was added to the plates. The cells were then stained with propidium iodide (5 mg/mL). The cell cycle distribution was analysed by observing DNA content using flow cytometry.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

In WEHI-3 cells, changes in mRNA expressions of PI3K, AKT, mTOR and PTEN following 72 h of treatment with 25, 30 and 50 µM of curcumin ester were assayed using RT-PCR. The RNA was isolated from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc) in accordance with the manual protocol. Then, cDNA was synthesized via reverse transcription with 1-µg samples of total RNA for 25 min at 37 ˚C using Primescript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Roche LightCycler®96 Real-time PCR system and SYBR Premix EX Taq Ⅱ kit (Takara, Biotechnology Co. Ltd) were used for RT-PCR. The 20-µL reaction mixture consisted of 0.8 µL of each of forward and reverse primer, 2 µL of cDNA, and 6.4 µL of purified water. The reaction amplification was carried out as follows: 2 min of pre-degeneration at 93 ˚C, 39 cycles of denaturation for 15 sec at 93 ˚C, and annealing for 25 sec at 58 ˚C. The expression of GAPDH was used as internal loading control.

Western blot analysis

The WEHI-3 cells were exposed to curcumin ester for 72, and then lysed with RIPA lysis buffer. The protein concentration in each lysate was determined using Bicinchoninic Acid Protein assay kit (Thermo Fisher Scientific, Inc.). Each protein sample (20 µg) was resolved on SDS-PAGE (10 - 12 %) via electrophoresis and subsequently transferred to PVDF membranes. The membranes were blocked with 5 % non-fat milk prior to their incubation with primary antibodies against PI3K, AKT, mTOR and PTEN overnight at 4˚C. Thereafter, the membranes were washed with PBS, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000 dilution) for 2 h at room temperature. Then, the protein bands were visualized using the ECL detection system (Pierce; Thermo Fisher Scientific, Inc.).

Examination of cells under transmission electron microscopy

The WEHI-3 cells were seeded into the 6-well plates at a density of 3 x 10^6 cells per well, and treated for 72 h with 25, 30 and 50 µM of curcumin ester at 37 ˚C. After harvesting, the cells were subjected to fixation with chilled glutaraldehyde (2.5 %) at 4 ˚C for 3 h, and then washed two times with 0.1 mol/L PBS solution. Following fixing with 1 % OsO_4 solution at 4 ˚C for 3 h, the cells were dehydrated using gradient ethanol and subsequently embedded in resin. The cell staining was performed using uranyl acetate over 45 min at room temperature. The cells were then examined under the transmission electron microscope (TEM-1011; JEOL, Ltd., Tokyo, Japan).

Statistical analysis

Data are presented as mean ± SD of three experiments. Statistical analysis was performed using SPSS (version 18.0; SPSS Inc., Chicago, IL, USA). Statistical differences amongst groups were determined using one-way analysis of variance (ANOVA), followed by Tukey's test. The differences were taken as statistically significant at $p < 0.05$.

RESULTS

Curcumin ester suppressed the proliferation of WEHI-3 and THP-1 cells

The results of CCK-8 assay revealed that curcumin ester treatment for 72 h caused significant and dose-dependent reductions in the proliferations of WEHI-3 and THP-1 cells (Figure 2). Curcumin ester decreased WEHI-3 cell proliferation to 94, 89, 77, 61, 53, 39 and 28 %, at doses of 5, 10, 15, 20, 25, 30 and 50 µM, respectively. The proliferation of THP-1 cells was decreased on exposure to 5, 10, 15, 20, 25, 30 and 50 µM of curcumin ester to 91, 85, 73, 58, 49, 41 and 32 %, respectively.

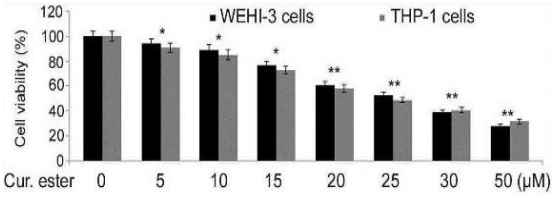

Figure 2: Effect of curcumin ester on the viabilities of leukemia cells. The viabilities cells exposed to 5, 10, 15, 20, 25, 30 and 50 µM of curcumin ester were analysed using CCK-8 assay. The results are expressed as mean ± SD of three independently performed experiments; *$p < 0.05$; **$p < 0.01$ versus cells in negative control group

Curcumin ester caused WEHI-3 cell cycle arrest in G1/Go phase

On exposure of WEHI-3 cells to curcumin ester for 72 h, cell cycle was arrested in G1/G0 phase (Figure 3). The WEHI-3 cell population in G1/G0 phase increased, whereas, cell populations in S and G2/M phases decreased significantly on treatment with 25, 30 and 50 µM of curcumin ester.

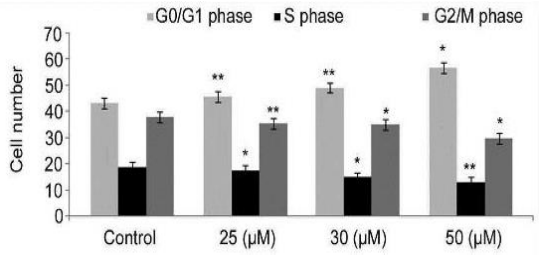

Figure 3: Effect of curcumin ester on cell cycle progression. The WEHI-3 cells, on exposure to 25, 30 and 50 μM curcumin ester for 72 h, were subjected to flow cytometric analysis. *p < 0.05; **p < 0.01 versus cells in negative control group

Curcumin ester treatment induced apoptosis in WEHI-3

Curcumin ester treatment led to induction of apoptosis in WEHI-3 cells in a dose-dependent manner (Figure 4). Flow cytometric analysis showed that curcumin ester at levels of 25, 30 and 50 μM enhanced apoptosis in WEHI-3 cells to 46, 58 and 64 %, respectively, relative to 2.36 % apoptosis in control.

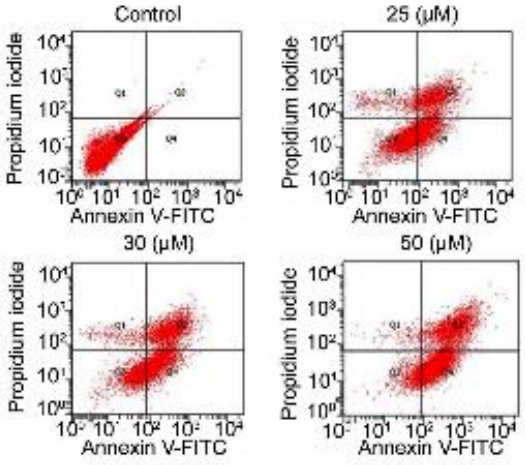

Figure 4: Effect of curcumin ester on apoptosis in WEHI-3 cells. Induction of apoptosis in WEHI-3 cells was assessed with flow cytometry using FITC-Annexin V and PI staining. Effect of curcumin ester was studied at doses of 25, 30 and 50 μM

Curcumin ester suppressed phosphorylation of PI3K, AKT and mTOR in WEHI-3 cells

The effect of curcumin ester on phosphorylation of PI3K, AKT, PTEN and mTOR was assessed with western blotting and RT-PCR assays. The results are shown in Figure 5. Curcumin ester suppressed the protein expression levels of p-PI3K, p-AKT and p-mTOR in WEHI-3 cells in a concentration-based manner (Figure 5 A). Moreover, RT-PCR assay confirmed that the mRNA levels of p-PI3K, p-AKT and p-mTOR were suppressed in WEHI-3 cells on treatment with curcumin ester (Figure 5 B). The level of p-PTEN was increased in WEHI-3 cells on treatment with curcumin ester.

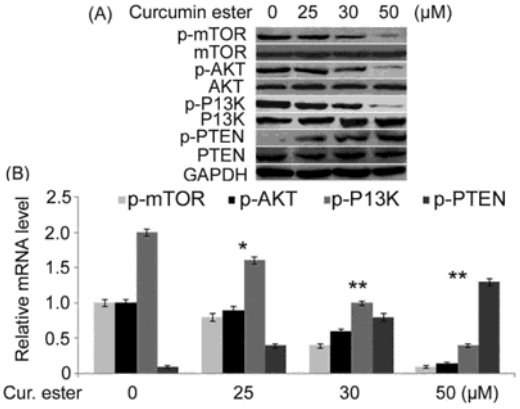

Figure 5: Effect of curcumin ester on p-PI3K, p-AKT, p-mTOR and p-PTEN levels in WEHI-3 cells. The cells were treated with curcumin ester for 72 h and then subjected to (A) western blot, and (B) RT-PCR assays for the expressions of PI3K, AKT, mTOR and PTEN. *p < 0.05; **p < 0.01 versus control

Morphology of WEHI-3 cells

Treatment of WEHI-3 cells with curcumin ester significantly increased the presence of apoptotic bodies in concentration-dependent manner (Figure 6). The presence of apoptotic bodies confirmed that curcumin ester induced apoptosis in WEHI-3 cells.

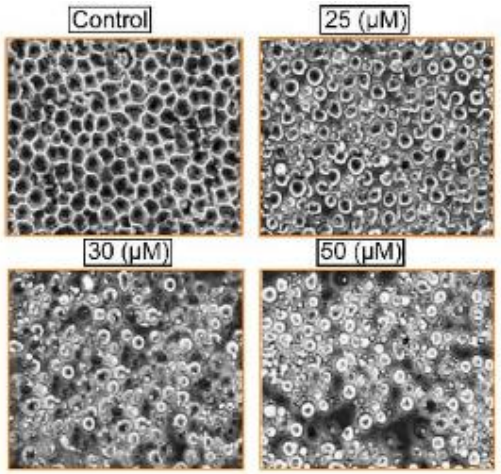

Figure 6: Scanning electron micrograph (SEM) of WEHI-3 cells following curcumin ester treatment. The cells, after treatment with 25, 30 and 50 μM of curcumin ester for 72 h, were examined under the electron microscope for apoptotic changes

DISCUSSION

The present study investigated the effect of curcumin ester on proliferation of leukemia cells *in vitro*. The study demonstrated that curcumin ester exhibited inhibitory effect on leukemia cell viability by apoptosis induction, cell cycle arrest in G1/G0 phase and inhibition of PI3K, AKT and mTOR expression. Curcumin has been found to exhibit growth inhibitory effects on various types of carcinoma cells [11,12]. Studies have shown that in malignant pleural mesothelioma cells, curcumin exposure caused cell death through apoptosis, pyroptosis and autophagy [13,14]. The present study determined the effect of curcumin ester on WEHI-3 and THP-1 cell proliferation. The CCK-8 assay revealed that exposure of WEHI-3 and THP-1 cells to curcumin ester suppressed proliferation concentration-dependently. Thus, initial results suggest that curcumin ester exerts inhibitory effects on the viabilities of leukemia cells. Chemotherapeutic agents generally inhibit carcinoma cell proliferation by activating apoptosis and autophagy [15,16].

It has been reported that anti-neoplastic drugs suppress proliferation of carcinoma cells via activation of programmed cell death pathway (apoptosis) [17]. In the present study, exposure of WEHI-3 cells to curcumin ester caused apoptosis induction in a concentration-dependent manner. The proportion of apoptotic WEHI-3 cells was raised significantly with increase in the concentration of curcumin ester. Therefore, the curcumin ester-mediated suppression of leukemia cell viability was associated with activation of apoptotic pathway. One of the main pathways involved in transferring intracellular signals is known as PI3K/AKT/mTOR pathway. Studies on various types of carcinoma have shown that PI3K/AKT/mTOR pathway is up-regulated in cancer cells [18,19].

Activation and translocation of PI3K to plasma membrane is associated with the conversion of phosphatidylinositol 4,5-bisphosphate to its tri-phosphate form [20]. Studies have shown that failure of the cells to regulate PI3K/AKT/mTOR pathway is responsible for the development of many types of carcinoma [20]. Activation of PI3K/AKT/mTOR pathway may be linked to mutation in tyrosine kinase or c-kit tyrosine kinase receptor in the cells [21]. The PI3K/AKT/mTOR pathway is down-regulated by activation of a tumor suppressor protein, PTEN [22]. In the present study curcumin ester treatment of WEHI-3 cells inhibited the expressions of PI3K, AKT and mTOR in a concentration-dependent manner. The levels of protein and mRNA corresponding to PI3K, AKT and mTOR were significantly decreased in WEHI-3 cells treated with curcumin ester, while the expression of tumor suppressor gene, PTEN was markedly upregulated.

CONCLUSION

The results obtained in this study clearly suggest that curcumin ester inhibits leukemia cell proliferation by inducing apoptosis and cell cycle arrest in G1/G0 phase. Moreover, the findings indicate that curcumin ester downregulates PI3K/AKT/mTOR pathway and promotes PTEN in leukemia cells. Thus, curcumin ester may be used for the development of effective treatment strategy for leukemia.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yao Xiaodan designed the study and wrote the paper. Guo Ting, Wu Jin, Ding Ting, Wang Hongxiang and Xu Liwen performed the experimental work, carried out the literature study and compiled the data. Guo Ting, Wu Jin and Ding Ting performed literature survey, analyzed the data and compiled the data. Guo Ting and Wu Jin contributed to this work equally. The research article was thoroughly read by all the authors and approved for publication.

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