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Curcumin inhibits *WT1* gene expression in human leukemic K562 cells[1]

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Key words

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Abstract

Aim: Wilms' tumor1 (*WT1*) gene is highly expressed in leukemic blast cells of myeloid and lymphoid origin. Thus, *WT1* mRNA and protein serve as promising tumor markers for the detection of leukemia and monitoring of disease progression. The purpose of this study was to investigate the modulating effects of curcumin on *WT1* gene expression in the human leukemic cell line K562. **Methods:** The cytotoxicity of curcumin on the K562 cell line was evaluated by using 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The K562 cell line was treated with a non-cytotoxic dose of curcumin (5, 10, or 15 μmol/L) for 13 d. The expression levels of WT1 protein and *WT1* mRNA were assessed by Western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR), respectively. **Results:** Curcumin had a cytotoxic effect on K562 leukemic cells with an inhibitory concentration at 50% (IC_{50}) of approximately 20 μg/mL (54.3 μmol/L). Non-cytotoxic doses of curcumin, at concentrations of 5, 10, and 15 μmol/L for 2 d, decreased the level of WT1 protein and WT1 mRNA in the K562 cell line in a dose-dependent manner. Similarly, curcumin at a concentration of 10 μmol/L significantly decreased the level of WT1 protein and mRNA in a time-dependent manner. **Conclusion:** The inhibitory effects of curcumin are associated with a decrease in the levels of both WT1 protein and *WT1* mRNA. The current study provides a molecular basis for future clinical trials in leukemic patients. Thus, curcumin could be a promising chemotherapeutic agent for human leukemia.

Introduction

Leukemias comprise a group of clonal diseases characterized by an accumulation of abnormal blood cells, which are thought to derive from a single cell in the marrow that has undergone a genetic alteration. Leukemia is the most common childhood cancer. Malignant transformation occurs as a result of the accumulation of genetic mutations in cellular genes. Some cases of mutations in oncogenes have provided useful molecular markers for monitoring the course of disease during treatment. The abl translocation in chronic myelogenous leukemia is a good example. The detection of overexpression in specific oncogenes or tumor suppressor genes provides information that is useful in the diagnosis of leukemia and prognosis of the disease. The overexpression of Wilms' tumor (WT1) protein in leukemia is a promising biomarker. WT1 is expressed in stem cells of the bone marrow, but not in normal mature blood cells[1,2]. Many previous studies have demonstrated that the *WT1* gene is highly expressed in leukemic blast cells of myeloid and lymphoid origin, and thus *WT1* mRNA provides a novel tumor marker for the detection of minimal residual disease of leukemias and for monitoring disease progression of myelody-splastic syndromes[3–9]. It has been demonstrated that the *WT1* gene is expressed in leukemic cell lines K562 and HL60, and that differentiation of these cells in culture is accompanied by downregulation of WT1 protein levels[10,11]. K562 cells, which are derived from chronic myelocytic leukemia blastic crisis and express high levels of WT1, can be treated with nocodazole (40 ng/mL) to synchronously arrest the cell

cycle[12].

The *WT1* gene is defined as a tumor suppressor gene in childhood renal tumors. However, the wild-type *WT1* gene is highly expressed in leukemic blast cells. The *WT1* gene is expressed at high levels in various types of leukemias [acute myeloblastic leukemia (AML), acute lymphoblastic leukemia (ALL) and chronic myelocytic leukemia (CML)]. Miwa *et al*[13] and Miyaki *et al*[14] examined *WT1* gene expression in leukemias using Northern blot analysis and detected *WT1* gene expression in some cases of AML, ALL, and CML in the accelerated phase or blast crisis. Inoue *et al* provided a new insight into the significance of *WT1* gene expression in leukemias by quantifying the expression levels of the *WT1* gene by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)[15]. In all leukemia samples examined, including AML, ALL, and CML, significant levels of *WT1* gene expression were found, and the average levels were approximately 1000 and 100 000 times greater than those in normal bone marrow or peripheral blood cells, respectively[16]. Moreover, Bergmann *et al* reported a correlation between *WT1* mRNA levels and prognosis[17]. Taken together, all these findings demonstrate that *WT1* mRNA is a novel tumor marker in leukemic blast cells of almost all leukemias and that its expression level is a new prognostic factor for acute leukemia.

Curcuminoids are natural phenolic coloring compounds found in rhizomes of *Curcuma longa* Linn, a member of Zingiberaceae (ginger) family, and commonly known as turmeric. Curcuminoid content in turmeric varies from 1% to 5% of fresh turmeric rhizome, and has been identified as the major yellow pigment in turmeric. It has been widely used as a spice, to color cheese and butter, as a cosmetic, and in some medicinal preparations[18,19]. Curcuminoids include curcumin (curcumin I), demethoxycurcumin (curcumin II) and bisdemethoxycurcumin (curcumin III). All commercial curcuminoids sold as "curcumin" (eg ICN, GNC, and Sigma-Aldrich), are mixtures of the 3 curcuminoids. Curcumin has a wide range of biological and pharmacological activities, including antioxidant properties[20–22], anti-inflammatory properties[19], anti-mutagenic activity *in vitro*[23], anti-carcinogenic effects[24–26], hypocholesterolemic effects in rats[27], hypoglycemic effects in humans[28], and multidrug resistance (MDR) modulation effects[29]. The safety of curcumin has been studied in various animal models[30], and it is clear that turmeric is not toxic even at high doses in laboratory animals. A single feeding of a 30% turmeric diet to rats did not produce any toxic effects. In a 24-h acute toxicity study, mice were fed doses of 0.5, 1.0, and 3.0 g/kg of turmeric extract. There was no increase in mortality compared with controls in either study. A 90-d treatment with turmeric extract resulted in no significant weight gain[31].

Due to its wide range of biological and pharmacological effects and lack of toxicity in animal models, curcumin was selected for study in leukemia. In this paper we aimed to examine the modulating effect of curcumin on *WT1* gene expression in the K562 human leukemic cell line.

Materials and methods

Reagents Commercial grade curcumin (77% curcumin, 17% demethoxycurcumin and 3% bisdemethoxycurcumin), 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) dye, and dimethylsulfoxide (Me_2SO) were purchased from Sigma-Aldrich (St Louis, MO, USA). RPMI 1640, SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA polymerase reagent, Trizol reagent, penicillin-streptomycin, *L*-glutamine, and primers were purchased from Invitrogen Life Technology (Carlsbad, CA, USA). Primary mouse polyclonal anti-WT1 clone C-19 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was purchased from Promega (Madison, WI, USA). A SuperSignal detection kit was purchased from Pierce (Rockford, IL, USA).

Cells and cell cultures The erythroid leukemic cell line (K562) was a generous gift from Dr Chaisuree SUPAWILAI (Research Institute for Health Sciences, Chiang Mai, Thailand). This cell line was cultured in RPMI 1640 medium containing 10% fetal calf serum, 1 mmol/L *L*-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C. When the cells reached 80% confluency, they were harvested and plated for consequent passages or for curcumin treatment.

The effects of curcumin on cell growth were observed by examining the morphology of cultures with an inverted phase contrast microscope. The MTT test was used throughout all experiments to check cell viability.

MTT assay Cell survival was determined by using the MTT assay as described elsewhere[32]. The MTT assay was performed by plating cells in 96-well plates (3.0×10^5 cells/well) in 100 μL medium, and incubating them at 37 °C for 1 d before curcumin treatment. After 1 d, curcumin stocks prepared in Me_2SO were added to the culture medium (100 μL) at various concentrations and incubated in a humidified tissue-culture chamber (37 °C , 5% CO_2) for another day. The Me_2SO concentration was kept at 0.4%. The cell survival in each well was determined by the MTT assay and compared with that of untreated cells. Briefly, after removal of 100 μL medium, MTT stock dye solution was added (15 μL/100 μL

medium) to each well, and the plate was incubated at 37 °C in 5% CO_2 atmosphere. After 4 h, Me_2SO (100 mL) was added to each well and mixed thoroughly to dissolve the dye crystals. Absorbance at a wavelength of 570 nm was measured with an enzyme-linked immunosorbent assay (ELISA) plate reader with a reference wavelength of 630 nm. Fractional absorbance was calculated by using the following formula:

$$\% \text{ Cell survival} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

Western blot analysis Cell nuclear extracts were prepared as described previously[33]. The cell nuclear proteins (100 μg/lane) were separated by 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotted overnight onto nitrocellulose filters. The filters were incubated sequentially with primary mouse polyclonal anti-WT1 clone C-19 at a 1:1000 dilution, followed by a treatment with HRP-conjugated goat anti-mouse IgG at a 1:15 000 dilution. Proteins were visualized by using the SuperSignal protein detection kit and quantitated by using a scan densitometer.

RNA extraction and quantitative RT-PCR RNAs of K562 cells were isolated by using the Trizol reagent according to the manufacturer's instructions. RNaseOUT was added to the RNA extraction products for RNA protection (40 units per 20 μL of reaction mixture). The amount of RNA was determined by optical density (*OD*) measurement at a wavelength of 260 nm (one *OD* unit = 40 μg/mL). RT-PCR was performed using the SuperScript III One-step RT-PCR System with Platinum *Taq* DNA polymerase reagent. For *WT1*, the sense primer was 5'-GGCATCTGAGACCAGTGAGAA-3', and the antisense primer was 5'-GAGAGTCAGACTTG-AAAGCAGT-3', corresponding to residues 780–800 and residues 1232–1253, respectively, of the published cDNA sequence[34]. cDNA was synthesized from 1 μg of total RNA at 60 ºC for 30 min and denatured at 94 ºC for 2 min. PCR amplification was performed for 30 cycles of sequential denaturation (94 ºC, 1 min), annealing (60 ºC, 1 min), and extension (72 ºC, 1 min), which gave a 474 bp product. *β-Actin* gene expression, used as an internal control for RNA loading, was carried out by using the sense primer 5'-CAGAGCAA-GAGAGGCATCCT-3' and the antisense primer 5'-TTGAA-GGTCTCAAAC ATGAT-3' corresponding to residues 216–235 and residues 405–424, respectively. The *β-actin* cDNA was synthesized from 1 μg of total RNA at 55 ºC for 30 msin and denatured at 94 ºC for 2 min. PCR amplification was performed for 30 cycles of sequential denaturation (94 ºC, 1 min), annealing (55 ºC, 1 min), and extension (72 ºC, 1 min), which yielded a 201 bp product. For a negative control, water was amplified using a total of 30 cycles to detect any possible contamination. A total of 15 μL of each PCR product was electrophoresed on a 1% agarose gel, visualized with ethidium bromide staining (2 mg/mL), and quantitated using scan densitometry (Bio-Rad, Richmond, CA, USA).

Statistical analysis All data are expressed as mean±SD from triplicate samples of 3 independent experiments. Statistical differences between the means were analyzed by one-way ANOVA. $P<0.05$ was considered statistically significant.

Results

Cytotoxic effects of curcumin on the K562 leukemic cells Curcumin produced a cytotoxic effect on K562 cells with an inhibitory concentration at 50% (IC_{50}) of approximately 20 μg/mL (54.3 μmol/L) as shown in Figure 1.

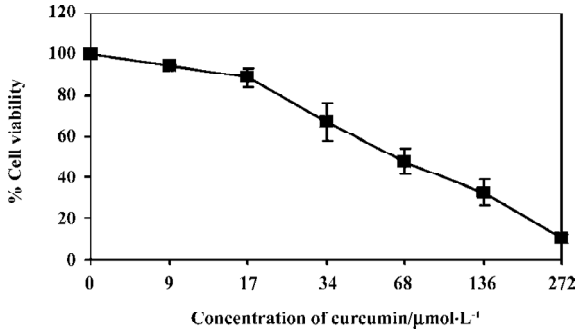

Figure 1. Cytotoxic effects of curcumin on K562 cells. Cells (3×10^5 cells/well), in 200 μL medium were grown in the presence of 0.4% DMSO (vehicle control) or various concentrations of curcumin for 2 d. The numbers of viable cells were determined by MTT assay. The number of viable cells is expressed as a percentage of vehicle control. Mean±SD of 3 independent experiments performed in triplicate.

WT1 protein and mRNA expression The K562 cells were treated with 5, 10, or 15 μmol/L curcumin for 2 d before cell harvesting and the nuclear proteins were analyzed by Western blotting. The levels of WT1 protein in the K562 cells were decreased by 10%, 29%, and 63% in response to treatment with 5, 10, and 15 μmol/L curcumin, respectively, compared with the vehicle control (Figure 2).

To verify if curcumin could modulate spontaneous *WT1* expression (mRNA) occurring *in vitro*, the same as WT1 protein expression, K562 cells were treated with curcumin (5, 10, or 15 μmol/L) for 2 d and expression of *WT1* mRNA was examined by RT-PCR. Values for the expression of WT1 mRNA (after normalization to β-actin expression) in K562 cells were shown to decrease by 17, 37, and 46%, respectively (Figure 3).

This result clearly shows that curcumin significantly decreased WT1 expression both at the mRNA and protein levels.

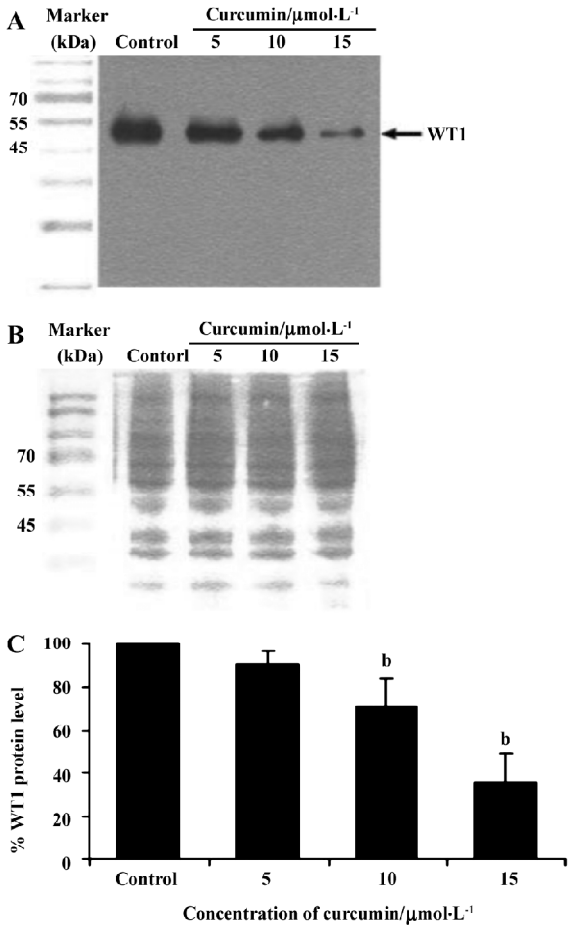

Figure 2. WT1 protein expression in K562 cells cultured in 5, 10, or 15 μmol/L curcumin for 2 d. Cell nuclear lysates from K562 cells were probed by immunoblotting with anti-WT1 antibody (A). Coomassie blue stain was used as a quantity control (B). The bands were quantified by scan densitometry (C). The control culture for this experiment was cells treated with 0.01% Me_2SO (vehicle control). Data are mean±SD of 3 independent experiments. $^bP<0.05$ *vs* control.

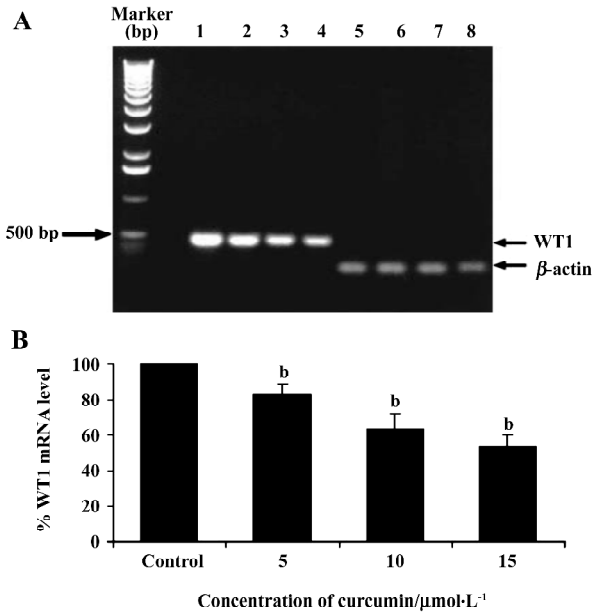

Figure 3. *WT1* mRNA level in K562 cells cultured in 5, 10, or 15 μmol/L curcumin for 2 d. The *WT1* mRNA level was determined by RT-PCR (A). The bands were quantified by scan densitometry (B). The control culture for this experiment was cells treated with 0.01% DMSO (vehicle control). Mean±SD in 3 independent experiments. $^bP<0.05$ *vs* control. Lane 1, untreated K562, control; lane 2, 5 μmol/L curcumin-treated K562; lane 3, 10 μmol/L curcumin-treated K562; lane 4, 15 μmol/L curcumin-treated K562; lanes 5–8, β-actin equivalents of lanes 1–4, respectively.

Therefore, subsequent experiments were designed to observe the modulating effects of curcumin on WT 1 expression after various incubation times. WT1 protein level was found to be decreased by 29%, 51%, and 99%, respectively, in response to 1, 2 and 3 d of treatment (Figure 4). Expression of *WT1* mRNA (after normalization to β-actin expression) in K562 cells after treatment with curcumin for 1, 2, and 3 d was decreased by 14%, 38%, and 63%, respectively (Figure 5).

Discussion

In anticancer drugs research, dietary plants, for example, turmeric, chili, ginger, pepper and garlic are of central interest in Thailand. Curcumin, a major active component of the food flavoring turmeric (*Curcuma longa* Linn) consists of 3 major active ingredients: curcumin, demethoxycurcumin and bisdemethoxycurcumin. Curcumin has numerous biological properties, including antioxidant and anti-inflammatory effects, as well as antimutagen and anticancer properties. Moreover, curcumin also inhibits oncogene expression and protein kinase C activation. The anticancer properties of curcumin have been described by many researchers, including our group. This inhibitory effect by curcumin regulates a wide variety of genes that require AP1 and NFkB activation, which promote cell proliferation and cell differentiation. WT1 protein has been reported to play an important role in early hematopoiesis, and also controls cell differentiation. These transcription factors are regulated by protein kinase C (PKC), which also regulates WT1 protein by phosphorylation at the C-terminal domain[35], and in turn regulates cell proliferation in leukemic cells. The expression of *STAT5* mRNA and protein in K562 cells was inhibited by curcumin and curcumin also inhibited K562 cell proliferation[36].

Recently, the *WT1* gene, a marker of leukemia, was shown to be overexpressed in leukemic cells, including the K562 and HL60 cell lines[37]. Thus, in the present study, we wanted to demonstrate the possible role of curcumin in the expres-

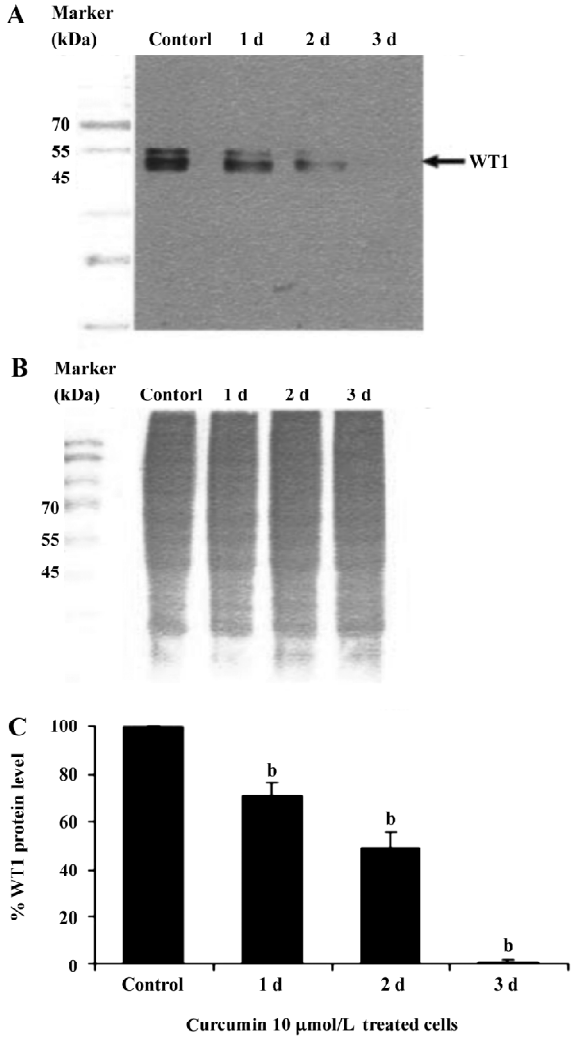

Figure 4. Effects of curcumin on WT1 protein expression in K562 cells. WT1 protein expression in K562 cells cultured in 10 μmol/L curcumin for 1, 2, and 3 d was determined by Western blotting, using anti-WT1 antibody (a). Coomassie blue stain was used as a quantity control (b). The bands were quantified by scan densitometry (c). The control culture for this experiment (0.01% DMSO) was harvested on d 3. Mean±SD of 3 independent experiments. $^bP<0.05$ *vs* control.

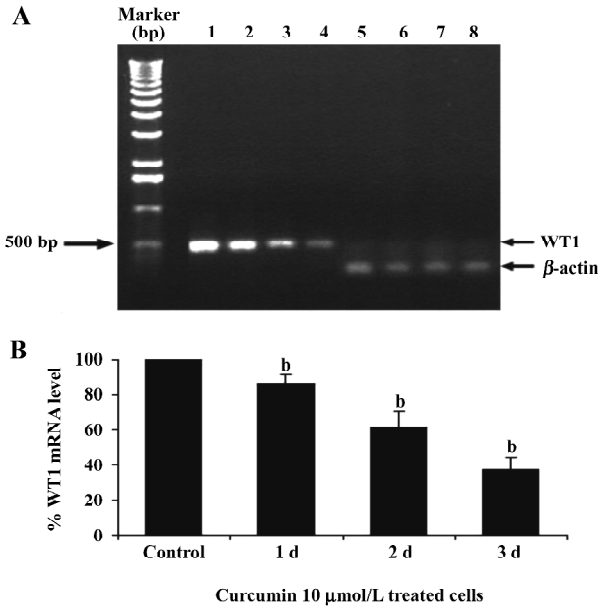

Figure 5. Effects of curcumin treatment on WT1 mRNA levels in K562 cells. WT1 mRNA levels in K562 cells cultured in 10 μmol/L curcumin for 1, 2, and 3 d were determined by RT-PCR. The PCR products (474 bp *WT1* and 201 bp *β-actin*) were run on a 1% agarose gel (A). The bands were quantified by scan densitometry (B). WT1 gene expression was measured and normalized to *β-actin* expression. $^bP<0.05$ *vs* control. Lane 1, untreated K562, vehicle control (0.01% Me_2SO); lanes 2–4, curcumin-treated K562 for 1–3 d, respectively; lanes 5–8, β-actin equivalents of lanes 1–4, respectively.

sion of WT 1 protein and *WT1* mRNA in K562 human leukemic cells. When 5, 10, or 15 μmol/L curcumin was added to K562 cells for 2 d, there was a decrease in WT1 expression with increasing curcumin concentration, indicating that curcumin reduces the level of immunoreactive WT1 protein observed in K562 cells and also reduces the level of *WT1* mRNA under the same conditions. The experimental results shown in Figures 4 and 5 also indicate the time-dependent inhibitory effect of curcumin treatment for 1–3 d on WT1. The mechanistic roles of curcumin in *WT1* gene promoter activity and signaling control are under extensive investigation in our laboratory.

In our experiment in which we evaluated the cytotoxicity of curcumin in human K562 leukemic cells (Figure 1), we found an IC_{50} value of 54 μmol/L. This indicates that curcumin is less toxic in K562 cells than in HL-60 and U937 leukemic cells, which have IC_{50} of 19 and 24 μmol/L, respectively (data not shown). The result is consistent with our previous observation demonstrating that curcumin affects proliferation to various degrees in different cancer cell lines, including Hep-2 (human larynx cancer), PC-9 and PC-14 (human lung cancer), Hep-1 (mouse hepatoma), F-25 (mutated H-ras transfected NIH mouse fibroblast)[38], and B-NHL cell line Raji cells[39]. Duvoix *et al* found that curcumin reduces the levels of GSTP1-1 mRNA as well as protein, which is related to its apoptotic effect on the K562 cell line[40].

Taken together, our data indicate that treatment of human K562 leukemic cells with non-cytotoxic concentrations (low doses) of curcumin inhibits *WT1* gene expression, whereas curcumin at a high dose induces cell cytotoxicity (IC_{50}=54 μmol/L). Our results suggest that curcumin could potentially be used as a chemotherapeutic agent for human leukemia. This research may lead to clinical trials in the future.

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