African Journal of Pharmacy and Pharmacology Vol. 5(16), pp. 1846-1856, 29 October, 2011
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.149
ISSN 1996-0816 ©2011 Academic Journals

Full Length Research Paper

Pure curcumin inhibits exogenous *Wilms' tumor (WT1)* (+/+) isoform protein via degradation pathway and protein kinase C in transfected U937 cells

Suwanna Semsri[1], Songyot Anuchapreeda[1]*, Nutjeera Intasai[1], Tanyarat Jomgeow[1], Singkome Tima[1], Colleen Sweeney[2] and Pornngarm Limtrakul[3]

[1]Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand.
[2]UC Davis Cancer Center, 4645 2nd Avenue, Sacramento, CA, USA.
[3]Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Accepted 13 October, 2011

***Wilms' tumor (WT1)* gene overexpresses in leukemic cells which is alternatively spliced at two sites, yielding four isoforms: WT1 (+/+), (+/-), (-/+), and (-/-). Curcumin is one of major active components of the spice turmeric, widely known as anticancer. This study investigated the effects and inhibitory mechanism of pure curcumin on WT1 isoform-transfected U937 cells. WT1 transfected U937 cells were initially treated for 24 h, with 10 µM pure curcumin. Pure curcumin exhibited a strong inhibitory effect on WT1 (+/+) mRNA level detected by real time PCR. Treatment of WT1 transfected U937 cells with non-cytotoxic doses (10, 15, and 17 µM) of pure curcumin decreased WT1 protein levels in a dose-dependent manner. Pure curcumin at the concentration of 15 µM significantly decreased the protein levels of the WT (+/+) isoform in a time-dependent manner. It also decreased exogenous WT1 (+/+) protein half-life. WT1 protein expression was inhibited by protein kinase C inhibitor (GF109203x) suggesting that pure curcumin decreased exogenous WT1 (+/+) expression in transfected U937 cells *via* protein kinase C during post-translational processing.**

Key words: Pure curcumin, Wilms' tumor (WT1), isoform, protein kinase C, transfected U937 cell line.

INTRODUCTION

Turmeric (*Curcuma longa* Linn) is commonly used as a spice in curries and other South Asian and Middle Eastern cuisine, for dyeing, and to impart color to mustard condiments. It is also a highly popular medicinal herb, due to its biological properties. Its active ingredient is curcuminoids. Curcuminoids, especially pure curcumin, have strong anti-cancer and anti-tumor progression properties (Azuine and Bhide, 1992; Huang et al., 1992; Nagabhushan and Bhide, 1992; Naama et al., 2010; Pandey et al., 2010).

The *WT1* gene encodes a 48 to 57 kDa nuclear protein that has four zinc fingers and acts as a transcriptional activator or repressor (Rauscher et al., 1990; Wang et al., 1993; Wang, 2000). The four different isoforms of WT1 have been reported in WT1-expressing cells (Haber et al., 1991). Alternative splice I inserts 17 amino acids (+17AA) in exon 5, between the amino terminus and the zinc finger domain, and alternative splice II inserts 3 amino acids (+KTS) in exon 9 which is located between zinc-Fingers 3 and 4, and yields for four isoforms: WT1 [17AA(+)KTS(+)] or WT1 (+/+), WT1 [(17AA(+)KTS(-)] or WT (+/-), WT1 [(17AA(-)KTS(+)] or WT1 (-/+), and WT1 [(17AA(-)KTS(-)] or WT1 (-/-). All of the four isoforms are expressed in primary human solid cancers (Oji et al., 2002) and human primary leukemia (Miwa et al., 1992). Among the four WT1 isoforms, the WT1 17AA(+)KTS(+) isoform is dominantly expressed in all of the above mentioned cancers (Haber et al., 1991; Gu et al., 2010). This suggests that the constitutive expression of the WT1 17AA(+)KTS(+) isoform could rescue the growth inhibitory effect of WT1 antisense oligomers in solid tumors (Oji et al., 1999) indicating the contribution of the WT1 17AA(+)KTS(+) isoform to the growth of cancer cells (Hubinger et al., 2001). The 17AA(-)KTS(-)WT1

*Corresponding author. E-mail: sanuchapreeda@gmail.com.

isoform induced G1 arrest in osteosarcoma cell lines (Englert et al., 1997) and inhibited G1/S progression and accelerated differentiation in 32D cl3 murine myeloid progenitor cells in response to G-CSF (Loeb et al., 2003), suggesting that is in some cases a tumor-suppression role. As for the WT1 17AA(-)KTS(+) isoform, its functions remains unclear (Ito et al., 2006). These results indicate that each of the WT1 isoforms has a separate function and that these play an important role in leukemogenesis and tumorigenesis. The *WT1* gene is involved in the promotion of the cell cycle, as supported by findings that suppression of the WT1 expression induced a G2/M or G1 block in human leukemia K562 cells (Yamagami et al., 1998; Tuna et al., 2005).

Protein kinase C (PKC) is a family of serine/threonine protein and plays important roles in several diseases such as cancer, diabetes, stroke (Koivunen et al., 2006; Griner and Kazanietz, 2007). Therefore, PKC has been subject to intensive research and drug development (Hofmann, 2004) particularly in cancer research.

Previous studies have demonstrated that pure curcumin inhibits both *WT1* gene and protein expression (Anuchapreeda et al., 2006a, 2006b, 2008). However, the inhibitory effect of pure curcumin on the various WT1 isoforms has not been reported. Moreover, the inhibitory mechanism of pure curcumin on WT1 protein expression in transfected U937 cells is still unclear. Thus, the aims of this study were to investigate the effect and inhibitory mechanism of pure curcumin on WT1 isoforms in WT1 transfected U937 cells.

MATERIALS AND METHODS

Cell culture

Human monoblastic U937 and K562 cell lines were cultured in RPMI 1640 (Invitrogen, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, USA) and incubated in a 5% humidified CO_2 atmosphere at 37°C.

Vector construction

The eukaryotic expression vector pcDNA 3.1 carrying the cDNA for WT1 [17AA(+)KTS (+)] or WT1 (+/+), WT1 [(17AA(+)KTS(-)] or WT (+/-), WT1 [(17AA(-)KTS (+)] or WT1 (-/+), or WT1 [(17AA(-)KTS (-)] or WT1 (-/-) and a mock control cell (empty vector) were kindly provided by Dr. Haruo Sugiyama and Dr. Yusuke Oji, Osaka University Graduate School of medicine, Osaka, Japan.

Transfection procedure

Before transfection, plasmid vectors were linearized by a restriction enzyme *Pvul*. U937 cells (5×10^4 cells/ml) and seeded in 24-well plates overnight. The next day, 5 ng of plasmid were added to 2 ml of cell suspension and introduced into U937 or K562 cells through lipofectin (Qiagen, USA) or liprofectamine LTX Plus. Afterward, the cells were incubated at 37°C, 5% CO_2, for 24 h. Next, the cells were washed with PBS and resuspended in fresh medium before further incubation at 37°C, 5% CO_2, for 24 h. The cells were cultured in 96-well plate at a density of 1×10^3 cells/well (100 µl). After 2 weeks, individual clones that grew in the presence of 500 µg/ml G418 (Invitrogen, USA) were expanded for WT1, GFP, ErbB2 (676), EbrB3, and myc tagged Lrig1expression.

Extraction and isolation of pure curcumin

Pure curcumin was purified from turmeric powder using column chromatography. Pure curcumin extraction and isolation were performed as previously described (Limtrakul et al., 2004).

Cytotoxicity of pure curcumin extract on WT1 isoform-transfected U937 clone by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cytotoxicity of the pure curcumin extract, on the WT1 isoform-expressing U937 clones, was detected by the MTT assay as previously described (Anuchapreeda et al., 2006b). Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich, USA). The reaction is catalyzed by mitochondrial succinate dehydrogenease and requires NADH, which must be supplied by the living cells.

RNA extraction, reverse transcriptase PCR (RT-PCR) and real time PCR

WT1 isoform-transfected U937 clones were harvested and RNA was isolated using a High pure RNA Isolation kit (Roche, Germany), according to the manufacturer's instructions. RNaseOUT™ (Invitrogen, USA) was added to the RNA extraction products for RNA protection. RT-PCR was performed using SuperScript™ III One-step RT-PCR System with Platinum® Taq DNA polymerase reagent (Invitrogen, USA). For WT1, the forward primer was 5'-GACCTGGAATCAGATGAACTTAG-3', and the reverse primer was 5'-TTCTGACAACTTGGCCACCG-3'. For GAPDH, the forward primer was 5'-CGAAGTCAACGGATTTGGTCGTAT-3' and the reverse primer was 5'-AGCCTTCTCGGTGGTGAAGAC-3'. The cDNA was synthesized from 1 µg of total RNA at 60°C for 30 min, and denatured at 94°C for 2 min. PCR amplification was performed for 35 cycles of sequential denaturation at 94°C for 1 min; annealing at 60°C for 1 min; and extension at 72°C for 1 min. For the negative control, water was amplified in a total of 35 cycles, to detect any possible contamination. A total of 10 µl of each PCR product was analyzed using a 2% agarose gel electrophoresis, visualized with ethidium bromide staining (2 mg/ml), and quantified using scan densitometry (BioRad, USA).

Real-time PCR was carried out using TaqMan probe-based chemistry (Operon Biotechnologies GMbH, Germany). The primers and probe for WT1 were 5′-GATAACCACACAACGCCCATC-3′ (forward), 5′-CACACGTCGCACATCCTGAAT-3′ (reverse), and 5′-[6-FAM]ACACCGTGCGTGTGTATTCTGTATTGG[TAMRA-6-FAM]-3′ (probe). β- actin primers were 5′-CCCAGCACAATGAAGATCAAGATC AT-3′ (forward), 5′-ATCTGCTGGAAGGTGGACAGCGA-3′ (reverse), and 5′-[6-FAM]TGAGCGCAAGTACTCCGTGTGGATCGGCG[TAM-RA-6-FAM]-3′ (probe). The amplification reactions were all performed in triplicate in a Chromo4 Real-Time PCR System with 50 cycles, which consisted of activated Taq DNA polymerase at 95°C for 10 min, denaturation at 95°C for 30 s, and annealing at 63°C for 1 min. Data were collected and analyzed using the Opticon Monitor 3 program (BioRad, USA). The mRNA expression levels of the *WT1* gene were presented as a ratio to that of β-actin, and the relative expression levels, based on the $\Delta\Delta C_T$ method (Ginzinger, 2002) were calculated. Normalization:

$\Delta C_T = C_T$(sample) - C_T (β-actin); $\Delta\Delta C$T = ΔC_T (Sample 1) - ΔC_T

(Sample 2). Relative quantification = $2^{-\Delta\Delta C}_{T}$.

Western blot analysis

Cells were washed twice with cold PBS and then washed once with hypotonic buffer (10 mM 2-hydroxyl piperazinyl ethanesulfonic acid [HEPES]-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol). Cells were lysed, on ice, with hypotonic buffer containing 0.1% Nonidet P40, for 30 min. Then crude nuclei and cytosolic fractions were separated by centrifugation at 15,000 x g for 10 min. The crude nuclear pellets were successively incubated in laemmli buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulphonylfluoride) for 15 min on ice. The supernatant nuclear proteins were collected by centrifugation at 15,000 x g for 15 min. The nuclear protein extracts (50 µg/lane) were heated in boiling water for 5 min. Samples were loadred onto 12% sodium dodecylsulfate (SDS)-polyacrylamide gels for electrophoresis. WT1 protein detection was performed using primary rabbit polyclonal anti-WT1 (C-19) and GAPDH (Santa Cruz, USA). HRP conjugated goat anti-rabbit IgG (Promega, USA) was used as a secondary antibody for WT1and GAPDH protein detection. Proteins were visualized using the SuperSignal® protein detection kit (Thermo Scientific, USA).

Immunoprecipitation

Nuclear extracts were removed by NE-PER® Nuclear and Cytoplasmic extraction reagent (Thermo Scientific, USA). Nuclear protein extracts (70 µg) were incubated with 10 µg of the agarose conjugated with rabbit polyclonal WT1-specific antibodies (Santa Cruz, USA) for 5 h at 4°C. The immunoprecipitated proteins were separated by 12% SDS-PAGE. The proteins on the membrane were detected with rabbit polyclonal anti-WT1 (C-19).

Assay for protein half life

Cells were incubated with cycloheximide (CHX: 50 µg/ml) in the presence or absence of 15 µM pure curcumin for 3, 6, 12 and 24 h. After that, the cells were lysed with lysis buffer (50 mM Tris, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA) and the crude protein lysates were resolved by SDS-PAGE and subjected to Western blot analysis. GAPDH was used as an internal protein loading control.

Proteasome inhibitor assay

Cells were incubated with or without 5 µM MG132, in the presence or absence of 15 µM pure curcumin, for 3 h. After that, the cells were lysed in lysis buffer and the crude protein lysates were evaluated using the Western blot analysis. GAPDH was used as an internal protein loading control.

PKC inhibitor assay

Cells were incubated with 2.5 µM GF109203x (PKCα,δ, β1, and ε inhibitors) for 1, 3, 5, 7 and 24 h. After that, the cells were lysed in lysis buffer and the crude protein lysates were evaluated using Western blot analysis. GAPDH was used as an internal protein loading control.

Statistical analysis

Statistical analysis was performed with SPSS software (version 10.0). All experiments were conducted in duplicate and repeated at least three times. The data were expressed as the mean ± standard error of sample mean (S.E.M.). Statistical differences between the means were tested by one-way ANOVA. Probability values $P<0.05$ were considered significant.

RESULTS

Establishment and characterization of U937 clones constitutively expressing WT1

To investigate the role of WT1 isoforms on cell proliferation in leukemic cells, stable clones of WT1-expressing U937 cells were established. The WT1 (+17AA/+KTS or +/+), WT1 (+17AA/-KTS or +/-), WT1 (-17AA/+KTS or -/+), and WT1 (-17AA/-KTS or -/-) constructs were transfected into U937 cells by lipofection. The overexpression of WT1 mRNA in different clones was detected using RT-PCR and real time PCR. Transfected U937 cells displayed high levels of WT1 isoform mRNA. WT1 (+/+) clones were designated as WT1 (+/+)/1C7, WT1 (+/+)/1G3, WT1 (+/+)/2E10, and WT1 (+/+)/1E3, whereas WT1 (+/-) clones were WT1 (+/-)/1C8, and WT1 (+/-)/1G9. WT1 (-/+) clones were WT1 (-/+)/1D3 and WT1 (-/+)/1D6. The WT1 (-/-) clone was WT1 (-/-)/1E9. Mock control cells were transfected with pcDNA 3.1 empty vectors. The control clones showed low levels of endogenous WT1 mRNA (Figure 1A and 1B). Moreover, when the expression of the four WT1 isoforms was found in WT1-tranfected U937, control cells (wild type U937), and mock control using immunoprecipitation and Western blot analysis, all four isoforms were clearly observed at higher levels in the transfected cells, than in those of the cell and mock controls (Figure 1C).

The effect of pure curcumin on WT1 isoform and WT1 overexpression reverses the effect of pure curcumin activity

Pure curcumin exhibited a cytotoxic effect on the U937 clones with inhibitory concentrations at 50% (IC_{50} value) of approximately 21 ± 1.0 µM (WT1 (+/+)/1C7), 19 ± 0.2 µM (WT1 (+/-)/1G9), 24 ± 0.6 µM (WT1 (-/+)/1D6), and 23 ± 0.4 µM (WT1(-/-)/1E9), respectively by MTT assay. The IC_{50} values of U937 control cells and mock control cells were 15 ± 1.0 and 17 ± 2.2 µM, respectively. The IC_{20} values of pure curcumin were used to investigate the inhibitory effect and mechanism of pure curcumin on the *WT1* gene expression. Indeed, WT1 mRNA undergoes alternative splicing at two sites resulting in four mRNA species and protein products; WT1 +/+, WT1 +/-, WT1 -/+, and WT1 -/- (Haber et al., 1991; Fraizer et al., 1994). WT1 reportedly plays an important role in leukemic cell proliferation in leukemogenesis. Recently, pure curcumin was shown to inhibit cell growth and repress WT1 expression (Anuchapreeda et al., 2008). For this expe-

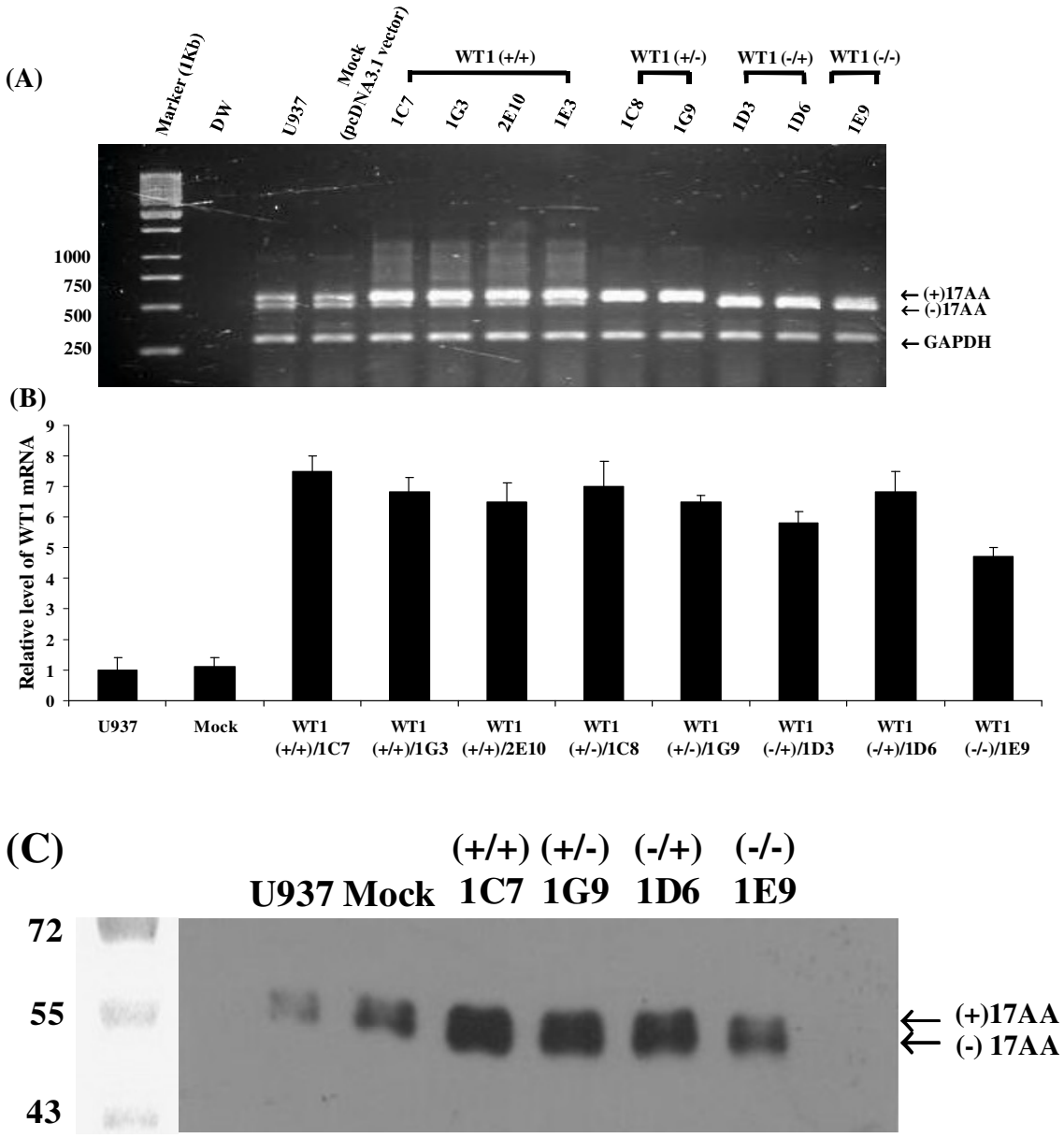

Figure 1. Expression of *WT1* gene and WT1 protein in transfected cells. (A) Expression of WT1 mRNA in WT1 isoform-transfected U937 cells was determined by RT-PCR and (B) Real time PCR. (C) WT1 was immunoprecipitated and detected with the specific anti-WT1 (C-19) antibody using SDS-PAGE (12% gel) and ECL analysis. Asterisks (*) denote values that were significantly different from the vehicle control ($P < 0.05$).

riment, it was determined whether pure curcumin could reverse the anti-proliferative effects by overexpressing exogenous WT1 in stably transfected U937 cells. Cells were then treated with 15 µM pure curcumin and the anti-proliferative effect was determined using the MTT assay. The overexpression of all four WT1 isoforms in stably transfected U937 cells showed their resistance to the anti-proliferative effects of pure curcumin was greater in comparison to the transfected vector control (Figure 2). Data demonstrates both of the transcriptional redundancy of WT1 isoforms and their regulatory function in cell proliferation. These findings provide key insights into the growth inhibitory mechanisms of pure curcumin.

The effect of pure curcumin on mRNA levels in four WT1 isoform-transfected cells

Previous studies have reported that pure curcumin was able to inhibit the endogenous *WT1* gene in K562, U937, HL60, and Molt4 cells and to inhibit protein expression in K562 and Molt4 cells (Anuchapreeda et al., 2008). Yet to

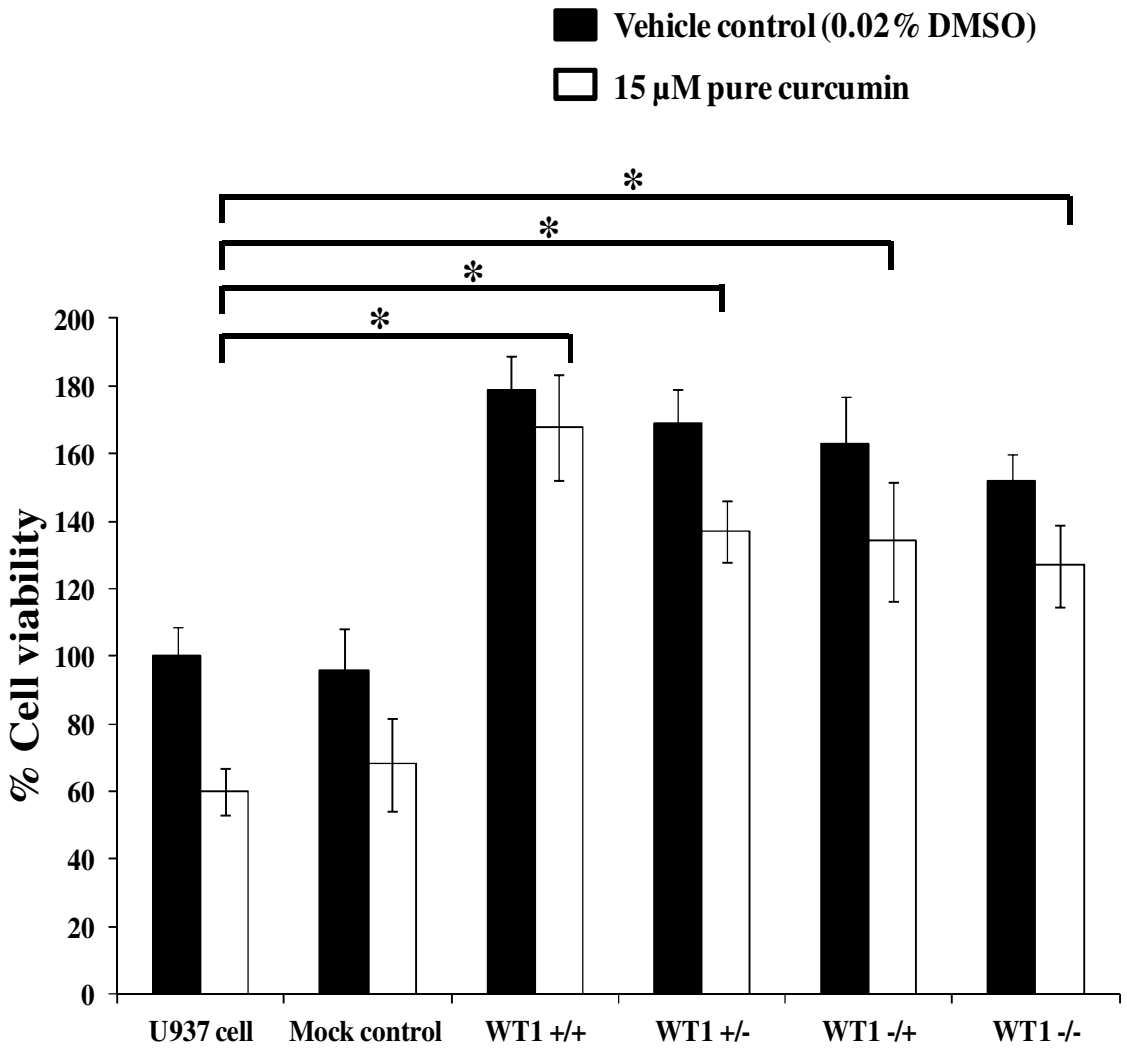

Figure 2. Cytotoxic effect of pure curcumin on WT1 isoform-transfected U937 cells. Data show the mean ± S.E.M. of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

date, the effect of pure curcumin on WT1 isoform expression has not been reported. Thus, the WT1 isoform-transfected cells, control cells, and mock control cells were treated with non-cytotoxic doses (IC_{20} value) of the pure curcumin, for 24 h. The concentrations, at IC_{20} value, of WT1 (+/+), (+/-), (-/+), (-/-), cell control, and pcDNA3.1 control vector were 15, 11, 13, 13, 9, and 9 µM, respectively. The expression of the exogenous WT1 (+/+) and WT1 (+/-) isoforms were significantly decreased by pure curcumin when compared to total endogenous WT1 mRNA levels in the cell control and pcDNA3.1 control vectors. However, pure curcumin did not inhibit WT1 expression in WT1 (-/+) and WT1 (-/-) isoform-transfected cells (Figure 3). Each treatment was compared to the vehicle control (0.02% DMSO alone without the pure curcumin in the culture medium).

The effect of pure curcumin on exogenous WT1 protein in WT1 (+/+) isoform-transfected U937 cells

To examine whether pure curcumin could inhibit WT1 (+/+) protein expression, WT1 protein levels were determined using the Western blot analysis. WT1 (+/+) isoform-transfected cells were treated with pure curcumin (10, 15, and 17 µM) for 24 h. The levels of WT1 (+/+) protein after pure curcumin treatment were 37 ± 3.7, 13 ± 1.2, and 11 ± 1.5%, respectively. Thus the exogenous WT1 (+/+) protein levels were significantly decreased by 63, 87 and 89%, respectively (P<0.05), when compared to vehicle control (Figure 4A and 4B). In addition, the levels of the exogenous WT1 (+/+) protein after 15 µM pure curcumin treatment for 3, 6, 12, and 24 h were 52 ± 6.7, 31 ± 2.7, 19 ± 4, and 12 ± 1.8%, respectively. Thus the WT1 (+/+) protein levels were significantly decreased

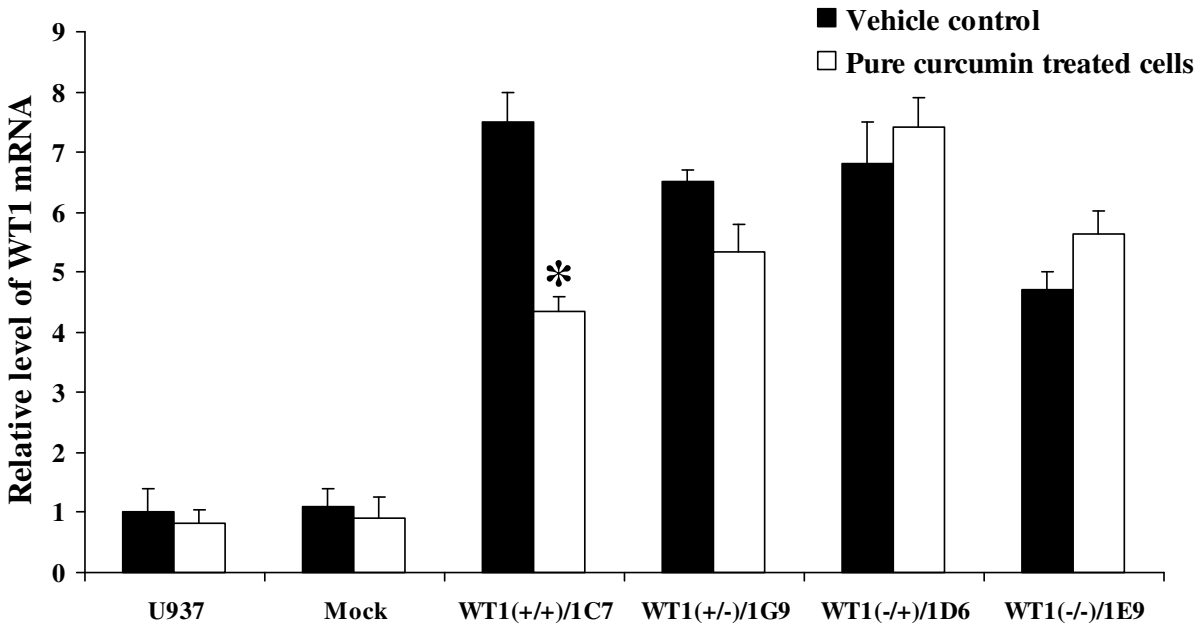

Figure 3. The relative level of WT1 mRNA in pure curcumin treated WT1 isoform-transfected U937 cells. Data show the mean ± S.E.M. of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

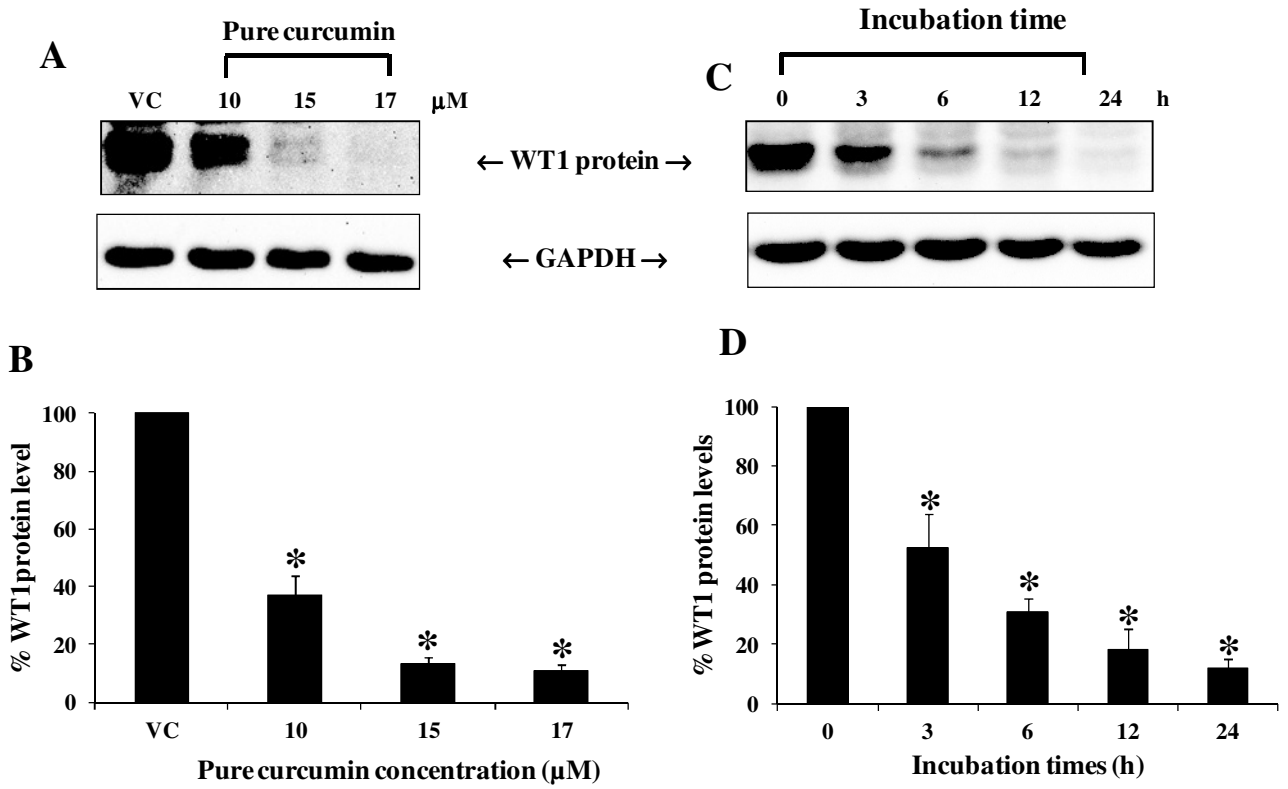

Figure 4. Effect of pure curcumin on WT1 protein levels in WT1 (+/+) transfetced U937 cells. (A) The WT1 protein levels were detected using the Western blot analysis. (B) The percentage of WT1 protein levels were determined by scanning densitometry and normalized to GAPDH. (C) WT1 proteins were detected by the Western blot analysis. (D) The percentages of WT1 protein were determined by scanning densitometry and normalized to GAPDH. Data show the mean ± S.E.M. of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

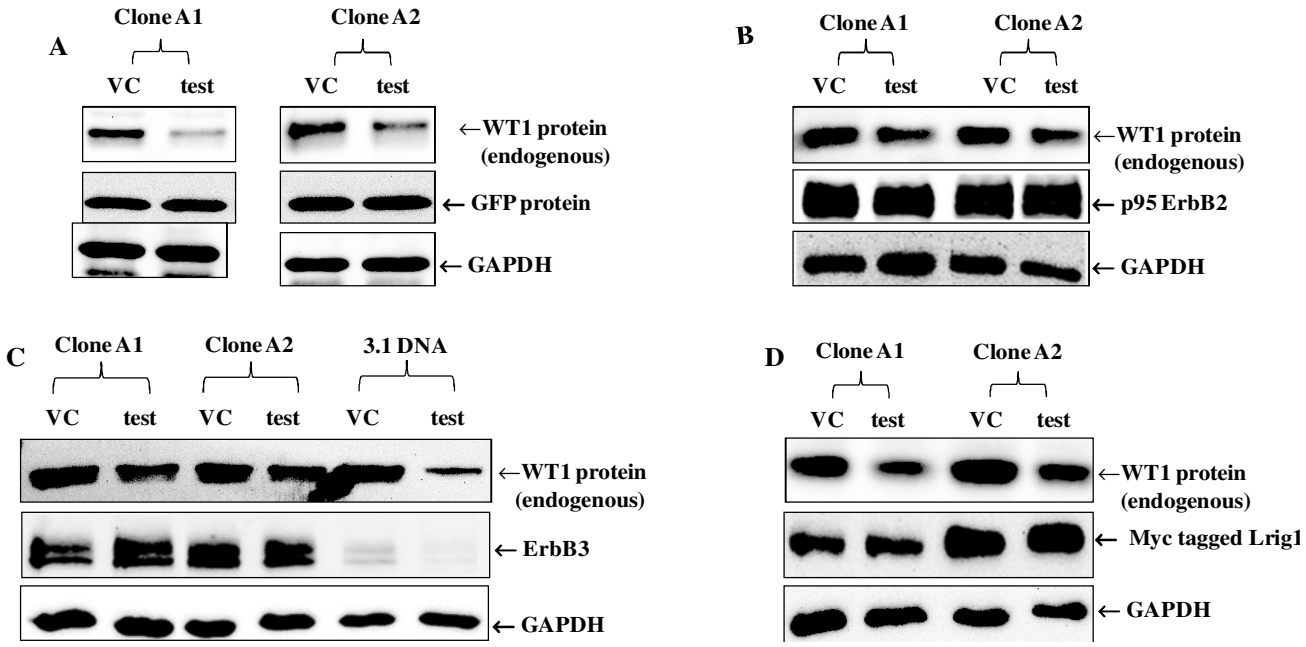

Figure 5. Effect of pure curucmin on other proteins driven by the pCMV promoter. The proteins were detected using the Western blot analysis. The membranes were probed with primary antibodies against (A) GFP, (B) p95 ErbB2, (C) ErbB3 or (D) myc-tagged Lrig1 proteins. The GAPDH proteins were used as a loading control.

by 48, 69, 81 and 88%, respectively ($P<0.05$) when compared to vehicle control (Figure 4C and 4D).

The effect of pure curcumin on exogenous WT1 is specific

To determine whether the effect of pure curcumin on exogenous WT1 expression was specific, we examined the impact of pure curcumin on the expression of four other exogenous proteins, driven by the pCMV promoter, in the same pcDNA3.1 vector as WT1. This experiment used GFP, p95 ErbB2, ErbB3, and myc-tagged Lrig1 genes. K562 cells were transfected as indicated in the materials and methods.

The cells were either treated with or without 15 µM of pure curcumin. While 15 µM pure curcumin decreased exogenous WT1 expression by 87% (Figure 3), the protein levels of GFP, p95 EbrB2, ErbB3, and myc-tagged Lrig1 were not decreased when compared to the vehicle control in transfected K562 cells (Figure 5). This indicates that pure curcumin specifically decreases WT1 expression. Moreover, it is not related to the pCMV promoter activity inhibition of the exogenous WT1 expression. The K562 cells were used for WT1 isoform transfections in this study in order to observe the effect of pure curcumin on the endogenous and exogenous *WT1* gene expression. Normally, the K562 cell itself constitutively overexpresses the endogenous WT1 protein. It could then be determined if the activity of pure curcumin effects the WT1 protein expression, but not pCMV promoter.

The effect of pure curcumin on exogenous WT1 protein half life in WT1 (+/+) isoform-transfected U937 cells

To investigate whether the decrease in the WT1 (+/+) protein isoform involved a degradation mechanism, WT1 (+/+) isoform-transfected U937 cells were treated with cycloheximide, to inhibit new protein synthesis, either with or without 15 µM of pure curcumin. WT1 protein expression was then followed over time to measure its half life. Exogenous WT1 (+/+) protein stability after pure curcumin treatment was significantly decreased when compared to control conditions. The half life of WT1 protein in the control treatment was 15 h, whereas in pure curcumin treatment was only 3.6 h. To examine whether pure curcumin promotes the proteasomal degradation of WT1, we determined if the MG132 proteasome inhibitor could eliminate the impact of pure curcumin on WT1. Indeed, in the presence of MG132, the suppressive effect of pure curcumin on WT1 expression was largely prevented (Figure 6). These results demonstrate that pure curcumin decreases WT1 expression, at least in part through the promotion of proteasomal degradation. However, it should also be noted that a decrease in WT1 (+/+) mRNA was observed following treatment with pure curcumin (Figure 3), so its effects on WT1 expression are

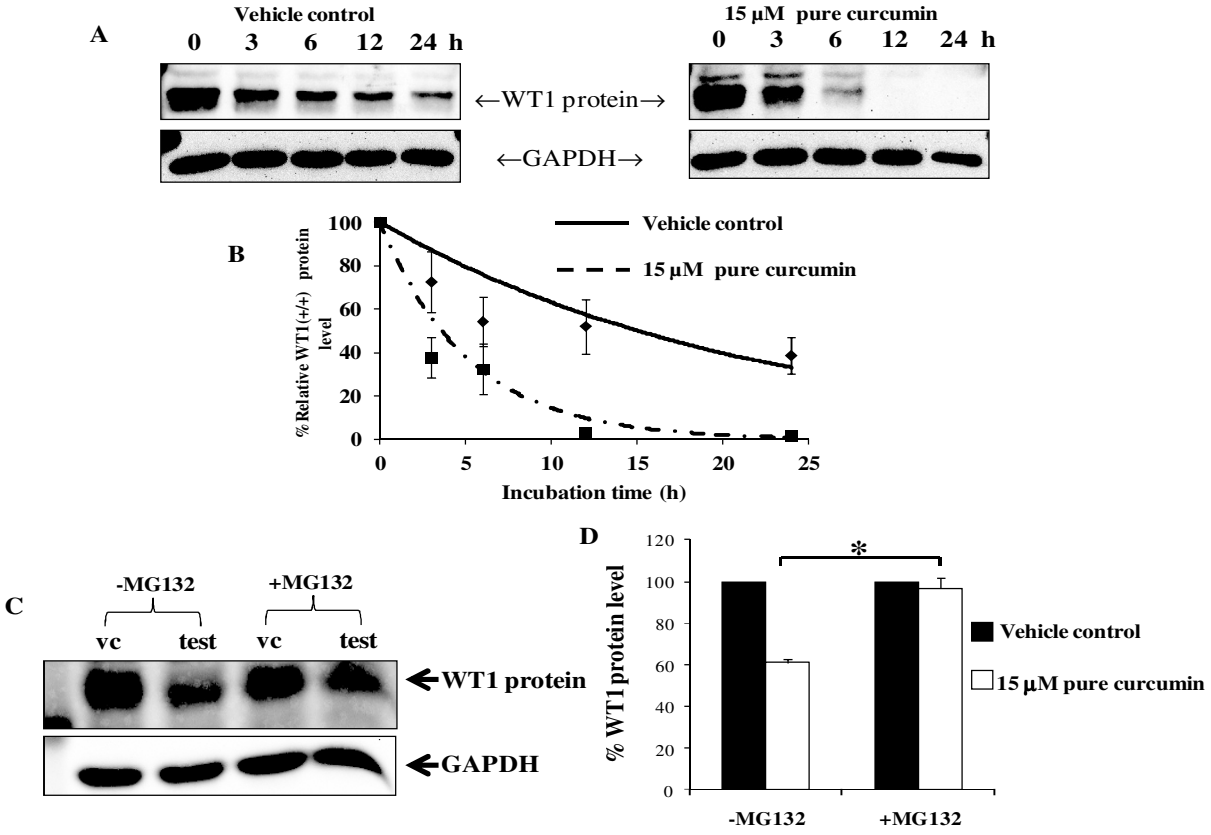

Figure 6. Effect of pure curcumin on WT1 (+/+) protein degradation in transfected U937 cells. (A) The WT1 protein half life using cyclohexamide was determined by Western blot analysis. (B) The half life of exogenous WT1 (+/+) proteins was determined through Western blot analysis. (C) Western blot analysis of the exogenous WT1 (+/+) protein levels when treated with MG 132. (D) Exogenous WT1 (+/+) protein levels, were measured and normalized with GAPDH protein levels. Data are the mean ± S.E.M. of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

likely to be multi-factorial.

The effect of PKC inhibitor on exogenous WT1 (+/+) protein expression in transfected U937 cells

According to the results presented in this paper, pure curcumin clearly and specifically decreases the exogenous WT1 (+/+) protein in transfected U937 cells in a process that is dependent upon the proteasome. Interestingly, phosphorylation of WT1 has been previously reported to shown that pure curcumin can decrease WT1 phosphorylation, by PKC (Jensen, 2006). Thus, this experiment aimed to investigate the effect of a PKC inhibitor (GF109203x) on the exogenous WT1 (+/+) protein expression in transfected U937 cells. Cells were incubated with 2.5 µM GF109203x for different lengths of time (1, 3, 5, 7, and 24 h). The levels of exogenous WT1 (+/+) protein were significantly decreased after 2.5 µM GF-109203 treatment for 3 and 5 h, when compared to the individual vehicle control (P<0.05). Moreover, an incubation time of 5 h treatment significantly decreased the exogenous WT1 (+/+) protein compared to those at 7 and 24 h (Figure 7).

DISCUSSION

Overexpression of *Wilms' tumor1* (*WT1*) gene is important in leukemogenesis because both the RNA and the protein levels of WT1 increase 1,000-10,000 fold in leukemic cells (Inoue et al., 1994; Menssen et al., 1995). This suggests it may play an important role in oncogenesis. Therefore, the over expression of *WT1* gene and WT1 protein may serve as a possible biological marker for monitoring leukemia. Chemical compounds extracted from the spices and/or medicinal plants, such as turmeric, ginger, garlic, chilli, pepper, and black pepper are becoming popular as chemotherapeutic drugs in anticancer drug research. In fact pure curcumin extracted from turmeric, a spice grown in tropical regions of Asia, has been shown to decrease WT1 mRNA and WT1 protein levels in human leukemic cell lines (Anuchapreeda et al., 2008). There are four WT1 isoforms (WT1 (+/+), WT1 (+/-), WT1 (-/+), WT1 (-/-).

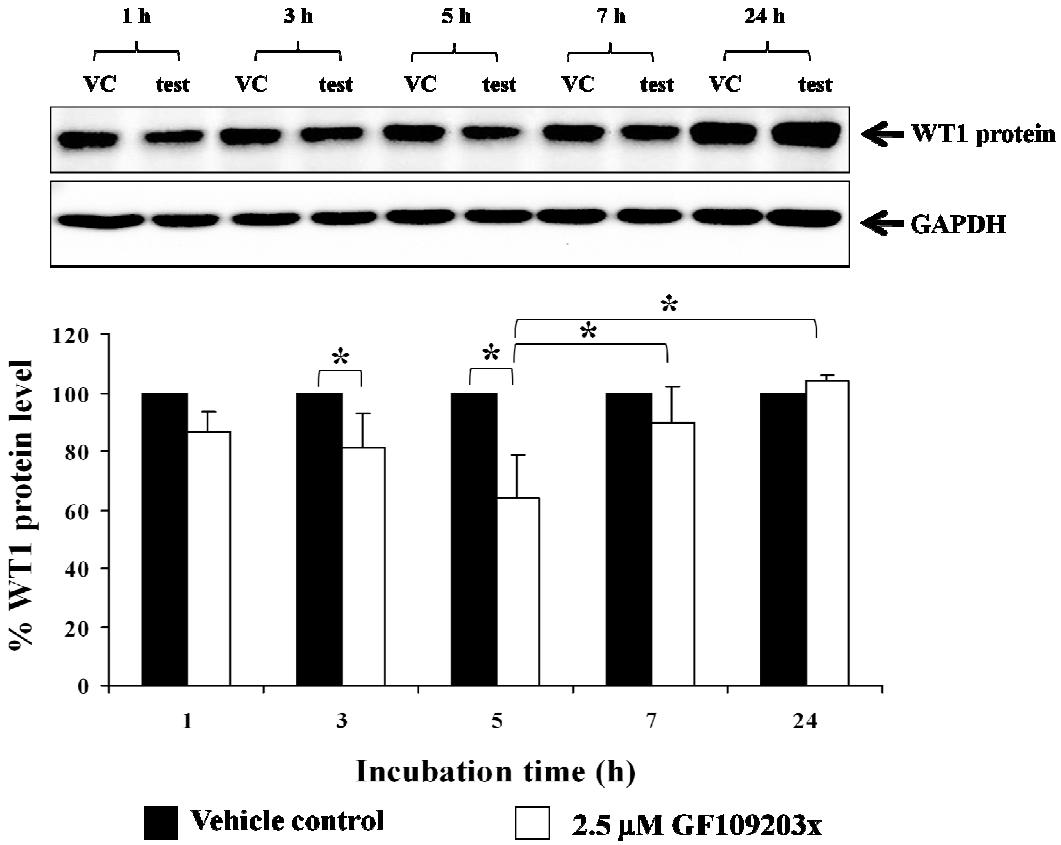

Figure 7. The effect of GF109203x on WT1 (+/+) protein expression in transfected U937 cells. Asterisks (*) denote values that were significantly different from the vehicle control ($P<0.05$).

However, the specific WT1 isoform that is inhibited by pure curcumin in leukemic cells is still unknown. This study aimed to address this question by identifying the WT1 isoform that is suppressed by pure curcumin. Stably transfected U937 cells expressing each of the WT1 isoforms were used as the experimental model because U937 cells express modest WT1 mRNA and WT1 protein levels (Svedberg et al., 1998). In this study, pure curcumin was cytotoxic toward wild type, vector control, and WT1 isoform transfected U937 cells. When non-cytotoxic doses of pure curcumin (IC_{20}) were used, WT1 (+/+) isoform-transfected cells clearly showed decreased WT1 mRNA and WT1 protein levels. This demonstrates that the WT1 (+/+) isoform is specifically suppressed by pure curcumin compared to other WT1 isoforms.

Curcumin also has numerous other biological properties, such as antioxidant effects and anti-inflammatory effects as well as anti-mutagen and anti-cancer properties. Additionally, curcumin inhibits oncogene expression and activation of PKC upon induction by TPA (Liu et al., 1993). The tumor promoter (example, TPA) activates PKC by reacting with the zinc-thiolates present within the regulatory domain of PKC. In contrast, the oxidized forms of some cancer preventive agents, such as polyphenolics (curcumin, ellagic acid, and 4-hydroxytamoxifen) and seleno compounds, can inactivate PKC by oxidizing the vicinal thiols present within the catalytic domain. This brings an efficient counteractive mechanism to block the signal transduction, induced by the tumor promoter, at the first step (Gopalakrishna and Jaken, 2000). The inhibitory effect of curcumin affects a wide variety of genes that require transcription factors, such as Activation-Protein 1 (AP1) and Nuclear Factor Kappa B (NFκB) (Singh and Aggarwal, 1995; Qi et al., 2009), which are regulated by PKC. WT1 protein has also been reported to be involved in early hematopoiesis and cell proliferation. WT1 protein is a transcription factor regulated by activated PKC that phosphorylates the C-terminal domain of WT1 regulating the proliferation in leukemic cells (Ye et al., 1996).

This study is the first to demonstrate the inhibitory properties of pure curcumin on the expression of the WT1 (+/+) isoform, in transfected U937 cells. However, pure curcumin did not effect the expression of four different reporter proteins (GFP, p95 EbrB2, ErbB3, and myc-tagged Lrig1) under the same pCMV promoter, highlighting the specificity of the action of pure curucmin on WT1. Therefore, the decreased amount of WT1 expression in WT1 (+/+) transfected U937 cells was not due to the

effect of pure curcumin on the pCMV promoter activity. Moreover, we found that it was related to the decreased stability of the WT1 protein in response to the treatment of pure curcumin. Inhibition of PKC-mediated phosphorylation of WT1 using the PKC inhibitor (GF109203x) led to the suppression of the WT1 (+/+) protein expression, suggesting that PKC signaling in the WT1 (+/+) transfected U937 cells is important for the stability of the WT1 protein. We propose that pure curcumin suppresses WT1 protein levels through the inhibition of PKC at post-translational level, thereby destabilizing the protein.

Conclusion

This study is the first to discover that pure curcumin could down-regulate exogenous WT1 (+/+) protein in transfected U937 cells. Previous studies reported that curcumin inhibited the phosphorylation capacity of PKC (Lin, 2004; Aggarwal et al., 2007; Anand et al., 2008). We suggested that pure curcumin suppressed the exogenous WT1 (+/+) protein level by inhibiting the activation of PKC, thereby destabilizing WT1 (+/+). This property of pure curcumin could be used as a possible anti-leukemic agent in human leukemic cancer.

ACKNOWLEDGEMENTS

This research was supported by Thailand Research Fund (TRF), the Royal Golden Jubilee (RGJ) PhD Program, the Thailand Government Budget, and National Research Council of Thailand (NRC). The authors would like to thank Dr. Haruo Sugiyama and Dr. Yusuke Oji for providing the vectors encoding the four WT1 isoforms. We gratefully acknowledge Dr. Dale E. Taneyhill and Dr. Lakmal Kotelawala for critically reading the manuscript.

REFERENCES

Aggarwal BB, Setthi G, Baladandayuthapani V, Krishnan S, Shisodia S (2007). Targeting cell signaling pathways for drug discovery: an old lock needs a new key. J. Cell Biochem., 102: 580-592.

Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB (2008). Curcumin and cancer: an "old-age" disease with an "age-old" solution. Cancer Lett., 267: 133-164.

Anuchapreeda S, Limtrrakul P, Thanarattanakorn P, Sittippeechacharn S, Chanarat P (2006a). Inhibitory effect of curcumin on WT1 gene expression in patient leukemic cells. Arch. Pharm. Res., 29: 80-87.

Anuchapreeda S, Thanarattanakorn P, Sittippeechacharn S, Chanarat P, Limtrrakul P (2006b). Curcumin inhibits WT1 gene expression in human leukemic K562 cells. Acta. Pharmacol. Sin., 27: 360-366.

Anuchapreeda S, Tima S, Duangrat C, Limtrakul P (2008). Effect of pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin on WT1 gene expression in leukemic cell lines. Cancer Chemother. Pharmacol., 62: 585-594.

Azuine MA, Bhide SV (1992). Chemopreventive effect of turmeric against stomach and skin tumors induced by chemical carcinogens in Swiss mice. Nutr. Cancer, 17: 77-83.

Englert C, Maheswaran S, Garvin AJ, Kreidberg J, Haber DA (1997). Induction of p21 by the Wilms' tumor suppressor gene WT1. Cancer Res., 57: 1429-1434.

Fraizer GC, Wu YJ, Hewitt SM, Maity T, Ton CC, Huff V, Saunders GF (1994). Transcriptional regulation of the human Wilms' tumor gene (WT1). Cell type-specific enhancer and promiscuous promoter. J. Biol. Chem., 269: 8892-8900.

Ginzinger DG (2002). Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp. Hematol., 30: 503-512.

Gopalakrishna R, Jaken S (2000). Protein kinase C signaling and oxidative stress. Free Radic. Biol. Med., 28: 1349-1361.

Griner EM, Kazanietz MG (2007). Protein kinase C and other diacylglycerol effectors in cancer. Nat. Rev. Cancer, 7: 281-294.

Gu W, Hu S, Chen Z, Qiu G, Cen J, He B, He J, Wu W (2010). High expression of WT1 gene in acute myeloid leukemias with more predominant WT1+17AA isoforms at relapse. Leuk. Res., 34: 46-49.

Haber DA, Sohin RL, Buckler AJ, Pelletier J, Call KM, Housman DE (1991). Alternative splicing and genomic structure of the Wilms tumor gene WT1. Proc. Natl. Acad. Sci. U S A., 88: 9618-9622.

Hofmann J (2004). Protein kinase C isozymes as potential targets for anticancer therapy. Curr. Cancer Drug Targets. 4: 125-146.

Huang MT, Wang ZY, Georgiadis CA, Laskin JD, Conney AH (1992). Inhibitory effects of curcumin on tumor initiation by benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene. Carcinogenesis, 13: 2183-2186.

Hubinger G, Schmid M, Linortner S, Manegld A, Bergmann L, Maurer U (2001). Ribozyme-mediated cleavage of wt1 transcripts suppresses growth of leukemia cells. Exp. Hematol., 29: 1226-1235.

Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kit K, Hiraoka A, Masaoka T, Nasu K, Et AL (1994). WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood, 84: 3071-3079.

Ito K, Oji Y, Tatsumi N, Shimizu S, Kanai Y, Nakazawa T, Asada M, Jomgeow T, Aoyagi S, Nakano Y, Tamaki H, Sakaguchi N, Shirakata T, Nishida S, Kawami M, Tsuboi, A, Oka Y, Tsujimoto Y, Sugiyama H (2006). Antiapoptotic function of 17AA(+)WT1 (Wilms' tumor gene) isoforms on the intrinsic apoptosis pathway. Oncogene, 25: 4217-4229.

Jensen ON (2006). Interpreting the protein language using proteomics. Nat. Rev. Mol. Cell Biol., 7: 391-403.

Koivunen J, Aaltonen V, Peltonen J (2006). Protein kinase C (PKC) family in cancer progression. Cancer Lett., 235: 1-10.

Limtrakul P, Anuchapreeda S, Buddhasukh D (2004). Modulation of human multidrug-resistance MDR-1 gene by natural curcuminoids. BMC Cancer, 4: 13.

Lin JK (2004). Suppression of protein kinase C and nuclear oncogene expression as possible action mechanisms of cancer chemoprevention by Curcumin. Arch. Pharm. Res., 27: 683-692.

Liu JY, Lin SJ, Lin JK (1993). Inhibitory effects of curcumin on protein kinase C activity induced by 12-O-tetradecanoyl-phorbol-13-acetate in NIH 3T3 cells. Carcinogenesis, 14: 857-861.

Loeb DM, Summers JL, Burwell EA, Korz D, Friedman AD, Sukumar S (2003). An isoform of the Wilms' tumor suppressor gene potentiates granulocytic differentiation. Leukemia, 17: 965-971.

Menssen HD, Renkl HJ, Rodeck U, Maurer J, Notter M, Schwartz S, Reinhardt R, Thiel E (1995). Presence of Wilms' tumor gene (WT1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. Leukemia, 9: 1060-1067.

Miwa H, Beran M, Saunders GF (1992). Expression of the Wilms' tumor gene (WT1) in human leukemias. Leukemia, 6: 405-409.

Naama JH, Al-Temimi AA, Al-Amiery AAH (2010). Study the anticancer activities of ethanolic curcumin extract. Afr. J. Pure Appl. Chem., 4: 68-73.

Nagabhushan M, Bhide SV (1992). Curcumin as an inhibitor of cancer. J. Am. Coll. Nutr., 11: 192-198.

Oji Y, Miyoshi S, Maeda H, Hayashi S, Tamaki H, Nakatsuka S, Yao M, Takahashi E, Nakano Y, Hirabayashi H, Shintani Y, Oka Y, Tsuboi A, Hosen N, Asada M, Fujioka T, Murakami M, Kanato K, Motomura M, Kim EH, Kawakami M, Ikegame K, Ogawa H, Aozasa K, Kawase I, Sugiyama H (2002). Overexpression of the Wilms' tumor gene WT1 in de novo lung cancers. Int. J. Cancer, 100: 297-303.

Oji Y, Ogawa H, Tamaki H, Oka Y, Tsuboi A, Kim EH, Soma T,

Tatekawa T, Kawakami M, Asada M, Kishimoto T, Sugiyama H (1999). Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. Jpn. J. Cancer Res., 90: 194-204.

Qi LL, Wang JB, Wang HZ, Luo YT, Wu TX (2009). Curcumin induces cleavage of beta-catenin by activation of capases and downregulates the beta-catenin/Tcf signaling pathway in HT-29 cells. Afr. J. Pharm. Pharmacol., 8: 5527-5533.

Pandey A, Garg P, Raja RB (2010). Anticarcinogenic effect of turmeric on processed food products containing acrylamide. Afr. J. Pharm. Pharmacol., 4: 841-850.

Rauscher FJ, Morris JF, Tournay OE, Cook DM, Curran T (1990). Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science, 250: 1259-1262.

Singh S, Aggarwal BB (1995). Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane). J. Biol. Chem., 270: 24995-25000.

Svedberg H, Chylicki K, Baldetorp B, Rauscher FJ 3rd, Gullberg U (1998). Constitutive expression of the Wilms' tumor gene (WT1) in the leukemic cell line U937 blocks parts of the differentiation program. Oncogene, 16: 925-932.

Tuna M, Chavez-reyes A, Tari AM (2005). HER2/neu increases the expression of Wilms' Tumor 1 (WT1) protein to stimulate S-phase proliferation and inhibit apoptosis in breast cancer cells. Oncogene, 24: 1648-1652.

Wang R (2000). WT1 Gene and Leukemia. Zhongguo Shi Yan Xue Ye Xue Za Zhi, 8: 307-311.

Wang ZY, Qiu QQ, Enger KT, Deuel TF (1993). A second transcriptionally active DNA-binding site for the Wilms tumor gene product, WT1. Proc. Natl. Acad. Sci. U S A., 90: 8896-8900.

Yamagami T, Ogawa H, Tamaki H, Oji Y, Soma T, Oka Y, Tatekawa T, Tsuboi A, Kim EH, Akiyama T, Sugiyama H (1998). Suppression of Wilms' tumor gene (WT1) expression induces G2/M arrest in leukemic cells. Leuk. Res., 22: 383-384.

Ye Y, Raychaudhuri B, Gurney A, Campbell CE, Williams BR (1996). Regulation of WT1 by phosphorylation: inhibition of DNA binding, alteration of transcriptional activity and cellular translocation. EMBO J., 15: 5606-5615.