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Co-activation of WT1 and AP-1 proteins on *WT1* gene promoter to induce *WT1* gene expression in K562 cells

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ABSTRACT

Earlier studies have revealed one function of the inhibitory mechanism of curcumin. Activating PKCα induces *WT1* gene expression via signalling through downstream JNK and c-JUN. In the present study, the effect of c-JUN/AP-1 binding and transcriptional regulation of the *WT1* gene promoter was investigated in K562 leukaemic cells. The non-cytotoxic dose (IC_{20} values) of curcumin (WT1 and AP-1 inhibitors) was employed to examine its effect on *WT1* gene-mediated WT1 and AP-1 protein expression. Non-cytotoxic doses of both tanshinone IIA (AP-1 DNA-binding inhibitor) and SP600125 (JNK inhibitor) were used to test the role of c-JUN/AP-1 in *WT1* gene expression. Curcumin, tanshinone IIA, and SP600125 inhibited WT1 protein expression in a dose-dependent manner (5–15 μM) at 24 h as shown by immunoblotting. A ChIP assay showed that curcumin and tanshinone IIA inhibited AP-1 and WT1 binding to the proximal WT1 promoter (−301 bp), and a luciferase reporter assay showed that the WT1 luciferase gene reporter activity was decreased after curcumin, tanshinone IIA, and SP600126 treatments. Furthermore, depletion of c-JUN abrogated *WT1* gene expression. In summary, AP-1 contributes to the WT1 autoregulation of *WT1* gene expression in leukaemic K562 cells.

1. Introduction

The *Wilms' tumour 1* (*WT1*) gene encodes a 48–57 kDa nuclear protein that binds to DNA sequences to activate target gene expression, depending on the cellular or chromosomal context. The *WT1* gene has been well characterized, and it contributes to cell growth regulation and differentiation [1–3] through downstream effectors. These effectors include platelet-derived growth factor A (PDGF-A) chain [4], c-Myc, Bcl-2 [5], colony-stimulating factor-1 (CSF-1) [6], transforming growth factor-β1 (TGF-β1) [7], insulin-like growth factor 1 receptor (IGF1R) [8], insulin-like growth factor II [9], retinoic acid receptor-α (RAR-α) [10], and RNA metabolism [11]. WT1 protein is highly expressed in most leukaemias [9,12–15], and its expression is further increased upon disease relapse [16].

Of the four known WT1 isoforms, the 17 amino acid and 3 amino acid (KTS) insertion or exclusion are important for generation of WT1 protein isoforms. WT1 17AA (+) KTS (+) isoform is dominantly expressed in all cancers examined. Previous studies using antisense oligomers to specifically block the WT1 17AA (+) KTS (+) isoform have shown that the growth effect of constitutive WT1 expression is reversed [17], indicating that WT1 17AA (+) KTS (+) is the dominant isoform actively promoting the growth of cancer cells [18]. Studies have shown that the other three WT1 isoforms, namely, WT1 17AA (+) KTS (−), WT1 17AA (−) KTS (+), and WT1 17AA (−) KTS (−), have less impact on cell growth. Ectopic WT1 isoform protein suppression has revealed the antiproliferative activity of curcumin. These four isoforms are reduced after curcumin treatment [19]. The WT1 17AA (−) KTS (+) and the WT1 17AA (−) KTS (−) isoforms induce G_1 arrest in osteosarcoma cell lines [20]. These results indicate that each of the WT1 isoforms may have diverse functions and play important roles in leukaemogenesis and tumourigenesis.

Additionally, our previous work reported that the WT1 17AA (+) KTS (+) isoform is expressed at high levels in K562 cells [19]. We previously demonstrated that curcumin-mediated inhibition of protein kinase Cα (PKCα) contributes to curcumin-driven WT1 downregulation [19]. Indeed, constitutive activation of PKCα blocks curcumin-

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mediated downregulation of WT1 transcription. PKCα signalling through c-JUN N-terminal kinase (JNK) activates the c-JUN protein, which then binds to c-Fos to form a heterodimeric complex called activated protein-1 (AP-1) [21]. The AP-1 transcription factor complex promotes proliferation and differentiation in cancer cells through regulation of target genes typically in concert with multiple transcription factors [22,23]. In the present study, curcumin was used as a WT1 and AP-1 inhibitor in K562 leukaemic cells. Studying curcumin pharmacology, Anuchapreeda et al. showed that all three curcuminoids (curcumin I, curcumin II, and curcumin III) affect cell viability and proliferation in three leukaemic cell lines (K562, U937, and HL-60) [24]. Curcumin has been reported to affect *WT1* gene expression in leukaemic cells [24–26]. However, further research is required as WT1 transcriptional regulation of the *WT1* gene promoter is still unclear. WT1 signalling has been previously reported to involve PKCα and JNK in K562 cells [19]. JNK is a member of the mitogen-activated protein kinase (MAPK) family that regulates a range of biological processes implicated in tumourigenesis, neurogenerative disorders [27], and cell apoptosis [28,29]. JNK activity in human leukaemic cells (U937, K562, HL60, and THP-1) is necessary to promote cell proliferation [30]. The Bcr/Abl oncogene in chronic myelocytic leukaemia (CML) has been reported to enhance JNK function as measured by transcription from c-JUN-responsive promoters and requires Ras and MAPK/ERK kinase kinase (MEK kinase) [31]. Knockdown of the expression of JNK1, JNK2, or c-JUN has been previously reported to diminish procyanidin-induced effects on cell proliferation and apoptosis of oesophageal adenocarcinoma cells [32]. The present study investigated the mechanism of JNK signalling promotion of *WT1* gene expression through WT1 and c-JUN/AP-1 using K562 as a leukaemic cell model.

2. Materials and methods

2.1. Reagents

Cell culture reagents, including RPMI-1640 medium, foetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin, were purchased from Invitrogen™ Life (Carlsbad, CA, USA). Curcumin, SP600125, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tanshinone IIA was purchased from Enzo Life Sciences (USA), and trypan blue solution was purchased from AMRESCO® (Ohio, USA).

2.2. Cell line and cell culture conditions

The erythroid leukaemic cell line, K562, was purchased from Riken (Japan). The K562 cell line was maintained in RPMI-1640 medium containing 10 mM HEPES, 1 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% foetal bovine serum. The cell line was incubated in 95% relative humidity and 5% CO_2 at 37 °C.

2.3. MTT cytotoxicity assay

Cell cytotoxicity was determined by the MTT method. K562 cells were cultured in 96-well plates (1.0×10^4 cells/well) containing 100 μl of medium prior to treatment with curcumin (WT1 and AP-1 inhibitors) [19,33], tanshinone IIA (AP-1 DNA-binding inhibitor) [33,34], and SP600125 (JNK inhibitor) [30] at 37 °C for 24 h. Subsequently, 100 μl of fresh medium containing various concentrations (3–100 μM) of curcumin, SP600125, and tanshinone IIA was added to each well and incubated for an additional 24 h or 48 h. The final concentration of DMSO in the culture medium was 0.2% (*v*/v). The metabolic activity of each well was determined by the MTT assay and compared to those of untreated cells. After removal of 100 μl of medium, MTT dye solution was added (15 μl/100 μl medium), and the plates were incubated at 37 °C for 4 h in a humidified 5% CO_2 atmosphere. Afterward, 200 μl of DMSO was added to each well and mixed thoroughly to dissolve dye crystals. The absorbance was measured using an ELISA plate reader (Biotek EL 311) at 570 nm with a reference wavelength of 630 nm. High optical density readings corresponded to a high intensity of dye colour, i.e., to a high number of viable cells able to metabolize MTT salts. The fractional absorbance was calculated by the following formula:

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

The average cell survival obtained from triplicate determinations at each concentration was plotted as a dose response curve. Three independent experiments were performed. The 50% inhibition concentration (IC_{50}) of the active substances was determined as the lowest concentration that reduced cell growth by 50% in treated compared to untreated or vehicle control (0.2% DMSO in culture medium). The IC_{50} values were represented as the mean ± standard error (SE) and compared for their activities.

2.4. Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged. To detect the leakage of LDH into the cell culture medium, a tetrazolium salt is used in this assay. Release of LDH activity was determined by the colorimetric method of Chan et al. [35]. K562 cells (1.0×10^4 cells/ml) were cultured in complete RPMI-1640 with 15 μM curcumin and 15 μM tanshinone IIA for 24 h. Idarubicin (2 μM) was used as a positive control. The culture supernatants were collected for LDH assay. The reaction was done by using LDH assay buffer (0.92 mM INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride), 0.39 mM PMS (*N*-methylphenazonium methyl sulfate), 1.75 mM NAD (nicotinamide adenine dinucleotide), and 74 mM lactic acid in 200 mM Tris buffer solution). Reaction was stopped by 0.1 N HCl. The absorbance was measured using an ELISA plate reader (Biotek EL 311) at 520 nm. The amount of formazan product can be colorimetrically quantified by standard spectroscopy.

2.5. Trypan blue exclusion assay

Trypan blue exclusion method is a typical method to measure cell viability [36]. The intact membrane of viable cells excludes the trypan blue dye. Trypan blue enters dead cells, which have permeable membranes. The trypan blue assay was used to evaluate cell viability as described previously [37]. This microscopic assay for cell death was performed by assessing the ability of live cells to exclude trypan blue dye. After curcumin, tanshinone IIA, and SP600125 treatment as described in Section 2.3, a 50 μl aliquot of the cells was stained with 50 μl of 0.2% trypan blue, and cells were observed under a light microscope (40× magnification). Stained cells were considered to be no longer viable.

2.6. Western blot analysis

K562 cells were treated with curcumin, tanshinone IIA, and SP600125 for 24 h. Treated cells were washed twice with ice-cold PBS and lysed with cold RIPA buffer (50 mM Tris, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, and 0.5 mM EDTA) containing protease inhibitors. Samples were loaded into 12% sodium dodecylsulfate (SDS)-polyacrylamide gels for electrophoresis. WT1, cyclin B, cdc2, and c-Jun protein detection was performed using the following primary antibodies: rabbit polyclonal anti-WT1 antibody (Santa Cruz, CA, USA), mouse monoclonal anti-cyclin B antibody (BD Transduction Laboratories™, USA), mouse monoclonal anti-CDK1 antibody (cdc2) (Abcam®, MA, USA), and rabbit polyclonal anti-c-Jun antibody (Santa Cruz, CA, USA). Rabbit polyclonal anti-GAPDH antibody (Santa Cruz, CA, USA) at 1:1000 followed by staining with HRP-conjugated goat anti-rabbit IgG antibody (Invitrogen™, CA, USA) at 1:10,000 was used for normalization. Immunoblotting was visualized using the Luminata™

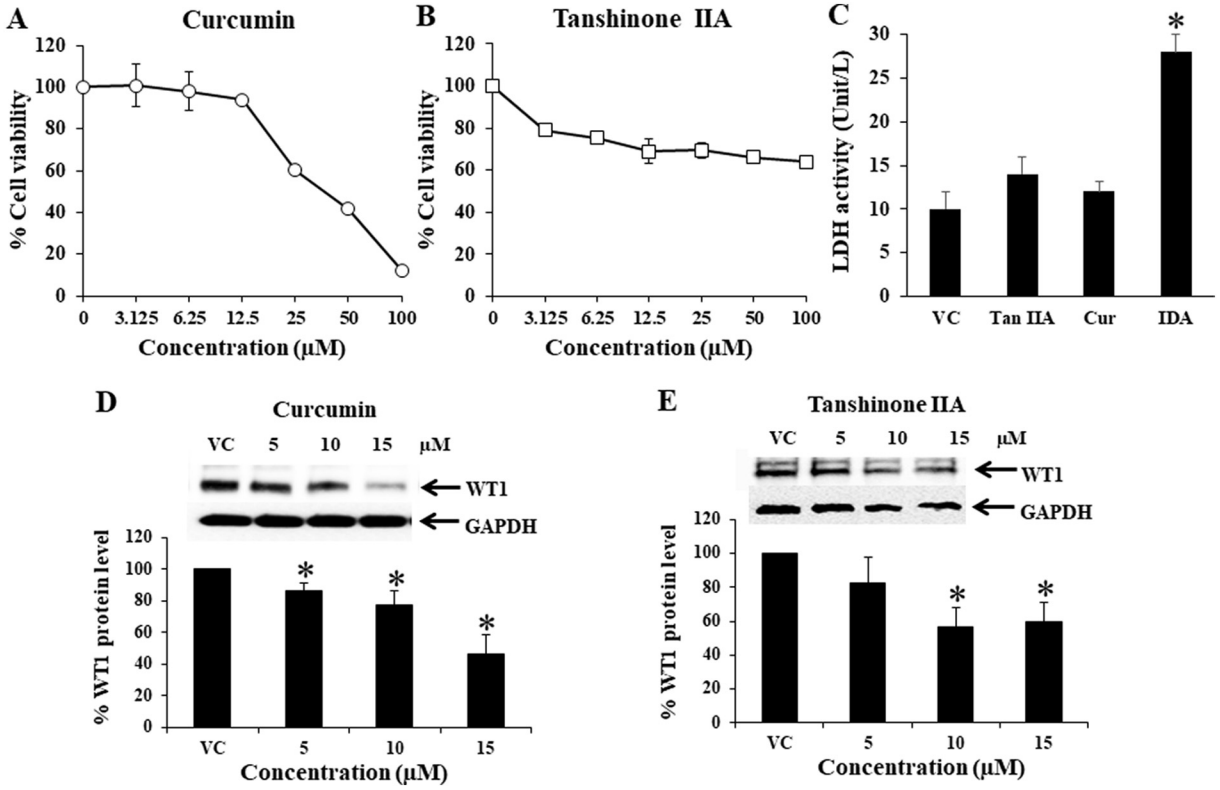

Fig. 1. Curcumin and tanshinone IIA induces cytotoxicity and suppresses WT1. (A) Cytotoxicity induced by curcumin (WT1 and AP-1 inhibitors) and (B) tanshinone IIA (AP-1 DNA-binding inhibitor) was examined in vitro by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays after treatment for 24 h in K562 cells at doses of 0, 3.125, 6.25, 12.5, 50, and 100 μM. (C) Non-cytotoxic dose at 15 μM of curcumin (Cur) and tanshinone IIA (Tan) were examined by LDH activity assay. Idarubicin (IDA) at the concentration of 2 μM was used as a positive control. Dose response for (D) curcumin and (E) tanshinone IIA on WT1 protein expression was determined by immunoblotting. K562 cells were incubated with (D) curcumin (5, 10, and 15 μM) and (E) tanshinone IIA (5, 10, and 15 μM) for 24 h. GAPDH was used as a loading control. Densitometry of protein levels was graphed as the percentage of vehicle control. Data represent the mean value ± SD of three independent experiments (*$P < 0.05$ vs. vehicle control, one-way ANOVA).

Forte Western HRP substrate protein detection kit (Merck Millipore) on an Alpha Innotech imaging system (Cell Biosciences, Inc., CA, USA).

2.7. Cell cycle analysis

K562 cells (5.0×10^5 cells/ml) were cultured in RPMI-1640 medium with non-cytotoxic concentrations (IC_{20}) of curcumin (5, 10, and 15 μM) for 24 h and fixed with ice-cold 70% ethanol for 30 min. Cell pellets were washed with ice-cold PBS and stained with propidium iodide (PI) solution (0.1% Triton X-100, 8 μg/ml RNase A, 2 mM EDTA, and 20 μg/ml PI) in the dark at 4 °C. Thereafter, red fluorescence was measured on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 25,000 events was collected per sample. The data were analysed using FlowJo 7.6.5™ software (FlowJo. LLC, OR, USA).

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP is a type of immunoprecipitation used to investigate the interaction between protein and DNA in the cell [38]. Briefly, cells were treated with 15 μM curcumin, 15 μM tanshinone IIA, or 3.5 μM mammea E/BB for 24 h. Cells were then crosslinked with 1% formaldehyde for 10 min and then treated with 0.125 M glycine to stop the crosslinking. Cells were resuspended in cell lysis buffer (5 mM PIPE, 85 mM KCl, 1% NP40, and protease inhibitors), incubated on ice for 15 min and centrifuged at 5000 rpm at 4 °C for 5 min. The nuclei pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 1% SDS; and protease inhibitors) and incubated on ice for 10 min before sonicating. The chromatin lysate was precleared with StaphA cells (Sigma) before immunoprecipitation at 4 °C overnight. The following antibodies were used: WT1 (C-19) (Santa Cruz, CA, USA), c-JUN (ACTIVE MOTIF®, USA), and normal rabbit IgG as a negative control. StaphA cells were then added to precipitate the IgG/protein/DNA complex. StaphA cell pellets were washed extensively followed by elution and reversal of protein-DNA crosslinking. Protein was removed from the DNA lysate using the Qiaquick PCR kit (Qiagen, CA, USA). Standard PCR was performed on the precipitated DNA template using Go Taq® DNA Polymerase (Promega, USA).

2.9. Primer design

The human WT1 promoter sequence was obtained from NCBI (accession # U77682). The following WT1 promoter region primers were designed utilizing Primer3 Input (version 0.4.0), UCSC Genome Browser and Vector NTI advance 10: WT1 promoter primer sequence No. 1 forward primer (CTGAACGGACTCTCCAGTG) and reverse primer (CGCTGCCTTGAACTCCTTAC); and WT1 promoter primer sequence No. 2 consisted of forward primer (GGCCCCTCTTATTTGAGCTT) and reverse primer (CAAGAGGAA GTCCAGGATCG).

2.10. Reporter gene assay

WT1 promoter vectors were a kind gift from Professor Dr. Takashi Murate (Department of Medical Technology, Nagoya University Graduate School of Heath Sciences, Japan). The WT1 promoter sequence, including the WT1 and AP-1-binding site, 301 bp reporter construct was inserted into the pGL3 basic vector. K562 cells were co-transfected with the vector and β-galactosidase for 24 h and then

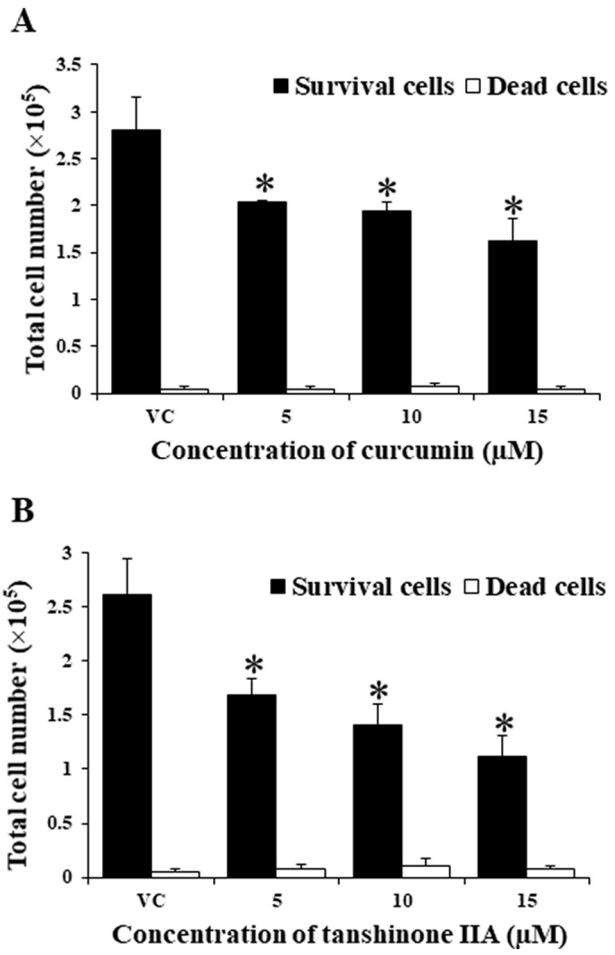

Fig. 2. Dose response effect of curcumin and tanshinone IIA on total cell number. (A) Curcumin and (B) tanshinone IIA decreased the proliferation of K562 cells in a dose-dependent manner after treatment for 24 h. The cell number was examined after treatments using the trypan blue exclusion method. Data are presented as the mean ± SD. *$P < 0.05$ vs. untreated control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treated with 15 μM curcumin, 15 μM tanshinone IIA, or 15 μM SP600125. The co-transfected cells were lysed using lysis buffer. Luciferase activity of the cell lysates was measured using a Dual-Luciferase Reporter Assay Kit (Promega, USA), β-galactosidase Assay Kit (Promega, USA) and a GloMax 96 Microplate Luminometer with dual injections (Promega, USA).

2.11. Generation of stable c-JUN knockdown cell lines by lentiviral transduction

c-JUN and scrambled control short hairpin RNA (shRNA) lentiviral particles were purchased from Santa Cruz Biotechnology. shRNA lentiviral particles were added to the medium of K562 cells in the presence of 10 μg/ml polybrene followed by selection with 10 μg/ml puromycin.

2.12. Statistical analysis

Statistical analysis was performed with SPSS software (version 10.0). Furthermore, all experiments were performed in duplicate and repeated at least three times. The data were expressed as the means ± standard deviation (SD). Statistical differences between the means were tested by one-way ANOVA. The probability values of $P < 0.05$ and $P < 0.001$ were considered significant.

3. Results

3.1. Curcumin and tanshinone IIA induces cytotoxicity and suppresses WT1 in K562 cells

To determine the non-cytotoxic doses for curcumin and tanshinone IIA, dose response curves were performed using K562 cells, and the IC_{50} values were 37.5 μM and > 100 μM, respectively, at 24 h (Fig. 1A and B). The IC_{20} doses, which were calculated at 5–15 μM for the three treatments, were used on all further experiments performed in the present study. However, 15 μM curcumin and 15 μM tanshinone IIA were determined to confirm non-cytotoxicity by an LDH activity assay. The result showed non-cytotoxicity of both curcumin and tanshinone IIA at the concentration of 15 μM on K562 cells when compared to the vehicle control. Idarubicin (2 μM) was used as a positive control (Fig. 1C).

The dose effect of both compounds on WT1 protein expression was tested at non-cytotoxic concentrations (5, 10, and 15 μM) for 24 h. As expected, curcumin significantly decreased WT1 protein levels by 13.3, 22.3, and 53.8%, respectively (Fig. 1C). Tanshinone IIA significantly decreased WT1 protein levels by 17.4, 43.2, and 40.1%, respectively (Fig. 1D). These results revealed that the inhibition of AP-1 binding to DNA leads to a decrease in WT1 protein levels, similar to what was observed with curcumin (WT1 and AP-1 inhibitors). These results suggested that AP-1 may regulate *WT1* gene expression in K562 leukaemic cells. Several reports have shown that curcumin has a repressive effect on AP-1 function and DNA binding. Curcumin simultaneously inhibits phosphorylation and translocation of c-JUN/AP-1 to the nucleus in cultured human promyelocytic leukaemia cells [39].

3.2. Dose response effect of curcumin and tanshinone IIA on total cell number

To determine the effect of curcumin and tanshinone IIA on the viability of K562 cells after treatment for 24 h, cell numbers were assessed. All three treatments decreased cell viability in a dose-dependent manner. Curcumin treatment at 5, 10, and 15 μM for 24 h decreased the total cell number by 27.1, 30.7, and 42.1%, respectively, as compared to control (Fig. 2A). Tanshinone IIA treatment at 5, 10, and 15 μM for 24 h significantly decreased cell numbers by 35.6, 46, and 57.1%, respectively (Fig. 2B). Furthermore, the percentage of dead cells was not significantly different between curcumin and tanshinone IIA treatments (Fig. 2A and B). These findings suggested that non-cytotoxic doses of curcumin and tanshinone IIA decrease WT1 protein expression and lead to decreased cell proliferation in K562 cells.

3.3. Effect of curcumin and tanshinone IIA on WT1 and c-JUN DNA binding to the WT1 gene promoter

To study the effects of curcumin and tanshinone IIA on the DNA binding of c-JUN and WT1 at the *WT1* gene promoter, a chromatin immunoprecipitation (ChIP) assay was employed. One AP-1 consensus sequence, TGAGTGA, was identified at the *WT1* gene promoter region (+144 to +150) relative to the putative transcription start site and next to the previously studied WT1-binding site (Supplementary Fig.) [19]. The effect of curcumin treatment on c-JUN and WT1 DNA binding at this region of the *WT1* gene promoter was tested. Unlike the control, curcumin significantly inhibited both WT1 and c-JUN DNA binding (Fig. 3A and B). Interestingly, Tanshinone IIA treatment reduced both c-JUN and WT1 DNA binding in K562 cells (Fig. 3C and D).

3.4. WT1 promoter reporter activity is repressed by curcumin and tanshinone IIA

Using a gene reporter consisting of a 301 bp region of the WT1 proximal promoter (−), we previously showed that curcumin interrupts

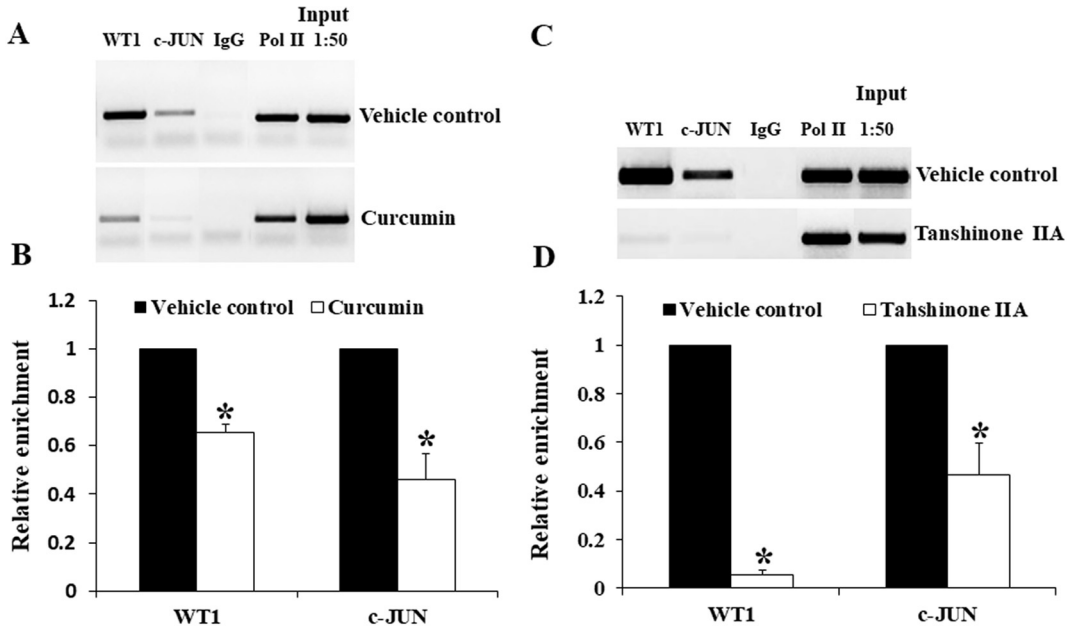

Fig. 3. Curcumin treatment attenuates WT1 and AP-1 binding to the proximal WT1 promoter. K562 cells were treated with (A) 15 μM curcumin and (C) 15 μM tanshinone IIA for 24 h, and ChIPs were performed. Chromatin lysates were immunoprecipitated with antibodies to WT1, AP-1 (c-Jun), Pol II (positive control) or IgG (negative control). ChIP lysates and input (1:50 dilution) were assayed by standard PCR using primers containing the consensus sequence for WT1 and AP-1 located at the WT1 proximal promoter. (B and D) The WT1 and c-JUN immunoprecipitated lysates from curcumin and vehicle control treatments were analysed by SYBR green RT-PCR and graphed as relative DNA enrichment over 1:50 input as percentage of vehicle treatment. Data represent the mean value ± SD of three independent experiments. Asterisks (*) denote values that are significantly different from the vehicle control ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

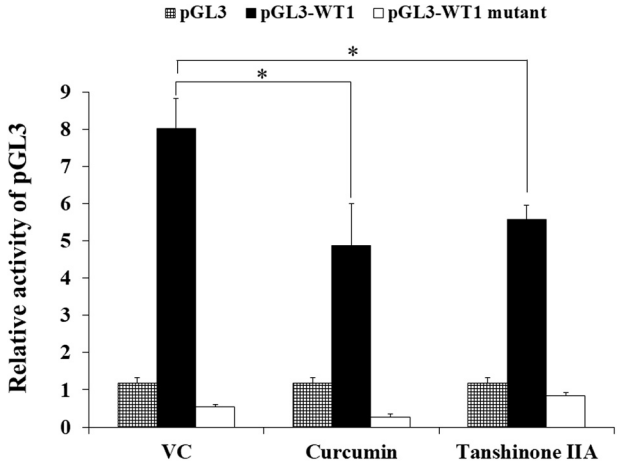

Fig. 4. WT1 promoter reporter activity is repressed by curcumin and tanshinone IIA. K562 cells were transfected with the pGL3_basic luciferase reporter vector containing 301 bp of the WT1 proximal promoter followed by 15 μM curcumin, 15 μM tanshinone IIA or vehicle treatment for 24 h. The firefly luciferase and β-galactosidase activities were assayed, and relative activities were graphed compared to the pGL3 basic vector. Site-directed mutagenesis of the WT1 consensus sequence (−50 to −39) abrogated WT1 promoter activity compared to the wild type WT1 promoter construct (301 bp WT1). Experiments were performed a minimum of three times, and representative graphs are shown. Data represent the mean value ± SD of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($P < 0.05$).

WT1 binding to its own promoter but has no effect on SP1 binding, thus demonstrating specificity of its action [19]. This 301 bp region contains consensus binding sites for both WT1 (−50 to −39) and c-JUN/AP-1 (+144 to +150) (Supplementary Fig.). Because curcumin interrupts c-JUN/AP1 binding to the WT1 proximal promoter (Fig. 3), the contribution of c-JUN/AP1 to WT1 expression was examined by mutating the WT1-binding site located at −50 to −39 [19]. Transfection of the wild type 301 bp promoter construct into K562 cells demonstrated high luciferase activity in vehicle control-treated K562 cells and a diminished response from curcumin- and tanshinone IIA-treated K562 cells (Fig. 4). In contrast, the reporter construct containing the mutated WT1 consensus site (negative control) showed reduced reporter activity compared to the parent vector under all treatment conditions. This result supported the ChIP binding data (Fig. 3) showing that reduction in c-JUN/AP-1 binding to its consensus site at the *WT1* gene promoter inhibits *WT1* gene activation after curcumin treatment. In summary, these data indicated that the curcumin-induced decrease of both WT1 and AP-1 binding contributes to the reduction of *WT1* expression.

3.5. Effect of SP600125 (JNK inhibitor) on cytotoxicity, WT1 protein expression, total cell number, and WT1 promoter reporter activity in K562 cells

To further support the effect of AP-1 on *WT1* gene expression, SP600125 (JNK inhibitor) was used to test the role of c-JUN/AP-1 in this regulation. The cytotoxicity of SP600125 on K562 cells was examined at 24 h in K562 cells using the MTT assay, which indicated an IC_{50} value of 62.5 μM for SP600125. Non-cytotoxic doses (5–20 μM) were then used in subsequent experiments (Fig. 5A). Treatment with SP600125 at 5, 10, and 15 μM for 24 h decreased WT1 protein levels by 7.9, 18.9, and 23.6%, respectively (Fig. 5B), and decreased cell numbers by 33, 38.4, and 41.5%, respectively (Fig. 5C). Thus, these results confirmed that the inhibition of JNK leads to a decrease in WT1 protein expression. Furthermore, the luciferase activity assay showed that SP600125 significantly diminished WT1 promoter activity by 79.1% compared to vehicle control ($P < 0.001$) (Fig. 5D).

3.6. Cell cycle is arrested after curcumin and SP600125 treatments

To further examine the hypothesis that inhibition of the WT1-AP-1 signalling pathway affects K562 cell proliferation by cell cycle arrest,

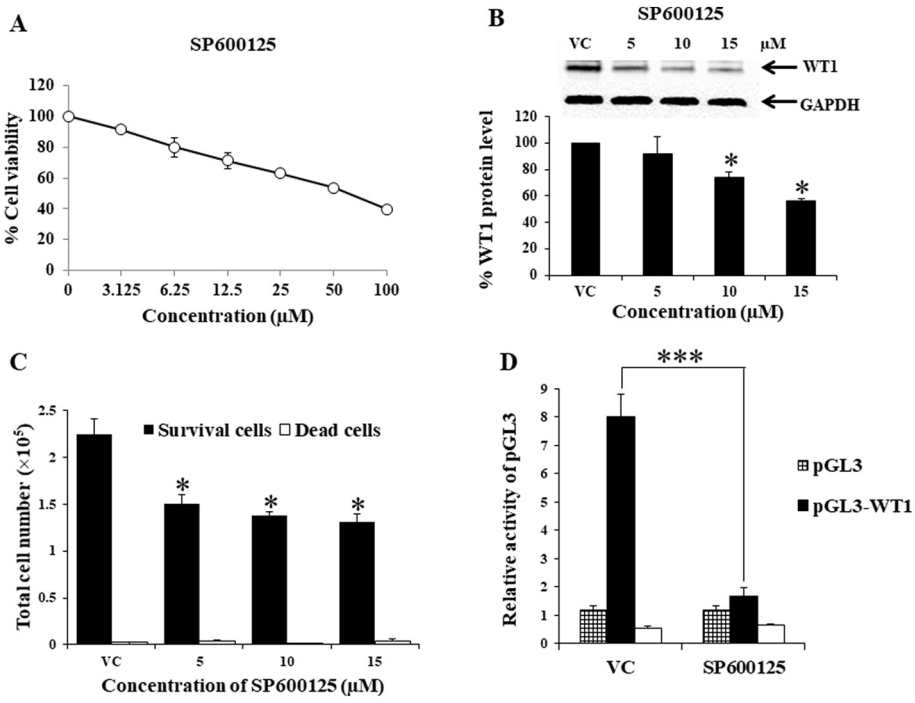

Fig. 5. Effect of SP600125 (JNK inhibitor) on cytotoxicity, WT1 protein expression, total cell number, and WT1 promoter reporter activity. (A) SP600125 cytotoxicity at 0, 3.125, 6.25, 12.5, 50, and 100 μM was examined in vitro by MTT after treating K562 cells for 24 h. (B) Dose response for SP600125 on WT1 protein expression was determined by Western blotting. K562 cells were incubated with SP600125 (5, 10, and 15 μM) for 24 h. GAPDH was used as a loading control. Densitometry of protein levels were graphed as the percentage of vehicle control. (C) SP600125 decreased K562 cell proliferation after 24 h. The cell number was examined after treatments using the trypan blue exclusion method. (D) SP600125 (15 μM) decreased luciferase activity in K562 cells. K562 cells were transfected with the pGL3_basic luciferase reporter vector containing 301 bp of the WT1 proximal promoter followed by treatment with 15 μM SP600125 or vehicle for 24 h. The firefly luciferase and β-galactosidase activities were assayed, and relative activities were graphed compared to the pGL3 basic vector. Experiments were performed a minimum of three times, and representative graphs are shown. Data represent the mean value ± SD of three independent experiments (***$P < 0.001$ vs. vehicle control, one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

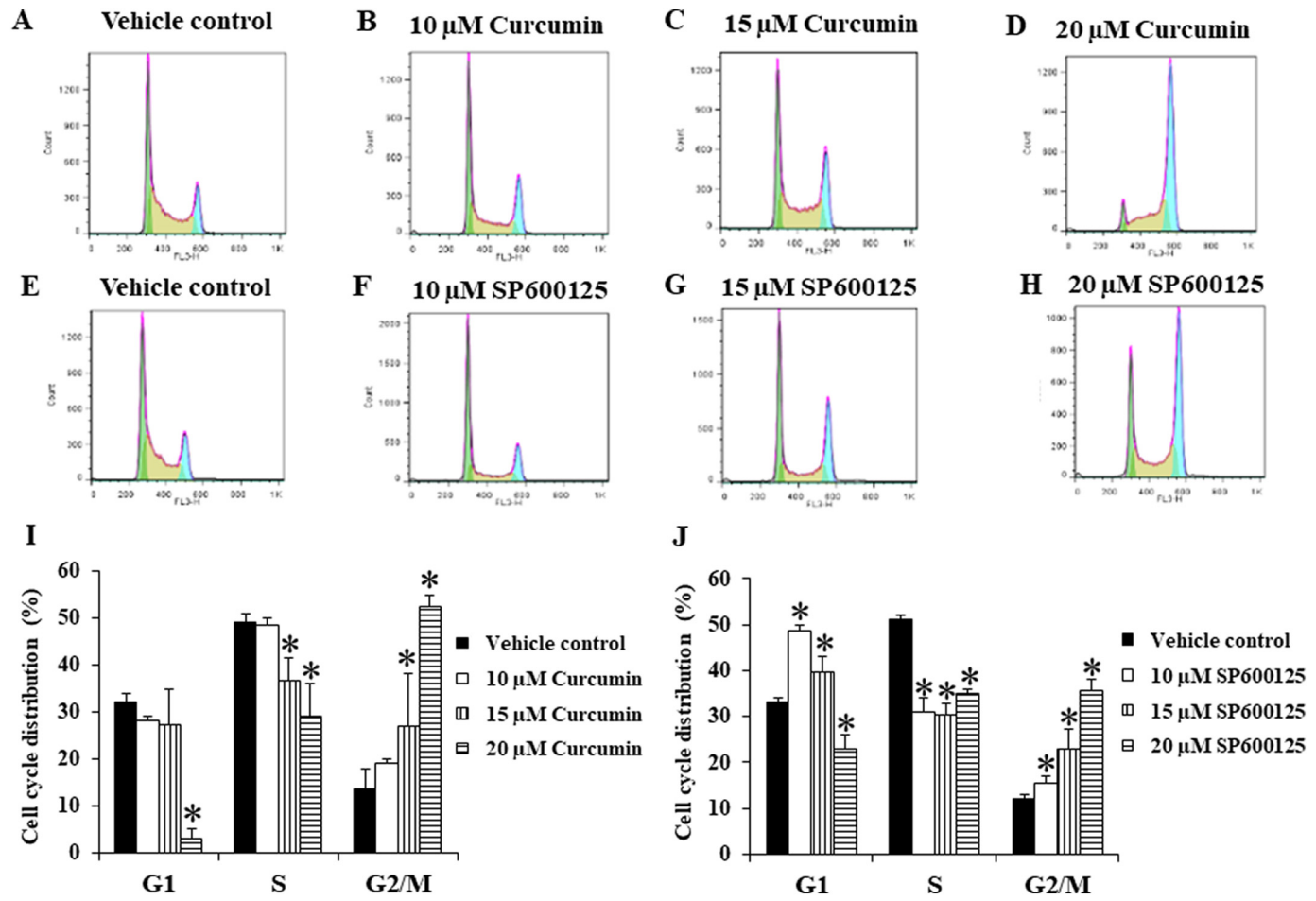

Fig. 6. Cell cycle was arrested after curcumin and SP600125 treatments for 24 h. (A–D) K562 cells (1.0×10^5 cells/ml) were treated with 10, 15, and 20 μM curcumin and (E–D) SP600125 for 24 h. After fixation in 75% ethanol, cells were stained with propidium iodide (PI), and cell cycle was analysed by flow cytometry. (I and J) Graphs present the percentages of cell cycle distribution after curcumin and SP600125 treatments. Data represent the mean value ± SD of three independent experiments (*$P < 0.05$ vs. vehicle control, one-way ANOVA).

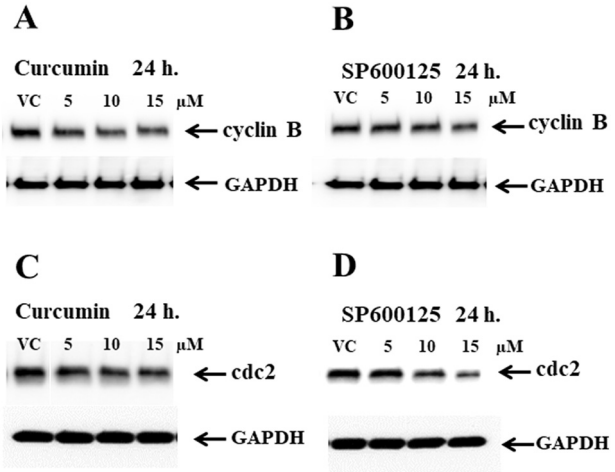

Fig. 7. Effect of curcumin and SP600125 on cyclin B and cdc2 protein expression levels in K562 cells. (A and B) Cyclin B and (C and D) cdc2 were examined as markers of G2/M phase cell cycle arrest. Cells were treated with 5, 10, and 15 μM of (A and C) curcumin and (B and D) SP600125 for 24 h. The protein expression levels of cyclin B and cdc2 in whole protein lysates were determined by immunoblotting.

flow cytometry was performed after curcumin and SP600125 treatments in K562 cells. Fig. 6 shows that the K562 cell population was arrested in the G2/M phase in a dose-dependent manner (10, 15, and 20 μM). The percentage of cell cycle distributions at the G2/M phase after curcumin treatment at 10, 15, and 20 μM significantly increased by 19, 27, and 52.2%, respectively, (Fig. 6B, C, D, and I) compared to the vehicle control (13.7%, Fig. 6A). SP600125 treatments at 10, 15, and 20 μM also increased cell cycle arrest at the G2/M phase in a dose-dependent manner with the cell cycle distribution percentages of 4, 11, and 24%, respectively, (Fig. 6F, G, H, and J) compared to the vehicle control (13.7%, Fig. 6E). As a control for the cell cycle arrest, the protein expression levels of cyclin B and cdc2, markers of the G2/M phase, were measured. Immunoblots showed that cyclin B and cdc2 levels were decreased in a dose-dependent manner (Fig. 7A–D). In summary, the cell cycle distribution measured by flow cytometry showed that K562 cells were arrested at the G2/M phase after the third

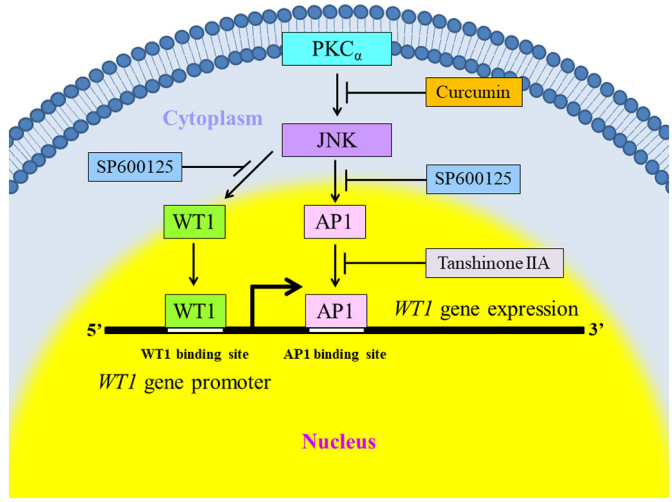

Fig. 9. Schematic of co-activation of WT1 and AP-1 proteins stimulates *WT1* gene expression.

20 μM curcumin treatment. The percentage of K562 cell population in the G2/M phase increased to 52.5%, while cyclin B and cdc2 were decreased.

3.7. Effect of c-JUN knockdown on WT1 protein expression and effect of c-Fos/AP-1 inhibitor (mammea E/BB) on WT1 and c-JUN DNA binding

To further support the effects of c-JUN/AP-1 on *WT1* gene regulation, c-JUN expression was silenced by treating K562 cells with c-JUN lentiviral shRNA. The protein level of c-JUN after silencing was decreased by 44.27% (Fig. 8A) as compared to vehicle control (VC). Interestingly, c-JUN silencing resulted in a significant decrease in WT1 protein expression (Fig. 8A). These results suggested that c-JUN/AP-1 plays a contributory role in the regulation of *WT1* gene expression in leukaemic K562 cells. Moreover, the effect of c-Fos/AP-1 on WT1 and c-JUN binding to the *WT1* gene promoter was examined using mammea E/BB (c-Fos inhibitor) [40]. Mammea E/BB (3.5 μM) inhibits c-Fos by disrupting c-Fos/AP-1 binding to the WT1 promoter [40]. In the present study, mammea E/BB significantly inhibited WT1 and c-JUN DNA

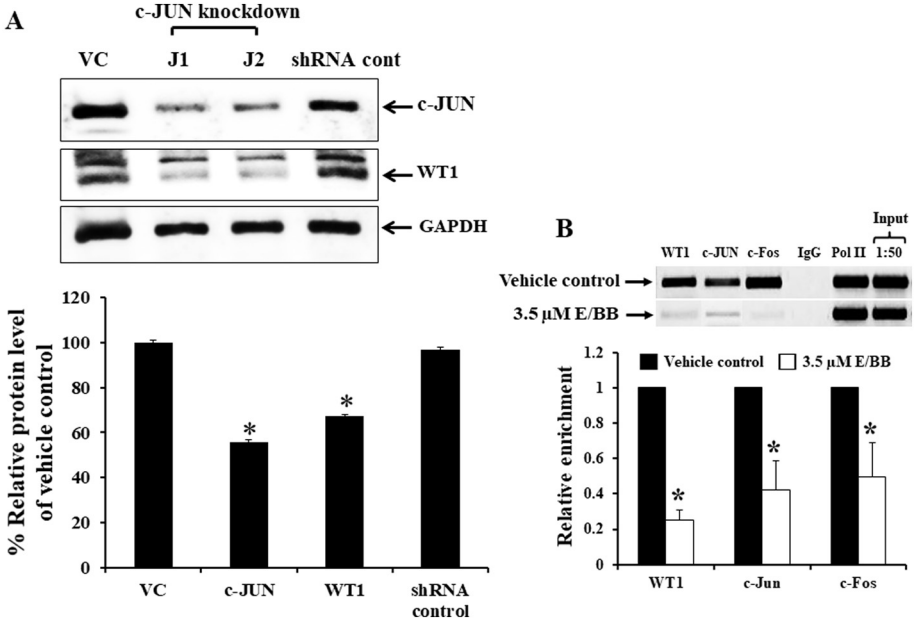

Fig. 8. Effect of c-JUN knockdown on WT1 protein expression and effect of c-Fos inhibitor (mammea E/BB) on WT1- and c-JUN-DNA binding. (A) *c-JUN* gene expression was suppressed by c-JUN shRNA lentiviral treatment of K562 cells. J1 and J2 indicate two independent c-JUN shRNA treatments. Experiments were performed a minimum of three times, and a representative graph is shown. (B) ChIP assay shows attenuated WT1 and AP-1 binding to the proximal WT1 promoter. K562 cells were treated with 3.5 μM mammea E/BB for 72 h, and ChIPs were performed. Chromatin lysates were immunoprecipitated with antibodies to WT1, c-Fos/AP-1, Pol II (positive control) or IgG (negative control). ChIP lysates and 1:50 dilution of input were assayed by standard PCR using primers containing the consensus sequence for WT1 located at the WT1 proximal promoter. The WT1 immunoprecipitated lysates from mammea E/BB or vehicle control treatment were analysed by SYBR green RT-PCR and graphed as relative DNA enrichment over 1:50 input as percentage of vehicle-treatment. Data represent the mean value ± SD of three independent experiments. Asterisks (*) denote values that are significantly different from the vehicle control ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

binding ($P < 0.05$) (Fig. 8B).

4. Discussion

WT1 protein plays critical roles in the normal development of several organs in vertebrates. Previous studies have shown that the *WT1* gene acts as an oncogene in many types of malignant tumours, especially leukaemia [41]. Our previous work showed that PKCα signalling promotes cell proliferation in the K562 leukaemia cell line through activation of the *WT1* gene. We also showed that the repressive effect of curcumin on *WT1* gene expression in the leukaemic K562 cell line is mediated through inhibition of PKCα signalling upstream of the WT1 transcription factor auto-regulatory function [19]. In the present study, we hypothesized that the downstream effects of PKCα/JNK signalling are mediated through effects on AP-1, which in turn transcriptionally impacts *WT1* gene expression.

To further understand the molecular mechanisms by which AP-1 is involved in *WT1* gene expression, inhibitors to block both AP-1 DNA binding (by curcumin and tanshinone IIA) and JNK signalling (SP600125) were used. The effect of tanshinone IIA (AP-1 DNA binding inhibitor) [34] and curcumin (WT1 and AP-1 inhibitors) on DNA binding and transcriptional regulation was tested. While curcumin is known for its ability to suppress cell proliferation, downregulate transcription factors, and inhibit the activity of JNK, protein tyrosine kinases, and protein serine/threonine kinases in a wide variety of tumour cells, the inhibitory effect(s) of curcumin in leukaemic cells remains less defined [42]. Recently, curcumin has been reported to inhibit WT1 in leukaemic K562 and Molt4 cell lines [24,43] as well as primary cells obtained from leukaemia patients [25]. In the present study, curcumin was used to investigate if AP-1 plays a role in mediating *WT1* gene expression in the K562 leukaemic cell line. The biological activities of curcumin include suppressing both WT1 and AP-1 in cell signalling [19,24,39].

In previous studies, we demonstrated that WT1 is suppressed by curcumin via the PKCα and JNK signalling pathway [19]. Other research has shown that curcumin treatment of mouse fibroblast cells inhibits c-JUN/AP-1 binding to its consensus motif, resulting in the inhibition of c-JUN/AP-1-mediated *WT1* gene expression [44]. In addition, curcumin has been reported to block AP-1 and NF-κB binding to GSTP1–1 promoters in K562 cells [45,46]. The present study showed that curcumin and tanshinone IIA inhibit *WT1* gene expression through suppression of both WT1 auto-regulation and c-JUN/AP-1 binding to its cognate consensus site at the proximal *WT1* gene promoter (see model in Fig. 9). Furthermore, after blocking JNK activity with SP600125, it was confirmed that WT1 promoter activity is involved upstream of both WT1 and AP-1. The results suggested and confirmed that WT1 and c-JUN/AP-1 decrease WT1 promoter activity after SP600125 treatment, leading to a decrease in WT1 protein level and total viable cell number.

Previous reports have shown that the *WT1* gene is involved in regulating cell proliferation by promoting the cell cycle. Furthermore, non-cytotoxic doses of curcumin have been reported to decrease leukaemic cell proliferation without activation of cell death pathways [47,48]. Thus, the inhibitory mechanisms of curcumin and SP600125 on the leukaemic cell might be through induction of cell cycle arrest. SP600125 was used to inhibit JNK upstream of both WT1 and c-JUN/AP-1. The present findings suggested that the curcumin and SP600125 suppression of *WT1* gene expression and reduction of cell proliferation are mediated through the induction of cell cycle arrest (Fig. 6 and 7).

WT1 gene expression has been associated with promotion of the cell cycle, and suppression of WT1 expression has been found to induce G2/M arrest in leukaemic cells and G1 arrest in HER2/neu-overexpressing breast cancer cells [47,48]. In addition, c-JUN/AP-1 has been reported to trigger cell cycle progression via the restriction point in leukaemia cells [42]. Therefore, we hypothesize that the inhibitory mechanism of WT1 and c-JUN/AP-1 by SP600125 (JNK inhibitors; upstream of c-JUN/AP-1) and curcumin (WT1 and AP-1 inhibitors) on leukaemic cell proliferation may be due to the induction of cell cycle arrest by inhibition of both WT1 and c-JUN/AP-1.

WT1 auto-regulation through c-JUN/AP-1 promoter regulation and downstream signalling through PKC/JNK pathways were investigated. The c-JUN/AP-1 DNA binding inhibitors, tanshinone IIA and curcumin, decreased both c-JUN/AP-1 and WT1 DNA binding at the *WT1* gene proximal promoter (Fig. 3) and decreased *WT1* gene promoter activity (Fig. 4). This result was confirmed by measuring *WT1* gene promoter activity in the presence of SP600126 (JNK inhibitor). Blocking JNK activity by SP600125 resulted in decreased WT1 promoter activity, WT1 protein expression, and total cell number. Thus, these results suggested that AP-1 binding may be necessary to promote WT1 binding or perhaps augment *WT1* gene autoregulation. Furthermore, c-JUN knockdown significantly decreased WT1 protein expression. Thus, these findings indicated that c-JUN/AP-1 plays a role in the regulation of *WT1* gene expression in leukaemic K562 cells.

AP-1 is generally found to be in a c-JUN and c-Fos heterodimer. In the present study, c-Fos was disrupted by mammea E/BB (a c-Fos inhibitor), which results in an inhibitory effect on WT1 protein expression. Mammea E/BB suppresses WT1 and c-FosDNA binding at the *WT1* gene promoter [40]. After c-Fos was disrupted by mammea E/BB, the binding of WT1 and c-JUN to the *WT1* gene promoter was decreased in the present study, indicating that blocking either c-JUN or c-Fos activities attenuates *WT1* gene expression. Further experiments are necessary to explore the underlying mechanism and if a co-regulatory relationship exists between the two transcription factors.

In conclusion, the present results indicated that co-activation of c-JUN/AP-1 and WT1 proteins downstream of the JNK signalling pathway results in the regulation of *WT1* gene expression and cell proliferation in K562 leukaemic cells.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

Disclosure statement

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.11.001.

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