CHAPTER IV

DISCUSSION

Expression of the *Wilms' tumor1* (*WT1*) gene product has been shown to increase 1,000-10,000 fold in leukemic cells (5, 38), suggesting that the *WT1* gene may play an important role in oncogenesis. Therefore, the overexpression of *WT1* gene and WT1 protein may serve as a possible biological marker for monitoring leukemia. In anticancer drug research, dietary and/or medicinal plants, such as ginger, garlic, chili, pepper, and turmeric are becoming popular as chemotherapeutic and/or chemopreventative nutracenticals. Curcumin is the main active ingredient of turmeric, a traditional herbal medicine and food of Southern Asia. It has been reported to affect multiple cell signaling pathways (274), and recent studies indicate that curcumin inhibits WT1 expression in various leukemic cell lines (8).

Curcumin also has numerous other biological properties, such as antioxidant, anti-inflammatory, anti-mutagen and anticancer properties. Additionally, curcumin inhibits oncogene expression and activation of PKC upon induction by TPA (198). Tumor promoter (example, TPA) activates PKC by reacting with zinc-thiolates present within the regulatory domain of PKC. In contrast, the oxidized forms of some cancer-preventive agents, such as polyphenolic- (curcumin, ellagic acid, and 4-hydroxytamoxifen) and seleno-compounds, can inactivate PKC by oxidizing the vicinal thiols present within the catalytic domain. This creates an efficient counteractive mechanism to block the signal transduction induced by tumor promoters

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at the first step (275). Curcumin exhibits anticancer activities both *in vitro* and *in vivo* through a variety of mechanisms. It inhibited cell proliferation and induced cell apoptosis in several cancer cell types *in vitro*. These included bladder, breast, lung, pancreas, prostate, cervix, head and neck, ovary, kidney, brain, blood, and skin cancer cells (140, 276). Studies of curcumin have evaluated its use *in vivo* for cancer prevention and therapy (276-278). The inhibitory effects of curcumin have a wide variety of gene defects that require transcription factors such as Activation protein 1 (AP1) and Nuclear Factor kappa B (NF-κB) (279) 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 the activated PKC which phosphorylates the C-terminal domain of WT1, regulating the proliferation in leukemic cells (280).

The activity of pure curcumin in K562 cells was examined by determining the inhibitory concentration at 20 and 50% growth (IC_{20} and IC_{50}) values. The results were in line with the previous reports of Anuchapreeda *et al.* (2008) (8). The IC_{20} and IC_{50} values of this experiment were 16.5 and 31.8 μM (approximately 6.07 and 11.7 μg/mL), respectively. Pure curcumin, a derivative of curcuminoids, was previously shown to suppress WT1 transcription mRNA and protein levels in several human leukemic cell lines, although the mechanisms were not explored (6, 8). This study confirmed the inhibitory effects of pure curcumin on WT1 (8). Pure curcumin showed the ability to down-regulate the *WT1* gene expression in a dose- and time-dependent manner in K562 cells.

The overexpression of WT1 in leukemic cells has been linked to cell proliferation. However, the specific WT1 isoform which has this effect has not yet

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been reported. The overexpression of four exogenous WT1 isoforms in K562 cells and stable clones of transfected U397 cells has been shown to reverse the cytotoxic activity of pure curcumin. Importantly, WT1 +/+ was the main isoform involved in reversing the inhibitory effects of pure curcumin. These findings provide key insight into the growth-inhibitory mechanisms of pure curcumin *via* WT1 +/+ isoform and also demonstrate the transcriptional redundancy of WT1 isoforms and their regulatory function in cell proliferation.

Interestingly, the WT1 isoforms that are suppressed by pure curcumin were further investigated in a stably transfected U937 cell line. Recently, pure curcumin has been shown to decrease WT1 mRNA and WT1 protein levels in human leukemic cell lines (8). There are four WT1 isoforms, WT1 +/+, +/-, -/+, -/-. However, the specific WT1 isoform that is inhibited by pure curcumin in leukemic cells is still unknown. This experiment constructed stably transfected U937 cells expressing each of the WT1 isoforms to use as experimental models because U937 cells themselves express modest WT1 protein levels (8, 281). In this study, pure curcumin was cytotoxic toward wild type, pcDNA3.1 control, and all WT1 isoform-transfected U937 cells. However, overexpression of WT1 isoforms could shift the IC_{20} and IC_{50} values such that U937 cells overexpressing WT1 isoforms were less sensitive to the cytotoxic effects of curcumin. This could have promising implications for the clinical use of pure curcumin. When non-cytotoxic doses (IC_{20} values) of pure curcumin were used, WT1 +/+ isoform-transfected cells clearly showed decreased levels of WT1 mRNA and WT1 protein, demonstrating that the WT1+/+ isoform of WT1 is specifically suppressed by pure curcumin.

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However, the suppression of exogenous WT1 +/+ protein in transfected U937 cells by pure curcumin depended on the protein degradation pathway. Furthermore, this study showed that pure curcumin did not affect the expression of four different reporter proteins (GFP, p95 ErbB2, ErbB3, and myc-tagged Lrig1) put under the control of the same pCMV promoter, suggesting the specificity of the action of pure curcumin 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, this study also showed that the decreased stability of the WT1 protein relates to pure curcumin treatment. The experiments using the PKC inhibitor GF109203x revealed that inhibition of PKC, and in turn PKC-mediated phosphorylation of WT1, leads to a suppression of WT1 +/+ protein expression. These results suggest that PKC signaling in WT1 +/+ transfected U937 cells is important for the maintenance of WT1 expression. The inhibition of PKC demonstrates destabilization of the WT1 protein. This study proposes that pure curcumin suppresses WT1 protein level through inhibition of PKC at the post-translational level there by destabilizing the protein.

The downregulation of endogenous *WT1* gene expression by pure curcumin was determined in this study. The results showed that endogenous WT1 was similarly suppressed by pure curcumin. To examine whether pure curcumin might suppress endogenous WT1 through a protein degradation pathway, WT1 half-life was examined using the protein synthesis inhibitor cycloheximide (CHX). The half-life of WT1 protein after treating the cells with 0.02% DMSO (vehicle control) was not significantly different compared to cells treated with 15 μM (approximately 5.5 μg/mL)

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pure curcumin. In addition, the proteasome inhibitor MG132 was used for further probe the effect of pure curcumin on WT1. WT1 protein expression was decreased in parallel with pure curcumin treatment in the presence and absence of MG132 or EGCG or lactacystin. This result indicates that the pure curcumin-mediated downregulation of endogenous WT1 protein was not through proteasomal degradation pathways. Since pure curcumin has been shown to decrease WT1 mRNA levels (6), the experiment was performed to determine whether WT1 mRNA stability was affected. Therefore, actinomycin D was used to inhibit the transcription process. The stability of WT1 mRNA was unchanged between vehicle control and pure curcumin-treated K562 cells. Taken together, these results demonstrate that the downregulation of *WT1* gene expression by pure curcumin was independent of protein or mRNA degradation pathways.

However, several previous studies have shown that curcumin affects multiple signaling pathways, including protein kinase C (274, 282-284). Preliminary data from phosphokinase arrays (R&D system, MN, USA) probed with K562 lysates indicated decreased phospho-c-Jun and phosho-JNKpan (PKC targets) from pure curcumin-treated cells, compared to the vehicle control. Then this study showed that the inhibitory effect of pure curcumin on *WT1* gene expression is mediated through the PKC signaling pathway. Treatment of K562 cells with GF109203x (PKC inhibitor) downregulated endogenous WT1 expression. The result mimicked the activity of pure curcumin on *WT1* gene expression. In addition, chronic activation of PKC by expression of Myr.PKCα demonstrated that PKCα activation rescued the pure curcumin inhibitory effect on WT1 expression. However, it has been reported that curcumin and its derivatives bind to the C1B subdomains of novel PKC, including δ,

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ε, η, and θ isoenzymes. It was shown that curcumin and its long-chain derivatives can bind to the activator binding domain of PKC by forming hydrogen bonds with the residues at the activator binding site. It was also indicated that curcumin and its derivatives can influence PKC activation and its membrane translocation properties differently, depending on the nature of the PKC subtype. In contrast to phorbol esters, which are tumorogenic, curcumin is non-toxic and has anti-cancer properties (285).

Indeed, the PKC signaling pathway could possibly be upstream of the JNK pathway, because PKC can induce JNK activity by phosphorylation (286, 287). In addition, PKC inhibitor GF109203x treatment blocks TPA-induced ERKs and JNKs protein phosphorylation, which indicates that activation of PKC locates upstream of MAPKs activation in TPA-treated HL-60 cells. TPA's protection against Baicalein (5, 6, 7-trihydroxyflavone; BE)-induced cell death is by the induction of JNK protein phosphorylation, which is suppressed by the JNK inhibitor, SP600125 (287). Moreover, there have been several studies showing PKC-dependency of Phosphatidylinositol 3-kinase (PI3K) and MAPK activation (288-290). Recently, Leitges *et al.* 2002 (291) disclosed that insulin activates atypical PKCs largely *via* PI3K-dependent increases in Phosphatidylinositol (4,4,5)-triphosphate (PIP3), which enhances PDK-1-dependent loop phosphorylation and subsequent autophosphorylation, and relieves pseudosubstrate-dependent autoinhibition in atypical PKCs (292). The results suggest that PKC and PI3K are crucial, but that extracellular signal-regulated kinase (ERK), also known as mitogen-activated protein kinase (MAPK), is less important for the mitogenic activity of CSB (293). Additionally, Chen *et al.* (1999) suggested that curcumin suppressed chemical carcinogenesis through inhibition of

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JNK (195), a kinase needed for AP1 activation. Hahm *et al.* (2002) demonstrated this through inhibition of the fos-jun-DNA complex as well (196). Then, the experiment investigated the upstream and downstream steps of the PKC signaling cascade to WT1 downregulation. Specific kinase inhibitors were used to determine the signaling cascade. The SP600125, LY294002, and U0126 are JNK, PI3K, and MEK inhibitors, respectively. The results showed that three inhibitors decreased WT1 mRNA expression, and that PI3K is upstream, while JNK and c-Jun are downstream of the PKC signaling cascade. Finally, these signaling molecules are presented upstream of the WT1 transcription factor, as indicated in Figure 52.

This study also showed that pure curcumin treatment with the concentration at IC_{20} value could enhance phospho-Chk2 kinase protein in K562 cells by human phospho-kinase array kit, thus the mechanism might be involved in cell cycle arrest. While the phospho-HSP27 kinase protein was decreased, it can be suggested that it did not relate to cell apoptotic signal cascade in this condition. High levels of HSP27 have been reported to inverse relation with cell proliferation, metastasis, and resistance to chemotherapy in breast cancer (294, 295). Moreover, it has also been shown that phospho-HSP27 is phosphorylated by PKC (296). The variability of phospho-HSP27 was suggested to have a biological significance and that it represented the variable expression or activity of PKC (297). The decrease of phospho-HSP27 by 15 μM pure curucmin was related to the dowonregulation of PKC activation that is involved in tumor cell proliferation.

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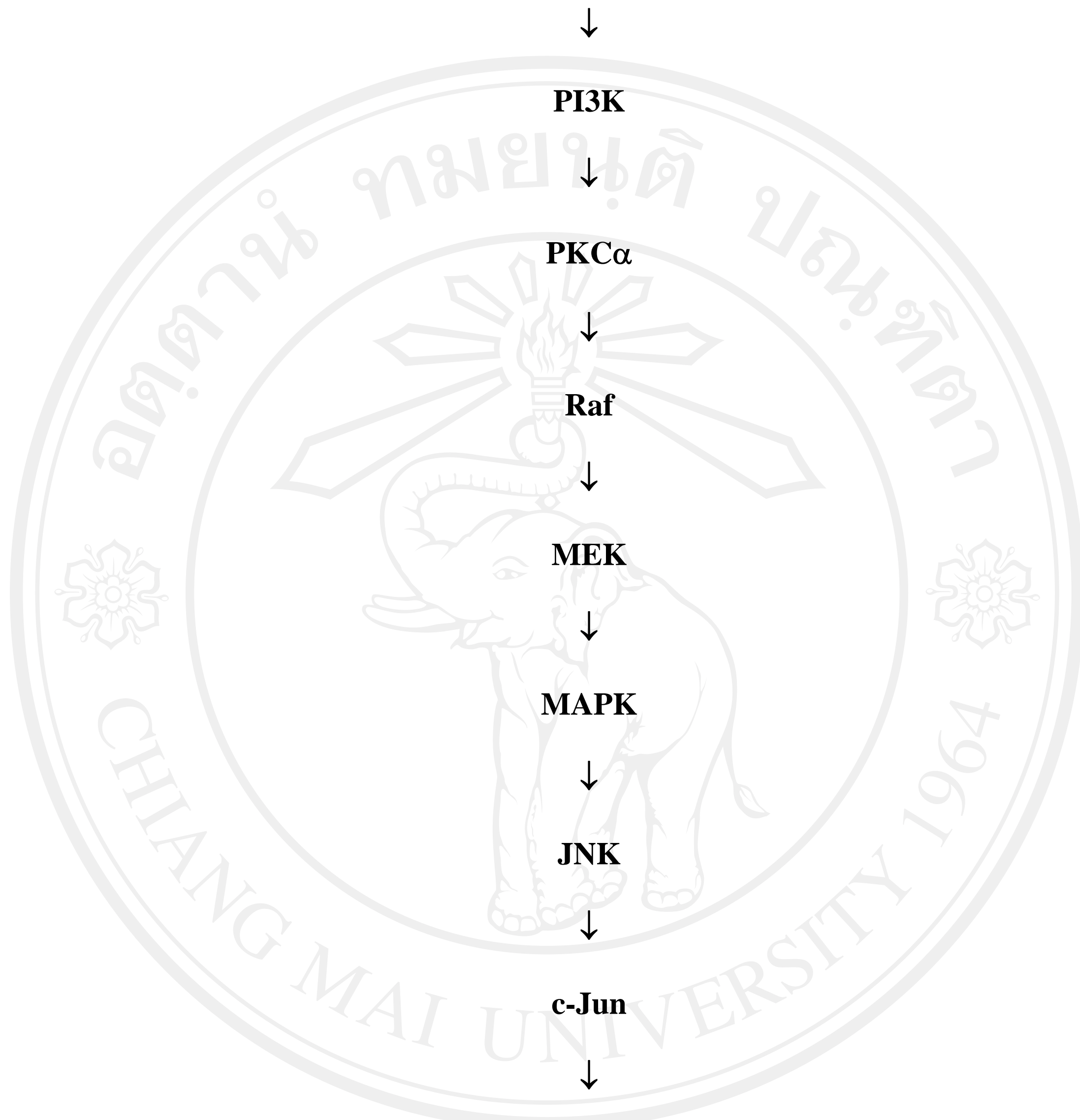

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Figure 52 Signaling cascade diagram proposing a relation to the WT1 transcription factor activity.

Ye *et al.* (1996) has demonstrated the role of phosphorylation in the regulation of DNA binding activity and the nuclear translocation of WT1 (280). Two

recombinant WT1 proteins containing the DNA binding domain with or without a three-amino (KTS) insertion (WT1ZF+KTS and WT1ZF-KTS) were strongly phosphoylated by protein kinase A (PKA) and PKC *in vitro*. Both PKA and PKC phosphorylation inhibited the ability of WT1ZF+KTS or WT1ZF-KTS to bind to a sequence derived from the WT1 promoter region in the gel mobility shift assays.

Most of the potential PKA and PKC phosphorylation sites within the DNA binding domain are highly conserved among WT1 proteins from different organisms (16, 17, 298, 299). Some of them are also conserved within the related proteins of the epidermal growth factor receptor (EGFR) family (300-304). Based on the crystal structure of the EGR1 protein, two of the serine residues, ser365 and ser393 located in zinc finger 2 and 3, respectively, are adjacent to two of the arginine residues involved in making contact with the guanine residues of the DNA binding site. In addition, at least one of the serine residues (Ser365) that is a potential target for phosphorylation by PKA, PKC, and CKII may also be involved in contacting the DNA backbone. A mutation affecting this same Ser residue has been reported in a human leukemia cell line, CEM. WT1-DNA binding activity studies have revealed that specific binding activities of this mutant protein were severely impaired (305).

This study also revealed that pure curcumin affected the interaction between WT1 transcription factor and *WT1* gene promoter, which led to downregulated *WT1* gene expression. The WT1 promoter has been extensively analysed (270) and shown to auto-regulate itself by the WT1 transcription factor (116) and by other transcription factors, including GATA1 (272, 306) and Sp1 (307). It also regulates the immune suppressant cytokine *interleukin-10* (*IL-10*) gene (308). K562 cells were treated with various doses of pure curcumin for 24 h and immunoblotted for WT1 and Sp1 protein

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levels. Pure curcumin at the concentrations of 10 and 15 μM showed down-regulation of WT1 protein levels. Then the effect of pure curcumin on the interaction between WT1 and Sp1 transcription factors and the WT1 proximal promoter was investigated by chromatin immunoprecipitation (ChIP) assay. The results clearly showed that pure curcumin could prevent the binding of WT1 transcription factor to the region containing a WT1 consensus sequence in the proximal promoter, as shown by standard PCR and ChIP qPCR. However, the pure curcumin treatment had no effect on the binding of Sp1 to the WT1 consensus sequence. This suggests that pure curcumin depletes WT1 through a loss of auto-regulatory function. These results also demonstrated that pure curcumin simultaneously impacted WT1 protein levels and WT1 protein-DNA interactions (Figure 35).

The relationship between PKC and WT1 proteins in the signal cascade was investigated using PKC inhibitor to determine whether the downregulation of *WT1* gene by pure curcumin involved the PKC signaling cascade. Both pure curcumin and PKC inhibitor treatment decreased WT1 expression. Overexpression of Myr.PKCα in transfected K562 cells increased WT1 protein levels and rescued the inhibitory effect of pure curcumin on WT1 expression. Protein-DNA binding is commonly regulated by phosphorylation of protein transcription factors. Regarding WT1 regulation, it has been reported that PKC phosphorylates WT1 protein at one of several potential sites, inhibiting DNA binding *in vitro* (280). These results suggested that PKCα phosphorylation may also enhance WT1-DNA binding. Interestingly, PKCα and pure curcumin have been shown to physically interact (283).

The results of the WT1 ChIP assay demonstrated that pure curcumin abrogated WT1 transcription factor binding to the WT1 proximal promoter. To

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further evaluate the effect of pure curcumin on the WT1 promoter auto-regulation, a luciferase reporter construct for the minimal proximal WT1 promoter sequence required for maximum response in K562 leukemic cells was used in this experiment. There are several potential WT1 and Sp1 consensus sites within the WT1 proximal promoter (270). The WT1 (-50 to -39) and Sp1 (-224 to -203) consensus binding sites included within the 301 bp reporter construct are indicated (Figure 33). Transfection of the 301 bp constructed into K562 cells demonstrated high luciferase activity with vehicle control-treated cells and a diminished response from pure curcumin-treated K562 cells. This experiment did not utilize an internal control for normalization of transfection efficiency; however a large number of independent experiments were conducted with similar results (n=7). Vidovic *et al.* (2010) has reported that WT1 systematically increases the expression of both SV40 (simian virus40) and TK (thymidine kinase)-driven renilla expression (309), suggesting that renilla luciferase is not a good internal control. To address that, this experiment was performed using β-galactosidase as an internal control for normalization. The results showed the same pattern as the results using the renilla for co-transfection. Thus, pure curcumin still abrogates WT1 promoter activity that is regulated by WT1 transcription factor.

Furthermore, this experiment also determined whether the luciferase activity was driven by WT1 protein binding to the proximal promoter. To examine this, the WT1 binding site located at -50 to -39 was mutated. Importantly, the luciferase construct containing the mutated WT1 binding site in the proximal WT1 promoter was completely unresponsive when transfected into K562 cells suggesting WT1 binding to the proximal promoter was driving firefly luciferase expression. The WT1 binding site included within the luciferase reporter construct and the ChIP-qPCR

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amplified region suggests that the inhibitory effect of pure curcumin on WT1 auto-regulation is mediated through the upstream WT1 binding site located at position -50 to -39 in the WT1 proximal promoter.

Finally, this study suggested that the signaling pathway of the inhibitory mechanism of pure curcumin on the endogenous *WT1* gene expression in K562 cell line clearly associated with PKCα, JNK, and c-Jun signaling pathway upstream of the WT1 transcription factor. As a result, it affects both WT1 protein-promoter binding and WT1 promoter function and leads to decreased WT1 mRNA and protein levels in K562 cells. However, the signal cascades between c-Jun-related AP1 and WT1 are not clear in this study; it should be further explored to reveal the complete signaling pathway of WT1. Moreover, this study also demonstrated the inhibitory mechanism of pure curcumin on exogenous WT1 protein expression involved in a protein degradation pathway *via* PKCα signaling cascade that was presented in schematic model (Figure 53). Interestingly, the inhibitory mechanisms of pure curucmin on exogenous WT1 +/+ protein were different from that of endogenous WT1, since the overexpresion of WT1 isoforms in U937 cells was auto-driven by pCMV promoter but not by the U937 cell itself that expresses low level of WT1 protein (281). It might produce unstable exogenous WT1 proteins in the WT1-transfected U937 cells after pure curcumin treatment due to the incomplete post-translational modification process since this process is important for protein function and stability (310, 311). These observations strongly suggest that exogenous and endogenous WT1 are regulated by pure curcumin through different mechanisms. Endogenous WT1 regulation involves transcriptional suppression while exogenous WT1 regulation involves protein degradation.

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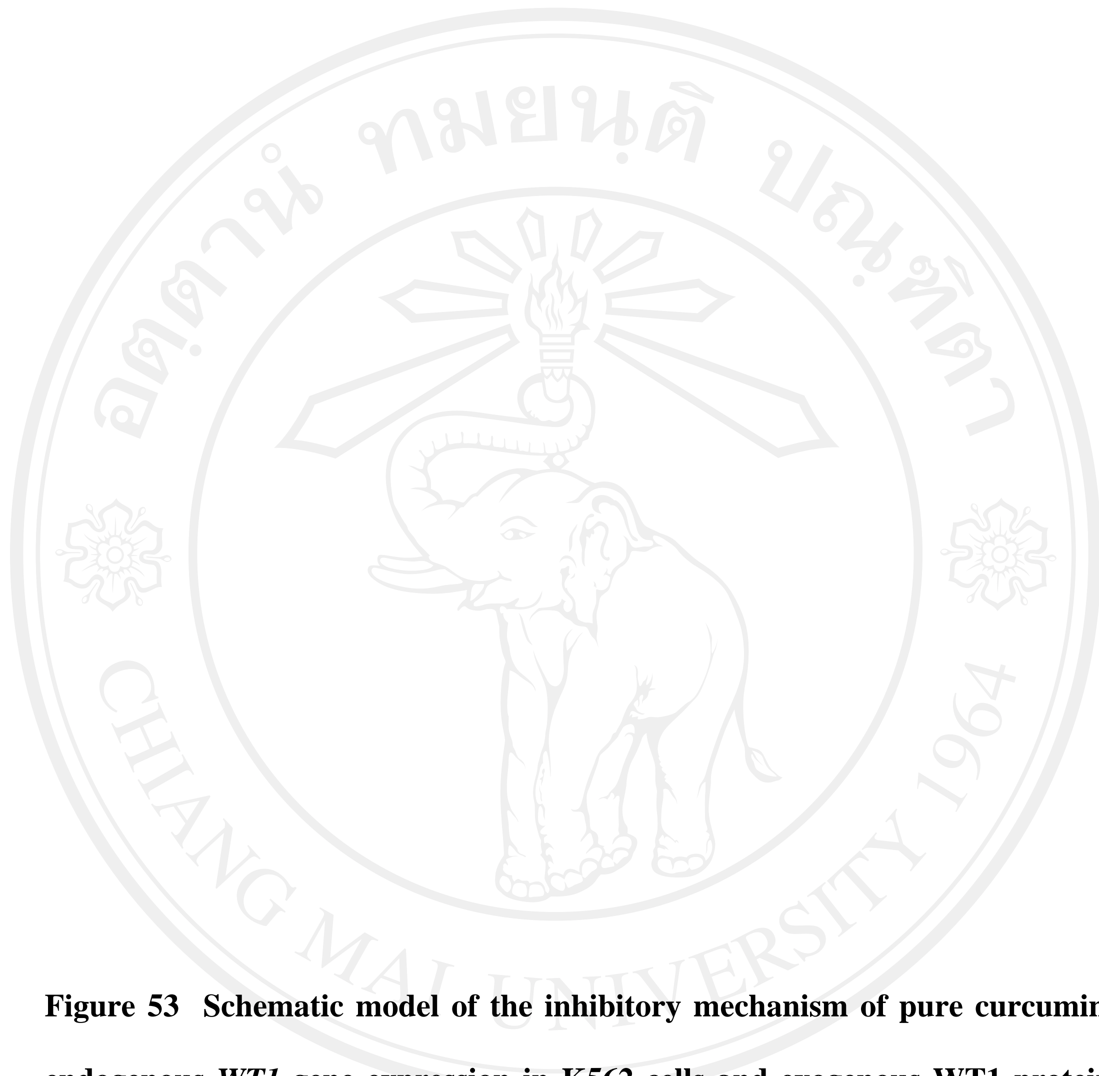

Figure 53 Schematic model of the inhibitory mechanism of pure curcumin on endogenous *WT1* gene expression in K562 cells and exogenous WT1 protein in transfected U937 cells.

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