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Chrysin inhibited stem cell factor (SCF)/c-Kit complex-induced cell proliferation in human myeloid leukemia cells

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ABSTRACT

Stem cell factor (SCF) has important roles in the proliferation and differentiation of hematopoietic stem cells. The complex of c-Kit and its ligand SCF induce hematopoiesis, melanogenesis, and gametogenesis. However, the mechanism by which SCF induces cell proliferation in the human megakaryoblastic leukemia cell line, MO7e, and the signaling molecules involved, especially in downstream signaling of c-Kit, remain unclear. Here, we show that pharmacological inhibition of the PI3K pathway inhibits SCF/c-Kit signaling and cell proliferation. In addition, we find that the Shc/PDK1/PKC/Akt/c-raf signaling cascade is essential for SCF/c-Kit signal pathway. Our results also suggest that ERK5 is activated and translocated to the nucleus, activating CREB and STAT3. Interestingly, chrysin shuts down the SCF/c-Kit complex-induced signaling cascade. Taken together, these studies give additional insight into the molecular mechanism of SCF/c-Kit-induced cell proliferation and its inverse agonist, chrysin. Finally, these findings enhance our understanding of MO7e cell proliferation.

1. Introduction

c-Kit is a receptor tyrosine kinase from a type III RTK subfamily which also includes receptors for platelet-derived growth factor (PDGF), colony-stimulating factor 1 (CSF-1), and flt-3 ligand. Moreover, c-Kit plays key roles in haemopoiesis, mast cell function, melanogenesis, fertility, and gut motility [1–3]. The c-Kit/SCF complex is essential for cell proliferation, survival, differentiation, and functional activation in a variety of cells [3,4]. Upon binding of the c-Kit ligand, SCF, the receptors homodimerize, activating the intrinsic tyrosine kinase and inducing autophosphorylation which generates binding sites for SH2 domain-containing proteins and activates the intracellular signaling cascade [5,6].

Recently, several research teams have analyzed c-Kit signaling in physiologically relevant, factor-dependent myeloid cell lines using systems where the cellular response can be strictly related to the presence of the introduced receptor [7,8]. SCF-induced c-Kit signaling functions through at least four intracellular pathways, including phosphatidylinositol-3-kinase (PI3K), the Janus family of protein tyrosine kinases (Jak), the Src family members, and the Ras-Raf-mitogen-activated protein (MAP) kinase cascade. These pathways mediate a number of cellular processes, including gene

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transcription, proliferation, differentiation, survival, and metabolic homeostasis [9–11]. Little is known about how ERK5, part of the MAP kinase cascade, functions in SCF-induced signaling. A recent report indicated that ERK5 plays an important role [12], but no comprehensive investigation has been done. In addition, there is no report regarding transcription factors in the SCF/c-Kit signaling cascade. Moreover, several reports show that the anti-angiogenic protein kinase inhibitors, SU5416 and SU6668, and a c-Kit inhibitor, Gleevec (STI-571), attenuate c-Kit phosphorylation and inhibit SCF-induced survival, migration, and tube formation in the human myeloid leukemia cell line MO7e and in HUVEC cells [13,14]. Chrysin, interestingly, has the ability to abolish SCF/c-Kit signaling. Chrysin (5,7-dihydroxyflavone) is a natural flavonoid contained in many plant extracts, honey, and propolis [15]. Kwon and co-workers have reported that chrysin engages in multiple biological activities, such as anti-inflammation, anti-cancer, and anti-oxidation effects [16]. In particular, flavonoids including chrysin have shown anti-cancer activity in leukemia cells [17–19]. However, the cellular and molecular mechanisms underlying chrysin-induced inhibition of SCF/c-Kit signaling in leukemia cells are not clear.

We now report detailed studies of intracellular signaling from the SCF/c-Kit complex to the PI3K/PKC/ERK5/CREB cascade. These signaling events are essential for SCF/c-Kit complex-induced cell proliferation. In addition, chrysin can attenuate this signaling cascade and cell proliferation. These results provide new insights into the proliferation of myeloid leukemia cells and may generate novel targets for therapeutic drug development.

2. Materials and methods

2.1. Materials

Chrysin was purchased from Sigma (St. Louis, MO) and other compounds were purchased from CalBiochem (EMD; San Diego, CA). The purity of Chrysin is about ≥96.0% (HPLC) (catalog No. #27214). We made it stock concentration of 10 mM in DMSO and diluted 2000 folds in incubation media (final concentration of DMSO: 0.05%). Thus, the final concentration of 3% undefined component(s) in chrysin compound accounts less than 1.5×10^{-5}% of the final culture volume, which may not affect physiological behavior. Human stem cell factor, anti-p85 antibody, anti-phospho-Tyr (pTyr100) antibody, anti-phospho-Shc (Tyr317) antibody, anti-phospho PDK1 (Ser241) antibody, anti-phospho-PKC (pan) (gamma Thr514) antibody, anti-phospho-Akt (Ser473) antibody, anti-phospho-c-Raf (Ser338) antibody, anti-phospho-MEK1/2 (Ser221) antibody, anti-phospho-ERK1/2 (Thr202/Tyr204) antibody, anti-phospho-ERK5 (Thr218/Tyr220), anti-phospho-CREB (Ser133) antibody, anti-phospho-STAT3 (Ser727) antibody, anti-phospho-c-Kit (Tyr719) antibody, and anti-β-actin antibody were purchased from Cell Signaling (Danvers, MA).

2.2. Cell lines

The human myeloid leukemia cell line, MO7e, obtained from Dr. Y. Lee (ChungBuk National University, Chungju, Korea), was cultured in RPMI 1640 medium with 20% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 ng/mL human granulocyte-macrophage colony-stimulating factor (GM-CSF). Before stimulation with stem cell factor (SCF), cells were factor starved for 16–18 h in RPMI 1640 supplemented with 0.5% bovine serum albumin (BSA). For intracellular signaling studies, serum and GM-CSF-starved cells were incubated with SCF. When inhibitors were used, cells were preincubated for one hour at 37 °C before SCF stimulation.

2.3. Western blotting

MO7e cells were grown in six-well plates. After SCF stimulation, cells were lysed with 2× lysis buffer (250 mM Tris–Cl pH 6.5, 2% SDS, 4% mercaptoethanol, 0.02% bromophenol blue, and 10% glycerol). Equal amounts of whole cell lysates were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk in Tris-buffered saline (50 mM Tris–Cl, pH 7.5, and 150 mM NaCl) for 30 min at room temperature, then incubated overnight with primary antibody in TTBS (0.5% Tween 20 in Tris-buffered saline). After washing with TTBS, the blot was further incubated for 45 min at room temperature with anti-rabbit or anti-mouse antibody (Cell Signaling) in TTBS, and visualized using the ECL system (Amersham Biosciences, Piscataway, NJ) and Las 3000 (Fuji, Japan).

2.4. Cell fractionation

MO7e cells were cultured in six-well plates. Cells were rinsed with ice-cold PBS and harvested with cell lysis buffer (50 mM Tris–Cl pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, and 0.5% NP-40). After incubation on ice for 30 min, samples were sonicated and centrifuged at 12,000 × *g* for 15 min at 4 °C. Supernatants were collected (cytosolic fraction). Nuclear pellets were resuspended in nuclear buffer (20 mM Tris–Cl pH 7.4, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT), vortexed, and then centrifuged. Supernatants were collected (nuclear fraction) and the protein concentration of each sample was measured.

2.5. Immunoprecipitation for PI3K

Cells were harvested by scraping into lysis buffer [25 mM HEPES, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, and protease inhibitor tablet-Complete Mini (Roche, Basel, Switzerland)] followed by sonication at 4 °C (four times for 5 s each) and centrifugation at 15,000 × *g* for 15 min. Supernatants from cell sonicates were used for immunoprecipitation. Protein concentrations were determined by Coomassie Plus™ protein assay (Pierce, Rockford, IL). Lysate samples (230 μL) were pre-cleared by rotating incubation with Gammabind G-Sepharose for 30 min at 4 °C. Following centrifugation, anti-p85 antibody was added to pre-cleared lysates and incubated overnight at 4 °C. The beads were pelleted and washed extensively with cell lysis buffer. Bound proteins were dissociated by boiling the samples in PAGE sample buffer, and whole samples were separated on SDS-PAGE. For the reprobing of PDVF membranes, the blots were

stripped by incubation in reprobe buffer (62.5 mM Tris–Cl pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol) at 55 °C for 30 min.

2.6. Cell proliferation assay

Cell proliferation was measured using a Cell-Counting Kit-8 (Dojindo, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, MO7e cells were prepared by placing 5,000 cells into each well of a 96-well plate, followed by incubation at 37 °C overnight in a CO_2 chamber. Various concentrations of several inhibitors were added to each well for 1 h prior to SCF treatment. After 48 h, 10 μL of CCK-8 was added to each well incubated at 37 °C for 1 h. Cell viablility was determined by o.d. measurement at 450 nm.

2.7. Modeling of the ligand binding pocket of c-Kit

The three-dimensional structures of chrysin and STI-571 were generated using SYBYL 7.0 (Tripos Associates). The structure of Chrysin was energy minimized using the Conjugate Gradient method. The coordinates of c-Kit were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/, entry code 1T46). The active sites of c-Kit were mapped and good RMS values obtained in docking experiments with STI3 (STI-571) using FlexX. FlexX was performed several times with different customized receptor description files (RDFs) of various combinations of charge and torsion angles of active site residues in order to find the best position. To increase binding interactions, torsion angles of side chains located within 5 Å of the ligands were adjusted manually.

3. Results

3.1. Effect of SCF stimulation on intracellular signaling

Many papers have reported that SCF is able to induce autophosphorylation of receptor tyrosine kinases and proliferation of MO7e cells [20–21,13]. To confirm whether SCF was able to activate these signaling events, we assayed SCF-induced PI3K activation. As shown in Fig. 1A, SCF phosphorylated p85, suggesting that SCF could induce PI3K activation. In fact, SCF is known to activate PI3K in several cell lines. However, the pathways linking PI3K to specific up- and down-stream signaling mediators are not fully understood. To obtain some insight into pathways up- and down-stream of PI3K that may be essential for SCF signaling, we examined the activation of several proteins using Western blot analysis with phospho-specific antibodies. As shown in Fig. 1B, SCF activated Shc, PDK1, PKC, Akt, c-raf, and the ERK1/2 MAP kinase signaling cascade. To determine whether this signaling pathway is controlled by PI3K, we used wortmannin, a known inhibitor of PI3K. Wortmannin abolished this signaling cascade, but did not affect Shc phosphorylation (Fig. 1B, right lane). Interestingly, SCF also activated ERK5. Like other MAP kinase family members, big MAP kinase 1 (BMK1)/ERK5 plays a significant role in cell growth and differentiation [22]. These results suggest that SCF activates PI3K via Shc and its down-stream proteins to convey signaling.

3.2. SCF induced ERK5 translocation to the nucleus

We also investigated the effect of SCF on ERK5 translocation to the nucleus, because it is required for both ERK5's transcriptional activity and its role as a signaling mediator. In the nuclear fraction from SCF-treated cells, there was a time-dependent increase in the phosphorylated form of ERK5, whereas a decrease was observed in the cytosolic fraction (Fig. 2. upper panel). CREB was used as control for the nuclear fraction (Fig. 2. lower panel). These results show that SCF induces ERK5 and ERK1/2 translocation in a time-dependent manner.

3.3. SCF-induced CREB and STAT3 activation is mediated by ERK1/2 and ERK5

To determine which proteins are involved downstream of ERK1/2 and ERK5 within the SCF signaling pathway, we investigated the phosphorylation of CREB as a downstream target of ERK5 [23], and of STAT3 as a downstream target of ERK1/2 [24]. As shown in Fig. 3A, SCF induced CREB and STAT3 phosphorylation in a time-dependent manner, which peaked following 15 and 30 min of SCF stimulation, respectively. In addition, to determine whether CREB and STAT3 activation was mediated by PI3K or ERK1/2 MAP kinase, we used wortmannin (100 nM), an inhibitor of PI3K, and U0126 (10 μM), an inhibitor of MEK1/2 Pretreatment with wortmannin or U0126 inhibited SCF-induced CREB and STAT3 phosphorylation. These results show that CREB and STAT3 appear to be closely related to SCF signaling, and that activation of these proteins is regulated by ERK1/2 and ERK5 MAP kinases.

3.4. Chrysin inhibited SCF-induced phosphorylation of c-Kit

Chrysin (5,7-dihydroxyflavone) is a natural flavonoid contained in many plant extracts [15]. Many polyphenolic compounds, including chrysin, are known to have multiple biological activities, such as anti-inflammation [25,26], anti-cancer [27,28], anti-oxidation [29,30], and estrogenic effects [29]. However, there is no report regarding the effect of chrysin on c-Kit/SCF signaling in leukemia cells. To investigate the ability of chrysin to inhibit the initial events in the c-Kit signaling pathway, SCF-induced tyrosine phosphorylation of c-Kit in the presence of varying concentrations of chrysin was examined by Western blot analysis with an anti-phospho c-Kit antibody (Tyr719). As shown in Fig. 4A, chrysin inhibited SCF-induced tyrosine phosphorylation of c-Kit in MO7e cells in a dose-dependent manner, with an IC_{50} of 2.74 μM. To examine whether this effect was due to the non-selective cytotoxicity of chrysin, we performed a cell viability assay. Chrysin did not affect cell viability in MO7e cells (Fig. 4B). In addition, an immunoprecipitation assay was performed with anti-p85 antibody to determine whether chrysin inhibits PI3K. As shown in Fig. 4C, p85 phosphorylation was abolished by chrysin. These results suggest that chrysin likely acts as an inhibitor of SCF-induced c-Kit signaling. We also evaluated several SCF-activated proteins (Fig. 1B) to determine whether they may be regulated by chrysin. As shown in Fig. 4D, chrysin inhibited Shc, PDK1, PKC, c-Raf, MEK1/2, and ERK5. Moreover,

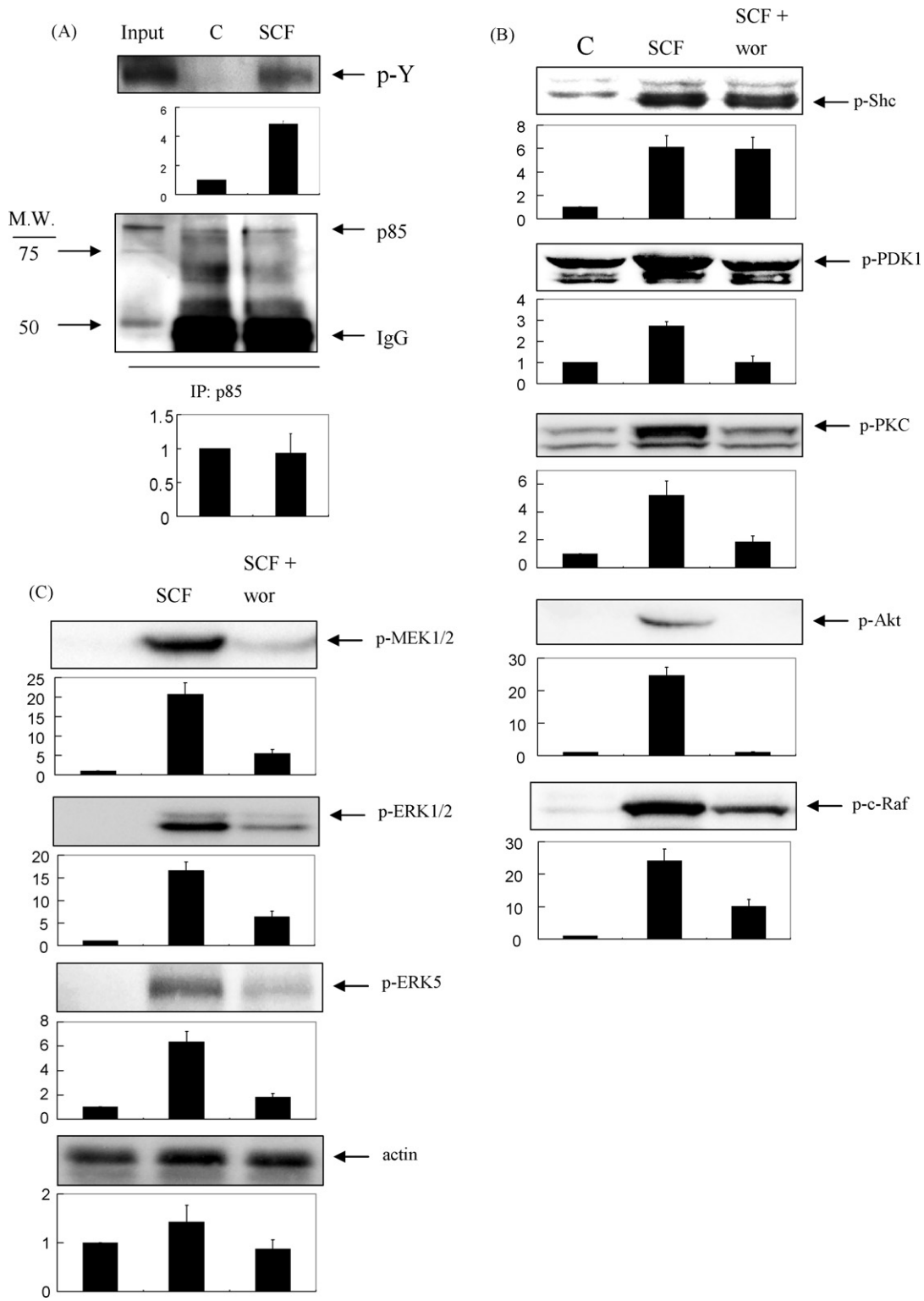

Fig. 1 – SCF activates PI3K and both its upstream and downstream proteins. (A) MO7e cells grown in 20% FBS serum were washed and exposed to serum-free media during SCF treatment (50 ng/mL) for 5 min. Cells were solubilized with IP lysis buffer and cell lysates were immunoprecipitated with anti-p85 antibody. After SDS-PAGE, proteins were immunoblotted with anti-phospho-tyrosine antibody (upper panel). The same membrane was reprobed with anti-p85 antibody (lower panel). The input lane contains one-tenth of the lysate volume used for immunoprecipitation. (B) Serum-free MO7e cells were pretreated with Wortmannin (100 nM) for 1 h, prior to treatment with SCF for 5 min. Cells were harvested, lysed, and immunoblotted with the indicated antibodies. Actin is used as a loading control. Representative results from three independent experiments are shown. M.W.: molecular weight. The Y-axis in graph is expressed as the fold over basal and is presented as the mean ± S.E. of three experiments.

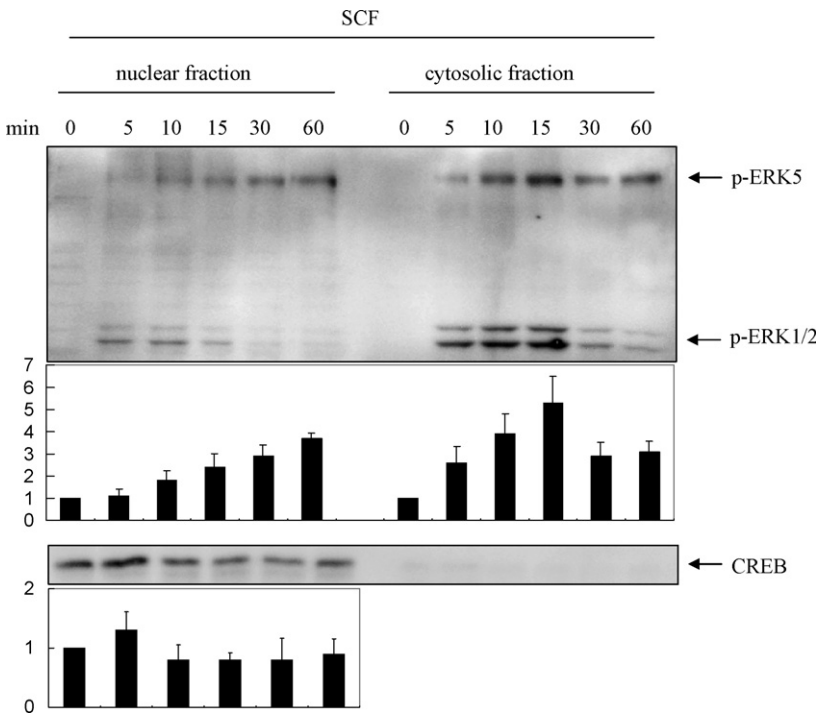

Fig. 2 – SCF induces translocation of ERK1/2 and ERK5 MAP kinases into the nucleus. Cells were treated with SCF for the indicated time. Cells were lysed, sonicated, and the cytosolic and nuclear fractions separated by centrifugation. After SDS-PAGE, proteins were immunoblotted with anti-phospho-ERK5 antibody (upper panel). This antibody also detects phospho-ERK1/2 MAP kinase (ref: manufacturer's instructions). The same membrane was reprobed with anti-CREB antibody (lower panel). The Y-axis in graph is expressed as the fold over basal and is presented as the mean ± S.E. of three experiments.

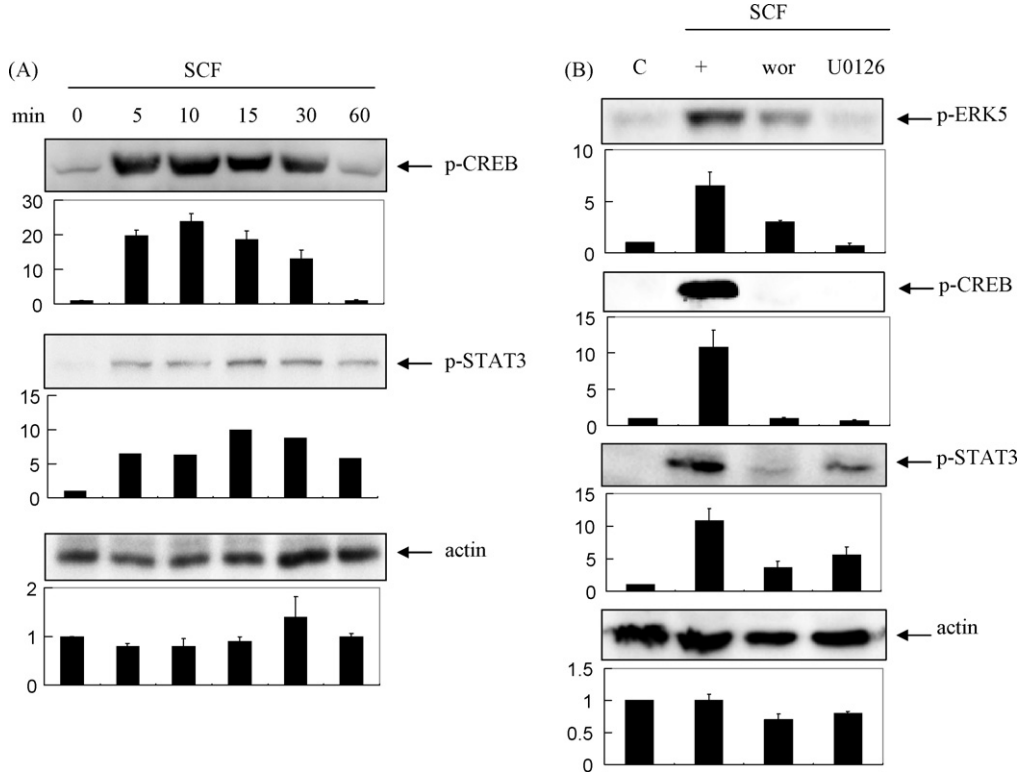

Fig. 3 – SCF induces the activation of transcription factors. (A) Cells were treated with SCF for the indicated times. Cells were lysed and immunoblotted with the indicated antibodies. (B) Serum-free MO7e cells were pretreated with Wortmannin (100 nM) or U0126 (10 μM) for 1 h, prior to treatment with SCF for 10 min. Cells were harvested, lysed, and immunoblotted with the indicated antibodies. Actin was used as a loading control. Representative results from three independent experiments are shown.

chrysin inhibited ERK5 and ERK1/2 translocation (data not shown). In addition, chrysin inhibited CREB and STAT3 (Fig. 4E), suggesting chrysin may be an inhibitor of c-Kit.

3.5. *Effect of chrysin on SCF/c-Kit complex-induced cell proliferation*

SCF has been reported to play a role as a survival factor in myeloid cells, preventing cell death via c-Kit [31]. Proliferation of MO7e cells cultured in the presence of SCF was measured. Cell numbers increased in a time-dependent manner (Fig. 5A). In addition, we examined whether chrysin, PI3-K inhibitor, or MEK inhibitor affects SCF-induced cell proliferation and discovered that they did. Of these, chrysin had more potent activity. These results suggest that chrysin inhibits cell proliferation in MO7e cells by blocking c-Kit phosphorylation. Cell proliferation in response to SCF appeared to be closely dependent on c-Kit activation, at least in part.

3.6. *Docking of chrysin in the binding pocket of c-Kit*

We used a molecular modeling strategy to dock chrysin and STI-571 into the ligand-binding pocket of c-Kit. The binding positions of chrysin were obtained by FlexX. Fig. 6A shows a

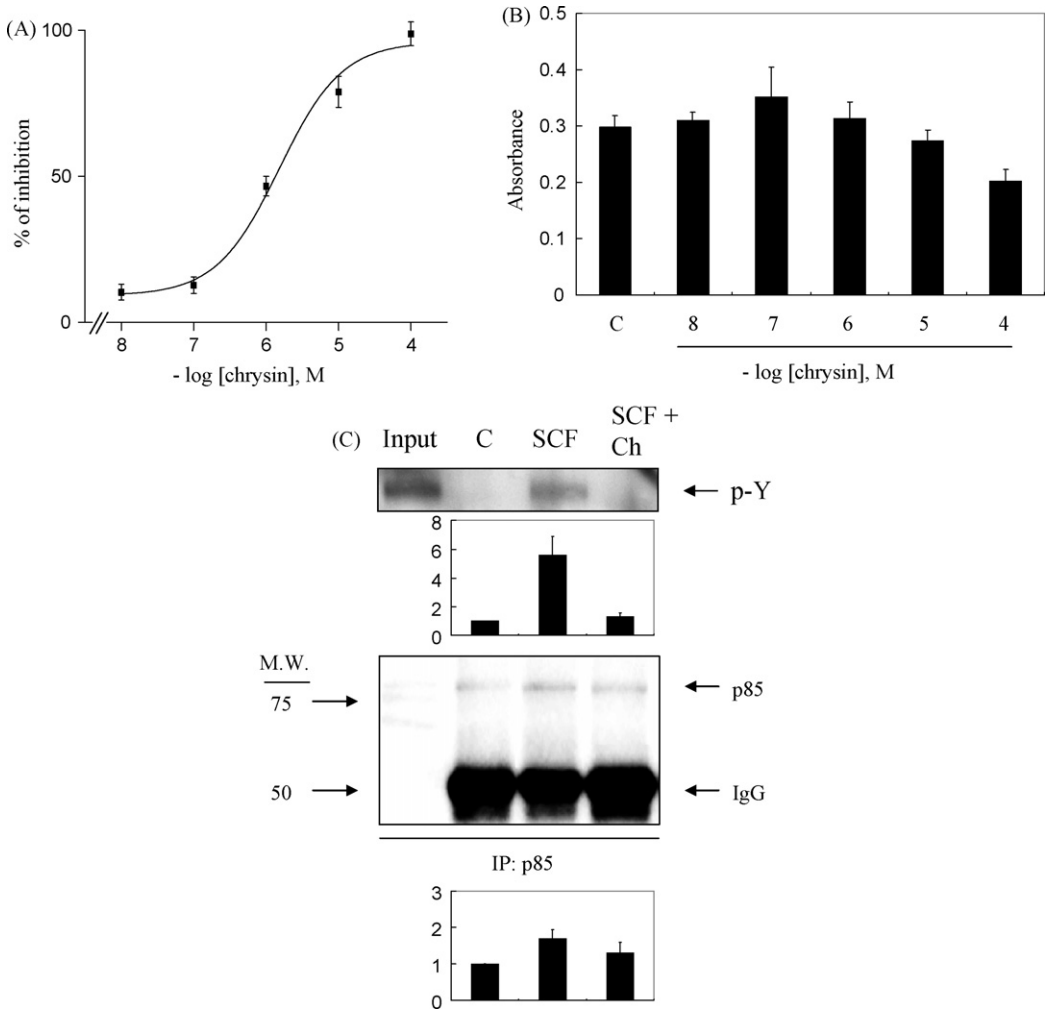

Fig. 4 – Chrysin inhibits SCF-induced phosphorylation of c-Kit. (A) Serum-free MO7e cells were pretreated with the indicated concentrations of chrysin for 1 h, prior to treatment with SCF for 5 min. Cells then were harvested, lysed, and immunoblotted with phospho c-Kit antibody. The inhibitory activity of chrysin on SCF-induced c-Kit phosphorylation are shown after normalization using total c-Kit antibody to standardize the values. (B) 5000 cells were incubated for 48 h with or without the indicated dose of chrysin. Cell proliferation was measured using a Cell-Counting Kit-8 according to the manufacturer's instructions. (C) Cells were pretreated with chrysin for 1 h (5 μM) prior to treatment with SCF. Cells were then solubilized with IP lysis buffer and cell lysates were immunoprecipitated with anti-p85 antibody. After SDS-PAGE, proteins were immunoblotted with anti-phospho-tyrosine antibody (upper panel). The same membrane was reprobed with anti-p85 antibody (lower panel). The input lane contains one-tenth of the lysate volume used for immunoprecipitation. Serum-free MO7e cells were pretreated with chrysin for 1 h, prior to treatment with SCF for 5 min (D) and for 10 min (E). Cells were harvested, lysed, and immunoblotted with the indicated antibodies. Actin is used as a loading control. Representative results from three independent experiments are shown.

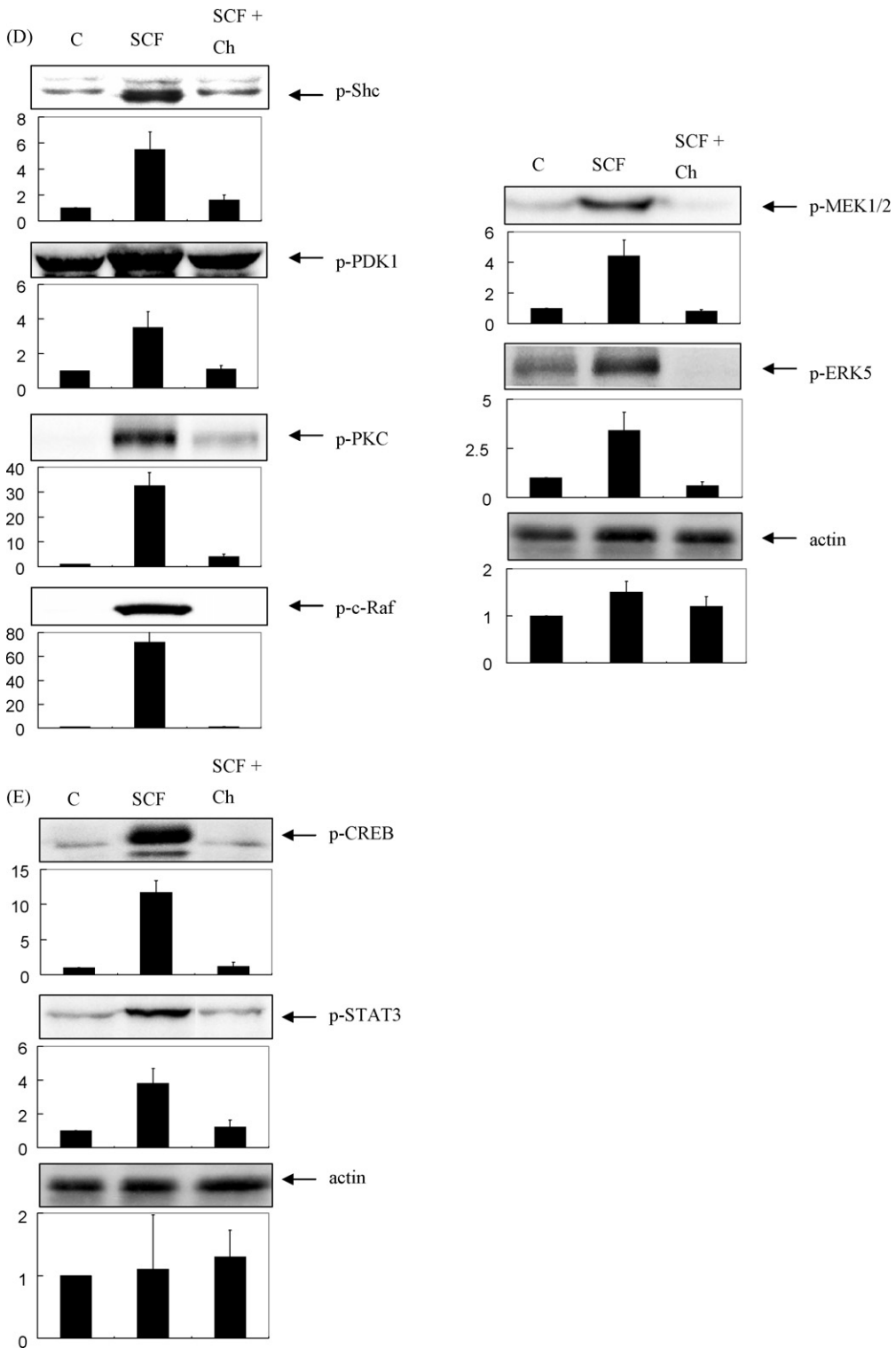

Fig. 4. (Continued).

stereoview of the docked model (for the sake of clarity, only the active site of c-Kit is shown.) The stereoview shows that the calculated model of chrysin has high structural similarity to STI-571. Chrysin appears to overlap on the pyridyl pyrimidine part of STI-571 (Fig. 6B). Whereas STI-571 has 4 hydrogen bonds to the active site of c-Kit, chrysin has only 2 hydrogen bonds (possibly at Leu595 and Cys673), presumably because the molecular weight and structure of chrysin are half that of STI-571. These hydrogen bonds may closely appear to stabilize the binding of chrysin.

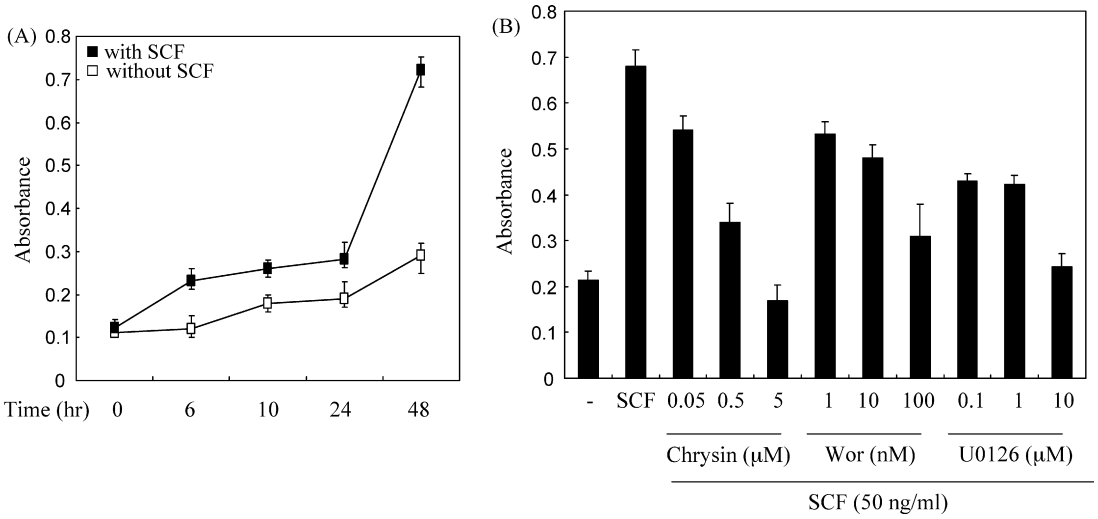

Fig. 5 – Chrysin inhibits SCF/c-Kit complex-induced cell proliferation in MO7e cells. (A) 5000 cells were incubated for the indicated times with or without SCF. Cell proliferation was measured using a Cell-Counting Kit-8 according to the manufacturer's instructions. (B) In serum-free conditions, 5000 MO7e cells were pretreated with the indicated concentrations of chrysin, wortmannin, and U0126 for 1 h, prior to treatment with SCF for 48 h. The values shown are means ± S.D. of experiments performed in triplicate.

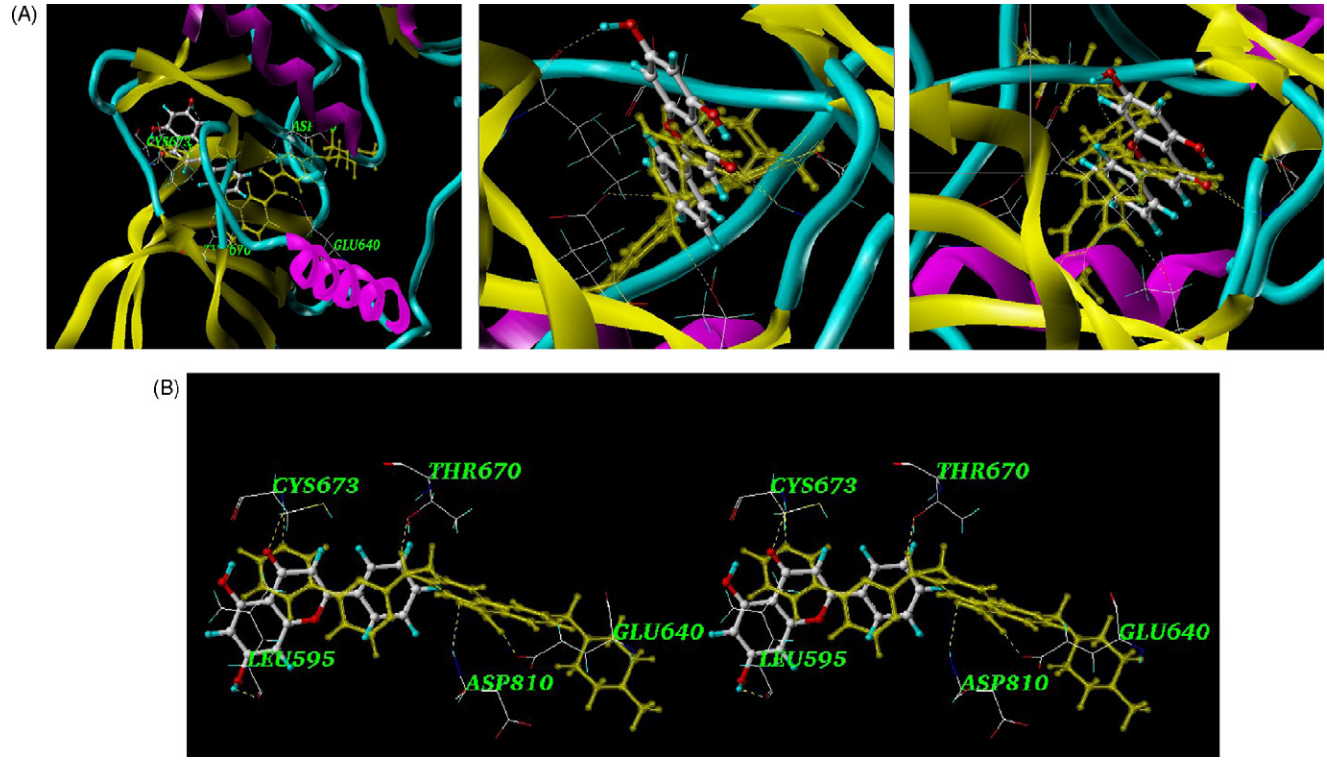

Fig. 6 – Docking model of chrysin and STI-571 in c-Kit active site using FlexX. (A) Stereoview of the docked model of chrysin and STI-571 in which active the site of c-Kit protein is shown. (B) STI-571 (yellow) and chrysin (ball and stick) were docked and interacting residues were checked. Yellow dotted lines indicate hydrogen bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

This study focused on the signaling network of the SCF/c-Kit complex and the action of chrysin. Our results suggest that SCF can activate intracellular signaling mediators in a Shc and PI3K-dependent manner. PI3K activation is one of the important pathways affected by SCF. Much effort has been put forth to clarify SCF's signal transduction pathways, but the

signaling mechanism of the SCF/c-Kit complex has not yet been fully elucidated, especially upstream and downstream of PI3K. In this study, we identify several critical mediators of SCF/c-Kit complex-induced cell proliferation.

First, we found that PI3K regulates SCF signaling in MO7e cells. It has been reported that PI3K has a critical role in SCF signaling [6,32–33]. In time course studies PI3K activation began increasing at 5 min and reached a plateau 15 min after SCF treatment (data not shown). Wortmannin did not inhibit Shc activation, suggesting that Shc is upstream of PI3K in SCF signaling (Fig. 1B). PI3K may then activate PDK1 and PKC to induce the activation of Akt. Although Ras is a well-known downstream target of Akt, SCF did not activate Ras (data not shown), suggesting that SCF signaling is a Ras-independent pathway. This result shows that Akt activation does not always guarantee Ras will be activated to induce cell proliferation. Given these findings, Ras and Raf appear to be irrelevant to SCF signaling under the conditions studied. Recently, several reports showed that there is cross-talk between the G-protein coupled receptor (GPCR) and receptor tyrosine kinase (RTK) signaling pathways which modulates physiological phenomena in several cancers [34–36]. In fact, Qian et al. assert that SCF could activate the cAMP pathway through direct and/or indirect mechanisms in bone marrow [37]. These reports showing that RTK signaling and G-protein signaling form a complexed network in several biological responses are intriguing. Such cross-talk between SCF-induced RTK activation and GPCRs may be present in this study and could be important in understanding signal pathways mediated by various cytokines in biological processes. It will be necessary to further explore this intersection of important signaling mechanisms.

Second, we demonstrated that SCF signaling mediates ERK5 MAP kinase activation. The ERK5 MAP kinase pathway contains MEK5 (MAP2K) and MEKK2/3 (MAP3K) as its upstream regulators. ERK5 can be activated by proliferative stimuli such as epidermal growth factor (EGF), serum, lysophosphatidic acid, neurotrophins, and phorbol ester, as well as by stress stimuli, such as sorbitol, H_2O_2, and UV irradiation [38]. However, the role of ERK5 MAP kinase in SCF signaling is unknown. Our results suggest that SCF might activate ERK5 and ERK1/2 MAP kinases at the same time and induce protein translocation to convey the signal to the nucleus. Interestingly, after translocation, more phosphorylated ERK5 was detected than phosphorylated ERK1/2 (Fig. 2). This result raises the possibilities that (1) ERK5 signaling is more prolonged than ERK1/2 signaling in the early stages of SCF/c-Kit complex-induced cell proliferation, and (2) ERK5 signaling is related to SCF/c-Kit complex signaling. Translocated ERK5 can activate CREB (Fig. 3A). Evidence that ERK5 contributes to the survival of neurons in the central nervous system via activation of CREB has been reported [23]. In our study, ERK5 appears to play a significant role in SCF/c-Kit complex-induced cell proliferation in leukemia cells.

Flavonoids are natural polyphenolic phytochemicals that are ubiquitous in plants and present in the common human diet. They may exert diverse beneficial effects, including antioxidant and anticarcinogenic activities [15–19]. Furthermore, Lin et al. reported that the flavonoid chrysin suppresses LPS-induced angiogenesis by downregulating the interaction of VEGF and VEGFR2, and of IL-6 and IL-6R [39]. Chrysin also has a potential for clinical and therapeutic applications against the physiological and biochemical effects of aging [40]. Nevertheless, little is known about the effect of chrysin on SCF/c-Kit complex-induced cell proliferation in MO7e cells. As shown in Figs. 4 and 5, chrysin inhibits both SCF-induced activation of c-Kit and its downstream signaling mediators and SCF/c-Kit-induced cell proliferation in MO7e cells at a concentration similar to that required to inhibit receptor phosphorylation in cells (Fig. 5). Interestingly, wortmannin inhibited SCF-induced proliferation at nanomolar concentrations, but it abolished intracellular PI3K activity, whereas chrysin inhibited receptor-mediated signal transduction at a much earlier stage. Based on these results, chrysin may be a more potent inhibitor of growth than wortmannin.

Many papers have reported that SCF induces c-Kit activation, triggering cell proliferation, differentiation, and melanogenesis. However, little is known about the SCF/c-Kit complex signaling pathway and how it induces cell proliferation. This study focused on identifying components of the SCF/c-Kit complex signaling pathway and chrysin's effects. To examine whether chrysin could bind to the ligand binding pocket of c-Kit receptor, we carried out molecular modeling. Since the main structure of Chrysin is a flavonoid, three-dimensional structures of flavonoids were generated using SYBYL 7.1 (Tripos Associates, http://www.tripos.com/). Thirty flavonoid compounds were energy minimized by the Conjugate Gradient method and evaluated based on their flavon structure. The coordinates of c-Kit were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/, entry code 1T46). Compounds were docked into c-Kit by FlexX. The flavonoids appeared to overlap with the pyridyl pyrimidine moiety of STI-571. The modeling of a flavonoid into the binding pocket of c-Kit demonstrated that the flavonoid would stably form several hydrogen bonds with key active site residues. These results suggest that the chrysin may bind to the interaction pocket of c-Kit as well as gleevec, even though they have different structures.

These results show (1) identification of SCF/c-Kit signaling from receptor to transcription factors, and (2) inhibition of SCF/c-Kit signaling by chrysin. Taken together, chrysin appears as a likely candidate drug for modulating c-Kit function(s) in order to mediate SCF/c-Kit complex-induced cell proliferation in the acute myeloid leukemia model. These findings provide new insight into the potential action of chrysin in leukemia and may generate novel strategies for therapeutic drug development.

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REFERENCES

[1] Qiu FH, Ray P, Brown K, Barker PE, Jhanwar S, Ruddle FH, et al. Primary structure of c-Kit: relationship with the

CSF-1/PDGF receptor kinase family-oncogenic activation of v-Kit involves deletion of extracellular domain and C terminus. EMBO J 1998;7:1003–11.
[2] Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, et al. Human proto-oncogene c-Kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J 1987;6:3341–51.
[3] Ashman LK. The biology of stem cell factor and its receptor C-Kit. Int J Biochem Cell Biol 1999;31:1037–51.
[4] Broudy VC. Stem cell factor and hematopoiesis. Blood 1997;90:1345–64.
[5] Duronio V, Welham MJ, Abraham S, Dryden P. Schrader JW. p21ras activation via hemopoietin receptors and c-Kit requires tyrosine kinase activity but not tyrosine phosphorylation of p21ras GTPase-activating protein. Proc Natl Acad Sci USA 1992;89:1587–91.
[6] Serve H, Hsu YC, Besmer P. Tyrosine residue 719 of the c-Kit receptor is essential for binding of the P85 subunit of phosphatidylinositol (PI) 3-kinase and for c-Kit-associated PI 3-kinase activity in COS-1 cells. J Biol Chem 1994;269:6026–30.
[7] Blume-Jensen P, Jiang G, Hyman R, Lee KF, O'Gorman S, Hunter T. Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3′-kinase is essential for male fertility. Nat Genet 2000;24:157–62.
[8] Kissel H, Timokhina I, Hardy MP, Rothschild G, Tajima Y, Soares V, et al. Point mutation in kit receptor tyrosine kinase reveals essential roles for kit signaling in spermatogenesis and oogenesis without affecting other kit responses. EMBO J 2000;19:1312–26.
[9] Hemesath TJ, Price ER, Takemoto C, Badalian T, Fisher DE. MAP kinase links the transcription factor Microphthalmia to c-Kit signalling in melanocytes. Nature 1998;391:298–301.
[10] Broudy VC, Lin NL, Liles WC, Corey SJ, O'Laughlin B, Mou S, et al. Signaling via Src family kinases is required for normal internalization of the receptor c-Kit. Blood 1999;94:1979–86.
[11] Van Dijk TB, Van Den Akker E, Amelsvoort MP, Mano H, Lowenberg B, von Lindern M. Stem cell factor induces phosphatidylinositol 3-kinase-dependent Lyn/Tec/Dok-1 complex formation in hematopoietic cells. Blood 2000;96:3406–13.
[12] Garrington TP, Ishizuka T, Papst PJ, Chayama K, Webb S, Yujiri T, et al. MEKK2 gene disruption causes loss of cytokine production in response to IgE and c-Kit ligand stimulation of ES cell-derived mast cells. EMBO J 2000;19:5387–95.
[13] Smolich BD, Yuen HA, West KA, Giles FJ, Albitar M, Cherrington JM. The antiangiogenic protein kinase inhibitors SU5416 and SU6668 inhibit the SCF receptor (c-kit) in a human myeloid leukemia cell line and in acute myeloid leukemia blasts. Blood 2001;97:1413–21.
[14] Matsui J, Wakabayashi T, Asada M, Yoshimatsu K, Okada M. Stem cell factor/c-kit signaling promotes the survival, migration, and capillary tube formation of human umbilical vein endothelial cells. J Biol Chem 2004;279:18600–7.
[15] Williams CA, Harborne JB, Newman M, Greenham J, Eagles J. Chrysin and other leaf exudate flavonoids in the genus Pelargonium. Phytochemistry 1997;46:1349–53.
[16] Woo KJ, Jeong YJ, Inoue H, Park JW, Kwon TK. Chrysin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression through the inhibition of nuclear factor for IL-6 (NF-IL6) DNA-binding activity. FEBS Lett 2005;579:705–11.
[17] Monasterio A, Urdaci MC, Pinchuk IV, Lopez-Moratalla N, Martinez-Irujo JJ. Flavonoids induce apoptosis in human leukemia U937 cells through caspase- and caspase-calpain-dependent pathways. Nutr Cancer 2004;50:90–100.
[18] Woo KJ, Jeong YJ, Park JW, Kwon TK. Chrysin-induced apoptosis is mediated through caspase activation and Akt inactivation in U937 leukemia cells. Biochem Biophys Res Commun 2004;325:1215–22.
[19] Cipak L, Rauko P, Miadokova E, Cipakova I, Novotny L. Effects of flavonoids on cisplatin-induced apoptosis of HL-60 and L1210 leukemia cells. Leuk Res 2003;27:65–72.
[20] Ikeda H, Kanakura Y, Tamaki T, Kuriu A, Ishikawa H, Kanayama Y, et al. Expression and functional role of the proto-oncogene c-kit in acute myeloblastic leukemia cells. Blood 1991;78:2962–8.
[21] Kuriu A, Ikeda H, Kanakura Y, Griffin JD, Druker B, Yagura H, et al. Proliferation of human myeloid leukemia cell line associated with the tyrosine-phosphorylation and activation of the proto-oncogene c-kit product. Blood 1991;78:2834–40.
[22] Cameron SJ, Abe J, Malik S, Che W, Yang J. Differential role of MEK5alpha and MEK5beta in BMK1/ERK5 activation. J Biol Chem 2004;279:1506–12.
[23] Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, Segal RA. Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat Neurosci 2001;4:981–8.
[24] Levy O, Granot Y. Arginine-vasopressin activates the JAK-STAT pathway in vascular smooth muscle cells. J Biol Chem 2006;281:15597–604.
[25] Ko HH, Tsao LT, Yu KL, Liu CT, Wang JP, Lin CN. Structure-activity relationship studies on chalcone derivatives. The potent inhibition of chemical mediators release. Bioorg Med Chem 2003;11:105–11.
[26] Kim EJ, Kwon KJ, Park JY, Lee SH, Moon CH, Baik EJ. Effects of peroxisome proliferator-activated receptor agonists on LPS-induced neuronal death in mixed cortical neurons: associated with iNOS and COX-2. Brain Res 2002;941:1–10.
[27] Koganov MM, Dueva OV, Tsorin BL. Activities of plant-derived phenols in a fibroblast cell culture model. J Nat Prod 1999;62:481–3.
[28] Habtemariam S. Flavonoids as inhibitors or enhancers of the cytotoxicity of tumor necrosis factor-alpha in L-929 tumor cells. J Nat Prod 1997;60:775–8.
[29] Machala M, Kubinova R, Suchy V. Chemoprotective potentials of homoisoflavonoids and chalcones of Dracaena cinnabari: modulations of drug-metabolizing enzymes and antioxidant activity. Phytother Res 2001;15:114–8.
[30] Lapidot T, Walker MD, Kanner J. Antioxidant and prooxidant effects of phenolics on pancreatic beta-cells in vitro. J Agric Food Chem 2002;50:7220–5.
[31] Meyer C, Drexler HG. FLT3 ligand inhibits apoptosis and promotes survival of myeloid leukemia cell lines. Leuk Lymphoma 1999;32:577–81.
[32] Ueda S, Mizuki M, Ikeda H, Tsujimura T, Matsumura I, Nakano K, et al. Critical roles of c-Kit tyrosine residues 567 and 719 in stem cell factor-induced chemotaxis: contribution of src family kinase and PI3-kinase on calcium mobilization and cell migration. Blood 2002;99:3342–9.
[33] Young SM, Cambareri AC, Ashman LK. Role of c-KIT expression level and phosphatidylinositol 3-kinase activation in survival and proliferative responses of early myeloid cells. Cell Signal 2006;18:608–20.
[34] Thomas SM, Bhola NE, Zhang Q, Contrucci SC, Wentzel AL, Freilino ML, et al. Cross-talk between G protein-coupled receptor and epidermal growth factor receptor signaling pathways contributes to growth and invasion of head and neck squamous cell carcinoma. Cancer Res 2006;66:11831–9.
[35] Lui VW, Thomas SM, Zhang Q, Wentzel AL, Siegfried JM, Li JY, et al. Mitogenic effects of gastrin-releasing peptide in head and neck squamous cancer cells are mediated by activation of the epidermal growth factor receptor. Oncogene 2003;22:6183–93.

[36] Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nat Med 2002;8:289–93.
[37] Qian J, Yehia G, Molina CA, Fernandes A, Donnelly RJ, Anjaria DJ, et al. Cloning of Human Preprotachykinin-I promoter and the role of cyclic adenosine 5′-monophosphate response elements in its expression by IL-1 and stem cell factor. J Immunol 2001;166:2553–61.
[38] Xu BE, Stippec S, Lenertz L, Lee BH, Zhang W, Lee YK, et al. WNK1 activates ERK5 by a MEKK2/3-dependent mechanism. J Biol Chem 2004;279:7826–31.
[39] Lin CM, Chang H, Li SY, Wu IH, Chiu JH. Chrysin inhibits lipopolysaccharide-induced angiogenesis via down-regulation of VEGF/VEGFR-2 (KDR) and IL-6/IL-6R pathways. Planta Med 2006;72:708–14.
[40] Dhawan K, Kumar S, Sharma A. Beneficial effects of chrysin and benzoflavone on virility in 2-year-old male rats. J Med Food 2002;5:43–8.