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ORIGINAL ARTICLE

Trichosanthin down-regulated p210$^{Bcr-Abl}$ and enhanced imatinib-induced growth arrest in chronic myelogenous leukemia cell line K562

Kunzhong Zhang · Jianhua Xu · Xiuwang Huang · Lixian Wu · Caixia Wen · Yingying Hu · Yu Su · Yuanzhong Chen · Zhiqiang Zhang

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Abstract
Purpose Trichosanthin (TCS), an active component extracted from the root tubers of traditional Chinese medical herb Tian-Hua-Fen of the Cucurbitaceae family, has long been used for medical purpose in China; there is increasing interest in developing TCS as cancer therapeutic agents. The present study was to investigate the growth arrest of K562 cells and its molecular mechanisms, which the drugs induced by TCS and the possible functional interaction of TCS with imatinib (STI571) to K562 cells.
Methods Trypan blue exclusive staining was used to access the cell growth inhibition; western blot was used to evaluate the p210$^{Bcr-Abl}$, phosphorylated tyrosine kinase (PTK), and some signaling molecules involving in cell proliferation and apoptosis in K562 cells.
Results TCS and imatinib inhibited K562 cells at a time- and dose-dependent manners, respectively; TCS down-regulated p210$^{Bcr-Abl}$ at a time- and dose-dependent manners; TCS synergistically enhanced imatinib-induced K562 cell growth arrest and down-regulation of p210$^{Bcr-Abl}$, PTK activities, procaspase-3, Hsp90,NF-κB and PKC.
Conclusion The results suggest that TCS not only by itself involves but also synergizes activities of imatinib to induce K562 cell growth arrest, down-regulation of p210$^{Bcr-Abl}$ and its downstream signals and to stimulate the effect of the tyrosine kinase inhibition.

K. Zhang · J. Xu (✉) · X. Huang · L. Wu · C. Wen · Y. Hu · Y. Su · Z. Zhang
Institute of Clinical Pharmacology,
School of Pharmacy, Fujian Medical University,
Fujian 350004, People's Republic China
e-mail: xjh@mail.fjmu.edu.cn

Y. Chen
Fujian Institute of Hemetology,
Fujian 350004, People's Republic China

Keywords Combination drug therapy · Trichosanthin · Imatinib · Cell growth · p210$^{Bcr-Abl}$ · Chronic myelogenous leukemia · K562 cell line

Introduction

Trichosanthin (TCS), a type I ribosome-inactivating protein (RIP), is an active component extracted from the root tubers of Chinese medical herb Tian-Hua-Fen (*Trichosanthes kirilowii*) of the Cucurbitaceae family [1]. It has been traditionally used for mid-term abortion in ancient Chinese society for hundreds of years [2]. Until 1970s, TCS was isolated from *T. kirilowii* and has been used to in ectopic pregnancies, hydatidiform moles, and trophoblastic tumors [3, 4]. In the early 1990s, the primary and three-dimensional structures of TCS had been elucidated [1, 5–7] (Fig. 1), and TCS was applied in the treatment of patients with AIDS or AIDS-related complex in phase I and II studies [8, 9]. Recently, TCS has been found to induce apoptosis in virto of anti-cancer and was found to induce apoptosis in chronic myelogenous leukemia (CML) cell lines K562 [10–13]. But the mechanisms induced apoptosis in CML has not been well elucidated.

Chronic myelogenous leukemia is characterized by a reciprocal t (9; 22) chromosomal translocation, known as the Philadelphia chromosome, which fuses parts of the c-abl gene, located on chromosome 22, is the hallmark of CML [14]. The fusion gene is called bcr-abl. After transcription and translation of the bcr-abl, the constitutively activated p210$^{Bcr-Abl}$ oncoprotein is formed. This leads to uncontrolled activation of the ABL tyrosin kinase. Expression of the p210$^{Bcr-Abl}$ oncoprotein is sufficient and necessary for the development of a CML phenotype [15]. Thus, p210$^{Bcr-Abl}$ is one of the most intensively studied proteins in cancer research.

Fig. 1 Three-dimensional structure of trichosanthin (Shaw et al. 1994)

The most remarkable specificity and efficacy in treating CML is imatinib (STI571, formerly CGP57418B; Novartis Pharmaceutics, Basel, Switzerland), an inhibitor of the tyrosine kinase (TK), which selectively suppresses the growth of the CML primary cells and cell lines in vitro by blocking kinase activities and inducing cell apoptosis [16, 17]. However, follow-up of patients treated with imatinib has demonstrated that imatinib seems unable to eradicate the malignant progenitors and a significant portion of patients will develop resistance to imatinib with progression of their leukemia after long-time use [18–22]. In patients with advanced phase of CML, imatinib is less effective and its response duration is short. Despite of the excellent clinical results with imatinib in chronic myeloid leukemia, most patients have minimal residual disease and others will develop resistance, which may eventually reccur [23]. Thus, developing other approaches to overcome and to prevent the resistance to imatinib is necessary [24].

As the type I RIP, we considered that TCS might take part in the down-regulation of $p210^{Bcr\text{-}Abl}$, and may synergize imatinib in treating CML, since there were no reports upon the actions at our present understanding, we were interested in identifying potential mechanisms of TCS on K562 cells, and whether or not TCS could enhance the effect with imatinib in treating CML. In this study, $p210^{Bcr\text{-}Abl}$-positive K562 cell line was used as a cellular model of CML for drug screening.

Materials and methods

Cells and cell culture

The human CML cell line K562 (Ph^+) (purchased from Shanghai Institute of Biochemistry and Cell Biology, China) was cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and gentamicin (80 U/100 ml) in a humidified atmosphere with 5% CO_2 at 37°C.

Drugs and anti-bodies

Trichosanthin was purchased from Shanghai Jinshan Medicine Co., Ltd., China (1.2 mg/ml); imatinib was kindly provided by Novartis Pharmaceutics and prepared as 10 mmol/l stock solution in DMSO at −20°C. Stock solutions were diluted in RPMI 1640 medium to achieve the final concentration. Anti-C-Abl, Anti-PTK, Anti-procaspase-3, Anti-Hsp90, Anti-NF-κB, Anti-PKC, Anti-β-actin and the second anti-bodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell growth inhibition assay

K562 cells were plated in a final volume 0.2 ml in triplicate at a density of $(1–2) \times 10^8$/l in 96-well plates and were exposed to TCS, imatinib and their combinations. After the indicated time, cells were incubated with trypan blue and the number of live cells were counted.

Protein extraction and western blotting

After treatment with drugs, cells were collected by centrifugation and washed thrice in ice-cold phosphate-buffered saline (PBS, pH 7.2). The cell pellets were resuspended in lysis buffer [Tris–HCl 50 mmol/l, pH 8.0, NaCl 150 mmol/l, dithiothreitol 1 mmol/l, edetic acid 0.5 mmol/l, nonidet P40 0.1% (v/v), sodium dodecylsulfate 0.1% (w/v)] containing protease inhibitors (aprotinin 1 mg/l, leupeptin 2 mg/l, sodium orthovanadate 100 μmol/l and phenylmethylsulfonly fluoride 10%). The protein concentration of each sample was estimated using the Coomassie brilliant blue kit (Nanjing Jiancheng Biotechnology, Jiangsu, China). After boiling for 5 min, lysates were subjected to electrophoresis on 8% polyacrylamide gels and transferred to Hybond-C membranes (Amersham, Arlington Heights, IL, USA) in transfer buffer [Tris 25 mmol/l, glycine 190 mmol/l, methanol 20% (v/v)] using a Transfer Apparatus at 150 mA for 2 h. The membranes were blocked with blocking-buffer [5% (g/v) non-fat milk] at 4°C overnight and were incubated with primary anti-bodies: C-Abl, phosphorylated tyrosine kinase (PTK), procaspase-3, Hsp90, NF-κB, PKC and β-actin anti-bodies for 2 h at room temperature, washed four times in TBST [Tris-buffered saline supplemented with 0.03% (*v/v*) Tween-20], and incubated with the second antibody (1:500) for 1.5 h at room temperature, washed four times with Tris–HCl (pH 7.2), the membranes then were detected with substrate according to product kits (Gene Lab., Singapore). All experiments were repeated at least three times and yielded similar results.

Statistical analysis

Data were expressed as mean ± SD. Jin's formula [25] was used to evaluate the synergistic effects between drugs. The formula is: $Q = E_{a+b}/(E_a + E_b - E_a \times E_b)$; Q is the combination index; E_{a+b} which represents the cell proliferative inhibition rate of the combined drug; E_a and E_b are signs of the cell proliferative inhibition rate of each drug. After calculation: $Q = 0.85$–1.15, means indication of simple addiction (+); $Q = 1.15$–$2.0 \rightarrow$ synergism (+ +); $Q > 2.0 \rightarrow$ significant synergism (+ + +); $Q = 0.85$–$0.55 \rightarrow$ antagonistic effect (−); $Q < 0.55 \rightarrow$ significant antagonistic effect (− −). The differences between treatment and control groups were evaluated with Student's t-test. The differences were considered significant at $P < 0.05$.

Results

Inhibition of TCS and/or imatinib on K562 cell growth

To evaluate the effects of TCS and imatinib on proliferation of $p210^{Bcr\text{-}Abl}$-expressing cell lines, we measured time- and dose-response curves of TCS and imatinib on K562 cells. The results indicated that 0.4 μM TCS caused the inhibitory rate of K562 cell growth by 38, 75, and 86% after cells exposed at 24, 48, and 72 h; the increasing concentrations (0.2–6.4 μM) of TCS caused dose-dependent increase on the inhibition of K562 cells for 24 h, 3.2 μM TCS which was nearly efficient to reach maximum inhibition, the IC_{50} of TCS for 24 h was about 0.8 μM (Fig. 2a, b), meanwhile, 0.2 μM imatinib caused the inhibitory rate of cell growth by 48, 77, and 93% after cells exposed at 24, 48, and 72 h. The increasing concentrations (0.1–0.8 μM) of imatinib caused dose-dependent increase on the inhibition of K562 cells for 24 h. About 0.8 μM imatinib was almost efficient to cause maximum inhibition, the IC_{50} of imatinib for 24 h was about 0.2 μM (Fig. 2c, d).

Since TCS is a RIP while imatinib is an inhibitor of the TK, they inhibit K562 cells by different mechanisms. We supposed whether or not there was a synergism between TCS and imatinib-mediated cell inhibition. The results showed that 0.1, 0.2, 0.4, and 0.8 μM imatinib for 24 h alone caused the inhibitory rate of cell growth by 18.7, 49.6, 71.8, and 94%, respectively; 0.4 μM TCS alone caused the inhibitory rate of 38%; however, after TCS was

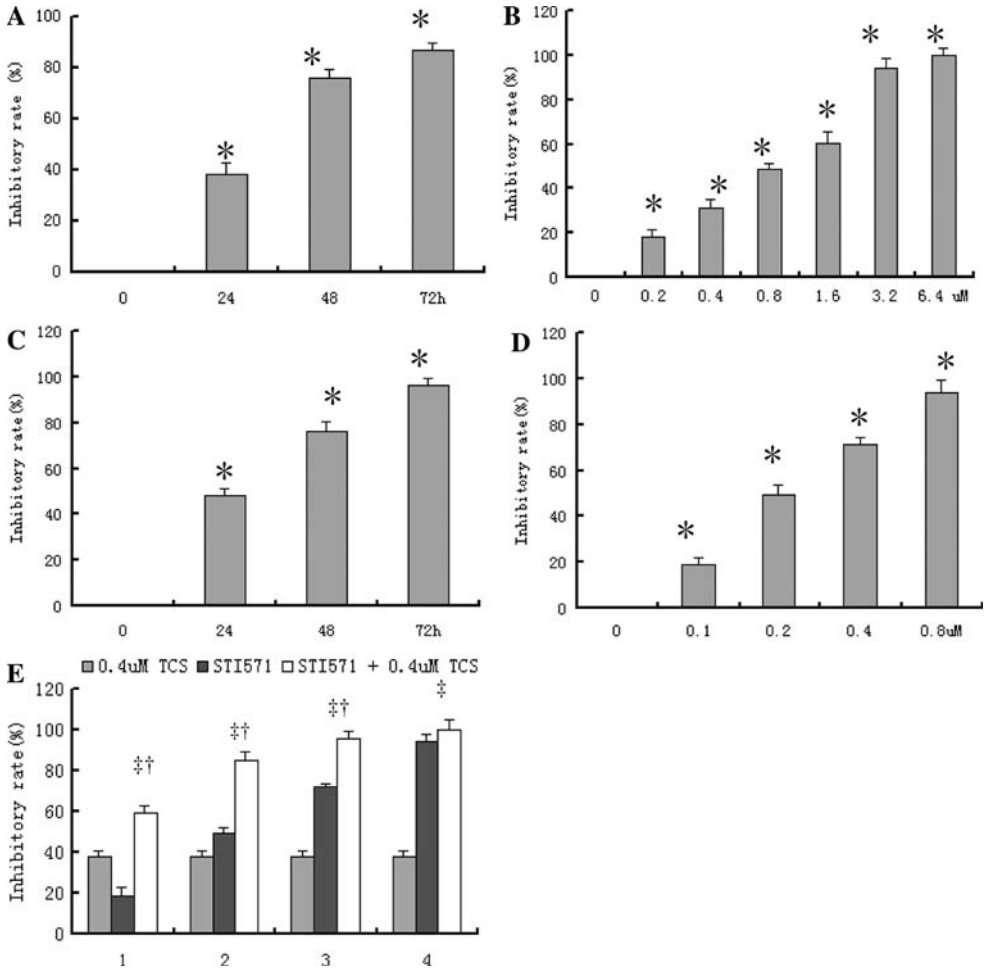

Fig. 2 Effects of TCS and/or STI 571 on K562 cells. **a** Cells were exposed to 0.4 μM TCS at different times. **b** Cells were exposed to TCS at different concentrations for 24 h. **c** Cells were exposed to 0.2 μM STI571 at different times. **d** Cells were exposed to STI571 at different concentrations for 24 h. **e** Cells were exposed to different concentrations of STI571 [(1) 0.1 μM, (2) 0.2 μM, (3) 0.4 μM, and (4) 0.8 μM] and TCS 0.4 μM for 24 h. After the indicated time, cells were counted triplicate, as described in Materials and methods. Results were calculated as the percent of values obtained with untreated cells and represent mean ± SD. *Asterisk* represents $P < 0.05$ vs. control. *Double dagger* represents $P < 0.05$ vs. 0.4 μM TCS; *single dagger* represents $P < 0.05$ vs. STI571

combined to the above concentrations of imatinib, the inhibitory rate of cell growth was increased at 59, 85, 96, and 100% ($P < 0.05$). The combined index was 1.189, 1.236, 1.163, and 1.038, respectively (Fig. 2e).

Effect of TCS on p210$^{Bcr-Abl}$, PTK

To further investigate the molecular and biological basis for the effects of TCS on K562 in Fig. 2, we examined the effect of TCS on p210$^{Bcr-Abl}$, PTK. Cells that treated with 0.4 μM TCS at different times (24, 48, and 72 h) or doses (0.4, 0.8, and 1.6 μM) showed gradual and progressive decreases of p210$^{Bcr-Abl}$ (Fig. 3a). This was also seen in the effect of TCS on PTK at a time- and dose-dependent manners in Fig. 3b.

Co-treatment with TCS enhanced imatinib-induced down-regulation of p210$^{Bcr-Abl}$, PTK and its downstream signals

p210$^{Bcr-Abl}$, a product of bcr-abl, is a unique opportunity to rationally design target-directed therapy [26], which serves as a biologically relevant function in vitro assay in K562 cells. As shown in Fig. 3c, no or gradual changes of p210$^{Bcr-Abl}$ with treatment of imatinib alone; a gradual decrease was observed with treatment of 0.4 μM TCS. However, the combination of 0.4 μM TCS and the increasing concentrations of imatinib (0.2–0.8 μM) caused significant progressive decrease of p210$^{Bcr-Abl}$. Comparatively, with the doses of imatinib increase, the levels of tyrosine phosphorylation decreased gradually which almost disappeared at the dose of 0.8 μM imatinib. However, cotreatment with 0.4 μM TCS, the levels of tyrosine phosphorylation decreased significantly at the dose of 0.2 μM imatinib, and almost disappeared at the dose of 0.4 μM imatinib (Fig. 3d).

We further detected some signaling molecules involving in cell proliferation and apoptosis in K562 cells. The results of the immunoblot analyses in Fig. 4 showed that increasing concentrations (0.2–0.8 μM) of imatinib caused slight or modest decrease of the apoptotic signaling molecules such as procaspase-3, Hsp90, PKC, NF-κB; 0.4 μM TCS caused obvious decreases of PKC and NF-κB but not procaspase-3 and Hsp90; after co-treatment of imatinib with 0.4 μM TCS, these signaling molecules decrease significantly.

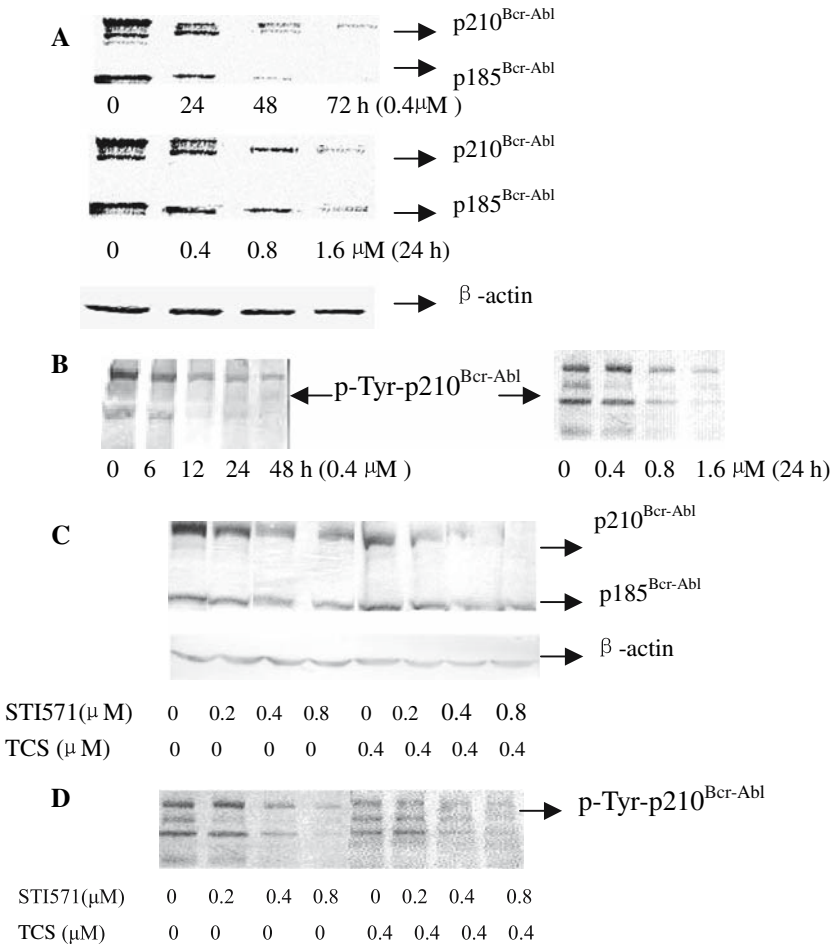

Fig. 3 Effects of TCS and/or STI 571 on p210$^{Bcr-Abl}$ and p-Tyr-p210$^{Bcr-Abl}$ in K562 cells. **a**. Cells were exposed to 0.4 μM TCS at different times and at different concentrations for 24 h. Multiple lower molecular bands represent products of p210$^{Bcr-Abl}$ down-regulation in TCS-treated cells protein. **b** Cells were exposed to 0.4 μM TCS at different times and at different concentrations for 24 h. The lower molecular bands represent products of p-Tyr-p210$^{Bcr-Abl}$ down-regulation in TCS-treated cells protein. **c** Cells were exposed to different concentrations of STI571, 0.4 μM TCS and their combinations for 24 h. The lower molecular bands represent products of p210$^{Bcr-Abl}$ down-regulation in STI571 combined with TCS treated cells protein. **d** Cells were exposed to different concentrations of STI571, 0.4 μM TCS and their combinations for 24 h. The lower intensity of molecular bands represent products of p210$^{Bcr-Abl}$ down-regulation in STI571 combined with TCS treated cells protein

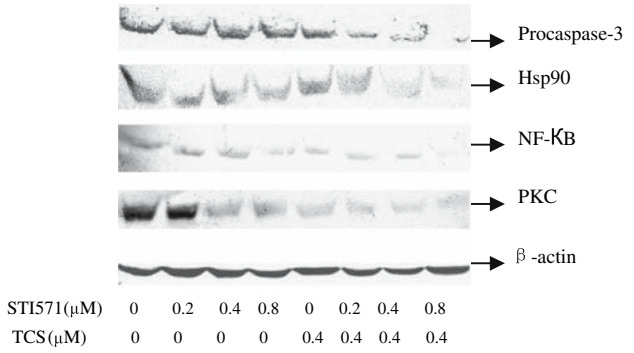

Fig. 4 Effect of TCS and STI571 on signaling molecules in K562 cells. Cells were exposed to different concentrations of STI571, 0.4 μM TCS and their combinations for 24 h. After the indicated time, protein were extracted and then immunoblot for Procaspase-3, Hsp90, NF-κB, PKC, and β-actin were performed as described in Materials and methods

Discussion

Previous reports had documented the apoptosis in cancers induced by TCS [10–13]. In our previous studies, TCS showed higher specificity in the inhibition of HL60, which does not express $p210^{Bcr\text{-}Abl}$ in comparing with K562 cells (Data not shown). In this studies, we demonstrated that TCS was capable of inducing growth arrest in K562 cells. In addition, TCS down-regulated $p210^{Bcr\text{-}Abl}$ protein level at a time- and dose-dependent manners. In recent years, inhibition of protein synthesis by cleavage of the N-glycosidic bond of a specific adenine of 28 S rRNA has been accepted as the mechanism by which plant RIPs cause cytotoxicity [27–29] (Fig. 5). Since TCS is a type I RIP, we suppose that TCS down-regulates $p210^{Bcr\text{-}Abl}$ may be similar to the mechanism of RIPs, thus reduces the uncontrolled activation of the ABL tyrosin kinase and $p210^{Bcr\text{-}Abl}$ downstream signals, limits the overgrowth of K562, finally inhibits the cell growth of K562.

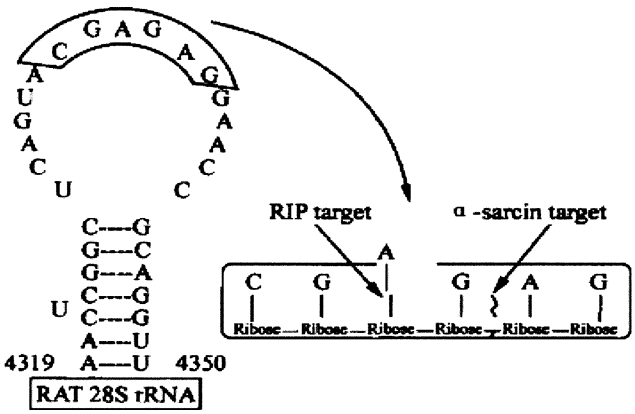

Fig. 5 The mechanism of ribosome-inactivating protein (Stirpe et al. 1992)

In this study, 0.4 μM TCS was combined with different concentrations (0.1–0.8 μM) of imatinib, the combined index ranged from 1.163 to 1.236 except 0.4 μM TCS and 0.8 μM imatinib (Fig. 2e). Our previous study also showed that the combination of lower concentrations (<0.4 μM) of TCS and imatinib synergistically inhibited cell growth and inducing apoptosis, down-regulated the $p210^{Bcr\text{-}Abl}$ (data not shown). In addiction, imatinib as a reversible inhibitor that occupies the adenosine triphosphate-binding pocket of $p210^{Bcr\text{-}Abl}$ and stabilizes the kinase in an inactive conformation [30], it did not exert significant effect on $p210^{Bcr\text{-}Abl}$ protein directly (Fig. 3c), this was consistent with the report that imatinib had milder effect or a small reduction on $p210^{Bcr\text{-}Abl}$ level [31]. In contrast, $p210^{Bcr\text{-}Abl}$ was down-regulated by TCS at a time- and dose-dependent manners (Fig. 3a). As the molecular basis of CML is the $p210^{Bcr\text{-}Abl}$ protein which induces deregulation of cell proliferation, cell apoptosis and adhesion abnormality to marrow stroma, moreover, some important downstream pathways have been significantly altered [32], therefore, the application of imatinib in CML has been considered as a molecular triumph of cancer research in the genomic era. On the other hand, resistance to imatinib in a number of patients after long-time of using ascribed to an elevated expression of the Bcr-Abl gene or to the mutation at key positions of the Bcr-Abl coding sequence around the ATP-binding domain [22]. The strategies have focused on inhibiting $p210^{Bcr\text{-}Abl}$ expression in addition to lowering its TK activity [33–36]. Interestingly, the results showed that TCS down-regulated $p210^{Bcr\text{-}Abl}$ protein level at a time- and dose-dependent manners (Fig. 3a), this indicated that one of the targets of TCS is $p210^{Bcr\text{-}Abl}$. Unlike imatinib, which inhibits the TK activity of BCR-ABL but apparently does not affect the turnover of the protein [31], TCS lowers the level of $p210^{Bcr\text{-}Abl}$, accompanied by a reduction of the TK activity (Fig. 3b). In summary, cotreatment with TCS sensitized K562 cells to imatinib-induced growth arrest. This sensitizing effect could be correlated with TCS-mediated down-regulation of activities of Bcr-Abl TK and the lowering of $p210^{Bcr\text{-}Abl}$ level as well as the signaling molecules such as PKC and NF-κB. Our results showed that reduction of PKC and NF-κB was obvious induced by TCS and imatinib (Fig. 4). PKC, a serine/threonine kinase that plays an essential role in the phosphorylation of proteins involved in signal transduction pathways that regulate cell proliferation and differentiation [37]. Our results indicated that inhibition of PKC activity might be a potential mechanism to induce apoptosis in K562 cell line; NF-κB was a dimmer consisting of p50 and p65 subunits, it located in cytoplasm and hang together with its inhibitor I-κB when not being activated. Research confirmed that the downstream cascade of PKC signal conduction was related to the activation of NF-κB and indicated that NF-κB participated in the signal

conduction of PKC to regulate the proliferation and apoptosis [38]. On the other hand, TCS alone did not lower Hsp90 level as well as procaspase-3 (Fig. 4), however, it is interesting that when both of TCS and imatinib were combined, Hsp90 and procaspase-3 level were lowered significantly, Hsp90, the molecular chaperone heat shock protein 90, is important in maintaining the conformation, stability, and function of $p210^{Bcr\text{-}Abl}$ [39]; Procaspase-3, an inactive 32 kDa proenzyme, is considered to be a downstream or "effector" enzyme. In agreement with previous results [40–43], we imagine that the synergism of TCS and imatinib on Hsp90 and Procaspase-3 may ascribes to the mechanism that imatinib induced apoptosis in K562 cells and sensitized K562 cells to TCS, but this is needed for further investigation.

Although it is still unknown whether or not TCS influences the level of $p210^{Bcr\text{-}Abl}$ in a direct or indirect way, its mode of action seems to be quite different, but highly complementary to that of imatinib. So the combination of imatinib and TCS represents a new model of synergistic targeting at the molecular level and might be a promising approach to improve the response rates and to prevent resistance.

To summarize, our results show a potent in vitro effects that TCS not only by itself involves but also synergizes activities of imatinib to induce K562 cell growth arrest, down-regulation of $p210^{Bcr\text{-}Abl}$ and its downstream signals and to stimulate the effect of the TK inhibition. Clinically, whether or not TCS could be used in combination with imatinib to prevent development of resistance or will translate into a better response and increase survival rates of patients with CML need further research.

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