Inhibitory effect of curcumin on proliferation of K562 cells involves down-regulation of $p210^{bcr/abl}$-initiated Ras signal transduction pathway[1]

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KEY WORDS curcumin; K562 cells; signal transduction; Bcr-Abl fusion proteins

ABSTRACT

AIM: To investigate the effects of curcumin (Cur) on proliferation of K562 cells and the relationship between these effects and Ras signal transduction pathway activated by $p210^{bcr/abl}$. **METHODS:** K562 cell line was used as a $p210^{bcr/abl}$-positive cell system and HL-60 cell line as a $p210^{bcr/abl}$-negative control; etoposide (VP-16), which has no influence on $p210^{bcr/abl}$ and has resistance to K562 cells[1], was used as an anticancer drug control to compare with curcumin. MTT was used to determine the proliferative effects of drugs on K562 and HL-60 cells. Western blot and flow cytometry were used to examine the abundance of signal protein molecules expressed in tumor cells. **RESULTS:** An exposure of K562 cells or HL-60 cells to Cur produced both concentration- and time-dependent increase in the anti-proliferative rate. Moreover, both cell lines had the same sensitivity to Cur ($P>0.05$). In contrast, HL-60 cells had more sensitivity to VP-16 than K562 cells in anti-proliferative effect ($P<0.01$). The abundance of $p210^{bcr/abl}$ as well as MEK-1 and c-JUN proteins were strongly down-regulated in curcumin-treated $p210^{bcr/abl}$-positive K562 cells while c-JUN and MEK-1 proteins were only slightly down-regulated in $p210^{bcr/abl}$-negative HL-60 cells. **CONCLUSION:** Curcumin inhibited the proliferation of K562 cells and the inhibitory effect was correlated with down-regulation of the abundance of $p210^{bcr/abl}$, which may ultimately lead to retard the Ras signal transduction pathway. Curcumin might be worthy of being evaluated as a potential chemotherapeutic agent to CML.

INTRODUCTION

Curcumin (diferuloylmethane, Cur)[2] is a polyphenol compound isolated from the rhizome of turmeric (*Curcuma longa L*) and is a major component of various recipes for curry. Cur has been reported to have several pharmacological effects including anti-tumor, anti-inflammatory, and anti-oxidant properties[3]. It has been well known that curcumin is a powerful inhibitor of proliferation of several tumor cells. Curcumin has been considered by oncologists as a potential third generation cancer chemopreventive agent, and clinical trials using it have been carried out in several laboratories[4]. However, the molecular basis of the anti-pro-

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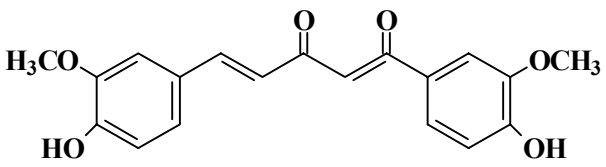
liferative effect of curcumin has not been investigated in detail. It has been reported that curcumin was a potent inhibitor of protein kinase C, EGF-receptor tyrosine kinase, and IkappaB kinase [5]. In addition, curcumin inhibits the activation of NFkappaB and the expression of c-jun, c-fos, c-myc, and iNOS[6]. Many cellular and biochemical effects of curcumin in mouse fibroblast cells have also been reported, such as inhibition of protein kinase C (PKC) activity induced by phorbol 12-myristate 13-acetate treatment, inhibition of tyrosine protein kinase activity, and inhibition of arachidonic acid (AA) metabolism[6]. Therefore, it is proposed that curcumin may suppress tumor promotion by blocking signal transduction pathways in the target cells.



Structure of curcumin ($C_{21}H_{20}O_6$, M_r 368.4)

$p210^{bcr/abl}$ encoded by Bcr-Abl fusion gene, which is present in almost all cases of human chronic myelogenous leukemia (CML), is a cytoplasmic protein that exhibits constitutive tyrosine kinase activity and initiates signaling through multiple pathways, including the ras/raf/mitogen-activated protein kinase pathway[7-9]. This $p210^{bcr/abl}$-initiated signaling decreases the ability of a variety of stimuli to induce apoptosis *in vitro*. Therefore, it is a new and attractive therapeutic strategy to target $p210^{bcr/abl}$ and $p210^{bcr/abl}$-initiated signaling. Moreover, because the $p210^{bcr/abl}$ is unique to the CML progenitor, strategies aimed at $p210^{bcr/abl}$ will not affect normal cells. Our previous work was the first to show that Cur also inhibited the activity of $p210^{bcr/abl}$ tyrosine kinase[10], therefore it may influence the downstream of this signal transduction pathway. While curcumin has several different molecular targets within the MAPK and PI3K/PKB signaling pathways that could contribute to inhibition of proliferation and induction of apoptosis[6,11], inhibition of $p210^{bcr/abl}$-initiated signaling has not been reported so far. Therefore, the ability of curcumin to modulate $p210^{bcr/abl}$-initiated signaling pathways that might contribute to K562 cell survival was investigated.

MATERIALS AND METHODS

Drugs and antibodies Curcumin was extracted from *Curcuma longa* L growing in Jianyang County, Fujian province; its purity was 97 %. Anti-$p210^{bcr/abl}$, anti-MEK-1, and anti-c-JUN monoclonal antibodies were purchased from Santa Cruz Biotechnology. Western blot kit was from Promega.

Cell culture The human leukemia cell lines K562 and HL-60 were maintained in RPMI-1640 medium supplemented with 10 % (v/v) fetal calf serum, streptomycin 100 mg/L, benzylpenicillin 100 kU/L at 37 ºC in a humidified 5 % CO_2. After incubation for 24 h, exponentially growing cells (1×10^9/L) were treated with curcumin of different concentrations (2.5, 5.0, 10 mg/L) or etoposide (VP-16) 10 mg/L for 24 h. MTT was used to determine the proliferative effects of drugs on tumor cells.

Flow cytometry (FCM) Curcumin-treated cells and control cells were collected, wased with phosphate buffered saline (PBS, dibasic sodium phosphate 9.1 mmol/L, monobasic sodium phosphate 1.7 mmol/L, and NaCl 150 mmol/L. pH was adjusted to 7.4 with NaOH), and resuspended in a final volume of 100 μL of ice-PBS. One milliliter of 70 % (v/v) ethanol in PBS was added to the resuspended cells with vigorous mixing. Fixed cells were incubated with primary Ab (anti-$p210^{bcr/abl}$, 1:400 dilution) for 30 min and then with FITC-labeled secondary IgG antibody before flow cytometry analysis. Flow cytometry measurements were made on a FACSCalibur machine, and date was analyzed with WinBryte software (Becton Dickinson).

Western blot analysis Protein was extracted from curcumin-treated cells with 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%, sodium dodecylsulfate 0.1 %, phenylmethylsulfonly fluoride 100 mg/L) supplemented with proteinase inhibitors: aprotinin 1 mg/L, leupeptin 2 mg/L, and sodium orthovanadate 100 μmol/L. Appropriate protein amounts (20 μg) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to nitrocellulose membrane (150 mA; 4 ºC) for 1.5 h. The blots were blocked in blocking-buffer (1 % BSA, Tris-HCl 20 mmol/L, pH 7.5, NaCl 150 mmol/L, 0.05 % Tween-20) at room temperature for 1 h and was followed by incubation with primary Abs (antip$210^{bcr/abl}$, antic-JUN or antiMEK-1 mAb, 1:1000 dilution) at room temperature

for 1 h and then with antirrabit or antimouse peroxidase-conjugated secondary IgG antibodies and developed with substrate, resulting in a visible color reaction on the membrane. Amount of protein bands were quantificated by scanning densitometry (Gel-Doc 1000 model, BIO-RAD).

Statistical analysis Data were expressed as mean±SD. The difference between treatment and control groups was evaluated using Student's *t*-test.

RESULTS

Effects of Cur on K562 cells or HL-60 cells Treatment with Cur at different concentrations for 24 h inhibited the proliferation of K562 cells and HL-60 cells in a concentration-dependent manner (Fig 1), Cur 10 mg/L for 24 h inhibited the proliferation of K562 and HL-60 cell lines by 54.5 % and 49.8 %, respectively. An exposure of K562 and HL-60 cell lines to Cur 10 mg/L for 0 h to 48 h resulted in time-dependent inhibition curves for both cell lines (Fig 2), the highest inhibitory rate for K562 and HL-60 was 81.9 % and 80.1 %, respectively. There was no significantly different effect of Cur on the two cell lines ($P>0.05$), which suggested that Cur had the same sensitivity to K562 as to HL-60 cells.

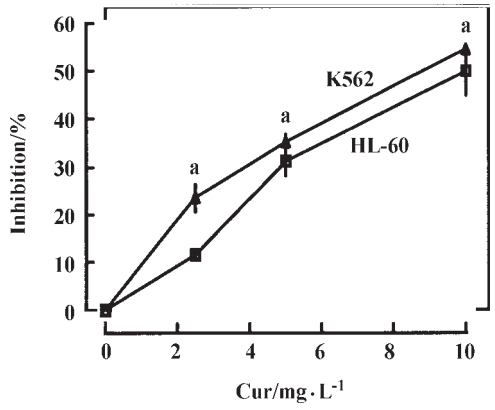


Fig 1. Concentration-dependent curve. K562 or HL-60 cells exposed to curcumin (Cur) in different concentrations (2.5, 5, 10 mg/L) for 24 h. *n*=3 assays. Mean±SD. [a]*P*>0.05 *vs* HL-60.

Effects of VP-16 on K562 cells or HL-60 cells Treatment with etoposide (VP-16) for 24 h inhibited the proliferation of K562 cells and HL-60 cells in a concentration-dependent manner (Fig 3). The effect of VP-16 on HL-60 cells was more sensitive than on K562

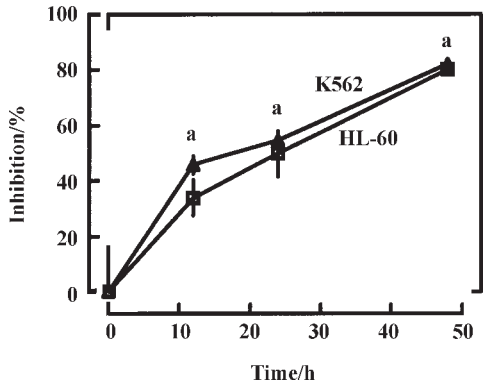


Fig 2. Time-dependent curve. K562 or HL-60 cells exposed to curcumin 10 mg/L for 12, 24, 48 h. *n*=3 assays. Mean±SD. [a]*P*>0.05 *vs* HL-60.

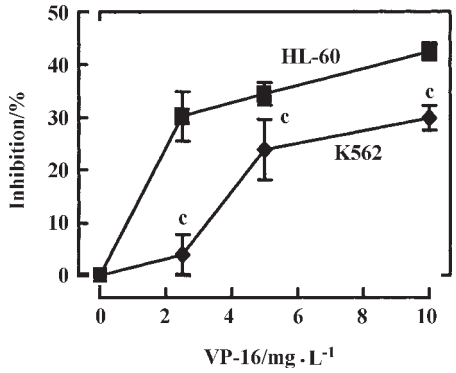


Fig 3. K562 or HL-60 cells exposed to VP-16 in different concentrations (2.5, 5, 10 mg/L) for 24 h. *n*=3 assays. Mean±SD. [c]*P*<0.01 *vs* HL-60.

cells ($P<0.01$). The different effects between curcumin and VP-16 on $p210^{bcr/abl}$-positive K562 cells and $p210^{bcr/abl}$- negative HL-60 cells provided us some evidence that curcumin inhibited the growth of K562 cells by down-regulation of $p210^{bcr/abl}$.

Effects of Cur on $p210^{bcr/abl}$ Treatment of K562 cells with curcumin resulted in a marked reduction of $p210^{bcr/abl}$ in a concentration-dependent manner after correction for differences in loading, the abundance of $p210^{bcr/abl}$ was decreased significantly ($P<0.01$) after treatment with curcumin from 0 mg/L to10 mg/L for 24 h; in contrast, VP-16 had no apparent effect on $p210^{bcr/abl}$ ($P>0.05$, Fig 4, 5).

Effects of Cur on Ras signal transducton pathway Scanning of the blots showed that treatment of $p210^{bcr/abl}$-positive k562 cells with curcumin resulted in a marked reduction of MEK-1 (Fig 5) from 19.6±1.2 to 0; and c-JUN (Fig 5) from 20.4±2.0 to 7.9±0.8 when treated with curcumin from 0 mg/L to10 mg/L for 24 h

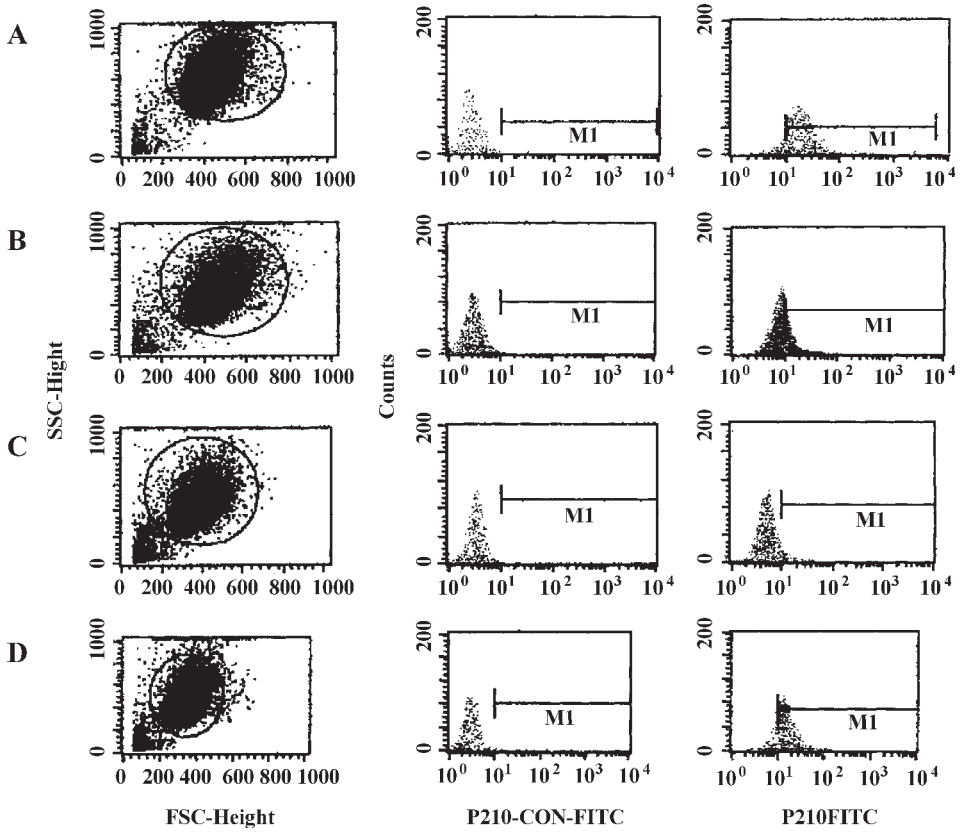


Fig 4. The positive rate of p210$^{bcr/abl}$ in K562 cells by FCM. A: Control, 90.0 %; B: Cur 5 mg/L, 5.06 %; C: Cur 10 mg/L, 1.84 %; D: Vp-16 10 mg/L, 98.51 %.

after correction for differences in loading , in contrast, MEK-1 and c-JUN were only slightly deregulated in p210$^{bcr/abl}$-negative HL-60 cells (Fig 5). VP-16 10 mg/L also deregulated MEK-1 and c-Jun in both cells.

DISCUSSION

Curcumin has been shown to inhibit proliferation of many tumor cells but its effect on K562 cells has not been investigated in detail. Results in this study showed that Cur had almost the same anti-proliferatory effect on p210$^{bcr/abl}$-positive K562 cells and p210$^{bcr/abl}$-negative HL-60 cells (P>0.05). In contrast, VP-16 had less effect on K562 cells than on HL-60 cells (P<0.01), which was consistent with the reports that p210$^{bcr/abl}$-positive K562 cells were resistant to the induction of apoptosis by VP-16[1,12]. The results implied that curcumin could reverse the ability of K562 cells to resist to chemotherapeutic agent. This effects of curcumin on K562 cells may target *bcr-abl* fusion gene or its product p210$^{bcr/abl}$, because it has been known that p210$^{bcr/abl}$, is responsible for most cases of chronic myelogenous leukemia (CML) and protection of CML cells from apoptosis by a variety of chemotherapeutic agents and this resistance to apoptosis is specifically reversed by treatment with antisense oligonucleotides that inhibit synthesis of the bcr/abl kinase[13,14]. p210$^{bcr/abl}$ is a cytoplasmic protein that exhibits a constitutive tyrosine kinase activity and initiates signaling through multiple pathways, including the ras/raf/mitogen-activated protein kinase pathway[7-9]. This p210$^{bcr/abl}$-initiated signaling decreases the ability of a variety of stimuli to induce apoptosis *in vitro*. Our results were the first to show that the abundance of p210$^{bcr/abl}$ as well as c-JUN and MEK-1 proteins were strongly deregulated in curcumin-treated p210$^{bcr/abl}$-positive K562 cells while c-JUN and MEK-1 proteins were only slightly deregulated in p210$^{bcr/abl}$-negative HL-60 cells. The result suggested that curcumin could only directly reduce c-JUN and MEK-1 proteins in p210$^{bcr/abl}$-negative HL-60 cells; while in p210$^{bcr/abl}$-positive K562 cells, in addition to direct reduction of c-JUN and MEK-1 proteins, indirect deregulation of them through reduction of their upstream p210$^{bcr/abl}$ was also significant. And as p210$^{bcr/abl}$-negative control, how were HL-60 cells inhibited by curcumin? It was reported that *bcl*-2 gene family participated in the regulatory pro-

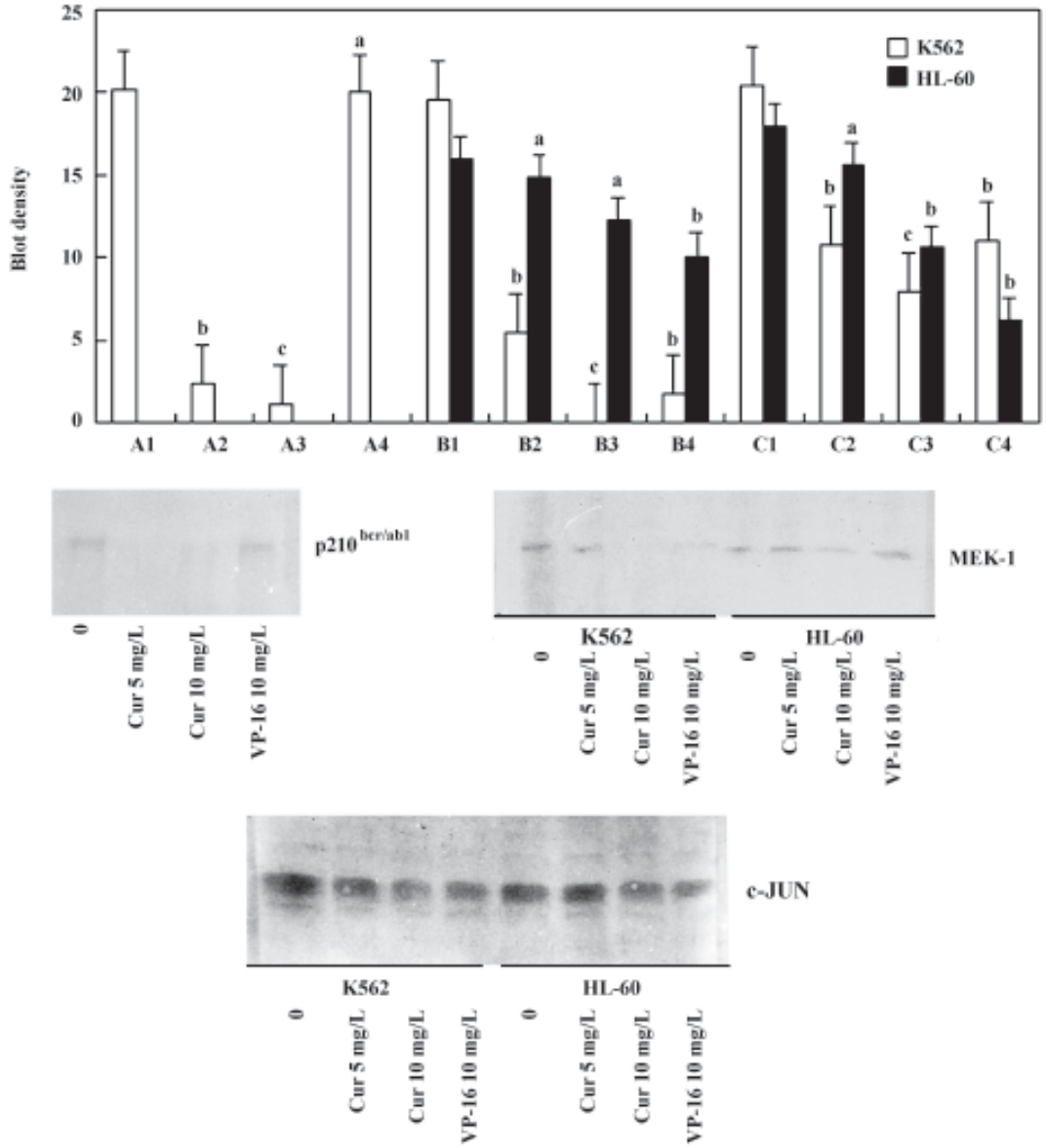


Fig 5. Effects of Cur on p210$^{bcr/abl}$ (A1-A4), MEK-1 (B1-B4), and c-JUN(C1-C4) in K562 and HL-60 cells by Western blot. The density of the blots was scanned. A1, B1, and C1 : control; A2, B2, and C2: Cur 5 mg/L; A3, B3, and C3: Cur 10 mg/L; A4, B4, and C4: VP-16 10 mg/L. *n*=3 assays. Mean±SD. $^{a}P>0.05$, $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

cess of apoptosis induced by curcumin in HL-60 cells; the level of Mcl-1 was down-regulated and that of Bax up-regulated in a time-dependent manner[15].

In general, present study demonstrated that curcumin inhibited the proliferation of K562 cells through targeting p210$^{bcr/abl}$, which, in turn, resulted in down-regulation of p210$^{bcr/abl}$-initiated Ras signaling pathway.

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