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# Down-regulation of p210$^{bcr/abl}$ by curcumin involves disrupting molecular chaperone functions of Hsp90[1]

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## Key words

curcumin; molecular chaperones; bcr-abl fusion proteins; heat shock protein 90

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## Abstract

**Aim:** To investigate the effects of curcumin (Cur) on p210$^{bcr/abl}$ level in K562 cells, and the relationship between these effects and the molecular chaperone functions of heat shock protein 90 (Hsp90). **Methods:** Flow cytometry and Western blot were used to examine the abundance of p210$^{bcr/abl}$, Hsp90, p23, Hsp70, and p60$^{Hop}$ in K562 cells treated with Cur. Reverse transcription polymerase chain reaction (RT-PCR) was used to determine the bcr-abl mRNA level in K562 cells treated with Cur. After co-immunoprecipitation of p210$^{bcr/abl}$ and its molecular chaperones, the immunoprecipitate was then subjected to Western blot analysis with anti-Hsp90, anti-Hsp70, anti-p23, and anti-p60$^{Hop}$mAb. **Results:** An exposure of K562 cells to Cur produced time-dependent down-regulation of p210$^{bcr/abl}$, the inhibition rate of p210$^{bcr/abl}$ in K562 cells determined by flow cytometry after treatment with Cur 27.2 μmol/L for 1 h, 6 h, 12 h and 24 h was 31.2%, 63.7%, 81.3% and 94.5%, respectively. In contrast, Cur had almost no influence on bcr-abl mRNA level. Treatment with Cur for 24 h reduced the association of p210$^{bcr/abl}$ with Hsp90/p23 complex, while increasing the association of p210$^{bcr/abl}$ with Hsp70/p60$^{Hop}$ complex; however, the total protein abundance of Hsp90, p23, and p60$^{Hop}$ in K562 cells had no apparent change, while Hsp70 increased greatly. **Conclusion:** Down-regulation of p210$^{bcr/abl}$ by Cur involves dissociating the binding of p210$^{bcr/abl}$ with Hsp90/p23 complex. In contrast, the association of p210$^{bcr/abl}$ with Hsp70/p60$^{Hop}$ complex increased.

## Introduction

Molecular chaperones are proteins that are responsible for maintaining the correct folding, function and stability of client proteins. Of these, heat shock protein 90 (Hsp90) has recently emerged as a focus of interest because of its role in regulating proteins that are responsible for malignant transformation. Approximately 50 proteins have been identified as clients of Hsp90[1]. Most of these proteins play important roles in the control of cell cycle, growth and apoptosis and their dysregulated function might lead to transformation. Examples include bcr-abl, Her2, Raf-1, Akt, Cdk4, mutant p53, estrogen and androgen receptors. In the absence of Hsp90, or when the function of Hsp90 is disrupted, multiple client proteins are targeted for ubiquitination and proteasomal degradation. This, in turn, leads to growth arrest and apoptosis in cancer cells *in vitro*, and to inhibition or regression of tumor growth in animals[2].

Hsp90 is present in cells in equilibrium between a low chaperoning activity 'latent state' and an 'activated state', with increased chaperoning efficiency. The shift in equilibrium might be dictated by the amount of 'stress' on the system, such as mutated and dysregulated proteins[3]. Thus, the effects of inhibiting Hsp90 function could depend more on the 'activity' and degree of involvement of the co-chaperone-protein-Hsp90 complexes and less on its cellular levels. Hsp90 participates in at least two multimolecular chaperone complexes that then associate with client proteins. One such complex comprises Hsp90, p23, and p50 (immunophilin in the case of steroid receptors), and the other consists of

Hsp90, Hsp70, and $p60^{Hop}$. Association of client proteins with a P23-containing Hsp90 complex correlates with functionality; however, association with a complex containing Hsp70 and $p60^{Hop}$ may not[4]. Collectively, Hsp90 inhibitors will disrupt crucial chaperone functions in a transformed cell, which might not be toxic to normal cells. These studies confirm that Hsp90 is a promising target for novel cancer therapeutics and pave the road for the introduction of Hsp90 inhibitors in the treatment of cancers. Recently, attention has been directed at the development of pharmacological Hsp90 inhibitors as chemotherapeutic agents. Such efforts have focused on the ansamycin antibiotics, including geldanamycin (GA) and its closely related analogue, 17-AAG[5].

Chronic myelogenous leukemia (CML) is a myeloproliferative disease characterized by a well-defined genetic abnormality involving the bcr-abl translocation, which occurs in the Philadelphia (Ph) chromosome. This genetic alteration results from a chromosome 9:22 translocation that leads to expression of a chimeric fusion protein, bcr-abl, with deregulated tyrosine kinase activity[6]. The bcr-abl fusion protein and its constitutively-activated tyrosine kinase activity are essential for malignant progression in CML. Because of this characteristic feature of CML, the bcr-abl kinase is a good candidate for molecular-targeted chemotherapy. Earlier studies have demonstrated that multiple signal transduction pathways are involved in abnormal growth signaling by bcr-abl, including Ras, Stat5, and phosphatidylinositol-3 kinase[7].

Because $p210^{bcr/abl}$ is one of the client proteins of Hsp90, disruption of the chaperone functions of Hsp90 may reduce the protein level of $p210^{bcr/abl}$, and therefore potentially retard several signal transduction pathways initiated by $p210^{bcr/abl}$.

Curcumin (Cur), a natural compound present in turmeric, possessing both anti-inflammatory and antioxidant effects, has been studied vigorously as a chemopreventative agent in several cancer models[8]. Because curcumin has already been shown to have low systemic toxicity in animal and human studies[9], we explored the effect of curcumin on K562, a human CML cell line that expresses $p210^{bcr/abl}$. Our previously published work was the first to show that curcumin inhibited the proliferation of K562 cells and the inhibition effect was correlated with down-regulation of $p210^{bcr/abl}$/Ras/Raf/MEK-1/ERK/Elk-1 and $p210^{bcr/abl}$/Ras/MEKK/SEK/JNK/c-Jun signal transduction pathway[10]. But why was curcumin able to block several signal pathways at the same time? How dose curcumin down-regulate the $p210^{bcr/abl}$ protein level? By decreasing its synthesis or by increasing its degradation? Will curcumin be able to influence the chaperone function of Hsp90? With these questions in mind, we have now examined the effects of curcumin on $p210^{bcr/abl}$ and the functions of its molecular chaperone, Hsp90, in more detail.

In present study, we have demonstrated that curcumin time-dependently depleted $p210^{bcr/abl}$ by disrupting its binding with the molecular chaperone, Hsp90. The present study suggests that curcumin could be worthy of being evaluated as a potential chemotherapeutic agent to CML.

## Materials and methods

**Drugs and antibodies** Curcumin was extracted and purified from *Curcuma longa* L growing in Jianyang County, Fujian province; its purity was 97%. Anti-$p210^{bcr/abl}$, anti-Hsp90, and anti-Hsp70 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin monoclonal antibody was purchased from NeoMarkers (Fremant, CA), anti-$p60^{Hop}$ and anti-p23 monoclonal antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Protein A-Sepharose was purchased from BOEHRINGER MANNHEIM (Gmbh, Germany).

**Cell culture** The CML cell line K562 was maintained in RPMI-1640 medium supplemented with 10% (*v*/*v*) fetal calf serum, streptomycin 100 mg/L, penicillin 100 IU/L at 37 ºC in humidified 5% $CO_2$. After incubating for 24 h, exponentially growing cells ($1\times10^9$/L) were treated with curcumin of different concentrations (13.6 or 27.2 μmol/L) or GA 5 μmol/L for the indicated length of time.

**Flow cytometry** Cells ($1\times10^7$/aliquot) treated with curcumin for different lengths of time were collected, washed 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.) 3 times, 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 and was mixed vigorously. Cells were then fixed overnight. Fixed cells were incubated with primary Abs (anti$p210^{bcr/abl}$, 1:400 dilution) for 1 h before being washed with PBS 3 times, and then incubated with Fluorescein (mistakenly abbreviated by its commonly-used reactive isothiocyanate form, FITC) -labeled secondary IgG antibodies for 30 min before flow cytometry analysis. K562 cells without curcumin treatment were used as $p210^{bcr/abl}$ positive control, and K562 cells incubated with secondary IgG antibodies directly but without primary Abs as negative control. Flow cytometry measurements were made on a FACSCalibur machine, and the data was analyzed with WinBryte software (Becton Dickinson).

**Western blot analysis** Protein was extracted from

curcumin-treated cells with a 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%, phenylmethylsulfonyl 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) for 1 h at room temperature, which was followed by incubation with primary Abs (anti-$p210^{bcr/abl}$, anti-Hsp90, anti-Hsp70, anti-$p60^{Hop}$, anti-p23 or anti-Actin mAb, 1:1000 dilution) for 1 h at room temperature and then with antimouse peroxidase-conjugated secondary IgG antibodies and developed with substrate, resulting in a visible color reaction on the membrane.

**Co-immunoprecipitation[5] and Western blot** Curcumin-treating cells ($1\times10^7$/aliquot) and control cells were washed twice with ice-cold PBS and resuspended in 1 mL of ice-cold lysis buffer. All further steps were performed at 4 ºC. After a 15-min incubation, samples were sedimented at 12 000×*g* for 5 min. Supernatants were left to react overnight with 5 μg of monoclonal anti-$p210^{bcr/abl}$ antibody, diluted with 30 μl of pre-swollen protein A-Sepharose beads, and incubated for an additional 2 h with gentle agitation. The beads were sedimented at 3200×*g* for 2 min, washed 4 times with 1 mL aliquots of wash buffer [150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.5), 1 mmol/L sodium orthovanadate, 10% (*v*/*v*) glycerol, 0.1% (*w*/*v*) Triton X-100, and 1% (*w*/*v*) thiodiglycol], and eluted by heating for 20 min at 65 ºC with 50 μL of SDS sample buffer consisting of 2% (*w*/*v*) SDS, 62.5 mmol/L Tris-HCl (pH 6.8), 1mmol/L EDTA, and 5% (*v*/*v*) β-mercaptoethanol. The immunoprecipitate was subjected to electrophoresis and immunoblotting as described previously.

**$p210^{bcr/abl}$ mRNA expression**[11] Total cellular RNA was isolated from untreated and curcumin-treated k562 cells using TRIzol according to the manufacturer's instruction. First strand cDNA synthesis was performed using random hexamers. The sequence of primers are as follows: bcr-abl sense 5'-CTCCAGACTGTCCACAGCATTCCG-3'; anti sense 5'-TCAGACCCTGSGGCTCAAAGTC-3'; β-actin sense 5'-TACCTCATGAAGATCCTCA-3'; antisense 5'-TTCGTGGATGCCACAGGAC- 3'. The reactions were denatured for 1 min at 95 ºC, annealed for 2 min at 55 ºC, and extended at 72 ºC for 1.5 min in a Perkin-Elmer Thermal Cycler 480 (Branchburg, NJ, USA). PCR products were separated on 2% agarose gels containing ethidium bromide and were photographed under UV light.

## Results

**Curcumin time-dependently depleted $P210^{bcr/abl}$** Treatment with Cur inhibited the proliferation of K562 cells in a concentration- and time-dependent manner, Cur 27.2 μmol/L for 24 h inhibited the proliferation of K562 by 54.5%[10]. When K562 cells were treated with Cur 27.2 μmol/L, down-regulation of $p210^{bcr/abl}$ (Figure 1) was observed from 1 to 24 h. The inhibition rate of 1 h, 6 h, 12 h, and 24 h was 31.2%, 63.7%, 81.3%, and 94.5%, respectively. This result demonstrated that curcumin was able to deplete the abundance of $p210^{bcr/abl}$ quickly. The half-life of $p210^{bcr/abl}$ in K562 cells after curcumin treatment was estimated to be 5 h.

**Effects of curcumin on $p210^{bcr/abl}$ mRNA expression levels** To further elucidate the mechanism responsible for the changes in amounts of $p210^{bcr/abl}$ protein, the levels of $p210^{bcr/abl}$ mRNA were determined. In contrast to the protein levels, the $p210^{bcr/abl}$ mRNA expression levels had no apparent change after treatment with Cur 27.2 μmol/L for 1 h, 6 h, 12 h, 24 h, or 48 h (Figure 2).

**Effects of curcumin on the Hsp90/$p210^{bcr/abl}$ complex** When K562 cells were treated with Cur for 24 h, total lysate was prepared, resolved by SDS-PAGE, and analyzed by Western blotting. Although Cur treatment induced obvious down-regulation of $p210^{bcr/abl}$, the drug induced no clear decrease in Hsp90, p23, and $p60^{Hop}$; while the abundance of Hsp70 protein increased significantly (Figure 3). Next $p210^{bcr/abl}$ was immunoprecipitated from untreated cells or Cur 27.2 μmol/L for 24 h to examine the effects of drug exposure on the association of $p210^{bcr/abl}$ with Hsp90 and other associated chaperones (Figure 4). After immunoprecipitation with anti-$p210^{bcr/abl}$ and blotting with anti-Hsp90, anti-p23 and anti-Hsp70, the abundance of Hsp90 or p23 protein binding with $p210^{bcr/abl}$ decreased significantly, in contrast, Hsp70 binding with $p210^{bcr/abl}$ increased dramatically, while the amounts of $p60^{Hop}$ protein binding with $p210^{bcr/abl}$ had no apparent change. These data show that the exposure of K562 cells to Cur 27.2 μmol/L for 24 h dissociated $p210^{bcr/abl}$ from Hsp90/p23 complexes, but drug treatment increased the association of the protein with Hsp70/ $p60^{Hop}$ complexes.

## Discussion

Curcumin (diferuloylmethane) is a polyphenol derived from the plant *Curcuma longa*, which is commonly used as a yellow coloring and flavoring agent in foods. Extensive published research over the last 50 years has indicated that this polyphenol can both prevent and treat cancer[8].

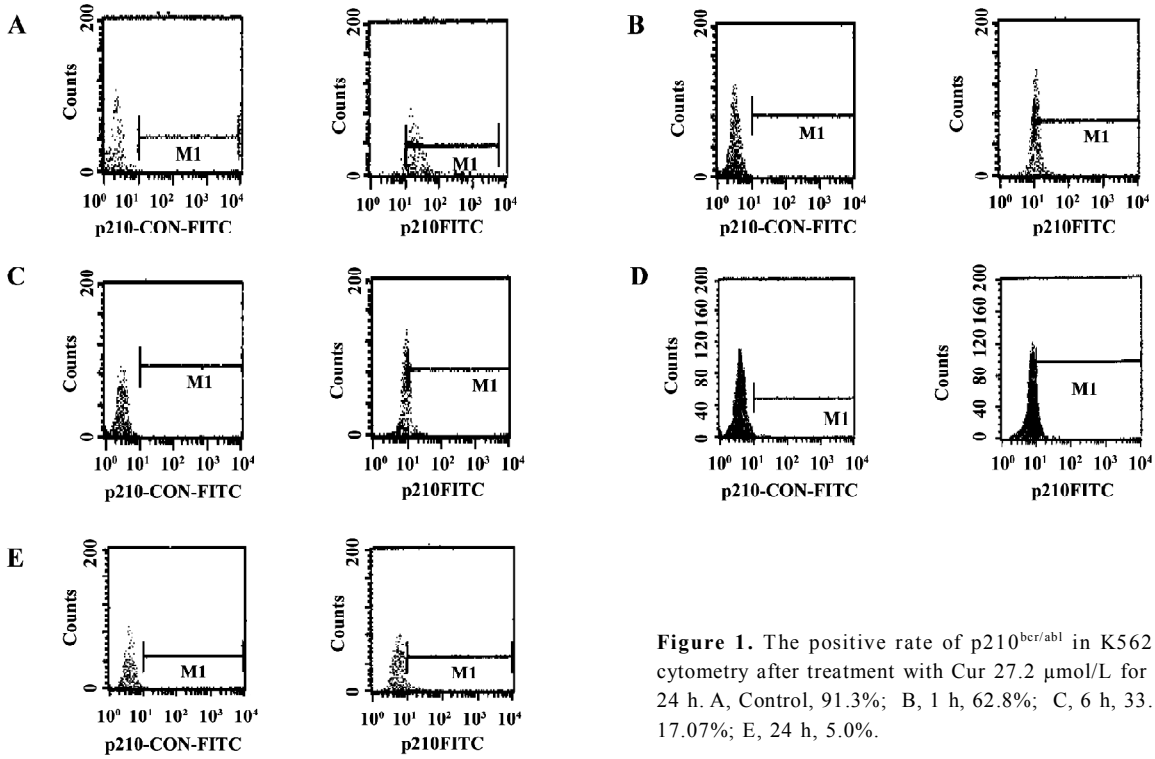


**Figure 1.** The positive rate of p210$^{bcr/abl}$ in K562 cells by flow cytometry after treatment with Cur 27.2 μmol/L for 1 h, 6 h, 12 h, 24 h. A, Control, 91.3%; B, 1 h, 62.8%; C, 6 h, 33.14%; D, 12 h, 17.07%; E, 24 h, 5.0%.

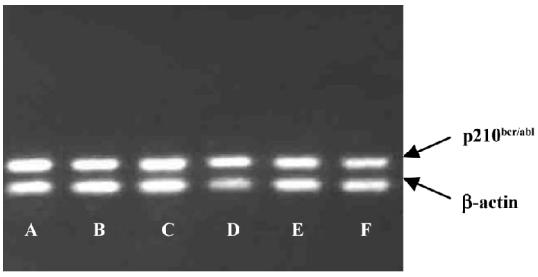


**Figure 2.** The mRNA expression levels of p210$^{bcr/abl}$ in K562 cells with RT-PCR after treatment with Cur 27.2 μmol/L for 1 h (B), 6 h (C), 12 h (D), 24 h (E), 48 h (F). A, Control.

Recently, curcumin has been considered by oncologists as a potential third generation cancer chemopreventive agent, and clinical trials have been carried out in several laboratories. It has been shown to be a potent inhibitor of protein kinase C, EGF-receptor tyrosine kinase, c-Jun N-terminal kinase, protein tyrosine kinases, protein serine/threonine kinases and IkappaB kinase[12]. In addition, curcumin inhibits the activation of NFkappaB and the expression of c-jun, c-fos, c-myc, cyclin D1, NIK, MAPK, ERK, ELK, PI3K, Akt, CDK, HER2 and iNOS[13]. Curcumin inhibited p185$^{neu}$ *in vitro* and depleted p185$^{neu}$ protein *in vivo* by disrupting its binding with the molecular chaperone GRP94 (glucose-regulated

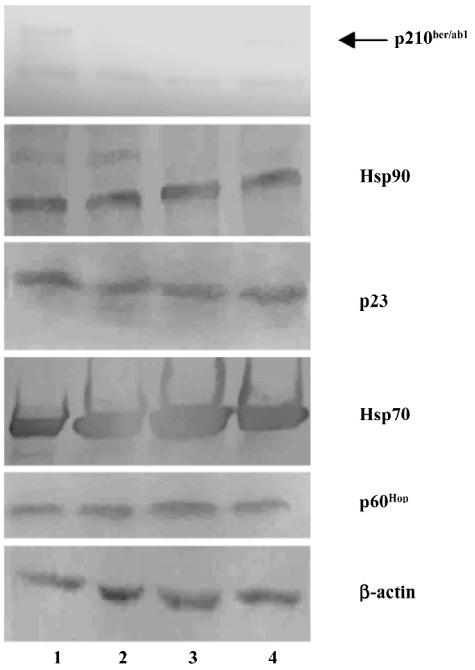


**Figure 3.** Effects of Cur on Hsps in K562 cells. Lysates of K562 CML cells were immunoblotted with the appropriate antibodies. 1, control; 2, Cur 13.6 μmol/L; 3, Cur 27.2 μmol/L; 4, GA 5 μmol/L. This result showed that Cur treatment down-regulated p210$^{bcr/abl}$ markedly, and increased the protein level of Hsp70 significantly. The amounts of Hsp90, p23, and p60$^{Hop}$ in total lysates had no apparent changes.

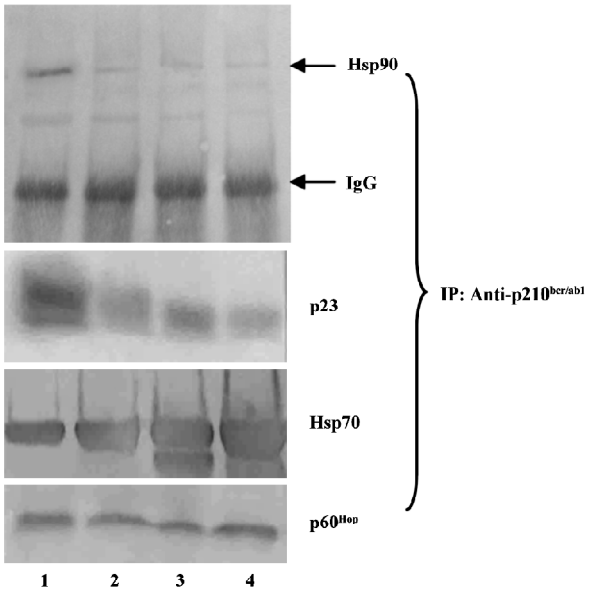


**Figure 4.** Treatment with Cur altered the composition of multimolecular chaperone complexes associated with $p210^{bcr-abl}$. Lysates of K562 CML cells were immunoprecipitated with $p210^{bcr/abl}$ antibody. Coprecipitation of members of Hsp90 multimolecular complexes was detected using immunoblotting with the appropriate antibodies. 1, control; 2, Cur 13.6 μmol/L; 3, Cur 27.2 μmol/L; 4, GA 5 μmol/L. This result showed that Cur treatment shifted the binding of $p210^{bcr/abl}$ from Hsp90/p23 to Hsp70/$p60^{Hop}$.

protein)[14].

Results in this study showed that curcumin reduced the protein level of $p210^{bcr/abl}$ in a time-dependent manner. After curcumin treatment for 6 h, the inhibition rate of $p210^{bcr/abl}$ was 63.7%. It has been reported that the normal half-life of $p210^{bcr/abl}$ protein is in excess of 24 h[15]; our data showed that curcumin treatment significantly decreased the half-life of $p210^{bcr/abl}$ to about 5 h. Additionally, the $p210^{bcr/abl}$ mRNA expression levels had no apparent change after treatment with Cur 27.2 μmol/L. Collectively, these data showed that the ability of curcumin to deplete $p210^{bcr/abl}$ within 24 h might not be a result of the inhibition of bcr/abl mRNA synthesis but is, at least in part, a result of an increase in protein degradation.

Why was curcumin able to down-regulate a variety of transcription factors and signaling protein kinases? Moreover, many of these proteins are the client proteins of Hsp90. Our data in the present study is the first to demonstrate that curcumin is able to disrupt the molecular chaperone functions of Hsp90. This is a newly discovered mechanism of the anti-cancer effect of curcumin. The result of co-immunoprecipitation analysis showed that the $p210^{bcr/abl}$ protein formed a stable complex with Hsp90 and p23 in the absence of curcumin (Figure 4). This demonstrates that most of the $p210^{bcr/abl}$ protein in untreated K562 cells exist in the mature Hsp90 complex. More importantly, after the addition of curcumin, $p210^{bcr/abl}$ protein was dissociated from an Hsp90/p23 chaperone complex and associated instead with Hsp70/$p60^{Hop}$ (Figure 4). Disruption of Hsp90/p23/ $p210^{bcr/abl}$ complexes may be followed rapidly by the proteolytic degradation of $p210^{bcr/abl}$. An ubiquitin-dependent proteasome-mediated pathway may be implicated with this phenomenon because most client proteins have been reported to be degraded by the proteasome system[5]. Further study is required to examine this phenomenon and to systematically test this possibility. Another possibility, that some $p210^{bcr/abl}$ may be degraded in a Hsps-independent pathway, such as the disruption of post-transcriptional activity of bcr/abl mRNA, also requires further study. Our results were similar to the research of Blagosklonny *et al*[16], who showed that composition of the multichaperone complexes associated with $p210^{bcr/abl}$ was altered by geldanamycin before destabilization of these kinases, so that p23 association was lost and Hsp70/$p60^{Hop}$ association either increased or was unaffected. All of these data suggest that curcumin inhibits the transition of $p210^{bcr/abl}$ from an immature $p60^{Hop}$-Hsp70 complex to a complex including Hsp90-p23 that allows the chaperoned protein to acquire a functional and perhaps stable conformation. Although the function of the $p60^{Hop}$-Hsp70 complex remains vague, it has been proposed to mediate proteolytic degradation of its associated client protein. The study of Schneider *et al* showed that Hsp90, in cooperation with Hsp70, p60 and other factors, functioned as a quality control system in the refolding or degradation of client proteins[17]. These specific Hsp90 substrates may have an intrinsic structural instability under normal cellular conditions, and this may render them especially sensitive to Hsp90 inhibitors. Normal dissociation of Hsp90 from client proteins depends on the ATPase activity of Hsp70 and is mediated by p60[17].

In conclusion, we offered evidence that curcumin inhibited $p210^{bcr/abl}$, dissociated the binding of $p210^{bcr/abl}$ with Hsp90/p23 complex. In contrast, association of $p210^{bcr/abl}$ with Hsp70/$p60^{Hop}$ complex increased. Pharmacologically, curcumin has been found to be safe. Human clinical trials indicate no dose-limiting toxicity when administered at doses of up to 10 g/d[8]. All of these studies suggest that curcumin has enormous potential in the therapy of CML and other cancers.

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