

Full Length Research Paper

Antitumor effects of cucurmosin in human chronic myeloid leukemia occur through cell cycle arrest and decrease the Bcl-2/Bax ratio to induce apoptosis

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Accepted 1 June, 2011

Cucurmosin, a novel type 1 ribosome-inactivating protein, induced cell death in various cell types including several tumor cell lines. However, the mechanism remained largely uncharacterized. In this study, we investigated the possible mechanism underlying its cytotoxicity by using K562 cells. In the study, typical characteristics of apoptosis, such as cell shrinkage, apoptotic bodies, cell cycle arrest and DNA fragmentation, were observed in cucurmosin treatment. The loss of the MMP ($\geq \psi_m$) and the content of cytochrome c in cytolis were increased in cucurmosin treatment. Cucurmosin-treated activated caspase-3, down regulated the expression of anti-apoptotic Bcl-2 protein and upregulated the expression of pro-apoptotic Bax protein, eventually leading to a decrease of the Bcl-2/Bax ratio in K562 cells. Down-regulation of p210^{Bcr-Abl} protein level in cucurmosin treatment was observed. In addition, Hsp90, the molecular chaperone of p210^{bcr/abl} was also down-regulated in cucurmosin treatment. Our data indicated that cucurmosin induced apoptosis of K562 cells by mitochondria dysfunction and the down-regulation of P210^{bcr/abl} by disrupting molecular chaperone functions of Hsp90 might be also one of the mechanisms implicated in the cucurmosin-mediated apoptosis in the K562 cells. Thus, this study provided the rationale to investigate cucurmosin as a potential therapeutic agent for leukemia cells.

Key words: Cucurmosin, chronic myeloid leukemia, k562 cells, apoptosis.

INTRODUCTION

Cucurmosin is an active component extracted from the sarcocarp of *Cucurbita moschata* (pumpkin) which is a kind of vegetable and has long been used for medicinal purpose in China. Recently, it has been proved to be one of type 1 ribosome-inactivating proteins (RIPs). The complete mature protein sequence is obtained from N-terminal protein sequencing and partial DNA sequencing. The structure has been determined by high

resolution crystal structure analysis at 1.04 Å resolution. It contains two domains: a larger N-terminal domain of seven α -helices and eight β -strands and a smaller C-terminal domain consisting of three α -helices and two β -strands (Hou et al., 2008).

Our previous studies indicated that cucurmosin possessed an rRNA N-glycosidase activity (Chen et al., 2000) and had potential anti-tumor effect on several cell lines of tumor, especially strong for K562 cells. The IC₅₀ values were 14.84, 3.25, 0.94 μ g/ml, respectively, for 24, 48 and 72 h in K562 cells, a human chronic myeloid leukemia (CML) cell line expressing p210^{bcr/abl} (data not shown). However, the underlying mechanism remained unknown. In this study, we investigated the effects of

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cucurmosin on K562 cell apoptosis and its molecular mechanism. The p210^{bcr-abl}-positive K562 cell line was used as a cellular model of CML for drug screening.

MATERIALS AND METHODS

Cells culture and cucurmosin treatment

Cucurmosin (98% purity) was isolated from the sarcocarp of *Cucurbita moschata* (pumpkin) by this laboratory (3.68 mg/ml) and prepared as 10 mmol/l stock solution in double distilled water at -20°C. Stock solutions were diluted in RPMI 1640 medium (Gibco BRL, USA) to achieve the final concentration. K562 cells (Ph⁺) (Shanghai Institute of Biochemistry and Cell Biology, China) were suspended in complete medium. RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma, USA) and gentamicin (80 U/100 ml). They were cultured in 6-well culture plates at 37°C 5% CO₂ humidified atmosphere, with the density of cells 2×10³ /ml. Three wells composed one treatment group. There were several treatment groups, with the treatment of 2.5 µg/ml cucurmosin for 24, 48 and 72 h. K562 cells without the treatment of cucurmosin were provided as a negative control. At the end of culture, the cells were harvested and the following assays were performed.

Ethidium bromide and acridine orange staining

K562 cells were suspended in complete medium at 1×10⁶ /ml. 100 µl cell suspension was placed on the cover slip and 4 µl of ethidium bromide and acridine orange (AO/EB; Beyotime, China) staining mixture (Sigma, USA) was added. After being incubated at 37°C for 2 mins, the stained nuclei was viewed under an Olympus microscope (Hatagaya, Tokyo, Japan).

Transmission electron microscope examination (TEM)

After exposure to cucurmosin, cells were fixed in ice-cold 2.5% glutaraldehyde and preserved at 4°C for further processing. When processing resumed, cells were post fixed in 1% osmium tetroxide in the same buffer, dehydrated in graded alcohols, embedded in absolute resin at 60°C, sectioned with an ultramicrotome, then stained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (Philips CM10, the Netherlands).

DNA ladder agarose-gel electrophoresis assay

Fragmented DNA was isolated using a DNA extraction kit according to the manufacturer's instructions (Beyotime, China). DNA samples were analyzed by electrophoresis using a 1.5% agarose gel containing 0.2 µg/ml ethidium bromide and visualized under UV illumination.

Flow cytometry analysis of apoptosis and cell cycle

Cells were washed twice with ice-cold phosphate-buffered saline (PBS), and fixed with 70% ethanol at 4°C overnight. After washing with PBS, cells were incubated in 0.5 ml PBS containing 50 µg/ml RNase A for 30 min at 37°C, and then added propidium iodide (PI; Sigma, USA) to achieve the final concentration of 50 µg/ml for 30 min on ice in the dark. The resultant cell suspension was then

subjected to flow cytometry analysis (Epics XL; Beckman Coulter, USA). Cells with fractional DNA content that located on DNA frequency histograms to the left of the G₁ peak (sub-G₁ cells) were identified as apoptotic cells. The percentage of apoptotic cells and cells in G₀/G₁, S and G₂/M phases were calculated.

Mitochondrial membrane potential (MMP) loss assessment

The mitochondrial membrane potential indicator rhodamine123 (Beyotime, China) (50 nmol/L final concentration) was added to K562 cell suspension (4×10⁵ cells/ml) and incubated at 37°C for 20 min. The loss of the MMP (ΔΨ_m) was assessed with flow cytometry.

Preparation of cytosolic fractions

The cells were harvested and rinsed with ice-cold PBS, and the cell pellets were resuspended in 300 µl of buffer A [20mM HEPES-KOH (pH 7.5), 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT] containing 250 mM sucrose and the proteinase inhibitors. After homogenization, the unbroken cells, the large plasma membrane and the nuclei were removed by centrifugation at 1,000 × g for 1 min at 4°C. The supernatant was then centrifuged at 10,000 × g for 20 min at 4°C. The pellet fraction containing the mitochondria was dissolved in 50 µl of the TNC buffer [10 mM Tris acetate (pH 8.0), 0.5% NP40, and 5 mM CaCl₂]. The supernatant was then centrifuged at 50,000 × g for 5 min to obtain the cytosol, which was immediately subjected to Western blot analysis.

Western blot analysis

Briefly, the cells were collected and lysed. Protein concentration was determined by the Lowry method using BSA as a standard. Equal amounts of protein were separated on 6 to 12% SDS/PAGE gels. The protein was then electrophoretically blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were hybridized with primary antibodies and then with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Sigma, USA). The primary antibodies were antibodies against the human cytochrome c, Bax, bcl-2, cleaved caspase 3 (P20), Hsp90, abl, and β-actin (Santa Cruz, USA). The immune blots were developed using enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Statistical analysis

All experiments were performed in triplicate. The data were expressed as mean ± SD, and Student's t-test was used to determine the significance of differences in multiple comparisons p<0.05 was considered to be statistically significant.

RESULTS

AO/EB fluorescent staining

After treatment with cucurmosin for 48 h, the cells were stained by AO/EB. The results demonstrated that apoptotic morphological changes could be detected (Figure 1).

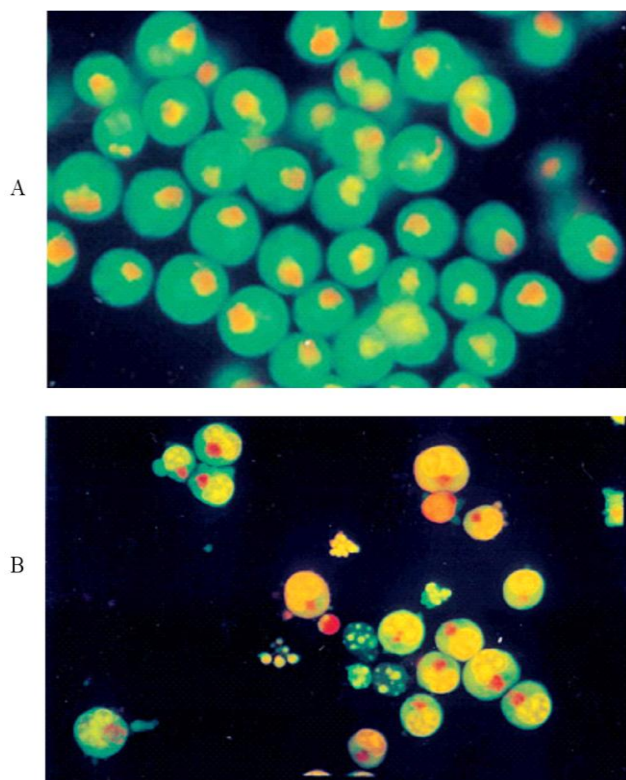


Figure 1. Ethidium bromide and acridine orange staining. K562 cells were exposed to solvent or 2.5 µg/ml curcumin for 48 h, then analysed directly under fluorescence microscope (A. control, B. drug treatment, magnification×400).

Transmission electron microscopy assay

Cellular morphological changes were also investigated by Transmission electron microscope. As shown in Figure 2, K562 cells after curcumin treatment displayed the characteristics of apoptosis, including chromatin condensation, nuclear shrinkage and “apoptotic body”.

DNA ladder agarose-gel electrophoresis assay

As shown in Figure 3, the analysis of the DNA fragmentation showed curcumin increased the cellular DNA degradation in time-dependent manner.

Cell apoptosis and cell cycle analysis

As shown in Table 1, after being cultured with 2.5 µg/ml curcumin for 24, 48 and 72 h, the percentage of sub-G₁ was 17.2±2.9, 35.5±3.3 and 47.9±2.4%, respectively, much higher than that (1.1±0.7%) in the control. Cell cycle analysis showed similar results. When K562 cells were treated with 2.5 µg/ml curcumin for 24, 48 or 72 h, an

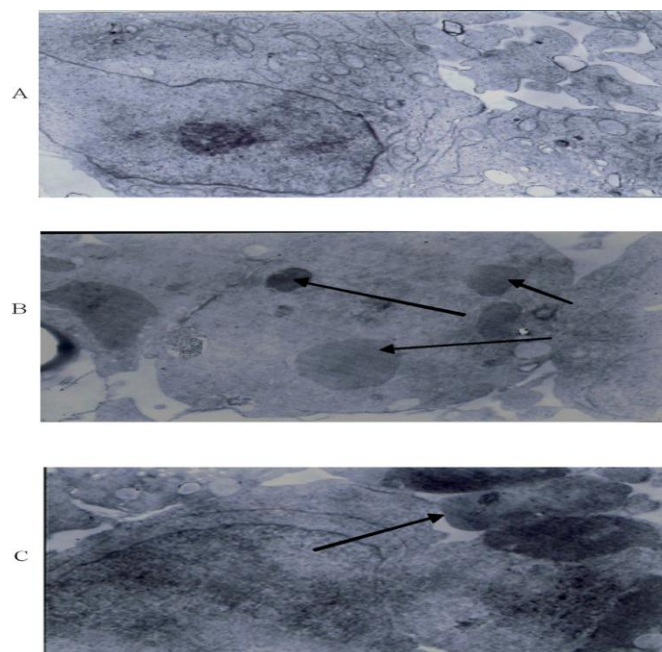


Figure 2. Effect of curcumin in inducing apoptosis as measured by Transmission electron microscope. K562 cells were exposed to solvent or 2.5 µg/ml curcumin for 0, 24, 48 h, then analysed under transmission electron microscope (A) control, (B) 24 h, (C) 48 h, magnification×8000), (arrows represent nuclear shrinkage and apoptotic body for B and C, respectively).

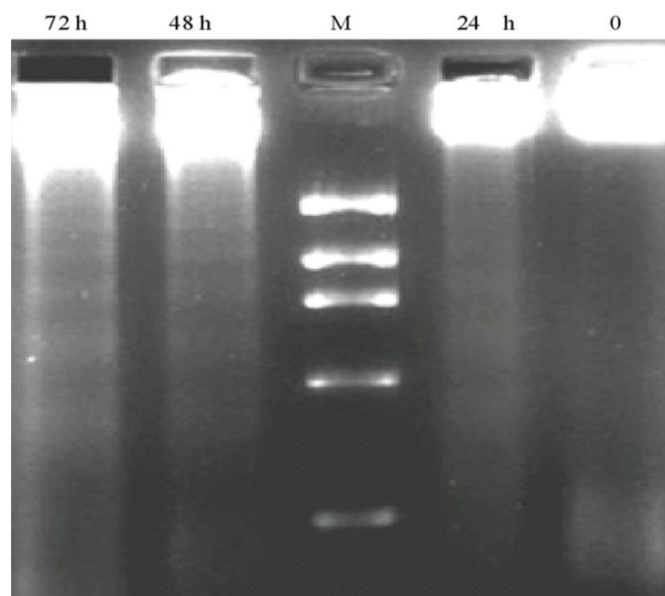


Figure 3. Effect of curcumin in inducing apoptosis as measured by the DNA fragmentation assay. K562 cells were exposed to 2.5 µg/ml curcumin for 0, 24, 48 and 72 h, respectively, after which the total genomic DNA was extracted and resolved on 1.5% agarose gel. The apoptotic DNA fragmentation was visualized by ethidium bromide staining and photographed under UV illumination. Lane M. DNA marker.

Table 1. Cell apoptosis of cucurmosin-treated K562 cells by flow cytometric analysis (n = 3). At the indicated time points after treatment with 2.5 µg/ml cucurmosin the cells were harvested and fixed in 70% ethanol. After stained with propidium iodide, DNA content was analysed by flow cytometry. The percentage of cells in the sub-G1 phase was regarded as the apoptotic rate *p < 0.05 vs control.

| Group | Sub-G ₁ (%) |
|---------------|------------------------|
| Control | 1.1±0.7 |
| 2.5µg/ml, 24h | 17.2±2.9* |
| 2.5µg/ml, 48h | 35.5±3.3* |
| 2.5µg/ml, 72h | 47.9±2.4* |

Table 2. Cell cycle distribution of cucurmosin-treated K562 cells by flow cytometric analysis (n=3). K562 cells were treated with 2.5 µg/ml cucurmosin for 0, 24, 48 and 72 h, respectively. The percentage of the cell population found within the G0/G1, S and G2/M phases was identified according to the DNA content quantified by flow cytometry after propidium iodide staining. Data represent the means ± SD among counts of three independent experiments *p < 0.05 vs control.

| Group | G1/G0 (%) | S (%) | G2/M (%) |
|---------------|-----------|-----------|----------|
| Control | 30.7±2.1 | 57.9±1.9 | 11.4±2.2 |
| 2.5µg/ml, 24h | 44.1±1.7* | 45.7±2.4* | 10.2±1.8 |
| 2.5µg/ml, 48h | 49.1±1.9* | 38.2±2.3* | 12.6±2.4 |
| 2.5µg/ml, 72h | 51.5±3.4* | 36.6±2.7* | 11.9±2.1 |

increase in the number of cells in the G0/G1 and a decrease in the number of cells in the S phases were evident, compared with control group (Table 2).

Effect of cucurmosin on MMP

The amount of MMP disruption was assessed after cucurmosin treatment by measuring the mitochondrial uptake of a membrane potential-sensitive dye, rhodamine123, in K562 cells. When the cells were treated with 2.5 µg/ml cucurmosin for 24, 48 and 72 h, the percentage of cells losing MMP was 57.6, 77.1 and 91.2%, respectively, compared with control group (0.7%) (Figure 4). This suggested that an apoptosis-inducing mechanism triggered by cucurmosin operated via the mitochondria in K562 cells.

Effect of cucurmosin on the content of cytochrome c

Perturbations in MMP and the release of cytochrome c could be one of the most conspicuous manifestations of apoptosis. To analyze the quantity of cytochrome c in cytosol, the protein of cytosolic fractions was extracted.

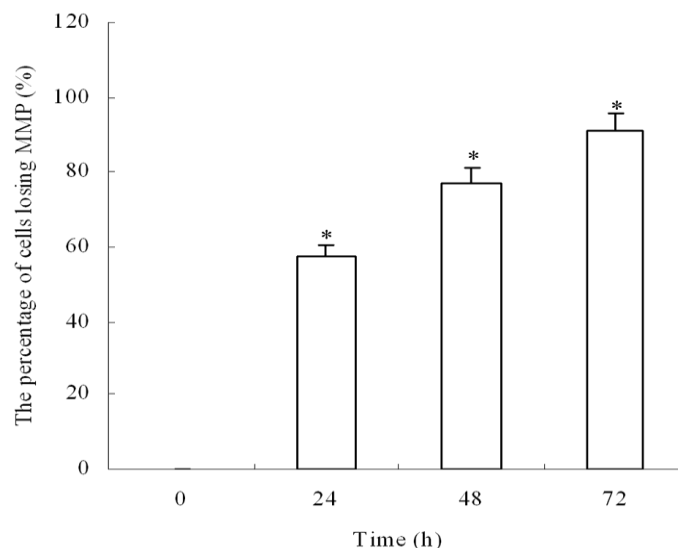


Figure 4. Cucurmosin induces the mitochondrial membrane damage. K562 cells were exposed to 2.5 µg/ml of cucurmosin for the indicated times, after which the loss of the mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by flow cytometry, as described in "Materials and Methods". The values were expressed as the mean percentage of cells exhibiting a low MMP, as reflected by reduced levels of Rhodamine123 uptake.

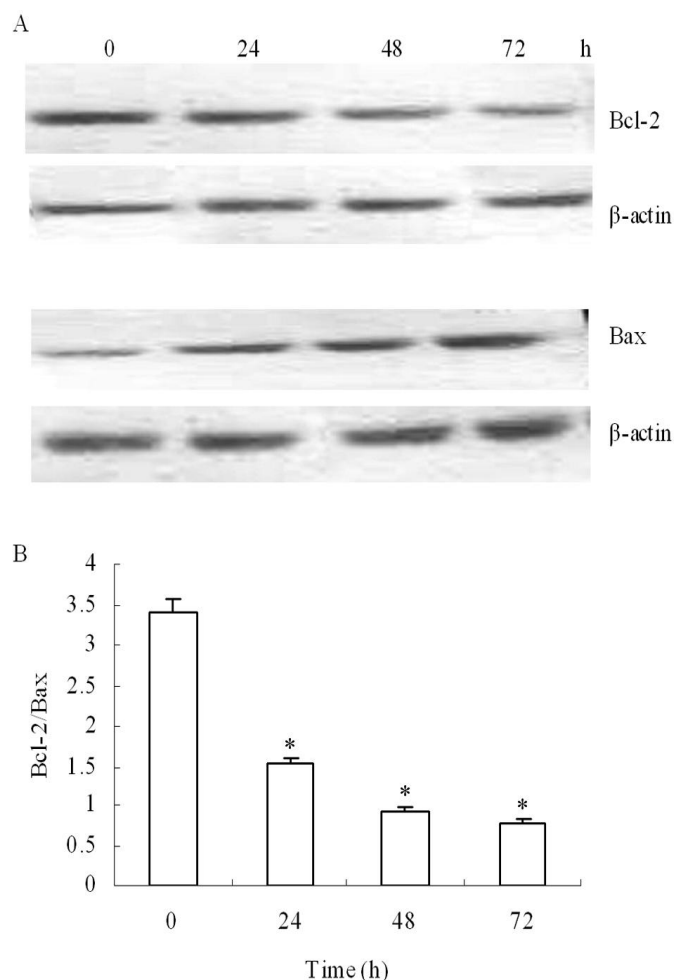


Figure 5. Effect of cucurmosin treatment on the Bcl-2/Bax ratio. K562 cells were exposed to 2.5 $\mu\text{g/ml}$ of cucurmosin for 0, 24, 48 and 72 h. After the indicated time, protein were extracted. The proteins were subjected to Western blot analysis using the indicated antibodies. (A) Expression of Bcl-2 and Bax in tumor cells after treatment with cucurmosin for the indicated time. The results are representative of three independent experiments performed in duplicate. β -actin was used as a loading control. (B) Error bars represent the means \pm SD of three independent experiments * $p < 0.05$ vs control.

Western blot analysis showed that the content of cytochrome c was increased markedly in cytolis after treatment of cucurmosin as time increased (Figure 6).

Effect of cucurmosin on apoptosis-associated proteins in K562 cells

We examined the effects of cucurmosin on the constitutive protein levels of Bcl-2 and Bax in K562 cells. The Western blot analysis exhibited a significant increase in the protein expression of Bax in cucurmosin-treated

K562 cells. In contrast, the protein expression of Bcl-2 was significantly decreased by cucurmosin treatment (Figure 5A). A significant shift in the ratio of Bax and Bcl-2 was observed after cucurmosin treatment (Figure 5B).

Caspase-3 was an important effector protein in the process of apoptosis and was responsible for the proteolytic cleavage of many key cellular proteins. Therefore, activation of caspase-3 (proteolytic cleavage of caspase-3) in cucurmosin-treated K562 cells was assessed by Western blot. These results showed that cucurmosin induced the activation of the pro-apoptotic protein caspase-3 in K562 cells (Figure 6).

Heat shock protein 90 (Hsp90) had recently emerged as a focus of interest because of its role in regulating proteins that were responsible for malignant transformation. We found the expression level of Hsp90 was also reduced in cucurmosin-treated K562 cells (Figure 6).

Effect of cucurmosin on the p210^{bcr/abl} expression levels

The changes in the level of the Bcr-Abl molecule after treatment with cucurmosin were evaluated because cucurmosin induced apoptosis in the Bcr-Abl-positive leukemia K562 cells. As shown in Figure 7, the p210^{Bcr-Abl} protein levels were decreased in time- and dose-dependent manners when K562 cells were treated with 2.5 $\mu\text{g/ml}$ cucurmosin at the different time (0, 24, 48, and 72 h) or doses (0, 1.25, 2.5, and 5 $\mu\text{g/ml}$) of cucurmosin for 48 h.

DISCUSSION

Although our previous studies showed a potent *in vitro* effects that cucurmosin not only by itself involved but also synergized activities of imatinib to induce K562 cell growth arrest (Hou et al., 2008; Sun et al., 2008), no effort was made to explore its underlying molecular mechanisms. Various studies showed the involvement of cell cycle regulation-mediated apoptosis as a mechanism of cell growth inhibition (Kweon, 2007; Sarfaraz et al., 2006; Tian et al., 2008; Sato et al., 2007). Therefore, we hypothesized that inhibition of growth by cucurmosin likely resulted from both an arrest in cell cycle progression and an increase in apoptosis.

To test our hypothesis, the cell cycle profile and apoptosis-inducing were analyzed in K562 cells with or without cucurmosin treatment by flow cytometry. We found that the cell cycle progression of cucurmosin-treated cells was arrested. In comparison to controls, the percentage of cells in the G0/G1 peak increased and the percentage of cells in the S peak decreased significantly in a time-dependent manner

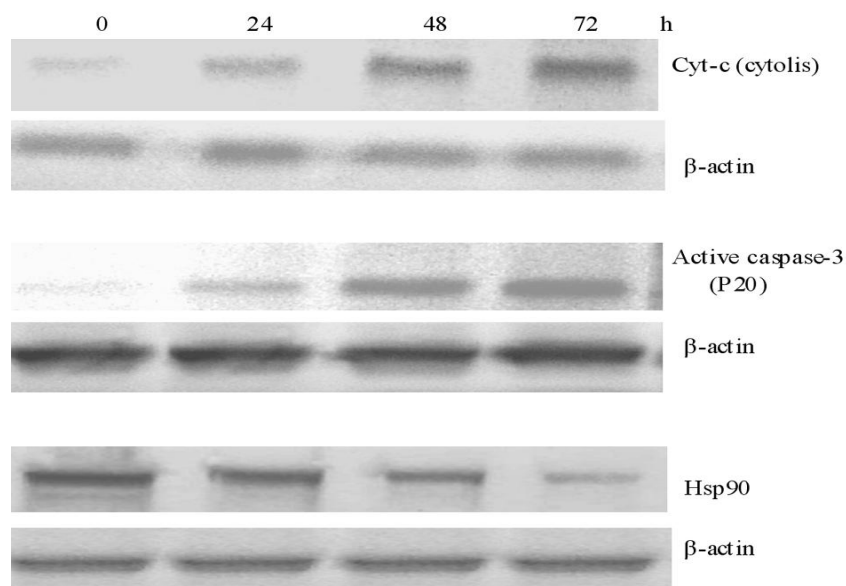


Figure 6. Effect of cucurmosin on signaling molecules in K562 cells. K562 cells were exposed to 2.5 $\mu\text{g/ml}$ of cucurmosin for 0, 24, 48 and 72 h. After the indicated time, protein were extracted and then immunoblot for cleaved caspase-3 (p20), Hsp90, cytochrome c and β -actin were performed as described in Materials and methods. The results of a representative study were shown; two additional experiments produced equivalent results.

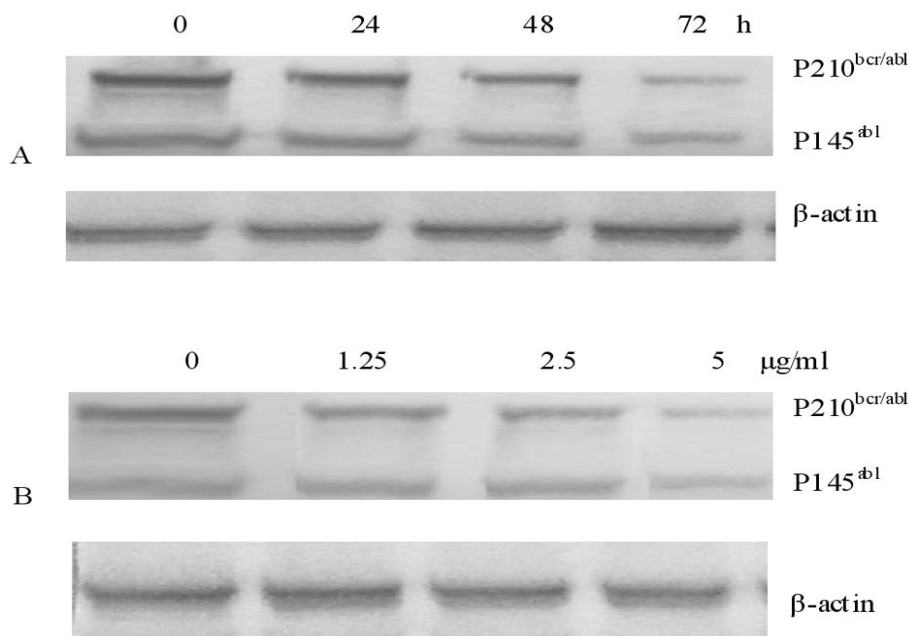


Figure 7. Effect of cucurmosin on the p210^{bcr-abl} protein in K562 cells. For the dose-dependent study, K562 cells were exposed to 0, 1.25, 2.5 and 5 $\mu\text{g/ml}$ cucurmosin for 48 h. For the time-dependent study, 2.5 $\mu\text{g/ml}$ of cucurmosin was used, and incubation was carried out at 37°C for 0, 24, 48 and 72 h, respectively. The p210^{bcr-abl} protein expression was examined by Western blot analysis using c-Abl antibody and AP-conjugated secondary antibody. Each lane was loaded with 20 μg of protein, β -actin levels were used as a control for protein loading. The result of a representative study was shown, three additional experiments yielded equivalent results.

(Table 2). In addition, we observed typical characteristics of apoptosis after cucurmosin treatment, such as cell shrinkage, the formation of apoptotic bodies and DNA fragmentation (Figures 1 to 3). These data suggested that the antitumor activities of cucurmosin were largely due to cell cycle regulation-mediated apoptosis in K562 cells.

The imbalance of expression of antiapoptotic and proapoptotic proteins after the stimulus was one of the major mechanisms underlying the ultimate fate of cells in the apoptotic process (Zhou et al., 2010). The Bcl-2 gene family included a number of anti-apoptotic proteins, including the Bcl-2 protein (Hong et al., 2002). Bcl-2 was located primarily in the outer mitochondrial membrane and blocked apoptosis by preventing cytochrome c release from the mitochondria, as well as by inhibiting caspase-3 activity. The Bax and BH-3 gene families consisted of pro-apoptotic proteins, including Bax, which translocated from the cytosol to the mitochondria and interacted with outer membrane proteins to form pores, thereby releasing cytochrome c and triggering caspase-mediated apoptotic cell death. Compared to other members of the Bcl-2 family, the relative levels of Bcl-2 and Bax proteins had been shown to be the most highly predictive of apoptosis and the most sensitive to apoptotic agents (Wesche-Soldato et al., 2007; Mackey et al., 1998; Rasiova, 2001). Bcl-2 and Bax were, therefore, attractive targets for the design of new anticancer drugs (Rasiova, 2001; Lohmann et al., 2000). In our study, we found that cucurmosin resulted in the downregulation of Bcl-2 and the upregulation of Bax in tumor cells, lowering the Bcl-2/Bax ratio and increasing the activation of caspase-3 (Figures 5 and 6). These suggested that Bcl-2 family participated in cucurmosin-induced apoptosis.

Mitochondria dysfunction was a critical event that caused the release of cytochrome c and subsequent activated caspases, a group of enzymes that executed apoptosis (Bednarz et al., 2007; Lin and Beal, 2006). Evaluation of the mitochondrial function during the induction of apoptosis was recorded through changes in its decreased MMP, as monitored with a fluorescent probe, which had generally been adopted as an indicator of cell apoptosis (Bolduc et al., 2004; Nicholls and Ward, 2000). Interestingly, our results showed that cucurmosin markedly induced the collapse of MMP and increased cytochrome c levels in cytolis. These results further implied that mitochondria dysfunction may be one of mechanisms in cucurmosin-induced apoptosis of K562 cells.

It was reported that the K562 cell line displayed a relatively high level of resistance to most cytotoxic drugs, which was probably because of a combination of Bcr-Abl oncogene expression (McGahon et al., 1994; Martin et al., 1998). In our study, down-regulating of p210^{Bcr-Abl} protein level at time- and dose-dependent manners by cucurmosin was observed. Although it was still unknown whether or not cucurmosin influenced the level of p210^{Bcr-Abl}

in a direct or indirect way, this indicated that one of the targets of cucurmosin was p210^{Bcr-Abl}.

Finally, it was worth mentioning that we found that cucurmosin down-regulated the level of Hsp90 in time-dependent manner. Hsp90, the molecular chaperone heat shock protein 90, was important in maintaining the conformation, stability, and function of p210^{Bcr-Abl} (Nicolas et al., 1997; An et al., 2000). Therefore, it seemed reasonable to speculate that the down-regulation of p210^{Bcr-Abl} was largely due to the decrease of Hsp90 in cucurmosin-induced apoptosis in K562 cells.

In summary, we confirmed that cucurmosin occurred through cell cycle arrest and decrease of the Bcl-2/Bax ratio to induce apoptosis. In addition, mitochondria dysfunction and the degradation of P210^{Bcr-Abl} by down-regulating its molecular chaperone Hsp90 may be also one of the important factors in cucurmosin-induced apoptosis in K562 cells.

ACKNOWLEDGEMENTS

This work was supported by National Nature Science Fund of China (30772587), Provincial Nature Science Fund of Fujian (C0510012), Scientific Foundation of Fujian Medical University (FJGXY04019) and Key Scientific Projects of Fujian Medical University (09ZD012).

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