Compound Bieshe Kang’ai inhibits proliferation and induces apoptosis in HCT116 human colorectal cancer cells

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INTRODUCTION

Apoptosis is programmed cell death mainly via intrinsic pathway [1]. While apoptotic pathway is activated by various stimuli, family members of B-cell lymphoma 2 are down regulated, which renders pro-apoptotic members to perturb the mitochondria. Subsequently, mitochondria releases cytochrome c, resulting in assembly of apoptotic protease-activating factor-1 and procaspase-9 to form apoptosomes. Consequently, caspase-9 is activated to cleave downstream targets including executioner caspase-3 and caspase-7 [2].
It is now known that reduced apoptosis and over proliferation are associated with initiation and progression of divers cancer types, which are characterized by impaired apoptotic signaling. To date, a strategy for targeting apoptotic molecules and inducing apoptosis appears to be a therapeutic approach for cancer.

Compound Bieshe Kang’ai (CBK) is a traditional Chinese medicinal formula containing Carapax trionycis (Bie Jia in Chinese), Hedysotis diffusa Wild (Bai Hua She She Cao in Chinese), Rhizoma curcumae (E Zhu in Chinese) and Radix astragali (Huang Qi in Chinese). CBK is broadly utilized in China for medication with high performance for various solid cancers, involving colorectal cancer, hepatoma, gastric cancer and breast cancer. However, the mechanism for the action of CBK on cancer has so far remained elusive.

Here, the function of CBK on proliferation and apoptosis in colorectal cancer cells and underlying mechanism of action were investigated, so as to support clinical application of CBK.

EXPERIMENTAL

Drugs and reagents

Authentic Chinese medicinal materials of CBK were purchased from Tongrentang (Beijing, China). American Type Culture Collection supplied HCT116 colorectal cancer cells and FHs 74 Int intestinal cells. Caspase-9 assay kit and caspase-3 assay kit were supplied by Beyotime (Nantong, China). The enzyme-linked immunosorbent assay (ELISA) kits for Bcl-2 and BAX (Bcl-2-associated X) were bought from Elabsciene Biotechnology (Wuhan, China). QIAGEN (Maryland, USA) provided RNeasy mini kit and quantitative RT-PCR kit.

Preparation of ethanol extract of Compound Bieshe Kang’ai (CBK)

Briefly, 100 g of CBK consisting of Carapax trionycis, Hedysotis diffusa Wild, Rhizoma curcumae and Radix astragali in the weight ratio of 1:1:0.9:0.9 were mashed and extracted with 1000 mL of 85 % ethanol using a refluxing method and filtered, followed by condensation with a rotary evaporator (Bilon, Type RE-52AA, Shanghai, China). Then, the ethanol solvent was evaporated, and the extract was dried at 60 °C, followed by dissolution in 40 % DMSO to a concentration of 1 g of crude CBK per milliliter. Prior to the tests below, the extract was diluted with cell culture medium to indicated final concentrations containing less than 0.1 % DMSO, and filtrated through a 0.22 microns cellulose ester membrane (Millipore, Massachusetts, USA) for sterilization.

Treatment of cells with CBK

HCT116 cells were grown with McCoy’s 5A medium containing 0.22 % sodium bicarbonate, 10 % FBS (fetal bovine serum), 100 μg/mL of streptomycin, 100 U/mL of penicillin. FHs 74 Int cells were maintained with Dulbecco’s Modified Eagle’s Medium containing 10 % of FBS, 30 ng/mL epidermal growth factor, 100 μg/mL of streptomycin, 100 U/mL of penicillin. HCT116 cells or FHs 74 Int cells were treated by CBK at diverse concentrations for various time intervals, followed by the tests as detailed below.

MTT assay of cell proliferation

After treatment of HCT116 cells or FHs 74 Int cells with CBK at different concentrations for various durations, MTT solution was aseptically added, followed by 4-hour incubation. After removal of the culture medium, MTT solvent was added, followed by gentle stirring to dissolve MTT formazan crystals, and colorimetric readout with a microplate reader (Thermo Scientific Varioskan Flash, Type 3001) set to 570 nm. The MTT assay was performed twice in quadruplicate.

Caspase activity assay

Following treatment of HCT116 cells grown in a 6-well plate with CBK for 48 h at 125, 250 and 500 μg/mL, cells were collected for examination of caspase activity with caspase-9 assay kit and caspase-3 assay kit respectively, in accordance with the user’s manual. Shortly, the protein concentration of whole cell lysate from HCT116 cells was determined with Bradford protein assay. Approximately 20 μg of lysate were mixed with 2 nmol of Ac-LEHD-pNA (caspase-9) or Ac-DEVD-pNA (caspase-3) and incubated for 90 min, followed by spectrophotometric measurement of A405 with Thermo Scientific Varioskan Flash (Type 3001). The A405 value was converted to enzyme activity on the basis of the constructed standard curve. The caspase activity assay was performed twice in triplicate.

Protein expression assay

After 48-hour treatment of HCT116 cells with CBK at 125, 250 and 500 μg/mL, cell culture supernatant was retrieved for examining protein levels of Bcl-2 and BAX using ELISA kits for Bcl-2 and BAX, as per the manufacturer’s
instructions. Basically, samples were added to micro ELISA plates pre-coated with antibodies to Bcl-2 or BAX. After 90-min incubation at 37 °C, biotinylated antibody to Bcl-2 or BAX and avidin-horseradish peroxidase conjugate were added and incubated, followed by addition of the substrate. After incubation for 15 min, the enzyme-substrate reaction was stopped, followed by spectrophotometric determination of OD_{450nm}. Finally, OD_{450nm} value was converted to protein level according to the standard curve. The assay was conducted triplicately for 2 times.

Quantitative RT-PCR analysis

After 24-hour treatment with CBK at 125, 250 and 500 μg/mL, HCT116 cells were processed for RNA extraction with RNeasy mini kit, followed by reverse transcription (RT) of 500ng of RNA per sample to cDNA with reverse transcription kit. The resulting cDNA was then analyzed using SYBR green PCR kit on iCycler Real-Time PCR System, as per the manufacturer's instructions. Primer sequences were depicted in the table below.

| Gene    | Sequence                                      | PCR product |
|---------|-----------------------------------------------|-------------|
| Caspase -9 | 5'-AGCAGAAAGACCATGGGTTTGAGG-3' (forward)     | 197 bp      |
|         | 5'-CCTCAGGAACCAAAACCTGGGA-3' (reverse)       |             |
| Caspase -3 | 5'-GGGTTCAGTTAGCTTCATAACAGC-3' (forward)     | 166 bp      |
|         | 5'-TGGGTCATGTGTGAGGAG-3' (reverse)           |             |
| Bcl-2   | 5'-TAACACAGGGCATTCCACG-3' (forward)          | 139 bp      |
|         | 5'-ATGATTGCCGCGTGAGCAG-3' (reverse)          |             |
| BAX     | 5'-ACAGGGCTTTGACGCAGTTGACAGC-3' (forward)    | 142 bp      |
|         | 5'-CCCTGGACTTTGAGCAGAAGA-3' (reverse)        |             |
| β-actin | 5'-ACTCCATGGCCAGAAGGA-3' (forward)           | 153 bp      |

ΔCt was calculated by subtracting Ct of β-actin, for normalization of mRNA levels in HCT116 cells. The data were shown as fold change relative to the control. The test was performed in duplicate for 3 times.

Staining with Hoechst 33342

Following 48-h treatment with CBK at 125, 250 and 500 μg/mL, HCT116 cells were fixed for 10 min with methanol, followed by Hoechst 33342 staining in the dark. After mounting with Antifade PVP medium (Beyotime, Nantong, China), cells were observed with a Leica fluorescence microscope (Type DMI3000B). The test was conducted twice in triplicate.

Statistical analysis

One-way analysis of variance (ANOVA) and Student's t-test were performed with SPSS software for statistical analysis, and the results shown as mean ± SEM (standard error of the mean). Statistical significance was set at $p < 0.05$.

RESULTS

CBK suppresses proliferation in HCT116 cells

Except at 31.25 μg/mL, CBK at other concentrations, including 62.5, 125, 250, 500 and 1000 μg/mL, concentration-dependently inhibited the proliferation of HCT116 cells (Figure 1 A). Therefore, CBK at 125, 250 and 500 μg/mL were selected for subsequent tests. Furthermore, treatment of HCT116 cells with CBK at 250 μg/mL for 12, 24, 36 and 48 h inhibited proliferation, which was time dependent (Figure 1 B).

![Figure 1](CBK suppresses proliferation in HCT116 cells. *$p < 0.05$ and **$p < 0.01$, verse Control)

CBK does not interfere with proliferation in FHs 74 Int cells

Next, CBK cytotoxicity to normal intestinal epithelial cells was explored. Intriguingly, CBK at all tested concentrations, including 31.25, 62.5, 125, 250, 500 and 1000 μg/mL, did not suppress the proliferation of FHs 74 Int cells (Figure 2 A). Also, viability of FHs 74 Int cells was not affected.
by treatment with CBK at 250 μg/mL for 12, 24, 36 and 48 h (Figure 2B).

**Figure 2**: CBK does not interfere with proliferation in FHs 74 Int cells

**CBK promotes caspase-9 activity and caspase-3 activity of HCT116 cells**

Treatment of HCT116 cells for 48 h with CBK at 125, 250 and 500 μg/mL enhanced caspase-9 activity, which was concentration dependent (Figure 3A). Moreover, CBK at 125, 250 and 500 μg/mL heightened caspase-3 activity in HCT116 cells in a concentration-dependent pattern (Figure 3B).

**Figure 3**: CBK promotes caspase-9 activity and caspase-3 activity. *p < 0.05 and **p < 0.01, verse Control

**CBK reduces Bcl-2 protein level and increases the expression of BAX protein in HCT116 cells**

Except at 125 μg/mL, the protein translation of Bcl-2 in HCT116 cells was significantly reduced by 48 hour of treatment with CBK at 250 and 500 μg/mL (Figure 4A). Additionally, 48-hour treatment of HCT116 cells with CBK at 125, 250 and 500 μg/mL increased BAX protein expression, which was concentration dependent (Figure 4B).

**Figure 4**: CBK reduces Bcl-2 protein level and increased BAX protein level. *p < 0.05 and **p < 0.01, verse Control

**CBK influences mRNA transcription of caspase-9, caspase-3, Bcl-2 and BAX in HCT116 cells**

Treatment of HCT116 cells for 24 h with CBK at 125, 250 and 500 μg/mL all drastically increased mRNA transcription of caspase-9 (Figure 5A), caspase-3 (Figure 5B) and BAX (Figure 5D). However, Bcl-2 mRNA level of HCT116 cells was sharply decreased by 24 h of treatment with CBK at 250 and 500 μg/mL (Figure 5C). All of these effects of CBK were concentration dependent.

**Figure 5**: CBK influences mRNA transcription of caspase-9, caspase-3, Bcl-2 and BAX. *p < 0.05 and **p < 0.01, verse Control

**CBK induces apoptosis in HCT116 cells**

With Hoechst 33342 staining and fluorescence microscopy, morphologic characteristics for apoptosis of HCT116 cells treated for 48 h by CBK at 125, 250 and 500 μg/mL were noted. The apoptotic HCT116 cells induced by CBK manifested cell shrinkage, chromatin condensation and nuclear fragmentation, which were at the initial process of apoptosis (Figure 6).

**Figure 6**: CBK induces apoptosis in HCT116 cells. A, the control; B, CBK at 125 μg/mL; C, CBK at 250 μg/mL; D, CBK at 500 μg/mL.
DISCUSSION

Mounting evidence reveals that traditional Chinese medicines have some advantages, such as acting on multiple pathological targets and possessing reduced adverse reactions, over modern chemotherapeutic agent for many types of diseases [3,4], particularly involving cancers [5]. Here, we present a Chinese medicinal formula CBK that is efficacious to varieties of cancers in clinical practice, and the underlying mechanism to be delineated.

Firstly, the study showed that CBK suppressed the proliferation in HCT116 cells in a concentration- and time-dependent manner, which coincided with other reports in which Hedyotis diffusa Willd, a Chinese herb of CBK, suppresses colorectal cancer growth through multiple cellular pathways [6], and Radix Astragali that helped carboplatin to inhibit B16 tumor cell growth [7]. Intriguingly, the cytotoxicity of CDK was specific to cancer cells, because CDK did not influence the viability of FHs 74 Int cells, suggesting that CBK may cause less adverse events in clinical settings. In addition, it is reported that Carapax Trionycis inhibits fibrogenesis of activated hepatic stellate cells [8], and Radix Astragali protects against podocyte injury in nephrotic syndrome [9]. The above data demonstrate that CBK is potentially superior to some chemotherapeutic anti-cancer agents, in terms of causing side effects.

In an attempt to provide novel mechanistic insights into the anti-cancer property of CBK, we set to explore whether or not CBK influenced the activities of apoptosis-mediating caspases, which serve as executioners in apoptotic process by cleaving cellular components. Of direct clinical significance is evidence that human tumors harbor divers point mutations in caspase-9, which renders caspase-9 inactive and incapable of inducing apoptosis [10]. Here, CBK elevated caspase-9 activity and caspase-3 activity in HCT116 cells, as is reported in the study suggesting that Hedyotis diffusa Willd causes apoptosis of A2780 ovarian cancer cells via activation of caspase 9/3 [11].

It is known that the ratio of Bcl-2 level to BAX level plays a crucial role in propagation of apoptotic cascade [12]. In this study, CBK decreased protein translation of Bcl-2 and increased that of BAX in HCT116 cells, which was consistent with another report that Hedyotis diffusa Willd promotes apoptosis of HT-29 colorectal cancer cells by downregulating Bcl-2 level and upregulating BAX level [13]. Additionally, CBK concentration-dependently enhanced the mRNA levels of caspase-9, caspase-3 and BAX, and lessened Bcl-2 mRNA transcription in HCT116 cells, which further illuminated the mechanism for action of CBK on inducing apoptosis of cancer cells and coincided with other study in which Hedyotis diffusa Willd increases BAX mRNA level and lowers Bcl-2 mRNA level in HepG2 carcinoma cells [14].

Apoptotic cells manifest distinct morphological traits, including chromatin condensation, cell shrinkage and membrane blebbing, accompanied by cleaving of lamins and actin filaments in cytoskeleton by caspases. Then, cellular contents become encased in ‘apoptotic bodies’, with the translocation of phosphatidylserine from the inside to the outer surface, which promotes phagocytosis of the apoptotic bodies by nearby macrophages [15]. CBK remarkably induced the morphologic changes for early apoptosis in HCT 116 cells, involving cell shrinkage, chromatin condensation and nuclear fragmentation. This was supported by previous reports that Hedyotis diffusa Willd and Rhizoma curcumae of CBK can induce apoptosis in HL-60 cells, THP-1 cells, SPC-1-A, Bcap37 and HepG2 cancer cells through intrinsic mitochondrial pathway [16].

This pharmacological study presents encouraging experimental evidence, underpinning the clinical utilization of CBK for various cancers, which merits further intense investigations. Efforts should be made to exploit the potency of CBK on extrinsic apoptotic pathway and the influence of CBK on the expression of p53, which can activate the appropriate cellular signaling cascades to execute apoptosis.

CONCLUSION

The findings show that CBK lessens proliferation and causes apoptosis in HCT116 cells by regulating the expression and function of some apoptosis-associated genes, such as caspase-9, caspase-3, Bcl-2 and BAX. As reduced apoptosis is a basic aspect of cancer biology and most cytotoxic anticancer therapies ultimately induce apoptosis of malignant cells, CBK with high potency of inducing apoptosis and less adverse effects may be a potent agent for cancers.

DECLARATIONS

Acknowledgement

This work was sponsored by National Natural Science Foundation of China (nos. 81173598)
and 81573813), Science and Technology Foundation for Returned Overseas Chinese Scholars of the Ministry of Human Resources and Social Security of China (no. 2011508), Scientific Research Foundation for Returned Overseas Chinese Scholars of the Ministry of Education of China (no. 2013693), Specialized Research Fund for Doctoral Program of Higher Education of the Ministry of Education of China (no. 20115132120010), Natural Science Foundation of Chengdu University of Traditional Chinese Medicine (no. 314-062).

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. HBX, KY and XLM conceived and designed the study. SW, LT, NL and YRS carried out the experiments. MH, MZ, LJZ, LWJ and HBX analyzed the data. SW and HBX wrote the manuscript. All authors read and approved the manuscript for publication.

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