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CTAB induced mitochondrial apoptosis by activating the AMPK-p53 pathway in hepatocarcinoma cells

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Abstract

The reprogramming of energy metabolism as a new concept is emerging and is one of the hallmarks of cancer. CTAB, known as a quaternary ammonium compound with the activity of inhibiting mitochondrial H-ATP synthase, has shown the potential to influence cell energy metabolism. In this study, we investigated the effects and the underlying mechanisms of CTAB on liver cancer cells. The results showed that CTAB reduced the cell viability of various hepatocarcinoma cells in dose- and time-dependent manner. The results showed that CTAB induced mitochondrial apoptosis in human hepatocarcinoma HepG2 cells by activating AMPK and p53 signaling pathways. Our study shed the light on CTAB as a promising cancer therapeutic candidate.

1. Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide. Globally, the incidence of liver cancer is approximately 750,000 new cases per year.[1, 2] Up to date, the maintain therapies for HCC are surgery and hepatic transportation. However, a well-established anticancer chemotherapeutic approach is still an urgent need.[3] Numerous research studies have demonstrated that several small molecule compounds with quaternary ammonium moiety have excellent anti-tumor effect *in vitro* and *in vivo*, such as berberine, emodin and curcumin.[4, 5] CTAB (cetyltrimethylammonium bromide), which also possesses a group of quaternary ammonium with the molecular weight 369.45, is a common template agent for nanoparticles synthesis, especially in the synthesis of mesoporous carriers.[6, 7] However, the anticancer effect of CTAB on HCC and underlying mechanism cell remain unclear.

A new concept was emerging that reprogramming energy metabolism is one of the hallmarks in cancer development. Cancer cell has a distinct metabolic advantage over normal cell since it can acquire rapid ATP production by aerobic glycolysis and synthesize sufficient biomolecules to support its proliferation and metastasis. CTAB has previously been reported to compromise bio-energetic homeostasis by inhibiting H^+-ATP synthase to prevent the mitochondrial repolarization, which leads to a progressive reduction in intracellular ATP levels in human head and neck cancer.[8] The AMP-activated protein kinase (AMPK) was sensitive to the intracellular change of the AMP to ATP ratio, and sustained cellular and whole-body energy homeostasis by regulating metabolic pathways.[9] Meanwhile, it is shown that AMPK is involved in the regulation of cancer cell growth, proliferation, autophagy under a variety of physiological and pathological stresses.[10, 11] Recently, AMPK has been regarded as a novel therapeutic target in cancer treatment. Therefore, it was necessary to investigate the relationship between CTAB and AMPK in cancer cells.

CTAB has a smart structure with a positively charged polar head and a nonpolar hydrophobic tail.[8] The trait of amphiphathic molecule causes the disruption of lipid

bilayers and the increase in cell membrane permeability, due to nanoscale holes formation by the quaternary ammonium cations attachment onto the membrane. The increase of cell membrane permeability is a pivotal event in apoptosis initiation.[12] Two major apoptosis pathways have been defined in mammalian cells, one receiving and processing extracellular death-inducing signals (such as Fas ligand/Fas receptor), and the other sensing and integrating a variety of intrinsic signals (example for the mitochondria dependent pathway).[13] Currently, Apoptosis mediated by AMPK is focused on the balance regulation between a number of tumor suppressor genes including LKB1, p53, mTOR with oncogenes such as PI3K, Akt and ERK.[14, 15]

In present study we tried to investigate the tumoricidal potential of CTAB in HCC cell lines and elucidate the mechanisms underlying AMPK in CTAB-inducing apoptosis. This study will give a novel clue in CTAB as a promising cancer therapeutic candidate.

2. Materials and Methods

2.1 Chemicals and reagents

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) and Compound C were purchased from Sigma Aldrich. 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin and BCA protein assay kit were bought from Beyotime Institute of Biotechnology (Jiangsu, China). The primary antibodies were diluted 1:1000 before use, including AMPK (Cat. # sc-25792, Santa Cruz Biotechnologies), p-AMPK (Cat. # sc-33524, Santa Cruz Biotechnologies), p53 (Cat. # 10442-1-AP, Proteintech), Bax (Cat. # sc-7480, Santa Cruz Biotechnologies), Cytochrome c (Cat. # sc-13561, Santa Cruz Biotechnologies), Caspase 9 (Cat. # 842, Cell signaling), Caspase 3 (Cat. # 836, Cell signaling), PARP (Cat. # 1442, Cell signaling) and GAPDH (Cat. # sc-25778, Santa Cruz Biotechnologies). All the chemical compounds were analytically pure reagents.

2.2 Cell culture and MTT assay

Hep3B, HepG2, Bel-7402 and Bel-7404 were maintained in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin,

and 100 μg/mL streptomycin in an atmosphere of 95% air and 5% CO2 at 37□. HepG2 was from a 15-year-old white liver cancer tissue. Hep3B was from a-7- year old black boy patients with hepatocellular carcinoma. Bel-7402 cell line was established from clinical surgical specimens of liver cancer in 1974. MTT assay to assess cell viability was performed in 96-well plates in octuplicate. Cells were seeded at a density of 5×10^3 cells/well overnight, and treated with CTAB at final concentrations of 0, 0.2, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25 μg/mL and/or 0.3 μmol/L Compound C were added for 24 h. After 12 h incubation with indicated reagents, 20 μL MTT (5 mg/mL) were added to each well for at the last 3 h. Afterwards, the cell supernatants were discarded, the MTT crystals were dissolved with DMSO and the optical densities were measured. The absorption density was measured by the absorbance at 570 nm.

2.3 Apoptosis detection by flow cytometry

Cell death and apoptosis were detected with the use of annexin V- FITC apoptotic detection kit and flow cytometry. In brief, after treating with 0, 1.5, 3, 6, 8, 12 μM CTAB for 24 h, $HepG_2$ cells were harvested and washed twice with cold PBS. Then the cell pellets were resuspended with binding buffer to cell suspension at a density of $1\times l0^6$ cells/mL. Next, 5 μL of FITC-conjugated annexin V was added to the suspension and incubation was made for 15 min at 4°C in the dark. After that, 5 μL of propidium iodide (PI) was injected into the mixture and incubated with cells for 5 min. The samples were subsequently analyzed by flow cytometry.

2.4 Western Blot Analysis

HepG2 were grown in DMEM containing 12% fetal bovine serum. Then the cells were plated into 6-well tissue culture plates. After the cells reached confluence at Day 3, the medium was replaced by DMEM supplemented with 0.2% BSA. After 12 h, the medium was removed and the medium containing CTAB with the final concentrations of 0, 3, 6, 8, 12 μg/mL and/or 0.3 μmol/L Compound C was added, respectively. Cells were collected by centrifugation and washed twice with cold PBS. The cell pellets were suspended in 200 μL RIPA lysis buffer for 30 min at 4°C, vortexed every 10 min, and then centrifuged at 12,000×g for 15 min. The supernatants containing the total

protein extracts were collected. In some experiments, nuclear protein extract was collected by nuclear and cytoplasmic protein extraction kit according to manufactory's protocol. Protein concentration was measured by the Bradford protein assay kit. The immunoblotting analysis was performed with 20 μg sample proteins on a 12% SDS–polyacrylamide electrophoresis gel (SDS–PAGE). The electrophoresis was carried out first at 80 V for 20 min and followed by 180 V for 60–85 min. The separated proteins using SDS-PAGE gel are transferred to PVDF. The transferred membranes were incubated for 2 h in blocking buffer, TBST (10 mM Tris–HCl, 150 mM NaCl and 0.1% Tween-20) containing 5% non-fat milk powder and then incubated overnight with primary antibody (1:800 or 1:1000) in 10 ml TBST with gentle agitation at 4 ℃. The membranes were then washed and incubated with second antibody (1:2000) at room temperature for 1 h. Bands were visualized by an ECL Western blotting detection system (Tanon 4200).

2.5. Statistical analysis

All data were expressed as mean ± SD. Statistical significances among groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post. A value with $p < 0.05$ was considered as statistically significant.

3. Result

3.1 CTAB decreases cell viability in a dose- and time- dependent manner

To evaluate the cytotoxicity of CTAB on cancer cell lines, cell viability on several cancer cell lines, including HepG2, Hep3B, Bel-7402 and HL-7702 cells, was assessed by MTT assay after incubation of 24 h or 48 h（Fig. 1）. As shown in Figs AD, CTAB less than 12.5 μg/mL provoked a marked reduction in cell viability in a dose- and time-dependent manner. As shown in Fig.1D and Fig.S1, there are significant differences in cell viability between cancer cell line HepG2 and normal cell line HL-7702 after the treatment of CTAB with the concentrations from 0.39μg/ml to 6.25μg/ml. In addition, the IC_{50} values of HepG2, Hep3B, Bel-7402 and HL-7702 are 3.81, 5.93, 5.38 and 6.08 μg/mL for a-24h CTAB treatment and 1.71,

2.71, 3.81 and 5.38 μg/mL for a-48h CTAB treatment, respectively. The IC_{50} value of HL-7702 cell for a 24 h CTAB treatment is 6.08μg/mL.

3.2 CTAB induces cell apoptosis

Cell death and apoptosis were detected with the use of Annexin V- FITC apoptotic detection kit and flow cytometry (FCM). As depicted in Fig.2, the apoptotic rate of HepG2 cells increased with the increase of CTAB concentration after 24 h treatment. Apoptotic rates calculated by the sum of early apoptosis (Q_4) and late apoptosis (Q_2) are 5.75%, 10.32%, 12.0%, 17.05, 21.6%, 34.4% and 44.27% , respectively, with the treatment of CTAB for 24 h (Table 1).

3.3 CTAB activates AMPK-p53 pathway

To investigate the mechanisms of the CTAB induced apoptosis on HepG2 cells, the protein expression of AMPK and P53 were determined. Phosphor-AMPK expression significantly increased when the concentration of CTAB was more than 3 μg / mL (Fig 3). P53 was detected 24 h after CTAB exposure and its protein expression increased in a dose-dependent manner and is in line with Phosphor-AMPK. We also found that CTAB activated Bax in HepG2 cells (Fig 6). To further elucidate the relationship between AMPK and p53, AMPK inhibitor Compound C, was used before CTAB exposure. However, we found that compound C, a competitive inhibitor of AMPK, specifically reduced the CTAB-induced HepG2 cells death, the concentration of CTAB was 8 μg/mL and the concentration of Compound C was 0.3 μM (Fig. 4). By treating of HepG2 cells with a combination of compound C， we found that it did not affect the cell viability of HepG2 cells at a concentration of 0.3 μM (Fig. 5). Treatment of HepG2 cells with a combination of compound C and CTAB caused a significant reduction of p-AMPK and p53 (Fig. 3). These findings suggested that CTAB activates p53-Bax pathway via AMPK.

3.4 CTAB induces mitochondria-dependent apoptosis

In chemical-induced apoptosis, mitochondria play an important role in the commitment of cells to apoptosis through cytochrome c-dependent or -independent pathways. To elucidate the molecular mechanism of CTAB-induced apoptosis in HepG2 cells, we examined the expression of proteins associated with the mitochondria dependent pathway. HepG2 cells were exposed to the indicated concentrations of CTAB for 24 h. Our data showed that CTAB induced the release of cytochrome c from mitochondria to the cytosol. Casepase-9, Caspase-3 and poly (ADP-ribose) polymerase (PARP), which are all well-known downstream molecules of cytochrome c, were also cleaved in concentration-dependent manners (shown in Fig. 6) and cleaved caspase-9 and Bax reduced with the addition of Compound C. These data suggest that CTAB induces the mitochondria-dependent apoptosis pathway.

4. Discussion

Recently, the altered metabolism of tumor cells is widely recognized as an emerging hallmark. The significant difference between the normal cells and cancer cells is that the normal cells produce ATP through oxidative phosphorylation in mitochondria while the cancer cells prefer to utilize glycolysis as energy source even if they are under normal aerobic conditions.[16] This 'aerobic glycolysis' was also called 'Warburg effect', which increase cell glucose uptake, and subsequently induces the cancer cell proliferation, metastasis and drug resistance.[17] Therefore, blocking Warburg effect may be the potential effective strategy in cancer therapy.

It has demonstrated that AMPK was involved in the modulation of Warburg effect in cellular energetics.[16] AMPK negatively regulates the Warburg effect in cancer cells and suppresses tumor growth.[18] Furthermore, activators of AMPK have been used as an adjunct to cancer therapy through regulating the Warburg effect.[17] In our study, CTAB decreased the cell viability and induced apoptosis by in a dose- and time-dependent manner in HCC cell lines. When CTAB concentrations ranged from 0.39 μg/ml to 6.25 μg/ml, it showed the selective killing effect on cancer cell line HepG2 comparing with normal cell line HL-7702. Noticeably, the selectivity on tumor cells disappeared when CTAB concentration is more than 12.5 μg /mL (Fig.1D

and S1). This suggests that the dosage of CTAB is one of the critical factors in cancer therapy. Moreover, the protein levels of phosphorylated AMPK were increased with the apoptosis induced by CTAB, and the cytotoxicity of CTAB was reversed by AMPK inhibitor Compound C, indicating that antitumor effect of CTAB is AMPK dependent. As a result, the cytotoxic effect of CTAB is probably due to blocking Warburg effect by the activation of AMPK in HCC cells.

AMPK is not only a sensor of cellular energy, but also a crosstalk protein involved in located apoptotic signaling pathway, such as LKB1- AMPK, Myc-AMPK, AMPK-SIRT1, AMPK-FOXO3, AMPK-p53 etc.[15, 19-21] Our results showed that AMPK activation is consistence to the increase of p53 expression with the treatment of CTAB, and AMPK inhibitor compound C alleviated p53 activation, indicating that anti-cancer effect of CTAB is mediated by AMPK-P53 pathway. As we know the modification and nuclear accumulation of p53 play a pivotal role in apoptosis execution.[22] It is reported that AMPK activation is associated with the phosphorylation of p53 at Ser15 and Ser20, which is coupled to the acetylation of p53 at Lys382 in HCC cells.[23] SIRT1 mediated the effect of AMPK regulating p53.[24] Furthermore, inhibition of AMPK by Compound C led to increased p53 deacetylation through phosphorylates SIRT1 on T344, inhibiting p53 function and promoting cell survival. [25] In our study, we did not know the p53 protein modification state, because of the lack using phosphorylated or acetylated antibodies.[26] However, the discordant results also showed that CTAB-treated wild-type [p53(+/+)] and mutant [p53(-/-)] colon cancer HCT116 cells have similar sensitivity to CTAB treatment, which suggests that CTAB toxicity on some cancer cells is independent of p53 status.[8, 27] Therefore, the mechanism of CTAB-induced apoptosis should be considered in various types of cancer cells.

Many studies have demonstrated that p53 mediated apoptosis by transcriptional activation of pro-apoptotic genes like BH3-only proteins Noxa and Puma, Bax, p21, AIP1, Apaf-1, or by transcriptional repression of Bcl2 and IAPs.[28, 29] p53 functioned as apoptosis suppressor predominantly via the mitochondrial dependent apoptosis pathway through activating Bcl-2 family proteins.[30-32] Our results showed that the

protein expression of Bax and cytochrome C increased alone with the increment of CTAB dosage, while Compound C significantly inhibited the activation of Bax.[33] Moreover, the activation of cleaved caspase-9, cleaved caspase-3 and cleaved PARP are closely relative to Bax expression (Fig. 6).[34, 35] These results indicated that CTAB-induced mitochondria-dependent apoptosis is mediated by AMPK-p53 activation in HCC cells.

In summary, this study shed the light on CTAB as a promising cancer therapeutic candidate, and CTAB probably disturbs the Warburg effect in tumor cells by inducing AMPK-P53 activation, subsequently triggers mitochondria-dependent apoptosis by Bax activation.

Conflict of Interest

The authors report no conflict of interest.

Acknowledgment

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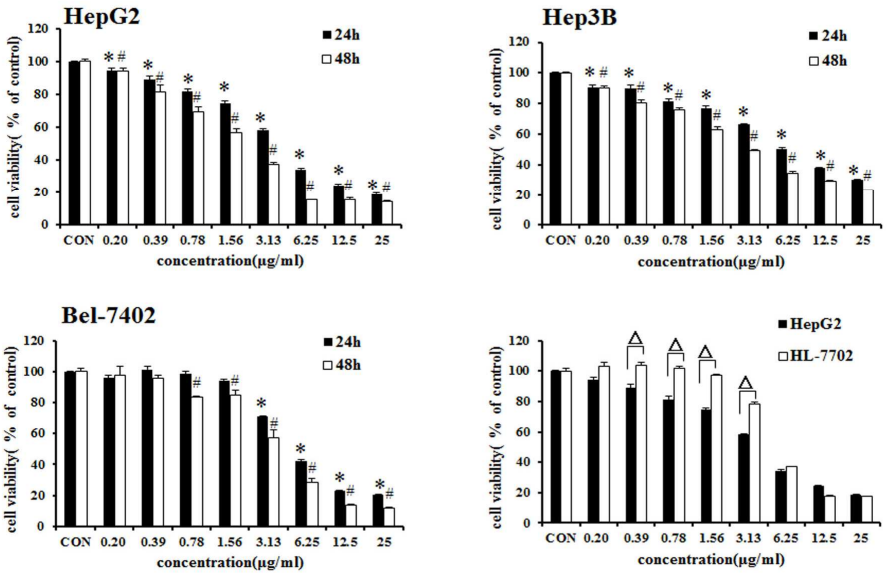

Fig. 1 Effect of CTAB on cell viability on HepG2 cells (A) Hep3B cell (B) Bel-7402 cell (C) and HL-7702 cell (D). The experiments were performed in triplicate. Data presented as means ± SD of three independent experiments. $^{*}P<0.05$, cells treated with different concentration of CTAB for 24 h compared with control group. $^{\#}$ $P<0.05$, cells treated with different concentration of CTAB for 48 h compared with control group. △ $P<0.05$，HL-7702 cells treated with different concentration of CTAB for 24h vs. HepG2 cells treated with different concentration of CTAB for 24 h.

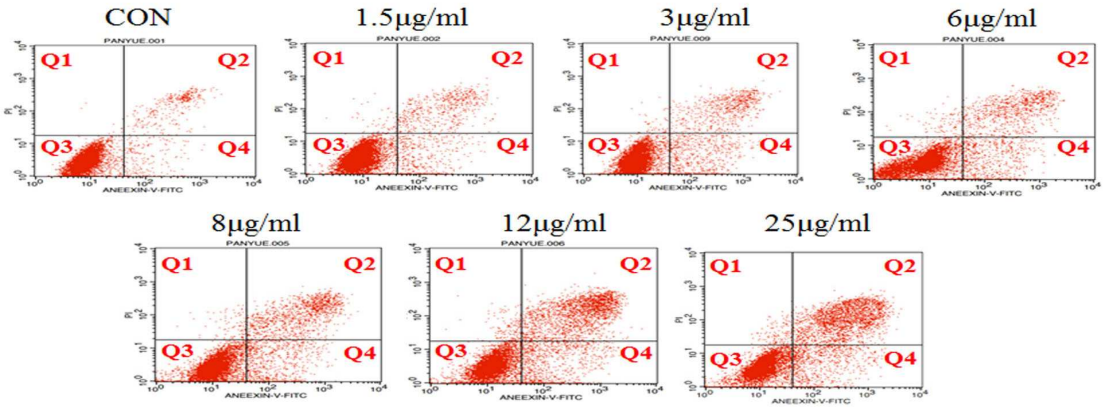

Table 1 the percentage of cell population in four quadrats

	CON	1.5 μg/ml	3 μg/ml	6 μg/ml	8 μg/ml	12 μg/ml	25 μg/ml
Q1(%)	0.1	0.49	0.47	1.01	0.95	1.57	1.64
Q2(%)	3.51	5.57	5.20	8.88	12.65	23.49	33.19
Q3(%)	94.15	89.19	87.53	81.94	77.45	64.05	54.09
Q4(%)	2.24	4.75	6.80	8.17	8.95	10.71	11.08

Fig. 2 Apoptosis induced by CTAB in HepG2 cells. HepG2 cells were treated with different concentration of CTAB for 24 h. Apoptotic rates were determined by flow cytometry. One of three independent experiments is shown. Data presented as means ± SD of three independent experiments, when compared with control group.

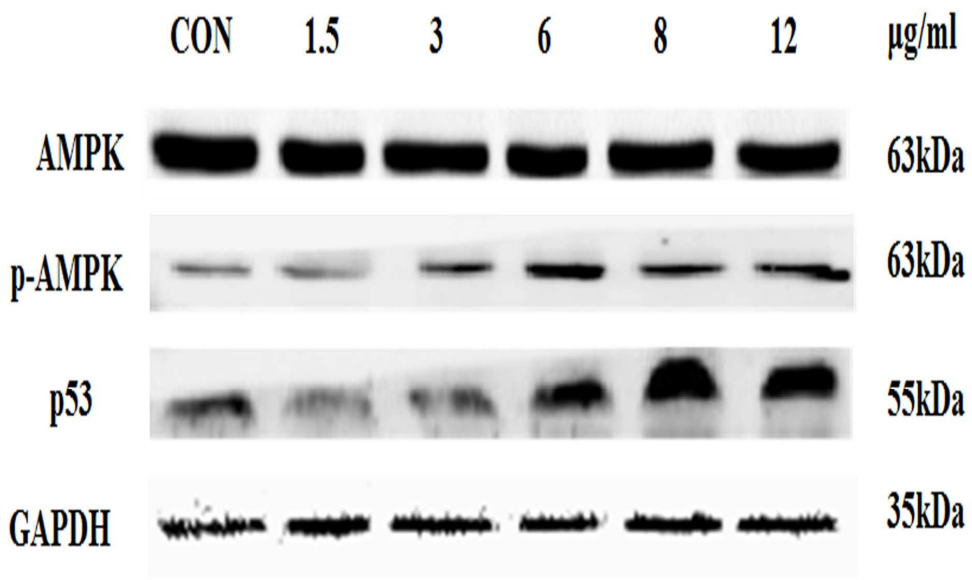

Fig. 3 AMPK and p53 protein expression activation by different concentration of CTAB treatment.

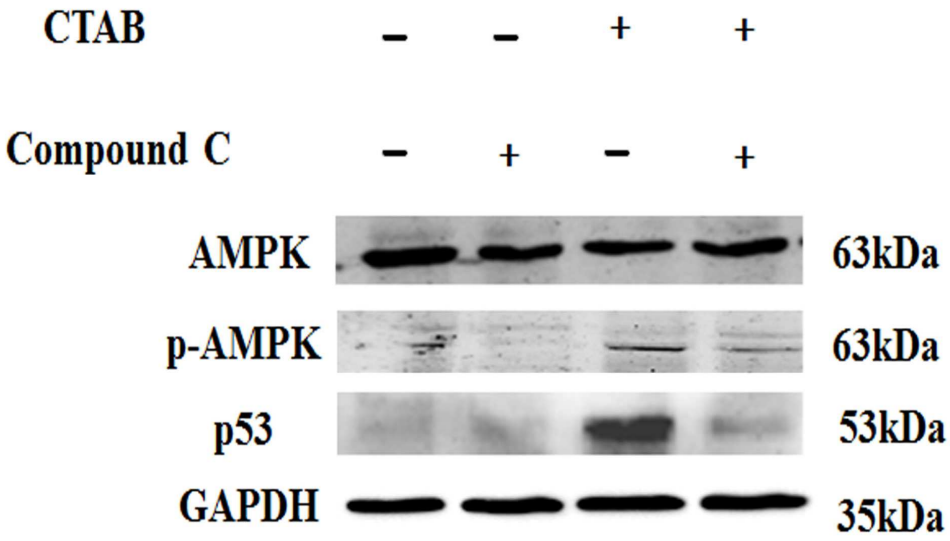

Fig. 4 p53 protein expression after CTAB treatment or CTAB combined Compound C treatment.

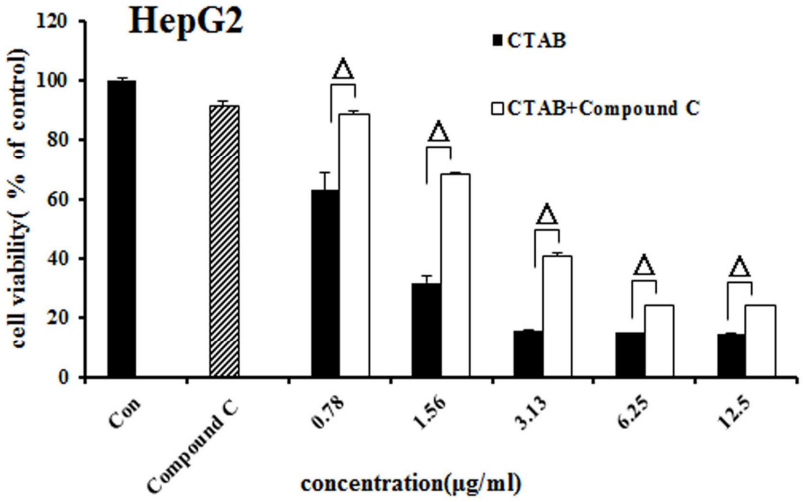

Fig. 5 Cell viability in HepG2 cells after CTAB treatment or CTAB combined Compound C treatment. The experiments were performed in triplicate. Data presented as means ± SD of three independent experiments. △ $P < 0.05$，comparisons between the co-treatment groups of HepG2 cells with CTAB at the indicated concentrations plus 0.3μM Compound C and the corresponding groups treated with CTAB alone, at 24 h after treatment.

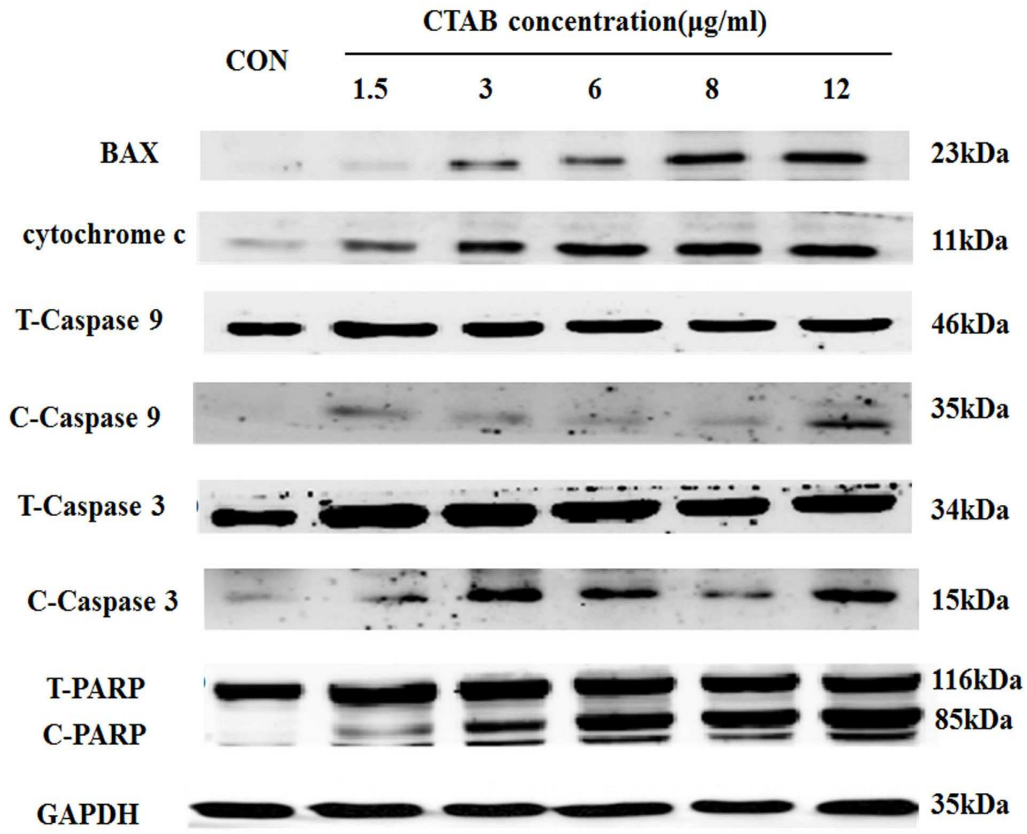

Fig. 6 Proteins expression on CTAB induced mitochondria-dependent apoptosis pathway. BAX, Cytochrome C, caspase 9, caspase 3 and PARP protein expression in HepG2 cells for 12 h. T-means the total protein, C-means the cleaved form of the protein.