MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 5211-5218
DOI: 10.12659/MSM.915840

Received: 2019.02.22
Accepted: 2019.03.14
Published: 2019.07.13

Diacetyl Hexamethylene Diamine (CAHB) Exerts Pro-Apoptotic and Anti-Proliferative Function in Leukemic T Lymphocytes via Downregulating PI3K/Akt Signaling

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BC Ye Hong
C Jia Zhang
CD Qunyi Guo
BD Min Zhu
CD Baoguo Chen
AG Wenda Luo

Department of Hematology and Oncology, Taizhou Hospital of Zhejiang Province, Wenzhou Medical University, Taizhou, Zhejiang, P.R. China

Corresponding Author: Wenda Luo, e-mail: luowendalsll@163.com
Source of support: This work was supported by Zhejiang Province Public Welfare Technology Application Research (No. 2016C33233) and the Zhejiang Medical and Health Research Fund Project (No. 2017KY166)

Background: T cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy caused by abnormal proliferation of immature T cell progenitors. Chemotherapy of T-ALL usually consists of induction, consolidation, and long-term maintenance. Diacetyl hexamethylene diamine (CAHB) is a newly developed agent that induces the differentiation of malignant cells and deprives their clonal growth ability. Since its effect on T-ALL has not been previously determined, we evaluated its potential function in the Jurkat cell line.

Material/Methods: MTT assay was conducted to evaluate the cytotoxicity and anti-proliferative effect of CAHB. The apoptosis level of CAHB-treated Jurkat cells was evaluated using flow cytometry via staining with Annexin V/PI or cleaved-caspase-3. The alteration of mitochondrial membrane potential was determined by flow cytometry. The expression of Bax and Bcl-2 was evaluated by RT-PCR and Western blot. Western blot was also used to assess the activation of Akt.

Results: CAHB inhibited the proliferation and promoted the apoptosis of Jurkat cells in a time- and dose-dependent manner by decreasing activation of Akt, reducing the mitochondrial membrane potential, and downregulating the Bcl-2/Bax ratio.

Conclusions: Our data suggest that CAHB might be regarded as a novel treatment agent for T-ALL since it can induce apoptosis and inhibit proliferation of the T-ALL cell line at a relatively low level.

MeSH Keywords: Cell Proliferation • Diacetyl • Precursor T-Cell Lymphoblastic Leukemia-Lymphoma • Proto-Oncogene Proteins c-akt

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/915840

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Background

Acute lymphoblastic leukemia (ALL) is a group of hematologic malignancies featuring impaired differentiation and high proliferation of a clonal population of lymphoid progenitor cells in bone marrow, peripheral blood, and extramedullary sites [1]. ALL is often considered a pediatric disease, as 80% of ALL cases are children, but it can also be a devastating disease in adults [2]. ALL is classified into 3 types according to the morphology and cytogenetic profiles: B lymphoblastic, T lymphoblastic, and Burkitt-cell leukemia [3]. T cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy due to the aberrant cell growth and proliferation of immature T cell progenitors. T-ALL is twice as prevalent in males as in females and accounts for approximately 15% of pediatric and 25% of adult ALL cases [4].

Although CAR-T therapy has shed new light on B-ALL treatment in recent years, chemotherapy is still the main treatment method in T-ALL. Chemotherapy usually consists of induction, consolidation, and long-term maintenance. Induction therapy aims to establish normal hematopoiesis [5,6]. Diacetyl hexamethylene diamine (CAHB) is a newly developed hexamethylene bisacetamide (HMBA) analogue that induces the differentiation of malignant cells and abrogates their clonal growth ability [7]. HMBA has been carried out in clinical trials for a variety of solid tumors, including non-small cell lung cancer, myelodysplastic syndrome (MDS), refractory AML, and other hematologic malignancies, and preliminary results show its anti-tumor effects. However, the high neurotoxicity and blood toxicity of HMBA largely hinder its further application. As an HMBA analogue, CAHB displays the characteristics of low toxicity and high efficacy. In addition, Phase I and II-A clinical trials indicated that CAHB has effects on MDS, with only mild and reversible adverse reactions in patients [8,9]. Therefore, it was suggested to expand indications for the treatment of relapsed refractory leukemia and lung cancer [8].

Our research group has focused on the effect and mechanism of CAHB in malignancy. Research on CAHB in treating T-ALL has not been reported previously. Here, we demonstrate the function of CAHB in the Jurkat T-ALL cell line, aiming to provide a new perspective on T-ALL treatment strategies.

Material and Methods

Cell culture and treatment

Jurkat cells (acute T cell lymphoblastic leukemia) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. Jurkat cells were cultured at 37°C and 5% CO_2 in RPMI 1640 medium (Hyclone, USA) with 10% fetal bovine serum (Hyclone, USA), streptomycin (100 mg/mL), and penicillin (100 U/mL). At 80% confluence, cells were digested by trypsinization (0.5% trypsin/2.6 mM EDTA) (HyClone, USA), collected, and sub-cultured according to experimental requirements.

CAHB was purchased from Wuxi Shanhe Pharmaceutical Co. (China). We dissolved 1 g CAHB in 5 ml saline at 56°C and filtered it with 0.22-μm Millex filters (Millipore, USA). The CAHB stocking solution was stored at 4°C. Then, the CAHB solution was diluted into the indicated concentrations and added into the culture medium.

Cell viability assay

We seeded 5×10^5 cells into a 96-wells plate (Corning, USA) and treated them with the indicated concentrations of CAHB, followed by incubation for 12–72 h. We followed the instructions in the manual of the Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTT) kit. OD_{490nm} was measured to determine the inhibition rate of cell proliferation.

Flow cytometry

For the apoptosis assay, Jurkat cells were treated with CAHB for 24 h. Then, 5×10^5 cells in each group were collected and washed with PBS. Cells were double-stained with Annexin V-FITC and PI (Biovision, USA) following the method in the product manual and incubated at room temperature for 10 min in the dark. To assess mitochondrial membrane potential ($\Delta\Psi m$), the cells were treated with CAHB (0, 5, 10, 20, 40 mmol/L) for 24 h, then harvested and washed with HBSS, incubated for 30 min in the dark with 25 nM TMRM (MCE, USA), and analyzed by flow cytometry.

RNA extraction and RT-PCR

We seeded 5×10^6 cells and treated them with CAHB. After 24-h incubation, the cells were washed with PBS and harvested. Total RNA was extracted from the cells by use of Trizol (Invitrogen, USA). A Nanodrop spectrophotometer (Thermo Scientific, USA) was used to determine the concentration of RNA before reverse transcription. The cDNA was synthesized by reverse transcription from total RNA using RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas, USA). The expression of Bax, Bcl-2, and caspase-3 at the mRNA level was determined by RT-PCR using Taq DNA polymerase (Generay, China). The amplified DNA products were analyzed by electrophoresis and imaged with Bio-Rad Gel 2000, then semi-quantified using Quantity One software.

Primer:
β-actin F: 5'-AAGATGACCCAGATCATGTTTGA-3'
R: 5'-TTAATGTCACGCACGATTTCC-3'

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Bcl-2 F: 5'-CAGCGACTTCTCCCGCCGCTACCGC-3'
R: 5'- CCGCATGCTGGGGCCGTACAGTTCC-3'
Bax F: 5'-TGGCAGCTGACATGTTTTCTGAC-3'
R: 5'-CGTCCCAACCACCCTGGTCT-3',

Western blot analysis

After incubation with CAHB for 24 h, Jurkat cells were washed with PBS, harvested, and lysed in RIPA cell lysis buffer. Quantification of total protein was performed using a BCA kit (Thermo Fisher, USA). We separated 50 μg proteins in each group using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred them to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked by 5% skimmed milk for 1 h at room temperature. The primary antibodies (Cell Signaling Technology, USA) were diluted into indicated concentration (Bcl-2 1: 500, Bax 1: 1000, Akt 1: 1000, p-Akt 1: 1000, GAPDH 1: 1000 A) in 5% skimmed milk and incubated with the PVDF membrane at room temperature for 2 h. After washing 3 times with TBST for 10 min, the membrane was incubated with HRP-linked secondary antibody (1: 2000 dilution) (Cell Signaling Technology, USA), at room temperature for 1 h. Then, the membrane was washed again with TBST 3 times and the signal was detected using an ECL Plus Kit (Applied Biosystems). Quantity One was used for grayscale value calculation of Western blot images.

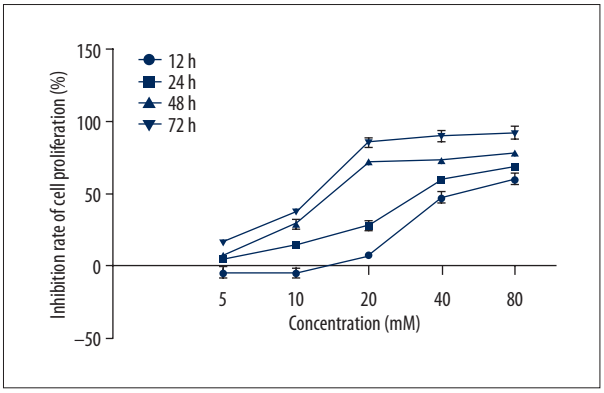

Figure 1. CAHB inhibits the proliferation in Jurkat cells. Jurkat cells were treated with 0-80mM CAHB for 12, 24, 48, 72 hours, respectively. MTS assay were established to determine the cell viability.

Statistical analysis

Data are shown as the Mean ± Standard error (SEM). Group differences were analyzed by unpaired *t* test from 3 independent experiments and the final data were summarized using GraphPad Prism. $p \leq 0.05$ was considered significant.

Results

CAHB inhibits proliferation in Jurkat cells.

To determine the effect of CAHB on T-ALL, we first treated the T-ALL cell line Jurkat with CAHB of different concentrations (5–80 mM). MTT assay was performed. We found that when treated with CAHB, cell proliferation was significantly inhibited. This inhibitory effect was both time- and dose-dependent (Figure 1, Table1). We also observed that the inhibitory function of CAHB was most prominent at 24 h, so in the follow-up experiments we stimulated Jurkat cells with CAHB for 24 h. These results show that CAHB inhibits the proliferation of Jurkat cells in a time- and dose-dependent manner.

CAHB upregulates apoptosis of Jurkat cells.

We explored whether CAHB can induce apoptosis of Jurkat cells. Jurkat cells were treated with 20 mM CAHB for 24 h and stained with DAPI. Compared with the control group, which showed complete nuclear morphology and uniform chromatin, cells in the 20 mM group underwent significant apoptosis with decreased nuclear volume, chromatin condensation, nuclear fragmentation, and apoptosis corpuscles formation (Figure 2A). To further demonstrate the effect of CAHB on cell apoptosis, we treated Jurkat cells with 0–40 mM CAHB for 24 h, double-stained them with Annexin V and PI and analyzed cell apoptosis by flow cytometry. After co-culturing with 10 mM,

Table 1. CAHB inhibits the proliferation in Jurkat cells.

Concentration (mM)	12 h	24 h	48 h	72 h
5	−5.1±2.8	3.8±1.5	6.3±2.1	15.4±1.5
10	−5.3±3.0	13.9±1.0*	28.7±3.0	37.1±0.9*
20	7.3±1.6*	27.4±2.9*	71.0±2.2*	84.5±2.5*
40	47.2±3.1*	59.2±1.5*	72.3±2.1*	88.9±3.9*
80	59.5±3.7*	67.6±2.1*	77.8±2.1*	91.2±4.7*

Statistical results of Figure 1. The final data were summarized from 3 independent experiments. * $p \leq 0.05$ was considered significant. Data are expressed as mean ±SEM.

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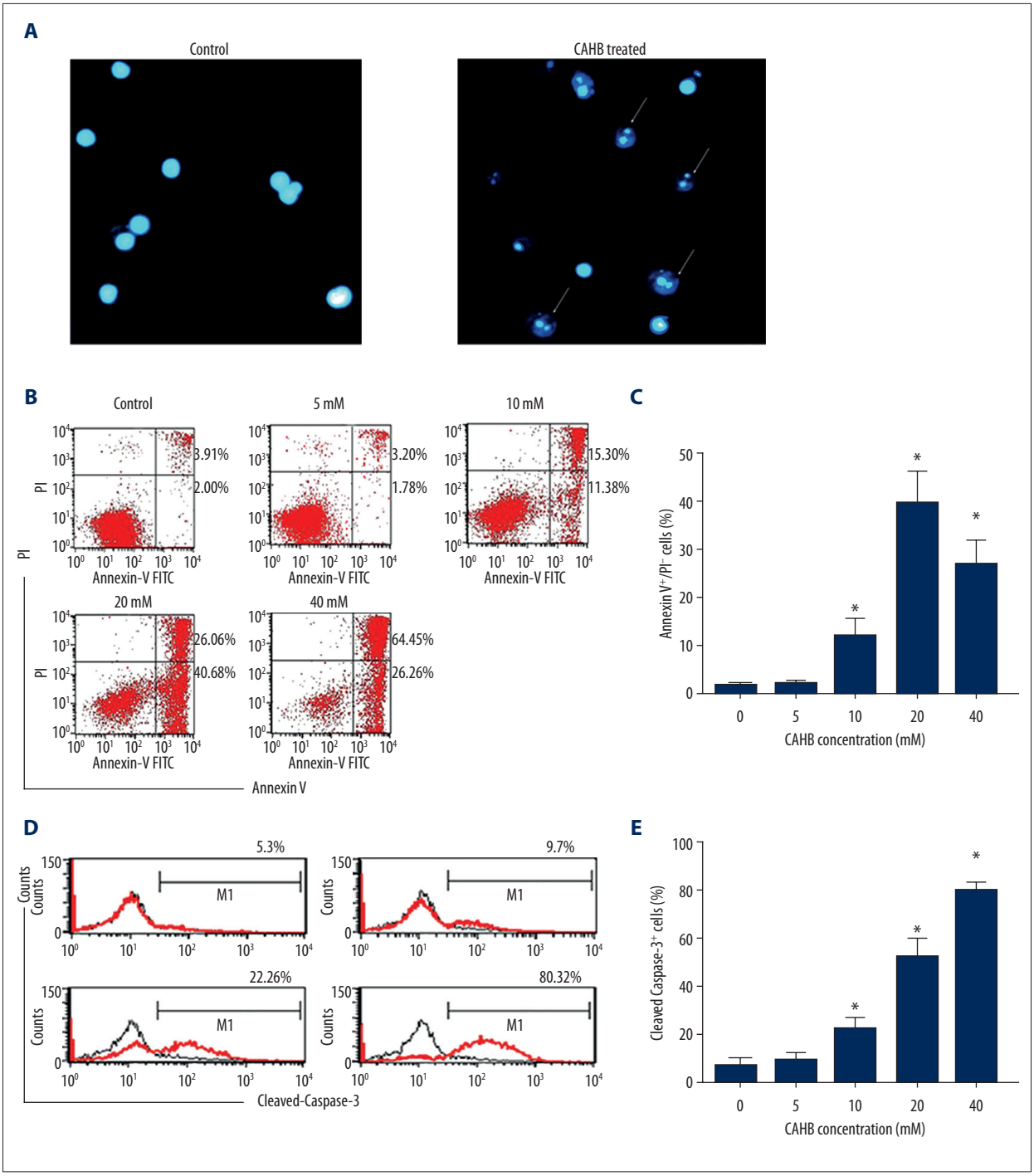

Figure 2. CAHB upregulates apoptosis in Jurkat cells. (**A**) After treatment with saline or 20 mM CAHB for 24 h, Jurkat cells were stained with DAPI and observed under a fluorescence microscope. (**B**) Jurkat cells were treated with saline or 5 mM, 10 mM, 20 mM, and 40 mM CAHB for 24 h and double-stained with Annexin V and PI. Representative results of apoptotic cells (Annexin V^+/PI^-) are shown. (**C**) Statistical results of **B**. The final data were summarized from 3 independent experiments. * $p \leq 0.05$ was considered significant. Data are expressed as Mean±SEM. (**D**) Jurkat cells were treated as **B** and stained with cleaved-Caspase-3. Representative results of cleaved-Caspase-3^+ cells are shown. (**E**) Statistical results of **D**. The final data were summarized from 3 independent experiments. * $p \leq 0.05$ was considered significant. Data are expressed as mean ±SEM.

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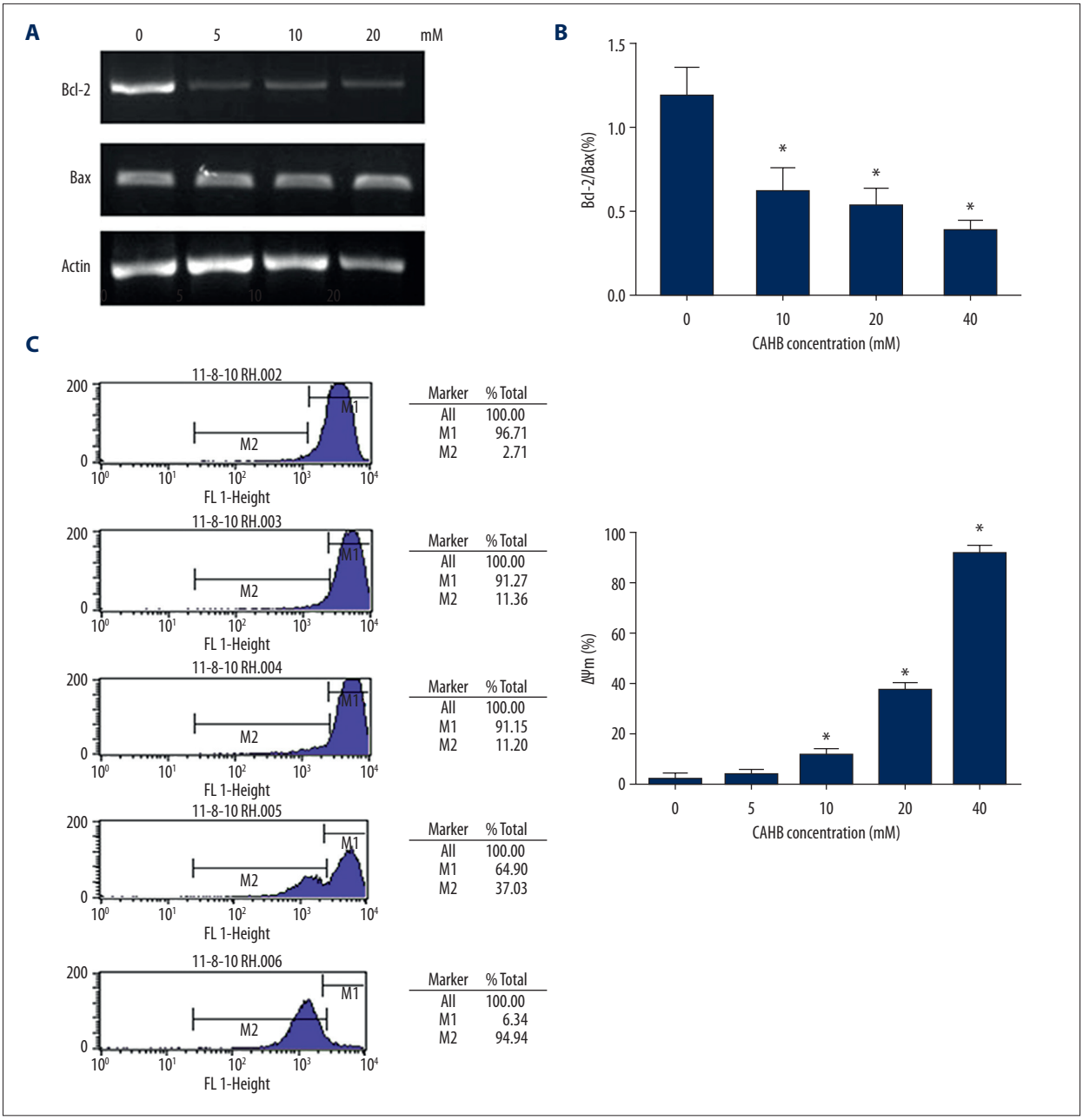

Figure 3. CAHB decreases the cell viability via Bcl-2 and mitochondrial membrane potential. (**A**) Jurkat cells were treated with saline or 5 mM, 10 mM, and 20 mM CAHB for 24 h. mRNA expression was determined by RT-PCR. (**B**) Statistical results of DNA band density in **A**. The final data were summarized from 3 independent experiments. * p≤0.05 was considered significant. Data are expressed as mean ±SEM. (**C**) Jurkat cells were treated as **A**. The flow cytometry representative results of mitochondrial membrane potential (ΔΨm) are shown. (**D**) Statistical results of **C**. The final data were summarized from 3 independent experiments. * p≤0.05 was considered significant. Data are expressed as mean ±SEM.

20 mM, and 40 mM CAHB, the percentages of Annexin V^+/PI^- apoptotic cells were significantly increased (Figure 2B, 2C). We also detected cleaved-caspase-3^+ cells after incubation with CAHB. Consistent with the results above, the percentage of cleaved-caspase-3^+ cells were significantly increased compared to the untreated group, in a dose-dependent manner (Figure 2D, 2E), showing that CAHB also exerts a pro-apoptotic function in Jurkat cells.

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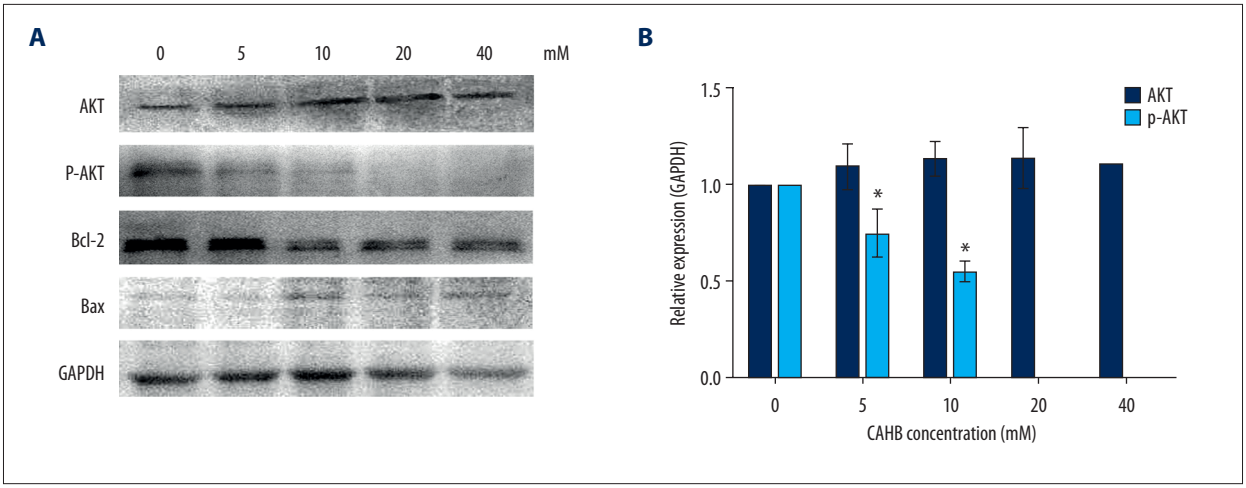

Figure 4. CAHB inhibits the phosphorylation of Akt and downregulates PI3K/Akt signaling pathway. (**A**) Jurkat cells were treated with saline or 5 mM, 10 mM, and 20 mM CAHB for 24 h. Total Akt, phosphorylated Akt, Bcl-2, and Bax were determined by Western blot. (**B**) Statistical results of protein band density in **A**. The final data were summarized from 3 independent experiments. * $p \leq 0.05$ was considered significant. Data are expressed as mean ±SEM.

CAHB decreases cell viability via downregulating Bcl-2 and mitochondrial membrane potential

Previous studies indicated that Bcl-2 family members play crucial roles in the anti-apoptosis signaling pathway. Bax and Bcl-2 can form the heterodimer and decrease the level of free Bcl-2 and abrogate its anti-apoptotic function. Thus, the ratio of Bcl-2/Bax indicates anti-apoptotic ability. Thus, we next detected their expression in CAHB-treated cells. RT-PCR results indicated that after 0–20 mM CAHB treatment, the ratio of Bcl-2/Bax mRNA expression was significantly decreased compared with the control group, and the effect was dose-dependent (Figure 3A, 3B). Moreover, the downregulated ratio of Bcl-2/bax often leads to decreased mitochondrial membrane potential ($\Delta\Psi m$). Thus, we determined the $\Delta\Psi m$ level in the CAHB-treated cells by use of flow cytometry. After 24 h of CAHB treatment at concentrations of 0 mM, 5 mM, 10 mM, 20 mM, and 40 mM, the proportion of $\Delta\Psi m$ decrease was (3.1±1.0)%, (5.1±0.7)%, (12.2±1.9)%, (38.4±1.3)%, and (91.8±2.8)%, respectively (Figure 3C, 3D). The effect was also dose-dependent. In summary, these results illustrate that CAHB downregulates the Bcl-2/Bax ratio and leads to alteration of mitochondrial membrane potential, thus contributing to the apoptosis of Jurkat cells.

CAHB inhibits phosphorylation of Akt and downregulates the PI3K/Akt signaling pathway

The PI3K/Akt signaling pathway is the upstream signal of the Bcl-2 family. Aberrant Akt activation is observed in many malignant tumors, including T-ALL. Phosphorylation of Akt in CAHB-treated Jurkat cells was determined by Western blot analysis. First, the Bcl-2/Bax expression ratio was also decreased at the protein level, which is in accordance with the mRNA level we illustrated above (Figure 4A). Furthermore, Akt phosphorylation was lower in the CAHB-treated group, also in a dose-dependent manner (Figure 4A, 4B). Hence, these results show that the CAHB inhibits the phosphorylation and activation of Akt, thus promoting the apoptosis of Jurkat cells.

Discussion

Our results suggest that CAHB significantly inhibits the proliferation of Jurkat cells, and the effect is dose-dependent. However, the results also suggest that low-concentration treatment displays a certain effect on promoting cell proliferation at the early stage, but the mechanism is unknown and further study is required. Our group has conducted *in vitro* experiments on human small cell lung cancer NCI-H446 cells to test the inhibitory function of CAHB, but the effect was not obvious. The anti-proliferative effect of CAHB in Jurkat cells is more obvious than that in NCI-H446 cells. After 20-mM CAHB treatment for 24 h, apoptotic body formation was observed in many cells, and dead cells appeared in the 40 mM group. This phenomenon was also observed by detecting Annexin V^+/PI^- cells. In the 20 mM group, the early apoptosis rate reached 39±6.3%, but in the 40 mM group the early apoptosis rate was decreased because the numbers of late apoptotic cells and dead cells increased. To sum up, the excessive cell death in the experiments above was probably caused by the high concentration of CAHB. Guo et al. first discovered that after 40 Mm CAHB treatment for 24 h, apoptosis of MDS cell line MUTZ-1 cells was observed initially, and the apoptosis was significantly increased after 72 h [9]. Therefore, compared to MDS and SCLC cell lines, the T-ALL cell line Jurkat is more sensitive to CAHB,

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with higher proliferation inhibition and apoptosis promotion. Hence, these results indicate CAHB might be suitable for use in treatment of T-ALL.

Apoptosis involves a series of signaling pathway activation and gene expression. Recent studies indicate the PI3K/Akt signal pathway is aberrantly activated in a variety of tumors, and is closely related to cancer development [10–12]. Alejandro et al. found that excessive activation of the PI3K/Akt pathway occurs in patients with T-ALL [12]. Akt is a vital target protein in the PI3K/Akt signaling pathway. Akt is phosphorylated at Thr308 and Ser473, and Akt was activated only with both sites phosphorylated [13]. Morishita et al. reported that phosphorylation of Akt might be the cause of pre-B-ALL drug resistance, which suggested that Akt could be considered as a new target for therapy of relapsed and refractory pre-B-ALL [14]. In addition, PTEN plays a vital role in the diagnosis and prognosis of many tumors, as an important negative regulator in the PI3K/Akt signaling pathway [15]. Deletion or downregulation of PTEN gene often leads to abnormal activation of the PI3K/Akt signaling pathway. A previous study found that the expression of PTEN gene was absent in Jurkat cells, resulting in an abnormal increase of p-Akt expression [16]. The results of our study show that Akt was highly phosphorylated in Jurkat cells, which were similar to results in previous studies. Phosphorylation of Akt was also significantly downregulated after CAHB treatment in our experiments, suggesting that the CAHB-induced apoptosis of Jurkat cells might be caused by blocking the phosphorylation of Akt and thereby downregulating activity of the PI3K/Akt signaling pathway.

The mechanism of the PI3K/Akt signaling pathway in apoptosis is not clear. Multiple downstream factors might contribute to the apoptosis. Henshall et al. found that apoptosis might be mediated via downstream Bcl-2 family [17]. Bax forms the heterodimer with Bcl-2 or Bcl-xl in quiescent conditions. Activated PI3K/Akt pathway leads to the phosphorylation of Bax serine residues, which causes the conformation change. Thus, Bax depolymerizes with Bcl-2 or Bcl-xl. Free Bcl-2 can exert its anti-apoptosis function, and the heterodimer formed by Bax and Bcl-2 can inhibit the anti-apoptosis effect of Bcl-2. The decrease of Bcl-2/Bax ratio negatively regulates mitochondrial membrane potential, which leads to the release of cytochrome C, combined with ATP and apoptotic protease activator (apaf-1), thus successively activating caspase-9 and caspase-3, and finally leading to apoptosis. On the contrary, an upregulated Bcl-2/Bax ratio inhibited apoptosis. Activated Akt might inhibit caspase-3 activation directly [18], or phosphorylate the Ser196 site of caspase-9 precursor [19], thereby inhibiting caspase-3 activation and preventing the latter from promoting apoptosis.

Ruefli et al. reported that the expression of Bcl-2 protein was downregulated and the activation of caspase-3 was increased in human acute T-lymphocytic leukemia cell lines treated by CAHB analogue HMBA [2]0. Palumbo et al. also reported similar findings in HMBA-induced apoptosis of malignant mesothelioma cells *in vitro* [21]. In the present study, we found that the ratio of Bcl-2/Bax was downregulated and the caspase-3 activation rate was increased in Jurkat cells after CAHB treatment, which is consistent with previous studies. We also observed that Bax expression was significantly increased at the protein level after CAHB treatment, but there was no significant change at the mRNA level, suggesting that its regulation might occur at the protein level.

Conclusions

We discovered that one of the CAHB anti-tumor mechanisms is inhibiting the phosphorylation of Akt, downregulating the activity of the PI3K/Akt signaling pathway, and then acting on the downstream Bcl-2 family-related factors and activating caspase-3.

Conflict of interests

None.

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