Effects of chebulinic acid on differentiation of human leukemia K562 cells[1]

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KEY WORDS K562 cells; chebulinic acid; butyrates; hemin; glycophorin; CD61 antigen; gene expression regulation; cell differentiation

ABSTRACT

AIM: To study effects of chebulinic acid on erythroid and megakaryocytic differentiation in K562 cells. **METHODS:** The benzidine staining method was used to evaluate hemoglobin synthesis; the expression of erythroid specific glycophorin A (GPA) protein and megakaryocytic surface marker CD61 was determined by flow cytometry using fluorescence labeled antibodies; erythroid and megakaryocytic mRNA expression was analyzed by RT-PCR. **RESULTS:** During erythroid differentiation induced by butyric acid (BA) or hemin, chebulinic acid not only inhibited the hemoglobin synthesis of BA- and hemin-treated K562 cells in concentration-dependant manner with IC_{50} of 4 μmol/L and 40 μmol/L respectively, but also inhibited another erythroid differentiation marker acetylcholinesterase at the concentration of 50 μmol/L in the cells either treated or untreated with each erythroid differentiation inducers, whereas chebulinic acid 50 μmol/L did not change GPA protein expression in these cells significantly. When K562 cells were treated with TPA 50 μg/L for 72 h to induce megakaryocytic differentiation, the presence of chebulinic acid 50 μmol/L slightly provoked the decrease of GPA protein expression induced by TPA. Chebulinic acid did not change the TPA-induced CD61 expression at the same concentration. Chebulinic acid also reduced the mRNA levels of erythroid relative genes including γ-globin, PBGD, NF-E2, and GATA-1 genes in K562 cells either treated or untreated with BA, whereas chebulinic acid upregulated the mRNA levels of GATA-2 transcription factor in these cells. **CONCLUSION:** Chebulinic acid had inhibitory effect on erythroid differentiation likely through changing transcriptional activation of differentiation relative genes, which suggests that chebulinic acid or other tannins might influence the efficiency of some anti-tumor drugs-induced differentiation or the hematopoiesis processes.

INTRODUCTION

Tannins are distributed in almost all plants utilized as foods and medicinal plants[1]. In addition, tannins have been considered to be food additives. It was estimated that people in the United States ingested each day 1 g of tannic acid (a tannin)[2]. Tannins have been well shown to possess a variety of pharmacological activities such as antiviral, antimicrobial, antioxidant, and hepatoprotective activities and antitumor effects [3]. More attention should be paid to the adverse health effects of tannins.

It has been extensively demonstrated that human chronic myelogenous leukemia K562 cells can be induced to differentiate towards erythroid and megakaryo-

[1] Project supported by Tsinghua University 985 Project, the China Postdoctoral Science Foundation and the National Natural Science Foundation of China (No 30271593).
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Received 2003-01-22 Accepted 2003-07-14

cytic lineages by various differentiation inducers[4]. Erythroid differentiation of K562 cells could be achieved by exposure to several pharmacological agents, including hemin[5], arabinofuranosylcytosine[6], anthracyclines, butyric acid (BA), and doxorubicin[7], while 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced K562 cells to differentiate towards the megakaryocyte lineage[8]. K562 cells have severed as a model to screen anti-tumor drugs and to research hematological cell differentiation.

Recently, a subchronic toxicity study showed that gallic acid by ig could induce reduction of hemoglobin concentration, hematocrit, and red blood cell counts in whole blood of F344 rats[9]. Although the author thought that the development of anemia induced by gallic acid was hemolytic, we presumed that this hydrolysate (gallic acid) of many tannins and tannins *per se* could interfere with hematopoiesis. Chebulinic acid is a ellagitannin widely present in plants such as *Terminalia chebula* fruit. Chebulinic acid showed many bioactivities including inhibition of cancer cell growth[10], anti-*neisseria gonorrhoeae* activity[11], inhibiting the contractile responses of cardiovascular muscles[12], and blocking the binding of HIV rgp120 to CD4[13].

However, there has been no experimental evidence about the effect of chebulinic acid on hematological cell differentiation. In present study, the effect of chebulinic acid on chemistry agent-induced erythroid and megakaryocytic differentiation was investigated in K562 cells.

MATERIALS AND METHODS

Reagents RPMI-1640 medium was obtained from Gibco BRL; fetal bovine serum (FBS) was from HyClone; acetylthiocholine iodide, BA, 5,5'-dithiobis-(2-nitrobenzoic acid), hemin, MTT, penicillin, streptomycin, and TPA were from Sigma; fluorescein isothiocyanate (FITC)-conjugated anti-CD61 antibodies and mouse isotype IgG_1-FITC were from Becton-Dickinson; phycoerythrin (PE)-conjugated anti-GPA antibodies and mouse isotype IgG_1-PE antibodies were from Caltag; UNIQ-10 Total RNA Minipreps Classic Kit and 2-N First Strand cDNA Synthesis Kit were purchased from Sangon (Shanghai, China); chebulinic acid was kindly provided by Prof Yan-Ze LIU (He-nan College of Traditional Chinese Medicine, Zhengzhou, He-nan Province, China).

Cell culture K562 cells were grown in suspension in RPMI-1640 medium supplemented with 10 % (v/v) FBS, penicillin 100 kU/L, and streptomycin 100 mg/L in a 5 % (v/v) CO_2 humidified atmosphere at 37 ºC. Exponentially growing K562 cells were used for the experiments.

Benzidine staining and determination The percentage of cells staining for hemoglobin was estimated by staining with benzidine/H_2O_2 essentially as previously described[14]. The blue-stained hemoglobin-positive cells were counted in a hemacytometer on a microscope. At least 500 cells were counted for each sample.

Acetylcholinesterase activity The method used to determine acetylcholinesterase (AChE) activity was modified from that of Ellman *et al*[15]. Briefly, the enzyme reaction was undergone at 37 ºC in a 1-mL reaction system containing 0.5×10^6 K562 cells, 0.1 mol/L potassium phosphate buffer (pH 8.0), 0.6 mmol/L 5,5'-dithiobis-(2-nitrobenzoic acid), and 0.75 mmol/L acetylthiocholine iodide substrate. After 20 min, each sample was centrifuged at 4 ºC (3000 rpm, 5 min). The absorbance of the supernatant was measured spectrophotometrically at 405 nm.

Flow cytometric analysis The expression of erythroid and megakaryocytic antigens was determined by direct immunofluorescence using the following conjugated antibodies: FITC-conjugated anti-CD61 antibodies, and PE-conjugated anti-GPA antibodies. Mouse isotype IgG_1-FITC and IgG_1-PE antibodies served as controls respectively. Flow cytometric analysis was performed using the FACScan instruments (Coulter, Epics Elite, USA).

Isolation of total RNA UNIQ-10 Total RNA Minipreps Classic Kit was used to extract total RNA from cultured cells. Cells 1×10^7 were collected by centrifugation and 0.5 mL of Trizol was added. After the cells were lysed, 100 μL of chloroform/isoamyl alcohol (24:1, v/v) was added, and the sample was shaken vigorously. The suspension was centrifuged at 12 000 rpm for 5 min at room temperature. The RNA in the aqueous phase was transferred to an RNase-free tube. water-free ethanol 150 μL was added and mixed. Then, the RNA solution was transferred to UNIQ-10 Column, stored for 2 min at room temperature, and centrifuged at 10 000 rpm for 1 min at room temperature. The UNIQ-10 Column was washed (centrifugation at 10 000 rpm for 1 min at room temperature) with solution RW (Sangon) twice. The UNIQ-10 Column was placed into an RNase-free tube, mixed with 50 μL of DEPC-treated water, placed at 65 ºC for 2 min, and centrifuged at 10 000 rpm for 1 min at room temperature.

The solution in the collection tube was RNA sample. The RNA quantity was calculated from the absorbance at 260 nm.

RT-PCR 2-N First Strand cDNA Synthesis Kit was used to synthesize cDNA. PCR was carried out using synthesized cDNA and specific primers sets for each gene (Tab 1) in a 50 μL reaction with UNOII Thermocycler (Biometra). The reactions were initiated by a step for 5 min at 95 ºC, and then subjected to 30 cycles of primer annealing at annealing temperature (Tab 1) for 45 s, and DNA extension at 72 ºC for 45 s, and denaturation at 95 ºC for 1 min. β-Actin served as a control and was amplified for 25 cycles. PCR products were analyzed on 2 % agarose gel, stained with ethidium bromide and photographed with ImageMaster Video Documentation System (Pharmacia Biotech).

Data analysis Student's *t*-test was used to determine the statistical significance. $P<0.05$ was considered statistically significant.

RESULTS

Inhibition of hemoglobin synthesis and AChE activity in K562 cell by chebulinic acid Firstly, hemoglobin synthesis of K562 cells was studied as the percentage of benzidine-positive cells induced by hemin or BA. Both hemin (40 μmol/L) and BA (0.5 mmol/L) could induce hemoglobin synthesis in K562 cells as expected**.** However, when chebulinic acid was added, the percentage of benzidine-positive cells was decreased in the concentration-dependant manners (r_{BA}=-0.944, $P<0.01$; r_{hemin}=-0.972, $P<0.01$). In K562 cells co-treated with hemin (40 μmol/L) and chebulinic acid for 72 h, the concentration of chebulinic acid at which the percentage of differentiated cells was inhibited by approximately 50 % was 40 μmol/L (Fig 1A). In K562 cells co-treated with BA (0.5 mmol/L) and chebulinic acid for 72 h, chebulinic acid 4 μmol/L reduced the differentiation of BA-treated cells by approximately 50 % (Fig 1B). As shown in Fig 1B, when the concentration of chebulinic acid was up to 10 μmol/L, the percentage of benzidine-positive cells was decreased to less than 5 %, and a complete inhibition of benzidine-positive cells was obtained in the presence of chebulinic acid 50 μmol/L. These results demonstrated that chebulinic acid had inhibitory effect on hemoglobin synthesis of K562 cells induced by hemin or BA, but the sensitivity of BA-treated cells responsive to chebulinic acid was higher than that of hemin-treated cells.

After K562 cells were treated with chebulinic acid 50 μmol/L for 72 h, the AChE activity of chebulinic acid-treated cells was significantly lower than that of the control cells ($P<0.05$). Chebulinic acid also significantly inhibited the AChE activity in the cells treated with hemin 40 μmol/L or BA 0.5 mmol/L for 72 h ($P<0.01$ or 0.05 respectively, Fig 2).

No significant effects of chebulinic acid on expression of erythroid and megakaryocytic surface markers K562 cells were treated with hemin 40 μmol/L or BA 0.5 mmol/L for 72 h in the presence of chebulinic acid 50 μmol/L, and the expression of erythroid (GPA) was measured by flow cytometry. Chebulinic acid only

Tab 1. Primers used for PCR amplification.

Primers	Sequences	Annealing temperature/ºC
γ-Globin	Sense: ACAAGCCTGTGGGGCAA Antisense: GCCATGTGCCTTGACTTT	56
PBGD	Sense: GGTCCTACTATCGCCTCCCTC Antisense: CCAGCCTCTGTCCCCTCCAGC	60
GATA-1	Sense: CAGTCTTTCAGGTGTACCC Antisense: GAGTGATGAAGGCAGTGCAG	60
NF-E2	Sense: ATTTGAGCCCCAAGCCCCAGC Antisense: CCAGCCTCTGTCCCCTCCAGC	62
GATA-2	Sense: ATCAAGCCCAAGCGAAGACTG Antisense: ACATTGTGCAGCTTGTAGTAGAGGC	60
c-*mpl*	Sense: GCACTGTGATGCTTTATGCAAC Antisense: TGAACGGTTTAGAGGATGAGGA	56
β-Actin	Sense: TGGACTTCGAGCAAGAGATGG Antisense: ATCTCCTTCTGCATCCTGTCG	60

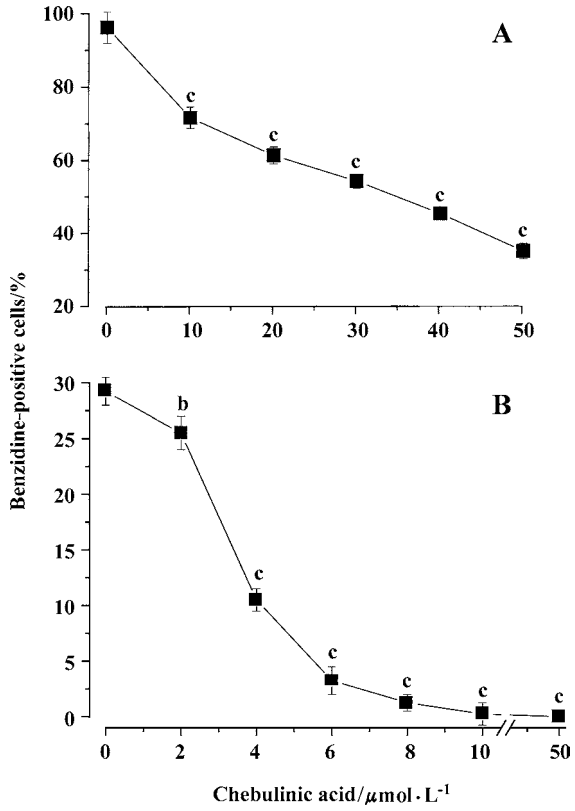

Fig 1. Inhibition of chebulinic acid on K562 cell hemoglobin synthesis induced by hemin and BA. The K562 cells were simultaneously treated with hemin 40 μmol/L (A) or BA 0.5 mmol/L (B) combined with different concentrations of chebulinic acid for 72 h. After treatment, benzidine/H_2O_2 was used to stain the cells containing hemoglobin. The benzidine-positive cells were counted out of 500 cells per sample. Data represent the mean±SD from three independent experiments. $^bP<0.05$, $^cP<0.01$ *vs* the control cells without treatment of chebulinic acid.

slightly inhibited GPA expression in the K562 cells not treated with any inducers (from 92.5 % down to 84.2 %), and in hemin-treated cells (from 90.6 % down to 78.9 %), whereas chebulinic acid did not affect GPA expression induced by BA (Fig 3A).

In addition, we also evaluated the effects of chebulinic acid on megakaryocytic differentiation induced by TPA, a megakaryocytic differentiation inducer. Chebulinic acid 50 μmol/L could slightly provoke the inhibitory effect of TPA 50 μg/L on GPA expression to a little extent (from 87.3 % down to 77.5 %, Fig 3A). When the cells were treated with TPA 50 μg/L for 72 h, the CD61-positive percentage of K562 cells was increased to 60.2 % from 4.8 %, whereas chebulinic acid did not affect CD61 expression induced by TPA (Fig 3B).

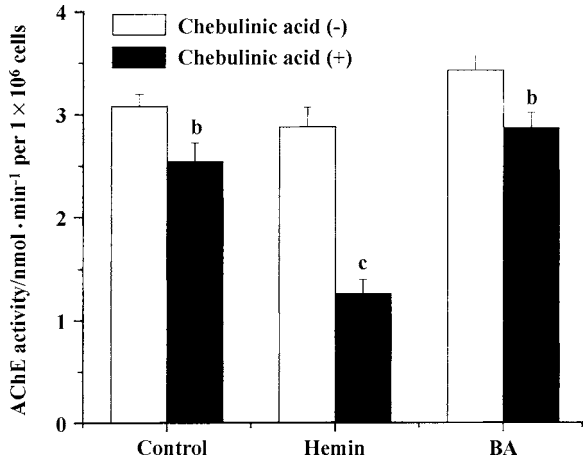

Fig 2. The effect of chebulinic acid on AChE activity in K562 cells. The K562 cells were simultaneously treated with hemin 40 μmol/L or BA 0.5 mmol/L combined with chebulinic acid 50 μmol/L for 72 h. Then AChE activity was determined by Ellman's method. Data represent the mean±SD from five independent experiments. $^bP<0.05$, $^cP<0.01$ *vs* the cells without treatment of chebulinic acid.

Effects of chebulinic acid on erythroid and megakaryocytic mRNA expression In K562 cells only treated with chebulinic acid 50 μmol/L for 72 h, the levels of γ-globin and PBGD mRNA as well that of mRNA for erythroid transcription factor NF-E2 were obviously decreased, the level of mRNA for GATA-1, another erythroid transcription factor, was slightly reduced, the level of mRNA for GATA-2, a megakaryocytic transcription factor, was obviously induced, whereas the level of mRNA for the megakaryocytic relative gene c-*mpl* was not affected. Similarly, chebulinic acid significantly reduced mRNA levels of γ-globin, PBGD, NF-E2, and GATA-1 genes in BA-treated K562 cells, while chebulinic acid promoted the level of mRNA for GATA-2 in these cells. It suggested the transcription changes of these genes might contribute to the inhibitory effect of chebulinic acid on erythroid differentiation of K562 cells (Fig 4).

DISCUSSION

In present study, we demonstrated that chebulinic acid inhibited the hemoglobin synthesis induced by hemin and BA in K562 cells. Moreover, chebulinic acid obviously inhibited AChE activities of K562 cells. In addition, we found that another tannin tellimagrandin I exerted similar inhibitory effects on erythroid differentiation (data not shown). It has shown that gallic acid, the hydroly-

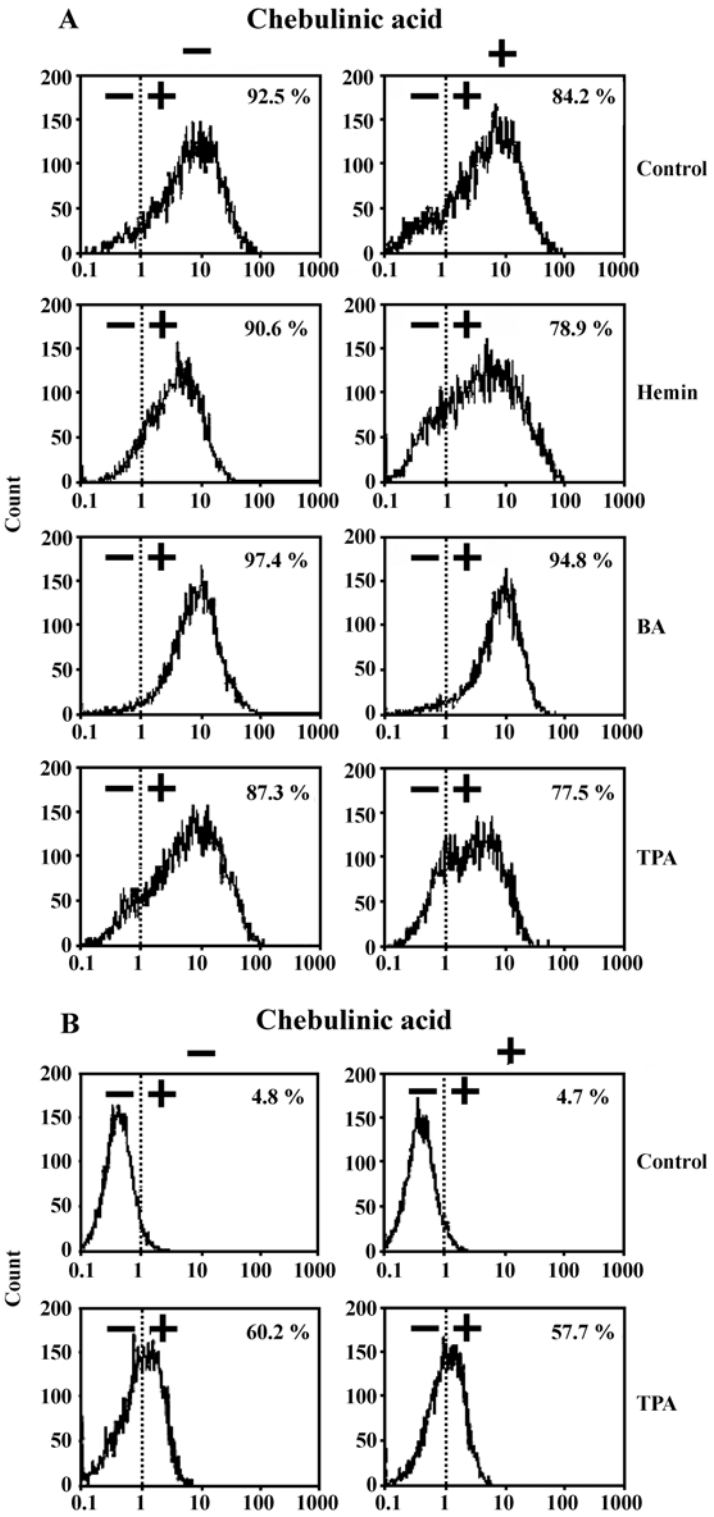

Fig 3. Expression of erythroid and megakaryocytic surface markers in K562 cells. The K562 cells were simultaneously treated with 40 μmol/L of hemin, 0.5 mmol/L of BA or 50 μg/L of TPA combining with 50 μmol/L of chebulinic acid for 72 h. Then, the cells were stained with direct immunofluorescence using FITC-conjugated anti-CD61 antibodies or PE-conjugated anti-GPA antibodies. Expression of erythroid marker GPA protein (A) and megakaryocytic marker CD61 protein (B) was analyzed by flow cytometry. Data from a typical experiment representative of three.

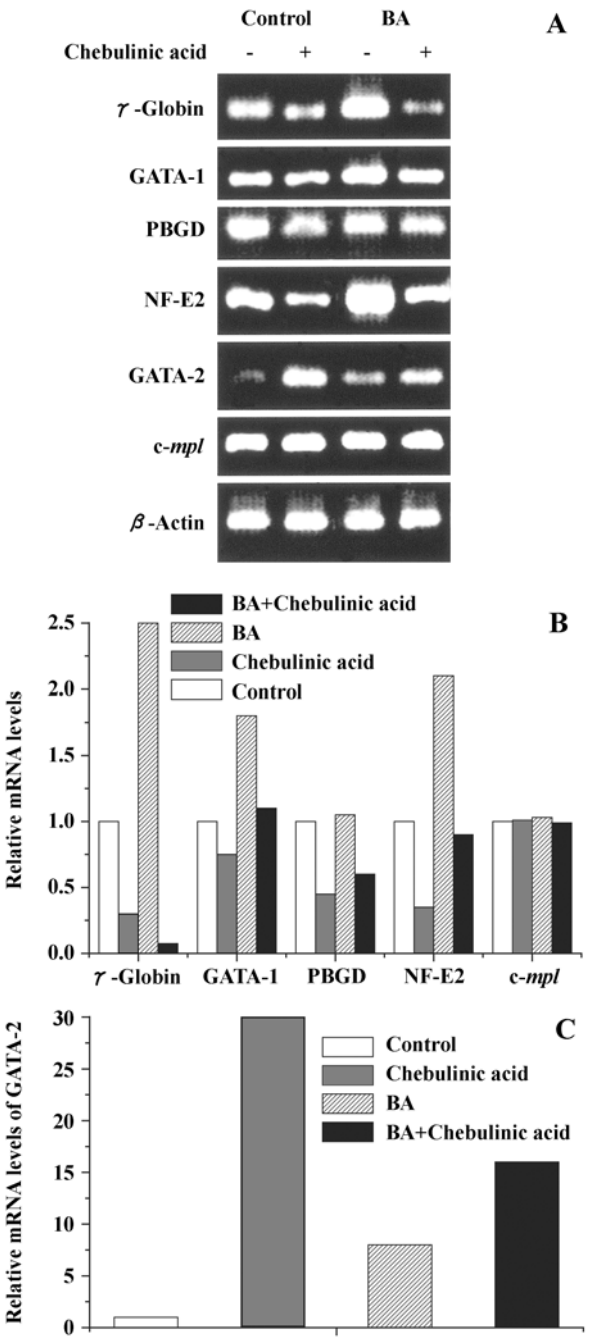

Fig 4. Effects of chebulinic acid on mRNA expression of erythroid and megakaryocytic relative genes in K562 cell. The K562 cells were simultaneously treated with BA 0.5 mmol/L combined with chebulinic acid 50 μmol/L for 72 h. Then the total RNA was extracted from the cells, and RT-PCR was used to analyze mRNA expression level of genes. PCR products were analyzed on 2 % agarose gel stained with ethidium bromide (A). Software band leader 3.00 was used to analyze the relative mRNA levels for γ-globin, PBGD, NF-E2, GATA-1, and c-*mpl* genes (B), and GATA-2 (C) compared with β-actin. β-Actin serves as a control. Data from a typical experiment representative of two.

sate of almost tannins, could induce anemia in F344 rats[9]. These results suggested tannins such as chebulinic acid and tellimagrandin I might influence hematopoiesis. Furthermore, there are many anticancer drugs that exert effects on cancer cell through inducing differentiation. For example, arabinofuranosyl cytosine, anthracyclines, BA, and doxorubicin all can induce erythroid differentiation of K562 cell. Therefore, chebulinic acid is likely to disturb the anticancer effects of these drugs.

Erythroid differentiation mechanisms are extremely complex and involve multiple regulatory events depending on the inducer used. Detailed pathways remain unknown, and no common explanation is available for different inducers. Short-chain fatty acids such as BA and propionate are effective suppressors of colorectal cancer. In such cell types, these compounds have been shown to increase cellular peroxide generation[16]. Recently, It was shown that BA-induced oxidative stress was evidenced by changes in GSSG and GSH levels, the decrease in cellular antioxidant enzyme activities (CAT, GPx, GRase, CuZnSOD, and particularly mitochondrial MnSOD), and the oxidation of the dihydroethidium probe. The addition of NAC, PDTC, or quercetin considerably decreased the differentiation, suggesting that ROS production was involved in this process[17]. Hemin was regarded as a nonoxidative stress-related and reversible inducer[18,19]. Treatment of K562 cells with hemin induced differentiation and related intracellular modifications such as an increase in heat shock gene expression and thioredoxin expression, without any reduction of glutathione level, suggesting that oxidative stress was not involved in this process[18]. Chénais *et al* found that among the antioxidants used, PDTC and quercetin have no effect on hemin-induced erythroid differentiation, and only NAC at a high dose inhibited hemin-induced differentiation, indicating that the mechanism of hemin is not linked to a radical process[17]. Similar to other tannins, chebulinic acid also belongs to polyphenol compounds with strong antioxidant activity[20]. In our study, the results showed that chebulinic acid inhibited erythroid differentiation with different IC_{50} for different inducer-treated cells (40 μmol/L for hemin-induced cells and 4 μmol/L for BA-induced cells). The marked inhibitory effect of chebulinic acid on BA-induced differentiation could be related to its antioxidant activity. The alterative phathway could be involved in the inhibitory effect of chebulinic acid on hemin-induced

differentiation.

The transcriptional activation of the γ-globin gene has been demonstrated in BA-induced K562 cells[21,22]. This has been related to the enhancement of DNA-binding activity and the expression of GATA-1 and NF-E2 transcription factors[21,23]. In addition, the heme synthesis pathway enzymes PBGD were also shown to be up-regulated at the transcriptional level in response to BA[21]. Hemin-induced differentiation did not involve the overexpression of GATA-1 and NF-E2 transcription factors[24]. In our study, chebulinic acid inhibited the mRNA level of γ-globin and PBGD genes not only in BA-indued K562 cells but also in uninduced K562 cells, whereas chebulinic acid inhibited the transcriptional activations of GATA-1 and NF-E2 genes. These results suggested that the decrease of γ-globin and PBGD mRNA level was likely due to the down-regulation of these transcription factors, thus resulting in the decrease of hemoglobin synthesis. In addition, although GATA-1 and GATA-2 transcription factors present overlapping yet distinct patterns of expression[25], the over-expression of GATA-2 would inhibit the erythroid differentiation[26,27]. We found that chebulinic acid induced the increase of GATA-2 gene mRNA level in BA-treated and untreated K562 cells. Therefore, GATA-2 up-regulation could also attribute to inhibitory effect of chebulinic acid on erythroid differentiation.

In conclusion, we demonstrated that chebulinic acid had inhibitory effects on erythroid differentiation of inducer-treated K562 cells. The further studies are to be preformed to establish whether the inhibitory effects could disturb the efficiency of anticancer drugs and affect the hematopoiesis *in vivo*.

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