Emodin and AZT synergistically inhibit the proliferation and induce the apoptosis of leukemia K562 cells through the *EGR1* and the Wnt/β-catenin pathway

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Abstract. The aim of the present study was to investigate the synergistic antitumor effects of emodin and 3'-azido-3'-deoxythymidine (AZT) on human chronic myeloid leukemia cells and to explore the possible underlying mechanisms. The K562 cells were treated with emodin and AZT, and the rates of cell inhibition and apoptosis were determined by MTT assay and flow cytometry, respectively. The mRNA expression of *EGR1* was detected by reverse transcription-polymerase chain reaction (RT-PCR) analysis. The expression of *EGR1* was silenced using siRNA, and then protein expression of β-catenin was detected by western blotting. The results demonstrated that AZT enhanced the inhibitory effect of emodin in K562 cells. The IC_{50} of the emodin/AZT combination at 24, 48 or 72 h was 23.6/235.6, 10.2/101.6 or 5.9/58.5 μmol/l, respectively, which was significantly lower compared with the IC_{50} of emodin (all >32 μmol/l) or AZT (all >320 μmol/l) alone. There was a dose-dependent response to the combined emodin and AZT treatment, and the calculation of the combination index yielded values <1, demonstrating the synergistic effect of the combined treatment compared with the control ($P<0.05$). Furthermore, the combination of emodin and AZT increased apoptosis in K562 cells ($P<0.05$). Apoptosis was higher in the combination group compared with that of either treatment alone or control groups. The expression of early growth response-1 (*EGR1*) in K562 cells was upregulated in a time-dependent manner. The expression of *EGR1* was higher in the combination group compared with that in the emodin or AZT alone groups. The expression of the Wnt/β-catenin signaling pathway in the combination group was lower compared with that in the emodin or AZT alone groups. The expression of the Wnt/β-catenin signaling pathway was significantly increased following *EGR1* siRNA transfection. These data suggest that treating K562 cells with a combination of emodin and AZT exhibits reduced toxicity and improves therapeutic efficacy, and that the growth, inhibition, apoptosis and regulation of the Wnt/β-catenin signaling pathway in human chronic myeloid leukemia cells by emodin and AZT may be associated with the expression of *EGR1*.

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Introduction

Chronic myeloid leukemia (CML) is a malignant myeloproliferative disease, originating from pluripotent hematopoietic stem cells and comprising ~15% of leukemia cases (1). CML is characterized by increased proliferation of the granulocytic cell line and is associated with erythroid cells and platelet hyperplasia (2). Chemotherapy remains the first line of clinical treatment for CML. However, although chemotherapy improves the survival of CML patients, and despite the development of novel chemotherapeutic drugs and hematopoietic stem cell transplantation, 30% of CML patients relapse after remission. Tyrosine kinase inhibitors (TKIs), the first-line treatment drug for CML at present, are associated with a high rate of adverse effects and CML recurrence (3). Thus, in order to improve the outcome of CML patients, it is crucial to explore alternative therapeutic approaches with enhanced efficacy, low toxicity and multi-target combinations of anticancer drugs, possibly with different mechanisms of action (4).

Emodin (1,3,8-trihydroxy-6-methyl anthraquinone) is a traditional Chinese medicine isolated from rhubarb, with effective antitumor, antibacterial and antiaging properties (5,6). Emodin has been reported to be effective in treating a number of tumors that are insensitive to other chemotherapies, adding exciting new prospects to cancer treatment (7). However, it has also been reported (8) that emodin is potentially nephrotoxic by damaging proximal tubules, which limits its applicability in cancer therapy. However, it remains unknown whether a

combination of emodin and currently available chemotherapeutic drugs can exert synergistic anticancer effects while reducing side effects, such as nephrotoxicity.

Azidothymidine (AZT), a thymidine analogue and telomerase inhibitor mainly used in anti-HIV therapy, has been found to exert potent inhibitory effects on a variety of malignancies, including leukemia (9-11). However, the therapeutic dosage of AZT is usually associated with severe toxic side effects, which restrict the antitumor application of AZT in the clinical setting (12). Recent studies indicate that AZT exerts marked therapeutic effects acting synergistically with other antitumor drugs within a safe dosage range (13,14). This observation prompts further investigation to identify drugs optimally combined with AZT for the treatment of leukemia.

The gene encoding early growth response protein-1 (*EGR1*), a member of the immediate early gene family, is rapidly induced by extensive extracellular stimulation, and is a converging point for a number of intracellular signaling cascades that control tumor cell growth and proliferation, as well as other signaling cascades associated with cell death mechanisms. Accumulating evidence indicates that *EGR1* acts as a tumor suppressor (15).

The canonical Wnt/β-catenin pathway is characterized by activation of transcriptional activity mediated by β-catenin. Several components of this pathway are associated with a variety of human diseases, particularly cancer (16-21). The Wnt/β-catenin pathway has been proven to play an important role in the malignant transformation of hematopoietic cells and the development of hematological malignancies (22-24). In CML, the majority of the cases are associated with BCR-ABL oncoprotein mutations and BCR-ABL amplification. BCR-ABL fusion gene regulates β-catenin, and it may be associated with the high expression of β-catenin observed in CML (25). Therefore, the Wnt/β-catenin pathway appears to play a key role in regulating leukemia resistance and late recurrence, which provides a new approach to targeted treatment of leukemia (26).

EGR1 has been demonstrated to induce an increase in glycogen synthase kinase (GSK3) β activity. GSK3β phosphorylates β-catenin and induces its proteasomal degradation, which attenuates β-catenin expression and β-catenin-dependent T-cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional activity (27). The combination of bioinformatics, the NCBI database, Patch software, and KEGG database were utilized to identify the *EGR1* sequence, *EGR1* predicted target gene, and Wnt/β-catenin pathway genes. It was revealed that TCF, an important gene in the Wnt/β-catenin pathway, is also a target gene of *EGR1*. However, it remains unclear whether the Wnt/β-catenin signaling pathway is regulated by the *Egr-1* gene in CML.

Based on the abovementioned data, it is reasonable to hypothesize that enhancing the antitumor effect of drugs at a similar or lower dosage would be of great value. To the best of our knowledge, the effects of the combination of emodin and AZT on the inhibition of proliferation and induction of apoptosis in the human CML cell line K562 has not been determined. The present study was designed to investigate the effects of a combination of emodin and AZT on CML cell proliferation inhibition and apoptosis induction *in vitro*, explore the possible underlying mechanisms, and provide an experimental basis for further development and clinical application of emodin and AZT.

Materials and methods

Cell culture. The human CML K562 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). K562 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (0.292 mg/ml). These cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2. Cells in the logarithmic growth phase were harvested for experiments.

Chemicals and reagents. Emodin (purity >98%) was purchased from Biological Technology Development Co. Ltd. AZT, MTT and dimethyl sulfoxide were purchased from Sigma-Aldrich; Merck KGaA. RPMI-1640 was purchased from Gibco; Thermo Fisher Scientific, Inc. Fetal calf serum was purchased from Hangzhou Evergreen Co., Ltd. Gene primers and the reverse transcription kit were purchased from Beijing Tiangen Biotech Co., Ltd. The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was purchased from Nanjing KGI Biological Ltd. The Nucleofector™ nuclear transfection instrument was purchased from Germany LONZA Group, Ltd.

Cell proliferation assay. K562 cells were plated at a concentration of $1x10^5$/ml in 96-well plates in 100 μl of culture medium per well. The cells were treated with emodin (8, 16 and 32 μM), AZT (80, 160 and 320 μM) and a combination of emodin and AZT (8/80, 16/160 and 32/320 μM) for 24, 48 and 72 h. The experimental cells were incubated with 10 ml MTT (5 mg/ml in serum-free medium) for 4 h, followed by addition of 100 μl lysis solution (10% SDS in 0.01 M HCl) per well and incubation for a further 24 h. The cell survival rate was measured using optical density (OD) at 490 nm on a microplate reader (Ultramark™ Microplate system; Bio-Rad Laboratories, Inc.) in triplicate. The experiment was repeated three times. The inhibition rate (IR) was calculated as follows: IR (%)=[1-Mean OD of experiment)/Mean OD of control] x100%.

Synergy determination. The synergistic effects of emodin and AZT at a fixed concentration ratio (emodin: AZT=1:10) and at different concentrations were analyzed using the CalcuSyn 2.0 software (Biosoft). The drug half maximal inhibitory concentration (IC_{50}) and combination index (CI) were calculated. A CI value of 1 indicates an additive effect, values <1 indicate synergistic action, and values >1 indicate antagonism.

Detection of apoptotic cells. Using flow cytometry, the apoptosis analysis was performed as described in the Annexin V-FITC/PI apoptosis detection kit manual. The harvested K562 cells were resuspended in fresh medium, adjusting cell density to $1x10^5$/ml. A total of 2.5 ml cell suspension per well were added into 6-well plates. The cells were treated with emodin at a final concentration of 32 μM, AZT at a final concentration of 320 μM, and a combination of the two at a final concentration of 32 μM (emodin)/320 μM (AZT) for 24, 48 and 72 h. Equal volumes of RPMI-1640 were used as control. Cells were collected by centrifugation (111.8 x g,

5 min, 37˚C) and resuspended in 500 μl of 1X binding buffer in tubes. Annexin V-FITC (5 μl) and PI (5 μl) were added, the tubes were incubated at room temperature for 5 min in the dark, and the samples were then examined by flow cytometry. The experiment was repeated three times.

Detection of cell cycle distribution. The harvested K562 cells were resuspended in fresh medium, adjusting cell density to $3x10^5$/ml. A total of 2.5 ml cell suspension per well were added into 6-well plates. The cells were treated with emodin at a final concentration of 32 μM, AZT at a final concentration of 320 μM, and a combination of the two at a final concentration of 32 μM (emodin)/320 μM (AZT) for 24, 48 and 72 h. The cells were collected by centrifugation (111.8 x g, 5 min, 37˚C), washed twice with PBS, fixed with 70% iced ethanol overnight, stained in the dark for 30 min with 0.6 ml PI, and examined by flow cytometry. The experiment was repeated three times.

Detection of EGR1 mRNA expression. The harvested K562 cells were resuspended in fresh medium, adjusting cell density to $1x10^6$/ml. A total of 5 ml cell suspension per well was added into 6-well plates. The cells were treated with emodin at a final concentration of 32 μM, AZT at a final concentration of 320 μM, and a combination of the two at a final concentration of 32 μM (emodin)/320 μM (AZT) for 0, 0.5, 1, 2, 4 or 8 h; subsequently, the K562 cells were subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total cellular RNA at different time points was obtained using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, RNA concentration was measured by UV spectrophotometry. RNA was reverse-transcribed using random primers and AMV reverse transcriptase (Tiangen) for 5 min at 70˚C, 5 min on ice, and 60 min at 37˚C. The single-stranded cDNA was amplified by PCR using GoTaq DNA polymerase (Tiangen). PCR of the *EGR1* gene was performed under the following conditions: 45 sec at 94˚C, 45 sec at 55˚C and 45 sec at 72˚C, for a total of 30 cycles. After PCR of the *EGR1* gene, equal amounts of RT-PCR products were loaded on 1.0% agarose gels. The results were analyzed using Quantity One software (V4.6.6; Premier Biosoft, Palo Alto, CA, USA). The primers for PCR are shown in Table I.

Cell transfection. For the *EGR1* gene mRNA, a specific base sequence was selected as the interference target, and the siRNA fragment was used as follows: si-*EGR1* sense, 5'-CCC GGUUACUACCUCUUAUTT-3' and antisense, 5'-AUAAGA GGUAGUAACCGGGTT-3'. Target-free siRNAs with fluorescently labeled homologous sequences were used as negative controls (NC). K562 cells were transfected by electroporation. Nucleofector I nuclear transfection apparatus and Amaxa® Cell Line Nucleofector® (Lonza, Cologne, Germany) were used. K562 cells in the logarithmic growth phase were collected, the cell density was adjusted to $1\text{-}2x10^6$/ml, and the cells were divided into a blank control group (K562, only subjected to electroporation), a non-specific control group (K562/NC, transfected with the FAM-labeled universal random negative control NC-siRNA), and the target siRNA group (K562/siRNA, transfected with Egr-1 siRNA). The transfection methods and procedures were performed in accordance with the kit instructions. Transfected cells emitting green fluorescence were observed under a fluorescence microscope, and RT-PCR was used to determine the integration of *EGR1* mRNA expression in the transfected K562 cells. Each set of experiments was repeated 3 times.

Detection of β-catenin protein expression in the Wnt/β-catenin pathway by western blotting. K562 cells in the logarithmic growth phase and K562 cells transfected with siRNA were resuspended in fresh medium to adjust the cell density to $1x10^6$/ml. A total of 5 ml cell suspension per well was added to a 6-well plate. The final concentration of emodin was 32 μM, the final concentration of AZT was 320 μM, and the final concentration of emodin/AZT was 32/320 μM for 72 h. The total protein concentration was determined by the BCA method. Electrophoresis was performed using a 10% polyacrylamide gel, and western blotting was conducted in a conventional manner. Finally, color development was performed by ECL chemiluminescence, and imaging was performed using a gel imager. Each set of experiments was repeated 3 times.

Bioinformatics analysis. The *EGR1* transcription factor sequence was downloaded from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov), and the *EGR1* target gene was predicted by Patch software (http://gene-regulation.com/cgi-bin/pub/program/patch/bin/patch.cgi). The KEGG database (https://www.genome.jp/kegg/) was used to search for all genes of the WNT/β-catenin pathway. The intersection of the target genes predicted by *EGR1* and the genes in the WNT/β-catenin pathway was selected.

Statistical analysis. The dose inhibition effect of each drug alone and the effects of the combination of the two drugs were calculated using CalcuSyn 2.0 statistical software (Premier Biosoft). Experimental data are shown as means ± standard deviation. Comparisons were performed using Student's-test and one-way ANOVA, and the multiple test were performed with post hoc test. All data were processed using SPSS 13.0 (SPSS, Inc.).

Results

Inhibitory effects of emodin, AZT and their combination on the proliferation of the K562 cells. The proliferation of K562 cells treated with AZT (Fig. 1A), emodin (Fig. 1B) and the combination of emodin/AZT (Fig. 1C) at three different concentration levels for 24, 48 or 72 h was decreased in a dose-and time-dependent manner. The inhibition rates of the combination of emodin and AZT in K562 cells (Fig. 1C) were significantly higher compared with those of either AZT or emodin alone at the same concentrations and time ($P<0.01$).

Analysis of the effects of emodin, AZT and their combination Associations between inhibitory effects and the dose of emodin, AZT and their combination. K562 cells were treated with emodin and/or AZT for 24, 48 and 72 h. The IC_{50} values for emodin in K562 cells at 24, 48 and 72 h were all

Table I. Primer sequences, and length of RT-PCR.

Gene	Primer sequences (5'→3')	Length
EGR1	Upstream: TTCGCTAACCCCTCTGTCTACTACTATT	
	Downstream: GACTCCACTGGGCAAGCGTAA	180 bp
β-actin	Upstream: GACTCCACTGGGCAAGCGTAA	
	Downstream: GTGGGGCGCCCCAGGCACCA	548 bp

EGR1, early growth response -1.

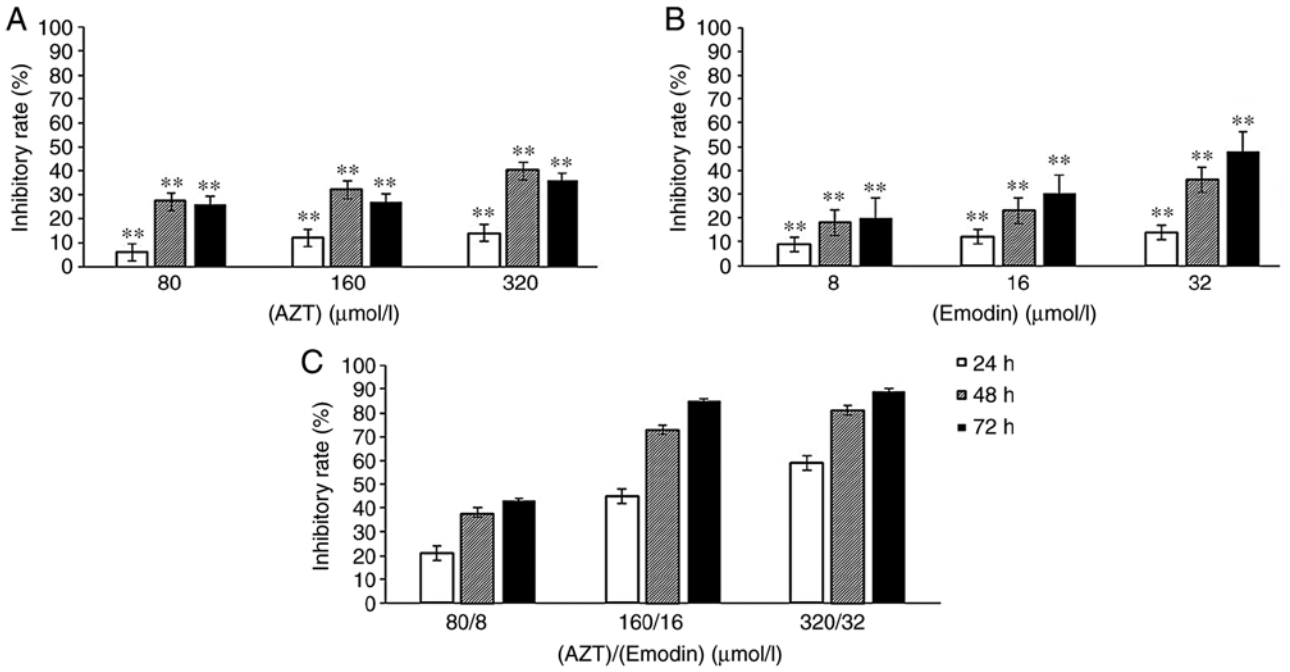

Figure 1. Proliferation inhibition effects of AZT, emodin and the combination on K562 cells (n=3). (A) K562 cells were treated with AZT at 80, 160 and 320 μmol/l for 24, 48 and 72 h. (B) K562 cells were treated with emodin at 8, 16 and 32 μmol/l for 24, 48 and 72 h. (C) K562 cells were treated with emodin and AZT at similar concentrations for 24, 48 and 72 h. **P<0.01, compared with the combination of emodin and AZT at the same concentrations and time. AZT, azidothymidine.

>32 μmol/l, and the IC_{50} values for AZT in K562 cells at 24, 48 and 72 h were all >320 μmol/l; however, the IC_{50} values of the emodin/AZT combination in K562 cells at 24, 48 and 72 h were 22.43/224.31, 10.53/105.26, and 7.31/73.07 μmol/l, respectively (Table II).

CI of emodin and AZT in the K562 cells. As calculated by CalcuSyn 2.0 statistical software, the CI values of the combination of emodin and AZT at a fixed concentration ratio in K562 cells treated for 24, 48 and 72 h were all <1 (Table III). These results indicate a strongly synergistic inhibitory effect of the emodin + AZT combination on the CML cell line K562.

Apoptosis and cell cycle

Apoptosis rates of the K562 cells. The apoptosis rates of K562 cells treated with emodin and/or AZT were increased when compared with the control (Fig. 2) in an overall time-dependent manner; however, the increase in apoptosis rates at 48 h was the most significant. The apoptosis rates in the combination treatment group were higher compared with those in the control and single-agent groups, and the differences were statistically significant (P<0.05) (Table IV).

K562 cell cycle distribution. Emodin, AZT and their combination exerted inhibitory effects on cell cycle progression in K562 cells, as determined by flow cytometry. After K562 cells were treated with the combination of emodin and AZT for 48 h, the percentage of cells in the G_0/G_1 phase was increased, and that of cells in the G_2/M and S phases was decreased. Therefore, emodin/AZT combination treatment arrested cells in the G_0/G_1 phase (Table V).

Effects of the combination of emodin and AZT on EGR1 mRNA expression. It was demonstrated by RT-PCR (Fig. 3) that the transcriptional levels of *EGR1* in K562 cells treated with emodin, AZT or a combination of the two quickly increased to a peak at 0.5 h, and then gradually declined. With prolongation of the processing time, the expression of *EGR1* in K562 cells decreased to a minimum at 8 h, which was similar

Table II. IC_{50} of emodin, AZT and the combination in regards to the growth of K562 cells.

	IC_{50} (μM) at:		
Treatment	24 h	48 h	72 h
Emodin	>32	>32	>32
AZT	>320	>320	>320
Emodin + AZT	22.43/224.31	10.53/105.26	7.31/73.07

IC_{50}, half maximal inhibitory concentration; AZT, 3'-azido-3'-deoxythymidine. μM is equivalent to μmol/l.

Table III. CI values at different treatment times.

Treatment time (h)	AZT (μmol/l)	Emodin (μmol/l)	Fa	CI
24	80	8	0.221224	0.221
	160	16	0.435789	0.102
	320	32	0.592201	0.087
48	80	8	0.338468	0.789
	160	16	0.741626	0.107
	320	32	0.813899	0.111
72	80	8	0.424044	0.302
	160	16	0.853403	0.047
	320	32	0.872798	0.077

AZT, 3'-azido-3'-deoxythymidine; CI, combination index. μM is equivalent to μmol/l.

Table IV. Apoptosis rates in the different treatment groups (n=3).

	Apoptosis rate (%) at:		
Group (μmol/l)	24 h	48 h	72 h
Control	1.04±0.22	1.19±0.25	1.31±0.37
Emodin 32	1.47±0.23	2.1±0.57	1.58±0.41
AZT 320	1.11±0.32	7.1±1.42	5.98±1.45
Emodin 32 + AZT 320	1.75±0.29[b,c,f]	9.59±2.16[b,d,e]	7.03±1.27[b,d,e]

[a]P<0.05, [b]P<0.01, compared with the control; [c]P<0.05, [d]P<0.01, compared with emodin alone; [e]P<0.05, [f]P<0.01, compared with AZT alone. AZT, 3'-azido-3'-deoxythymidine. μM is equivalent to μmol/l.

to the levels in untreated levels. When the cells were treated with emodin or AZT alone for 0.5 h, the *EGR1* gene expression in K562 cells increased by 48.33 or 14.1%, respectively, while treatment of K562 cells with the combination of emodin and AZT for 0.5 h increased the expression of *EGR1* gene by 128.64%, which was significantly higher compared with the effect of emodin or AZT alone (Fig. 3).

Cell transfection. The transfection efficiency was observed with FAM fluorescently labeled siRNA, and it was found that the transfected cells emitted green fluorescence under a fluorescence microscope. The transfection efficiency was >75%, and the cells were used in subsequent experiments (Fig. 4). After trypan blue staining, with a count of live cells up to 75%, the cells were used for follow-up studies. The results of the PCR analysis demonstrated that, compared with the non-specific control and the blank control groups, the expression level of *EGR1* in the siRNA group was decreased at 24 h after transfection, and the difference was statistically significant (P<0.01); there was no significant difference with the

Table V. Effect of the different treatments on the cell cycle distribution of K562 cells (n=3).

Treatment (μmol/l)	Time (h)	Percentage of cells (%)		
		G_0/G_1 phase	S phase	G_2/M phase
AZT 320	24	65.63±2.66	24.79±1.22	0.54±0.04
	48	65.35±2.58	28.92±0.98	2.74±0.12
	72	75.86±4.04	18.43±1.78	0.29±0.03
Emodin 32	24	39.12±3.12	29.97±2.03	26.28±1.36
	48	43.19±1.85	35.04±0.97	17.15±0.96
	72	41.26±1.35	36.06±1.14	15.28±0.88
Emodin 32 + AZT 320	24	70.54±2.44[b,d]	22.94±0.95[a,d]	1.85±0.08[a,d]
	48	77.5±2.98[b,d]	18.87±0.88[b,d]	0.07±0.01[b,d]
	72	77.38±3.01[d]	16.24±0.86[a,d]	0.5±0.04[a,d]

[a]P<0.05, [b]P<0.01, compared with emodin alone; [c]P<0.05, [d]P<0.01 compared with AZT alone. AZT, 3'-azido-3'-deoxythymidine. μM is equivalent to μmol/l.

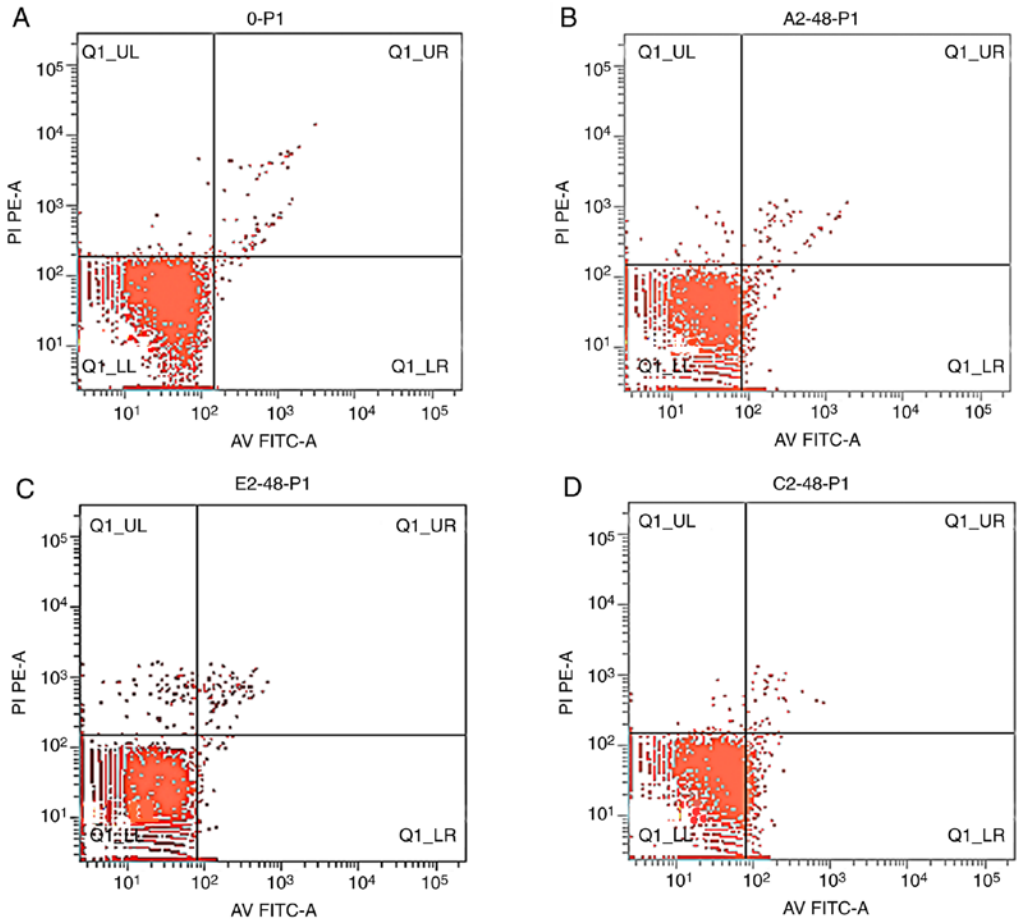

Figure 2. Apoptosis of K562 cells. (A) K562 cells were treated without emodin or AZT. (B) K562 cells were treated with AZT (320 μmol/l) for 48 h. (C) K562 cells were treated with emodin (32 μmol/l) for 48 h. (D) K562 cells were treated with a combination of emodin (32 μmol/l) and AZT (320 μmol/l) for 48 h. Apoptosis was determined by flow cytometry. AZT, azidothymidine.

specific control group. This indicates that siRNA blocks the expression of *EGR1*. After 24 h of transfection (Fig. 5), the expression of *EGR1* was significantly lower compared with that prior to transfection, and the difference was statistically significant (P<0.01).

Detection of β-catenin protein expression in the Wnt/β-catenin pathway by western blotting. The results of western blotting are shown in Fig. 6A and B. Compared with the blank control group, the protein expression of β-catenin in K562 cells treated with emodin (32 μM) and AZT (320 μM) was

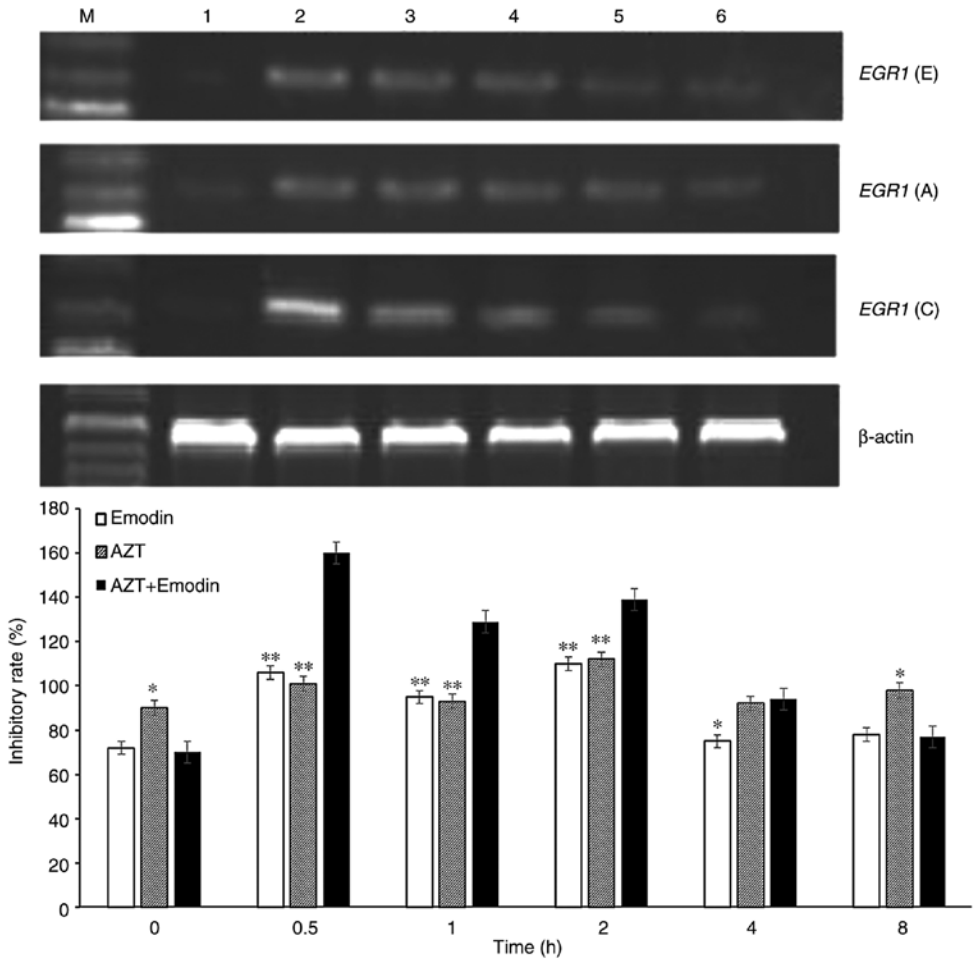

Figure 3. Expression of *EGR1* mRNA in K562 cells treated with emodin alone, AZT alone and a combination of emodin and AZT for 0, 0.5, 1, 2, 4 and 8 h. Lanes; M, DNA marker lane; lane 1, blank control; lane 2, 0.5 h; lane 3, 1 h; lane 4, 2 h; lane 5, 4 h; and lane 6, 8 h. *EGR1* (E): 32 μmol/l emodin; *EGR1* (A): 320 μmol/l AZT; *EGR1* (C): 32 μmol/l emodin + 320 μmol/l AZT. *P<0.05, **P<0.01 compared with the combination of emodin and AZT. *EGR1*, early growth response -1; AZT, azidothymidine.

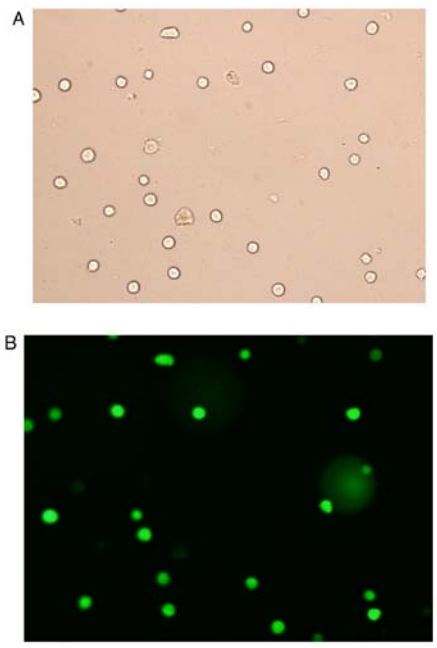

Figure 4. K562 cells were transfected with *EGR1*-siRNA. (A) Cells observed under a phase contrast microscope; magnification x200. (B) Cells observed under a fluorescence microscope at 6 h; magnification x200. The transfected cells emitted green fluorescence. *EGR1*, early growth response -1.

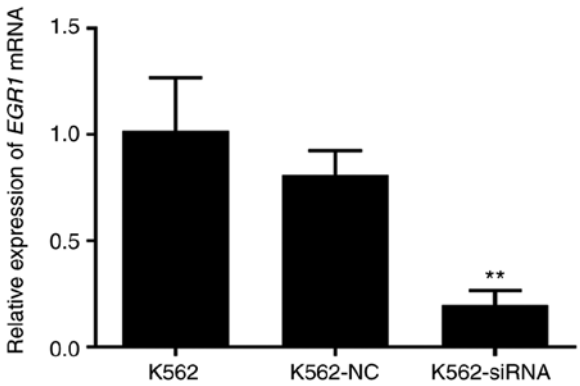

Figure 5. Relative expression of *EGR1* mRNA in the K562 cells transfected with siRNA-NC or *EGR1*-siRNA (n=3). **P<0.01 compared with the K562 or K562-NC groups. Groups: K562, the blank control group where K562 cells were only subjected to electroporation; K562-NC, a non-specific control group transfected with the FAM-labeled universal random negative control NC-siRNA; K562-siRNA, K562 cells transfected with *EGR1*-siRNA. *EGR1*, early growth response -1.

decreased, and the difference was statistically significant (P<0.05). Compared with the single-drug and the control groups, the expression level of the β-catenin protein in K562

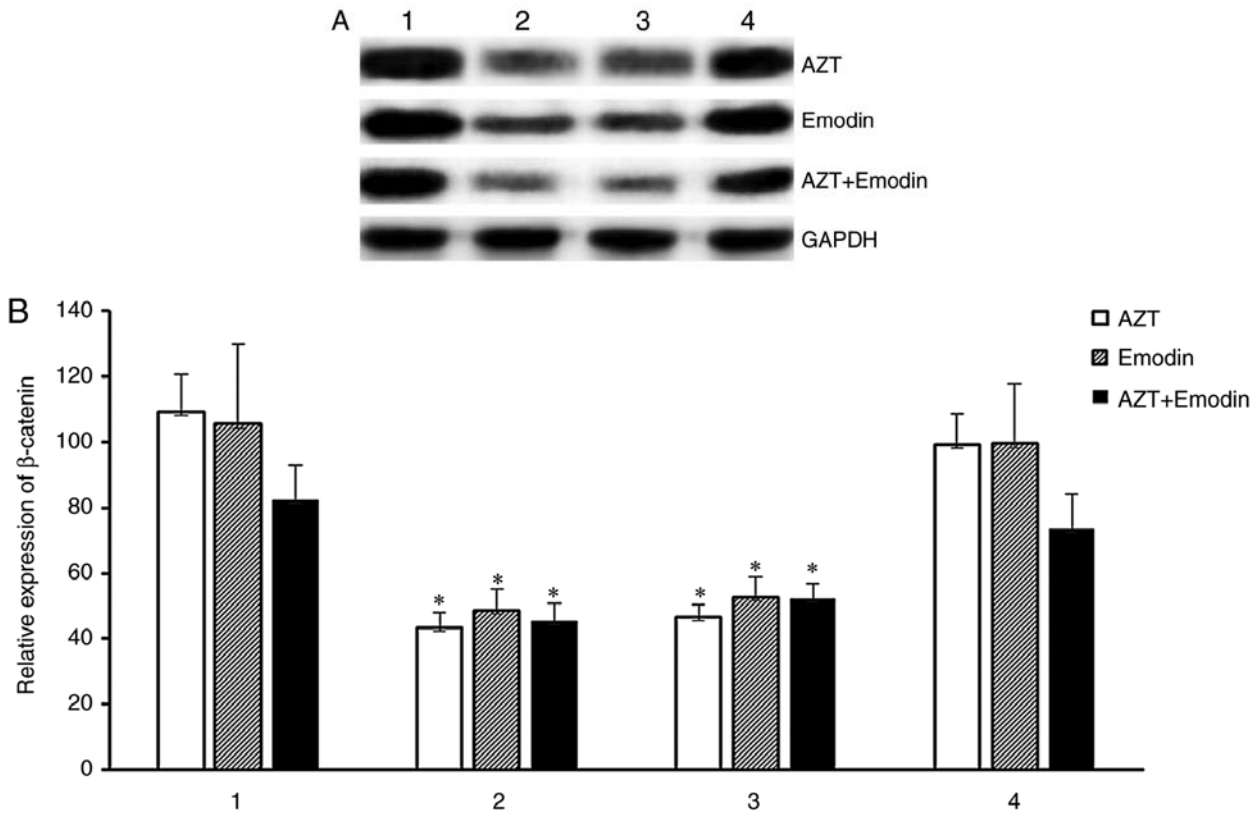

Figure 6. (A) Western blot assay for the expression of β-catenin protein after administration and transfection of *EGR1* siRNA (n=3). Lane 1, blank control group; lane 2, normal dosing group [AZT (320 μmol/l); emodin (32 μmo1/l); emodin + AZT (32/320 μmol/L)]; lane 3, negative transfection (K562-NC) + drug group [AZT (320 μmol/l); emodin (32 μmo1/l); emodin + AZT (32/320 μmol/l)]; lane 4, positive transfection (K562-siRNA) + drug group [AZT (320 μmol/l); emodin (32 μmo1/l); emodin + AZT (32/320 μmol/l)]. (B) Quantification of β-catenin protein. *P<0.05, compared with the blank control group.

cells treated by the combination of emodin (32 μM) and AZT (320 μM) was significantly decreased (P<0.05). Compared with untransfected K562 cells, the expression of the β-catenin protein was significantly higher in the three groups with positively transfected siRNA, and the differences were statistically significant (P<0.05). There was no significant difference in the expression level of the β-catenin protein between the three groups of negatively transfected siRNA and untransfected K562 cells (P>0.05).

Discussion

The results of the present study indicated that the combination of emodin and AZT exerted obvious synergistic inhibitory effects (CI <1) on leukemia K562 cells *in vitro*. The synergy between the two drugs exhibited a type of concentration dependent trend. The combination of emodin and AZT at higher concentrations exhibited more significant synergy. Compared with emodin or AZT alone, the combination of emodin and AZT at lower doses produced proliferation inhibition and apoptosis induction similar to those achieved by higher doses of emodin or AZT alone. This indicated that the effective combination of the two drugs may be associated with lower toxicity and can significantly improve therapeutic efficacy. In addition, the proliferation inhibition is more extensive than the apoptosis rate, which may be caused by AZT or emodin inducing autophagy, and autophagy is a protective mechanism for the apoptosis of leukemia K562 cells (28-31). The evaluation of the synergy between emodin and AZT may provide evidence for clinical application and future treatment studies.

The combination of telomerase inhibitors with small-molecule substances, such as anthraquinone, quinoline, berberine and their analogues, has become an important focus of anti-tumor drug research in recent years (32).

As a natural monomer supplementary chemotherapy drug, emodin may inhibit tumor cell growth in pancreatic, prostate and colorectal cancer, as well as other malignant tumors (33). Emodin may induce apoptosis in leukemia cells through Bcl-2/Bax, the caspase-3 protein family, the human telomerase reverse transcriptase Htert, c-Myc, and other apoptosis-related molecules (34). Emodin may also reverse multi-drug resistance of tumor cells and increase the body's sensitivity to antitumor drugs. It was previously demonstrated that emodin can reduce the expression of the multi-drug resistance gene MRP1 through the PI3K/AKT pathway (35) and reverse the multi-drug resistance of CML cells (36). In addition, emodin was shown to markedly enhance the sensitivity of tumor cells to arsenic trioxide (37), 5-fluorouracil (38), platinum (39), paclitaxel (40), gemcitabine (41) and curcumin (42), as well as other drugs, in a variety of cancers.

The present results confirmed the synergistic interaction between emodin and AZT, thus supporting the use of this combination at low doses with low toxicity. Administration of emodin in combination with a telomerase inhibitor may be an effective approach to the treatment of refractory and recurrent leukemia.

In recent years, *EGR1*, a tumor suppressor gene, has been increasingly attracting research attention due to its role in the occurrence and development of tumors. *EGR1* is one of the most important members of the immediate early gene family. The expression of *EGR1* decreases or even disappears in a variety of human malignancies, and its expression level is associated with tumor sensitivity to chemotherapy (43). Apoptosis induction by *EGR1* may be relevant to its broad spectrum multi-directional regulation and cell signaling pathway network. It is generally believed that *EGR1* acts as a tumor suppressor by regulating the expression of downstream target genes, such as transforming growth factor-β, cyclin D1, c-Jun, phosphatase and tensin homolog, p53 and p21 (44,45). In the present study, changes in the transcriptional level of the *EGR1* gene were observed using RT-PCR analysis. It was observed that the gene expression of *EGR1* was increased in K562 cells that were treated with emodin or AZT alone, or the combination of emodin and AZT, and that the increase in the expression of the *EGR1* gene induced by the combination of the two drugs was more significant compared with that by either drug alone; furthermore, this effect was time-dependent. Western blotting demonstrated that the protein expression of β-catenin in the Wnt/β-catenin signaling pathway that was promoted by the combination of emodin and AZT was significantly higher compared with that of either drug alone. After transfection of siRNA with the *EGR1* gene, the expression of the β-catenin protein in the Wnt/β-catenin signaling pathway was increased, indicating that the *EGR1* gene may regulate the Wnt/β-catenin signaling pathway. The difference between the expected and the actual result may be due to the higher expression of *EGR1* induced by the two-drug combination, which may exert antitumor effects directly or through regulating other downstream target genes not investigated in the present study. We will assess the expression of other related proteins in the Wnt/β-catenin signaling pathway in our subsequent research.

In summary, the present study demonstrated that the combination of emodin and AZT can enhance tumor cell proliferation inhibition, apoptosis induction and decrease the expression of the Wnt/β-catenin signaling pathway more efficiently compared with emodin or AZT alone, which may be mediated by increased expression of *EGR1*. However, it is unclear whether the therapeutic dose in the combination therapy of the two drugs is a safe dose, and the exact underlying mechanism has not been fully elucidated. We will determine therapeutic dosage and explore the underlying mechanism in CML clinical samples in our future research.

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Availability of data and materials

All the datasets generated and analyzed in the present study are included in this published article.

Authors' contributions

CC contributed to the experimental design. WM and LY contributed significantly to conducting the experiments. FL performed the data analyses and wrote the manuscript. CZ helped perform the analysis with constructive discussion. All the authors have read and approved the final version of this manuscript for publication and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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