In Vitro Investigation of The Cytotoxic Activity of E35 on Multiple Myeloma Cell Lines

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Research

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Abstract

Background: Bortezomib is used for the treatment of multiple myeloma (MM); however, it has significant adverse effects. Emodin has been reported to exhibit inhibitory effects on MM cell lines. Here, we investigated the efficacy of E35, an emodin derivative, using U266 and MM1s cell lines in the treatment of MM and the efficacy of the combination of bortezomib and E35.

Methods: MTT assays were used to observe the effects of E35 on MM cell growth. The effects on cellular apoptosis were observed using the Annexin V/propidium iodide (PI) staining assay. The expression of apoptosis-related genes, including the caspase family, was also examined. The efficacy of the combination of bortezomib and E35 was investigated by examining the expression of the Akt/mTOR/4EBP1 signaling pathway-related proteins.

Results: We found that E35 inhibited the growth of the U266 and MM1s cells by inducing cellular apoptosis. E35 also downregulated the expression of the apoptosis-related genes and suppressed the phosphorylation of the Akt/mTOR/4EBP1 signaling pathway-related genes, exhibiting synergistic effects with bortezomib. All the observed effects were dose-dependent.

Conclusion: The results of this study showed that E35 exhibited cytotoxic effects in MM cell lines. Thus, E35, especially in combination with bortezomib, may be considered as a promising treatment for MM. However, this requires further investigation *in vivo*.

Background

Multiple myeloma (MM) is the second leading hematological malignancy, accounting for approximately 10% of all hematological tumors [1]. Despite some progress in therapy based on new understanding of the molecular mechanisms, MM remains generally incurable, with a poor median survival. The crucial role of the NF- κ B signaling pathway, considered as one of the most important inflammatory pathways involved in the onset of MM [1], in the development and progression of MM has been increasingly investigated. Hence, suppressing the abnormal activation of inflammatory pathways has been an important therapeutic approach.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a natural anthraquinone compound extracted from *Radix et Rhizoma Rhei*. Its biological activities are complex and multifaceted but include anti-inflammatory and immunomodulatory effects. Emodin has been reported to exhibit therapeutic effects on hematological tumor cell lines. A previous study reported that emodin contributes to the suppression of cellular proliferation [2] and induce apoptosis [3] in a number of leukemic cell lines via inhibiting inflammatory signaling pathways [4]. However, there is a paucity of studies on the effects of emodin on MM.

In 2007, Muto et al. found that emodin suppresses interleukin-6-related JAK2/STAT3 pathway and causes apoptosis in MM cells by downregulating myeloid cell leukemia 1 (Mcl-1) expression [5]. In a previous

study, we found that E11, an emodin derivative, significantly inhibits proliferation and induces apoptosis of RPMI8226 and U266 MM cell lines [6].

Our laboratory has investigated the effects of 16 kinds of emodin derivatives on cell lines of hematological tumors [6, 7]. One emodin derivative, E35 ($C_{34}H_{50}BrNO_5 \cdot H_2O$; molecular weight, 631.29; Fig. 1A) has been shown to downregulate TP53 protein expression and decrease PI3K/Akt protein phosphorylation in diffuse large B cell lymphoma cells [8] while downstreaming Crk, Akt/mTOR, and MEK/ERK pathways in 32Dp210-T315I leukemia cells [9] and inhibiting the cellular growth and inducing apoptosis in leukemia cells [10]. However, since suppressing the phosphorylation of Akt/mTOR/4EBP1 signaling pathway has been a therapeutic target in MM [11], the effects of E35 on MM have not been investigated. Bortezomib has been regarded as an effective strategy in treating MM [12], the most important mechanism being the suppression of the NF- κ B signaling pathway [13, 14] and retention of some tumor suppressor proteins [13]. Other mechanisms include promoting the apoptosis of MM cells [15] and inhibiting the Akt/mTOR activity [16]. However, bortezomib is associated with a number of adverse effects, the most common of which are fatigue, nausea, diarrhea, constipation, peripheral neuropathy, arthralgia, insomnia, headache, limb pain, thrombocytopenia, and upper respiratory tract infection [17-19]. The most common cause of bortezomib discontinuation is peripheral neuropathy [20, 21]. These adverse effects may reduce the quality of life in some patients, restricting bortezomib use in such patients. Hence, the use of certain products in combination with bortezomib to achieve similar efficacy but with a reduced incidence of adverse effects is on the increase. Based on this, we hypothesize that E35 can induce apoptosis in MM cells, suppressing the activation and phosphorylation of Akt/mTOR/4EBP1 signaling pathway, and may present synergistic effects with bortezomib.

In the present study, we employed two MM cell lines, U266 and MM1s cells, to investigate our hypothesis. We investigated the efficacy of E35 on MM cells, exploring its potential mechanisms via the inflammatory signaling pathway.

Materials And Methods

Myeloma cell lines

U266 and MM1s myeloma cell lines were obtained from the Chinese Academy of Medical Sciences, Tianjin Institute of Hematology. The cells were cultured in RPMI1640 medium (Gibco, NY, USA) with 10% fetal bovine serum (Hao Yang, China), incubated at 37°C under saturated humidity, and passaged once every 2 days. Cells that were at the logarithmic growth phase were used for the subsequent experiments.

Preparation of the E35 solution

Sterile emodin ($C_{15}H_{10}O_5$) was purchased from a pharmaceutical company (Qingze, China). E35, a novel emodin derivative ($C_{34}H_{50}BrNO_5H_2O$, Fig. 1A), was designed and synthesized. The purity was 98%, determined using a high-performance liquid chromatography. The agent was dissolved in the

dimethyl sulfoxide (DMSO) (Sigma, USA) in 50,000 μM as a primary solution and stored at -20°C before use.

Cytotoxicity assays

The MTT assay was employed to measure the viability and proliferation of the U266 and MM1s cell lines after E35 administration. The U266 and MM1s cells were seeded into 96-well plates at a density of 2.0×10^5 per well. The E35 dilution was subsequently added. The final concentrations of E35 in the wells were 0, 0.5, 1, 2, 4, and 8 $\mu\text{mol/L}$, respectively. A control group was added with the same amount of DMSO as the highest concentration group. Then, 10 μL of the combined MTS/PMS solution (5 mg/mL MTT) were added into each well, and the plates were incubated for 4 hours. Then, the cells were incubated in 5% CO_2 incubator at 37°C for various durations (24, 48, 72, and 96 hours). The optical density was measured with a STAT FAX-2100 spectrophotometer (Awareness Tech, USA) in 490 nm at test. The reference wavelength was 630 nm. The proliferation inhibitory rate (%) and IC_{50} values were calculated by the CalcuSyn Statistical software.

Annexin V/propidium iodide apoptosis assay

The U266 and MM1s cells were seeded into 96-well plates. Then, the E35 dilution was added. The final concentration of E35 in the wells was 1.2 μmol . After incubation with E35 for 12 hours, the cells were harvested and washed with PBS twice and then stained with Annexin V-FITC/propidium iodide (PI) (Becton-Dickinson, NJ, USA) as per the manufacturer's instructions. The early apoptotic cells were quantified by a BD FACSCanto II cytometer (BD Biosciences, USA).

Real-time polymerase chain reaction analysis

The mRNA expression of the apoptosis-related genes, namely, C-Myc, Bcl-2, Mcl-1, and Pim2, was evaluated by real-time polymerase chain reaction (RT-PCR). The U266 and MM1s cells were pretreated with different concentrations of E35 or RPMI1640 for 48 hours. The total mRNA was extracted and reverse-transcribed. The transcription levels of C-Myc, Bcl-2, Mcl-1, and Pim2 were evaluated by quantitative RT-PCR using the iCycler real-time detection system (Bio-Rad, USA) in a two-step method. The hot-start enzyme was activated at 95°C for 5 minutes. Subsequently, cDNA was amplified for 40 cycles, which consisted of denaturation at 95°C for 15 seconds and annealing/extension at 58°C for 30 seconds. A melt curve analysis was then performed (55°C for 1 minute and then increased by 0.5°C every 10 seconds) to detect the formation of primer-derived trimmers and dimmers. The primer sequences were listed as follows: C-Myc: forward, 5'-TCCTGGCAAAAGGTCAGAGT-3', and reverse, 5'-TTGTGTGTTGCGCTCTTGA-3'; Bcl-2: forward, 5'-CGACGACTTCTCCCGCCGCTACCGC-3', and reverse, 5'-CCGCATGCTGGGGCCGTACAGTTCC-3'; Mcl-1: forward, 5'-ATCTCTCGGTACCTTCGGGAGC-3', and reverse, 5'-CCTG ATGCCACCTTCTAGGTCC-3'; Pim2: forward, 5'-CAGCCATCCAGCA CTGCCATTTC-3', and reverse, 5'-AGTCTGGGGAGACATGGGCTGG -3'; β -actin: forward, 5'-GGCATGGGTGAGAAGGATTCC-3', and reverse, 5'-ATGTCACGCACGATTTCCCGC-3'. β -Actin was used as the internal control. All experiments were performed in triplicates, and the data were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method.

Western blotting analysis

Western blotting analysis was employed to measure changes of the protein expression associated with the treatments. The cells were exposed to E35 at varying concentrations for 48 hours and then harvested and lysed. The protein concentrations were measured using the DCTM protein assay kit (Bio-Rad, USA). Electrophoresis was performed in 8%–12% sodium dodecyl sulfate polyacrylamide gel, and the samples were subsequently transferred onto a polyvinylidene fluoride membrane. Western blotting analysis was conducted as per the kit instructions. The apoptosis-related and the Akt/mTOR/4EBP1 signaling pathway-related proteins were detected in U266 and MM1s cells, and the investigation with bortezomib was carried out in U266 cells. The following primary antibodies were used for the analysis: C-Myc, Bcl-2, Mcl-1, Pim2, poly(ADP-ribose) polymerase (PARP), caspase-3, Akt, p-Akt, mTOR, p-mTOR, 4-EBP1, p-4EBP1, EIF4E, p-EIF4E, NF- κ B (Danvers, USA), and β -actin (Fremont, USA) (internal reference). The primary antibodies were incubated overnight at 4°C. The membranes were probed with secondary antibodies (goat anti-mouse or goat anti-rabbit IgG) for 1 hour at room temperature. Finally, an enhanced chemiluminescence detection system (Pierce, USA) was used for the exposure. Quantitative analyses of protein expression were performed using an Image-Pro Plus system (Media Cybernetics, USA).

Statistical analysis

Data were expressed as the mean $\pm$ standard deviation (SD) calculated by the data from at least three independent experiments. The SPSS software (v19.0.0, IBM, IL, USA) was used for the statistical analysis. One-way analysis of variance followed by a Dunnett's post hoc test was used for multiple comparisons. We selected $p < 0.05$ as a level of significance.

Results

Effects of emodin derivative (E35) on myeloma cell lines

The results of the MTT assay show that E35 strongly inhibited the U266 and MM1s cells based on time (Fig. 1B) and concentration (Figs. 1C and D) independently. The IC₅₀ at 48 hours in U266 was 1.82 ± 0.07 μ mol/L (Fig. 1C) and in MM1s was 2.01 ± 0.10 μ mol/L (Fig. 1D).

E35 induced cell apoptosis in U266 and MM1s cells

Fig. 2 shows the results of the Annexin V/PI staining assay. After 12-hour exposure of E35, cellular apoptosis was observed in U266 (Fig. 2A) and MM1s cells (Fig. 2B). The apoptotic rate in control was $5.37\% \pm 1.53\%$ in U266 and $6.60\% \pm 0.92\%$ in MM1s. Exposure of the E35 (1 μ M) was $15.7\% \pm 1.65\%$ in U266 and $16.47\% \pm 1.14\%$ in MM1s, whereas exposure of the E35 (2 μ M) was $24.10\% \pm 2.21\%$ in U266 and $25.10\% \pm 1.48\%$ in MM1s (Fig. 2C). Thus, exposure of E35 significantly enhanced the apoptotic rate in both MM cell lines, and dose-effect relationship was presented.

mRNA expression of apoptosis-related genes affected by E35 exposure

Fig. 3 shows the mRNA expression of apoptosis-related genes affected by E35. The U266 and MM1s exhibited the same tendency. Exposure of E35 for 48 hours downregulated the mRNA expression of the apoptosis-related genes. A high dose (2 μ M) induced significant downregulation ($p < 0.05$) in all the genes, while a low dose (1 μ M) only significantly downregulated the C-Myc expression (Fig. 3A).

Protein expression of the apoptosis-related proteins affected by E35 exposure

Fig. 4 shows the expression of the apoptosis-related proteins, including caspase family affected by E35 exposure. The U266 and MM1s cells exhibited the same tendency. Dose-effect relationship was also presented, and higher dose was associated with stronger effects. Exposure in low (1 μ M) and high (2 μ M) doses of E35 significantly downregulated the expression of C-Myc, Bcl-2, Mcl-1, and Pim2.

With respect to the caspase family, E35 exposure significantly downregulated the expression of full PARP and significantly upregulated the expression of cleaved PARP and cleaved caspase-3 (17 KD). As for the cleaved caspase-3 19 KD, E35 in both doses exhibited a significant downregulatory effect only in the MM1s cells, and no significant effect was found in the U266 cells.

Expression of the Akt/mTOR/4EBP1 signaling pathway-related proteins affected by E35 exposure

Fig. 5 shows the expression of the Akt/mTOR/4EBP1 signaling pathway-related proteins affected by E35 exposure. Exposure in low (1 μ M) and high (2 μ M) doses of E35 significantly downregulated the expression of p-Akt, p-mTOR, and p-EIF4E. The expression of Akt, mTOR, 4EBP1, and EIF4E was unchanged by E35 in both doses. p-4EBP1 was only downregulated by E35 in high dose (2 μ M) in both the U266 and MM1s cells. Fig. 6 demonstrates that E35 administration markedly abrogated the phosphorylation of these Akt/mTOR/4EBP1 signaling pathway-related proteins. These effects were also confirmed by administration of bortezomib, a proteasome inhibitor (Fig. 6). Bortezomib, E35, and bortezomib and E35 combination significantly downregulated the protein expression of C-Myc, NF- κ B, p-4EBP1, and p-EIF4E. Mcl-1 expression was significantly downregulated by bortezomib and bortezomib and E35 combination. The expression of 4EBP1 and EIF4E was unchanged by E35 and bortezomib (Fig. 6). The combination of E35 and bortezomib exhibited a stronger effect, confirming its synergistic effects.

Discussion

In the present study, cytotoxic activities of E35 on MM cells were investigated using U266 and MM1s cell lines. We found that E35 inhibited the growth of U266 and MM1s cells and induced cellular apoptosis by downregulating the expression of apoptosis-related genes (C-Myc, Bcl-2, Mcl-1, and Pim2). Caspase-3 expression (17 KD subunit) was significantly upregulated, the full PARP was significantly downregulated, and the cleaved PARP was upregulated by E35 treatment. Moreover, we found that E35 downregulated the phosphorylation of the Akt/mTOR/4EBP1 signaling pathway-related proteins. The downregulation and suppression of phosphorylation exhibited synergistic effects with bortezomib, a positive control. To the best of our knowledge, this is the first study to report on the effects and underlying mechanisms of

E35 on MM cells. These results present *in vitro* evidence for the value of E35 (as well as its combination with bortezomib) as a potential therapy for MM.

The results of the MTT assay shows that E35 plays an inhibitory role in the growth of U266 and MM1s cells. Moreover, such an inhibitory effect increased with an increase in the concentration and treatment duration (Figs. 1B–D), indicating a dose- and time-dependent capacity of E35. The results of Annexin V/PI staining assay indicated that E35 induced the apoptosis of MM cells (Fig. 2) and downregulated mRNA/protein expression of apoptosis-related genes, including C-Myc, Bcl-2, Mcl-1, and Pim2 (Figs. 3,4). These results were consistent with previous studies on the effects of E35 on non-Hodgkin's Lymphoma cells [8] and E11 on MM cells [6]. C-Myc and Bcl-2 play a key role in the regulation of apoptosis and the growth of malignancy [22–24]. Mcl-1 shares a homology domain with Bcl-2 and exerts an antiapoptotic effect [25]; Pim kinases that belong to a serine/tyrine kinase family and that has three forms within Pim2 were reported to be closely correlated with the development and progression of MM [26]. In addition, a synergistic function of Pim2 and C-Myc was found to have an antiapoptotic effect; Pim2 is recognized as a partner gene of C-Myc during the induction and development of tumor [27, 28]. In the cancer state, such genes are remarkably upregulated. Some authors believed that high expression of these genes is good for the proliferation of cancer cells, and suppression of their expression can be therefore considered as promising therapeutic target [26]. Our results of positive control, bortezomib, also show that these genes were significantly downregulated by bortezomib (Fig. 6). Moreover, the synergistic effects of the combination of E35 and bortezomib lend credence to this (Fig. 6). Taken together, this underscores the potential therapeutic value of E35 on MM cells.

The role of the caspase family in cellular apoptosis has been established. Activation of caspase-3 and degradation of PARP trigger cellular apoptosis. We found that the caspase-3 expression was significantly upregulated by E35, along with the full PARP being significantly downregulated. Meanwhile, cleaved PARP expression was significantly upregulated by E35 treatment (Fig. 4). A possible explanation is that, when apoptosis was triggered by E35, the activated caspase-3 was produced and subsequently sliced the other substrates of caspase-3, including PARP. The total PARP (116 KD) was spliced by caspase-3 between Asp216 and Gly217, generating two fragments (85 and 31 KD). The endonuclease, which is negatively regulated by PARP, was then activated and started degrading the DNA in the nucleosome, ultimately inducing apoptosis [29]. Interestingly, we found that the 19 KD subunit of caspase-3 was not increased, but reduced. The cause of this phenomenon is unknown, as such needs further investigation. However, these data suggested a crucial but complicated role of the caspase family in E35-mediated apoptosis in MM cells.

Another key finding of this study is that E35 suppressed the phosphorylation of the Akt/mTOR/4EBP1 signaling pathway-related proteins. The results of the western blot test showed that the expression of p-Akt, p-mTOR, p-EIF4E, and p-4EBP1 was significantly downregulated by E35 (p-4EBP1 only in high dose). Interestingly, however, the expression of Akt, mTOR, EIF4E, and 4EBP1 was unchanged (Fig. 5). These effects were replicated when bortezomib was administered. Specifically, the expression of p-4EBP1 and p-EIF4E was significantly suppressed by the combination of E35 and bortezomib. However, the expression

levels of 4EBP1 and EIF4E were unchanged (Fig. 6). These results are consistent with some previous studies [11, 16].

The Akt/mTOR/4EBP1 pathway has recently been recognized as one of key signal transduction pathways that suppress cellular apoptosis through phosphorylation and that subsequently induce the deactivation of downstream molecules of mTOR [30, 31]. 4EBP1 is a downstream protein of mTOR, which inhibits eIF4E (the subunit of eIF4F). When 4EBP1 is in a low phosphorylation state, it inhibits the translation of mRNA by binding tightly with eIF4E while reducing interaction with cap-binding protein eIF4G to form the initial complex of eIF4F [32]. It is known that eIF4F controls the expression of many proteins that are associated with cell proliferation, expansion, and apoptosis, such as Mcl-1 (antiapoptotic molecule), cyclin D1 and D3 (cell-cycle regulators), and C-Myc (oncoprotein) [33, 34]. On this note, the interpretation of our results becomes clearer. Specifically, the suppression of the expression of p-4EBP1 and p-EIF4E was associated with the downregulation of downstream eIF4F, subsequently suppressing the expression of more downstream genes (Mcl-1, C-Myc, etc.), leading to the suppression of cell proliferation, expansion, and induction of the cellular apoptosis. The synergistic effects of the combination of E35 and bortezomib generated a stronger effect on the suppression of phosphorylation (Fig. 6). Taken together, these results suggest the crucial role that the suppression of the phosphorylation of the Akt/mTOR/4EBP1 signaling pathway-related proteins plays in E35 mechanism of action. Additionally, the NF- κ B protein was downregulated by the simultaneous use of E35 and bortezomib, though to a stronger extent (Fig. 6). These results indicate that the suppression of the NF- κ B signaling pathway might play a role in the mechanism of action of E35 and/or its synergistic effects.

Conclusion

The present study found that E35 in U266 and MM1s MM cell lines exerted cytotoxic effects. E35 inhibited the growth of cells in a dose- and time-dependent manner, induced cellular apoptosis, and downregulated the expression of apoptosis-associated genes. Caspase-3 expression was significantly upregulated, along with the full PARP being significantly downregulated. The cleaved PARP was upregulated by E35 treatment, indicating a crucial but complicated role of the caspase family in E35-mediated apoptosis. Moreover, we found that E35 suppressed the phosphorylation of Akt/mTOR/4EBP1 signaling pathway-related genes, which is a key mechanism of the effects of E35. The synergistic effects of the combination of E35 and bortezomib and the dose-effect relationship supported this idea. Our results suggested that E35, particularly the combination of E35 with bortezomib, may be considered as a promising treatment for MM. However, this requires further investigation *in vivo*.

Abbreviations

DMSO: dimethyl sulfoxide; MM: multiple myeloma; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Mcl-1: myeloid cell leukemia 1; NF- κ B: nuclear factor-kappa B; LPS: lipopolysaccharide; PI: propidium iodide; RT-PCR: real-time polymerase chain reaction; SD: standard deviation;

Declarations

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Author Contributions

JZ, TA, and JH got the original ideas and designed the study, JZ, YYC, ZZ, YXC, YJC, WW, TA and JH performed the experiments. JZ and TA ran the statistics. JZ and TA drew the figures. JZ and TA wrote the first draft, all the authors revised and approval the final version. TA and JH supervised the study.

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Availability of data and materials

The data and materials used are included in the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Effects of emodin derivative (E35) on myeloma cell lines A. Chemical structure of E35 B. Inhibitory effects of E35 on U266 and MM1s cell growth after 48-hour incubation C. Growth curve of U266 cells in different E35 levels D. Growth curve of MM1s cells in different E35 levels Data are presented as means $\pm$ SD.

Figure 2

Results of the Annexin V/PI staining assay A. Representative images of the Annexin V/PI staining assay of E35 effects on U266 cells B. Representative images of the Annexin V/PI staining assay of E35 effects on MM1s cells C. Quantitative analysis of the Annexin V/PI staining assay of the apoptotic rate in the U266 and MM1s cell lines induced by E35 Data are presented as means $\pm$ SD. ** means $p < 0.01$ vs. control group.

Figure 3

Quantitative analysis of mRNA expression of C-Myc (A), Bcl-2 (B), Mcl-1 (C), and Pim2 (D) in U266 and MM1s cell lines treated by E35 Data are presented as means $\pm$ SD. * means $p < 0.05$ vs. control group.

Figure 4

Relative expression of apoptosis-related proteins and caspase family induced by E35 A. Representative images of western blot B. Quantitative results of relative expression of apoptosis-related proteins and caspase family in U266 cells induced by E35 treatment C. Quantitative results of relative expression of apoptosis-related proteins and caspase family in MM1s cells induced by E35 treatment Data are presented as means $\pm$ SD. * means $p < 0.05$; ** means $p < 0.01$ vs. control group.

Figure 5

Relative expression of the Akt/mTOR/4EBP1 signaling pathway-related proteins induced by E35 A. Representative images of western blot B. Quantitative results of relative expression of Akt/mTOR/4EBP1 signaling pathway-related proteins in U266 cells induced by E35 treatment C. Quantitative results of relative expression of Akt/mTOR/4EBP1 signaling pathway-related proteins in MM1s cells induced by E35 treatment Data are presented as means $\pm$ SD. * means p < 0.05; ** means p < 0.01 vs. control group.

Figure 6

Relative expression of C-Myc, Mcl-1, NF- κ B, 4EBP1, p-4EBP1, EIF4E, and p-EIF4E proteins affected by administration of E35 and bortezomib in U266 cells A. Representative images of western blot B. Quantitative results of relative expression of C-Myc, Mcl-1, NF- κ B, 4EBP1, p-4EBP1, EIF4E, and p-EIF4E proteins Data are presented as means $\pm$ SD. * means $p < 0.05$; ** means $p < 0.01$ vs. control group.