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ORIGINAL RESEARCH

Diosmetin Inhibits Cell Proliferation, Induces Cell Apoptosis and Cell Cycle Arrest in Liver Cancer

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Objective: Diosmetin (DIOS) has been confirmed to possess anti-cancer effects in some types of tumors. However, it remains unclear whether DIOS exerts anti-cancer effects on liver cancer. Thus, our purpose was to observe the effect of DIOS on cell proliferation, cell apoptosis and cell cycle arrest in human liver cancer cells.

Materials and Methods: The cell viability of HepG2 and HCC-LM3 cells under different concentrations of DIOS was detected using MTT assay. The cell apoptosis and cell cycle arrest were analyzed by flow cytometry. The expression levels of apoptosis/cell cycle-related proteins including P53, Bcl-2, Bax, cleaved-caspase3, cleaved-caspase8, cleaved-PARP, Bak, cdc2, cyclinB1 and P21 were measured using Western blot. HepG2 cells were transfected by checkpoint kinase 1 (Chk1)-small interfering RNA (siRNA) and checkpoint kinase 2 (Chk2)-siRNA, respectively. After that, cell cycle was detected.

Results: DIOS significantly suppressed cell proliferation and induced cell apoptosis of HepG2 cells and HCC-LM3 cells. Moreover, DIOS promoted cell cycle arrest in G2/M phase. Western blot results showed that DIOS significantly suppressed the expression levels of Bcl-2, cdc2, cyclinB1, and promoted the expression levels of Bax, cleaved-caspase3, cleaved-caspase8, cleaved-PARP, Bak, P53, and P21. The G2/M phase arrest was observed in HepG2 cells transfected with Chk2-siRNA, while the G2/M phase arrest was not obvious in HepG2 cells transfected with Chk1-siRNA.

Conclusion: Our findings revealed that DIOS could inhibit cell proliferation and promote cell apoptosis and cell cycle arrest in liver cancer. Furthermore, DIOS could induce G2/M cell cycle arrest in HepG2 cell via targeting Chk2.

Keywords: diosmetin, cell apoptosis, cell cycle arrest, liver cancer, HepG2 cell

Introduction

Liver cancer is one of the most common malignant tumors worldwide.[1–3] The number of patients who die of liver cancer worldwide is as high as 16 million per year.[4] China is a high-risk area of primary liver cancer in the world.[5] The occurrence and development of liver cancer is closely related to hepatitis B virus infection, long-term alcohol abuse, bad eating habits, and mildew food intake.[6] At present, the number of liver cancer patients in China accounts for about half of the total number of liver cancers in the world.[7] Liver cancer has become a malignant disease that seriously threatens people's health and life. The chemotherapy drugs for postoperative liver cancer have improved the treatment of liver cancer patients and improved their survival time.[8] The significance of the expected treatment is better when using these drugs for postoperative chemotherapy, but the main problem is that these chemotherapeutic drugs have a large cytotoxic effect, and

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the selectivity to tissue receptors is poor.[9] In recent years, targeted therapy of drugs has attracted attention due to specific receptor proteins and small side effects on normal cells.[1,10,11] Although the occurrence and development of tumors is an extremely complicated process of genetic alteration, most tumors are accompanied by a process of genetic mutations.[12] Even for the same type of tumor, malignant cell clusters are caused by changes in different genes leading to the formation of heterogeneous cells. Moreover, along with the misconfiguration of tumors, the mutated genes in tumor cells are constantly changing. So it is extremely difficult to inhibit the proliferation of tumor cells by targeting drugs targeting single gene.[13] Therefore, through the use of the differences between the biological metabolism of tumor cells and normal cells, further exploration of targeted specific anti-tumor drugs is a new strategy for the development of anti-tumor drugs.

In the field of drug development in the new era, natural product can enhance the anti-tumor effect of natural Chinese herbal medicine by improving the purity, efficiency and targeting of tumor receptors. These natural products include some semi-synthetic taxane derivatives and flavonoids.[14,15] Related studies have shown that most flavonoids have anti-tumor characteristics against multiple genes, multiple sites, and multiple pathways, and they also have low toxicity.[16,17] The pharmacological effects of flavonoids in anti-tumor, cell cycle arrest and cell apoptosis have been widely recognized.[18,19] DIOS is a flavonoid compound found mainly in the peels of oranges and lemons. It has anti-oxidant, anti-tumor and anti-mutagenic properties. Studies have found the anti-tumor effects of DIOS on inhibiting tumor cell proliferation.[20] However, the mechanism of anti-tumor activity of DIOS in liver cancer is poorly understood. In this study, we aimed to investigate the effects of DIOS on the cell viability, apoptosis and cell cycle arrest in human hepatoma HepG2 cells and HCC-LM3 cells.

Materials and Methods

Reagents

DIOS was purchased from Sigma (USA). AnnexinV-FITC/PI Apoptosis Assay Kit was purchased from BD (USA). Human p53, Bcl-2, Bax, cleaved-caspase3, cleaved-caspase8, cleaved-PARP, Bak, cdc2, cyclinB1 and p21 antibodies were purchased from CST (USA). Octamethylazozolium blue (MTT) and propidium iodide (PI) stains were purchased from Sigma. Cell protein extraction kit was purchased from Biyuntian (China).

Cell Culture

The human hepatoma cell lines including HepG2 and HCC-LM3 and human normal liver cell line LO2 were purchased from the Shanghai Cell Bank (China, Shanghai). The cells were cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Inc., USA) containing 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., USA), 100 U/mL penicillin, 100 mg/L streptomycin in 37°C incubator with 5% CO_2. The cells were passaged for 2 to 3 days, and the cells in the logarithmic growth phase were taken for further experiments.

MTT Assay

HepG2, HCC-LM3 and LO2 cells in the logarithmic growth phase were digested, counted, and mixed with 10% FBS to prepare 7.5×10^3 cells/well for inoculation in 96-well culture plates for 24 h. After the cells were attached, different concentrations of DIOS were added (final drug concentrations were 0, 1, 2, 5, 10, 15, 20, 25, 30 μg/mL). The cells were cultured for 6 h, 12 h, 24 h, 48 h, respectively. After that, the supernatant was discarded. One hundred μL DMSO was added to each well. After the crystals were sufficiently dissolved, the absorbance per well (A) was measured at a wavelength of 490 nm. The cell proliferation inhibition rate was calculated as follows: Inhibition rate = (control group A – test group A)/(control group A – blank group A) × 100%.

AnnexinV-FITC/PI Apoptosis Assay

Flow cytometry was used to detect cell apoptosis. Briefly, HepG2 and HCC-LM3 cells were treated with different concentrations of DIOS for 24 h, and the cell suspension was prepared. The cells were centrifuged for 5 min in pre-cooled phosphate buffer (PBS) to wash the cells. One hundred μL of the cell suspension was incubated with 5 μL of AnnexinV-FITC and PI in the dark for 20 min. Four hundred μL of buffer was added before operating the machine to measure the apoptosis rate by flow cytometry.

Cell Cycle Assay

HepG cells in logarithmic growth phase (concentration of 4.5×10^3 cells/mL) were treated with DISO (final concentrations of 0, 5, 10, 20 μg/mL). The cells were collected after 24 h, centrifuged at 1000 r/min for 5 min, washed

with pre-cooled PBS and then treated with 70% ethanol. After overnight fixation, the ethanol was removed by centrifugation, washed with pre-cooled PBS, stained with PI at 4°C for 30 min in the dark. The distribution of the cell cycle phase was detected by flow cytometry. The experiments were repeated at least three times independently.

Western Blot Assay

To detect the protein expression of cyclin p21, cdc2, cyclinB1, Bcl-2, Bax, cleaved-caspase3, cleaved-caspase8, cleaved-PARP and Bak protein, the HepG2 cells in logarithmic growth phase were collected and the total protein was extracted according to cellular protein extraction reagent. The protein content was determined by BCA method. The protein was denatured at 95°C for 10 min, and 20 μg protein sample was loaded on polyacrylamide gel for electrophoresis. Then, the protein separated from the gel was transferred onto the PVDF membrane. After 5% skim milk was blocked for 2 h, the primary antibodies were incubated at 4°C for 24 h, and the secondary antibodies were incubated at room temperature for 1 h. After washing with TBST solution, the expression of the target bands was detected by chemiluminescence kit (GE Healthcare Life Sciences, Chalfont, UK).

Cell Transfection

Chk1-siRNA, Chk2-siRNA, Bcl-2-siRNA, cleaved-caspase8-siRNA, and their negative control (NC) were synthesized and purchased from Ribobio (Guangzhou, China). Transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol.

Statistical Analysis

Statistical analyses were performed using SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA) and Graphpad Prism 7.0 (San Diego, CA, USA). Data were expressed as mean ± standard deviation (SD). All experiments were repeated at least three times. Comparisons were evaluated using student's *t* test or one-way analysis of variance. P<0.05 was considered statistically significant.

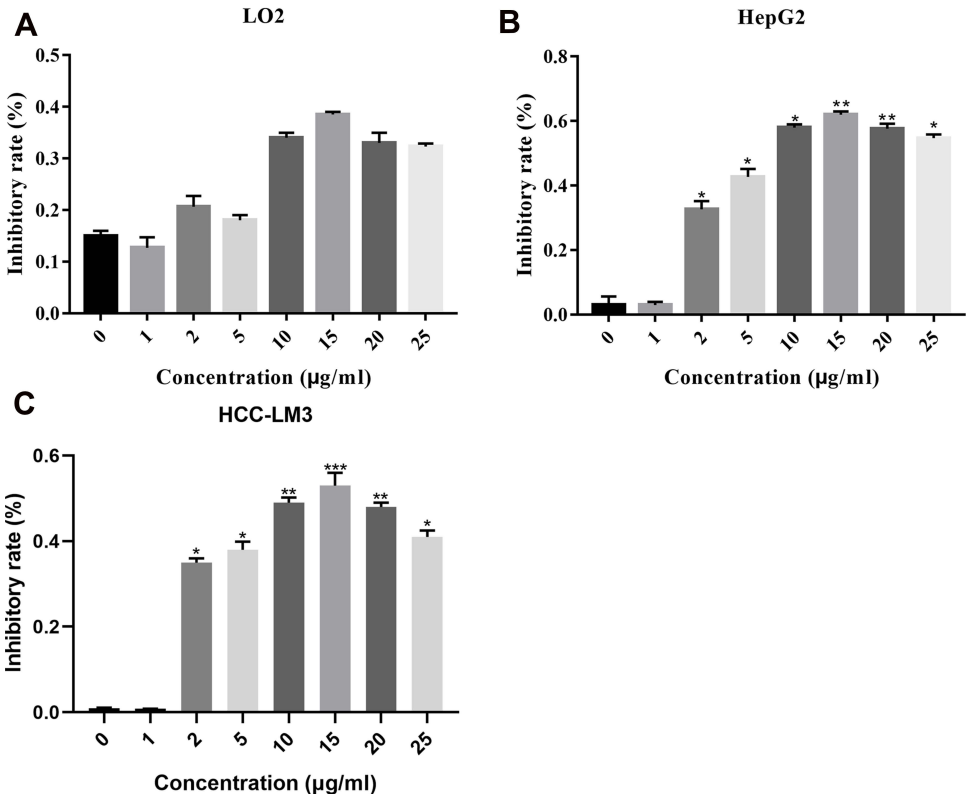


Figure 1 DIOS inhibits the cell viability of liver cancer cells using MTT assay. (**A**) The normal hepatocyte LO2 cells and liver cancer HepG2 (**B**) and HCC-LM3 (**C**) cells were treated with different concentrations of DIOS, respectively. The MTT assay was used to detect the cell viability. *P<0.05, **P<0.01 and ***P<0.001.
Abbreviations: DIOS, diosmetin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Results

DIOS Inhibits the Cell Viability of Liver Cancer Cells

The normal hepatocyte cell line LO2 and liver cancer cell line HepG2 and HCC-LM3 cells were treated with different concentrations of DIOS, respectively. MTT assay results showed that the cell viability of LO2 cells was not significantly inhibited under different concentrations of DIOS (Figure 1A). In contrast, we found that DIOS significantly suppressed the cell viability of HepG2 and HCC-LM3 cells, with a concentration-dependent manner (Figure 1B and C). Similarly, the results of the clone formation experiments showed that different concentrations of DIOS could not affect the proliferation of LO2 cells (Figure 2A and B). However, we found that DIOS significantly inhibited the proliferation of HepG2 and HCC-LM3 cells, with a concentration-dependent manner (Figure 2C–F). HepG2 cells were treated with different

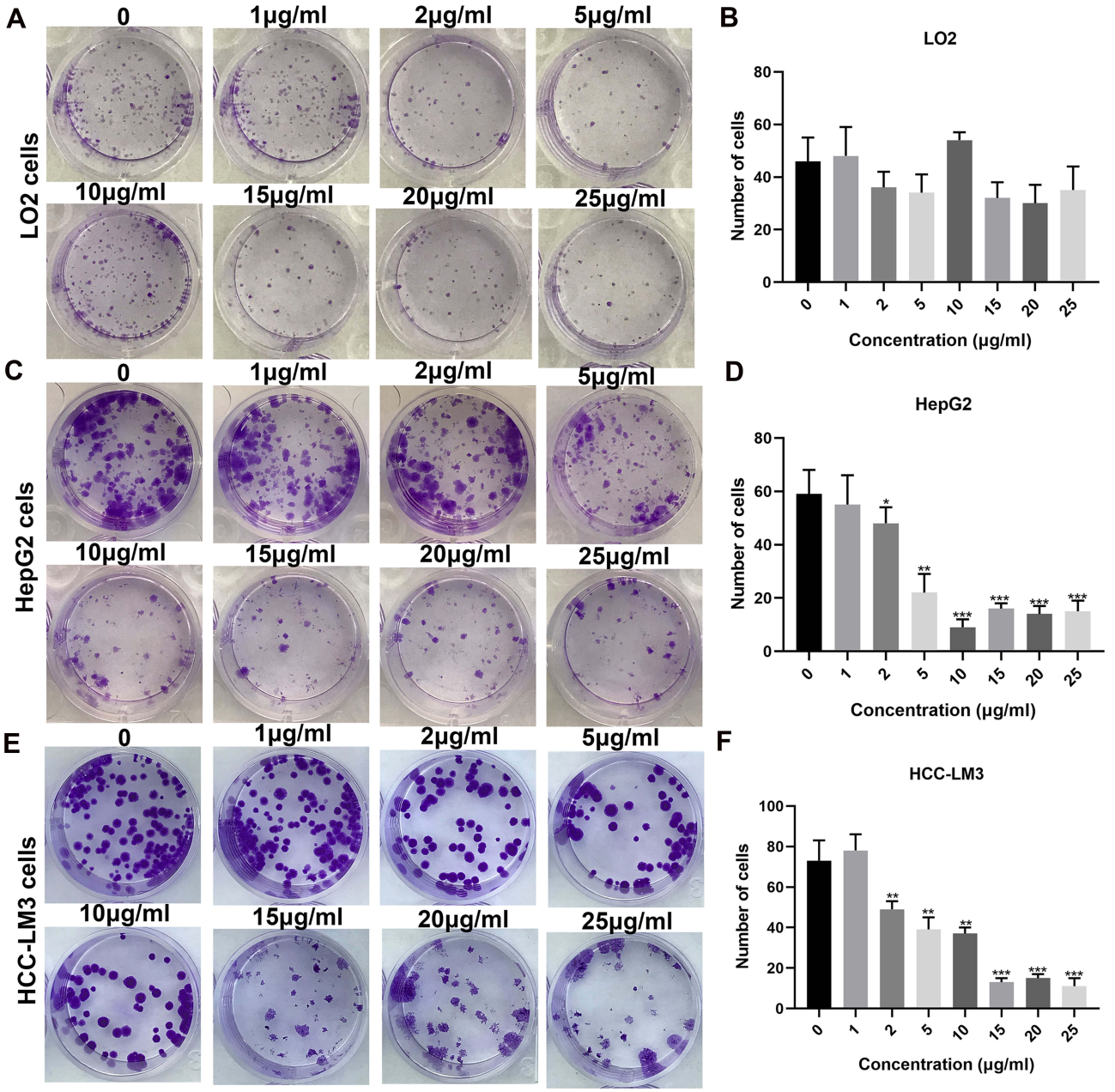


Figure 2 Clone formation assay results showing the inhibitory effects of different concentrations of DIOS on the proliferation of LO2 cells (**A, B**), HepG2 (**C, D**) and HCC-LM3 cells (**E, F**). *P<0.05, **P<0.01 and ***P<0.001.
Abbreviation: DIOS, diosmetin.

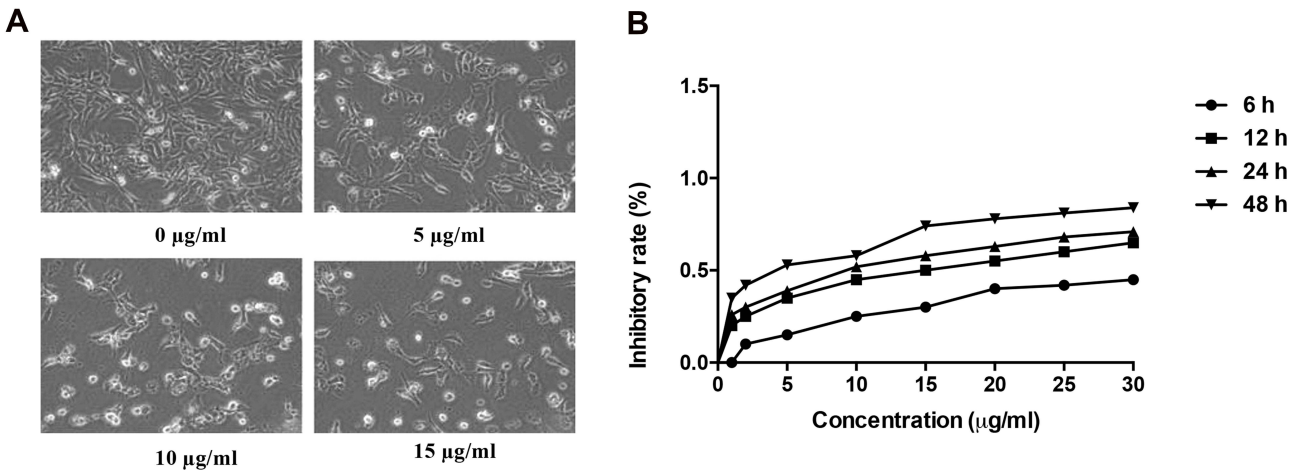


Figure 3 The cell morphology of HepG2 cells treated with DIOS. (**A**) HepG2 cells were treated with different concentrations (0, 5, 10, 15 μg/mL) of DIOS for 24 h, and the cell morphology was observed under light microscopy. (**B**) MTT assay was used to detect the effect of different concentrations of DIOS on cell viability at different times (6, 12, 24, 48 h).

Abbreviations: DIOS, diosmetin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

concentrations (0, 5, 10, 15 μg/mL) of DIOS for 24 h. Under the microscope, we found that the cells in the control group were slender, vigorously growing, regular in morphology, clear in cell contour, and large in size (Figure 3A). However, as for the HepG2 and HCC-LM3 cells treated with DIOS, the cells were irregular in shape, the cell morphology became round, the cell gap increased, some cells were floating, and the cell debris increased with the increase of concentrations (Figure 3A). Moreover, DIOS significantly decreased the cells viability of HepG2 and HCC-LM3 cells with concentration-dependent and time-dependent manners (Figure 3B).

DIOS Promotes Cell Cycle Arrest in G2/M and Cell Apoptosis of HepG2 Cells

HepG2 cells were treated with different concentrations (0, 5, 10, 15 μg/mL) for 24 h, and flow cytometry was employed to analyze the cell cycle change. As shown in Figure 4A and C, the cells were blocked in G2/M phase. Furthermore, DIOS promoted the proportion of G2/M phase, with a concentration-dependent manner. We also examined the cells apoptosis of HepG2 cells under different concentrations of DIOS. The results showed that DIOS significantly promoted cell apoptosis of HepG2 cells, with a concentration-dependent manner (Figure 4B and D). These results suggested that DIOS could induce cell cycle arrest in G2/M and cell apoptosis of HepG2 cells.

DIOS Is Involved in Regulating the Expression of Cell Cycle/Apoptosis-Associated Proteins in HepG2 Cells

After HepG2 cells treated with different concentrations of DIOS (0, 5, 10, 15 μg/mL) for 24 h, we examined the expression levels of cell cycle/apoptosis-associated proteins in HepG2 cells using Western blot. The results showed that DIOS significantly inhibited the expression levels of cell cycle-related proteins including cyclin B1 and cdc2, apoptosis-related proteins including Bcl-2 and cleaved-caspase8 in HepG2 cells, with a concentration-dependent manner (Figure 5A). Furthermore, DIOS significantly promoted the expression levels of P53, P21, cleaved-PARP, cleaved-caspase3, Bax, Bak, P-Chk1, P-cdc25c, P-Chk2 and P-cdk1 in HepG2 cells, with a concentration-dependent manner (Figure 5B).

DIOS Might Promote G2/M Arrest via Chk2 but Not Chk1 in HepG2 Cells

To further explore the mechanism by which DIOS might mediate G2/M cell cycle arrest, we knocked down the expression of Chk1 and Chk2 by the corresponding siRNAs in HepG2 cells, respectively (Figure 6A). We found that DIOS significantly promoted the proportion of G2/M phase in HepG2 cells. After inhibiting Chk2, the proportion of G2/M phase was significantly inhibited (Figure 6B). However, after inhibiting Chk1, the proportion

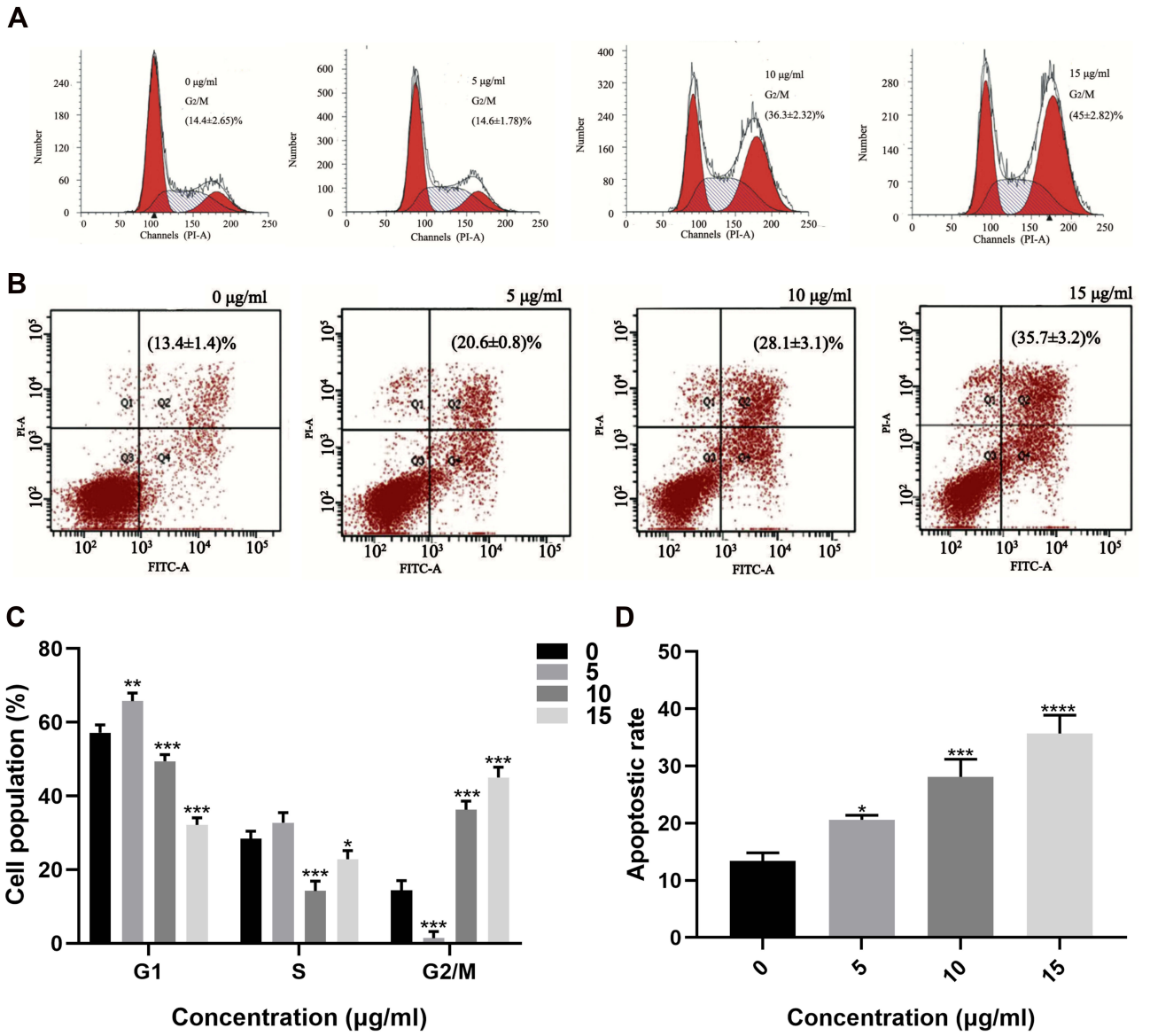


Figure 4 DIOS promotes cell cycle arrest in G2/M and cell apoptosis of HepG2 cells. (**A, C**) Flow cytometry was used to detect the cell cycle of HepG2 cells treated with different concentrations of DIOS (0, 5, 10, 15 μg/mL) for 24 h. (**B, D**) The apoptosis rate of HepG2 cells under different concentrations of DIOS (0, 5, 10, 15 μg/mL) for 24 h was detected using flow cytometry. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Abbreviations: PI-A, propidium iodide-area; G1, postsynthetic gap1 period; S, DNA synthesis phase; G2, postsynthetic gap2 period; M, mitotic phase; FITC-A, fluorescein isothiocyanate-area; DIOS, diosmetin.

of G2/M phase was not significantly inhibited (Figure 6B). DIOS might promote G2/M arrest via Chk2 but not Chk1 in HepG2 cells.

DIOS Might Promote HepG2 Cell Apoptosis by Bcl-2 and Cleaved-Caspase8

We observed whether DIOS might promote HepG2 cell apoptosis by apoptosis-related proteins including Bcl-2 and cleaved-caspase8. As shown in Figure 7A and B, Bcl-2 and cleaved-caspase8 were successfully silenced in HepG2 cells, respectively. Flow cytometry results suggested that DIOS significantly promoted HepG2 cell apoptosis. After inhibiting Bcl-2 or cleaved-caspase8, the inhibitory effects induced by DIOS were significantly reversed (Figure 7C). Thus, DIOS might promote HepG2 cell apoptosis by Bcl-2 and cleaved-caspase8.

Discussion

Liver cancer is a highly malignant and highly invasive solid tumor.[21,22] At present, there is no effective treatment.

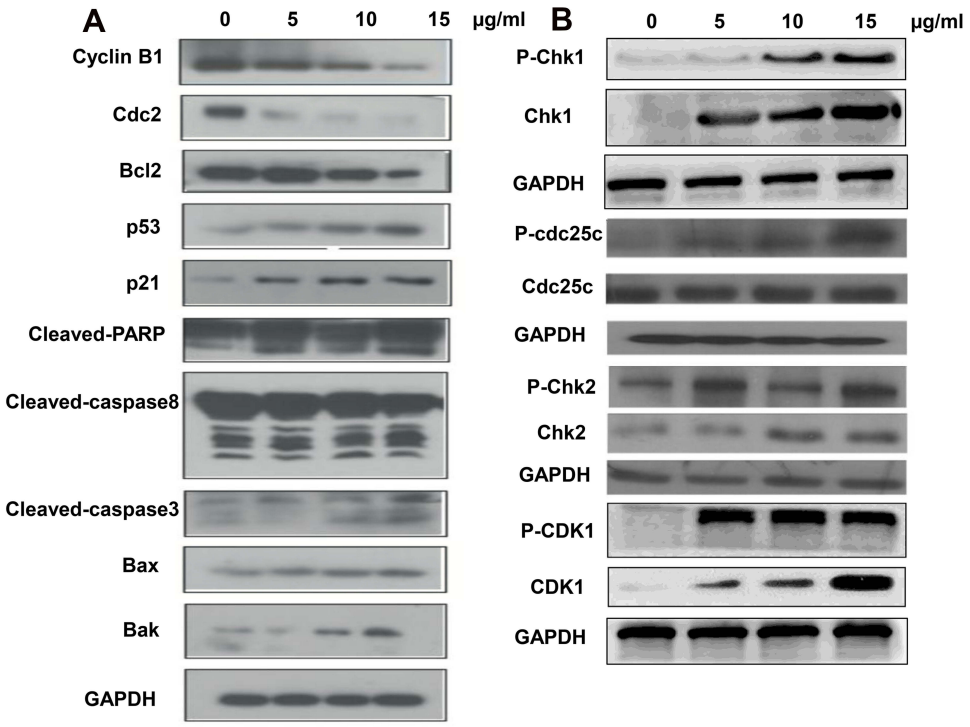


Figure 5 DIOS is involved in regulating the expression of cell cycle/apoptosis-associated proteins in HepG2 cells. HepG2 cells were treated with different concentrations of DIOS (0, 5, 10, 15 μg/mL) for 24 h, and the expression levels of cell cycle/apoptosis-associated proteins were detected by Western blot (**A, B**).
Abbreviations: Cdc, cell division cycle 2, Bcl, B-cell lymphoma; PARP, poly ADP-ribose polymerase; Bax, BCL2-associated X; Bak, BCL2-Associated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Chk, cyclin-dependent kinase; DIOS, diosmetin.

DIOS as a flavonoid anticancer drug provides a new idea for the current anticancer treatment.[23–25] In this study, we explored the anti-tumor mechanism of DIOS in human hepatoma HepG2 cells and HCC-LM3 cells.

We found that DIOS promoted G2/M phase arrest in HepG2 cells, while normal human liver cell LO2 had no significant changes after treatment with DIOS. It is well known that inhibition of the hepatoma cell cycle can induce the repression of liver cancer cell proliferation. The combination of cyclin and cyclin-dependent kinase (CDK) is closely related to cell cycle transition.[26] Our results showed that DIOS significantly inhibited the proliferation of hepatoma cells and promoted G2/M cell cycle arrest, which was in close association with Chk signaling pathway. However, there have been few studies on signal pathways in which DIOS is involved, especially in the Chk signal transduction pathway. Therefore, we explored the relationship between Chk signaling pathway and cell cycle regulated by DIOS. The transition from G1 to S causes a cascade of cyclinD/CDK4 complexes to bind to CDK6, and the cyclinB/CDK1 complex will shift from G2 to M during mitosis.[27] When DNA is damaged, the G2 phase-detection site will cause the cells to enter the mitosis phase for automatic repair. When cyclinB and cyclinA form a complex with CDK1, it is extremely important to generate an M-phase transition of the cascade activation pair.[28] In our experiments, it was found that DIOS down-regulated the protein expression of cyclinB/CDK1. There was no significant change in cyclinA expression after DIOS treatment. This indicates that DIOS may induce G2/M phase arrest by down-regulating cyclinB/CDK1. In addition, with the accumulation of p53, dissociation from the MDM2 binding site, p53 gene promotes p21Cip1 accumulation, which is an inhibitor of Cdc2.[29] This results in a decrease in the expression of cyclin B/cdc2; therefore, the DIOS-induced cell cycle arrest in G2/M phase may be through the induction of the cyclinB1/cdc2 pathway. Our study showed that the expression of p53 and p21 increased in DIOS-treated human hepatoma cell line HepG2, indicating that DIOS-induced cell cycle arrest may also be related to the p53 signaling pathway. Meanwhile, DIOS down-regulated Bcl-2 expression through P53 and mitochondrial apoptosis pathway and up-regulated the expression of Bax, Bak, cleaved-caspase3, cleaved-caspase8 and

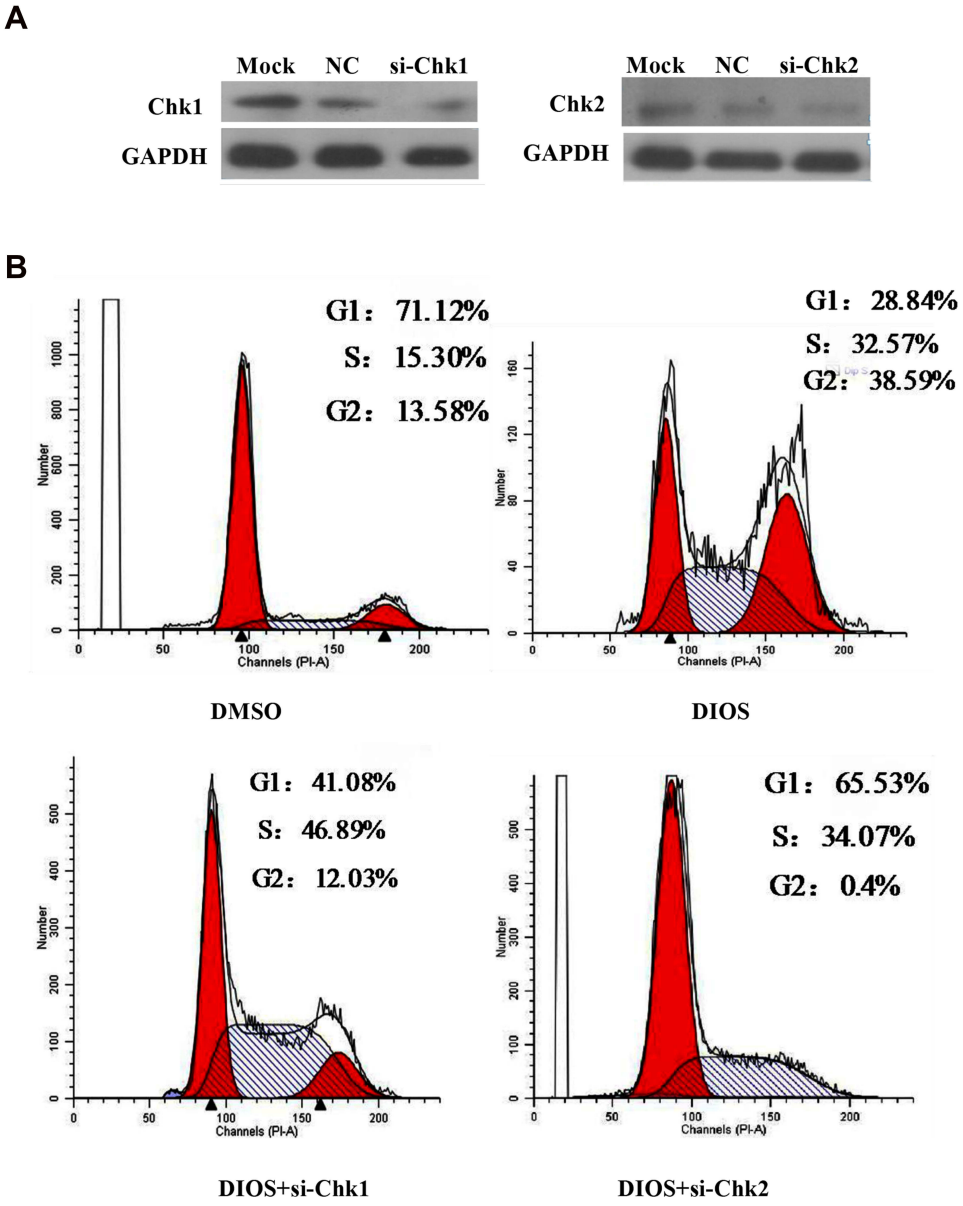


Figure 6 DIOS might promote G2/M arrest via Chk2 but not Chk1 in HepG2 cells. (**A**) Western blotting was used to detect the expression levels of cyclin Chk1 and Chk2 in HepG2 cells transfected with Chk1-siRNA and Chk2-siRNA. (**B**) Flow cytometry was used to detect the cell cycle changes in HepG2 cells transfected with Chk1-siRNA and Chk2-siRNA.

Abbreviations: PI-A, propidium iodide-area; DMSO, dimethyl sulfoxide; DIOS, diosmetin; Chk2, checkpoint kinase 2; siChk2, small interfering RNA-checkpoint kinase 2; NC, negative control; Chk1, checkpoint kinase 1; siChk1, small interfering RNA-checkpoint kinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G1, postsynthetic gap1 period; S, synthetic phase; G2, postsynthetic gap2 period; M, mitotic phase.

cleaved-PARP. Ultimately, DIOS promoted apoptosis and cell cycle arrest in G2/M phase through inducing the low expression of apoptosis-related proteins and cyclins.

DNA damage will result in the activation of ATM/ATR, which induces Cdc25c phosphorylation to inhibit the activation of Chk1 and Chk2.[30,31] Chk1 promotes phosphorylation by activating its ser317, ser345 and ser296 sites, and Chk2 activates its ser33/35, ser516, ser296, and Thr68 sites.[32,33] Our study found that DIOS can induce high expression of p-Chk1 (ser317, ser345) and p-Chk2 (ser33/35). To elucidate the intrinsic mechanism of DIOS-induced cell cycle arrest, we detected the expression of Chk1 and Chk2 in HepG2 cells transfected with Chk1-siRNA and Chk2-siRNA. We found that the change in G2 phase after Chk1-siRNA treatment was not obvious, but the proportion of G2 phase in HepG2 cells transfected with Chk2-siRNA was significantly decreased. This result demonstrated that the major signaling pathway regulating

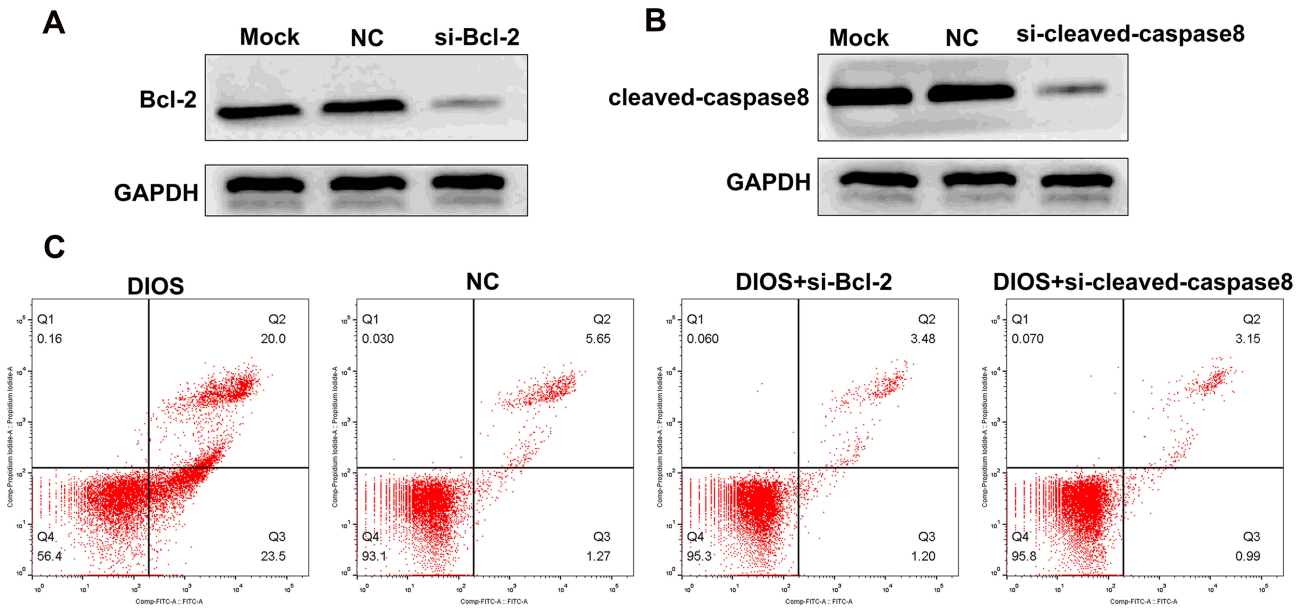


Figure 7 DIOS might promote HepG2 cell apoptosis by Bcl-2 and cleaved-caspase8. (**A, B**) The transfection effects of si-Bcl-2 and si-cleaved-caspase8 in HepG2 cells were detected using Western blot. (**C**) Flow cytometry was used to detect HepG2 cell apoptosis.

Abbreviations: NC, negative control; si-Bcl-2, small interfering RNA-B-cell lymphoma-2; Bcl-2, B-cell lymphoma-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si, small interfering RNA; FITC-A, fluorescein isothiocyanate-area; DIOS, diosmetin.

G2/M cell arrest could be the Chk2 pathway. The cyclinB/CDK and p53 signaling pathways are not fully involved in the regulation of G2/M cell arrest in HepG2 cells.

Taken together, our study showed that DIOS can cause proliferation inhibition, induce apoptosis and G2/M cell cycle arrest in human hepatoma HepG2 cells. Based on current research, DIOS may be a potential anti-tumor chemotherapeutic drug for liver cancer therapy. In future study, we will further clarify the role of DIOS in cell cycle arrest and signaling pathway in vitro and in vivo animal models, and provide new clinical treatment approaches for tumors.

Conclusion

In this study, our findings revealed that DIOS inhibited cell proliferation and promoted cell apoptosis in liver cancer cells. Furthermore, DIOS treatment induced G2/M cell cycle arrest in HepG2 cell by down-regulation of cell cycle-related protein cdc2, cyclinB1 and up-regulation of P53 and P21. Moreover, we also found that DIOS might promote HepG2 cell apoptosis by Bcl-2 and cleaved-caspase8. Our research provides novel insights into the mechanism of DIOS against liver cancer.

Abbreviations

DIOS, diosmetin; Chk1, checkpoint kinase 1; siRNA, small-interfering RNA; Chk2, checkpoint kinase 2.

Data Sharing Statement

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the manuscript, revising the manuscript critically, read and approve the final draft of the manuscript for submission, gave final approval of the manuscript version to be published and agreed to be accountable for every step of the work.

Disclosure

The authors declare no conflicts of interest.

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